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"Effects of sulphate-deficiency on cysteine metabolism in the green alga *Chlorella sorokiniana*"

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ABBREVIATIONS

ACN:	Acetonitrile
ADP:	Adenosine diphosphate
AMP:	Adenosine monophosphate
APS:	Adenosine- 5'- O- phosphosulphate
ATP:	Adenosine triphosphate
BCIP:	5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt
BSA:	Bovine serum albumin
CHES:	N-Cyclohexyl-2-aminoethanesulfonic acid
Chl:	Chlorophyll
CSC:	Cysteine synthase complex
Cys:	Cysteine
DD water:	Distilled, deionized water
DES:	Cysteine desulphydrase
DMPD:	N,N-dimethyl-p-phenylenediamine dihydrochloride
DTT:	Dithiothreitol
ECL:	Enhanced chemiluminescence
EDTA:	Ethylenediaminetetraacetic acid
Et-OH:	Ethanol
Fd:	Ferredoxin
Gg:	Gigagram
Glu:	Glutamic acid
Gly:	Glycine
GSH:	Reduced glutathione
GSSG:	Oxidized glutathione
HPLC:	High Performance Liquid Chromatography
IgG:	Immunoglobulin G

IPTG:	Isopropyl-ß-D-thiogalactopyranosid
kDa:	Kilodalton
MW:	Molecular weight
NADH:	Nicotinamide adenine dinucleotide
NADPH:	Nicotinamide adenine dinucleotide phosphate
NBT:	Nitro-blue tetrazolium chloride
nm:	Nanometer
OAS:	*O*-acetylserine
OASTL:	*O*-acetylserine(thiol)lyase
OD:	Optical density
*o***PA:**	*o*-phthaldialdehyde
PAPS:	3'-Phosphoadenosine-5'-phosphosulfate
PCV:	Packed cell volume
PLP:	Pyridoxal-phosphate
PMSF:	Phenylmethanesulfonylfluoride
Ppi:	Inorganic pyrophosphate
Psi:	Pound- force per square
PS-I:	Photosystem I
PS-II:	Photosystem II
Red-ox:	Oxidation-Reduction
ROS:	Reactive oxygen species
SAT:	Serine acetyltransferase
SDCs:	Sulphur-containing defence compounds
SDS:	Sodium dodecyl sulphate
Ser:	Serine
SULTR:	Sulphate transporter
TBS:	Tris buffered saline
TBS-T:	Tris buffer saline Tween20
TCA:	Tricloracetic acid

TEMED: N,N,N',N'-Tetramethylethylenediamine

FORMULAE

AgNO$_3$:   Silver nitrate

CaCl$_2$:   Calcium chloride

CuCl$_2$:   Copper(II) chloride

CuSO$_4$:   Copper sulphate

FeCl$_3$:   Iron(III) chloride

H$_3$BO$_3$:   Boric acid

HCl: Hydrochloridric acid

HCO$_3$:   Hydrogen carbonate

H$_2$O$_2$:   Hydrogen peroxide

H$_2$S: Hydrogen sulphide

KCl: Potassium chloride

MnCl$_2$:   Manganese chloride

Na$_2$CO$_3$:   Sodium carbonate

Na$_2$S: Sodium sulphyde

NH$_4$HCO$_3$:   Ammonium bicarbonate

(NH$_4$)$_6$Mo$_7$O$_{24}$: Ammonium heptamolybdate

O$_2$:   Oxygen

S: Sulphur

S^0:   Elemental sulphur

S^{2-}:   Sulphide

SO$_2$:   Sulphur dioxide

SO$_4$$^{2+}$:   Sulphate

ZnCl$_2$:   Zinc chloride

ZnSO$_4$:   Zinc sulphate

1 - INTRODUCTION

Plants, bacteria and fungi have the ability to assimilate inorganic sulphur and incorporate it into inorganic compounds. Animals on the contrary, do not assimilate inorganic sulphur; they require methionine as an essential amino acid for their source of sulphur nutrient.

The amino acid cysteine (Cys) is the first committed molecule in plant metabolism containing sulphur and it is the sulphide donor for the generation of methionine, glutathione (GSH), phytochelatins, iron-sulphur clusters, vitamins, cofactors, and multiple secondary metabolites; that's why the regulation of Cys-biosynthesis is critically important.

Figure 1: Chemical structure of *L*-cysteine.
Cysteine ($C_3H_7NO_2S$) is classified as a hydrophilic amino acid with a thiol side chain. The high reactivity of this thiol makes it an important structural and functional component of proteins, vitamins, cofactors and multiple secondary metabolites.

The aim of this research was to investigate the effects of sulphur-deficiency on plant cysteine metabolism and to characterize the *O*-acetylserine(thiol)lyase (OASTL), a key-enzyme in cysteine biosynthesis.

1.1 - Sulphur in the environment

Sulphur is an allotropic non-metallic element, which occurs in the environment in a variety of oxidative states that range from - 2 in its most reduced form (S^{2-}) to + 6 in its most oxidized form ($SO_4{}^{2+}$).

Sulphur is emitted to the atmosphere as a result of volcanic eruptions (mostly as H_2S), anthropogenic activities and decomposition of biological tissue (Lewandowska and Sirko, 2008).

Elemental sulphur isn't toxic, but some of its products such as sulphur dioxide (SO_2) and hydrogen sulphide (H_2S) are very dangerous for the health of organisms and the environment. In particular SO_2 in the stratosphere changes into hydrogen sulphide particles that reflect sun rays causing the greenhouse effect.

The local trends to reduce sulphur dioxide emissions all over the world vary from one region to another and are the results of environmental legislation imposed by local governments. In the majority of European countries, emissions have decreased by more than 60% from 1990-2004 (Vestreng et al., 2007, Lewandowska and Sirko, 2008) (Figure 2).

Figure 2: SO$_2$ emission maps.

Difference maps presenting the three European SO$_2$ emission reduction regimes. A) Reductions between 1980-1990; B) reductions between 1990-2000; C) and reductions between 2000-2004. Used unit: mg SO$_2$/grid cell (Vestreng et al., 2007).

In the countries that have accepted these set of rules, air pollution has decreased, however the concentration of sulphur-containing compounds both in soil and in the atmosphere have decreased as well. Sulphur-deficiency in the soil and in the atmosphere, leads plants to S-nutritional stress.

Sulphur deficiency in plant nutrition is an actual problem in European States (Yang et al., 2007, Lehman et al., 2008). It can be attributed to the decrease of atmospheric emissions and to less intensive application of mineral fertilizers.

	1980	1985	1990	1995	2000	2004
Albania*	72	73	74	14	32	32
Armenia	141	100	86	15	11	8
Austria	344	179	74	47	32	29
Azerbaijan	603	543	615	262	162	130
Belarus	*740*	*690*	*888*	*344*	*162*	*97*
Belgium	*828*	*400*	*361*	*262*	*171*	*154*
Bosnia and Herzegovina*	482	483	484	360	420	427
Bulgaria	2050	2314	2007	1477	918	929
Croatia	150	164	178	70	60	85
Cyprus	28	35	46	41	51	45
Czech Republic	2257	2277	1876	1090	264	227
Denmark	450	333	176	133	27	23
Estonia	287	254	274	117	96	90
Finland	584	382	259	95	74	83
France	3216	1496	1333	968	613	484
Georgia	230	273	43	6	7	5
Germany	7514	7732	5289	1708	630	559
Greece	400	500	487	536	493	537
Hungary	1633	1404	1011	705	486	240
Iceland	18	18	9	9	9	9
Ireland	222	140	186	161	131	71
Italy	*3437*	*2045*	*1795*	*1320*	*755*	*496*
Kazakhstan	639	575	651	528	506	425
Latvia	*96*	*97*	*97*	*47*	*10*	*4*
Lithuania	*311*	*304*	*263*	*92*	*43*	*40*
Luxembourg*	26	26	26	7	4	4
Malta	*29*	*29*	*29*	*33*	*26*	*17*
Netherlands	*490*	*258*	*189*	*127*	*72*	*66*
Norway	136	91	53	34	27	25
Poland	4100	4300	3278	2381	1507	1286
Portugal	266	198	317	332	306	203
Republic of Moldova	*308*	*282*	*175*	*94*	*13*	*15*
Romania	*1055*	*1255*	*1310*	*882*	*727*	*685*
Russian Federation*	7323	6350	6113	3101	2263	1858
Serbia and Montenegro*	406	478	593	428	396	341
Slovakia	780	613	542	239	127	97
Slovenia	234	241	198	127	99	55
Spain	*3024*	*2542*	*2103*	*1809*	*1479*	*1360*
Sweden	491	266	117	79	52	47
Switzerland	*116*	*76*	*42*	*28*	*19*	*17*
TFYR of Macedonia*	107	109	110	93	90	87
Turkey*	1030	1345	1519	1397	2122	1792
Ukraine	*3849*	*3463*	*3921*	*2342*	*1599*	*1145*
United Kingdom	4838	3714	3699	2343	1173	833
Grand Total	55340	48448	42896	26282	18263	15162

Table 1: Sulphur trends per European country 1980–2004.

The table shows EDGAR data, interpolation and extrapolation. Sulphur is expressed as Gg SO_2. Countries highlighted in grey: Officially reported data. *Bold italics*: Reported data completed by independent estimates. Stars(*): RAINS data, interpolation and extrapolation (Vestreng et al., 2007).

The total content of S in soil usually ranges between 0.01 and 0.1% (Stevenson and Cole, 1999; Balik et al., 2007), however the content of sulphur in plants varies from species to species, but generally it covers a range between 0.15 and 6% of plant dry weight.

In all plants, sulphur is included in amino acids (cysteine, methionine), hormones (ethylene, polyamines), vitamins (biotin, thiamine) and tripeptide glutathione.

S-compounds are involved in responses to abiotic stress, such as the tripeptide glutathione in the detoxification of reactive oxygen species and as substrate for synthesis of phytochelatins, or the glucosinolates in defence against herbivores and other pathogens (Davidian and Kopriva, 2010).

Insufficient sulphate nutrition reduces plant growth, vigor and resistance to abiotic and biotic stresses (Lewandowska and Sirko, 2008).

Symptoms of S- deficiency (Figure 3) in plants are difficult to verify, but in general the most evident and common are:

- Chlorosis, especially in young leaves;
- Slow growth;
- Small apical leaves;
- Anomalous leaf curling;
- White and ramose roots.

Figure 3: Symptoms of sulphur-deficiency in a higher plant.
A) Leaf of plant growth in presence of all nutrient; B-C) Effects of S-deficiency in leaves

1.2 - Sulphur assimilation as a platform for the biosynthesis of glutathione and sulphur-containing compounds

The role of sulphur in the resistance of crops against diseases became evident in Europe at the end of the 1980s when atmospheric S-depositions were greatly reduced, then S-deficiency became a wide problem in many countries (Booth et al., 1991; Knudsen and Pedersen, 1993; Kjellquist and Gruvaeus, 1995).

Figure 4: Sulphur-containing defence compounds synthesis.

After sulphate uptake, sulphate is largely transported to the shoot, where it becomes activated by ATP via ATP sulphurylase (2) in the leaves. The product, APS, is reduced by APS reductase (3), with the tripeptide glutathione (GSH) acting as an electron donor. Alternatively, APS is further activated by APS kinase (4) to form PAPS, which is required

for various sulphatation reactions, including the biosynthesis of glucosinolates. Sulphite is reduced by sulphite reductase (6) to H_2S, which is incorporated into *O*-acetylserine via OASTL (8) to form cysteine. The latter is incorporated into sulphur-rich proteins (SRPs; including thionins) and GSH. Furthermore, cysteine is the donor of reduced sulphur for glucosinolates biosynthesis and for the synthesis of phytoalexins (including camalexin). Finally, H_2S can be released from cysteine via the action of DES (9), whereas elemental sulphur, S^0, is possibly released from GSH. Note that via the activity of sulphite oxidase (5), excess sulphite is converted to sulphate, a reaction that uses O_2 as an electron acceptor, thereby releasing H_2O_2 (which could act as a defence signal). Sulphate assimilation [reactions (2), (3), (6), (8)] is localized in the plastids, whereas H_2S release occurs in plastids, mitochondria and cytosol. Sulphite oxidase is confined to peroxisomes.

Defence metabolites produced by plants can be classified in two groups: constitutive substances, which are involved in initial plant defence (Mohr and Schopfer, 1994) and induced metabolites, formed in response to an infection involving *de novo* enzyme synthesis (Van Etten et al., 1994).

Sulphur-containing defenced compounds (SDCs) (Figure 4) are involved in the constitutive and induced protection of plants against pests and disease (Bloem et al., 2005).

