Institut für Nutzpflanzenwissenschaften und Ressourcenschutz
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The effect of sulfur deficiency in pea (*Pisum sativum)* on factors related to nitrogen fixation and on sulfate transporters

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Dedicated to my son,Samyar

English abstract

Sulfur has a vital role in the plant life cycle. S deficiency reduces optimal yield and quality in legumes which lead to a reduction in nutritional value of food and feed. S deficiency also alters the symbiotic interaction between leguminous plants and soil nitrogen fixing bacteria which leads to a decline in N2 fixation rate. An efficient symbiotic nitrogen fixation (SNF) in the nodule demands a strong nutrient exchange between plant and bacterial cells. The plant symbiotic sulfate transporter (SST) supports adequate S supply for full activity of nodules. In the present study, I investigated how different levels of S supply affect the protein and mRNA expression of genes related to N2 fixation in Pisum sativum. Moreover, different sulfate transporter genes were identified in pea and the functional analysis was performed for the symbiotic sulfate transporter from pea after expression in yeast double mutant. Growth retardation was observed in both weak and severe S deficient plants while severe S deficiency led to a significant decline in total biomass and shoot dry weight. Chlorosis and yellowish leaves in severe S deficient plant were observed as a consequence of a 43% decline in the chlorophyll content of young leaves in comparison with the control plants. Both weak and severe S deficiency reduced nodule formation. Thus, nodules appeared smaller and green with less leghemoglobin content in the S deficient groups. Relative transcript abundance of leghemoglobin genes (PsLb5 and PsLb120) did not change significantly under S deficiency conditions in the vegetative phase, thus confirming that expression of both genes is essential to create a low O2 concentration in young nodules. In contrast, severe S deficiency during the generative phase reduced expression of leghemoglobin at mRNA and protein levels. Furthermore, the higher leghemoglobin content in the generative phase is indicative for a higher leghemoglobin demand of mature nodules to maintain a low O2 environment and to protect the nitrogenase for an effective SNF. Nitrogenase is encoded by the nifHDK gene cluster. Severe S deficiency reduced the relative transcript abundance of the β subunits (NifK gene) while expression of α subunits (NifD gene) was mostly unaffected. S deficiency conditions increased relative transcript abundance of ferredoxin to compensate the S limitation in the nodule. Furthermore, a higher level of ferredoxin expression in the generative phase compared to the vegetative phase might be accompanied by a more SNF activity in the generative phase. In contrast to the nodule, relative ferredoxin transcript abundance was reduced under severe S deficiency in the root and leaf tissues supporting sulfite reduction and NADP photo-reduction. Both severe and weak S deficiencies reduced the relative transcript abundance of ferredoxin in the young leaves more than the mature leaves because the mature leaves are the main site of sulfate storage and assimilation. Severe S deficiency reduced expression of the nodule-specific sulfate transporter (SST) in both vegetative and generative phase of plant life which confirms a disruption in sulfate transport in symbiosomes. Phylogenetic analysis of 14-full length sulfate transporter sequences from pea and other known sulfate transporters from the leguminous family revealed that they fall into five major groups. In conclusion, a reduced sulfate import into the nodule probably reduces the sulfate related metabolites and interrupts the expression and biosynthesis of nitrogenase, leghemoglobin and ferredoxin proteins and eventually leads to an interruption of SNF. Moreover, severe S deficiency could limit the S compounds and S containing amino acids essential for the synthesis of critical products such as chlorophyll, thus leading to a lower rate of photosynthesis and fewer energy sources for an efficient yield.

German abstract

Schwefel spielt eine bedeutende Rolle im Lebenszyklus von Pflanzen. Bei Leguminosen führt S-Mangel nicht nur zu Mindererträgen, sondern er wirkt sich auch negativ auf die Qualität der Samen aus. Des Weiteren resultiert eine suboptimale S-Versorgung in einer Beeinträchtigung der N_2-Fixierung. Eine effiziente symbiontische N_2-Fixierung (SNF) setzt einen Austausch von Nährstoffen zwischen Pflanzen- und Bakterienzellen voraus. Der Sulfattransporter (SST) trägt zur adäquaten S-Versorgung der Knöllchen bei. In den vorliegenden Untersuchungen wurde überprüft, wie sich eine unterschiedliche S-Versorgung auf die Expression von Genen auswirkt, die in Bezug zur N_2-Fixierung von *Pisum sativum* stehen. Ebenso wurden in Erbsen verschiedene Sulfattransporter-Gene untersucht und funktionelle Analysen für Sulfattransporter nach Expression in Doppelmutanten von Hefe durchgeführt. Bei schwachem und starkem S-Mangel wurden Wachstumsbeeinträchtigungen der Erbse beobachtet. Darüber hinaus resultierte starker S-Mangel in einem signifikanten Rückgang an Gesamt-Biomasse und Spross-Trockenmasse. Im Vergleich zur Kontrolle zeigten Pflanzen mit starkem S-Mangel chlorotische und gelbe Blätter als Folge eines um 43% geringeren Chlorophyllgehalts der jüngeren Blätter. Schwacher und starker Mangel führten zu einer verminderten Knöllchenbildung. Die Knöllchen waren kleiner und grün und enthielten weniger Leghämoglobin. Die relative Transkript-Abundanz von Leghämoglobingenen (PsLb5 und PsLb120) wurde während der vegetativen Phase unter S-Mangelbedingungen nicht signifikant beeinflusst, was bestätigt, dass die Expression beider Gene essentiell ist, um eine niedrige O_2-Konzentration in jungen Knöllchen zu gewährleisten. Im Gegensatz dazu resultierte ein starker S-Mangel während der generativen Phase in einer verminderten Expression von Leghämoglobin im mRNA- und Protein-Level. Weiterhin ist der hohe Leghämoglobingehalt während der generativen Phase ein Indikator für einen hohen Leghämoglobinbedarf reifer Knöllchen, um eine niedrige O_2-Umgebung und somit der Nitrogenase optimale Bedingungen für eine effektive SNF zu gewährleisten. Nitrogenase wird durch das nifHDK Gen-Cluster kodiert. Starker S-Mangel reduzierte die relative Transkript-Abundanz der β-Untereinheiten (Nifk Gene), während die Expression der α-Untereinheiten (NifD Gene) meist unbeeinflusst blieb. S-Mangel erhöhte die relative Transkript-Abundanz von Ferredoxin, um die S-Limitierung der Knöllchen zu kompensieren. Eine höhere Ferredoxin-Expression während der generativen Phase im Vergleich zur vegetativen Phase könnte durch eine höhere SNF-Aktivität in der generativen Phase begleitet werden. Im Gegensatz zu den Knöllchen war die relative Ferredoxin-Transkript-Abundanz in den Wurzeln und Blättern bei starkem S-Mangel reduziert. Starker und schwacher S-Mangel führten in jüngeren Blättern zu einer stärkeren Reduktion der relativen Transkript-Abundanz von Ferredoxin im Vergleich zu älteren Blättern, da letztere der Hauptort für die Sulfat-Speicherung und -Assimilation ist. Starker S-Mangel verminderte die Expression des knöllchenspezifischen Sulfattransporters (SST) sowohl während der vegetativen als auch während der generativen Phase, was die Unterbrechung des Sulfattransports in Symbiosome bestätigt. Phylogenetische Analysen von 14 Sulfattransportersequenzen von Erbsen und anderen von Leguminosenfamilien bekannten Sulfattransportern zeigen, dass sie in fünf Hauptgruppen eingeteilt werden können. Es kann geschlussfolgert werden, dass ein verminderter Sulfatimport in die Knöllchen wahrscheinlich die Synthese schwefelabhängiger Metabolite beeinträchtigt und die Expression und Biosynthese von Nitrogenase, Leghämoglobin und Ferredoxin unterbricht und folglich zur Unterbrechung der SNF führt. Des Weiteren kann starker S-Mangel die Bildung S haltiger Verbindungen und S haltiger Aminosäuren beeinträchtigen, die für die Synthese kritischer Produkte wie Chlorophyll erforderlich sind, wodurch die Photosynthese beeinträchtigt wird und somit weniger Energie für die Ertragsbildung zur Verfügung steht.

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Abbreviations

AmpR	Ampicillin resistance
AP	Adaptor primer
ATP	Adenosine triphosphate
AUAP	Abridged universal amplification primer
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
Bp	base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
chl	Chlorophyll
Cq	Quantification cycle
Ct	Threshold cycle
CTAB	Cetyltrimethylammonium bromide
ddH_20	Double deionized water
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTP	2'-Deoxynucleosidetriphosphate
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
Fig	Figure
FW	Fresh Weight
G	Gram
G	standard gravity (g = 9.81 m sec^{-2})
Gal	Galactose
HEPES	N-2-Hydroxyethylpiperazine-N-2-ethanesulfonic acid
IPTG	Isopropyl-β-D-1-thiogalctopyranoside
Kb	kilo base pairs
KDa	Kilodalton
LB	Luria-Bertani broth
mol%	% molar percentage
Motr	Molybdate transporters
NBT	Nitro blue tetrazolium
OD_{600}	Optical density measured at a wavelength of 600 nm

ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
RIN	RNA integrity number
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
Rpm	Rotations per minute
rRNA	ribosomal RNA
RT	Room temperature
Rts	Relative quantities
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SNF	Symbiotic nitrogen fixation
Sultr	Sulfate transporter
TBST	Tris Buffered Saline with Tween
TE	Tris–EDTA
Tm	Melting temperature
Tris-HCl	2-Amino-2-hydroxymethyl-propane-1,3-diol-hydrochloride
UV	Ultra violet light (1 nm – 380 nm)
(v/v) %	Percent volume per volume (mL per 100 mL)
(w/v) %	Percent weight per volume (g per 100 mL)
WT	Wild type
X-Gal	5-Bromo-4-chloro-3-indoxyl-β-D-galactopyranoside

1 Introduction

1.1 Role of sulfur in plants

Sulfur (S) belongs to the secondary macronutrients (calcium, sulfur, magnesium), which are essential for plant growth, together with the primary macronutrients (nitrogen, phosphorus and potassium) that are required in much higher amounts, and the micronutrients. In some plants such as oil crops, legumes and forages, S requirement is even more important than phosphorus for optimal yield and quality. Usually cereal crops need 15 to 30 kg S/ha but the requirement for legumes and oil seeds is 25 to 50 kg S/ha (Sarda et al., 2014).

Sulfur has a vital role in the plant life cycle and for yield due to the presence of a wide range of sulfur-containing compounds including amino acids (cysteine and methionine), Fe-S cluster enzymes, glutathionine (GSH), thiols, vitamins, cofactors such as thiamine and coenzyme A, and sulfolipids in chloroplast membranes (Hell, 1997; Dubuis et al., 2005; Noctor, 2006; Begueret et al., 2007; Popper et al., 2011). Moreover, sulfur-containing secondary metabolites such as phytochelatins, phytoalexins, alliins, thionins and glucosinolates that are found in some plant species play important roles in plant physiology and plant defence processes against pests, pathogens and abiotic stresses. The sulfur enhanced defense (SED) mechanism was demonstrated to be present in virus infected tobacco and to suppress virus accumulation in the presence of sufficient sulfate. Also elemental sulfur limited the spread of infection in tomato with *Verticillium dahliae* infection (Thomma et al., 2002; Cooper and Williams, 2004; Durenkamp and De Kok, 2004; Falk et al., 2007; Holler et al., 2010; Bai et al., 2013).

Sulfur improves the efficiency of other macronutrients such as phosphorus and potassium in plants. An adequate sulfur status is important for the uptake and assimilation of micronutrients such as iron. In graminaceous plants growing under S deficiency condition, synthesis and secretion of mugineic acids were decreased and consequently the plants showed lower tolerance to Fe deficiency (Astolfi et al., 2006).

In crop plants such as brown rice, sulfur together with calcium, iron and zinc are important for reducing cadmium (Cd) uptake and accumulation in Cd contaminated soils (Khan et al., 1996; Nikiforova et al., 2003; Astolfi et al., 2006; Fan et al., 2010).

1.2 Sulfur access and assimilation in plants

In the soil, there are two main sulfur sources including organic (90-95%) and inorganic (5-10%). Plants mainly take up sulfate (SO_4^{2-}), which represents only 1% of total sulfur in the soil. Soil organic sulfur must be converted to inorganic sulfate (SO_4^{2-}) through microbiological and biochemical reactions prior to uptake by the plant.

First, sulfate is absorbed by epidermal and cortical root cells and subsequently is transported to different tissues by different sulfate transporters (ST) (Scherer, 2001; Hawkesford, 2003; Rouached et al., 2009; Kulhanek et al., 2011).

During sulfate assimilation into organic compounds, sulfate is reduced to sulfide in different steps (Fig.1). First, sulfate is activated by ATP sulfurylase (ATPS) and converted to adenosine-5'-phosphosulfate (APS). Then APS is reduced stepwise to sulfite by adenosine 5'-phosphosulfate reductase (APR), and sulfite is subsequently reduced further to sulfide by sulfite reductase (SiR). To form cysteine, sulfide is transferred to O-acetyl serine (OAS). Cysteine is the most important precursor for other organic sulfur containing compounds such as glutathione, coenzymes and methionine in plants (Leustek and Saito, 1999; Durenkamp and De Kok, 2004; Davidian and Kopriva, 2010; Hubberten et al., 2012b).

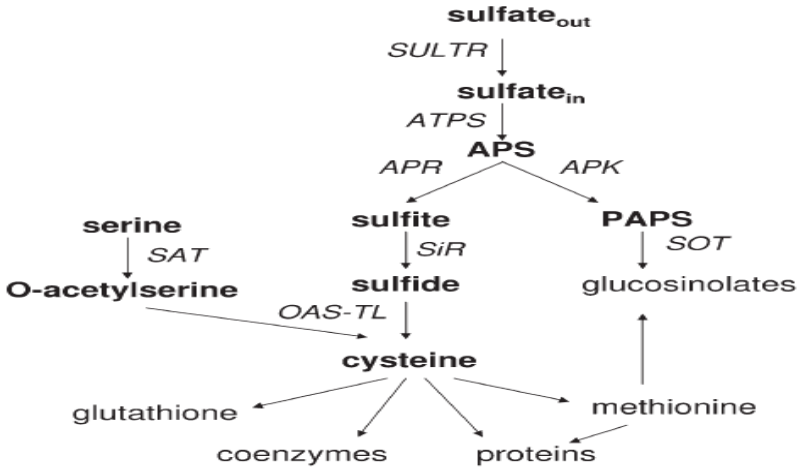


Fig. 1 Plant sulfate assimilation (Adopted from Davidian and Kopriva, 2010), ATPS: ATP sulfurylase, APS: adenosine 5'-phosphosulfate, APR: adenosine 5' -phosphosulfate reductase, APK: APS kinase, PAPS: 3'-phosphoadenosine 5' -phosphosulfate, SAT: serine acetyltransferase, SiR: sulfite reductase, OAS-TL: OAS (thiol) lyase OAS: O-acetylserine, SOT: sulfotransferase.

In addition to pedospheric sulfur (SO_4^{2-}), plants are able to use atmospheric sulfur (mainly SO_2 and H_2S) to support their growth. Atmospheric sulfur is reported to be a significant sulfur source for white clover legumes under low sulfur conditions. SO_2 and H_2S as sole sulfur sources are foliar absorbed via stomates. SO_2 is oxidized to sulfate via sulfite oxidase, while H_2S is reduced to cysteine via O-acetylserine (Durenkamp and De Kok, 2004; Durenkamp et al., 2007; Varin et al., 2013).

1.3 History of plant S deficiency

With Europe`s industrialization in the nineteenth-century, atmospheric sulfur dioxide (SO_2) emissions highly increased because of sulfur-containing fossil fuels, domestic coal burning and vehicles. Symptoms of industrialization were ecosystem problems such as acid rains and human health problems. In London during fog or smog, e.g. during the “pea souper” smog in 1959, SO_2 levels reached up to 3500 µg/m^3 and caused 4,000 deaths. Furthermore, it is reported from Chinese cities, that smog led to 50,000 premature deaths in 2000 (Hedley et al., 2002; Zhao et al., 2003; Dubuis et al., 2005).

In response to critical health problems, international agreements in the 1970s led to a significant decline in environmental pollutions such as SO_2. The consequence of decreased SO_2 emissions was an increase in the frequency of S deficiency in plants during the last decades. The current sulfur input from the atmosphere is reduced to about 10 kg ha^{-1} $year^{-1}$ in most Western European countries. Furthermore, the improvement of agricultural methods with less utilization of S-containing fertilizers, also the breeding of crops with higher yield and therefore higher S demand, promoted the occurrence of S deficiency (Dubuis et al., 2005; Scherer et al., 2008a; Howarth et al., 2009).

1.4 Plant responses to S deficiency

The average sulfur content in plants is about 0.2% to 0.5% of the dry matter. When plants are grown under less than the optimal conditions, deficiency symptoms appear. Visual S deficiency symptoms are chlorosis and yellow leaves (as a consequence of a decrease in chlorophyll content). Yellowing is followed by necrosis and death in long term S deficiency (Hawkesford, 2000; khan and Mazid 2011). S deficiency has a negative effect on the crop yield, protein concentration,

and total RNA content. S deficiency can reduce seed quality due to a shift in N to S ratio in plants (Drew, 1975; Zhao et al., 1999a; Nikiforova et al., 2005; Pacyna et al., 2006; Lunde et al., 2008)

A general response to nutritional limitation is root proliferation to increase the nutrient uptake (Lopez-Bucio et al., 2003; Bai et al., 2013). With increasing the root sink capacity, transport of sulfate via the xylem to young leaves is reduced and results in strong sulfur starvation in young leaves. Indeed, expression of internal sulfate transporter genes is changed under S deficiency conditions and sulfate is remobilized from mature leaves to the roots instead of young leaves. This is consistent with an enhanced sink capacity of the roots for sulfate (Clarkson et al., 1992; Abdallah et al., 2010; Honsel et al., 2012).

The negative effect of S deficiency on the shoot biomass is more pronounced as compared to the root biomass, resulting in a significant increase in the root to shoot ratio.(Lopez-Bucio et al., 2003; Buchner et al., 2004b; Hawkesford and De Kok, 2006; Varin et al., 2010; Ciaffi et al., 2013).

Another response to S deficiency is early flowering in plants. A rebalancing of metabolism and a general reduction in metabolic activity, i.e. decrease in the total RNA, chlorophyll, biomass and protein content, indicates the plant's priorities to save resources for survival. Consequently, S deficiency leads to earlier flowering although with retarded growth (Nikiforova et al., 2005; Hoefgen and Nikiforova, 2008; Watanabe et al., 2010).

1.5 S deficiency and nitrogen fixation of legumes

1.5.1 Leguminous plants and nitrogen fixation

Leguminous plants are cultivated approximately on 250 million ha worldwide. Symbiotic nitrogen fixation is one of the main characteristics of legumes. Bacterial symbiosis fixes about 9×10^{10} kg of N_2 per year which is about 80% of the total biologically fixed N_2 (Vance, 1998; Graham and Vance, 2000). Gram-negative bacteria in the soil like *Rhizobium*, *Mesorhizobium*, and *Allorhizobium* can undergo a symbiotic association with legumes by means of a molecular dialogue or cross talk between both partners. This leads to the formation of a new plant organ, the so-called nodule.

1.5.2 Nodule formation

In symbiotic associations with rhizobial bacteria (Fig. 2), plants secrete first flavonoids into the rhizosphere which triggers the release of rhizobial *Nod* factors (lipochitooligosaccharide signals). Then *Nod* factor signals are detected by receptor-like kinases *(Nod* factor receptors) in the root where they induce a number of physiological and morphological responses. Subsequently, cell division initiates nodule formation in the root cortex. Simultaneously, *Nod* factors stimulate the formation of an infection thread thorough root hair curling around the bacteria to encapsulate one or more bacteria. Afterwards bacteria enter the plant cell via an infection thread and are later surrounded by a plant plasma membrane-derived membrane in the symbiosome where they differentiate into their endosymbiotic form called bacteroids to fix nitrogen (Stougaard, 2000; Long, 2001; Oldroyd and Long, 2003; Oldroyd and Downie, 2008; Dolgikh et al., 2011; Oldroyd, 2013).

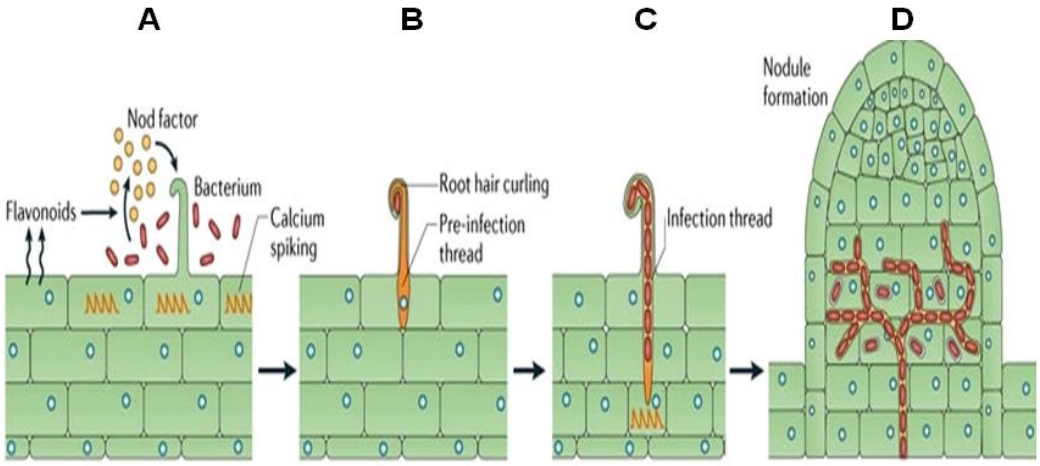


Fig. 2 Nodule formation (Oldroyd, 2013), **A**: The initial signaling in Rhizobium-legume interaction, **B:** Curled root hair formation and encapsulation of Rhizobium into root hair, **C**: Root hair invasion by development of the infection thread, **D**: Differentiated bacteroids and nodule formation.

Two main types of nodules have been described: determinate and indeterminate types. Determinate nodules lose meristematic activity with maturation and cannot be infected anymore by rhizobia. Determinate nodules grow due to cell expansion which leads to a spherical shape particularly in tropical legumes (for example in *Glycine* and *Phaseolus)*. Indeterminate nodules

have a persistent meristem and can continuously be infected by rhizobia. In indeterminate type nodules, new cells are produced over the lifespan of the nodules which leads to a cylindrical shape particularly in temperate legumes (for example in *Pisum* and *Medicago*).

Mature indeterminate nodules show different zones:

Zone I is an apical meristem zone that produces new tissue and later on differentiates into the other zones.

Zone II is the infection zone which is permeated with infection threads full of bacteria and cells are larger than the meristem zone.

Inter zone II–III is where the bacteria have entered the plant cells and start to differentiate into nitrogen-fixing bacteroids.

Zone III is the nitrogen fixation zone with plenty of differentiated bacteroids, being active in fixing nitrogen. Cells contain leghemoglobin and appear in pink color.

Zone IV is the senescent zone with cell and bacteroids degradation. Also the heme group is dissociated from the leghemoglobin protein and green color at the base of the nodule becomes visible (Vasse et al., 1990; Pérez Guerra et al., 2010; Popp and Ott, 2011).

1.5.3 Genes related to nitrogen fixation in legumes

A number of plant and bacteroid genes such as *Nif* (nitrogen fixation), Lb (leghemoglobin) and Fd (ferredoxin) participate in symbiotic nitrogen fixation (SNF). Symbiotic nitrogen fixation converts the unavailable atmospheric N_2 to NH_3 which is a metabolically available form of nitrogen for plants (Raymond et al., 2004). Nitrogen (N_2) is reduced to ammonia in a biochemical reduction where nitrogenase, a rhizobia specific enzyme, is involved.

$$N_2 + 8\ H^+ + 8e^- + 16\ ATP \longrightarrow 2NH_3 + H_2 + 16\ ADP + 16\ P_i$$

Nitrogenase is highly sensitive to oxygen. To protect nitrogenase from irreversible inactivation, different strategies have been developed (Becana and Rodriguez-Barrueco, 1989). For example, O_2 pressure is optimized by the plant leghemoglobin protein.

Converting N_2 to ammonia consumes high energy and the reduction of each N_2 molecule requires at least 16 ATP and eight electrons. Here, the plant ferredoxin proteins are the main electron donors.

1.5.3.1 Nif and Fix genes

Nitrogen fixation genes (Nif) in plants are homologous to the Nif genes in free living nitrogen fixing bacteria such as *Klebsiella pneumoniae*. Fix genes like Nif genes have a critical role in SNF although Fix genes do not have a homologous counterpart in *K. pneumoniae* (Fig. 3A).

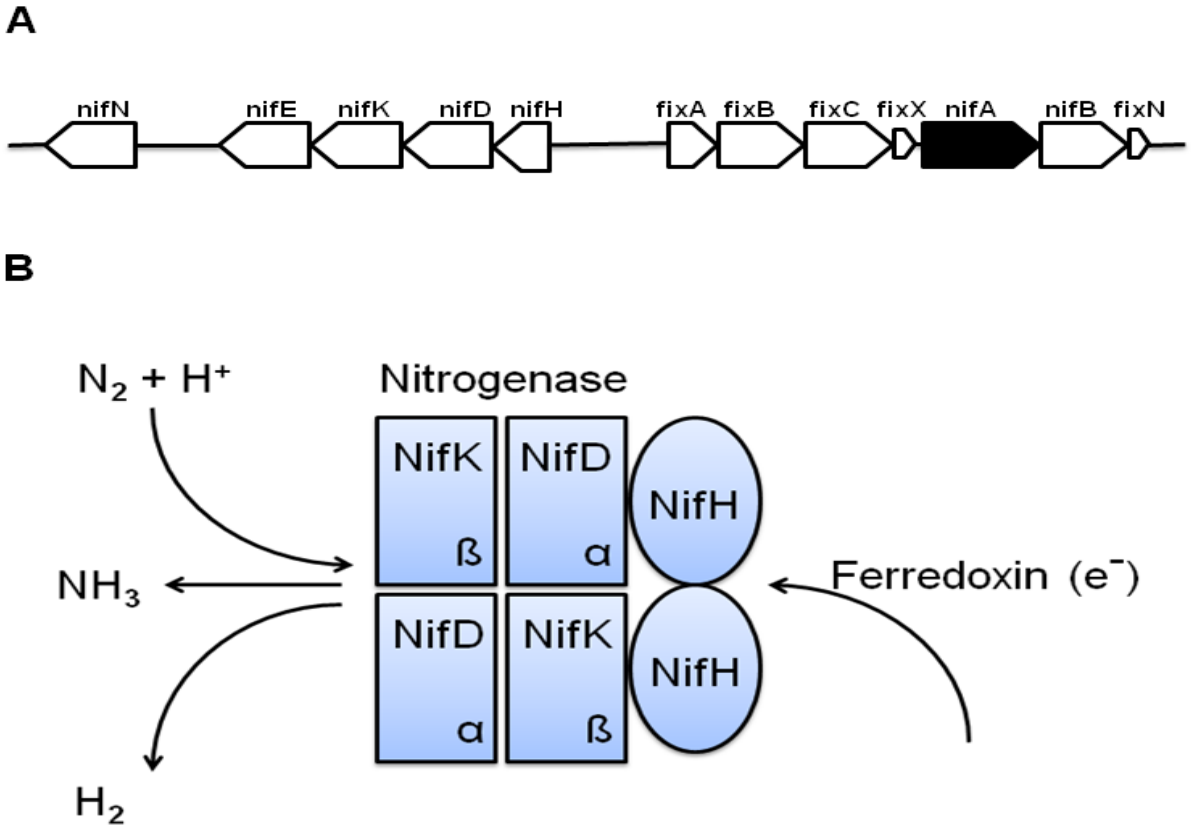


Fig. 3 A: Nif and Fix gene cluster in *Rhizobium* (Fischer, 1996), B: Nitrogenase enzyme contains two α and two β subunits which are encoded by *NifD* and *NifK* genes.

The N_2 fixing enzyme nitrogenase is a Fe-Mo protein and contains two α and two β subunits (Fig. 3B). Subunit α is encoded by the NifD gene and subunit β is encoded by the NifK gene. Many other Nif genes also participate in SNF such as NifH, NifS, NifB, NifN NifE and NifA (Fig. 3A). NifH encodes the Fe protein (nitrogenase reductase) while NifB, NifE and NifN are involved in the biosynthesis of cofactors for the nitrogenase protein. NifA is Positive regulator for Nif and Fix gene and oxygen regulated at the level of transcription while NifS encodes the cysteine desulfurase which releases sulfur for synthesis of metalloclusters (Fe-S) of nitrogenase (Holland et al., 1987; Fischer, 1994; Zheng et al., 1998; Raymond et al., 2004; Young et al., 2006).

1.5.3.2 Leghemoglobin

Hemoglobins (Hbs) are widespread hemoproteins in bacteria, yeast, plants, and animals. Human's hemoglobin and myoglobin are O_2 transporters and O_2 storage proteins. Hemoglobin proteins are also involved in nitric oxide (NO) metabolism and protect cells against oxidative nitrosative stress (Hebelstrup et al., 2007; Dmitryukova et al., 2011).

Plants contain two types of nonsymbiotic and symbiotic hemoglobin. Nonsymbiotic hemoglobins are stress induced proteins and participate in nitric oxide homeostasis using oxygen to convert nitric oxide to nitrate and to protect plants against oxidative nitrosative stress. Symbiotic hemoglobins are derived from the preexisting nonsymbiotic hemoglobins and are expressed exclusively in nodule tissue, e.g. hemoglobin in actinorhizal and leghemoglobin (Lb) in leguminous plants (Ott et al., 2005; Navascues et al., 2012).

Bacteroids in nodules continuously need a low oxygen concentration for efficient reduction of atmospheric nitrogen into ammonia because a high oxygen concentration inhibits nitrogenase activity. On the other hand, a low oxygen concentration is critical and is not sufficient to sustain oxidative respiration of bacteroids. Hence, leghemoglobin serves as an oxygen buffering system inside the nodules with an extremely fast O_2 association rate and rather slow O_2 dissociation rate (Fig. 4). Thus leghemoglobin keeps O_2 concentration in an optimal range to supply bacterial respiration while protect nitrogenase against oxygen damage (Kozik et al., 1996; Varin et al., 2010).

Sequencing homology in *Pisum sativum* reveals two distinct types of leghemoglobins, i.e. PsLbA (PsLb5 gene) and PsLbB (PsLb120 gene). PsLbA has higher O_2 binding affinity than PsLbB in SNF. Expression of PsLbA and PsLbB changes during nodule development. The PsLbA is expressed constantly in effective pea nodules while PsLbB is restrictively expressed in the early stage of SNF. In young circular nodules, both transcripts of PsLbA and PsLbB are distributed to the central part of the nodule while in older elongated nodules, leghemoglobin PsLbA is more distributed to the center of effective pea nodules tissue and PsLbB is restricted to the region from infection zone II to the distal part of nitrogen fixation zone III (Fig. 5) (Uheda and Syōno, 1982; Kawashima et al., 2001).

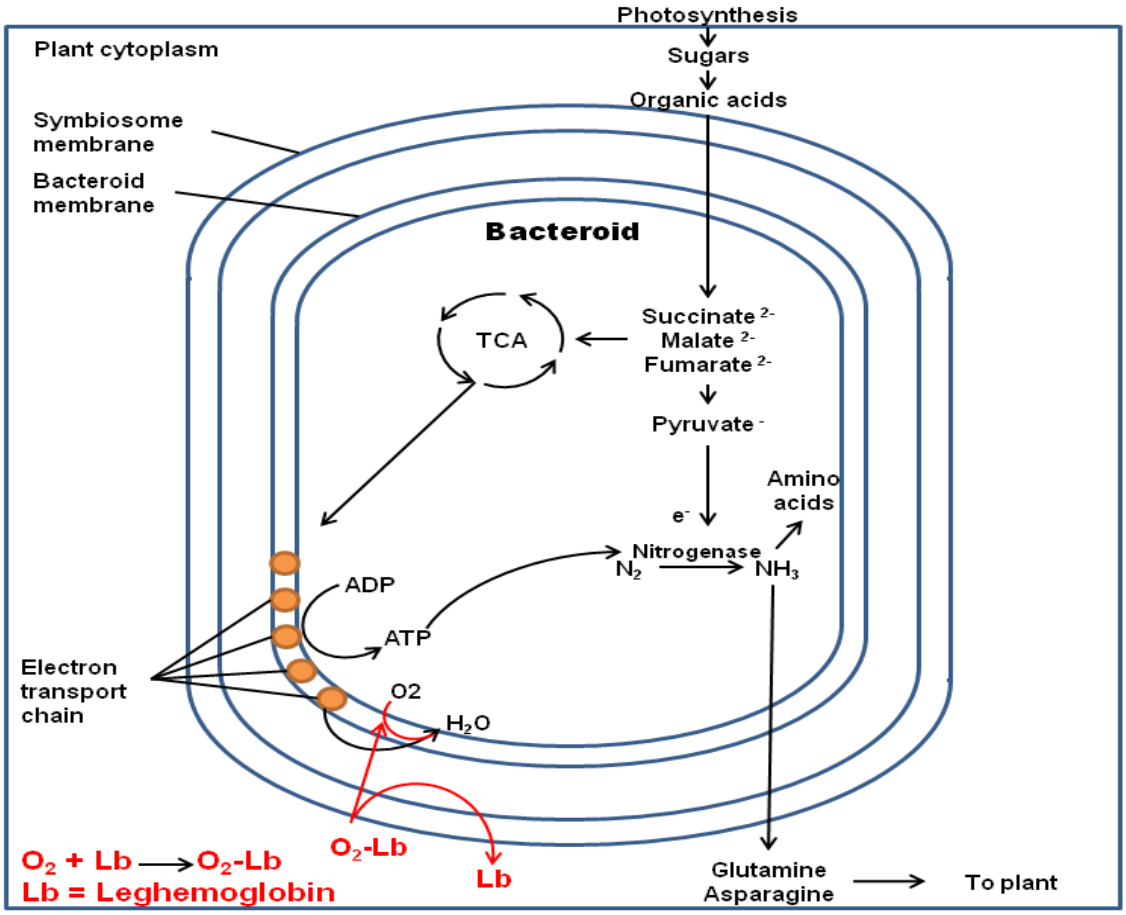


Fig. 4 Leghemoglobin serves as an oxygen buffering system in symbiotic bacteria, Leghemoglobin with fast O_2 association rate and slow O_2 dissociation rate keeps O_2 concentration in an optimal range to sustain bacterial respiration while protecting nitrogenase against oxygen damage.

The heterogeneity in Lbs expression is associated with a varying capacity in oxygen transport and is dependent on the nodule requirement for more effective N_2 fixation. Therefore, expression of both PsLbA and PsLbB genes are required in young nodules to keep the O_2 concentration low while bacteroids begin to actively fix nitrogen. However, in the nitrogen fixation zone III, only expression of LbA is sufficient to support nitrogenase activity and protect nitrogenase against O_2 damage because active bacteroids already consume oxygen and contribute to reduction of O_2 concentration inside the nodules (Kawashima et al., 2001).

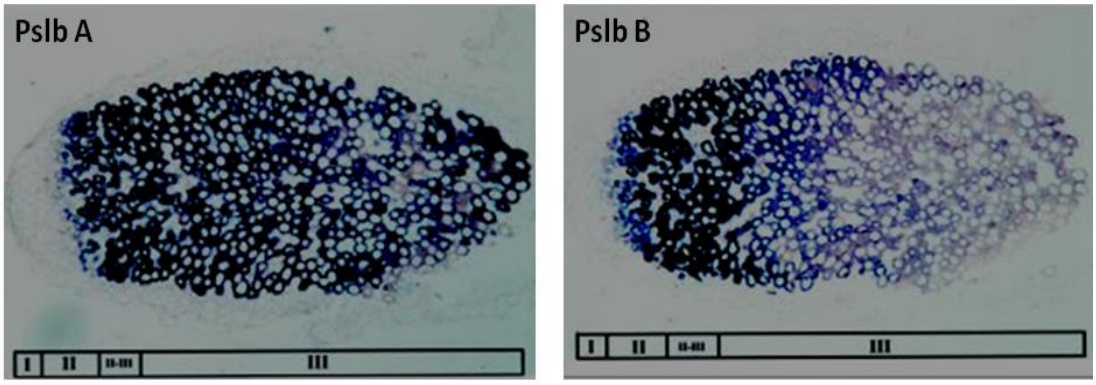


Fig. 5 In situ localization of leghemoglobin (PsLbA and PsLbB) in mature pea nodule (Kawashima et al., 2001), PsLbA is more distributed in the nitrogen fixation zone III while PsLbB is restricted to the region from infection zone II to the distal part of nitrogen fixation zone III.

1.5.3.3 Ferredoxin

Ferredoxin (Fd) is an iron-sulfur protein with multiple isoforms in plants, bacteria and algae. The iron-sulfur (2Fe-2S) group in ferredoxin is ligated by four highly conserved cysteine residues. Ferredoxin acts as a soluble electron carrier protein. Ferredoxin was first described in non-photosynthetic bacteria as an electron carrier protein involved in N_2 fixation . Now ferredoxin is a well-known protein in the light dependent reaction of photosynthesis in the chloroplast thylakoid membranes (Fig. 6A). Ferredoxin accepts electrons from photosystem I (PSI) and donates them to the enzyme ferredoxin-NADP-oxidoreductase (FNR) or to cytochrome b563 in cyclic photo-phosphorylation. In addition, ferredoxin can accept electrons from NADPH via FNR in a reversal of the photosynthetic reaction and can donate the electrons to different acceptors (under non-photosynthetic conditions) such as ferredoxin protein of nitrogenase enzyme complex in the root plastids (Fig. 6B) (Elliott et al., 1989a; Petracek et al., 1997; Hanke et al., 2004; Hanke and Hase, 2008; Blanco et al., 2013).

Ferredoxin donates an electron to many other plastid enzymes such as nitrite reductase in nitrogen assimilation, sulfite reductase in sulfur assimilation, glutamine oxoglutarate amino transferase in amino acid synthesis and fatty acid desaturase in fatty acid synthesis (Voss et al., 2011).

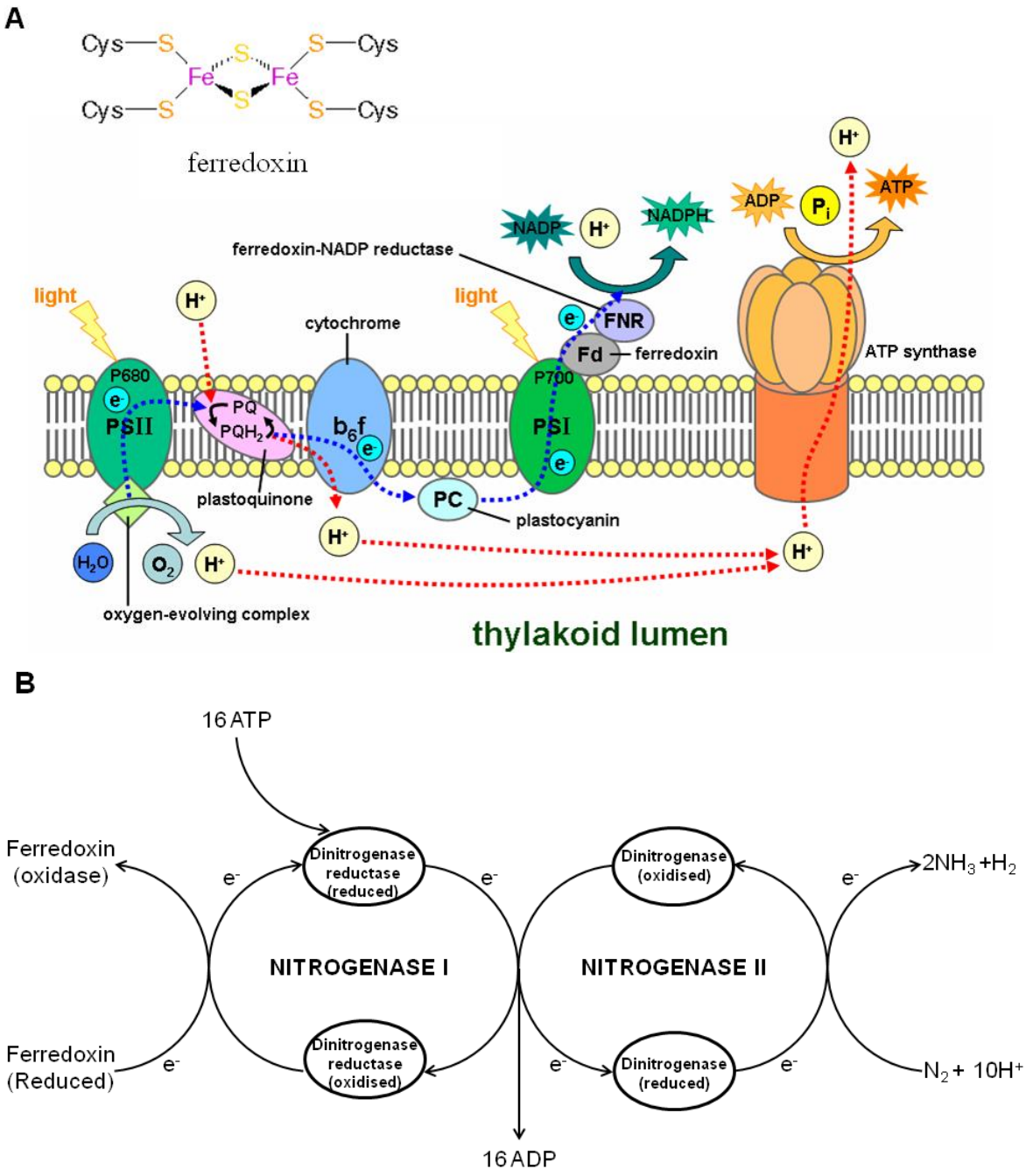


Fig. 6 A: Ferredoxin protein (Fd) is the final electron carrier in the light-dependent reactions of photosynthesis process (wikimedia.org/wiki/File:Thylakoid_membrane.png), B: Ferredoxin in nitrogen fixation process.

In higher plants, there are two main isoforms of ferredoxin i.e. the leaf type (photosynthetic) and the root type (non-photosynthetic) ferredoxin. Leaf type ferredoxin is light-dependent and is the most dominant form of ferredoxin in NADP-reduction. The root type ferredoxin is involved in nonphotosynthetic ferredoxin reduction and stimulates the activity of sulfite reductase (Mortenson et

al., 1962; Mortenson, 1964; Elliott et al., 1989a; Hanke et al., 2004; Higuchi-Takeuchi et al., 2011; Voss et al., 2011).

1.6 S deficiency responses of legumes

While oil seed plants have a highest sulfur content (1.1-1.7% dry matter), legume plants also have a high sulfur content (0.24-0.32% dry matter).S deficiency reduces legume biomass and seed yield while sulfur containing amino acids become limiting for protein synthesis (Liao et al., 2012).

In *Medicago*, S deficiency reduces shoot and root biomass. Moreover, sub-optimal sulfur supply leads to a significant decrease in both cysteine and methionine content in *Medicago* (DeBoer and Duke, 1982; Casieri et al., 2012).

Cysteine and methionine limitation reduces the nutritional value of food and feed legumes in regions suffering from S deficiency. Hence, application of sulfur containing fertilizers induces nitrogen percentage as well as yield in legume plants such as pulses (Khan and Mazid, 2011).

In *Pisum sativum*, S deficiency causes a reduction in the concentration of sulfur containing proteins i.e. albumin and legumin. So, the protein quality is improved due to an increase in the concentration of sulfur-containing amino acids in *Glycine max* (Sexton et al., 1998).

Sulfur supply in legumes is critical for the nitrogen fixation process. In soybean, both nodule quantity and quality significantly increases by adequate sulfur supply. Nodule formation in black gram (*Vigna mungo*) increases by sulfur fertilization. With insufficient sulfur supply to white clover (*Trifolium repens*), pale nodules appear in contrast to the normal pink color in adequate sulfur supply (Ganeshamurthy and Sammi Reddy, 2000).

In SNF, sulfur availability influences biosynthesis of the nitrogenase enzyme containing Fe-S cofactor. S deficiency affects C- and N-metabolism in *Vicia faba* and *Pisum sativum*. N_2 fixation significantly is reduced as a consequence of either low nodule formation or low nitrogenase activity (Scherer and Lange, 1996; Scherer, 2008b).

The leghemoglobin protein is crucial for SNF to establish the low oxygen concentration to maintain the nitrogenase activity. The leghemoglobin concentration in nodules is highly dependent on sulfate availability and decreases in S deficient legumes. Consequently, leghemoglobin reduction leads to low N_2

fixation due to higher O_2 concentration and inhibition of the nitrogenase enzyme. Finally, the availability of adequate sulfur amounts is vital for ferredoxin activity while this Fe-S protein plays an important role in SNF as well (Pacyna et al., 2006; Scherer et al., 2008a; Varin et al., 2010).

1.7 The sulfate transporter gene family in plants

Sulfate is transferred from the root (after uptake) to different organs through transmembrane (vascular) and intracellular transportation. Mainly sulfate is transferred during long distance transport from the root to the mature leaves as the main source of sulfate for assimilation and reduction. Furthermore, sulfate is re-exported from mature leaves to young leaves. Sulfate can return from leaves to root via the phloem. Intracellular transport is involved in moving sulfate into plastids or vacuoles. Moreover, reduced forms of sulfate (sulfite, sulfide) are stored in plastids and vacuoles which can redistributed to other cell compartments, tissues or to the seeds (Hawkesford, 2003; Buchner et al., 2004b; Kataoka et al., 2004; Ohkama-Ohtsu et al., 2004; Zuber et al., 2010).

Members of the sulfate transporter family control the distribution of sulfate in plants. The first classification of sulfate transporters was done by Smith et al in 1995b for the tropical forage legume, *Stylosanthes hamata,* and the sulfate transporter genes were divided into two high-affinity and a low-affinity H+/sulfate co-transporter. High-affinity sulfate transporters (SHST1 and SHST2) mediate sulfate uptake in plant roots but low-affinity sulfate transporters (SHST3) mediate the internal transport of sulfate between cellular or sub cellular compartments (Smith et al., 1978; Smith et al., 1995a; Smith et al., 1995b). In 2003, sulfate transporters were reclassified into five distinguished groups with specific functions (Fig. 7) (Hawkesford, 2003).

Group 1 (ST1) is responsible for primary uptake of sulfate by the root. ST1 transporters include SHST1 and SHST2 genes. Expression of ST1 transporters under sulfate starvation is de-repressed and repressed when sulfate supply is restored. ST1 members are mostly expressed in root tissue. However, expression of these genes in other tissues such as guard cells in leaf indicates additional functions of this group (Buchner et al., 2010; Takahashi et al., 2011).

Group 2 (ST2) has a low affinity for sulfate as compared to ST1 and proteins of this group are expressed mainly in vascular tissues of roots and

shoots. ST2 is responsible for both sulfate transport from roots to shoots via vascular bundles and transport of sulfur to the seed in siliques (Buchner et al., 2004b; Koralewska et al., 2007).

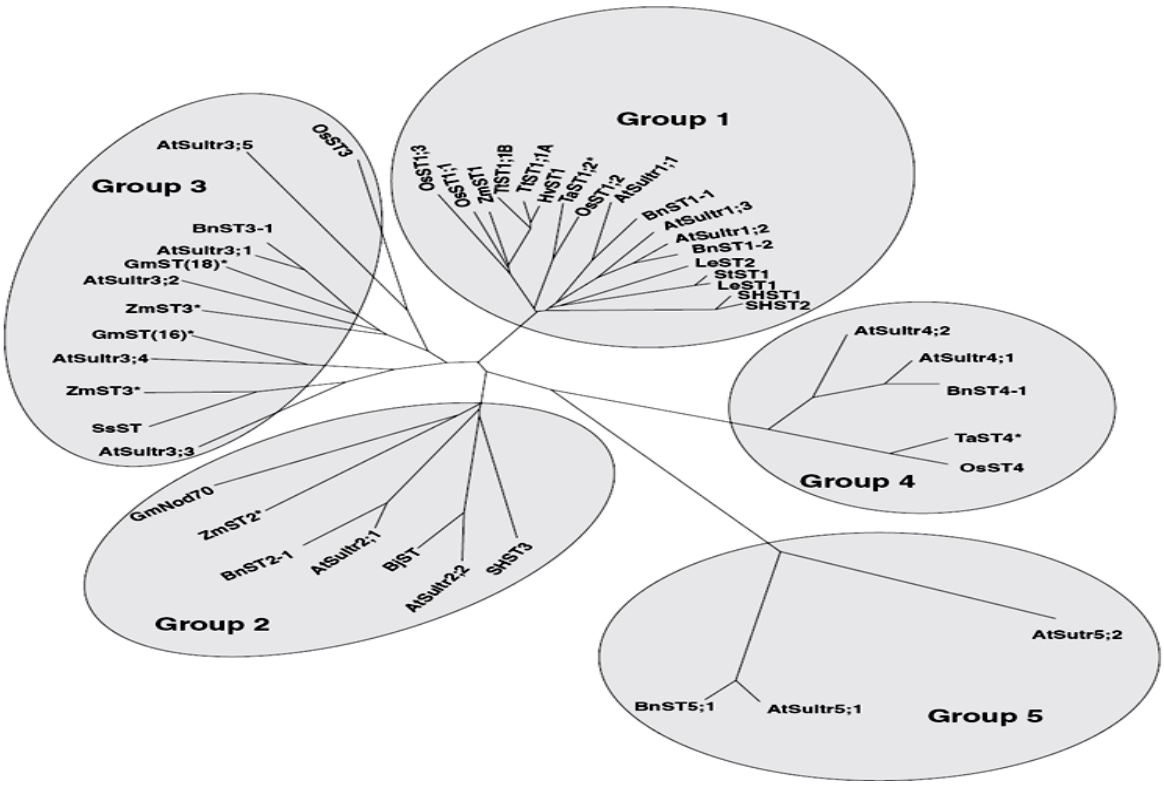


Fig. 7 Phylogenetic analysis of plant sulfate transporter (Hawkesford, 2003), Accession number: *Arabidopsis thaliana* (ST1-1: AB018695, ST1-2: AB042322, ST1-3: AB049624, ST2-1: AB003591, ST2-2: D85416, ST3-1: D89631, ST3-2: AB004060, ST3-3: AB023423, ST3-4: AB054645, ST3-5: AB061739, ST4-1: AB02342, ST4-2: AB052775, ST5-1: NP178147, ST5-1: NP180139), *Triticum tauschii* (TtST1,A: j238244 and TtST1, B: Aj238245), *Hordeum vulgare* (X96431), *Brasica napus* (BnST1-1: Aj416461, BnST1-2: Aj311388), *Zea mays* (ZmST1: AF355602), *Stylosanthes hamata* (SHST1: x82256, SHST2: X82256, SHST3: X82454), *Sporobolus stapfianus* (Sst: X96761), *Lycopersicon esculentum* (LeST1: AF347613, LeSt2: Af347614), *Solanum tuberosum* (StST1: AF309643), *Oryza sativa* (OsST1-1: AF493792, OsST3: BAB 68064.1, OsST4: AF493793).

The function of **Group 3 (ST3)** is still enigmatic in many plants. These proteins seem to have some redundancy and might be derived from gene duplications or during polyploidization. In legume plants, symbiotic sulfur transporter genes (SST like) are classified in the ST3 group and these transporters deliver sulfur to bacteroids which are critical for N_2 fixation (Hawkesford, 2003; Kataoka et al., 2004; Krusell et al., 2005; Momonoi et al., 2009).

Group (ST4) represents vacuolar sulfur efflux transporters in chloroplasts or tonoplast of leaves (Hawkesford, 2003; Buchner et al., 2010).

Group (ST5) shows functionally distinct transporters. ST5 is spec to plasma membranes for an additional role to take up other micronutrients with a similar charge, size and chemical properties to sulfate. ST5 transport oxyanions such as molybdenum and selenium which compete with bioavailable sulfate (Tomatsu et al., 2007; Fitzpatrick et al., 2008; Shinmachi et al., 2010; Cabannes et al., 2011).

2 Material and methods

2.1 Equipment and chemicals

2.1.1 Equipment

Instrument	Manufacture
Autoclave ZELB 3850	Tuttnauer Systec, Germany
Balance 770	Kern, Balingen-Frommern, Germany
Binocular microscope SZX16	Olympus, Hamburg, Germany
Bioanalyzer	Agilent Tec, Waldbronn, Germany
C/N/S-Elemental-Analyser	Euro EA, Milan, Italy
Camera DP7Z for microscope	Olympus, Hamburg, Germany
Centrifuge 5424	Eppendorf AG, Hamburg, Germany
Centrifuge 5810R	Eppendorf AG, Hamburg, Germany
Gel documentation system E-BOX	Peqlab, Erlangen, Germany
Heating Mixing Block MB-102	Bioer, Hangzhou, China
Homogeniser Precellys®24	Peqlab, Erlangen, Germany
Incubation shaker, Multitron 28570	Infors, Einsbach, Germany
Incubator, Kelvitron®t	Thermo Scientific Heraeus®, USA
Micro Pulser Electroporation	Bio-Rad, München, Germany
Microscope digital camera DP72	Olympus, Hamburg, Germany
Real-time PCR System-7300 USA	Applied Bioscience, Foster City, USA
Spectrophotometer Nano Drop 1000	Peqlab, Erlangen, Germany
Spectrophotometer Specord 205	Analytik Jena, Germany
Speed vacuum SPD121P	Thermo Savant, Germany
Sterile bench Model 1.8	Holten LaminAir, Denmark
Thermocycler T Professional	Biometra GmbH, Germany
Vortex Certomat®MV	Braun, Germany

2.1.2 Chemicals

Agarose	Peqlab, Germany
Ammonium persulfate (APS)	Bio-Rad, Germany
Ammonium nitrate	Bio-Rad, Germany
Bacto agar	Duchefa Biochemie, Netherlands
Bacto peptone	Duchefa Biochemie, Netherlands
Boric acid	Sigma, Germany
5-bromo-4-chloro-indolyl-β-D-galactopyranoside (X-gal)	AppliChem, Germany
Calcium carbonate	Sigma, Germany
Chloroform	Merck, Germany
Cobalt nitrate	Sigma, Germany
Copper(II) chloride	Sigma, Germany
Diethylpyrocarbonate (DEPC)	Sigma, Germany
Dipotassium phosphate	Sigma, Germany
Disodium hydrogen phosphate	Sigma, Germany
Monosodium phosphate	Merck, Germany
Ethanol	Merck, Germany
Ethidium bromide	Serva, Germany
Ethylenediaminetetraacetic acid (EDTA)	Roth, Germany
Ferric chloride	Sigma, Germany
Formaldehyde	AppliChem, Germany
Glucose	AppliChem, Germany
Glycine	AppliChem, Germany
4-(2-Hydroxyethyl)-1-piperazine-1-ethansulfonic acid (HEPES)	AppliChem, Germany
Isopropanol	AppliChem, Germany
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Duchefa Biochemie, Netherlands
Magnesium chloride	Sigma, Germany
Magnesium chloride hexahydrate	AppliChem, Germany
Magnesium sulfate anhydrous	Sigma, Germany
Monosodium phosphate	Sigma, Germany

β-Mercaptoethanol	Sigma, Germany
Murashige and Skoog medium	Duchefa Biochemie, Netherlands
Phyto agar	Duchefa Biochemie, Netherlands
Potassium chloride	Merck, Germany
Potassium sulfate	Sigma, Germany
Sodium chloride	Duchefa Biochemie, Netherlands
Sodium dodecyl sulfate (SDS)	AppliChem, Germany
Sodium molybdate	Sigma, Germany
Sorbitol	Duchefa Biochemie, Netherlands
Sucrose	Duchefa Biochemie, Netherlands
Tripotassium phosphate	Sigma, Germany
Dipotassium phosphate	Sigma, Germany
Triton X-100	Sigma, Germany
Tween-20	Sigma, Germany
Yeast extract	Duchefa Biochemie, Netherlands
Zinc chloride	Sigma, Germany

2.2 Enzymes

T4 DNA ligase	Biolab, New England
Taq DNA polymerase	Fermentas, Germany
Pfu DNA polymerase	Fermentas, Germany
Paq 5000 hot start PCR master mix	Agilent, Germany
MluI	Fermentas, Germany
XhoI	Fermentas, Germany
EcoRI	Fermentas, Germany
PstI	Fermentas, Germany
DNaseI	Fermentas, Germany

2.3 Molecular Biology Kits

Superscript ® III First Strand Kit	Invitrogen, Germany
NucleoSpin® Plasmid Quick Pure Kit	Macherey-Nagel, Germany
Gel/PCR DNA Fragment Extraction Kit	DNA cloning service, Germany
pGEM-T Easy Vector	Promega, Germany
Cloned JET PCR Cloning Kit	Fermentas, Germany
RevertAid™ First Strand cDNA Synthesis Kit	Fermentas, Germany
3' RACE system for Rapid Amplification of cDNA Ends	Invitrogen, Germany
5' RACE system for Rapid Amplification of cDNA Ends	Invitrogen, Germany
Universal RNA Purification Kit	Roboklon, Germany
RNA isolation from Plant	Macherey-Nagel, Germany
RNA 6000 Nano Kit	Agilent, Germany
Power SYBR®Green PCR Master Mix	Applied Biosystems, Warrington, England

2.4 Cultivation of *Pisum sativum* and growth conditions

2.4.1 Seed infection and germination

Pea seeds (*Pisum sativum* L., cv. Rocket) were inoculated with *Rhizobium leguminosarum* biovar viciae (Radicin No. 4; Jost, Germany) and germinated in quartz sand. Eight uniform 10 days old seedlings with two or three leaves were transferred into 10 liter pots containing perlite (Perligran G, Deutsche Perlite GmbH, Germany; granulometric composition 0–3 mm) and were cultivated in a greenhouse under natural conditions.