SDCs are crucial for the survival of plants under biotic and abiotic stress, its pool includes: glucosinolates, sulphur-rich proteins, phytoalexins, hydrogen sulphide and elementar sulphur and glutathione (Rausch and Wachter, 2005).

In plant cell, GSH is a relevant antioxidant preventing the damage of important cellular components, its reduced form (GSH) is a tripeptide (γ-Glu-Cys-Gly) (Figure 5) that exists interchangeably with the oxidized form (GSSG). Thus, GSH together with GSSG forms a sensitive red-ox-buffer that allows fine-tuning of the cellular red-ox-environment under normal conditions and under conditions of external environmental stresses (Meyer and Hell, 2005).

Figure 5: Glutathione structure.

GSH is a tripeptide synthesized from amino acids such as *L*-cysteine, *L*-glutamic acid and glycine. GSH is an antioxidant, preventing damage to important cellular components caused by reactive oxygen species as such as free radicals and peroxides.

In plants, the physiological significance of glutathione may be divided into two categories: sulphur metabolism and defence (Noctor and Foyer, 1998).

In plants, GSH represents the storage and transport form of reduced sulphur, controls sulphate influx into the organism at the level of sulphate uptake and xylem loading in the roots (Rennenberg and Herschbach, 1995). GSH is necessary for maintaining the red-ox balance, for xenobiotic and heavy metals detoxification (Rouhier et al., 2008), but also for protecting the cytosol and other cellular compartments against reactive oxygen species (ROS), which

have accumulated in response to abiotic and biotic stress (Mullineaux and Rausch, 2005; Rausch and Wachter, 2005).

Figure 6: GSH biosynthesis in *Arabidopsis thaliana*.

The localization of sulphate assimilation in the chloroplasts is highlighted by an orange box. Export of cysteine for protein-biosynthesis is suspected, but not confirmed experimentally (dashed arrow). Similarly, uptake of GSH into the plastids is suspected, but to date not supported by experimental evidence (dashed arrow) (Meyer and Hell, 2005).

1.3 - Sulphur metabolism in plants

Sulphur represents the ninth and least abundant essential macronutrient in plants, preceded by carbon, oxygen, hydrogen, nitrogen, potassium, calcium, magnesium and phosphorus (Saito, 2004).

For plants, the major source of this element is the inorganic sulphate present in the soil, although they are also able to use reduced sulphur

compounds absorbed from the atmosphere, by stomata, such as sulphur dioxide or hydrogen sulphide (Leustek et al., 2000).

S-assimilation is relatively well known in higher plants, but very little information exists on S-assimilation in lower plants and algae, this process in plant cell occurs in four steps:

1) Sulphate uptake;

2) Sulphate activation;

3) Reduction of activated sulphate;

4) Cysteine biosynthesis.

Figure 7: Sulphur assimilation in plant cell.

Green colour represents plastids, brown mitochondria and blue vacuoles. Numbers represent enzymes as follows: 1) Sulphate transporter; 2) ATP sulphurylase; 3) APS reductase; 4) Sulphite reductase; 5) Serine acetyltransferase; 6) *O*-acetylserine(thiol)lyase; 7) γ-glutamylcysteine synthetase; 8) Glutathione synthetase; 9) APS kinase; 10)

Sulphotransferase. Dashed lines represent multiple reaction steps; dotted lines indicate unconfirmed transport steps (Kopriva, 2006).

The sulphate assimilation pathway was first discovered in enteric bacteria, *Escherichia coli* and *Salmonella typhimurium* (Kredich NM, 1996) and it was subsequently characterized in plants.

In recent years many knowledge on sulphur metabolism in higher plants has come out, but very little is still known about this process in algae.

In all photosynthetic organisms, and therefore also in algae, S is assimilated as sulphate, which is usually easily accessible in soil and aquatic ecosystems (Giordano et al., 2005).

The transport of SO_4^{2-} occurs across several membranes (Figures 7-8):

1. Plasma membrane transporters in the root;

2. Plasma membrane transporters of vascular tissue for long-distance translocation and of leaf mesophyll cells for assimilation coupled with photosynthesis;

3. Inside the cells, transporters associated with organelle transport, in particular plastids and vacuoles (Hawkesford, 2003).

Being the sulphate one of major anion of vacuole, it is also transported by tonoplast.

Figure 8: Sulphate transport in plants.

Like nitrate, sulphate uptake across the plasma membrane is energized by an electrochemical gradient that is maintained by a proton-pumping ATPase. Sulphate is stored in vacuoles. Reduction of sulphate and its assimilation into cysteine take place in the plastids of root and leaf cells (Biochemistry & Molecular Biology of Plants, Buchanan, Gruissem and Jones).

Sulphate transport across plasma membrane occurs with protons at a ratio of 3 H^+:1 SO_4^{2-} (symport) and is driven by a proton ATPase (Leustek and Saito; 1999). In fact, the uptake of sulphate root-to-shoot is an energy-dependent mechanism co-transporters systems (Leustek et al. 2000; Saito, 2000).

Transport across the tonoplast is mediated by a uniport mechanism that is driven by the electrical gradient between vacuole sap and cytoplasm.

The movement of sulphate around the plant and between the cell compartments is facilitated by specific sulphate transporters (SULTR) (Figure 9), which are classified into five groups depending on their protein sequences and characteristics (Hawekesford, 2003), properties in translocation of sulphate, patterns of expression and tissue specificity.

Figure 9: Model for sulphate transport across the plasma membrane.

The transport of sulphate is powered by an electrochemical proton gradient generated by an ATPase that extrudes protons to the cell exterior. The sulphate transporter is able to couple the influx of protons to the transport of sulphate into the cell (Biochemistry & Molecular Biology of Plants, Buchanan, Gruissem and Jones).

In *Arabidopsis thaliana*, the family of sulphate transporters (encoded by 14 genes) is larger than that of transporters for nitrate (7 genes), inorganic phosphate (9 genes) or ammonium (6 genes) (Lewandowska and Sirko, 2008).

SULTR 1; 1 and SULTR 1; 2 (high affinity transporters) are the best characterized transporters, and are mainly expressed in the epidermis and cortex of root tissues. Transcripts accumulate under sulphate deprivation, indicating that these transporters have a specialized function to import sulphate from the environment to the roots (Takahashi et al., 2000; Shibagaki et al., 2002; Yoshimoto et al., 2002). SULTR 1; 3 is localized in the sieve element-companion cell complexes of the phloem and mediates the step of the source-to-sink translocation of sulphate through the plants (Yoshimoto et al., 2003).

In contrast to group 1, the members of group 2 are suggested to have low-affinity sulphate transport activity, however their precise functions in plants are unclear (Kataoka et al., 2004).

SULTR 2; 1 mRNA is abundantly expressed, particularly in the roots of sulphur-starved plants (Takahashi et al., 2000) and in the *sultr1;2* knockout (Maruyama-Nakashita et al., 2003), suggesting that the SULTR 2;1 transporter may participate in transporting sulphate to the xylem parenchyma cells.

SULTR 4;1 and SULTR 4; 2 are localized in tonoplast of pericycle and xylem parenchyma cells of root. They are responsible of sulphate efflux from the vacuole.

The role of transporters from group 3 and group 5 is still not fully clarified.

In the green alga *Chlamydomonas reinhardtii*, the sulphate anion is taken up first through the cell membrane, and then actively translocated into the chloroplast (Melis and Chen, 2005). In the chloroplast, it is first reduced to sulphide (S^{2-}) and then used in the synthesis of cysteine. This process is highly endergonic, with energy provided in the form of reductant and ATP by photosynthesis (Leustek et al., 2000).

In *Chlamydomonas r.,* two distinct sulphate transport systems are localized and function in the plasma membrane and chloroplast envelope, respectively (Figure 10) (Melis and Chen, 2005).

Figure 10: Sulphate uptake and assimilation pathway in *Chlamydomonas reinhardtii*. Sulphate anions are transported from the environment through the plasma membrane sulphate transport system to the cytosol. A chloroplast sulphate transport system is responsible for the subsequent sulphate transport from the cytosol to the chloroplast stroma. Sulphate assimilation occurs exclusively in the chloroplast of the green algae, leading to the biosynthesis of the S-amino acid cysteine (Melis and Chen, 2005).

The unicellular green alga *Chlamydomonas* has both H^+/SO_4^{2-} co-transporters (SULTR) and Na^+/SO_4^{2-} transporters (SLT), while vascular plants such as *Arabidopsis thaliana* only retained H^+/SO_4^{2-} co-transporters. This

finding suggests that *Chlamydomonas* diverged from the plants lineage (approximately 1 billion years ago) prior to the loss of the Na^+/SO_4^{2-} transporters (Pootakham, 2010).

For assimilation, sulphate must be activated, in fact the couple SO_4^{2-}/SO_3^{2-} has a very negative red-ox potential (about − 516 mV). The low reactivity of absorbed sulphate represents an obstacle for the assimilation, that's why sulphate can't be directly reduced by physiologic electron donors as Fd ox/red having a less negative red-ox potential (- 420 mV). This thermodynamic barrier can be crossed by activation of inorganic sulphate.

The activation process occurs in two reactions, catalyzed by specific enzymes:

1) ATP sulphurylase (EC 2.7.7.4.);

2) APS kinase (EC 2.7.1.25.).

Both APS and PAPS represent the two activated forms of sulphate.

In plants there are two enzymes able to metabolize APS, the first one is APS kinase, the second is APS-sulphotransferase (EC 1.8.9.9.2).

ATP sulphurylase is the metabolic entry point into the sulphur assimilation pathway. In its reaction, the inert sulphate is actived by covalent binding to ATP to form APS either in the cytosol or in the plastids.

$$SO_4^{2-} + ATP + H^+ \Longleftrightarrow APS + Ppi$$

Because this is an endergonic reaction, it is paired to a reaction having substrates Ppi or APS, producing energy.

Ppi is hydrolyzed by a pyrophosphatase. However, in the cytosol APS is phosphorylated to PAPS by APS kinase that uses ATP.

Genes encoding chloroplastic, mitochondrial and cytosolic ATP sulphurylase isoforms have been cloned from *Arabidopsis thaliana* and Soybean (Hatzfeld et al., 2000; Phartiyal et al., 2006).

Since chloroplasts are the primary site of reductive sulphate assimilation, the chloroplast isoform has been studied in detail. The respective role of cytosolic and mitochondrial isoenzymes in sulphate assimilation remains to be elucidated (Phartiyal et al., 2006).

$$APS + ATP \Longleftrightarrow PAPS + ADP + H^+$$

In the chloroplasts, sulphate bound toAPS is reduced to sulphite and subsequently to sulphide.

The sulphite reductase enzyme (EC 1.8.1.2.) catalyzes the reduction of sulphite to sulphide, using electrons that have come from reduced Fd.

$$SO_3^{2-} + Fd_{red} + H^+ \Longrightarrow S^{2-} + Fd_{ox} + H_2O$$

In photosynthetic cells, Fd_{red} derives from PS-I; however in non-photosynthetic cells, the electrons donor is NADPH which comes from the oxidative pentose phosphate pathway.

The last step of sulphur assimilation is the incorporation of inorganic sulphide into *L*-cysteine.

1.4 - Cysteine biosynthesis

Cysteine is the first sulphur-containing amino acid compound regarded as the terminal metabolite of sulphur assimilation and the starting point for production of methionine, glutathione and a variety of other sulphur metabolites (Saito, 2004).

The importance of cysteine formation in plant cells, is comparable to the ammonium fixation in the amide glutamine for protein biosynthesis.

In plants, algae and bacteria, cysteine biosynthesis proceeds by two interconnected and consecutive reactions catalyzed by two different enzymes: serine acetyltransferase (SAT, EC 2.3.1.30) and *O*-acetylserine(thiol)lyase (OASTL, EC 4.2.99.8).

SAT acetylates *L*-serine from acetyl-CoA to form *O*-acetylserine (OAS), which plays the role of substrate for the OASTL enzyme. The latter inserts sulphide into the carbon-skeleton of OAS to constitute the S-amino acid *L*-cysteine.

Figure 11: Biosynthesis of cysteine in *Arabidopsis thaliana.*

Primary sulphur assimilation and reduction takes place in plastids where is reduced to sulphide. The sulphide is incorporated to the carbon skeleton of OAS within the plastid, or diffuses to the cytosol and the mitochondria, to form the cysteine molecule. For each compartment, SAT and OASTL isoforms involved in the catalysis are shown (bold red font represents the major isoform) (Lòpez-Martìn et al., 2008).

In higher plants, both SAT and OASTL enzymes are found in plastids, mitochondria and cytosol (Lopez-Martin et al., 2008; Heeg et al., 2008).