2.4.2 Fertilization and treatments

Seedlings were supplied every 2nd day with all macro and micro nutrients in optimum concentration except sulfur and nitrogen (Table1). Nitrogen was supplied in the first two weeks after transplanting to cover requirements until the nitrogen fixation process started. All nutrients were applied in aqueous solution (stock solution) except $MgCl_2$ and $CaCO_3$ which were supplied as a dry powder. Fertilization was done over a period of 7 weeks after transplanting. Afterward, plants received only distilled water.

Sulfur was supplied as sulfate in 3 increments: 200 mg S pot $^{-1}$ (6.24 mM) as control, 25 mg S pot $^{-1}$ (0.78 mM) as weak S deficiency, 12.5 mg S pot^{-1} (0.39 mM) as severe S deficiency. Plants (6 biological replicates) were harvested both in the vegetative phase (6 weeks old plants) and in the generative phase (8 weeks old plants). After harvest, nodules, roots and Leaves were separated from each plant and were immediately frozen in liquid nitrogen. Tissues were stored at -80 ºC until analysis.

Table1 Nutrients supplies to plants

Nutrient	Component	Concentration (per pot)
Potassium in		
control	K_2HPO_4, K_2SO_4	1660 mg K
weak S deficiency	K_3PO_4, K_2SO_4	1660 mg K
severe S deficiency	K_3PO_4, K_2SO_4	1660 mg K
Phosphate in		
control	K_2HPO_4, Na_2HPO_4, NaH_2PO_4	654 mg P
weak S deficiency	K_3PO_4, Na_2HPO_4, NaH_2PO_4	654 mg P
severe S deficiency	K_3PO_4, Na_2HPO_4, NaH_2PO_4	654 mg P
Rest of nutrients (similar in all treatments)		
Nitrogen	NH_4NO_3	100 mg N
Magnesium	$MgCl_2$	500 mg Mg
Sodium	Na_2HPO_4, NaH_2PO_4	172 mg Na
Calcium	$CaCO_3$	600 mg Ca
Iron	$FeCl_3$	100 mg Fe
Copper	$CuCl_2$	20 mg Cu
Manganese	$Mncl_2$	20 mg Mn
Zink	$ZnCl_2$	20 mg Zn
Molybdenum	Na_2MoO_4	5 mg Mo
Cobalt	$Co(NO_3)_2$	5 mg Co
Boron	H_3BO_3	5 mg B

2.4.3 CNS measurement

Plant biomass from each pot was divided into the shoot and root tissue and oven dried at 70°C after each harvest. Dry samples were ground. Two mg vanadium pentoxide was added to 6 mg of ground tissue following the manufacturer's instructions and subsequently total carbon, nitrogen, and sulfur (CNS) quantities were measured by C/N/S-Elemental-Analyser (Euro EA, Milan, Italy).

2.5 mRNA quantification using quantitative real-time PCR

2.5.1 RNA isolation and purification

Total RNA was extracted from 100 mg of the nodule, root and leave tissue using a Gene MATRIX Universal RNA Purification Kit (Roboklone, Berlin, Germany) according to the manufacturer's guidelines. To avoid genomic DNA contamination, RNA purification was done using on-column DNase**I** digestion.

2.5.2 Quality controls

2.5.2.1 Total RNA quantity measurement

The total RNA concentration was measured by determining the optical density at 260 nm with a Nanodrop 1000 spectrophotometer. In Nanodrop, an absorbance of each signal (= 1.0) at 260 wavelengths (A_{260}) corresponds to 40 µg/mL of pure RNA.

2.5.2.2 Total RNA purity

The purity of total RNA was checked with $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios to uncover DNA, protein and phenol contamination. In high purity, RNA sample should have an $A_{260}/_{280}$ ratio between 1.8 and 2 and an $A_{260}/_{230}$ ratio close to 2.

2.5.2.3 Total RNA integrity

The integrity of the total RNA was checked on a formaldehyde agarose gel. For the preparation of the formaldehyde gel, agarose was mixed with DEPC water and melted in a microwave oven. Then MOPS buffer (pH= 8) was added and cooled down at room temperature following formaldehyde supplementation. The gel containing formaldehyde was immediately poured under the hood for solidification. 1 µg of RNA sample was mixed with DEPC water to obtain a total

volume of10 µL. Afterwards, 10 µL buffer was added to the RNA sample and it was incubated at 65 °C for 10 min. Then the sample was cooled down on ice for a few seconds and was loaded on the formaldehyde gel (30 min at 90 V). The total RNA was visualized under UV light (λ=302 nm) and high quality samples showed distinct ribosomal RNA bonds without any degradation.

RNA Sample Buffer	
Component	**Concentration**
Formamide	65% (v/v)
Formaldehyde	8% (v/v)
1 x MOPS pH 8	1.3% (w/v)
Ethidium bromide	54 µg/mL

10 x MOPS Buffer	
Component	**Concentration**
MOPS	0.2 M
sodium acetate	50 mM
Na-EDTA	10 mM
adjusted to pH 7 or pH 8 with NaOH	

Agarose gel	
Component	**Concentration**
Agarose	1.5% (w/v)
(in 1 x MOPS, pH 8)	
Formaldehyde	%6 (v/v)

RNA Running Buffer	
Component	**Concentration**
Formaldehyde	10% (v/v)
(in 1 x MOPS, pH 7)	

2.5.2.4 Determination of RNA integrity factor (RIN)

The plant cell has three different types of ribosomal RNA including cytosolic, chloroplastic and mitochondrial rRNA in different sizes from 5S to 25S. The integrity of these complex rRNA types was checked randomly with Agilent 2100 Bioanalyser using RNA 6000 Nano Kit. The RIN factor for quantitative real-time PCR (should be more than 7) was confirmed.

2.5.3 First Strand cDNA Synthesis

Reverse transcription of total RNA was done with a RevertAid^{TM} first strand cDNA synthesis Kit according to the manufacturer's description (Fermentas). 2 µg of DNase-treated total RNA was mixed with 2 µL of 100 µM primer (equal mixture of random hexamer and oligo dT) and filled up to 12 µL volume with distilled water. Afterwards, the reaction tube was heated for 5 min at 65 °C followed by chilling on ice. After spinning down, 4 µL of 5X reaction buffer, 1 µL of Ribolock^{TM} RNase inhibitor (20 U/µL), 2 µL dNTP mix (10 mM each) and 1 µL of RevertAid^{TM} reverse transcriptase (200 U/µL) were added to the reaction tube and it was

incubated for 5 min at 25 °C following 1 h at 50 °C. The reaction was terminated by heating at 70 °C for 5 min. Each run contained a negative template control and a no reverse transcriptase control. The cDNA were used as a template for RT-PCR and real-time qPCR amplification.

2.5.4 mRNA expression

The cDNA was diluted 1:12 to reach the highest efficiency in real-time PCR. The absence of genomic DNA was verified with control PCR using DNase-treated total RNA as a template.

All polymerase chain reactions were performed in a total volume of 20 µL containing 2 µL cDNA (16 ng of total RNA), 10 µL 2 X SYBR® Green Master Mix reagent and 2 µL primer mix (5µM of each primer) using a 96-well real-time PCR system (Applied Bioscience, Foster City, USA). The following standard thermal profile was used for amplification: 50°C for 2 min, 95°C for 10 min, 40 cycles of 95°C for 15 s and 60°C for 1 min. All plates contained a negative control for PCR template and a negative control for cDNA (no reverse transcriptom, and no RNA template). Dissociation curves were generated at the end of the PCR cycles from 60 to 95 °C to test for unspecific amplicons and primer dimmer peaks.

2.5.5 Data analysis of quantitative real-time PCR

Results were averaged over two technical replicates. For data analysis, 7300 SDS system software version 1.4 (Applied Bioscience, Foster City, USA) was used. Quantification cycle value (Cq) was transformed to relative quantity values (RQ_s) base on the efficiency of reaction for each single gene. Efficiency was calculated using 1 to 10 serial dilution standard curve derived from PCR amplicons. Finally, RQ_s were normalized using geometric means of three reference genes (Vandesompele et al., 2002).

2.5.6 Reference gene selection for normalization of genes of interest

The most stable reference genes were selected using geNorm (Vandesompele et al., 2002) out of a set of 7 reference genes i.e. ß-Tubulin, Transcription factor IIA (TransfactIIA), Phosphoprotein phosphatase 2A (PP2A), 18S ribosomal RNA (18S), Ubiquitin, Histone H3 and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as suggested by (Die et al., 2010).

Expression of the 7 candidate references genes was measured in selected samples from different tissues (root, leaves and nodules). Genes with the most stable expression were associated with the lowest M values. The most stable genes were TransfactII, PP2A and Histon H3 with M value ≤1.5 and V value ≤ 0.15.

Table.1 Primer characteristics

Gene name	Gene bank accession no.	Sequence
TransfactIIA[1]	GH720838	for GCAACCTCCTTCTCCTTGGAT rev TCTTCCCGTCCTTCCACATAA
PP2A[2]	Z25888	for CCACATTACCTGTATCGGATGACA rev GAGCCCAGAACAGGAGCTAACA
Histone H3	GH720808	for CAACTGTTCCTGGACGGAAT rev CAAAAGCCGCTCGTAAATCTG
ß-Tubulin	X54844	for GCTCCCAGCAGTACAGGACTCT rev TGGCATCCCACATTTGTTGA
Ubiquitin	L81140.1	for ACCACCACGGAGACGGAG rev ATGCAGATCTTTGTGAAGAC
18S[3]	u43011.1	for CCAGGTCCAGACATAGTAAG rev GTACAAAGGGCAGGGACGTA
GAPDH[4]	X73150.1	for TGGAAAGTTGACCGGTATGTCA rev CAAGCCTCACTGTAAGGTCAACA
Leghemoglobin A (psLb5)	AB010831.1	for CTGCTCAACTCCGAACAAAA rev TGTCTGCAGCAAAGCTTCTT
Leghemoglobin B (psLb120)	AB009844.1	for GATTCGGCAGGAGTAGTGGATAGT rev CATGCACCATTCCAAAAACCT
Ferredoxin	M31713.1	for CAGTCACCACCACAAAAGCATT rev CCACGTTTGAGACTGGTTTTCA
NifK[5]	NC_008381.1 Gene ID: 4403933	for GCTTCTGCGTTGGGAACAAT rev TGCGTCCTGGATGAACATGT
NifD[5]	NC_008381.1 Gene ID: 4403930	for CGCTGAACTTTTCCCGCTAA rev CCTCGATATCGTCGCCAATC
SST[6]	KP759572	for ACCAGTGGACCATTTTCCAAGA rev ACTGCTTGTACCACATTTGTCATTG

1. Transcription factor IIA
2. Phosphoprotein phosphatase 2A
3.18S ribosomal RNA
4. Glyceraldehyde-3-phosphate Dehydrogenase
5. Measured only in rhizobium
6. Symbiotic sulfate transporter

2.5.7 Gene of interest

Genes related to the nitrogen fixation complex such as nitrogenase (Nif D and Nif K), leghemoglobin (Lb A and Lb B), ferredoxin (Fd) and symbiotic sulfate transporter (SST) genes were investigated. Oligonucleotide primers were synthesized by Eurofines MWG operon (Table 1).

2.5.8 Statistics

Statistical analyses were done using SPSS (version 21). Data were tested for normality and for homogeneity of variances ($P < 0.05$) using levene's test. Data were analyzed using ANOVA and means of different levels of sulfur and time of harvest were compared using Tukey's HSD as a post-hoc test. Not normally distributed data or data with none homogeneous variances were subjected to the nonparametric Kruskal-Wallis and Mann-Whitney U tests.

2.6 Identification of the symbiotic sulfate transporter gene in *Pisum sativum*

2.6.1 Preparing electrocompetent cells of *Escherichia coli*

A single colony of *E. coli* was grown overnight in 20 mL LB medium at 37 °C while shaking at 200 rpm. Then 400 mL SOB medium was inoculated with the overnight culture and grown to an OD_{600}=0.5 while shaking at 180 rpm at 37 °C. Subsequently the cells were incubated in an ice bath for 30 min and poured into 50 mL sterile falcon tubes. Afterwards, the cells were harvested by centrifugation (6600 rpm, 10 min, 4 °C). After discarding the supernatant, the pellet was resuspended in 400 mL HEPES (1 mM). The centrifugation step was repeated three times with resuspension in 200, 100 and 50 mL cold distilled water. Immediately, the pellet was suspended in 20 mL of 20 % (v/v) glycerol and in 2 ml of 10 % (v/v) glycerol after the first and the second centrifugation step, respectively. The cell suspension was divided into 50 µl aliquots and was frozen in liquid nitrogen and was stored at -80 °C.

LB medium (pH 7.2)	
Component	**Concentration (1 L)**
Tryptone	10 g
Yeast extract	5 g
NaCl	10 g
For solid media 15 g Bacto Agar was added	

SOB medium	
Component	**Concentration (1 L)**
BD Bacto™Peptone	20 g
Yeast extract	5 g
NaCl	0.6 g
KCl	0.18 g
After autoclaving: added as sterile solutions	
$MgSO_4$	2 mM
$MgCl_2$	2 mM

2.6.2 Transformation of *Escherichia coli*

The frozen electrocompetent cells were thawed and subsequently mixed with a desalted ligation reaction mixture or with plasmid DNA. Then they were incubated in an ice bath for 2 min. The mixture was transferred to a cold 1 mm electroporation cuvette and electro shocked with a voltage pulse of 1250 V. Immediately, 600 µL cold LB medium without antibiotics was added to the transformation mixture and the bacteria regenerated at 37 °C for 40 min. Finally, bacterial cells were cultivated on LB medium with a selective antibiotic and incubated overnight at 37 °C.

2.6.3 Plasmid preparation from *Escherichia coli*

For mini plasmid extraction from *E. coli* cells, an overnight culture (1.5 mL) was harvested by centrifugation at 13000 rpm for 1 min. After discarding the supernatant, the pellet was suspended in 200 µL BF buffer containing lysozyme (1 mg/mL) and incubated for 1-2 min at room temperature. Cells were incubated on a heating block at 98 °C for 1 min and subsequently in the ice bath for 2 min.

Genomic DNA, proteins and residual cell debris were removed by centrifugation at 13000 rpm for 15 min. The supernatant was transferred into a new tube containing 480 µL of IS mix. The tube was converted slowly several times and incubated at room temperature for 2 min. Subsequently, the plasmid DNA was harvested by centrifugation for 20 min at 13000 rpm. The pellet was rinsed with 500 µL 75% ethanol with centrifugation for 5 min at 13000 rpm. After discarding the supernatant, the plasmid DNA was dried at 45 °C and dissolved in 80 µL ddH_2O with 1 µL RNaseA and stored at -20 °C.

BF buffer

Component	Concentration (1 L)
Sucrose	80 g
Triton X-100	5 g
EDTA (pH 8.0) [0.5 M]	100 mL
Tris-HCl (pH 8.0) [1 M]	10 mL

IS mix

Component	
Isopropanol	400 µL
Ammonium acetate [5 M]	80 µL

Highly purified plasmid DNA (for sequencing) was isolated with NucleoSpin® Plasmid Quick Pure Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.6.4 Degenerate primer designing

Sulfate transporter genes in some leguminous species (Table 2) were aligned to identify the highly homologous regions using multiple sequence alignment ClustalX versions 1.81. Degenerate primers were designed manually based on highly homologous regions and were used for symbiotic sulfate transporter gene identification.

Table.2 Aligned sulfate transporter genes in different plants

Plant	Gene bank accession no.
Astragalus drummondii	FN689520.1
Astragalus glycyphyllos	FN689515.1
Cicer arietinum	XM_004485500.1
Glycine max	XM_003526546.2
Glycine max	XM_006581488.1
Glycine max	XR_136691.1
Lotus japonicus	AM283536.1
Medicago truncatula	XM_003593117
Medicago truncatula	XM_003614920.1
Phaseolus vulgaris	XM_007136158.1
Stylosanthes hamata	X82454.1
Cicer arietinum	XM_004490308.1
Cicer arietinum	XM_00450522.1
Arabidopsis thaliana	AB018695.2

2.6.5 Isolation of partial cDNAs corresponding to the symbiotic sulfate transporter

Small fragments (200 bp) of symbiotic sulfate transporter (SST) were isolated by RT-PCR using cDNA from nodule tissues as template and the degenerated primers (Table. 3).

Table.3 Degenerated primers used for isolation of partial sulfate transporter gene

Forward	GTGGACCATTTTCSAAGACNGC
Reveres	CTCHRYRTCTCTRTATAWRCC

Note: N= (A+T+G+C), R= (A+G), S= (G+C), W= (A+T), Y=(C+T)

RT-PCR Reaction Mixture

Component	**Quantity in 50 µL**
cDNA	3 µL
Forward primer	2 µL (10 pmol μL^{-1})
Reverse primer	2 µL (10 pmol μL^{-1})
Buffer (10x)	5 µL
dNTP mix	1.5 µL(2.5 mM $nucleotide^{-1}$)
$MgCl_2$	1.5 µL (50 mM)
Taq-DNA-polymerase	1 µL (5 units μL^{-1})
H_2O	36 µL

RT-PCR Program

1- Predenaturation	94°C	3 minutes
2- Denaturation	94°C	45 seconds
3- Annealing	58°C	30 seconds
4- Elongation	72°C	1.5 minutes
5- Final elongation	75°C	10 minutes

Step 2-4 were repeated 40 times (cycles)

2.6.6 Cloning of partial cDNA of SST for sequencing

2.6.6.1 Extraction of partial cDNA from an agarose gel

The fragments (200 bp) corresponding to SST were separated on 1% agarose gel. The expected band was cut out and was extracted from the gel using the NucleoSpin® Extract II Kit (Macherey-Nagel) following the manufacturer's guidelines. The DNA was eluted in H_2O instead of elution buffer in the final step of the protocol to obtain salt free DNA samples.

2.6.6.2 Ligation of SST partial cDNA to pGEM®-T Easy vector

Linearized pGEM®-T Easy vector with single 3'-terminal thymidine overhangs at both ends can ligate with the sticky ends of a foreign DNA (with adenine overhangs). Sticky ends of cDNA fragments were obtained by using Taq

DNA polymerase. Partial cDNAs were ligated into pGEM®-T Easy vector following the manufacturer's instructions using T4 DNA ligase. For optimal efficiency in the ligation assay, the molecular ratio of the vector to insert was adjusted to 1:4. The ligation reaction was incubated at room temperature for one hour or alternatively incubated overnight at 4 °C to increase the efficiency.

2.6.6.3 Purification of ligation mixture

Buffer or salt containing DNA samples were desalted by the use of micro dialysis membranes. The sample was placed onto the membrane on ddH_2O for at least one hour. The constructs obtained by ligation and purification were used for the transformation of *E. coli* cells as described in section 2.6.2.

2.6.6.4 Blue/white screening

The pGEM®-T Easy vector contains the β-galactosidase gene (lacz) which breaks down artificial X-Gal and converts it into a compound with a blue color. Successful ligation with foreign DNA interrupts lacz function and results in colonies with a white phenotype. In addition, IPTG de-represses lacz expression. For transformed bacterial colonies containing the pGEM-T Easy vector, blue/white screening was used in LB medium containing Amp, IPTG and X-Gal. IPTG and X-Gal added to the plates before incubation.

X-Gal

Component	**Concentration (2 mL)**
5-bromo-4-chloro-3-indolyl-β-D-galactoside	0.1 g
N,N-dimethylformamide (DMF)	2 mL

Stored at -20 °C

IPTG

Component	**Concentration (5 mL)**
Isopropyl-β-D-thiogalactopyranoside	0.12 g
ddH_2O	5 mL

Filter sterilized and stored at -20 °C

2.6.6.5 Restriction analysis

A single white colony of *E. coli* was grown overnight in 1.5 mL LB+AMP medium at 37 °C. Subsequently plasmids were extracted from white transformed bacterial cells as described in section 2.6.3. Restriction endonucleases were used for digestion of the plasmid DNA to confirm successful transformation with the correct insertion (partial SST). PGEM-T Easy plasmids were cleaved with

EcoRI and PstI enzymes in tango buffer for 2 hours at 37 °C and were run in 1% agarose gel for verification.

Restriction Assay

Component	**Concentration (10 µL)**
Plasmid DNA	2 µL
10 x buffer	1 µL
EcoRI	0.2 µL
PstI	0.2 µL
ddH_2O	6.6 µL

2.6.7 Identification of a partial SST cDNA for designing specific sense and anti sense primers from partial cDNA

A plasmid carrying partial SST cDNA was sent for sequencing (LGC Genomic, Berlin, Germany). Blasting of the sequenced cDNA fragment showed a high homology of the partial SST to the sulfate transporter family in leguminous plants (i.e. 91 % similarity with *Medicago truncatula*). Finally, gene specific primers were designed based on the partial SST cDNA to be used in the RACE method.

2.6.8 Rapid amplification of cDNA 3´ ´End (3´-RACE)

The RACE system was used to produce nucleic acid sequences from 3´ and 5´ ends of a messenger RNA. The 3´ regions of the symbiotic sulfate transporter transcripts were isolated by 3´-RACE approach (Invitrogen 3´-RACE Kit) using specific oligonucleotide primers based on the sequencing results from the partial cDNA (200 bp) fragment (Fig. 8). Total RNA from nodule tissue was used for the first strand cDNA synthesis using Superscript ^{TM} II reverse transcriptase and an adaptor primer (AP) containing oligo dT and restriction endonucleases sites (Table 4). After cDNA synthesis, the template RNA was removed from the cDNA: RNA hybrid molecule by digestion with RNase H to increase the sensitivity of the PCR reaction. The fragments were amplified in different PCR reactions and nested-PCR systems. Abridged universal amplification primer (AUAP) was used as a sense primer. A gene specific primer was used as an antisense primer (Table 4). The amplification fragments from the 3´ region were gel extracted and were cloned into pGEM®-T Easy vector as it is described in section 2.6.7 for post amplification and verification. Plasmids eventually carrying the 3´ region of SST were sent for sequencing (LGC Genomic, Berlin, Germany) and the results were

blasted to find similarities to the other sulfate transporter families.

Fig. 8 Summary of the 3´-RACE system procedure

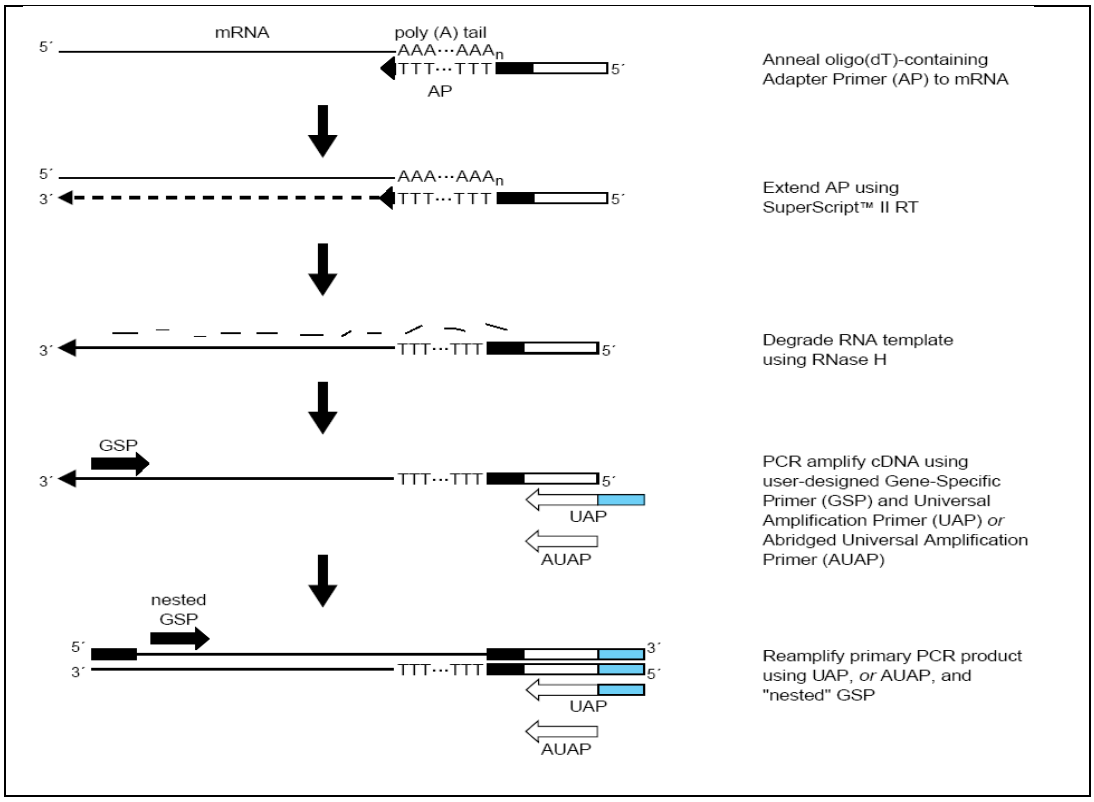


Table.4 Oligonucleotides used in 3´-RACE

Adaptor primer(AP)	GGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTT
Abridged universal amplification primer (AUAP)	GGCCACGCGTCGACTAGTAC
Gene specific primers (GSP1)	GGAGTATTAAATACTCGGGG
Gene specific primers(GSP2)	GGATTCTTACGTGAGTACGT

3´-RACE-PCR Reaction Mixture

Component	**Concentration (25 µL)**
cDNA	1 µL
GSP primmer	0.5 µL (10 µM)
AUAP	0.5 µL (10 µM)
PCR buffer (10x)	2.5 µL
dNTP mix	0.5 µL (10 mM nucleotide^{-1})
$MgCl_2$	1.5 µL (25 mM)
Taq-DNA-polymerase	0.5 µL (5 units µL^{-1})
ddH_2O	18 µL

3´-RACE -PCR Program

1- Predenaturation	94°C	3 minutes
2- Denaturation	94°C	45 seconds
3- Annealing	57°C	30 seconds
4- Elongation	72°C	2 minutes
5- Final elongation	75°C	10 minutes

Step 2-4 were repeated 40 times (cycles)

2.6.9 Rapid amplification of cDNA 5´End

Unknown sequences at the 5´end of the SST cDNA were identified using a 5´-RACE Kit following the manufacturer's instructions (Fig. 9). Total RNA extracted from nodule tissue was converted to first strand cDNA using Superscript ^{TM} II reverse transcriptase and GSP1 primer or adaptor primer containing oligo dT. The original RNA template was removed subsequently with RNase mix (the mixture of RNase H and RNase T1) and the first strand product was purified from unincorporated dNTP, GSP, and protein using a S.N.A.P column involved in RACE 5´ Kit. Afterward, the homopolymeric tail was added to the purified cDNA to bind the abridged anchor primer (AAP). Homopolymeric tail was added to the 3´ end of the cDNA using dCTP and terminal deoxynucleotidyl transferase (TdT). Tailed cDNA was amplified by PCR using gene specific primers (GSP2), AAP containing deoxyinosine and Abridged Universal Amplification primer (AUAP). Following the amplification steps, 5´-RACE products were cloned into pGEM®-T Easy vector as described in section 2.6.7 and were sent for sequencing. The products generated by 3´ and 5´-RACE were combined to produce a full length cDNA and the consensus sequence was used for functional proof of this gene.