The reaction that leads to cysteine synthesis is a convergence point between sulphur and nitrogen metabolism, which was found to be an important role in regulation (Kopriva et al., 2002).

Some years ago Lunn and co-workers (1990) suggested that endomembranes might be impermeable to cysteine due to the reactivity of the thiol group. These two enzymes, SAT and OASTL, were sublocalized in each of the three compartments where the protein synthesis occurred because the cysteine required for protein synthesis might be synthesized *in situ*.

The exclusive location of assimilatory sulphate reduction in plastids suggest that sulphide is able to diffuse through membrane (Mathai et al., 2009) to reach cytosol and mitochondria, as well as the exchange of sulphur-related metabolites between cytosol and plastids is required to coordinate methionine and GSH synthesis (Heeg et al., 2008).

Recently, specific transporters for cysteine were found in bacteria (Ohtsu et al., 2010). In plants why Cys synthesis occurs in three different cellular compartments remains still little clear.

Arabidopsis thaliana is the best investigated plant system on sulphur metabolism; its genome encodes five different SAT (Howarth et al., 2003) and eight OASTL isoforms (Wirtz et al., 2004; Alvarez et al., 2010).

The active form of OASTL enzyme is a homodimer of 60-70 kDa and has two molecules of the cofactor PLP (Barroso et al., 1997). In the unicellular green alga *Chlamydomonas reinhardtii* the molecular weight of OASTL was estimated to be 36 kDa (Ravina et al., 1999).

The major part of OASTL activity in leaves of *Datura innoxia* is located in the cytoplasm (~ 45%) and in the plastids (~ 45%), while only a minor part (about 5%) is associated with the mitochondrial fraction (Kuske et al., 1996). This pattern of distribution is confirmed by subcellular fractionation of leaves from *Spinacia oleracea* (Lunn et al., 1990) and Pisum *sativum* (Droux, 2003; Heeg et al., 2008).

By contrast, the major part of SAT activity resides in mitochondria (76-88%), while only residual amounts of SAT activity are found in cytoplasm (6-14%) and plastids (6-10%) from leaves of pea (Ruffet et al., 1995). This means that OASTL:SAT activity ratios are 200:1 in cytosol and 300:1 in plastids and only 4:1 in mitochondria, indicating that SAT is the rate-limiting step in cysteine synthesis (Heeg et al., 2008).

The subcellular distribution of SAT and OASTL seems to confirm that cysteine is synthesized in several locations in vascular plant cells. By contrast,

in *Chlamydomonas reinhardtii*, cysteine appears to be synthesized only in the chloroplasts (Ravina et al., 2002).

1.5 - Cysteine Synthase Complex

A property that defines an authentic OASTL enzyme has the ability to interact with SAT molecules to form a hetero-oligomeric complex named cysteine synthase complex (CSC) (Wirtz and Hell, 2006, Kumaran et al., 2009), which was first described in bacteria and has since been extensively studied in plants (Droux et al., 1998; Wirtz and Hell, 2006; Alvarez et al., 2010).

Protein-protein interactions between SAT and OASTL lead to the formation of CSC which plays an essential role in cysteine production.

Kredich and co-workers (1993), by ultracentrifugation and using recombinant proteins, determined a molecular weight of 310 kDa for CSC (Figure 12) purified from *Salmonella typhimurium*. They proposed that the complex contained two trimers of SAT and two dimers of OASTL.

Figure 12: CSC model by Kredich.

The cysteine synthase complex proposed by Kredich And co-workers (1993) contains two trimers of SAT enzymes and two dimers of OASTL enzymes.

Years later, with the use of analytical ultracentrifugation and size-exclusion chromatography analysis, Kumaran and co-workers (2009) suggested a new model of CSC in plants (Figure 13). They proposed that CSC contained a SAT trimer associated with three OASTL dimers but having the same molecular weight of 310 kDa.

Figure 13: CSC model by Kumaran.

Dimeric OASTL (violet) interacts with the C-terminal tail of a SAT trimer (orange) (Kumaran et al., 2009).

In the Kumaran proposed model, CSC differs from that which was originally proposed for the bacterial CSC because a hexameric SAT interacts with two OASTL dimmers. The variation may reflect differences in species and/or method of isolating the CSC (Kumaran et al., 2009).

The three–dimensional structures of SAT and OASTL from soybean (Figure 14), have been resolved using X-ray crystallography.

Figure 14: Structural overview of SAT and OASTL.

a) ribbon diagram of a homology model of the soybean SAT trimer, based on *E. coli* SAT. red sphere indicates the C-terminal tail of each monomer subunit. In the bacterial SAT structures, an additional 10–20 residues of each C-terminal tail are disordered beyond the residue indicated by the red sphere . b) ribbon diagram of *Arabidopsis. thaliana* OASTL (blue) with an *Arabidopsis t.* SAT C-terminal peptide (gold) bound at each active site (Kumaran et al., 2009).

In *Arabidopsis thaliana,* as described by Bogdanova and Hell (1997), the high conserved C-terminal region of the SAT enzyme would represent a bi-functional domain for transferase activity and for OASTL-SAT complex formation. Otherwise, the N- terminal region (α-helix cluster) would be involved in SAT-SAT interaction.

A regulatory function of the cysteine synthase complex in the rate of cysteine synthesis has been suggested which is based on the association/dissociation of the two enzymes, triggered by the availability of OAS and sulphide (Wirtz et al., 2001).

The rapid and stable formation of CSC would allow production of *O*-acetylserine to maintain intracellular cysteine levels during high demand conditions.

Plant and bacterial OASTL are dimers that are catalytically inactive in the CSC but become fully active upon dissociation of the complex by OAS (Wirtz et al., 2010). In fact, kinetic analyses on CSC revealed that the bound OASTL dimmers are catalytically almost inactive in the complex whereas SAT requires an excess of OASTL to gain full activity (Droux et al., 1998; Wirtz and Hell, 2007). On association in the complex, SAT is activated to yield a higher V_{max} and substrate affinities for OAS production, whereas OASTL is essentially inactivated and apparently acts as a regulator of SAT activity in the complex. Consequently, the intermediate OAS cannot be channeled within the complex but diffuses into the surrounding solution, where it reacts with free OASTL dimers and sulphide to form cysteine.

The levels of OAS and sulphide represent critical regulatory factors for the Cys-synthesis. According to literature, OAS trigged the dissociation of the complex, where sulphide compensates for this action, by promoting the binding of OASTL with SAT. It is believed that at low sulphide levels, OAS accumulates and hence slows its own synthesis by disrupting the CSC. In contrast, when sulphide builds up, OASTL binds to SAT increasing the production of OAS for efficient synthesis of cysteine (Droux et al., 1998).

1.6 - Cysteine Degradation

For many years the OASTL enzyme, responsible for the cysteine biosynthesis, was also considered to be involved in cysteine catabolism.

Recent literature breaks up this hypothesis, affirming that the OASTL enzyme is not able to perform cysteine degradation.

Unlike cysteine-biosynthesis, the cysteine-catabolism and the enzyme involved in this process are still not well understood.

The enzyme that plays a central role in the catabolism of the S-containing amino acid is the *L*-cysteine desulphydrase (DES; EC 4.4.1.1).

This enzyme catalyzes the enzymatic desulphuration of *L*-cysteine to sulphide, ammonia and pyruvate in a stoichiometric ratio of 1:1:1 and as similarity to the OASTL enzyme, PLP as its cofactor (Alvarez et al., 2010).

$$L\text{-Cysteine} + H_2O \xrightarrow{\text{DES}} \textbf{Pyruvate} + \textbf{Ammonia} + \textbf{Sulphide} + \textbf{H}^+$$

DES, found in higher plants (Bloem et al., 2004) shows a low affinity for OAS and a much higher affinity for *L*-Cys (Alvarez et al., 2010), which results in a poor or absent capacity to synthesize cysteine.

DES which was purified from *Leishmania major* by affinity chromatography: a single protein band with a relative molecular mass of 45 kDa was evidenced in SDS-PAGE (Marciano et al., 2010).

By gel filtration chromatography DES enzyme exhibited an elution volume which perfectly matched the value expected for a tetrameric protein. This result fit in well with the homotetrameric organization reported for the majority of members of the subfamily of Cys/Met-metabolism-PLP-dependent enzyme (Marciano et al., 2010).

In *Arabidopsis thaliana* this enzyme was found exclusively in the cytoplasm (Alvarez et al., 2010).

1.7 - *Chlorella sorokiniana* **as a model organism**

Chlorella sorokiniana is a single cell, fresh water green micro-algae, belonging to the *Chlorophyta* phylum.

Figure 15: *Chlorella sorokiniana* cells.
This alga is without flagella, spherical in shape (about 2 to 10 µm in diameter) and has a single and huge chloroplast in the shape of cup with a starch granule.

The name *Chlorella* is taken from the Greek word *chloros* meaning green and the Latin diminutive suffix *ella* meaning "small". German biochemist and cell physiologist Otto Heinrich Warburg awarded with the Nobel Prize in Physiology or Medicine for his research on cell respiration in 1931, also studied photosynthesis in *Chlorella*. In 1961 Melvin Calvin of the University of California received the Nobel Prize in Chemisty for his research on the pathways of carbon dioxide assimilation in plants using *Chlorella*.

This alga is without flagella and spherical in shape with a diameter between about 2 to 10 µm . *Chlorella* has few and small vacuoles in cytoplasm and a single and huge chloroplast in the shape of cup with a starch granule (Figure 16-17). The chloroplast contains the green photosynthetic pigments chlorophyll-a and –b, β-carotene and xantophille. *Chlorella* contains the highest level of chlorophyll in the world (Kerem et al., 2008).

Through photosynthesis *Chlorella* multiplies rapidly (about every 6 hours) requiring only carbon dioxide, water, sunlight, and a small amount of minerals to reproduce. *Chlorella sorokiniana* demonstrates optimal growth in the 37-39°C range, while most other *Chlorella* species, including *Chlorella vulgaris,* grow optimally at much lower temperatures.

Figure 16: Transmission electron micrograph of *Chlorella sorokiniana* cells.
Cells of *Chlorella sorokiniana* by transmission electron microscopy (TEM) FEI EM 2058.

Another distinctive characteristic of *Chlorella sorokiniana* is a strong cell wall filled of sporopollenin, which is a major component of the tough outer (exine) walls of spores and pollens grains. Sporopollenin is chemically very stable and is usually well preserved in soils and sediments.

Chlorella for its high protein content was considered to be a protein source and a "functional nutrient" first in Japan then in Europe and in America. Today it is accepted that *Chlorella* is rich in nutrient content. Active molecules of *Chlorella* are: 61.6% proteins, 12.5% fats, 13.7% carbohydrates, trace elements (Al, Ze, P, Ca, Mg, Mn, Ni, Se), vitamins (carotene, beta-carotene, thiamine, B1, B2, B6, C, D, E, K), nucleic acids (RNA and DNA), and various enzymes (Kerem et al., 2008).

2 - MATERIALS AND METHODS

2.1 - *Chlorella sorokiniana* strain and growth

All experiments were performed by using of *Chlorella sorokiniana* Shihira & Krauss, strain 211/8K (CCAP of Cambridge University).

The algae were grown in batches at 35°C, continuously stirred and illuminated (Philips TLD 30 W/55 fluorescent lamps, 250 µmol photons $m^{-2} s$). The cultures were insufflated with air containing CO_2 5% at a flow rate of 80-100 l h^{-1}. *Chlorella s*. was grown in different types of medium in order to analyze and compared results. An all nutrients containing medium (control or +S) and in condition of S-deficiency using a sulphate-free medium (-S).

In the control medium (Table 2) sulphate was supplied as 1.2 mM $MgSO_4$, nitrate was supplied as 5 mM KNO_3.

For further experiments cells were harvested when the culture was in exponential growth phase.

KH_2PO_4	13 mM
K_2HPO_4	4.3 mM
$CaCl_2$	0.18 mM
NaCl	0.35 mM
Fe/EDTA (+S)	$0.35 \cdot 10^{-3}$ mM
Oligoelements (+S)	0.5 mM
$MgSO_4$	1.2 mM
KNO_3	5 mM

Table 2: S-sufficient culture medium composition.
The medium (pH 6.5) contains all nutrients essential to algae growth.

The oligoelements mixture used to prepare the culture medium included:

- 14 mM $MnCl_2$;

- 46 mM H_3BO_3;

- 0.77 mM $ZnSO_4$;

- 0.32 mM $CuSO_4$;

- 0.12 mM $(NH_4)_6Mo_7O_{24}$.

To obtain S-starved cultures (-S), control cells were harvested by centrifugation (14 000 rpm for 10 min), washed with -S medium, centrifuged again and then the pellet was re-suspended in a sulphate-free medium (Table 3). The cells were grew in –S condition for a period of 24 h. Sulphate-free medium was prepared by replacing the S-salts with their chloride counterparts.

KH_2PO_4	13 Mm
K_2HPO_4	4.3 mM
$CaCl_2$	0.18 mM
NaCl	0.35 mM
Fe/EDTA (-S)	$0.35 \cdot 10^{-3}$ mM
Oligoelements (-S)	0.5 mM
$MgCl_2$	1 mM
KNO_3	2.5 mM

Table 3: S-free culture medium composition.

The –S medium (pH 6.5) was prepared replacing the S-salts with their chloride counterparts.

The oligoelements mixture for S-free medium was composed of:

- KCl 95 mg/L;

- H_3BO_3 80 mg/L;

- $CuCl_2$ 3 mg/L;

- $MnCl_2$ 24.7 mg/L;

- $ZnCl_2$ 6.8 mg/L;

- $(NH_4)_6Mo_7O_{24}$ 0.5 mg/L

2.2 – Determination of PCV

For PCV (Packed Cell Volume) measurement an aliquot of a well-mixed suspension culture (10 ml) was transferred into a graduated haematocrit tube. The tube was centrifuged whit a swinging-rotor for 10 min at 4000 g.

The packed cell volume was visually determined by taking a reading from the capillary graduation. The PCV was expressed as $\mu l \, ml^{-1}$.

2.3 – Measurement of total protein

Total proteins content (expressed as $mg \, ml^{-1}$) was determined by Bradford's method (1976) using Bio-Rad protein assay reagent and bovine serum albumin (BSA) as a standard protein.

Dye reagent was prepared by diluting 1 part of dye concentrate reagent with 4 parts of distilled, deionized water (DD). The solution was then filtred with Whatman filter (or equivalent) to remove particulates.

Variable volume of *Chlorella* crude extract was added to the diluted Bio-Rad solution (final volume 800 μl) and incubated at room temperature for at least 5 minutes, but no longer than 1 hour.

The absorbance was measured at 595 nm against blank.

2.4 - Spectrophotometric determination of total chlorophyll

To measure the total chlorophyll content, 5 ml of *Chlorella s.* culture were centrifuged at 4000 g for 10 min. The pellet was re-suspended in 5 ml of N, N-

dimethylformamide (DMF) and incubated in the dark at 4°C for at least 2 hours.

After, the absorbance of the mixture was analyzed by spectrophotometer at 647 and 664 nm. The total chlorophyll content was calculated using the next formula:

$$17.9 \cdot \text{Abs}_{647} + 8.08 \cdot \text{Abs}_{664} = \text{mg/ml of total chlorophyll}$$

2.5 - Serine and OAS intracellular determination

In order to measure intracellular levels of serine and OAS, 500 ml of cell suspension were harvested by centrifugation (4000 g for 10 min). The pellet was treated with 1 ml of cold 80% Et-OH, left for 10 min at 4°C, and centrifuged again. After filtration through Waters Sep-Pak Cartridges C18 light (Milan, Italy), the supernatant was used for amino acids analysis.

The amino acids pool was determined by HPLC after derivatization with o-phthaldialdehyde (oPA). All chromatographic equipments were from Gilson.

The analysis was made using a two-pump HPLC chromatograph (model 305-306) with a system controller programmer and a model 7125 sample injector valve. The o-PA-derivatized amino acids were separated on a reverse phase C18 ultrasphere. Solvent A consisted of 50 mM sodium acetate (pH 7) plus 1% tetrahydrofurane and solvent B was absolute methanol. An aliquot (50 µl) of the extract was derivatized for 1 min. A 20 µl sample of the mixture was injected and eluted at a flow rate of 1 ml min^{-1}. The eluted amino acids were detected by optical density at 340 nm with a model 115 wave-length detector.

Contents of serine and OAS were measured against a relative calibration curve and expressed as µmol ml^{-1} PCV.

2.6 – Cysteine and glutathione intracellular determination

Chlorella cells (500 ml of culture) were harvested by centrifugation (4000 *g* for 10 min) and the pellet frozen in liquid nitrogen for 90 seconds. To the pellet was added 2 ml of buffer containing 0.1 N HCl and 1 mM EDTA, and then the mixture was centrifuged (Sorvall RC5C centrifuge – Sorvall SS34 rotor) at 15000 rpm for 15 minutes at 4°C. The supernatant was the crude extract used to determine the Cys and GSH content.

According to Newton and co-workers (1981), cysteine and glutathione were quantified by reversed-phase HPLC after derivatization with monobromobimane.

Thiols were reduced at room temperature for 1 h, mixing 400 µl of extracted samples with 600 µl of 200 mM CHES (pH 9.2) and 100 µl of 3 mM DTT. An aliquot of 330 µl was derivatized for 15 min in the dark, adding 20 µl of 15 mM monobromobimane. The reaction was stopped by of 250 µl of 0.25% (v/v) methanesulfonic acid.

The instrument HPLC Gilson has an injector valve (model 7125) and two pumps (models 306 and 305).

The separation of GSH pick was realized by ultrasphere C18 inverse phase column (250×4.6mm) at flux of 1ml/min.

The solvent A was methanol 10% (v/v) and acetic acid 0.25% (v/v). The solvent B was methanol 90% (v/v) and acetic acid 0.25% (v/v). Both the eluants had pH 3.9 and filtered by filter Millipore 0.2 µm. The Gilson fluorimetric detector (mod 121) was furnished of filter having excitation length to 305-395 nm and emission length to 430-470 nm.

The Cys and GSH concentrations were expressed as µmol·ml^{-1} PCV.

Figure 17: HPLC-separation of Cys and GSH in *Chlorella sorokiniana* extracts. Conditions for separation as described in Materials and Methods section.

2.7 - OASTL activity assay

Chlorella sorokiniana cells (500 ml of culture) were harvested by centrifugation (4000 *g* for 10 minutes). The pellet was re-suspended in 10 ml of cold extraction buffer containing 50 mM of phosphate-buffer pH 7.5, 10 μM PLP and 1 mM DTT. The cells were broken by passing twice through a French pressure cell (1100 psi) and then centrifuged at 15000 rpm for 15 min at 4°C. The resulting supernatant as used as crude extract and assayed for enzyme activity.

According to Gaitonde (1967), the OASTL activity was colorimetrically determined as cysteine formation.

$$\textit{O-Acetilserine} + \textbf{Sulphide} \longrightarrow \textbf{L-Cysteine} + \textbf{Acetate}$$

The reaction mixture (final volume 100 μl) included:

1) 100 mM Hepes /KOH (pH 7.2);

2) 10 mM OAS;

3) 5 mM Na_2S;

4) 5 mM DTT;

5) Crude extract;

6) DD water.

The reaction was initiated by the addition of Na_2S and incubated in a thermo-mixer (5 min at 50°C), after which the reaction was terminated by adding 50 μl of TCA 20% (v/v). To the mixture, 100 μl of glacial acetic acid and 200 μl of acid ninhydrin reagent (0.12 gr of ninhydrin dissolved in 3 ml of glacial acetic acid and 2 ml of 37% chloridric acid) were added. The sample was incubated at 100° C for 5 minutes to allow the colour development and cooled on ice. The colour was stabilized by adding 0.55 ml of ethanol.

The spectral measurements of the reaction products were made against a blank with a Thermo Biomates 5 spectrophotometer at 560 nm.

One unit (U) of activity was the amount of the OASTL enzyme which catalyzes the formation of 1 μmol of cysteine min^{-1}.

$$\mu mol \text{ of cysteine } min^{-1} = \frac{\Delta 560 \cdot V_2 \cdot V_0}{6.4 \cdot t_1 \cdot V_1 \cdot V_E}$$

▪ $\Delta 560$ = Absorbance at 560 nm;

▪ V_2 = Final volume (ml) included ethanol;

▪ V_0 = Mixture incubated at 50°C;

- 6.4 = OAS molar extinction coefficient (mM $^{-1}$ cm^{-1});
- t_1 = time (min) of incubation at 100°C;
- V_1 = Mixture incubated at 50°C included TCA;
- V_E = Crude extract volume used for the assay.

2.8 - Partial Purification of OASTL enzyme from *Chlorella sorokiniana*

Chorella sorokiniana cells (2 litres of culture) were harvested by centrifugation (4000 *g* for 10 min), the pellet was re-suspended in the extraction buffer (50 mM phosphate-buffer pH 7.5, 1mM EDTA, 1 mM DTT, 10 μM PLP and 0.5 mM PMSF). The cells were broken by passing twice through a French pressure cell (1100 psi) and then centrifuged at 15000 rpm for 15 min at 4°C. The supernatant represented the crude extract.

Step 1: Heat treatment - Crude extract was incubated at 60°C for 30 min, then cooled and centrifuged at 15.000 rpm for 15 min at 4°C.

Step 2: Precipitation with ammonium sulphate - The supernatant was saturated with 30%- 80% of ammonium sulphate ((NH$_4$)$_2$SO$_4$), next centrifuged at 20 000 rpm for 15 min. The pellet was re-suspended in 2.5 ml of extraction buffer, centrifuged at 20 000 rpm for 15 min and next desalted by passing in Sephadex G-25 column (Pharmacia PD 10), which was previously equilibrated with the same extraction buffer.

Step 3: Ion-exchange chromatography - The extract was again partially purified by Q-Sepharose column (Pharmacia) (1.0 × 20 cm). The column was conditioned by 100 ml of buffer having the following composition: 50 mM phosphate-buffer pH 7.5, 1 mM DTT and 10 μM PLP.

The proteins of interest were eluted using a linear gradient of KCl 0-250

mmol/L in the same buffer (150+150 ml), obtained by a gradient mixer (Pharmacia GM-1).

The residual proteins were eluted with 50 ml of buffer containing 250 mmol/L of KCl. The flux was of 1 ml min^{-1} and fractions of 4 ml were collected. On every fraction was determined the proteins content and OASTL activity.

The fractions having OASTL activity were mixed and concentrated by Centriplus 30 tubes (Amicon Centriplus$^{®}$).

2.9 – Gel filtration chromatography

The molecular weight (MW) of *Chlorella sorokiniana* OASTL was determined by gel filtration chromatography. The column used for the gel filtration was a Superdex 200 HR 10/30 (GE Healthcare), which was equilibrated with 50 mM potassium phosphate buffer (pH 7.5) containing 0.1 M NaCl and 10 µM PLP. Elution was performed with the same buffer at a flow rate of 0.2 ml min^{-1}.

The calibration of the column (Figure 18) was carried out with standard molecular weight proteins such as:

- Cytochrome c: 12.4 kDa (25mg/ml);
- Carbonic anydrase: 29 kDa (25mg/ml);
- Ovalbumin: 44 kDa (25mg/ml);
- Transferrin: 81 kDa (25mg/ml).

The eluted fractions from Superdex were subjected to OASTL-ninhydrine assay.
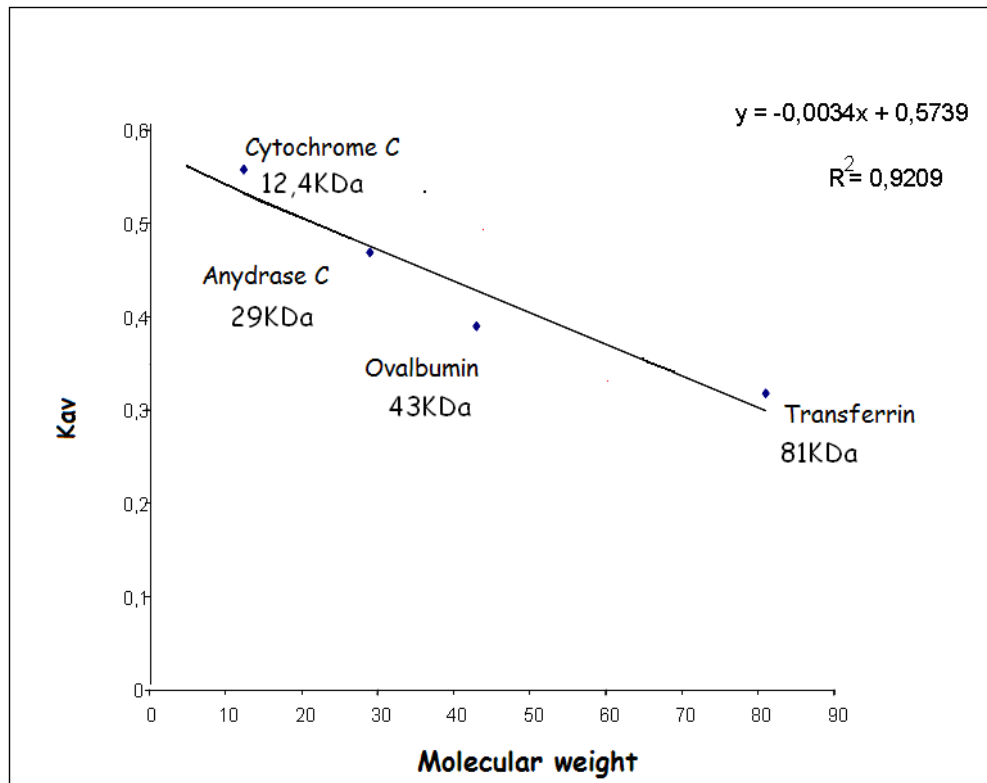
Figure 18: Superdex 200 HR calibration line.