Fig. 9 Summary of the 5´-RACE system procedure

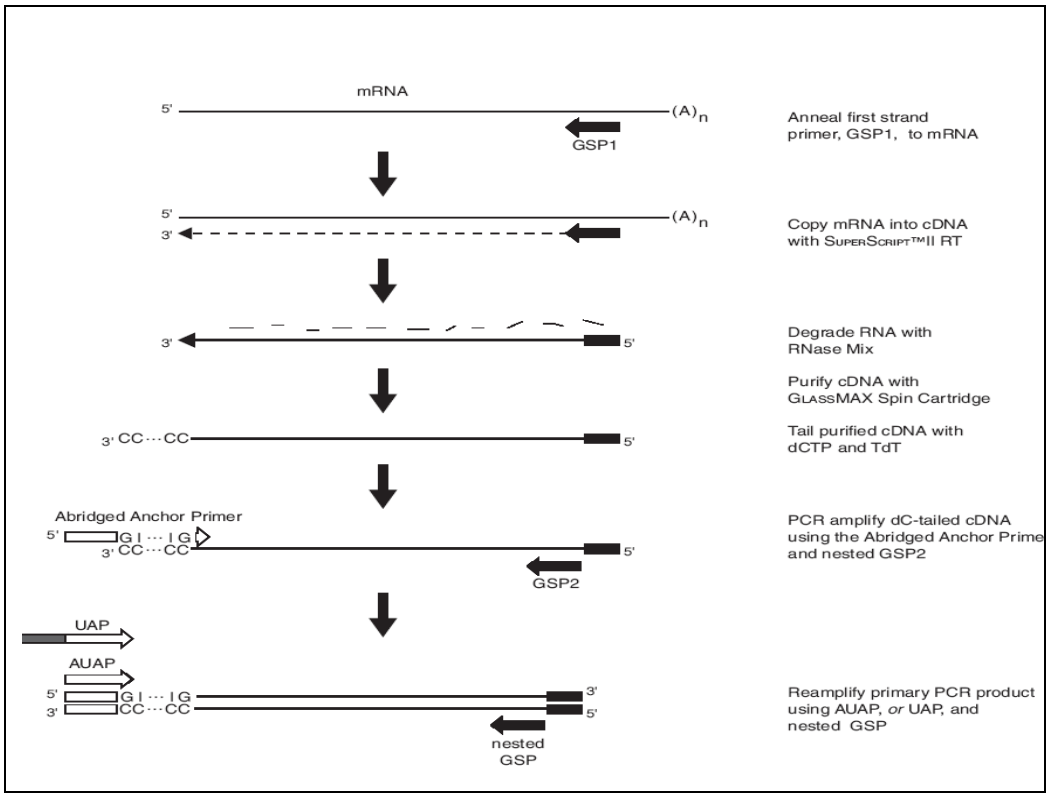


Table.5 Oligonucleotides primers used in 5´-RACE

Abridged anchor primer (AAP)	GGCCACGCGTCGACTAGTACGGGIIGGGIIGGGIIG
Abridged universal Amplification primer (AUAP)	GGCCACGCGTCGACTAGTAC
Gene specific primers (GSP1)	GAACCGATCTGGACGTAGTA
Gene specific primers(GSP2)	GGAATACTAGAGGCAACCTG

5´-RACE-PCR Reaction Mixture

Component	**Concentration (25 µL)**
cDNA	1 µL
GSP2 primmer	0.5 µL (10 µM)
AUAP	0.5 µL (10 µM)
PCR buffer (10x)	2.5 µL
dNTP mix	0.5 µL (10 mM nucleotide^{-1})
$MgCl_2$	1.5 µL (25 mM)
Taq-DNA-polymerase	0.5 µL (5 units µL^{-1})
ddH_2O	18 µL

5´-RACE-PCR Program

1- Predenaturation	95 °C	2 minutes
2- Denaturation	94 °C	30 seconds
3- Annealing	57 °C	30 seconds
4- Elongation	72 °C	1.5 minutes
5- Final elongation	72 °C	10 minutes

Step 2-4 were repeated 40 times (cycles)

2.7 Functional complementation of SST in the yeast double mutant CP154-7A

For functional proof of the symbiotic sulfate transporter (SST) from pea, the coding sequence (open read frame=ORF) was inserted into the *Saccharomyces cerevisiae* mutant strain CP154-7A (*Mataα, his3, leu2, ura3, ade2, trp1, sul1:LEU2, sul2:URA3*). The yeast double mutant CP154-7A is disrupted in two endogenous yeast sulfate transporters Sul1p and Sul2p (Cherest et al., 1997).

2.7.1 Amplification of ORF fragments

The coding sequence of SST (ORF=1920 bp) was amplified by PCR using oligonucleotide primers from 5´ and 3´ end of a full length cDNA. Primers were designed manually with overhangs including the restriction sites i.e. BamHI and SalI and were ordered from Eurofines MWG operon (Table.6). The proof-reading Pfu-DNA-polymerase was used for blunt end amplification.

Table.6 Oligonucleotides primers for ORF amplification

forward	ATGGATCCATGACGAAACTTGAAG
Reverse	ATGTCGACTTAAAGTGTCTCTTCG

PCR Reaction Mixture

Component	**Concentration (50 µL)**
cDNA	2 µL
Forward primer	2 µL (10 pmol μL^{-1})
Reverse primer	2 µL (10 pmol μL^{-1})
PCR buffer contain $MgCl_2$ (10x)	5 µL
dNTP mix	1 µL (10 mM $nucleotide^{-1}$)
Pfu-DNA-polymerase	1 µL (5 units μL^{-1})
H_2O	37 µL

2.7.2 Cloning of symbiotic sulfate transporter (SST)

2.7.3 Ligation of Blunt End SST ORF with the pJET 1.2 Cloning Vector

The blunt end SST ORF sequence including BamHI and SalI overhangs was extracted and purified from 1% agarose gel as described in section 2.6.7.1. The blunt end fragment was inserted into pJET 1.2 vector using T4 ligase. For optimal ligation efficiency, the molecular ratio of vector to insert was adjusted to 1:4. In the ligation assay, vector and insert (SST ORF) were mixed with 1 µL 10x ligation buffer and 0.5 µL T4 DNA ligase (10 U/µL). Then ddH_2O was added to

the 10 µL final volume. The ligation mixture was incubated for 30 minutes at room temperature (RT) subsequently purified with micro dialysis membranes. Finally, the purified ligation constructs were used for transformation of *E. coli* as described in section 2.6.2. Afterwards, plasmids were extracted from *E. coli* cells as described in section 2.6.3 and recombinant plasmids were restricted by BamHI and SalI endonucleases to confirm successful transformation of the SST ORF fragments.

Restriction Assay Component	**Concentration (10 µL)**
Plasmid DNA	2 µL
10x buffer	1 µL
EcoRI	0.2 µL
PstI	0.2 µL
ddH2O	6.6 µL

2.7.4 Ligation of SST ORF with the pESC_His yeast expression vector

The cloning of the full length SST coding sequence (ORF) into the pESC_His vector was done in three steps. In the first step, the ORF fragment was released from the pJET 1.2-SST vector using BamHI and SalI restriction enzymes and was purified with the DNA purification Kit. In the second step, the pESC_His vector was digested with the restriction enzymes BamHI and SalI and subsequently was purified. In the third step, the purified ORF fragment was ligated into the cleaved pESC_His for generation of pESC-SST. The ligation reaction was done using a combination of a vector and an insert (with 1:4 ratio), 1 µL 10x ligation buffer, 1 µL ATP, and T4 DNA ligase 0.5 µL (10 U/µL). Finally, ddH_2O was added to a final volume of 10 µL. The ligation mixture was incubated for 30 minutes at RT. After purification of the ligation mixture with micro dialysis membranes, the constructs were transformed into competent *E. coli* cells followed by plasmid DNA isolation and digestion with the same restriction enzymes to confirm the presence of the ORF fragment within the vector.

2.7.5 Generation of electrocompetent *Saccharomyces cerevisiae* cells

Electrocompetent cells were prepared from two different *y*east strains i.e. double mutant *S. cerevisiae CP* 154-7A and wild type *S. cerevisiae* INV*S*c1 (Mata*. his3D1, leu2, trp1-289, ura3-52*). A single colony of each strain was

cultivated in 5 mL YPD medium overnight at 28 °C and 200 rpm. 500 mL YPD medium was inoculated in the overnight culture and was grown to an OD_{600}=1.3. Afterwards the cells were harvested by centrifugation (10 min, 7000 rpm, 4 °C). The pellet was dissolved in 500 mL ice-cold sterile water and was centrifuged. Subsequently, the cells were washed again with 250 mL ice-cold sterile water and were centrifuged. The cell pellet was resuspended in 20 mL ice-cold 1 M sorbitol and centrifuged. Finally, the cells were dissolved in 1 mL of 1 M sorbitol with 10% (v/v) glycerol and aliquoted to 65 µL and frozen at -80 °C.

YPD Medium

Medium	**Concentration**
Yeast extract	1% (w/v)
Bacto-peptone	2% (w/v)
Glucose	2% (w/v)
Bacto agar for solidified medium	2% (w/v)

2.7.6 Transformation of *Saccharomyces cerevisiae*

The double mutant *S. cerevisiae* strain CP154-7A (Mata*α*, *his3, leu2, ura3, ade2, trp1, sul1:LEU2, sul2:URA3*) and wild type strain INVScI were transformed using yeast expression plasmid pESC-SST and empty vector pESC. 60 µL of competent yeast cells were mixed with desalted plasmid DNA and incubated in ice for 5 minutes. The mixture was transferred into a 1 mm sterile electroporation cuvette and electroporated with a voltage pulse of 750V. Immediately 1 mL of ice-cold sorbitol (1 M) was added and was incubated in ice for 2 minutes. Subsequently the cells were harvested using centrifugation (3500 rpm, 2 min) and then suspended in 100 µL of 1M sorbitol with 10% (v/v) glycerol. Transgenic yeast cells containing constructs of the pea symbiotic sulfate transporter were grown on a selective medium (Table 7) depleted in histidine at 28 °C for 2-3 days. Transformed colonies subsequently were grown in the synthetic minimal B medium (Cherest and Surdin-Kerjan, 1992) with sole sulfur sources (0.5 mM sulfate). B medium was filter sterilized and the sulfur source was added before use (Table 8).

Table.7 Selected Yeast Medium (-Histidine)

Component	**Concentration (1L)**
Dropout Powder	1.16 g
Galactose	2% (w/v)
YNB (yeast nitrogen base, without amino acids)	0.67% (w/v)
Leucine	60 mg/L
Tryptophane	40 mg/L
Uracil	20 mg/L
Bacto agar for solidification	2% (w/v)
Dropout Powder	
Component	**Concentration (1L)**
Adenine	2.5 g
L-arginine	1.2 g
L-aspartate	6 g
L-glutamate (sodium-salt)	6 g
L-lysine (HCl)	1.8 g
L-methionine	1.2 g
L-phenylalanine	3 g
L-serine	22.5 g
L-threonine	12 g
L-tyrosine	1.8 g
L-valine	9 g

Note: Mixture was ground to a fine powder.

Table.8 Minimal B medium

Mineral composition	**Concentration (1L)**
Ammonium chloride	5 mM
Monopotassium phosphate	6.6 mM
Dipotassium phosphate	0.5 mM
Sodium chloride	1.7 mM
Calcium chloride	0.7 mM
Magnesium chloride	2 mM
Oligo elements	**Concentration (1L)**
Boric acid	0.5 µg/mL
Copper chloride (1 H_2O)	0.04 µg/mL
Potassium iodide	0.1 µg/mL
Zinc chloride	0.19 µg/mL
Ferric chloride (6 H_2O)	0.05 µg/mL
Vitamin and growth factors	**Concentration (1L)**
Calcium pantothenate	2 µg/mL
Thiamine	2 µg/mL
Pyridoxine	2 µg/mL
Biotin	0.02 µg/mL
Inositol	20 µg/mL
Glucose	2%
Agarose for solidification	1%
Sulfate	0.5 mM added before used

2.8. Biochemical methods

2.8.1 Protein isolation

Protein was extracted from 100 mg leaf, root and nodule tissues according to the modified Cahoon method (Cahoon et al., 1992). The frozen tissue was homogenized using Precellys homogeniser (Peqlab, Erlangen, Germany) and filled up with 0.5 mL of the extraction buffer. After briefly vortexing, 0.5 mL phenol was added to the sample and it was centrifuged for 10 min at 3000 rpm. The upper phase was collected and transferred into a new eppendorf tube while the lower phase was mixed with 300 µL phenol and was centrifuged at 3000 rpm, for another 5 min. Once again the upper phase was collected and combined to the previously collected upper phase. For protein precipitation, 1.5 mL of 0.1 M ammonium acetate in methanol was added to the tube and after vortexing, it was stored over night at -20 °C. On the following day, the sample was centrifuged for 5 min at 13000 rpm and the pellet was washed with 100% acetone. Finally, the dried pellet was dissolved in 100 µL of 1% SDS.

Extraction buffer

Component	**Concentration (1L)**
Sucrose	0.7 M
Tris-HCl	0.5 M
EDTA	50 mM
KCl	0.1 M
ß-Mercaptoethanol	2% (v/v)

2.8.2 Protein quantification

Quantification of protein concentration was done by the bicinchoninic acid assay (BCA) based on the chemical reaction of peptide bonds with copper sulfate ($CuSO_4$). Bicinchoninic acid was mixed with copper sulfate (4%) to achieve a ratio of 49:1 and it was added to the protein sample.

Dilution series (0, 1, 2.5, 5, 7.5, 10 and 12.5 µg/µL) of bovine serum albumin (BSA) was used to create a standard curve. The appropriate amount of BSA stock solution (1µg/µL) was filled up with ddH_2O to 20 µL and was mixed with 200 µL BCA reagent. Also, 1 µL of each protein sample was mixed with 19 µL ddH_2O and 200 µL BCA reagents. The mixtures were incubated for 15 min at 60 °C. Following 2 min incubation in an ice bath, the samples were centrifuged at 11000 rpm for 5 min. Based on the generated standard curve, the protein quantity was calculated with the absorption at 550 nm using Nanodrop 1000 photometer.

2.8.3 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE)

Proteins were separated using discontinuous SDS PAGE containing 4% stacking and 10% separating gels. Sodium dodecyl sulfate (SDS) is a detergent that dissolves hydrophobic molecules and covers them with a negative charge. Following denaturation, the proteins were separated according to their size while running on a polyacrylamide gel under a constant electric field. Samples with 75 µg of protein were mixed with Laemmli loading buffer and heated for 15 min at 65 °C (Laemmli, 1970). Molecular protein marker (peqGold Protein Marker III, Peqlab) was also loaded onto the SDS-PAGE gels for protein size determination. Loaded samples and marker were separated in a gel chamber containing 1 x Tank buffer with current limit 30 mA constant.

4 Fold Laemmli buffer

Components	Concentration
Tris-HCl (pH 6.8)	200 mM
Dithiothreitol (DTT)	400 mM
Sodium dodecyl sulfate (SDS)	8%
Bromophenol blue	0.4%
Glycerol	40%
Adjust pH to 6.8 with HCl	

5x Tank buffer

Components	Concentration
Tris	125 mM
Glycine	960 mM
SDS	0.5% (w/v)

Stacking 4% gel (For 4 gels)

Components	Concentration
Acrylamide stock solution (30% acrylamide, 0.8% bisacrylamide)	2.66 mL
4x Stacking gel buffer (Tris-HCl, pH 6.8, 0.5 M)	5 mL
SDS-solution (100 mg/mL in H_2O)	0.2 mL
H_2O	12 mL
Tetramethylethylenediamine (TEMED)	10 µL
Ammonium persulfate (APS) solution (100 mg/mL in H_2O)	0.2 mL

Separation gel (10%) (for four gels)

Components	Concentration (50 mL)
Acrylamide stock solution (30% acrylamide, 0.8 % bisacrylamide)	17 mL
Tris-HCl (pH 8.8 ,1.5 M)	12.2 mL
SDS-solution (100 mg/mL in H_2O)	0.5 mL
H_2O	20 mL
Tetramethylethylenediamine (TEMED)	20 µL
Ammonium persulfate (APS) solution (100 mg/mL in H_2O)	0.3 mL

2.8.4 Western blotting and immunodetection

Proteins were transferred from the SDS gel onto a nitrocellulose membrane by semi-dry blotting (Towbin et al., 1979). The SDS PAGE gel was incubated in Towbin transfer buffer and due to the hydrophobic interactions with the membrane; proteins were immobilized onto the nitrocellulose membrane with current limit 130 mA constant for one hour. The nitrocellulose membrane was stained with Ponceau S red (0.2%) to check for the efficiency of proteins transfer to the blot and instantly used for the immunostaining with antibodies.

Towbin transfer buffer

Components	Concentration (1 L)
Tris	25 mM
Glycine	192 mM
Methanol	20%
SDS	0.1% (w/v)

For immunodetection, first the nitrocellulose membrane was incubated in blocking buffer overnight at 4 °C. Blocking buffer covers the empty places on the membrane to prevent nonspecific binding of antibodies. Next day, the membrane was incubated in the primary antibody solution (Rabbit polyclonal antibody raised against spinach ferredoxin protein) for 1 hour at room temperature while shaking. Subsequently, unbound antibodies were removed by washing (3 times 10 min) with TBST. Afterwards the membrane was incubated for 45 min with a conjugated secondary antibody (alkaline phosphatase conjugated anti-rabbit antibody) which binds to the first antibody. Finally, the membrane was washed with TBST (3 times each for 10 min) to remove the antibodies which were not bound to the first antibodies. Proteins were visualized with staining reaction i.e. NBT/BCIP.

Blocking buffer

Components	Concentration
Fat milk powder	2%
NaCl	150 mM
Tris-HCl pH 7.5	20 mM
Tween 20	0.1% (v/v)

Primary antibody solution (1:250)

Components	Concentration
Ferredoxin antibody	20µL
TBST	5mL

Secondary antibody solution(1:5000)

Components	Concentration
Anti-rabbit antibody	1µL
TBST	5mL

TBS

Components	Concentration
Tris-HCl pH 7.5	20 mM
NaCl	50 mM

TBST

Components	Concentration
TBS + Tween 20	0.05% (v/v)

Staining reaction

Components	Quality per blot
NBT Buffer (Nitro Blue Tetrazolium10 mg in Tris-HCl (pH 9.6,150 mM)	25 ml buffer
BCIP (0.5%)	250 µL BCIP
$MgCl_2$ 2M	50 µL $MgCl_2$

2.8.5 Chlorophyll determination

The accurate Chlorophyll a (chl *a*) and Chlorophyll b (chl *b*) concentrations were determined using 100 mg leaf tissue (Porra et al., 1989). The frozen tissue was homogenized using a Precellys homogeniser (Peqlab, Erlangen, Germany) and the extract filled up with 0.5 mL 80% (v/v) acetone which was followed by vigorously vortex and centrifugation (4 °C, 8000 rpm, 5 min). The samples were kept in ice without direct light; then the supernant was transferred into a new tube. Another 0.5 mL 80% acetone was added to the plant material and after centrifugation both supernatants were combined and were filled up to 1 mL with 80% acetone. After a short centrifugation to spin down the leaf particles, the supernatant was used for absorption measurement at 646.6 nm (chl *b*), 663.6 nm (chl *a*) and 750 nm (for background correction) by spectrophotometer. Chlorophyll pigment concentration was calculated as follows:

Chlorophyll a (µg/ mL)	Ca = 12.25 x A663.6 - 2.55 x A646.6
Chlorophyll b (µg/ mL)	Cb = 20.31 x A646.6 - 4.91 x A663.6
Total Chlorophyll (µg/mL)	Ca + Cb = 17.76 (A646.6) + 7.34 (A663.6)

2.8.6 Leghemoglobin measurement

The leghemoglobin content of nodules was determined using a Drabkin reagent Kit (Wilson and Reisenauer, 1963). Human standard hemoglobin protein (Sigma, Saint-Louis, USA) was used to create a standard curve. A serial dilution of a standard protein was prepared by 0, 6, 12 and 18 mg/mL cyanmethemoglobin in Drabkin solution. 100 mg frozen nodules were homogenized and mixed with 600 mL Drabkin solution. Briefly, the vortexed sample was centrifuged for 15 min at 3500 rpm. Subsequently the supernatant was transferred into a new tube and protein was extracted for another two times with 600 mL Drabkin solution. The combined supernatants were filled up to 2 mL with Drabkin solution and centrifuged for 30 min at 16000 rpm. Finally, leghemoglobin pigment absorbance was measured at 540 nm using the spectrophotometer. Protein quantity was calculated based on the generated human leghemoglobin standard curve.

Drabkin solution

Components	**Concentration (L)**
Potassium cyanide (KCN)	52 mg
Potassium ferricyanide [$K_3Fe(CN)_6$]	198 mg
Sodium bicarbonate ($NaHCO_3$)	1 g

All substances were dissolved in ddH_2o to final volume of 1000 mL

2.9 Next Generating Sequencing

2.9.1 Biological samples for transcriptional analysis

Pea (*Pisum sativum*) seeds were germinated on Murashige and Skoog medium for 2 weeks and plant samples (leaves and roots) were prepared under sterile conditions. Nodule samples were isolated from plants, cultivated under the natural condition and inoculated with *Rhizobium leguminosarum* as described in section 2.4.

Murashige and Skoog medium

Component	Concentration (L)
MS powder including vitamins	4.8 g
Sucrose	10.0 g
Plant agar	9.0 g

2.9.2 RNA Isolation and purification

Total RNA was extracted from leaves, roots and nodules respectively using a Macherey-Nagel plant RNA isolation Kit. DNA contaminations were removed by on-column digestion with rDNase (total RNA ≥ 20µg).

As RNA quality was critical for transcriptional sequencing, RNA integrity of all samples were checked for acceptable range by Agilent 2100 Bioanalyser using RNA 6000 Nano Kit (leaves and roots RIN≥9, nodules RIN≥7).Sequencing was done in BGI sequencing company (Hong Kong, China).

2.9.3 Transcriptome de novo assembly

All reads (100 bp paired end reads) resulting from sequencing of the roots (51,860,856 paired-end reads), leaves (52,407,480 paired-ends reads) and nodules (26,205,828 paired-end reads) were tested for quality, adapter and primer sequences to be prepared for trimming. All nucleotides having a quality lower than 0.05 and ambiguous nucleotides (>2 nucleotides) were trimmed. Meanwhile, the adapter- and primer-sequences were trimmed. Duplicate reads were omitted from the final reads that were used for the assembly. Further estimation of the paired distance range was done for all reads to adjust for the paired end reads alignment (maximum range including the reads was 308b).

The transcriptome *de novo* assembly was done using the following parameters: Mismatch cost = 2, Insertion cost = 3, Deletion cost = 3, Length fraction = 0.5, similarity fraction = 0.8. The minimum contig length was adjusted to 200 bp with the option of scaffolding contigs and automatic update of the resulted contigs. The processing of the reads and the assembly was done using CLC genomics workbench v7.0 (CLC Inc, Aarhus, Denmark).

2.9.4 Data analysis and sequence homology for transcriptome identification of the sulfate transporter gene family in *Pisum sativum*

The sequence homology was investigated in order to identify the sulfate transporter gene family in *Pisum sativum*. The test was done by nucleotide basic local alignment search tool (BLAST) of the whole transcriptome assembly once against the NCBI nucleotide collection (nr/nt) database and once against non-redundant protein sequences (nr). Best hits being determined by selecting sequences that had the lowest E-value and the highest bit score were extracted and each group of sequences was aligned to each corresponding contig. This test was done by the multiple alignment tool CLC genomics workbench (CLC Inc,). All sequences (assembled contigs) were compared with most known sulfate transporter genes in plants as reference plant and were scored by lowest E-value, greatest hit length and greatest identity. Finally, the best selected contigs were used to build a phylogenetic tree using CLC genomics workbench (CLC Inc).

3 Results

3.1 Sulfur supply and growth conditions

Initial symptoms of S deficiency in *Pisum sativum* are stunted growth and yellowish leaves (Fig.10). Both weak and severe S deficient (0.78 mM S and 0.39 mM in the fertilizer solution) plants were smaller than the control plants (6.24 mM S). Severe S deficient plants switched to the generative phase earlier than the control plants.

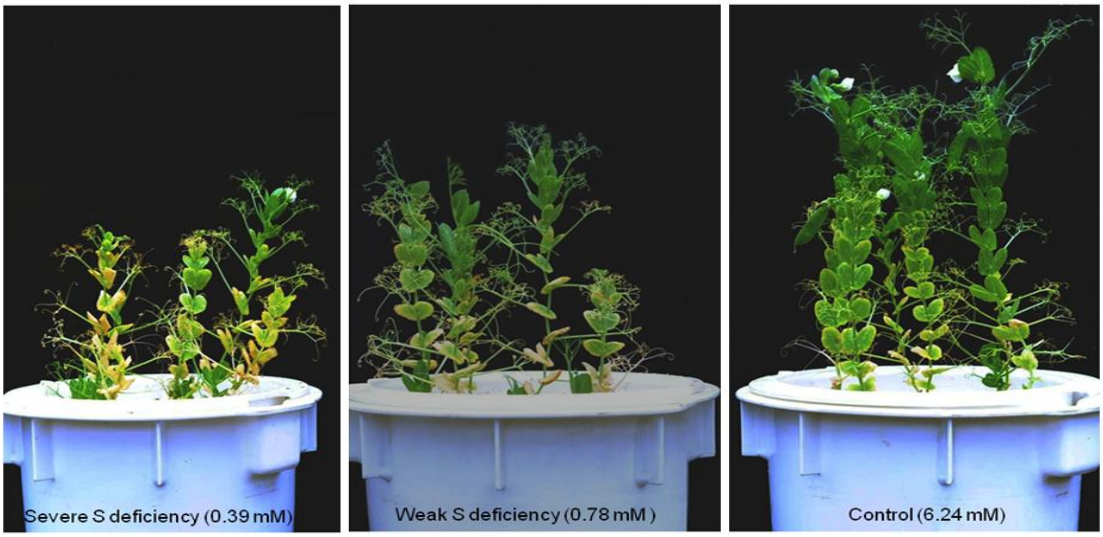


Fig. 10 *Pisum sativum* growth during the generative phase (8 weeks old plants), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe deficiency: 0.39 mM S.