Kav = distribution coefficient (the factor of pore volume available for a given molecule).

Kav = $(V\text{e} - V\text{o})/(V\text{t} - V\text{o})$.

2.10 - Purification of OASTL enzyme by Metal Chelate Affinity Chromatography

The purification of OASTL enzyme from *Chlorella sorokiniana* was carried out with the support of the Prof. Rüdiger Hell and his lab group (Institute of Plant Sciences, University of Heidelberg, Germany)

2.10.1 - Bacterial strain

Over-expression of the modified SAT was carried out using the HMS174 (DE3) bacteria.

HMS174 strains provide the recA mutation in a K-12 background. Like BLR, these strains may stabilize certain target genes whose products may cause the loss of the DE3 prophage. DE3 indicates that the host is a lysogen of lDE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under control of the lacUV5 promoter.

Such strains are suitable for production of protein from target genes cloned in pET vectors by induction with IPTG (Isopropil β-D-1-tiogalattopiranoside).

E. coli HMS174 (DE3) was cloned in the vector pET28a.

2.10.2 - Expression and preparation of target protein

Mature histidine-tagged AtSAT5 were expressed in HMS174(DE3).

Transformed bacteria were generously provided by R. Hell and M. Wirtz.

The bacteria were incubated overnight at 37°C in 25 ml of LB medium (Luria-Bertani broth, 1% tryptone, 0.5% yeast extract, 0.5% NaCl) containing kanamycin 2ml/L (Kanamycin solution from *Streptomyces kanamyceticus,* 50 mg/mL kanamycin in 0.9% NaCl, Sigma).

From overnight culture, a variable volume was collected and moved to in a flask containing 250 ml LB and 2 ml/L kanamycin. The new bacterial culture had an OD $_{600}$ of 0.1, which is what it should have been.
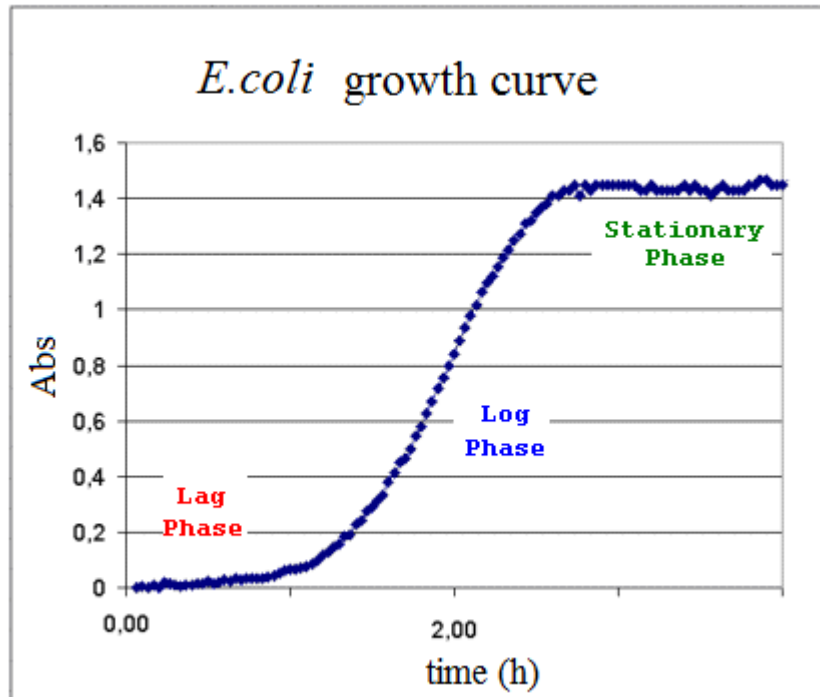
Figure 19: *E.coli* growth curve.

Lag Phase represents a period of slow growth during which the bacteria are adapting to the conditions in the medium. This is followed by a Log Phase in which growth is exponential, doubling every replication cycle. Stationary Phase occurs when the nutrients become limiting, and the rate of multiplication equals the rate of death.

When the bacterial growth was in the exponential growth phase (OD_{600} 0.6-0.8), the expression was induced by IPTG (Figure 20). After about 4 h from the induced expression, bacteria were collected by centrifugation (10.000 g for 10 min) and the pellet stored at -80°C.
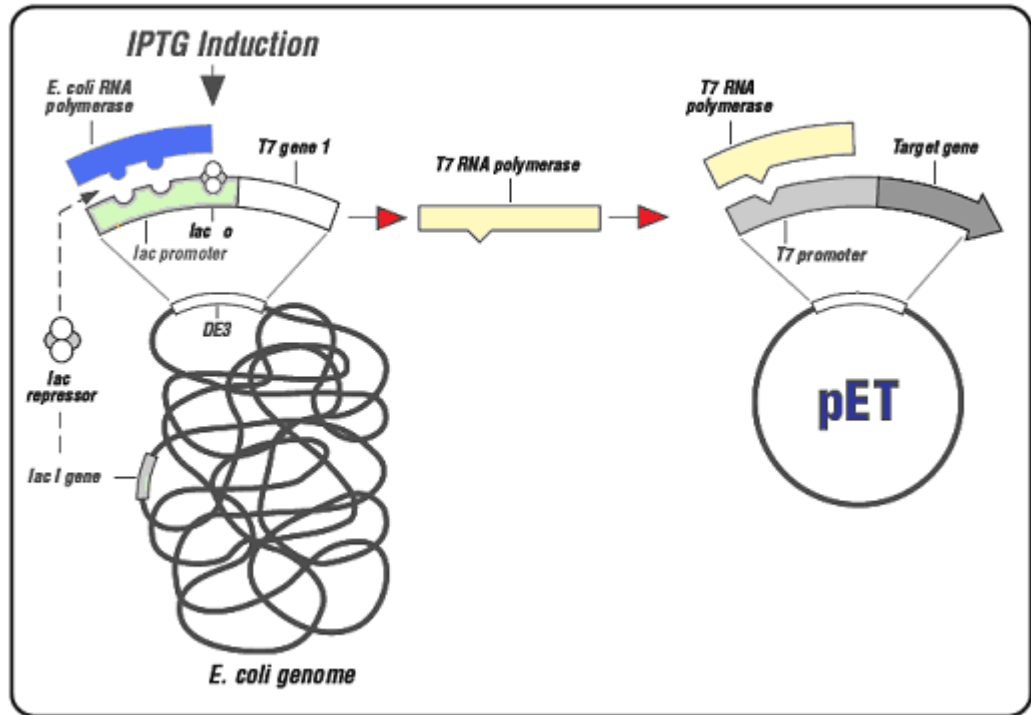
Figure 20: General scheme of gene expression in a host cell.

For protein production, a recombinant plasmid is transferred to an *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase. These hosts are lysogens of bacteriophage DE3, a lambda derivative that has the immunity region of phage *21* and carries a DNA fragment containing the *lacI* gene, the *lacUV5* promoter, and the gene for T7 RNA polymerase. This fragment is inserted into the *int* gene, preventing DE3 from integrating into or excising from the chromosome without a helper phage. Once a DE3 lysogen is formed, the only promoter known to direct transcription of the T7 RNA polymerase gene is the *lacUV5* promoter, which is inducible by IPTG (pET System Manual, Novagen).
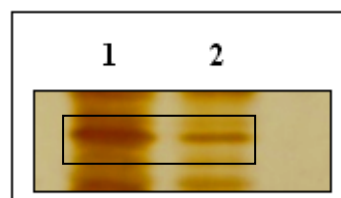
Figure 21: Expression of recombinant SAT.

SAT polypeptide production was 1mM IPTG induced in bacteria, and proteins were separated by 12.5% SDS-PAGE/Silver stain. Lane 1: induced bacteria; Lane 2: uninduced bacteria.

2.10.3 - Preparation of crude extract from *E.coli* cells

E.coli pET28-At SAT5 cells, harvested and stored at -80°C (for other information see the section 2.10.2), were re-suspended with 10 ml of SAT B buffer (50 mM Tris pH 8.0; 250 mM NaCl; 20 mM imidazole) and 25 μl of 200 mM PMSF.

Cells were lysed by passing twice through a French pressure cell (1100 psi) and pelleted by centrifugation at 15000 *g* for 15 min at 4°C. The supernatant was the bacterial extract.

2.10.4 – His-tag

Chlorella sorokiniana crude extract (protocol previously described in the section 2.7) was used for the purification of OASTL enzyme by metal chelate affinity chromatography.

According to literature, OASTL interacts with SAT enzymes to form the CSC, this property was exploited to construct an affinity purification columns with bound recombinant SAT 5 from *Arabidopsis thaliana*.
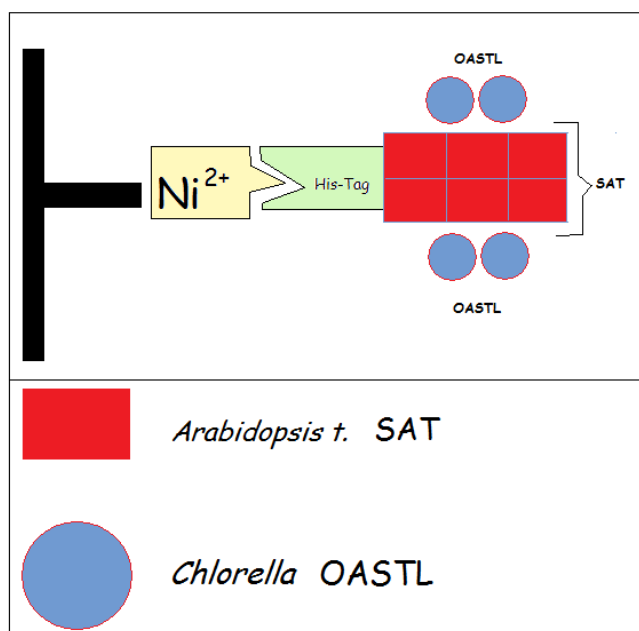
Figure 22: Scheme of OASTL purification by His-tag**.**

OASTL proteins from Chlorella *sorokiniana* interact with SAT in CSC, so this property was exploited to construct affinity purification columns with bound recombinant cytosolic SAT 5 from *Arabidopsis thaliana* to purify OASTL proteins from *Chlorella sorokiniana*.

Recombinant SAT5 proteins, expressed in *E. coli*, were immobilized in a nickel-loaded Hi-Trap column (1 ml, GE Healthcare). The purification was carried out as described by Heeg and co-workers (2008).

E.coli AtSAT5 crude extract was flowed into the Hi-trap column previously nickel-loaded for at least 1 hour. After a quick washing with washing buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 80 mM imidazole), bacterial OASTL which bound SAT-tag proteins was removed with elution buffer (50 mM Tris-HCl pH 8.0, 250 mM NaCl, 80 mM imidazole, 10 mM OAS). The column was then washed with washing buffer.

Chlorella crude extract was flowed through column for at least 1 hour and then OASTL proteins were eluted with 10 ml of buffer containing 0.5 M Tris pH 8.0, 250 mM NaCl and 50 mM OAS.

The purified fractions containing the enzyme OASTL were analyzed by SDS-PAGE.

2.11 - Vertical polyacrylamide gel electrophoresis (PAGE)

Polyacrylamide gels (native or denaturing) were prepared following the next protocols.

% RUNNING GEL	Running Buffer (ml)	H_2O (ml)	Acrylamide/Bis 30% (29:1) (ml)	0.4M Ammonium Persulphate (µl)	TEMED (µl)
8	5	9.52	5.37	100	10
12	5	7.15	7.65	100	10
% STACKING GEL	Stacking Buffer (ml)	H_2O (ml)	Acrylamide/Bis 30% (29:1) (ml)	0.4M Ammonium Persulphate (µl)	TEMED (µl)
3	1.89	4.065	0.75	37.5	7.5

Table 4: Protocol for running/stacking native – gel.

% RUNNING GEL	Running Buffer (ml)	H$_2$O (ml)	Acrylamide/Bis 30% (29:1) (ml)	10% SDS (µl)	0.4M Ammonium Persulphate (µl)	TEMED (µl)
10	5	8	6.6	200	100	10
12.5	5	6.4	8.30	200	100	10
% STACKING GEL	Stacking Buffer (ml)	H$_2$O (ml)	Acrylamide/Bis 30% (29:1) (ml)	10% SDS (µl)	0.4M Ammonium Persulphate (µl)	TEMED (µl)
4	1.89	4.5	0.75	75	37.5	7.5

Table 5: Protocol for running/stacking denaturing – gel.

Running (1) and stacking (2) buffer used in the gel preparation, contained:

1)   1.5 M Tris-HCl pH 8.0;
2)   0.5 M Tris-HCl pH 6.8.

2.12 - Silver staining

Following electrophoresis, gels were overnight incubated in fixative solution (30% of ethanol and 10% of acetic acid). The day after they were rinsed 3 times for 10 minutes with DD water and then soaked in sensitisation solution (0.8 mM sodium thiosulphate) for one minute. The sensitised gels were rinsed twice in DD water for 1 minute.

The rinsed gels were soaked in silver nitrate solution (70 µl formaldeyde 37%, 1.25 ml AgNO$_3$ 1N, 100 ml DD water) for 30 minutes and then washed for few seconds.

The gels were developed by a solution containing: 100 ml Na$_2$CO$_3$ 30%, 250 µl formaldeyde 37%, 125 µl sodium thiosulphate 10%, 1l DD water.

When brownish bands appeared on gel, the reaction was stopped by adding up acetic acid 1%.

2.13 – Western Blot analysis

Separated proteins by SDS-PAGE were overnight transferred to a nitrocellulose membrane (Hybond ECL, GE Healthcare) at a constant current of 160 mA.

The membrane was blocked, for at least 90 min, in 20 mM Tris/HCl (TBS buffer, pH 8.4) containing 5% BSA and then washed with TBS containing 0.1% of Tween-20 (TBS-T buffer). The blot was incubated for at least 90 min in TBS buffer with 2% BSA (1:10) containing rabbit primary antibody (1:10000 dilution) against the purified OASTL proteins from *Arabidopsis thaliana*. OASTL antibodies were produced against cytosolic (A), plastidial (B) and mitochondrial (C) isoforms.

The membrane was subsequently washed two times for 5 min with TBS-T buffer and incubated for 90 min with TBS buffer with 2% BSA (1:10) containing anti-rabbit IgG horseradish peroxidase conjugate (Sigma) (1:10 000 dilution). After rinsing in TBS buffer for 5 min (two times), membranes were developed with Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Little Chalfont, UK) and Lumi-film chemiluminescent detection film (Roche, Basel, Switzerland) according to the manual.

2.14 –In-gel digestion for mass spectrometry

OASTL protein sequence from +S cells was identified by MALDI-TOF mass spectrometry.

OASTL proteic bands coming from SDS-PAGE on purified fraction were excised and destained twice with 200 µl of destain-solution (100 mM NH$_4$HCO$_3$ pH 8.0 in 50% ACN (v/v)) for 40 min at 37ºC.

The gel was then re-hydrate with 50 µl of 100% ACN for 10 min at room temperature. The solution was then removed and the gel pieces were dried fully in the SpeedVac.

To each gel sample, 50 µl of 10 mM DTT and 50 mM of ammonium acetate were added for 30 min at 37°C, and then the solution was removed and replaced with the same volume of iodoacetamide in 50 mM of ammonium acetate for 50 min in the dark. This solution was removed and 100% ACN was added for 10 min, and then the gel dried in the SpeedVac.

Trypsin was added to each sample and put in ice for at least 10 min.

The trypsin digestion was realized overnight by adding up to the samples (40 µl) 50 mM of NH$_4$HCO$_3$ and 0.5 mM of CaCl$_2$. After overnight digestion, trypsin, NH$_4$HCO$_3$ and CaCl$_2$ were removed. The gel pieces were incubated for 20 minutes in 50 µl of 50 mM NH$_4$HCO$_3$ and 30% ACN. This solution was removed and added to the digestion solution.

To the gel pieces 50 µl of 50 mM NH$_4$HCO$_3$ in 60% ACN was added and stirred for 20min. This solution was removed and added to the previous extraction solution. All solutions were fully dried in a concentrator, and frozen in -20°C.

The peptides obtained with in-gel-digestion were cleaned bi ZipTip C$_{18}$ (Millipore, UK), then by MALDI-TOF the OASTL protein sequence was determined.
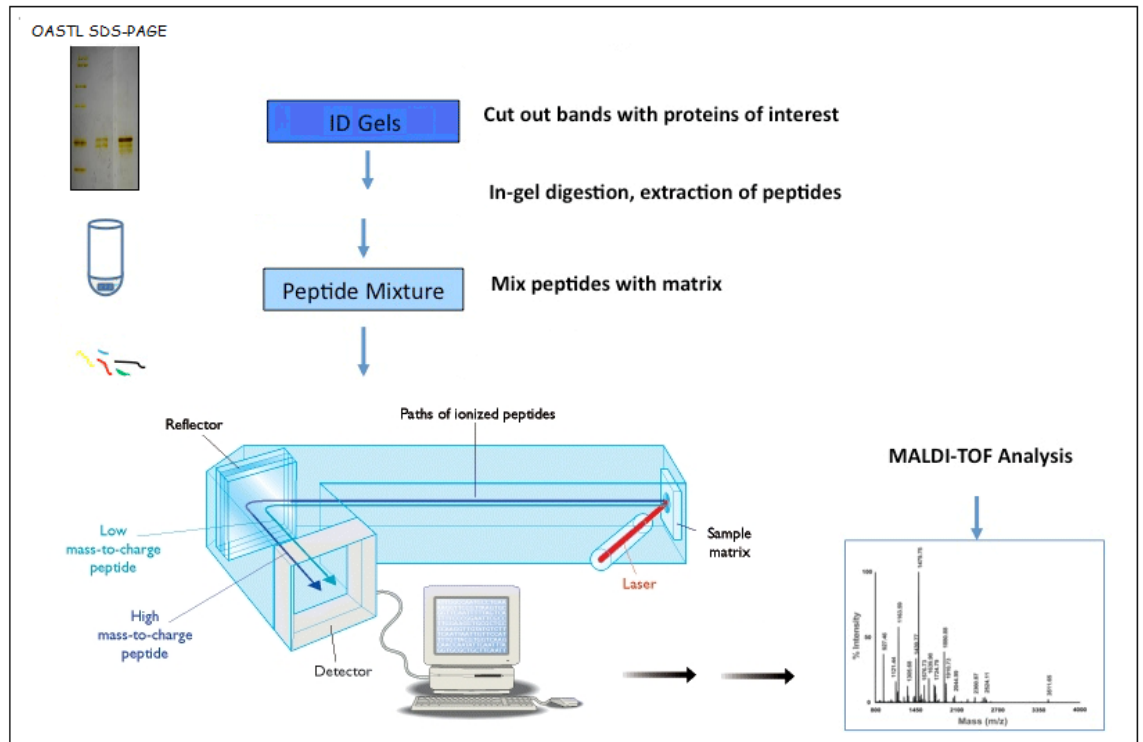
Figure 23: Scheme for the mass spectrometry.

After 1D gel electrophoresis a protein of interest is excised from the gel and digested with a protease such as trypsin, which cuts immediately after arginine or lysine amino acids. This cleaves the protein into a series of peptides which can be analyzed by MALDI-TOF. In the mass spectrometer the peptides are ionized by a pulse of energy from a laser and then accelerated down the column to the reflector and into the detector. The time of flight of each peptide depends on its mass-to-charge ratio. The data are visualized as a spectrum. The computer contains a database of the predicted molecular weights of every trypsin fragment of every protein encoded by the genome of the organism under study. The computer compares the masses of the detected peptides with the database and identifies the most likely source protein.

2.15 - Bioinformatic analysis

Protein sequences were compared with the non-redundant (nr) sequence database of BLASTP (protein against protein database). However multiple

sequence alignments were performed by ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

2.16 – *L*-cysteine desulphydrase (DES) activity assay

Chlorella cells (500 ml of culture) were harvested by centrifugation (4000 g for 10 min) and the pellet was re-suspended in 10 ml of extraction buffer (20 mM Tris-HCl, pH 8.0). The cells were broken by passing twice through a French pressure cell (1100 psi). After centrifugation at 15000 g at 4°C for 15 min the obtained supernatant represents the crude extract.

According to Alvarez and co-workers (2010), the DES activity was measured on the basis of the formation of methylene blue.

The reaction mixture, having final volume of 1 ml, was composed of:

1) 100 mM Tris-HCl pH 8.0;

2) 2.5 mM DTT;

3) 0.8 mM Cys;

4) extract (variable volume);

5) DD water.

The mixture was incubated at 37° C for 15 min and then the reaction was stopped by adding 0.1 ml of 20 mM DMP (dissolved in 7.2 N HCl) and 0.1 ml of 30 mM $FeCl_3$ (dissolved in 1.2 N HCl). After 15 minutes of incubation at room temperature and in the darkness, methylene blue formation was measured spectrophotometrically at 670 nm. The used molar extinction coefficient was $1.5 \cdot 10^6$ cm^2 mol^{-1}. One unit (U) of activity was the amount of the DES enzyme which catalyzes the release of 1 μmol of sulphide min^{-1}.

2.17 - DES activity stain

Chlorella sorokiniana crude extract was loaded in 12% native-gel (10 x 10 cm) and the electrophoresis carried out at constant current of 45 mA for 2h at 4°C.

Following electrophoresis, DES active proteins were detected soaking the gel for 3 min in a pre-warmed (30° C) reaction mix containing: 200 mM Na-Bicine pH 8.5, 50 µM PLP, 0.25 mg/ml BSA, 10 mM Cys. After 3 minutes to the reaction mixture was added 100µl of 100 mM lead acetate. The H_2S, released from DES reaction, reacted with lead acetate forming brownish bands on the gel.
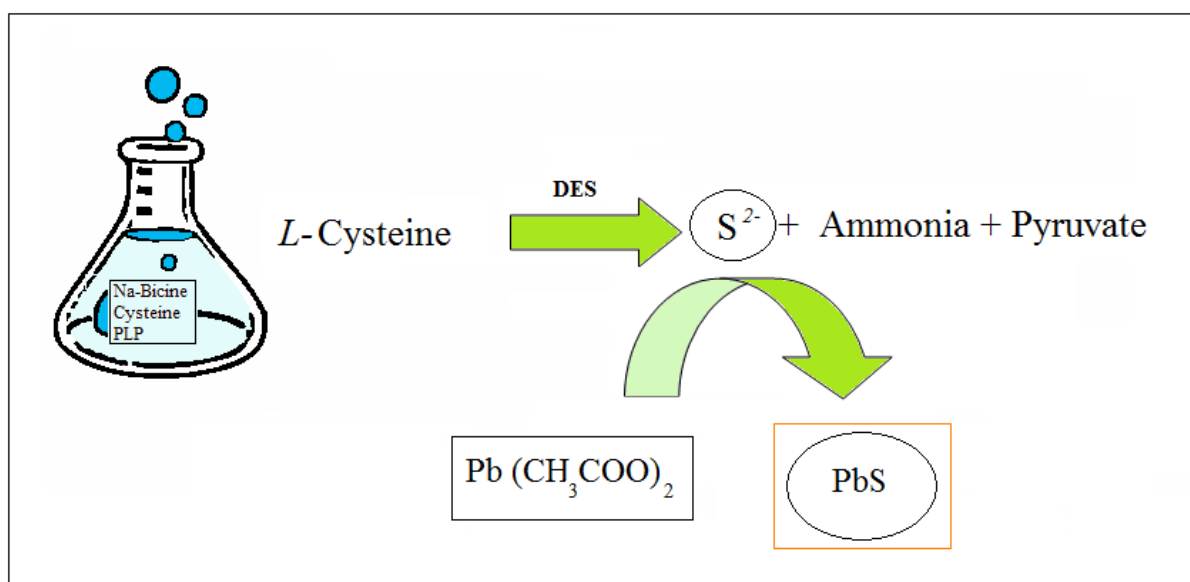
Figure 24: DES activity stain.
Chlorella sorokiniana crude extract was loaded in native-PAGE 12%, following electrophoresis the gel was stained using DES activity stain. Active DES proteins in native gels were detected by lead acetate. The reaction of H_2S with lead acetate was seen as brownish bands.

2.18 - Passive elution of proteins from polyacrylamide gel

DES activity bands from native-page (see paragraph 2.17) were excised and placed in clean micro-centrifuge tubes.