Severe S deficiency resulted in reduced total biomass ($P<0.06$) while weak S deficient plants grew more similarly to the control plants (Fig. 11). In contrast to the root dry weight, the shoot dry weight was affected by S deficiency. The shoot dry weight of severe S deficient plants was significantly reduced compared to the weak S deficient and the control plants (Fig. 11). As a consequence, the root to shoot ratio increased significantly during severe S deficiency compared to the control plants and was slightly increased compared to weak S deficient plants ($P<0.1$) (Fig. 11).

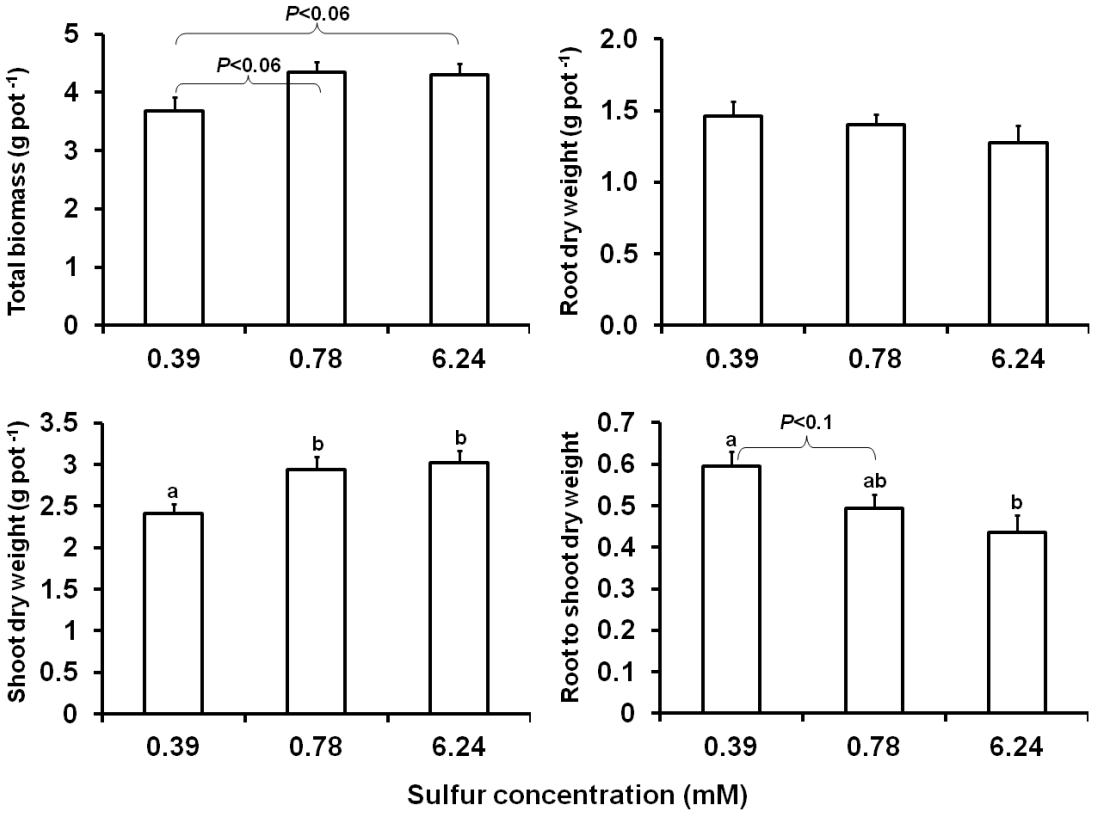


Fig. 11 Influence of S deficiency on total biomass, shoot dry weight, root dry weight and root to shoot ratio (means ± SE), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Different letters within one graph represent significant differences at P<0.05. A trend is defined when the P value is (0.05<P<0.1).

3.2 Sulfur content of plants

The S concentration in the fertilizer influenced S concentration in different tissues. Independent of the concentration of S in different treatments, S content in the shoot tissue was significantly higher than in the root tissue (P<0.001) (Table 9).

During both vegetative and generative phases, S content of the shoot tissue was significantly lower in severe and weak S deficient plants compared to the control plants. During the generative phase, S content of the shoot tissue was significantly reduced in severe S deficient plants vs. weak S deficient plants. After switching from the vegetative phase to the generative phase, S content of the shoot tissues did not change significantly under both S deficiency conditions. However, in the shoot tissue of the control plants, S concentration increased during the transition form the vegetative to the generative phase (P<0.05).

During the vegetative phase, the S concentration of root tissue was not changed although it was decreased as a trend in severe S deficient plants compared to the control plants ($P<0.08$). However, during the generative phase, the S content of the root tissue decreased significantly by weak or severe S deficiency.

The S concentration of root tissue during the generative phase was significantly higher than during the vegetative phase only in the control and the weak S deficiency plants (0.78 mM).

Table.9 Total S concentration in the shoot and root tissues of *Pisum sativum* in the vegetative and the generative phases

Tissue	S application	Total S uptake (mg pot^{-1})	
		vegetative phase	generative phase
Shoot	Severe S deficient (0.39 mM)	3.47 ^{a}	4.1 ^{a}
	Weak S deficient (0.78 mM)	5.49 ^{a}	7.41 ^{b}
	Control (6.24 mM)	9.21 $^{c\ *}$	14.28 ^{c}
Root	Severe S deficient (0.39 mM)	1.92 ^{a}	1.88 ^{a}
	Weak S deficient (0.78 mM)	2.52 $^{a\ *}$	3.43 ^{b}
	Control (6.24 mM)	3.03 $^{a\ *}$	4.66 ^{c}

Different letters indicate a significant difference between S treatments in each tissue ($P<0.05$). Asterisks indicate a significant difference between the vegetative and the generative phases ($P<0.05$). Independent of the level of S treatments, S content in the shoot tissue was significantly higher than the root tissue ($P<0.001$).

3.3 Sulfur supply and nodule development

Sulfur availability influenced nodule quality and quantity. In S deficient plants, nodules appeared to be phenotypically more pale and green in contrast to the pink nodules in the control plants (Fig.12). As compared to the control plants, severe and weak S deficiency resulted in less and smaller nodules. Nodule quantity (fresh weight) was significantly reduced under S deficiency conditions (Fig.13).

Fig.12 Root nodules of *Pisum sativum* during S starvation, control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 Mm S.

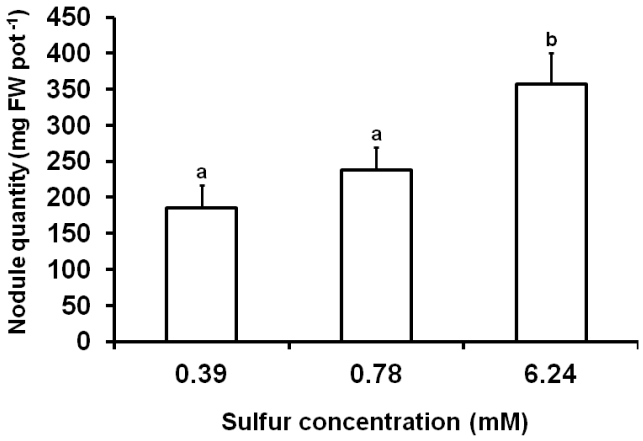


Fig.13 Nodule quantity in *Pisum sativum* (means ±SE), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Different letters represent a significant difference at $P<0.05$. FW = fresh weight.

3.4 Sulfur supply and chlorophyll content

The impact of S supply on the chlorophyll content of young and mature leaves was investigated separately. The chlorophyll content of young leaves during the vegetative phase was not changed noticeably by either weak or severe S deficiencies (Fig.14). In the generative phase, severe S deficiency significantly reduced the chlorophyll content of young leaves while weak S deficiency had no significant effect on the chlorophyll content of young leaves.

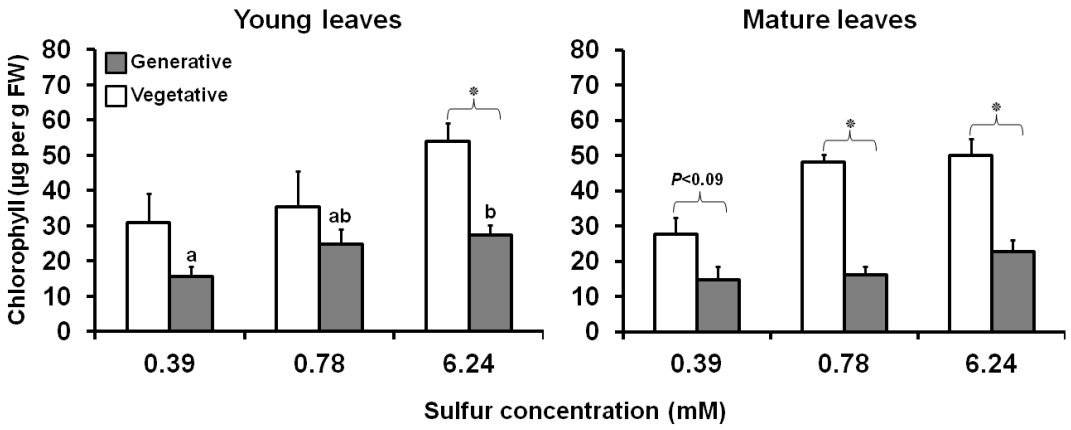


Fig.14 Total chlorophyll content in the young and mature leaves of *Pisum sativum* (means ±SE), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Chlorophyll was measured photometrically. Different letters indicate a significant difference between treatments (P<0.05). Asterisks indicate a significant difference between the vegetative and the generative phases (P<0.05). FW = fresh weight.

Mature leaves showed more tolerance to the weak and severe S deficiency and did not change noticeably in the vegetative and the generative phases (Fig.14). Nevertheless, the chlorophyll content of mature leaves significantly was dropped by switching from the vegetative to the generative phase.

3.5 Sulfur supply and leghemoglobin content in nodules

The leghemoglobin content of nodules was not affected by S deficiency in the vegetative phase. Nevertheless, the leghemoglobin content of nodules was significantly decreased in the generative phase under both S deficiency conditions compared with the control plants (Fig.15).

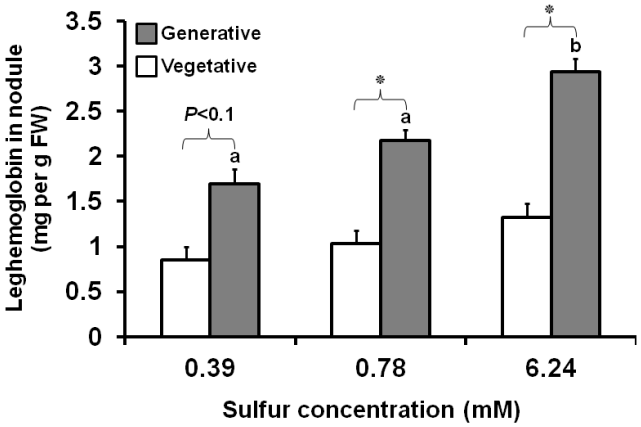


Fig.15 Nodule leghemoglobin protein content in *Pisum sativum*, control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Leghemoglobin was measured photometrically. Different letters indicate a significant difference between treatments (P<0.05). Asterisks indicate a significant difference between the vegetative and the generative phases *(P<0.05).* Fw = fresh weight.

Moreover, comparing the two S deficient conditions, the leghemoglobin content did not change noticeably in the generative phase. As compared to the vegetative phase, the leghemoglobin content in the generative phase was significantly higher in the control and in the weak S deficient plants and only increased as a trend in severe S deficient plants (P<0.1).

3.6 Expression of symbiotic nitrogen fixation (SNF) related genes

3.6.1 Expression of leghemoglobin genes in the nodule tissue

First, expression of two distinct types of leghemoglobin genes, i.e. PsLbA (PsLb5) and PsLbB (PsLb120), in *Pisum sativum* was investigated under different regimes of S deficiency (weak and severe). Gene expression as described in this paragraph was measured using qPCR thus the relative mRNA abundance values are not absolute quantities. In the generative phase, Pslb5 relative transcript abundance was significantly lower in severe S deficiency as compared to the control, while weak S deficient plants had similar relative transcript abundance like the other two treatments (Fig. 16). A lower relative transcript abundance of Pslb5 was observed in the generative phase as compared to the vegetative phase exclusively under severe S deficient condition.

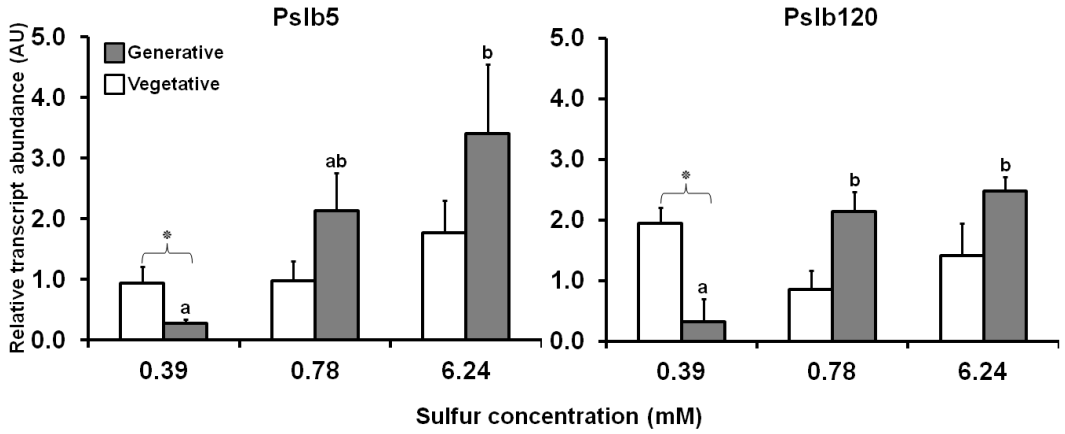


Fig.16 Leghemoglobin (Pslb5 and Pslb120) relative transcript abundance (means ± SE) in the nodule tissue of *Pisum sativum*, control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Transcripts were measured by qPCR. Different letters indicate a significant difference between treatments (P<0.05). Asterisks indicate a significant difference between the vegetative and the generative phases (P<0.05).

Relative transcript abundance of PsLb120 was not altered by S deficiency in the vegetative phase. However, in the generative phase, relative transcript abundance of PsLb120 declined under severe S deficiency compared to weak S

deficiency and control plants. Moreover, solely under the severe S deficiency, Pslb120 relative transcript abundance was reduced during the transition from the vegetative phase to the generative phase.

3.6.2 Expression of the symbiotic sulfate transporter (SST) gene in nodule tissue

The SST gene was identified in pea based on the sequence described in section 3.8, and subsequently oligonucleotide primers were designed and relative transcript abundance of SST was measured using qPCR. As expected, the amount of S supplied affected the SST relative transcript abundance (Fig. 17). In the vegetative phase, SST exhibited a trend of lower relative transcript abundance in the severe S deficient plants compared to the control plants ($P<0.08$) while the weak S deficiency did not noticeably change the relative abundance of SST transcript. In the generative phase, severe S deficiency resulted in a lower relative transcript abundance of SST as compared to weak S deficiency and to the control plants ($P<0.05$). Under severe S deficiency conditions, relative transcript abundance of SST significantly decreased when the plant switched from the vegetative phase to the generative phase and this was not repeated in the other S supplementation concentrations.

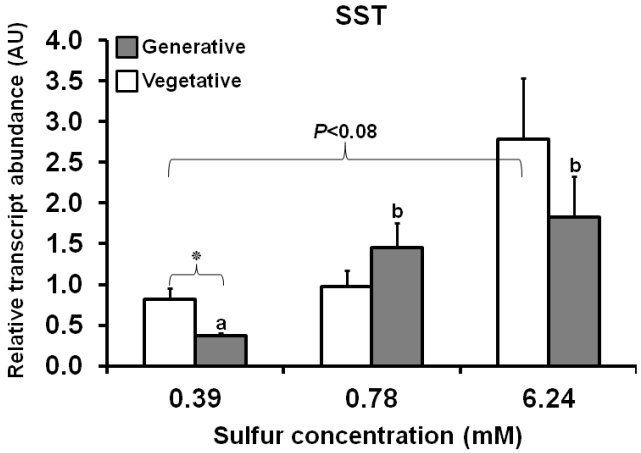


Fig.17 Symbiotic sulfate transporter (SST) relative transcript abundance (means ±SE) in nodule tissue of *Pisum sativum*, control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Relative transcript abundance was determined by qPCR. Different letters indicate a significant difference between treatments ($P<0.05$). Asterisks indicate a significant difference between the vegetative and the generative phases ($P<0.05$).

3.6.3 Expression of the nitrogenase (Nif) genes in the nodule tissue

The nitrogenase contains two α subunits which are encoded by the NifD gene and two β subunits which are encoded by the NifK gene. The relative

transcript abundance of the two genes was investigated using qPCR.

Relative transcript abundance of NifD was not affected by S deficiency in the vegetative phase as well as in the generative phase (Fig. 18). Also no remarkable difference was observed between the vegetative phase and the generative phase in the control plants and weak S deficient plants. Nevertheless, in severe S deficient plants, relative transcript abundance of NifD tended to decrease in the generative phase as compared to the vegetative phase (P<0.1).

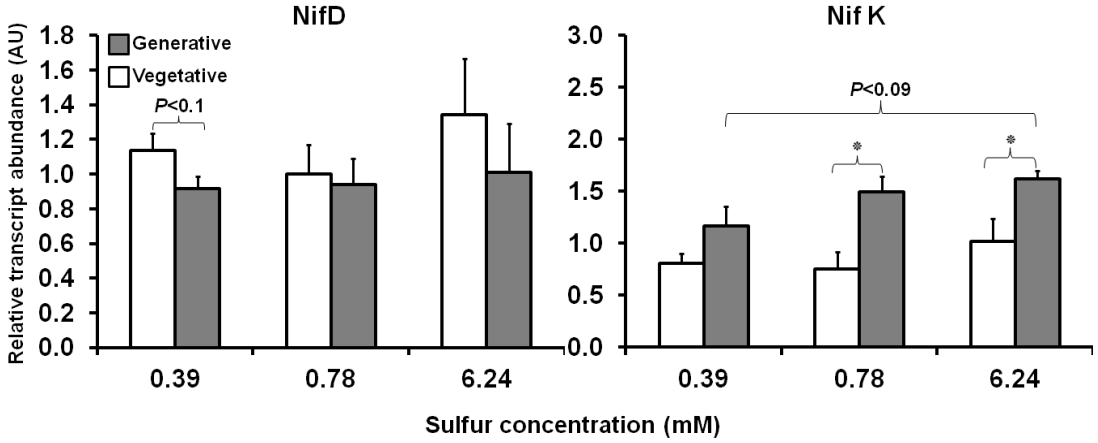


Fig.18 Nitrogenase (NifD and NifK) relative transcript abundance in the nodule tissue of *Pisum sativum* (means ±SE), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Transcripts were quantified by qPCR. Asterisks indicate a significant difference between the vegetative and the generative phases (P<0.05).

The relative transcript abundance of NifK (Fig. 18) changed more than NifD under S deficiency and tended to decrease under severe S deficiency condition as compared to the control S supply (P<0.09). In addition, in the control plants and in the weak S deficiency, relative transcript abundance of NifK significantly increased in the generative phase as compared to the vegetative phase.

3.6.4 Expression of the ferredoxin gene in the root, nodule and leaf tissues

Root ferredoxin relative transcript quantity significantly decreased in the vegetative phase under severe S deficiency as compared to the control and weak S deficiency conditions (Fig. 19). However, S deficiency did not change the relative transcript abundance of root ferredoxin in the generative phase. Furthermore, relative transcript abundance of ferredoxin in the root tissue was not significantly different between the generative phase and the vegetative phase in severe and weak S deficient plants while in control plants, relative transcript abundance of ferredoxin in the generative phase was lower than in the vegetative

phase.

The ferredoxin relative transcript abundance in the nodule tissue was remarkably increased under severe and weak S deficiency in the vegetative phase (Fig.19). In the generative phase, ferredoxin relative transcript abundance increased only as a trend in severe S deficiency plants in comparison to the control plants. In contrast to the root tissue, nodule ferredoxin relative transcript abundance in the generative phase was higher than in the vegetative phase in both control ($p<0.05$) and severe S deficient plants ($p<0.1$).

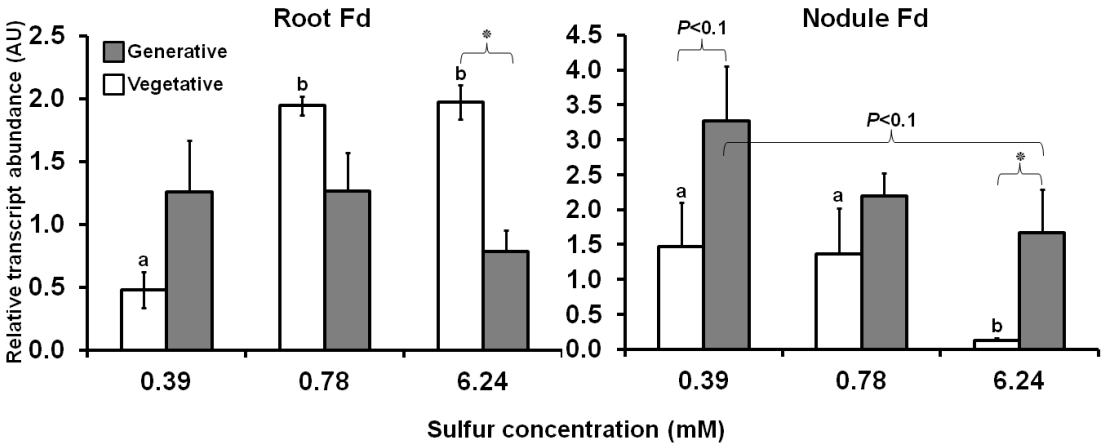


Fig.19 Ferredoxin (Fd) relative transcript abundance in the root and in the nodule tissue of *Pisum sativum* (means ± SE), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Relative transcript abundance was measured by qPCR. Different letters indicate a significant difference between S treatments ($P<0.05$). Asterisks indicate a significant difference between the vegetative and the generative phases ($P<0.05$).

In young leaves, ferredoxin relative transcript abundance decreased significantly under both deficiency conditions as compared to the control plants in the vegetative phase (Fig. 20). Moreover, in the control plants relative transcript quantity of ferredoxin decreased in the generative phase as compared to the vegetative phase. In mature leaves, ferredoxin relative transcript abundance was not affected by S deficiency. However, independent of the S supply, ferredoxin relative transcript abundance significantly decreased in the generative phase compared to vegetative phase (Fig. 20).

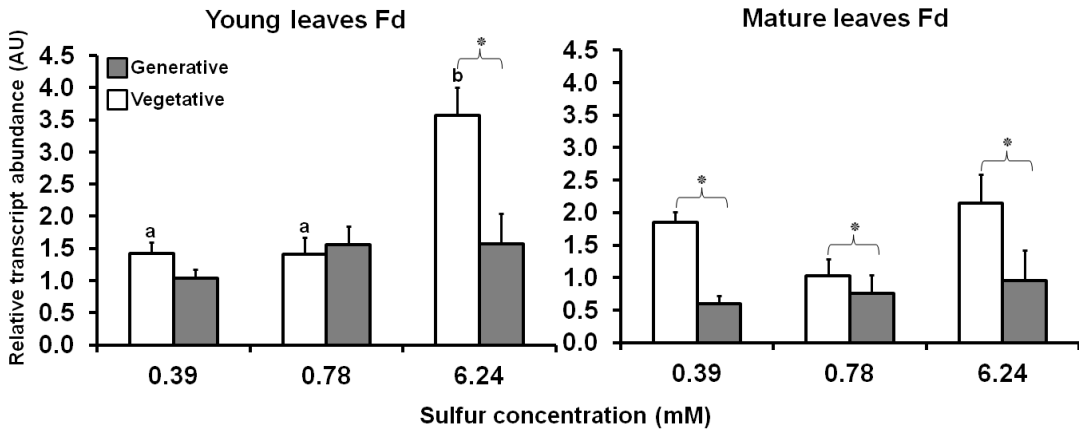


Fig. 20 Ferredoxin (Fd) relative transcript abundance in the young and mature leaves of *Pisum sativum* (means ± SE), control plants: 6.24 mM S; weak S deficiency: 0.78 mM S; severe S deficiency: 0.39 mM S. Different letters indicate a significant difference between S treatments ($P<0.05$). Asterisks indicate a significant difference between the vegetative and the generative phases ($P<0.05$).

3.7 Western blotting and immunodetection of ferredoxin

The influence of S deficiency on ferredoxin protein abundance was analyzed by western blotting of proteins extracted from 100 mg leaf, root and nodule tissues. The western blot results illustrated that the size of ferredoxin protein in *Pisum sativum* is around 24 KDa which is larger than the spinach ferredoxin protein which was used as a positive control. Immunodetection of the ferredoxin protein in the root tissue did not reveal differences for ferredoxin abundance between the control, severe and weak S deficient plants in either the generative or the vegetative phases (Fig. 21).

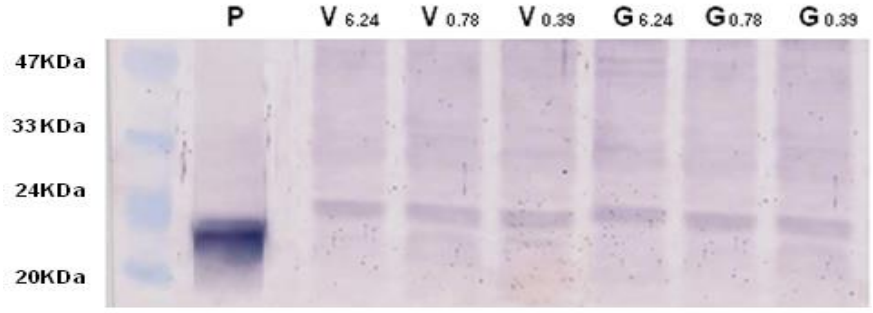


Fig. 21 Western blot of root ferredoxin protein detected with anti-ferredoxin antibodies, 70 µg total protein was loaded in each lane. **P**: positive control (standard spinach ferredoxin), **V6.24:** control plants (6.24 mM S) in the vegetative phase, **V0.78:** weak S deficient plants (0.78 mM S) in the vegetative phase, **V0.39:** severe S deficient plants (0.39 mM S) in the vegetative phase, **G6.24:** control plants (6.24 mM S) in the generative phase, **G0.78:** severe S deficient plants (0.78 mM S) in the generative phase**, G0.39:** weak S deficient plants (0.39 mM S) in the generative phase.