The elution buffer (50 mM Tris-HCl, 150 mM NaCl, and 0.1 mM EDTA; pH 7.5) was added (0.5-1 ml) to the tubes so that the gel pieces are completely immersed.

Gel pieces were crushed using a clean pestle and incubated in a rotary shaker at 30°C overnight. Then the tubes were centrifuged at 10.000 g for 10 min and carefully the supernatants were pipetted into the new micro-centrifuge tubes. The supernatant represented the extract.

2.19 - Statistical analysis

Sigma plot 11 software was used for data analysis. Data of mean ± ES of three independent experiments were presented. The statistical analysis was performed by one-way ANOVA analysis to determine differences between the S-sufficient and S-starved algae, $P < 0.05$ as significant.

3 - RESULTS

3.1- Content of metabolites in S-sufficient and S-starved cells of *Chlorella sorokiniana*

3.1.1-Total proteins content

Cells of *Chlorella sorokiniana* growth in +S medium showed soluble proteins content of 20.6 ± 1.7 mg ml^{-1} PCV. However, in cells S-starved for 24 h (-S), the soluble proteins content was decreased a lot (4.2 ± 0.15 mg ml^{-1} PCV) (Figure 25).
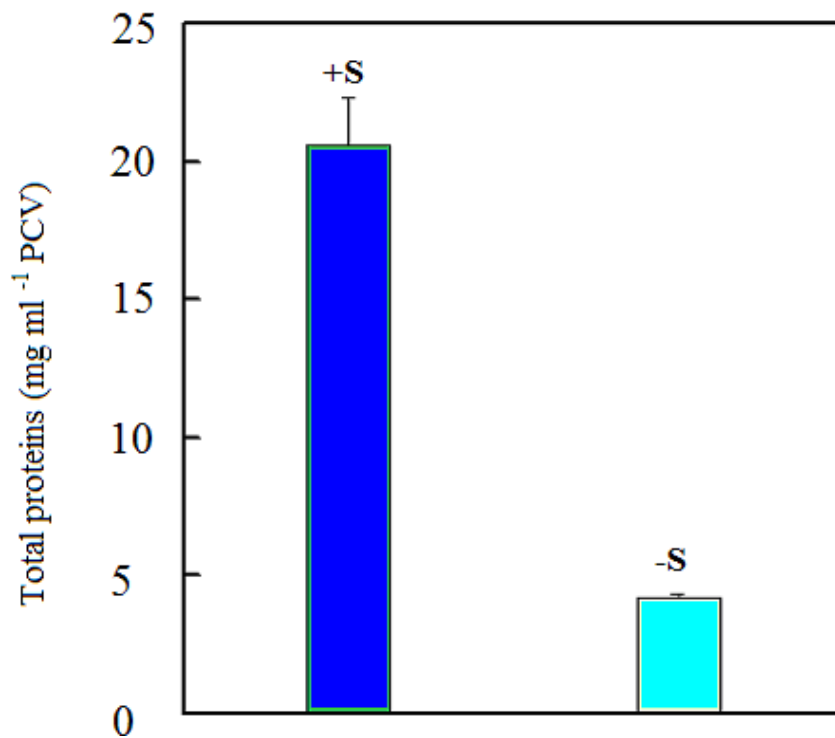
Figure 25: Total proteins content.

Effect of sulphate deficiency on soluble proteins content in *Chlorella sorokiniana* cells. The dark blue histogram represents + S cells, light blue histogram represents –S cells. Significant differences, using one-way ANOVA, was analyzed in S-starved cells respect to S-sufficient cells ($P < 0.001$). Sulphate was given to the cultures as $MgSO_4$ 1.2 mM.

3.1.2 - Intracellular levels of serine and *O*-acetylserine

S-starved cells (-S) contained levels of serine 3-fold higher than S-sufficient cells (Figure 26). The level of OAS, the immediate N-precursor of cysteine, also increased during S-starvation reaching a value that was 2.5-fold higher than S-sufficient cells (Figure 26).
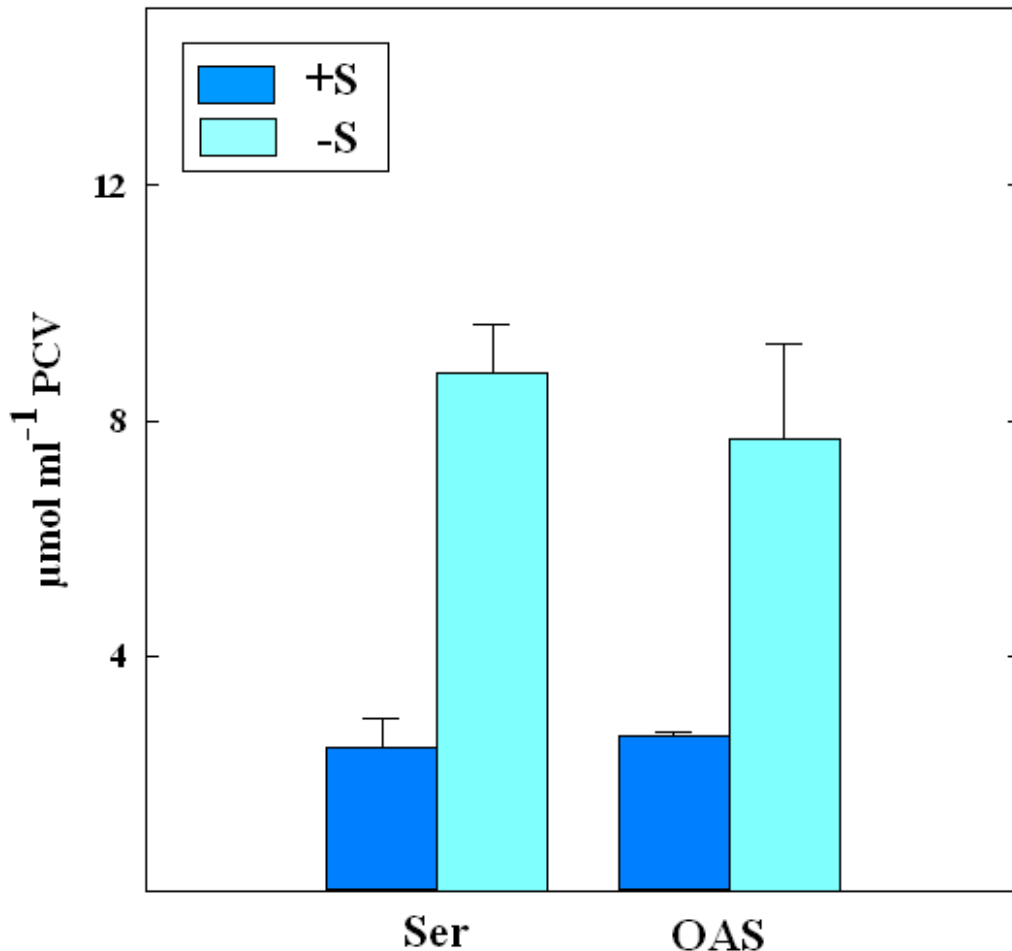
Figure 26: Intracellular levels of Ser and OAS.

Content of serine and OAS (μmol mL^{-1} PCV) in cells of *Chlorella sorokiniana* S- sufficient and S-starved for 24 h. Significant differences, using one-way ANOVA, was analyzed in S-starved cells respect to S-sufficient cells (P<0.001). Sulphate was given to the cultures as $MgSO_4$ 1.2 mM.

3.1.3 -Total cysteine and glutathione content

The Figure 27 shows the contents of total cysteine and glutathione in S-sufficient and S-deprived *Chlorella sorokiniana* cells.

In the S-sufficient cultures the cysteine content was 0.4 ± 0.02 μmol ml^{-1} PCV, while glutathione content was 1.24 ± 0.04 μmol ml^{-1} PCV.

When *Chlorella sorokiniana* cells were S-starved, the falling of cysteine and glutathione levels was very evident. The lowest values were showed off after 24 h of sulphate deprivation (Figure 28).
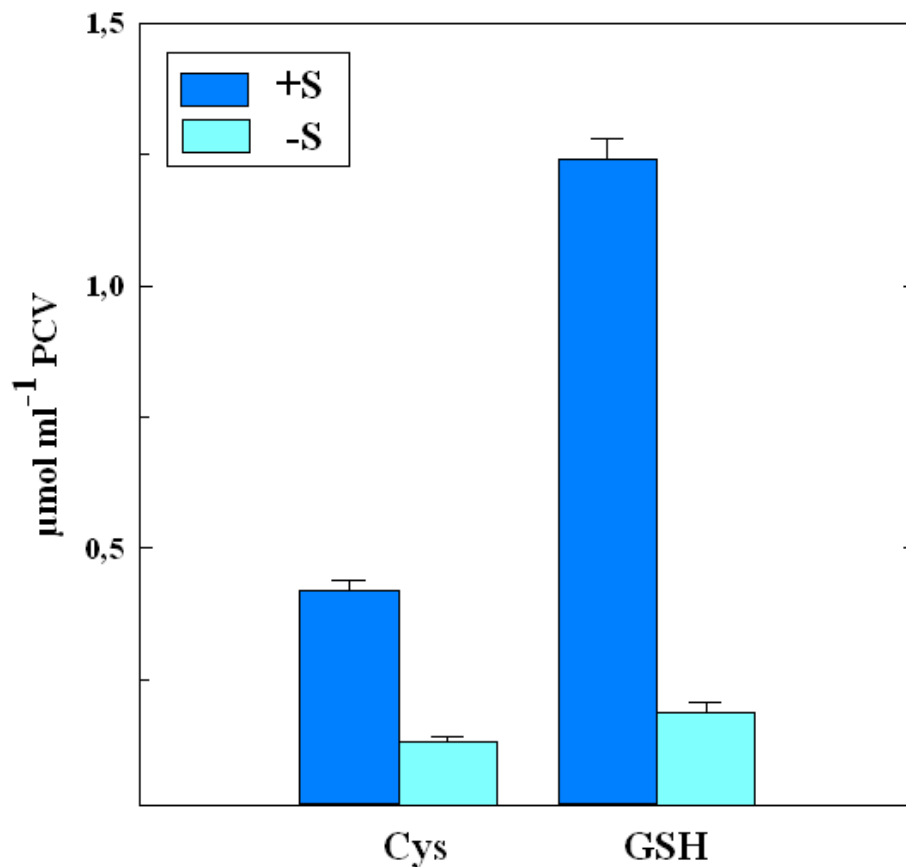
Figure 27: Total Cys and GSH content.

Content of cysteine and glutathione (μmol mL^{-1} PCV) in cells of *C. sorokiniana* S- sufficient and S-starved for 24 h. The dark blue histogram represents + S cells, light blue histogram represents –S cells. Sulphate was given to the cultures as $MgSO_4$ 1.2 mM. Significant differences, using one-way ANOVA, was analyzed in S-starved cells respect to S-sufficient cells ($P < 0.001$). Sulphate was given to the cultures as $MgSO_4$ 1.2 mM.
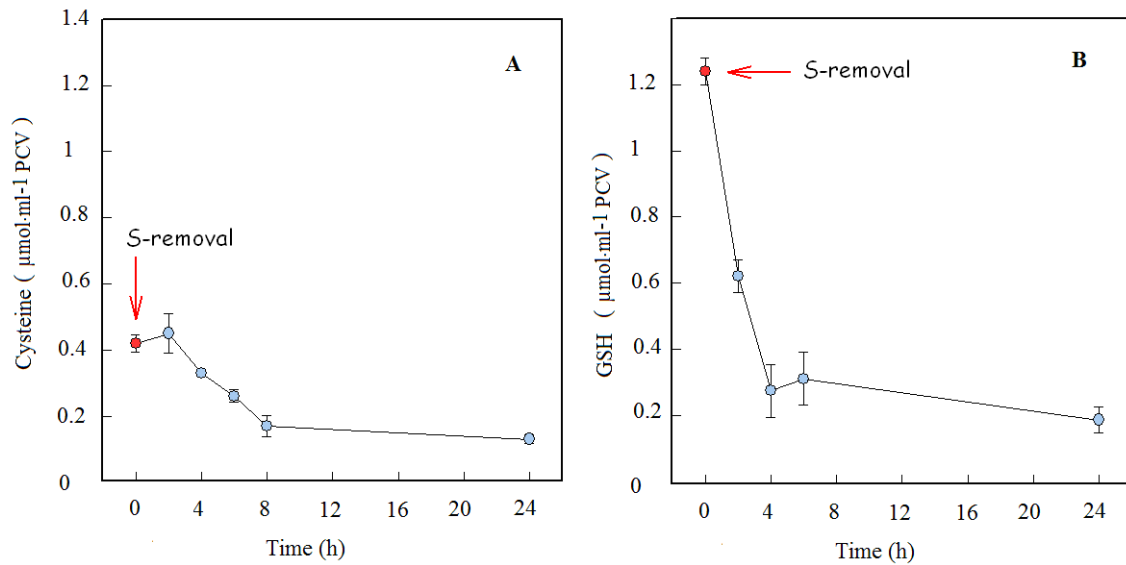
Figure 28: Time-dependent decrease of Cys and GSH content.
Time-dependent variations of cysteine (A) and GSH (B) content (μmol mL^{-1} PCV) in cells of *Chlorella sorokiniana* during S-starvation. The red circle represents S-sufficient cell in the moment of sulphate removal. Light blue circles represent –S cells at different times of S-starvation.