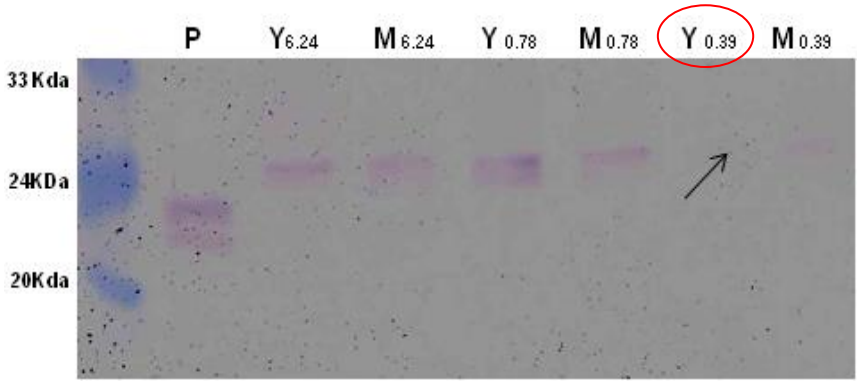


Fig. 22 Western blot of leaf ferredoxin protein in the vegetative phase using anti ferredoxin antibodies, 70 µg total protein was loaded in each lane. **P**: positive control (standard spinach ferredoxin), **Y6.24:** young leaves of the control plants (6.24 mM S), **M6.24:** mature leaves of the control plants (6.24mM S), **Y0.78:** young leaves of the weak S deficient plants (0.78 mM S), **M0.78:** mature leaves of the weak S deficient plants (0.78 mM S), **Y0.39:** young leaves of the severe S deficient plants (0.39 mM S). **M0.39:** mature leaves of the severe S deficient plants (0.39 mM S).

Immunodetection of ferredoxin protein in leaves demonstrated that the integrity of ferredoxin protein was not affected by weak S deficiency in the young and mature leaves as compared to the control plants in the vegetative phase (Fig. 22). However, the ferredoxin abundance was lower under severe S deficiency particularly in the young leaves. Nevertheless, the amount of ferredoxin protein did not seem to be affected by S deficiency in the generative phase (data not shown).

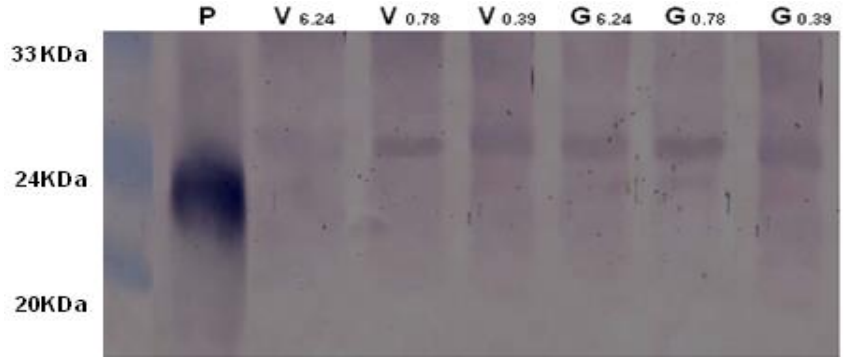


Fig. 23 Western blot of nodule ferredoxin protein prepared and incubated with anti-ferredoxin antibodies. 70 µg total protein was loaded in each lane. **P**: positive control (standard spinach ferredoxin), **V6.24:** control plants (6.24 mM S) in the vegetative phase, **V0.78:** weak S deficient plants (0.78 mM S) in the vegetative phase, **V0.39:** severe S deficient plants (0.39 mM S) in the vegetative phase, **G6.24:** control plants (6.24 mM S) in the generative phase, **G0.78:** weak S deficient plants (0.78 mM S) in the generative phase**, G0.39:** severe S deficient plants (0.39 mM S) in the generative phase.

In nodules, western blotting and immunodetection results demonstrated that the amount of ferredoxin protein was not affected by severe and weak S

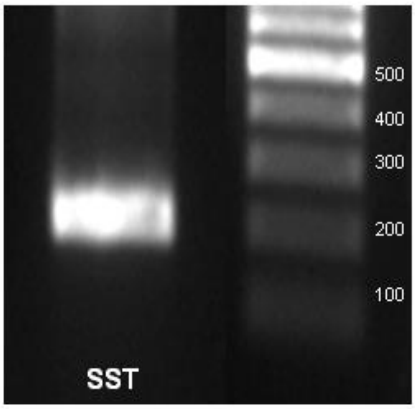
deficiency as compared to the control plants in the vegetative phase (Fig. 23). Similarly, in the generative phase, S deficiency did not change the integrity of ferredoxin protein in nodules.

3.8 Identification of a symbiotic sulfate transporter cDNA in *Pisum sativum*

3.8.1 Isolation of a partial cDNA corresponding to the symbiotic sulfate transporter

A complete cDNA gene of symbiotic sulfate transporter (SST) was identified in different steps using the RACE method and next generation sequencing. Small fragments of SST (267 bp, Fig. 24) were detected by RT-PCR using nodule cDNA as template and degenerate primers as described in section 2.6.5. Subsequently, a partial cDNA of SST was cloned into the pGEM®-T Easy vector in different steps as described in section 2.6.7. Plasmid DNA carrying the partial SST cDNA was submitted for sequencing. The partial pea SST sequence (267 bp) was highly similar (97 %) to the SST sequences from other legumes (*Medicago truncatula*, *Glycine max* and *Phaseolus vulgaris*).

A



B

CAGCGTCTCTGTATATACCAGAATCAGATAATTTTCCAAGTTTGCATGATGCAGGTCTAGCCAA
GTAAATTAGTCCTCTAATCAAACCTAGTCCAACGGAGATCATAAGGCCCATGTCCATGCTTATA
AAGGCAACACCTAAGAATGCACTCATGCAAATAACAAAATCAAATTTATCAACTTTAAAGAGGTA
AATAGCTTCCTCATAATTTATGAGCCCCAACATTGCAGACACAATAATAGCTGATAGAGCAACAA
GAGGGGTGA

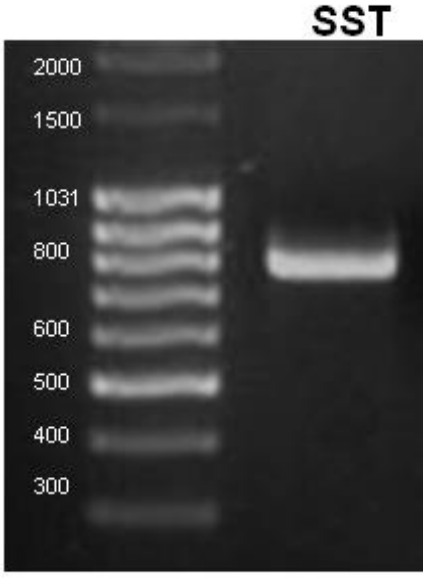
Fig. 24 Partial cDNA corresponding to symbiotic sulfate transporter (SST) gene from pea, A: The partial SST amplified fragment. RT-PCR was performed using nodule cDNA as template and the degenerate primers. The amplified fragment was separated in agarose gels and stained with ethidium bromide. **B:** Nucleotide sequence of partial SST (267 bp). The amplified fragment was cloned in pGEM®-T Easy vector then plasmid carrying DNA was sequenced.

3.8.2 Isolation of the 3´ end of the SST from pea by 3´- RACE

Sense and antisense primers were designed from a partial SST cDNA (267

bp). Subsequently, the 3´ regions of the SST transcript were isolated by the3´-RACE Kit as described in section 2.6.9 (Fig. 25A). The amplified fragments of 3´ SST end was gel extracted and cloned into pGEM®-T Easy vector and plasmids carrying 3´ region of SST were sent for sequencing. The nucleotide sequence (804 bp, Fig. 25B) was verified by sequence comparison using BLAST to the other sulfate transporter genes in leguminous. The 804 bp pea cDNA showed 94% homology to *Medicago truncatula*, 78% to *Cicer arietinum* and 92% to *Lotus japonicus* SST sequences.

A



B

GATTGGCCACGCGTCGACTAGTACTTTTTTTTTTTTTTTTTTTAGTAAACACATTCTCAATT
CCATAAAAAGTAGTGTTATACAACATTGATACAAAAAGATTAAAACAATCAAAATGTGTATAA
CCTAATTTAGATTTATTGATGTATATATGGGTATGACAAGTCTCTTAAAGTGTCTCTTCGTTAT
TATTTGTCTTAGATGTTCTGAGTGAATATTGACTTGCCATCACTGCATCGTCCAAATTTAGAT
AGAATTTTTCCTTTCCAATCTTGTCAACAAACTTTGATACTATTAGCTTCTCCATAACCTCTA
GCCTTGGATTTACTAATGACATCTGAATTCCTTTTCTTTCCAACACTTTATTTGTCTCCAACA
ATCCTTCAATACCAGTTGTGTCAATGGAAGTTACTCCTGTAAAATCGAGTATAACATGTTCG
ATATCATCTCCGGAAGAGCTTTGTTCGCTTTTAACATATCTCAAAATCCTTTCTTTGATGTAA
GTAGAATTTGAAAAATAAACAGGAGAACCAATTTGTAAAGCTAAAACTCCTGGAACTCTTGA
AGCATTAGAATATCGTTCAACATCTCTGTATATACCAGAATCAGATAATTTTCCAAGTTTGCA
TGATGCAGGTCTAGCCAAATAAATTAGTCCTCTAATCAAACCTAGTCCAACGGAGATCATAA
GGCCCATGTCCATGCTTATAAAGGCAACACCTAAGAATGCACTCATGCAAATAACAAATCAA
ATTTATCAACTTTAAAGAGGTAAATAGCTTCCTCATAATTTATGAGCCCC

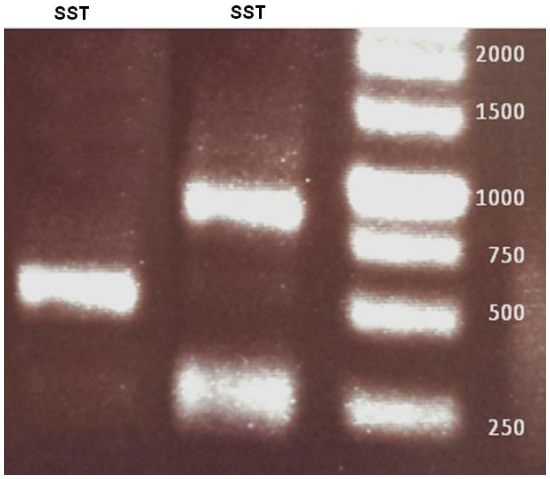
Fig.25 3' cDNA fragment of pea SST, A: The amplified fragment in 3´ end of pea SST. 3´ -RACE PCR was performed using nodule cDNA as template, abridged universal amplification primer (AUAP) as sense primer and a gene specific primer as antisense primers. The amplified fragment was separated in an agarose gel and stained with ethidium bromide. **B:** Nucleotide sequence of the 3´ end of SST (804 bp). The amplified fragment was cloned in pGEM®-T Easy vector and plasmid carrying DNA was sequenced.

3.8.3 Isolation of the 5´ end of the SST from pea by 5´-RACE

The 5´ regions of the SST transcripts were isolated in several steps by using the

5´-RACE Kit as described in section 2.6.10. The sizes of the 5' amplification products differed between 250 to 874 bp (Fig. 26). The largest amplified products were gel extracted and cloned into pGEM®-T Easy vector and sent for sequencing. The sequencing results confirmed the high homology to the other sulfate transporter genes in leguminous.

A



B

504 bs

TCAATTCTATATTTGAATTTTGATAGTAAAAATTTACCACCGGGGTTAAAAGCTGGTTTAATCACT
GGAGTTTTGCTTCTTTTAAACACAAAACTTTCAAGGAATCTTTTTTGTTATTGATAATACACCTCA
TGATGGAAACAAAAAGATGATAGCTTTTGGACTTATGAATTTTTTTGGTTCTTTTACCTCATGTT
ACTTAACCAGTGGACCATTTTCCAAGACTGCCTGAATTATAAAGCAGGGTGTAAAATTGCAATG
ACAAATGTGGGACAAGCAGTTTTAATGGCCCTTACCCTACAATTTTTGGCACCATTTTTGGCAA
CACACCACTTGTTGCTCTATCAGCTATTATTGTGTCTGCAATGTTGGGGCTCATAAATTATGAG
GAAGCTATTTACCTCTTTAAAGTTGATAAATTTGATTTTGTTATTTGCATGAGTGCATTCTTAGGT
GTTGCCTTTAAAAGCATGGACATGGGCCTTATGATCTCCGTTGGAC

874 bs

ACCCCTCTTGTTGCTCTCTCAGCTATTATTGTGTCTGCAATGTTGGGGCTCATAAATTATGAGG
AAGCTATTTACCTCTTTAAAGTTGATAAATTTGATTTTGTTATTTGCATGAGTGCATTCTTAGGTG
TTGCCTTTATAAGCATGGACATGGGCCTTATGATCTCCGTTGGACTAGGTTTGATTAGAGGACT
AATTTATTTGGCTAGACCTGCATCATGCAAACTTGGAAAATTATCTGATTCTGGTATATACAGAG
ATGTTGAACAATATTCTAATGCTTCAAGAGTTCCAGGAGTTTTAGCTTTACAAATTGGTTCTCCT
GTTTATTTTTCAAATTCTACTTACATCAAAGAAAGGATTTTGAGATATGTTAAAAGCGAACAAAG
CTCTTCCGGAGATGATATCGAACATGTTATACTCGATTTTACAGGAGTAACTTCCATTGACACAA
CTGGTATTGAAGGATTGTTGGAGACAAATAAAGTGTTGGAAAGAAAAGGAATTCAGATGTCATT
AGTAAATCCAAGGCTAGAGGTTATGGAGAAGCTAATAGTATCTAAGTTTGTTGACAAGATTGGA
AAGGAAAAATTCTATCTAAATTTGGACGATGCAGTGATGGCGAGTCAATATTCACTCAGAACAT
CTAAGACAAATAATAACGAAGAGACACTTTAAGAGACTTGTCATACCCATATATACATCAATAAAT
CTAAATTAGGTTATACACATTTTGATTGTTTTAATCTTTTTGTATCAATGTTGTATAACACTACTTT
TTATGGAATTGAGAATGTGTTTACTAAAAAAAAAAAAAAAAAAAAAGTACTAGTCGACGCGTGA
CCACTGATAGAGCAACAAGTGGTGTGATCTT

Fig. 26 5' cDNA fragment of pea SST, A: The amplified **5'** fragment of SST. 5´-RACE PCR was performed using nodule cDNA as template, a gene specific primer as sense primer and abridged anchor primer (AAP) as antisense primers. The amplified fragments were separated in agarose gel and stained with ethidium bromide. **B:** Nucleotide sequences of the 5´end SST cDNA (505 and 874 bp). The amplified products were cloned in pGEM®-T Easy vector and plasmid carrying DNA were sequenced.

Finally, all products from 3´ and 5´RACE were combined electronically to generate a full length cDNA sequence. A consensus sequence was obtained which covers 70% of the open reading frame of the SST in *Pisum sativum* (Fig. 27) as deduced from the lengths of the ORF sequences of other legumes. In parallel, the SST complete cDNA was identified from a *Pisum sativum* transcriptome library obtained by next generation sequencing as described in section 3.10.The complete SST cDNA sequence (2377 bp) was submitted to NCBI with NIH gene bank accession number of KP759572. At the same time, the full length of putative SST cDNA sequence from *Pisum sativum* cultivar Finale (2178 bp) was published in NCBI by a Russian Research Institute with accession number KC008603.1. This sequence showed 90% query cover and 100% homology with our sequence (*Pisum sativum* cultivar Rocket).

AACCCTTGGTCAATTCAATATTTGAATTTTGACAGTAGATATTTACCAGCGGTGTTAAGAGCT
GGTTTAATCACTGGAGTTTTGTCATTAGCGGAAGGAATAGCCATAGGAAGAAGCTTTTCTGTT
ACTGATAATACACCTCATGATGGAAACAAAGAGATGATAGCTTTTGGACTTATGAATTTATTTG
GTTCTTTTACATCATGTTACTTAACCAGTGGACCATTTTCCAAGACTGCCGTGAATTATAATGC
AGGGTGTAAAAGTGCAATGACAAATGTGGTACAAGCAGTTTTAATGGCTCTTACACTACAATT
TTTGGCACCATTATTTGGCAACACACCACTTGTTGCTCTATCAGCTATTATTGTGTCTGCAAT
GTTGGGGCTCATAAATTATGAGGAAGCTATTTACCTCTTTAAAGTTGATAAATTTGATTTTGTT
ATTTGCATGAGTGCATTCTTAGGTGTTGCCTTTATAAGCATGGACATGGGCCTTATGATCTCC
GTTGGACTAGGTTTGATTAGAGGACTAATTTATTTGGCTAGACCTGCATCATGCAAACTTGGA
AAATTATCTGATTCTGGTATATACAGAGATGTTGAACRATATTCTAATGCTTCAAGAGTTCCAG
GAGTTTTAGCTTTACAAATTGGTTCTCCTGTTTATTTTTCAAATTCTACTTACATCAAAGAAAG
GATTTTGAGATATGTTAAAAGCGAACAAAGCTCTTCCGGAGATGATATCGAACATGTTATACT
CGATTTTACAGGAGTAACTTCCATTGACACAACTGGTATTGAAGGATTGTTGGAGACAAATAA
AGTGTTGGAAAGAAAAGGAATTCAGATGTCATTAGTAAATCCAAGGCTAGAGGTTATGGAGA
AGCTAATAGTATCWAAGTTTGTTGACAAGATTGGAAAGGAAAAATTCTATCTAAATTTGGACG
ATGCAGTGATGGCRAGTCAATATTCACTCAGAACATCTAAGACAAATAATAACGAAGAGACAC
TTTAAGAGACTTGTCATACCCATATATACATCAATAAATCTAAATTAGGTTATACACATTTTGAT
TGTTTTAATCTTTTTGTATCAATGTTGTATAACACTACTTTTTATGGAATTGAGAATGTGTTTAC
TAAAAAAAAAAAAAAAAAAAAAGTACTAGTCGACGCGTGRCCACTGATAGAGCAACAAGTGG
TGTGAAAAAAAAAAAAAAA

Fig. 27 Symbiotic sulfate transporter consensus sequence from the generated product with 3´ and 5´ RACE (1224 bp).

3.9 Functional complementation of symbiotic sulfate transporter in a yeast double mutant

The coding sequence of SST (ORF=1923 bp) was amplified by PCR using nodule cDNA as template and oligonucleotide primers from the 5´ and 3´ ends of ORF with the addition of restriction site overhangs (BamHI and SalI). First, the SST ORF was ligated into the pJET 1.2 vector used for transformation into E. coli cells. Subsequently, recombinant plasmids were extracted from E. coli cells and restricted by BamHI and SalI endonucleases. Fragments were separated in agarose gels to confirm a successful transformation of the SST gene into E. coli (Fig. 28).

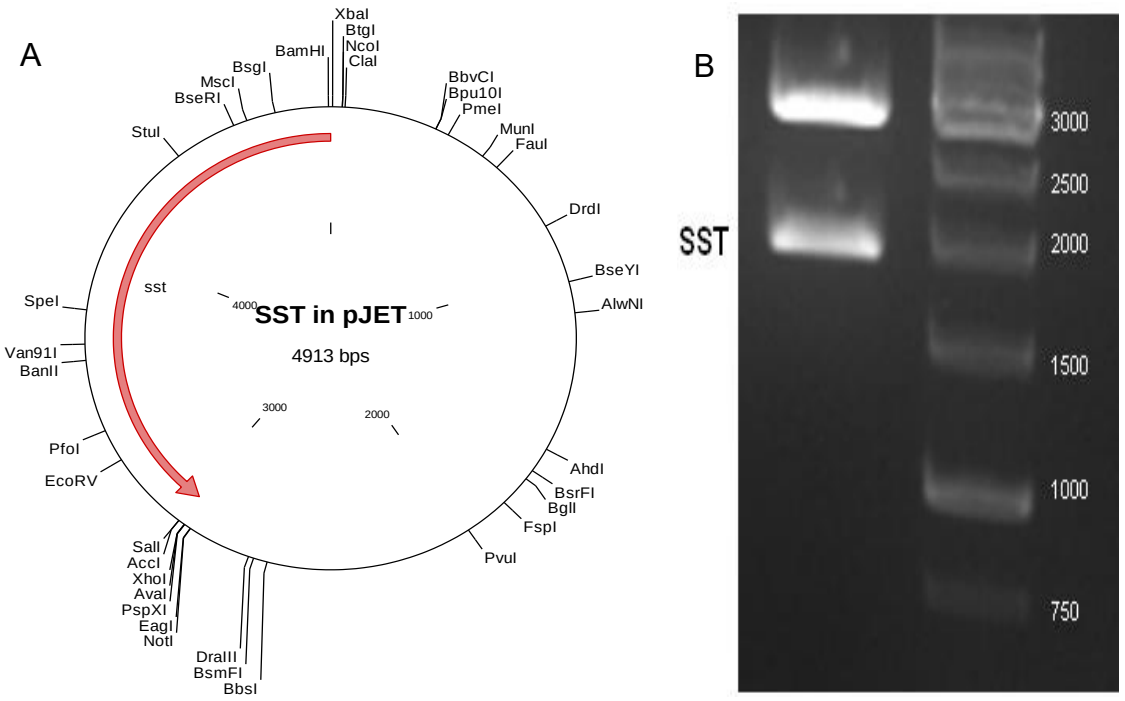


Fig. 28 Recombinant plasmid pJET –SST, A: The cDNA of SST in the pJET 1.2 vector. RT-PCR was performed using nodule cDNA as template and primers from 5´ and 3´ end of SST ORF contained the restriction site overhangs (BamHI and SalI).T4 ligase was used for insertion of SST fragment including BamHI and SalI sites. **B:** The SST fragment which was restricted by BamHI and SalI endonucleases. The upper band belongs to the pJET1.2 vector (2947 bp) and the lower band belongs to SST (1939 bp = ORF + restriction site overhangs).

In the next step, the ORF of SST was ligated into the yeast expression pESC_His vector. Hence the SST fragment (ORF) was released from pJET 1.2-SST vector using BamHI and SalI restriction enzyme. The pESC_His vector was digested with the same restriction enzymes. The purified ORF fragment was

ligated into the cleaved pESC_His vector for generation pESC- SST (Fig. 29). Finally, the recombinant plasmid (pESC- SST) was transformed into *E. coli* for post amplification and verification.

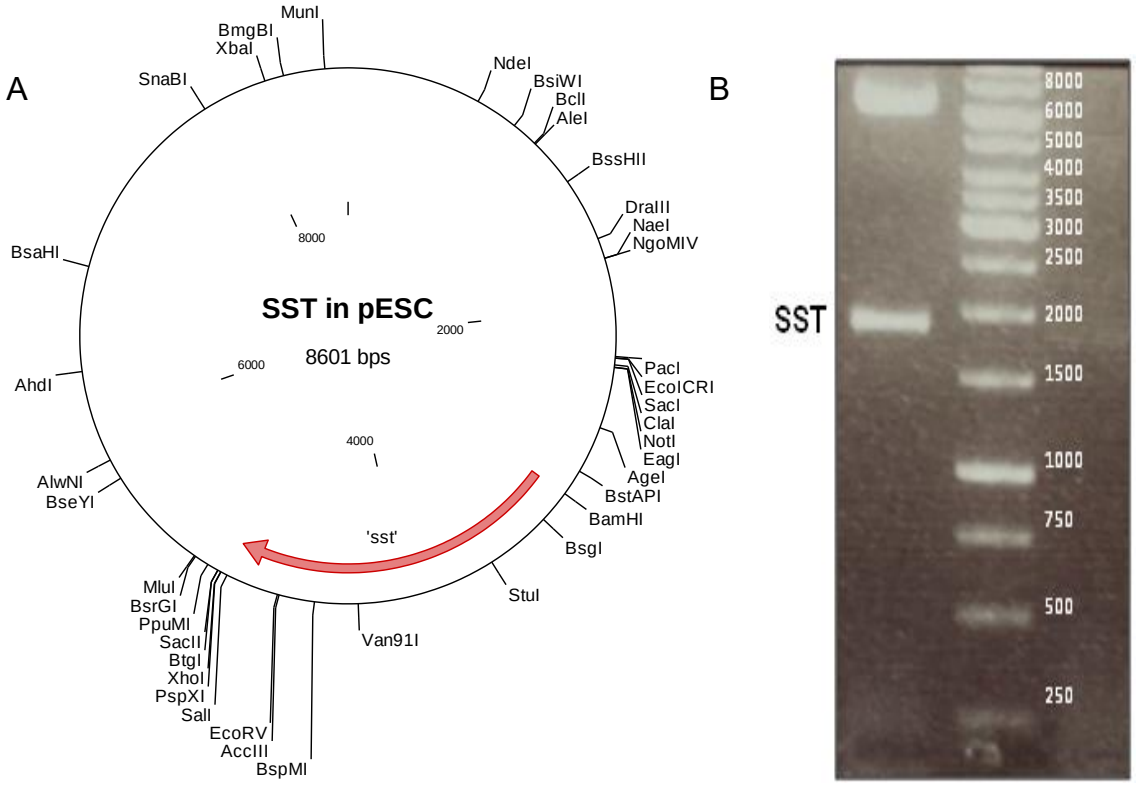


Fig. 29 The recombinant plasmid pESC-SST, A: The Constructs of SST in pESC_His vector. T4 ligase was used for insertion of the SST cDNA covering the ORF sequence using BamHI and SalI overhangs. **B:** SST ligations construct which was restricted by BamHI and SalI endonucleases. Upper band belongs to pJET1.2 vector (6672 bp) and lower band belongs to SST (1929 bp = ORF + restriction site overhangs).

For the functional proof of the enzymatic activity of the symbiotic sulfate transporter, the double mutant *Saccharomyces cerevisiae* strain CP154-7A (Mataα, his3, leu2, ura3, ade2, trp1, sul1:LEU2, sul2:URA3) and the yeast wild type strain INVSc1 (Mata. his3D1, leu2, trp1-289, ura3-52) were used. The yeast double mutant CP154-7A contains insertions in the two yeast sulfate transporters sul1 and sul2, therefore is unable to grow on low sulfur. The double mutant CP154-7A and the wild type INVSc1 were transformed using the yeast expression recombinant plasmid pESC-SST and the empty vector pESC-His as described in section 2.7.6. Transgenic yeast cells were selected on medium depleted in histidine. Subsequently, the selected colonies were grown in a synthetic minimal B medium with 0.5 mM magnesium sulfate as a sole sulfur source (Fig .30).

The expression of SST in the yeast mutant was able to complement the yeast mutant phenotype. Moreover, the growth of transgenic colonies (CP154-7A + pESC-SST) on the minimal B medium with 0.5 mM magnesium sulfate confirmed the function of the symbiotic sulfate transporter (SST) gene.

As a positive control, the empty vector pESC-His was transformed into the wild-type-like strain INVSc1 and transgenic colonies could grow on the selective medium without histidine. Furthermore, an empty vector was transformed into mutant strain CP154-7A as a negative control and the transgenic colonies could not grow on the B medium.

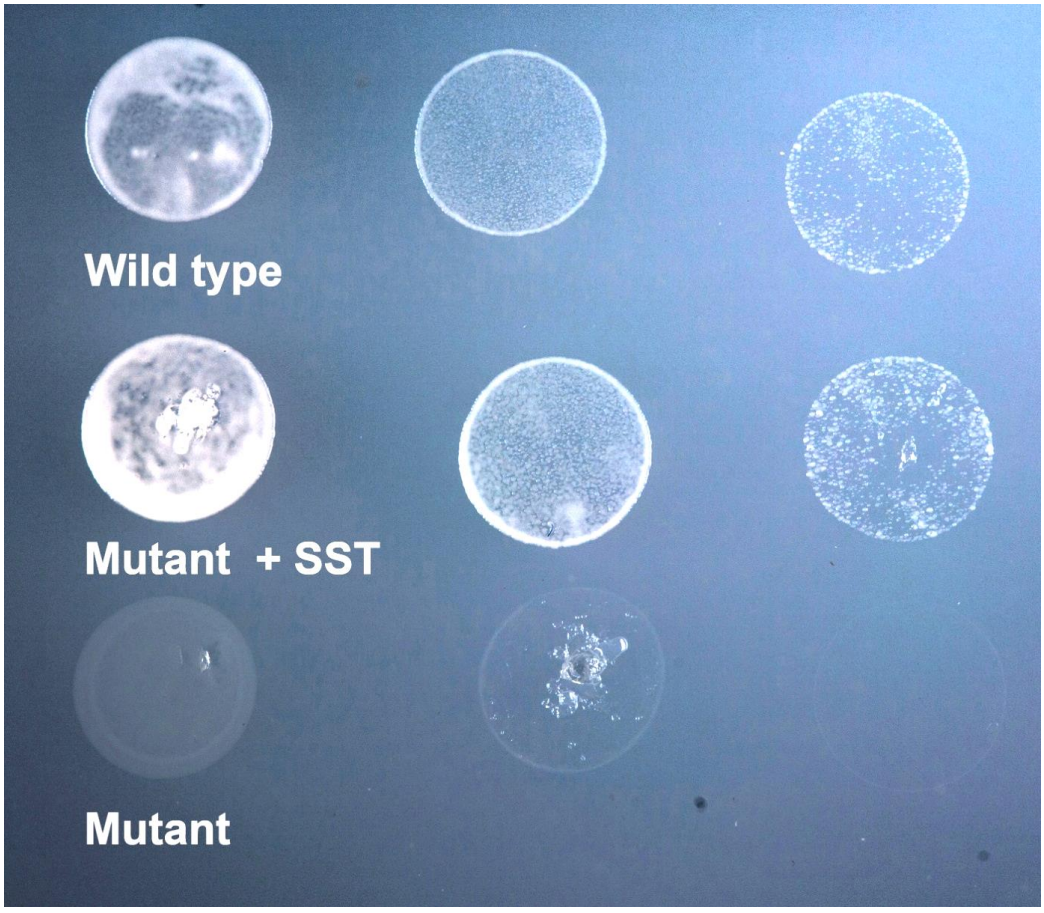


Fig. 30 Functional complementation of yeast sulfate transporter double mutant CP154-7A by *Pisum sativum* symbiotic sulfate transporter, yeast cells were grown on the synthetic minimal B medium with 0.5 mM magnesium sulfate as sole sulfur with 10-fold dilution series from left to right. Top row: The wild-type strain transformed with empty expression vector pESC-HIS was included as a positive control. Middle row: The mutant strain transformed with vector pESC-SST confirming functional complementation of SST. Bottom row: The mutant strain containing the empty vector pESC-HIS was included as negative control.

3.10 Identification of the sulfate and molybdate transporter gene family in *Pisum sativum* by transcriptome identification

In order to identify the pea sulfate transporter gene family, high quality RNA was extracted from the root, leaf and nodule tissues. RNA samples (one replicate for each tissue) were sent for RNA sequencing (BGI sequencing company, China). Large quantities of reads were obtained i.e. 52,407,480 paired-end reads for leaves, 51,860,856 paired-end reads for roots and 26,205,828 paired-end reads for nodule tissue. All reads were analyzed in order to trim low quality bases (<0.05), ambiguous nucleotides (more than 2 ambiguous bases) and the adapter sequences. Subsequently, de novo assembly of transcriptome was done for trimmed reads (Table 10). The processing of the reads and the assembly was done using CLC genomics workbench v7.0 (CLC Inc, Aarhus, Denmark) in collaboration with Molecular Phytomedicine Institute in the University of Bonn.

Table.10 Statistics summary of all transcriptome assembly

	Count	Average length	Total bases
Reads	154,373,710	90	13,893,633,900
Matched	141,912,204	90	12,772,098,360
Not matched	12,461,506	90	1,121,535,540
Contigs	69,875	797	55,700,349

The whole transcriptome assembly was used for identification of sequences of the pea sulfate transporter gene family using BLAST (with query sequence of most known sulfate transporter genes in plants) followed by multiple alignments of selected sequences with known sulfate transporters to test for the overall sequence homology. The comparison of the transcriptome assembly against the known sulfate transporter genes in other plants is described in section 2.9.4, which resulted in the identification of fourteen different sulfate transporter transcripts in *Pisum sativum* (Table 11) (see appendix, chapter 6.1 for complete sequences).

Sulfate transporter genes in pea and the related plants in the leguminous family were used to build a phylogenetic tree (Fig. 31). Phylogenetic analysis showed that sulfate transporters in *Pisum sativum* belong to five distinguished groups. Groups 1, 2, 3, and 4 are the main sulfate transporter groups in plants. While group 5 (Pssultr5) contains a distinct transporter which takes up other

micronutrients with similarity to sulfate such as molybdate. All sequences in group 5 accordingly are named as molybdate transporters (Psmotr).

Table.11 Sulfate transporter assembly characterized

Gene Id	Consensus length	Total read count	Average coverage
sultr1.1[1]	2289 bp	2151	73.21815408
sultr1.3a	2524 bp	869	30.01307448
sultr1.3b	2417 bp	2262	83.57757551
sultr2 2	2490 bp	1533	50.58660508
sultr3.1a	2319 bp	1959	75.77015955
sultr3.1b	2536 bp	4473	158.0406151
sultr3.3	2539 bp	3031	106.6494683
sultr3.4	2189 bp	581	23.71128369
sultr3.5	2377 bp	96905	3656.043753
sultr4.1	2441 bp	3733	230.9306237
motr1a[2](sultr5.1)	1838 bp	1838	20.91893362
motr1b(sultr5.1)	1589 bp	1185	66.86595343
motr1c(sultr5.1)	1854 bp	18308	885.6294498
motr2(sultr5.2)	2082 bp	5951	256.1791547

[1] sultr: sulfate transporter

[2] motr: molybdate transporters

In most of the groups, more than one sequence was identified and all sequences were submitted to NCBI with different NIH GenBank accession numbers (Table12).

Table.12 Pea sulfate transporter sequences submitted to NCBI with different NIH GenBank accession numbers

Gene name	NIH GenBank accession number	Gene Id	Complete cDNA (bp)
Pssultr1.1-like	KP759563.1	sultr1.1 [1](CRL28302 [4])	2273 bp
Pssultr1.3-likea	KP759565.1	sultr1.3(CNRL31544)	2417 bp
Pssultr1.3-likeb	KP759564.1	sultr1.3(CNRL20470 [5])	2524 bp
Pssultr2.2-like	KP759566.1	sultr2.2(CRL23196)	2490 bp
Pssultr3.1a-like	KP759567.1	sultr3.1(CNRL 17055)	2319 bp
Pssultr3.1b-like	KP759568.1	sultr3.1(CNRL 35817)	2536 bp
Pssultr3.3-like	KP759570.1	sultr3.3(CNRL 7291)	2539 bp
Pssultr3.4-like	KP759571.1	sultr3.4(CNRL 8499)	2189 bp
Pssultr3.5-like	KP759572.1	sultr3.5(CNRL423)	2377 bp
Pssultr4.1-like	KP759573.1	sultr4.1(CL 3117[3])	2441 bp
Psmotr1-likea(Sultr5.1)	KP759574.1	motr1 [2] (CNRL6468)	1838 bp
Psmotr1-likeb(Sultr5.1)	KP759576.1	motr1(CNRL1193)	1589 bp
Psmotr1-likec(Sultr5.1)	KP759577.1	motr1(CNRL2061)	1854 bp
Psmotr2-like(Sultr5.2)	KP759575.1	motr2(CNRL2300)	2082 bp

[1] sultr: sulfate transporter

[2] motr: molybdate transporters

[3] CL: extracted contig from leave mRNA library

[4] CRL: extracted contig from root and leave mRNA library

[5] CNRL: extracted contig from nodule, root and leave mRNA library

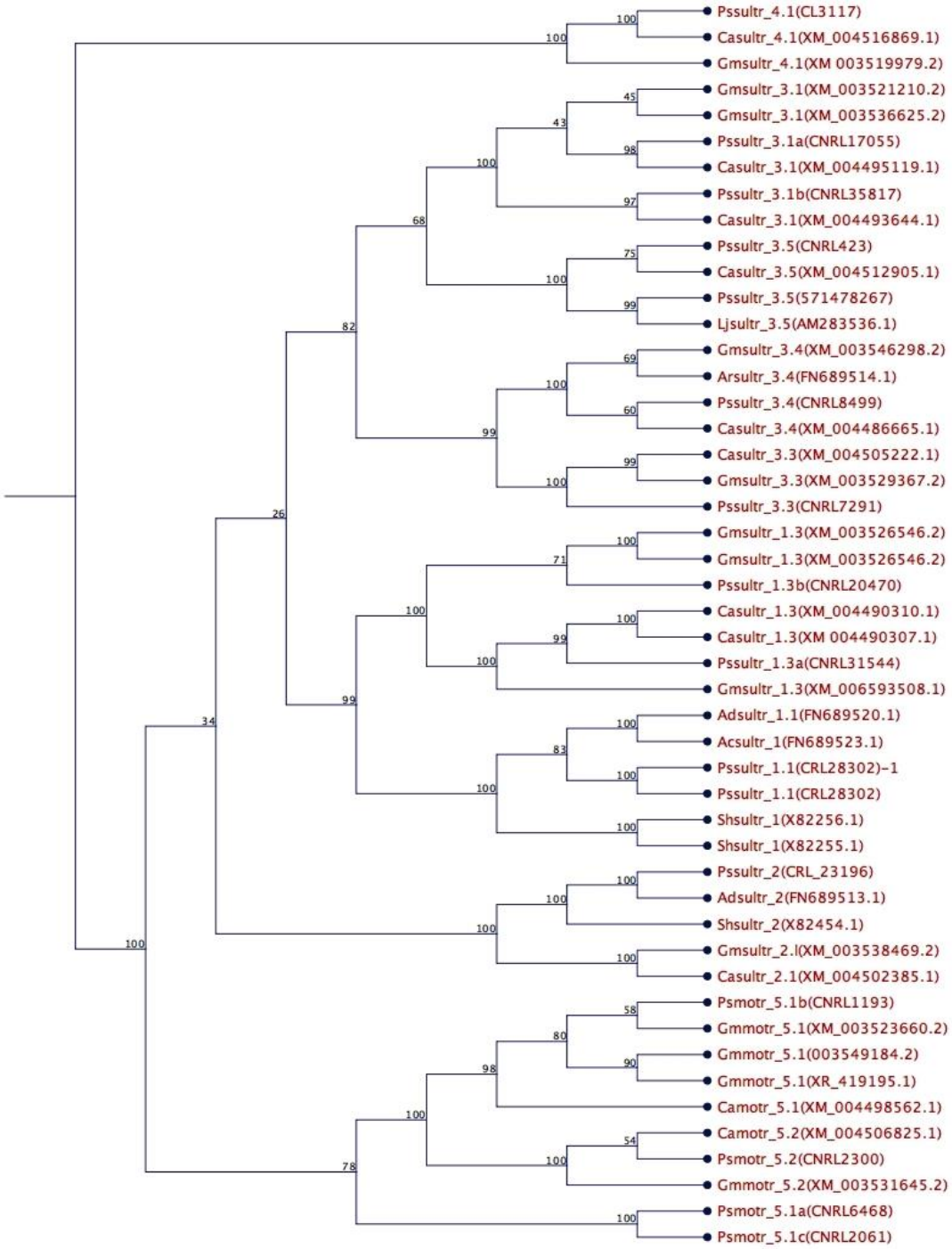


Fig. 31 Phylogenetic analysis of sulfate transporter genes in leguminous, A cladogram of a neighbor joining tree with 1000 bootstrap made out of *Pisum sativum* (Ps) sulfate transporter transcript and their homologues within *Astragalus drummondii* (Ad), *Astragalus crotalariae* (Ac), *Stylosanthes hamata* (Sh), *Cicer arietinum* (Ca), *Lotus japonicus* (lj), and *Glycine max (Gm).* The transcript IDs (in *Pisum sativum*) and accession numbers are indicated in brackets .The bootstrap value that were obtained from replicate trees is shown as a percentage beside each of the corresponding branch.

4 Discussion

S deficiency alters the symbiotic interaction between leguminous plants and soil nitrogen fixation bacteria which leads to a decline in N_2 fixation rate. In the present study, we investigated how different levels of S deficiency affect the factors related to N_2 fixation in *Pisum sativum*. Thus, the hypotheses were that S deficiency changes the phenotype and growth characteristics of the pea and that S deficiency affects the protein and mRNA expression of genes related to N_2 fixation. Moreover, we identified a symbiotic sulfate transporter (SST) in *Pisum sativum* and performed the functional analysis of the SST in yeast double mutant. Furthermore, we generated large quantities of RNA sequencing data from the root, leaf and nodule tissues in *Pisum sativum*. The sequence homology was investigated in the whole transcriptome assembly in order to identify the pea sulfate transporter gene family.

4.1 Plant performance and changes in the contents of chlorophyll, leghemoglobin and sulfur to S deficiency

Severe S deficiency led to a dwarf phenotype in *Pisum sativum* while weak S deficiency did not remarkably restrict plant growth (Fig. 10 and 11). Growth retardation in S deficient plants followed by an earlier flowering is a consequence of the reduction in proteins, chlorophyll and biomass (Nikiforova et al., 2005).

Biosynthesis of chlorophyll is decreased by N starvation which is a consequence of low nitrogen fixation rate in S deficiency condition. S deficiency reduces the biosynthesis of S compounds such as S-adenosyl methionine (SAM) which donates a methyl-group during chlorophyll synthesis. Therefore, a decline in the SAM reduces leaf chlorophyll content and induces chlorosis (Zhao et al., 1999a; Hoefgen and Nikiforova, 2008). Also S containing amino acids (methionine and cysteine) decreases with S deficiency and subsequently reduces protein synthesis (Khan et al., 1996; Scherer, 2001; Nikiforova et al., 2003; Khan and Mazid, 2011; Chorianopoulou et al., 2012). As a consequence of a decrease in protein synthesis, particularly the proteins involved in the photosynthetic process, loss of chlorophyll protein happens. Our data confirmed that severe S deficiency in the generative phase had no effect on chlorophyll content of mature leaves (Fig.10). Concomitantly, severe S deficiency declines the chlorophyll content of young leaves by 43% in comparison with the control plants (Fig. 14)

resulting in chlorosis and yellowish leaves. It is coincident with sulfate remobilization from mature leaves to roots to support root proliferation (Abdallah et al., 2010; Honsel et al., 2012). The decline in total chlorophyll in mature leaves when switching from the vegetative to the generative phase might be the consequence of a high sulfate remobilization into generative organs such as silique and seeds (Liao et al., 2012). Moreover, the decline in chlorophyll content of mature leaves is likely due to chlorophyll breakdown in the senescent leaves.

Weak S deficiency did not affect the shoot and root biomass in contrast to severe S deficiency (Fig. 11). The significant decline of shoot biomass in severe S deficient plants is probably the result of a decline in photosynthesis and nitrogen fixation rate. A decline in photosynthesis rate and in energy source is associated with less chlorophyll content of leaves in S deficient plants. Moreover, S limitation reduces the presence of proteins with high S content in the chloroplast leading to impairment in all types of photosynthetic activities (Terry, 1976; Astolfi et al., 2006). Furthermore, a decline in nitrogen fixation leads to a lower protein content and accumulation of nonprotein nitrogen compounds and subsequently decreases yield (Lange,1998; Pacyna et al., 2006; Khan and Mazid, 2011)

Root proliferation is a general response to the nutrient deficiency in plants in order to increase nutrient uptake. However, in the present investigations with pea, root biomass did not respond to S deficiency (Fig. 11). Different response of the root and shoot tissue to S deficiency led to a significant increase in the root to shoot ratio (Fig.11) which is consistent with previous results (Lopez-Bucio et al., 2003; Hawkesford and De Kok, 2006; Abdallah et al., 2010; Varin et al., 2010; Ciaffi et al., 2013).

Disruption of N_2 fixation is the most critical consequence of S deficiency in the legume. S deficiency may reduce N_2 fixation due to less nodule development, leghemoglobin reduction and nitrogenase deactivation (Scherer and Lange, 1996; Pacyna et al., 2006; Scherer et al., 2008b; Khan and Mazid, 2011, 2011). During this study, both weak and severe S deficiency in pea reduced the quality and quantity of nodules (Fig. 12 and 13). Weak S deficiency resulted in small nodules with green color at the base. Severe S deficiency had stronger effects and resulted in faint, green and premature senescence nodules (Fig.12). Sufficient leghemoglobin represents a pink color in nodules while green color is a

consequence of a modified leghemoglobin protein in the senescence nodules. During aging or stressful conditions of nodules, accumulation of NO_2 and a pH decrease down to 5.5 results in nitration reactions in leghemoglobin in which nitrated heme group [heme-NO_2] leads to green and aberrant O_2 binding leghemoglobins (Jun et al., 1994; Navascues et al., 2012)

Our data confirmed the importance of S for an effective nodule formation and is in line with previous reports showing the highest S content of the nodule compared to the root and shoot tissues. In other words, S content of ineffective nodules is considerably lower than the effective nodules (Zhao et al., 1999b; Pacyna et al., 2006; Scherer et al., 2006; Varin et al., 2010).

Severe S deficiency reduced leghemoglobin content of nodules in the generative phase (Fig.15) which probably leads to nitrogenase deactivation because of a high oxygen concentration and a disruption in the oxygen buffering system of leghemoglobin. Indeed, S deficiency deactivates influence of iron-sulfur cluster in the active site of nitrogenase enzyme and leads to less N_2 fixation (Nikiforova et al., 2003). Also, our result is consistent well with the previous result which showed symbiotic leghemoglobin is crucial for SNF and disruption in leghemoglobin synthesis leads to loss of bacterial nitrogenase protein and SNF (Ott et al., 2005).

S deficiency influenced S concentration of the root and shoot tissues. The S content of the shoot changed more than of the root in the vegetative phase and decreased significantly by both weak and severe S deficiency (Table 9). In the root tissue, we observed a trend of S decrease ($P<0.08$) which in line with a higher tolerance of root tissue to S deficiency because of root proliferation and sulfate remobilization from the mature leaves to the root tissue (Abdallah et al., 2010; Honsel et al., 2012). In contrast to the vegetative phase, in the generative phase S content of both tissues (root and shoot) in both severe and weak deficiency decreased in comparison to the control plants. This implies that long term limitation influences S content of both tissues. The leaf vacuole is the main storage site for sulfate after uptake by the root and the *chloroplast* is the main site for sulfate reduction and assimilation (Buchner et al., 2004a). Therefore, S content of the shoot tissue should be relatively higher than the root tissue which is reflecting our results (Table 9) and is in agreement with previous results (Varin et al., 2010; Honsel et al., 2012; Ciaffi et al., 2013).

4.2 Expression of SNF related genes under S deficiency condition

4.2.1 Leghemoglobin

In SNF process of the pea, leghemoglobin isoproteins with two high and low O_2 binding affinities protect the nitrogenase enzyme against O_2 damage (Uheda and Syōno, 1982; Kawashima et al., 2001). Under S deficiency conditions, the low leghemoglobin content in nodules has been reported previously (Appleby, 1984; Scherer et al., 2008b; Varin et al., 2010). Nevertheless, our mRNA data provide new evidence for leghemoglobin content changes under S deficiency condition. Transcript and protein abundance of the two leghemoglobin genes (PsLb5 and PsLb120) showed a similar response to S deficiency in vegetative phase and did not change significantly in response to weak and severe deficiencies (Fig. 15), confirming previous data (Kawashima et al., 2001) showing expression of the two genes is essential to create a low O_2 concentration in young nodules. In contrast to vegetative phase, long term severe S deficiency reduced expression of Pslb5 and Pslb120 in the generative phase of pea (at mRNA and protein level). Our result is different than another research showing that expression of Pslb5 increases under S depletion (Pacyna, 2005). Kawashima et al. (2001) suggested expression of the low O_2 affinity leghemoglobin (Pslb120) is limited to the initial stage of SNF in young nodules and reduces noticeably in mature nodules. In contrast, we found that Pslb120 is also expressed in the generative phase (accompanied with mature nodules) similar to Pslb5 and decreased only with sever S deficiency. This implies that expression of both Pslb5 and Pslb120 is critical in pea nodules to support an effective SNF.

Furthermore, the higher leghemoglobin content of nodules in the generative phase (Fig. 15) may represent a higher leghemoglobin demand of mature nodules to adjust a constant nodule free O_2 level in an effective SNF (Ott et al., 2005). The leghemoglobin content of severely S deficient plants tended to increase in the generative phase compared to vegetative phase, although mRNA expression of both Psl5 and Pslb120 genes was decreased in generative phase (Fig. 15 and 16). This might be due to the poor relationship between protein and mRNA data under severe S deficient conditions and remained to be clarified.

4.2.2 Ferredoxin

Ferredoxin is one of the important iron-sulfur proteins and is the main electron donor for the N_2 reduction in SNF. Ferredoxin shows a tissue specific expression pattern. Ferredoxin relative transcript abundance is strongly light dependent. In pea, a single ferredoxin gene has a different expression in etiolated and green tissues (Elliott et al., 1989a; Elliott et al., 1989b; Gallo-Meagher et al., 1992a; Gallo-Meagher et al., 1992b; Hansen et al., 2001).The two main isoforms of plant ferredoxin are known as root type ferredoxin (non-photosynthetic) and leaf type ferredoxin (photosynthetic), respectively. The root type ferredoxin mainly mediates electron flow from ferredoxin-NADP reductase (FNR) to sulfite reductase (SiR) in sulfate assimilation (Yonekura-Sakakibara et al., 2000). Severe S deficiency reduced relative transcript abundance of ferredoxin in roots of our cultivated pea in the vegetative phase (Fig. 19). It is likely that pea non-photosynthetic ferredoxin (root type) which supports sulfite reduction (SiR) is transcribed in a lower degree during severe S deficiency. Consequently, the relative transcript of root ferredoxin exhibited a significant reduction in shifting from the vegetative to the generative phase in control plants. This reduction did not occur in S deficient plants probably caused by the suppressing effect of S deficiency on ferredoxin transcript abundance in the vegetative phase. However, it is peculiar why the transcript fluctuations did not reflect ferredoxin protein expression in the root illustrating a stable protein expression (Fig. 21).

In contrast to the root, the relative transcript of ferredoxin was up regulated with the severe S deficiency in both vegetative and generative phases in the nodule (Fig.19). Even the weak S deficiency induced the ferredoxin transcript in the vegetative phase. These inductions represent a higher ferredoxin requirement to support an adequate electron supply for N_2 reduction (Mortenson, 1964). Therefore, expression of ferredoxin in the nodule increases in S deficiency conditions to compensate S limitation for the synthesis of adequate iron-sulfur ferredoxin proteins. Similarly, the higher level of ferredoxin expression in generative phase compared to vegetative phase could be accompanied by more SNF activity in the generative phase to support a higher electron supply.

Nevertheless, the transcript abundance pattern of ferredoxin in nodules was not reflected on the western blot analysis (Fig. 23). The amount of ferredoxin

protein remained fairly constant and did not follow the transcript levels in both vegetative and generative plants. This behavior shows that ferredoxin is an extremely stable protein in agreement with the response of the plant type ferredoxin of *Chlamydomonas reinhardtii* to S deficiency (Jacobs et al., 2009). Nevertheless, such kind of discrepancies between mRNA and protein abundance has been reported for other proteins such as the cytochrome response to copper deficiency (Quinn et al., 2003). The different patterns of ferredoxin expression between root and nodule tissue might be related to a difference in their post transcriptional regulation in the nodule.

Expression of leaf type ferredoxin is strongly light-dependent and this protein supports NADP photo reduction in the chloroplast (Blanco et al., 2011). Under adverse environmental conditions such as iron deficiency, ferredoxin transcripts and polypeptides decrease and leads to decreased CO_2 assimilation, leaf chlorosis and growth arrest (Thimm et al., 2001; Mazouni et al., 2003; Blanco et al., 2011).In our cultivated pea, S deficiency influenced relative transcript abundance of ferredoxin in the young leaves more than the mature leaves (Fig. 20). Ferredoxin in mature leaves did not respond to S deficiency because mature leaves are the main site of sulfate storage, assimilation and reduction (Buchner et al., 2004a; Davidian and Kopriva, 2010). Ferredoxin in young leaves decreased under S deficiency because sulfate remobilization under S deficiency condition occurs from mature leaves to roots instead of young leaves (Honsel et al., 2012). Therefore, the insufficient sulfate content in young leaves can interrupt the biosynthesis of iron-sulfur clusters in ferredoxin proteins (Touraine et al., 2004). As well as insufficient sulfate can reduce cysteine biosynthesis while cysteine is required for ferredoxin production because there are four highly conserved cysteine residues in ferredoxin proteins. Accordingly, both severe and weak S deficiency reduced the ferredoxin relative transcript abundance of young leaves in the vegetative phase (Fig. 20). Based on the immunodetection results, ferredoxin abundance of young leaves decreased with severe S deficiency which confirms the mRNA expression data (Fig. 22).

The reduction in ferredoxin relative transcript of mature leaves in the generative phase as compared to the vegetative phase (Fig. 20) is likely due to lower chlorophyll content (Fig. 15) and subsequently less ferredoxin is needed for NADP photo reduction of photosynthetic activity in mature or senescence leaves.

4.2.3 Nitrogenase

Nitrogenase (heterotetrameric dinitrogenase) enzyme is a highly conserved protein which is coded by a large nifHDK gene cluster in most of the nitrogen-fixing bacteria. Every active nitrogenase complex contains a lot of S-containing amino acids in its structure indicating to a high sulfur demand to accomplish the full enzyme activity. Dinitrogenase protein contains two pairs α β subunits with Fe-Mo-cofactors (7Fe-9S-Mo-C-homocitrate) in each α subunits and Fe-S cluster (8Fe:7S) between each α and ß subunits. NifH protein (dinitrogenase reductase) contains (Fe4-S4) cofactor transferring electrons from the ferredoxin (2Fe-2S) to the dinitrogenase (Schlessman et al., 1998; Thiel and Pratte, 2014; Zhang et al., 2015). Long term S deficiency in pea led to a lower sulfur S supply for biosynthesis of Fe-S cluster in the nitrogenase subunits and subsequently reduced the expression of nitrogenase (Fig.18). Alternatively, S limitation could reduce nitrogenase complex activity through lower concentration of NifH protein which is observed in *lotus japonicus* (Krusell et al., 2005).

In the current study, relative transcript abundance of α subunits (NifD gene) was mostly constant with S deficiency, while relative transcript abundance of β subunits (NifK gene) tended to decrease with severe S deficiency in the generative phase (Fig.18). Moreover, a higher relative transcript abundance of NifK in the generative phase (accompanied with mature nodules) compared to the vegetative phase (accompanied with young nodules) indicated a higher nitrogenase activity and SNF in mature nodules which is in agreement with a previous report (Fischinger and Schulze, 2010). However, this induction was not observed in sever S deficiency and confirmed a less nitrogenase activity of inefficient nodule. Furthermore, relative transcript abundance of α subunits (NifD gene) decreased in sever S deficiency condition in the generative phase leading to a less nitrogenase activity in inefficient nodules (Fig.18).

4.3 Identification of sulfate transporter genes in *Pisum sativum*

4.3.1 Symbiotic sulfate transporter in *Pisum sativum*

An efficient SNF in the nodule demands a strong nutrient exchange between plant and bacterial cells. Nodule function is strongly dependent on S supply by plant cells. In *Pisum sativum*, S content of the nodule is considerably higher than the root and shoot tissues even under S deficiency conditions (Zhao

et al., 1999b; Pacyna et al., 2006) confirming the high S demand of the nodules. Leghemoglobin and ferredoxin are the most dominant proteins in nodules which are extremely sensitive to S supply. Likewise, nitrogenase is a conserved protein with lots of Fe-S clusters in all nitrogen-fixing bacteria. Therefore adequate S supply for full activity of nodules requires the presence of a specific sulfate transporter.

The symbiotic sulfate transporter (SST) is a nodule-specific sulfate transporter with the ability to transfer sulfate from the plant cell cytoplasm to the rhizobia. The SST was first reported in *Lotus japonicus* as a crucial gene in SNF (Krusell et al., 2005). Transcript abundant of SST in the root and leaf was reported at 1% and 4% of SST transcript abundant in the nodule, respectively, which confirms that SST is a nodule specific gene. Furthermore, the SST location in nodule´s symbiosome membrane indicates that it is involved in sulfate transfer from the host cell to the bacteria cells (Wienkoop and Saalbach, 2003). Here, we identified and sequenced a cDNA region of SST in *Pisum sativum* (Pssul3.5) using two different methods i.e. RACE and Next Generation Sequencing. The SST sequence of *Pisum sativum* showed a high homology with other SST genes in leguminous plants, and its function was confirmed by expression in the yeast sulfate transporter mutant CP154-7A (Cherest and Surdin-Kerjan, 1992) (Fig. 30).

Many sulfate transporter genes have been reported in different plants. The sulfate transporters can be divided into two high-affinity groups and a low-affinity group (Smith et al., 1995a). Currently, phylogeny analysis displayed at least 4 distinct groups of sulfate transporters in higher plants. Group 1 with high affinity to sulfate contains the main transporters in the root for soil sulfate uptake while group 2 is known to contain a low affinity transporters to mediate internal sulfate transfer in the root and shoot tissues (Yoshimoto et al., 2002; Buchner et al., 2004b; Buchner et al., 2010; Takahashi, 2010). Group 3 is the largest group and the majority of the members seem to indicate some be redundant because the sequences are highly similar. These sequences might be derived from gene duplication or polyploidization (Hawkesford, 2003). Finally, group 4 which does not belong to the plasma membrane transporters is located on vacuolar tonoplast and is involved in remobilizing vacuole-stored sulfate (Kataoka et al., 2004; Buchner et al., 2010; Zuber et al., 2010).

Phylogenic analysis revealed that the SST gene in *Pisum sativum* (Pssul3.5) belongs to group 3 (Fig. 31) and is placed as a sister group to *Cicer arietinum* and *Lotus japonicus* which confirms its close relationship with symbiotic sulfate transporter gene in other legumes.

A mutation in SST gene in *Lotus japonicus* leads to a low concentration of NifH protein in the nitrogenase complex (Krusell et al., 2005). In the present investigations, severe S deficiency resulted in a lower transcript abundance of SST in pea and a disruption in sulfate transport into symbiosomes (Fig. 17). Therefore, reduced sulfate import into the nodule probably results in lower expression of the nitrogenase gene (NifK) and eventual interruption of SNF.

4.3.2 Sulfate and molybdate transporter genes in *Pisum sativum*

Different groups in the sulfate transporter family correspond to specific functions or redundancy in patterns. Overall uptake, storage and remobilization of sulfate to the cells are coordinated by different sulfate transporter members. Sulfate transporters are differentially expressed according to the development stage and the availability of S as a nutrient (Buchner et al., 2010; Davidian and Kopriva, 2010). In the present study, 10 sulfate transporters (Pssul) and 4 molybdate transporters (Psmo) were identified in *Pisum sativum* using next generation sequencing (Table 12).

Phylogenetic analysis of full length sulfate transporter sequences from pea (Pssul and Psmo) and known sulfate transporters (sultr) from leguminous family revealed that they fall into five major groups.

Sulfate transporters in group 1 are plasma membrane transporters with high affinity to sulfate. Group 1 mediates primary sulfate uptake into the root (sultr-1.1, sultr-1.2) and a sulfur transfer from the source organs to the sink organs (sultr-1.3) (Yoshimoto et al., 2002; Yoshimoto et al., 2003; Rouached et al., 2009). Sufficient sulfate represses S uptake because a re-supply of sulfur to S deficient plants rapidly reduces the relative transcript abundance of group 1 (Smith et al., 1997; Buchner et al., 2004a). Therefore, rapid mRNA turnover is one of the regular characteristics of group 1 sulfate transporters in the root and shoot tissues. Our phylogenic result (Fig. 31) revealed only one sultr-1.1 and sultr-1.3 genes, while sultr-1.2 were not identified in pea. Pssul-1.1 was placed as a sister group to the high-affinity sulfate transporter of *Stylosanthes hamata* and two

Pssul-1.3 genes were placed as sister groups to *Glycine max* and *Cicer arietinum*. We did not prove functionality of putative sulfate transporters in pea except sultr3.5 but a close phylogenetic relationship between pea sulfate transporters and the known sulfate transporters in the leguminous family suggests a similar function for the pea sulfate transporter homologs. Therefore, Pssul-1.1 might be the main high affinity sulfate transporter in the pea and might control sulfate uptake in the root and sulfate re-distribution from the shoot to the root tissue in order to enhance the root proliferation under S deficiency conditions. A similar behavior is reported in different species for higher expression of sul-1.1 and sul-1.3 under S deficiency conditions (Yoshimoto et al., 2007; Casieri et al., 2012; Hubberten et al., 2012a; Gallardo et al., 2014).

Sulfate transporters in group 2 belong to the low affinity transporters with internal sulfate transfer from the root to the leaves. The sulfate transporters in group 2 mediate loading and uploading of sulfate via the xylem, phloem and leaf bundle sheath cells (Takahashi et al., 2000). Similar to wheat and *Brachypodium*, we found only one putative sultr-2 like (Fig. 31). Pssul-2.2 like was placed as a sister group to the low-affinity transporter in *S. hamata* and probably is expressed in the root and shoot tissues for a long distance sulfate transport.

Sulfate transporters in group 3 are the largest group in the plants with multiple specialties and redundancy functions. Transcript of group 3 is reported in different developmental stages of seeds and is responsible for sulfate translocation in seeds (Zuber et al., 2010). Abiotic stress regulates mRNA expression of sulfate transporters in group 3. Expression of sulfate transporter 3.1 in *Medicago truncatula* increased in response to the drought and salt stress and was strongly associated with abscisic acid (ABA) biosynthesis in the root plastids. Therefore, sulfate transporter 3.1 provides sulfate for biosynthesis of cysteine, which serves as a sulfur donor in ABA biosynthesis (Gallardo et al., 2014). The other ortholog of group 3 is sulfate transporter 3.5 which plays an additional role in leguminous nodules. The sulfate transporter 3.5 is known as symbiotic sulfate transporter to supply adequate sulfur for efficient SNF (Krusell et al., 2005; Varin et al., 2010). The symbiotic sulfate transporter in pea (Pssul-3.5) was placed as a sister group to sultr-3.5 groups in *Cicer arietinum* and *Lotus japonicus*. Expression of sul3.5 in the root and leave of wheat is extremely lower than the other sulfate transporters and transcript level of wheat sul3.5 does not

change significantly under S deficiency condition (Buchner et al., 2010). Krusell et al. (2005) demonstrated that the expression of sulrt3.5 in *Lotus japonicus* is relatively low in the leaf and root tissues and in contrast is extremely high in the nodule confirming a critical role of this gene in SNF process. To our knowledge, there is no information available about nodule sultr-3.5 responses to S deficiency condition. Here, we showed that relative transcript abundance of Pssul-3.5 was reduced extremely under severe S deficiency (Fig. 17) and might explain the SNF interruption. Lower expression of sultr-3.5 under S deficiency conditions results in less S supply in the nodule and interruption in biosynthesis of sulfate related metabolites such as nitrogenase, leghemoglobin and ferredoxin proteins.

In vacuoles as the main sulfate storage pool, sulfate is transferred to the cytoplasm by sulfate transporters of group 4 (Kataoka et al., 2004; Ohkama-Ohtsu et al., 2004; Buchner et al., 2010). Limitation in cytoplasmic sulfate induces expression of both sulfate transporter group 1 and 4. The high-affinity plasma membrane transporter (sultr-1like) induces a maximum sulfate uptake. Vacuolar efflux transporter (sultr-4like) releases vacuole-stored sulfate and remobilizes it to the cells (Buchner et al., 2010). Phylogenetic analysis revealed that Pssul 4.1 was placed as a sister group to *Glycine ma*x and *Cicer arietinum* transporters (Fig. 31) and is likely induced under S deficiency condition as is known in the other legumes. (Kataoka et al., 2004; Casieri et al., 2012).

Sulfate transporters in group 5 are functionally distinct from the other sulfate transporter groups. The sultr-5 (also named motr) members participate in uptake and translocation of micronutrients (selenate and molybdate) with a similar charge and size like the sulfate ion. Four different pea molybdate transporter sequences show a close phylogenetic relationship with *Glycine max* and *Cicer arietinum* molybdate transporters (Fig. 31). Molybdate transporters could participate in uptake and translocation of either molybdate or sulfate (Tomatsu et al., 2007; Fitzpatrick et al., 2008) or participate in a specific transport and accumulation of molybdate in the shoot tissue (Baxter et al., 2008). Reduced amounts of available sulfate in the soil favor selenate and molybdate uptake which leads to a higher accumulation of selenate and molybdate in the root and shoot tissue although the plant's requirement for this micronutrients is scarce (Shinmachi et al., 2010). Moreover, S deficiency induces selenate and molybdate

uptake because higher expression of different sulfate transporters would concomitantly transport these anions (Cabannes et al., 2011).

5 Outlook

The experiments presented in this thesis are based on the analysis of the effects of different levels of S deficiency on N_2 fixation in *Pisum sativum*. In this regard, it will be interesting to remove all sulfur sources completely (tp zero) and in parallel to control atmospheric sulfur (mainly SO_2 and H_2S which can be absorbed by plants through stomates) to study the effect of the absence of sulfur on pea growth and development and N_2 fixation.

Disruption of N_2 fixation is the most critical consequence of S deficiency in legumes. We showed how different S deficiency conditions changed the expression of some genes related to the SNF process. We found that a single ferredoxin gene in pea has a different expression in the root and leaf tissues. Furthermore, ferredoxin expression in the root nodules was noticeably different than root tissue. It is likely that in addition to eukaryotic ferredoxin derived from to plant cells, prokaryotic ferredoxin belonging to the bacteroid in the nodule tissue might influence or interfere with plant-type ferredoxin. Therefore, in future research, it might be interesting to study two different ferredoxins under the same condition to unravel the differences and interferences and to distinguish between plant and bacteroid ferredoxins in SNF.

In the SNF process, we only measured the expression of two major genes related to the α β subunit of the nitrogenase protein (NifK and NifD). It should be informative to investigate the S deficiency effect on the expression of other genes coding for subunits of then nitrogenase protein complex such as NifH (encoding the nitrogenase reductase) and NifS (encoding the cysteine desulfurase which releases sulfur for the synthesis of Fe-S clusters of nitrogenase).

Based on RNA sequencing data of the root, leaf and nodule tissues in *Pisum sativum*, the sequence homology was investigated in the whole transcriptome assembly to identify the pea sulfate and molybdate transporter gene family. Moreover, our RNA sequencing library would be a valuable research tool to improve the quality of *Pisum sativum* sequence data and to identify unknown genes.

Among new sulfate transporters genes of *Pisum sativum* (10 sulfate transporters and 4 molybdate transporters) identified in the present study, only one symbiotic sulfate transporter was functionally tested in the yeast double mutant. Thus, the others sulfate and molybdate transporter genes can be subject

of future research in terms of functionality assessment.

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7 Appendix
7.1 Sulfate and molybdate transporter gene sequences from *Pisum sativum*

Pisum sativum sulfate transporter 1.1-like protein (Sultr1.1) mRNA, complete cds
GenBank: KP759563.1

TTTGGTGATTGATTAAAATGAGTCAAGTTTTTAGTGATGGAGTTGTGGCAAGAGCCACTGAGGAAATAAA
TAGCAATCCTCCTTCACGTAGACATGCTGAAAACATTCTTCCACATAGGCACAAAGTTGCATCTCCTCCA
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ATTCCTCAGGATATTGCATATGCAAAGCTTGCAAATTTGGAACCACAGTATGCACTATATACAAGTTTTG
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TCAAGGACAGAATATTGAAGTGGCTGACTGATGAAGAGGCTCAAAGGACAGCAAGTGAATTTGCCAGTAT
ACAGTATCTCATCGTTGAAATGTCGCCTGTTACTGATATTGACACTAGTGGCATTCATGCCTTAGAAGAT
TTACTCAAGAGCCTCAAGAAAAAAGATGTGCAGCTTCTTGTGGCGAATCCGGGACCGATTGTAATTGAGA
AGCTCCATGCATCAGAGTTATCAGGTGTAATTGGGGAAGATAAAATATTTCTAACAGTAGGTGATGCTGT
TGCAACTTTTGGTCCAAAGGGTGTAGATTCTTGAACAACATGATAAACTGTTCTAATTGAAGAAAAAGGG
TGTGTGAAGGTGTTAATGAAGCTCTTAGGAACAAGGAAAGGGCATGTAGATTTGTTTTCCTTAGTTTTTG
TTTGATACTCGTTTATTTTTAATTTCAATTGATGTCATGTAGAAAGTCATAAGGGAAAAAATGTGTTTTT
TATGTTGATTTTATTATATTATTATACCTCTTGTTTATGGTCTTATTTTGGTGTGGAAGTTAATATGAAT
ATTCTTTTGTGAATTATATATACTCTATGATTG

Pisum sativum sulfate transporter 1.3-like protein (Sultr1.3) mRNA, complete cds
GenBank: KP759565.1

AATTGGATTTCTACATGAGAACCTTAATTTCTAAGTCTTCCATAAGCTCCAGCACCAACTTTTGACTTGA
GTTATATAAAGCAAAGTAGCTTCTCAACAAGTCTTGAAAGTTCTGACCAGTTAGTTACACTCTTCTGTGA
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TAAAGAAATAAAAGAACTTAGTTAATTGTTACAAAAA

Pisum sativum sulfate transporter 1.3-like protein (Sultr1.3b) mRNA, complete cds GenBank: KP759564.1

GTGTTACAGTTCATATTTATTGCCATCACTGGTTATGTTATGACTTTTCTTTGTATAATATAACATAGTG
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TTTTGTATGATGTATGATGCAACAAAGTTAATTTTTTTTTTCATTTCATGTTTGCAATTCTATTTGAAGT
AAGG

Pisum sativum sulfate transporter 2.2-like protein (Sultr2.2) mRNA, complete cds
GenBank: KP759566.1

AAAAAGTAAAAATTGATGAGCATAAATAGAAGGGGATTTATACTCATCTTCACAAGCTACACAAATTCCC
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Pisum sativum sulfate transporter 3.1-like protein (Sultr3.1) mRNA, complete cds
GenBank: KP759567.1

TCAACACCCCCTTCATCCTTTTCATTTATCTTCTCTTTGTTCCTTCATCCCTATCTCATTCACTTCAACC
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TAATTCATA

Pisum sativum sulfate transporter 3.1-like protein (Sultr3.1b) mRNA, complete cd
GenBank: KP759568.1

CATCTGTGTAACTTGCCTCCTCTTCCTTCTTCCTTATCCCTTCTCACTTCCCAACTCTTCTCAATCCAAC
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CTTATTCTTATCAAAA

Pisum sativum sulfate transporter 3.3-like protein (Sultr3.3) mRNA, complete cds
GenBank: KP759570.1

GCATAATTTATTGTTCTGATGCTTTTGTCCTCTAGTTAACATAGGTATAGTCTTGTTTTATAGCGGTTTT
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TCCTTAATGCCAAAAAAAA

Pisum sativum sulfate transporter 3.4-like protein (Sultr3.4) mRNA, complete cds
GenBank: KP759571.1

GAAAGAGTCATTAGAGAAACATGGTTATGAATTGCAACAGAGTAGAACACTTTGAAGAATCCACCATGAA
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CTATTTGATTAGAGGGAAAAAGTGTTCCTAAGAGAGACATGTTTTGGCATCAGAAATGAAAGTATTATTG
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Pisum sativum symbiotic sulfate transporter 3.5-like protein (Ssultr3.5) mRNA, cc
GenBank: KP759572.1

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Pisum sativum sulfate transporter 4.1-chloroplastic-like protein (Sultr4.1) mRNA, cds
GenBank: KP759573.1

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GTTGTAAATTGTAAAATATACTACATGAAATTTTATGAAAGAATTTTTAGTGTAAAAAAAA

Pisum sativum molybdate transporter 1-like protein (Motr1a) mRNA, complete cd
GenBank: KP759574.1

CGGCGCTGGATAATCATGTTTGCTGAGAAATTTTTGGTTGGATTAGTTATGGTGATTGTGAGTCCCAAAA
CAATCAAAAACAAATTTTCCATGGCAAACCAAAACCCTCCTTCAATTCCCATATCAAACTCTGAAACCAC
CATAACTAATCCAACCAAAAATTTCTCAGCAAACATGATTATCCAGAAAGTGAAAACCAACTTGGTTTTA
CATTCAAAATGGGGTGAACTAAATGGTGCCATGGGTGACCTTGGCACCTATATACCCATAGTTCTTTCTT
TGACTCTATCAAAGAATCTCAACCTTGGCACCACATTAATCTTCACAGGTGTATACAACATTCTCACTGG
TGTCATTTATGGGGTCCCTATGCCGGTCCAGCCCATGAAGTCCATTGCCGCGGAGGCCTTATCGGATACC
ACCTTCGGTGTACCGGAGATCATGGCAGCCGGAATCTTGACTAGTGTCGCGGTGTTTTTATTGGGTGCCA
CCGGGTTGATGCAGCTGGTGTACAAGTTTATTCCTTTATCTGTTGTGAGGGGAATTCAACTAGCACAAGG
TTTATCATTTGCTTTAACTGCTGTTAAATATGTGAGAAAAATGCAAGATCTTCCAAAGTCTAAAGCTTTG
GGTCCAAGGCCTTGGTTTGGGTTAGATGGGTTGGTTTTAGCCATTGTTTGTGTTTGTTTTATTGTTATTG
TGAATGGTGCTGGTGAGAGTGAAAATCATCATAGGTGTTGTAATGGTGAAGAAAATGGTGAAAAAAAGAG
GACTAGTAGAGTGAGGAGAATTGTTTTTTCACTTCCTTCTGCTTTTATAATCTTTGTTTTAGGTGTTATT
TTGGCGTTCATAAAAAAGCCAAATGTGGTTCATGAAATCAAATTTGGACCCTCTTCAATAGAATTGGTGA
AGTTCACTAAACAAGCATGGAAGAAAGGTTTTGTGAAAGGTACAATTCCTCAACTACCTTTGACAATCTT
GAACTCTGTGATAGCTGTTTGTAAGCTGTCAAAGGATTTGTTTCCTGAAAAGGACTTTTCAGTCACTTCA
CTTTCAGTGACGGTTGGGTTGATGAATTTTGTTGGTTGTTGGTTTGGTGCTATGCCATGTTGTCATGGTG
CTGGTGGACTTGCAGGACAGTATAAATTTGGTGGAAGGAGTGGAGGATGTGTGGTACTTCTTGGTGTTGC
AAAGTTGATATTGGGATTGGTTTTAGGAACATCTTTGGCACACATATTGAAGATGTTTCCTGTTGGGATA
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TGGAATGGTTGTGTATTTGCTTCTTAAGCTAAGGAGTTGGACAAAAGATAAACCATAATATAGGTCCATA
CAAGAGATTTGGATTTGTTAGGGACACAAATTCTCACATTTTCGTAATCAACATGTTCGTTGGATCAAGA
TCATACGGTACATAAAGAAAGCAAATAGATTTTGATATTTTAAGACGGTAAAATATCAAAATTAAAATTT
AAACCGTTTGATCTTGATTCAACTATTATCAATACGTTGACCGCGAGAATGTGTAGGATTTGTGACCGCA
CACAATTCAAATTCTCGTCAAAGTACCAAGTATCGAGTAACTATAGCTATCCTTAATGTTGTTTGTTATT
TAATGTAGTTTTAAATTGTGTCCCACAACTTTTTTTTTTTTAAGAATATCATGCATTGTTGTGTATGTAA
TGCTCAACTAAGTGATGG

Pisum sativum molybdate transporter 1-like protein (Motr1b) mRNA, complete cd
GenBank: KP759576.1

CTCATAAGAGCCAATTAACCAAAACTTACTTCAAAACCTCACTCACTCTTCCATTCATGGCATCAAACTC

CCAACCTCTCCCCACCACCACCACTACCACCACTCCCACCACTAGACTCACCACCATACAAAAAATAAAA
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CAATAGTACTCTCACTTACTCTTTCCAAAAACCTCAACCTTGGAACAACTTTGCTTTTCACTGGCTTCTA
TAACTTCCTCACAGGTGCCATGTATGGTGTTCCTATGCCAGTTCAGCCCATGAAATCAATAGCTGCCGTC
GCACTCTCGGACCCCTCGTTCGGTATCCCGGAGATCATGGCTTCCGGTATCTTAACCGGAGCGGTTTTAT
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TCAGCTTGCACAGGGTTTGTCCTTTGCTTTAACTGCTGTTAAGTATGTTAAAAAAGTTCAGGATTTACCA
AAGTCTAAATCTTTGAATAACAGGGATTGGCTCGGGTTTGATGGGTTGGTTTTAGCTATTGTTTGTGTTG
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TTATTTCCCACCAAGGACTTTTCAGTAACTTCACTATCAGTGACAGTTGGGTTAATGAATCTTTTGGGTG
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GCGCATTTCTTCAAACAGTTTCCGGTTGGGATTTTAGGGGTGTTGTTGCTGTTTGCTGGGATTGAATTGG
CTATGGCTTGTAGGGATATGAATAAGAAAGAGGATTGTTTTGTTATGCTTATTTGTACAGCTGTTTCACT
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Pisum sativum molybdate transporter 1-like protein (Motr1c) mRNA, complete cd
GenBank: KP759577.1

ATGCACTGCATCTTTGGCGGTGATAAATCATATTATTTTCTTCCATTAGTTAAACCTCTCTATTAGCCAC
TATACATATTCTATATGGAAAACCAAAACCCTTCTTTAATTCAACCATCAGATCCTGAAGTACCTGAAAT
ATCTCCAACTGCTGTAACTAACCCTTCATCCACAACAAGCTTTTTCACCGCCAAAGATGCAGTGCATAAA
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TGTGTACAACATCATCACTGGTATCGTTTATGGGGTCCCTATGCCTGTCCAGCCTATGAAGGCAATTGCC
GCTGTGGCCTTATCAGACAAAGAATTCGGCGTACCCGAGATCATGGCTGCAGGAATCTTGACCGGCAGTG
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CTTCCTAGGTCTAAAGCTTTAGGTGAAAGACCATGGTTTGGGTTAGATGGGTTGGTTCTAGCTATCTTTT
GTGCTTTTTTTATTGTGATTGTGAATGGAGCCGGTGAACAGAATCGTGGAGGTTGCGGTGTTGTTGCAAA
TGGCGATGATTTAGACGAACAAAGAAACAATAGGATCAATAGATTGAGAAAGATTGTTTTTTCACTTCCT
TCTGCATTTTTAGTCTTTGTGTTGGGGATTGTTTTGGTTTTTATAAGAAACCACAAAGTTGCGCGCGAAG
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CTTTTTCCTGAAAGGGAATTTTCAGTTACTTCAATTTCAATAACAGTTGGACTAATGAATTTGGTTGGAT
GTTGGTTTGGTGCTGTACCAACTTGTCATGGTGCCGGTGGACTTGCGGGACAATATAAATTTGGTGGAAG
GAGTGGAGGGTGTGTAGTACTTCTTGGTGTTGGAAAATTGGTATTAGGGTTGGTGCTAGGAACTTCTTTG
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CATTGTGTGCTAGGGACATGAATAGCAAGCAAGATTTCTTTGTGGCACTTATTTGCACTGCTGTTTCTTT
AGTTGGATCTAGTGCATCACTTGGATTTTTAATTGGAATGATTGTTTATGTCTTGTTTAAGCTAAGGAGT
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AAGAATCATCTGTGCACAAATGTAGAGTCATATTAGTGTAATATTTTCAAAATTATGTTAATTTTTTGGG
AAGTTTGAATTTTTCTTTCTAAAAAATTGTTTTTTAAGTTGGTATTTGATTTCAAATGAGACTTTGAAGT
GATTCAAGTTAAAGAGGACTCTTTATATTGTAATTGTAAATAGAATGTTGCTGGATTTTTCTTTCCCTTT
TGTATGCTATCGCATACACAAAGCTTATAGTGTCAATAATGGAACAAAATAATTCAATCCAGTTTCAAGA
AGTTCTTATAATAGAGTTTTATTGTCAGTTGCGG

Pisum sativum molybdate transporter 2-like protein (Motr2) mRNA, complete cds
GenBank: KP759575.1

TTATTTATTTATTTATTTTGTGCATATATATGTAATGACAAAAAGCATTATTCACTCCATAGGTTCTTAG
TAACACCACAAAAATATTAAGCAACCACATCGACTAATTAAACCTCCAACCACCATGGCACACGACATCT
CCAACGACCAACCACCACCGGAAACCTCCACAACAACAACACCTCTCCTCCGCCGCAACTTCTTCCGTCT
CCCATCAACCCTCAAACTCAAAACCTCCCTCTGGTCAGAACTCTCCGGCGCCGTCGGCGACTTAGGAACA
TACATCCCAATCGTACTAGCTCTATCATTAGTCAACAATCTCGATCTAACAACAACTCTCATCTTCACTT

CTCTCTACAACATCATCACCGGAGTTTTCTTCGGGCTTCCTATGCCAGTCCAGCCTATGAAATCCATCGC
CGCCGTCGCCATCTCCGAATCGCCACCTCTCACAATTCCTCAAATCTCTGCCGCCGGTTTATCCGTCGCG
GCTGTCCTCCTCGTTCTCGGAACAACCGGTCTCATGTCTTTTCTCTACCGTTACCTACCTCTCCCGGTAG
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ACGAGCCAGAAGAAAATGAGTATGATAGTGACGATAGAGTTGATAATCTTCGACGAAATAAAATTCGTCG
AAGATTGAAGATACTATCAATGGTTCCCTCGGCTTTGATTGTGTTTTTATTTGGACTATTGTTGTGTTTT
ATTCGCGATCCTTCAATTTTTCATGATTTGCAATTTGGTCCTTCTAGGATTAAGTTGATTAGAATCACAT
GGGATGATTTTAAAGTTGGATTTGTCAGGGCTGCAATTCCGCAAATTCCGTTATCAATTTTGAATTCGGT
TATTGCGGTTTGTAAACTTTCCGGGGACTTGTTTCCGGAAAGAGAAGCTTCAGCTATGAAGGTTTCAGTG
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TGGCTGGTCAGTATAGGTTTGGAGGTAGGAGTGGTGCTTCTATTGTGTTTCTTGGGATTGGGAAATTGTT
GATTGCTTTGGTTTTTGGGAACTCTTTTGGGAGGATTTTGGGTCAGTTTCCTATTGGGATACTTGGTGTG
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AAACATATATGTATGTGTATTTTATATATTTATAGTTAAAGTTCATAATCCTTTTGAGTGTGGGACTTTT
TGCAAAATGTGTTGGTTGGTTCAATGGGGAACATGGTTTTGTAGGACCCTTCTATACTCTTGACTCAAAT
CAAGTGTTTGGATGAGTACATGCTTAATAAGGATTCATGTTATGATTCATAA

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