3.2 - Levels of total chlorophyll during S-starvation

The total chlorophyll content was determined in cells of *Chlorella sorokiniana* during S-deficiency (Figure 29). After two hours from S-deprivation was already observed a decrease of total chlorophyll from 37.7 mg ml^{-1} PCV to 35.7 mg ml^{-1} PCV. The lowering in the content of chlorophyll continued until 24 h when the value arrived to 11.5 mg ml^{-1} PCV (Figure 29).
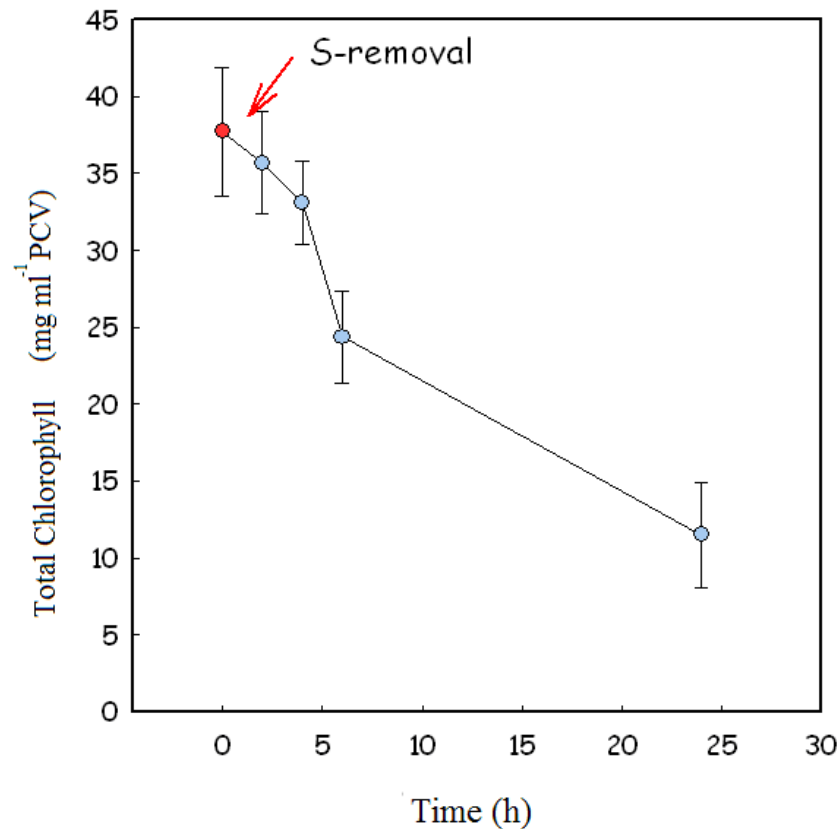
Figure 29: Levels of total chlorophyll during S-starvation.

Time-dependent variation of total chlorophyll content in cells of *Chlorella sorokiniana* during sulphate deficiency. The values were expressed as mg ml^{-1} PCV. The red circle represents S-sufficient cell when sulphate was removal. Light blue circles represent −S cells at different times of S-starvation.

3.3 - Sulphate availability and OASTL activity

OASTL activity was measured spectrophotometrically at selected time points of S-starvation until to 24 h.

In *Chlorella sorokiniana* cells, sulphate starvation caused a marked time-dependent increase of OASTL specific activity up to 49.8 ± 9.9 U mg^{-1} protein after 24 hours (Figure 30 panel A).

Considering that S-deprivation leaded to a reduction of the proteins content, (see above figure 25), the OASTL activity was also expressed respect to PCV and total chlorophyll (Figure 30 panel B), demonstrating that raising of OASTL activity was effective.
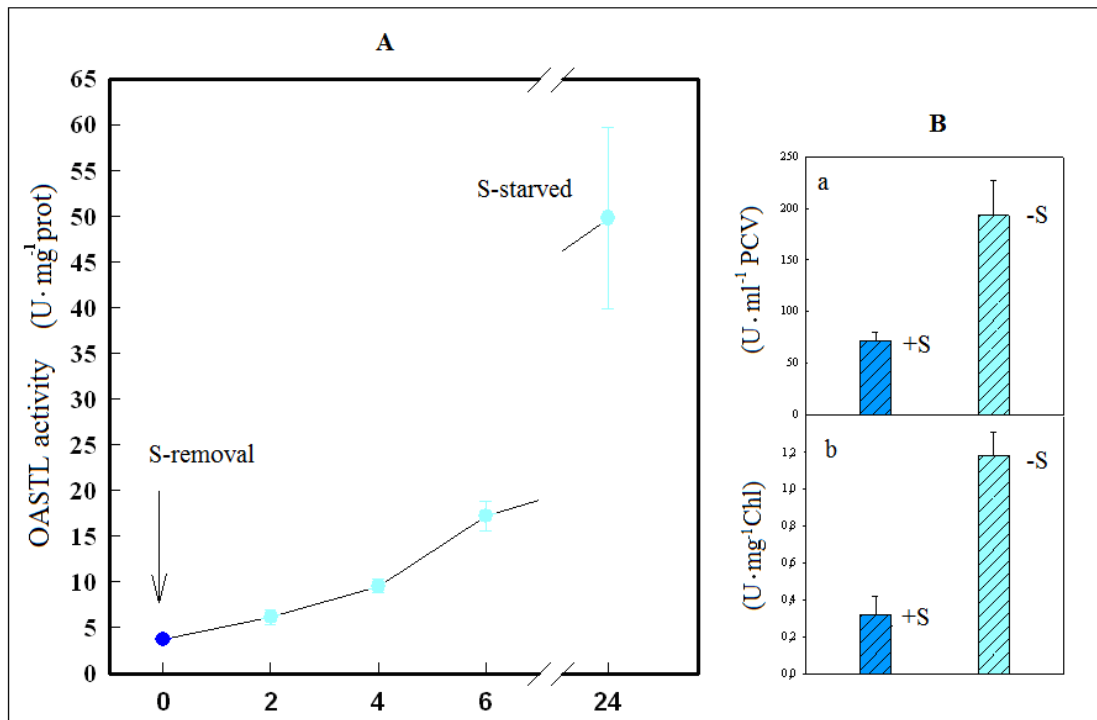
Figure 30: Sulphate availability and OASTL activity.

Effects of sulphate deprivation on OASTL activity in cells of *Chlorella sorokiniana*. At the indicated times, samples were assayed for OASTL activity. The activity was expressed as U mg^{-1} prot (panel A) and respect to chlorophyll and PCV (panel B). Significant differences, using one-way ANOVA, was analyzed in S-starved cells respect to S-sufficient cells (P <0,001). The values reported are means ± SE (n=3). Values are the average of three independent experiments.

3.4 - Determination of molecular weight of native OASTL enzyme

The native molecular weight of OASTL from *Chlorella sorokiniana* was determined by gel filtration chromatography using a Superdex 200 HR column. The native OASTL molecular mass was estimated to be 66.15 kDa (Figure31). This value was exactly double respect to the OASTL molecular weight obtained by SDS-PAGE on purified fractions (32-34 kDa). These results clearly indicated that, the active and functional form of OASTL from *Chlorella sorokiniana* is a dimer (Figure 31).
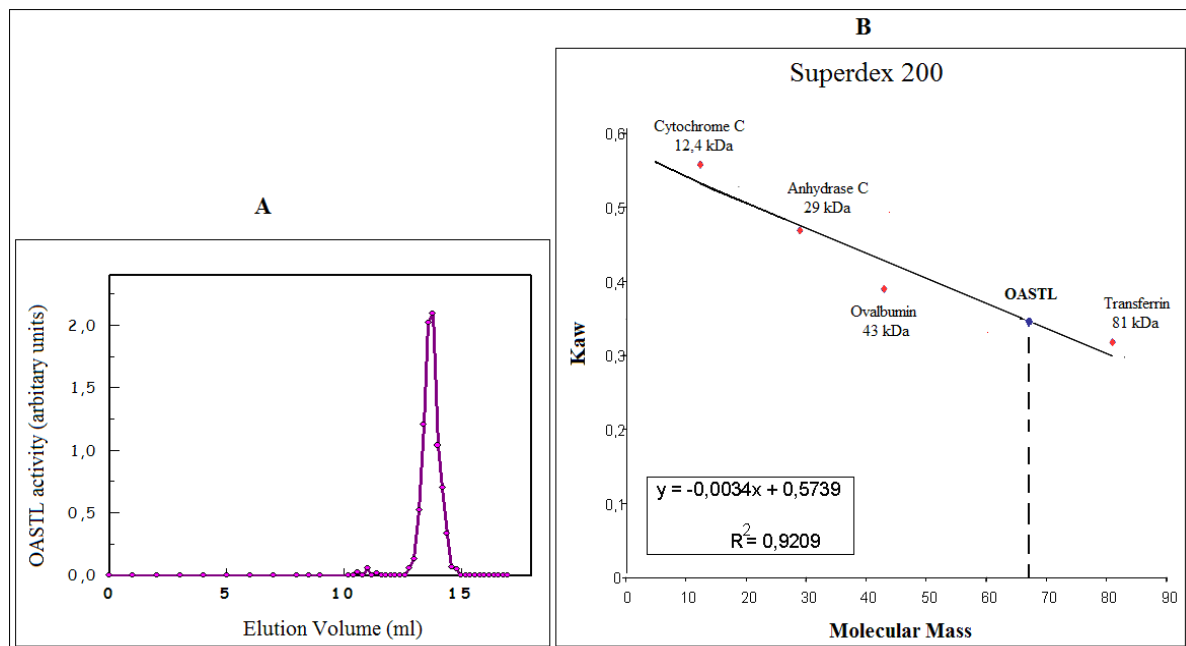
Figure 31: Gel filtration of OASTL from *Chlorella sorokiniana*.

A) Elution profile of purified OASTL. The purity of the enzyme was analyzed with an analytical grade gel filtration column, Superdex 200 HR; B) Measurement of molecular weight of native OASTL enzyme of *Chlorella sorokiniana* by gel filtration on Superdex 200 HR: standard proteins (red circles); *Chlorella s.* OASTL (blue circle).

3.5 - OASTL purification from *Chlorella sorokiniana* cells

The enzyme OASTL was purified from +S and –S *Chlorella sorokiniana* cells by Metal Chelate Affinity Chromatography.

Recombinant SAT5, expressed in *E. coli* were immobilized on a nickel-loaded Hi-Trap column, in fact is well known that tag-proteins have high affinity toward divalent ions of transition metals.

OASTL was purified 29 times from S-sufficient cells and 41 times in S starved with a recovery of 77% and 60% respectively (Table 6 and 7).

Step	Total protein (mg)	Total activity (U ml)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	4.8	24.85	5.18	100	1
His-tag	0.129	19.22	149	77	29

Table 6: His-tag on *Chlorella sorokiniana* +S crude extract.

Table of purification of OASTL from *Chlorella sorokiniana* S-sufficient cells by Metal Chelate Affinity Chromatography.

Step	Total protein (mg)	Total activity (U ml)	Specific activity (U/mg)	Yield (%)	Purification factor
Crude extract	1.4	35	25	100	1
His-tag	0.02	20.4	1020	60	41

Table 7: His-tag on *Chlorella sorokiniana* -S crude extract.

Table of purification of OASTL from *Chlorella sorokiniana* S-starved cells by Metal Chelate Affinity Chromatography.

The purity of OASTL protein was examined by denaturing-SDS-PAGE (Figure 32).

S-sufficient and S-starved purified extracts showed different patterns. OASTL purified proteins from S-sufficient cells migrated as two sharp bands corresponding to molecular mass of approximately 33.8 and 32.7 kDa; in S-starved cells the bands were not less than three, whereas the upper band (33.8 kDa estimated) was very deep and the lowest was very weak with an estimated mass molecular of 31.9 kDa. Therefore, proteins from S-deprived cells showed one band more than S sufficient cells (Figure 32).
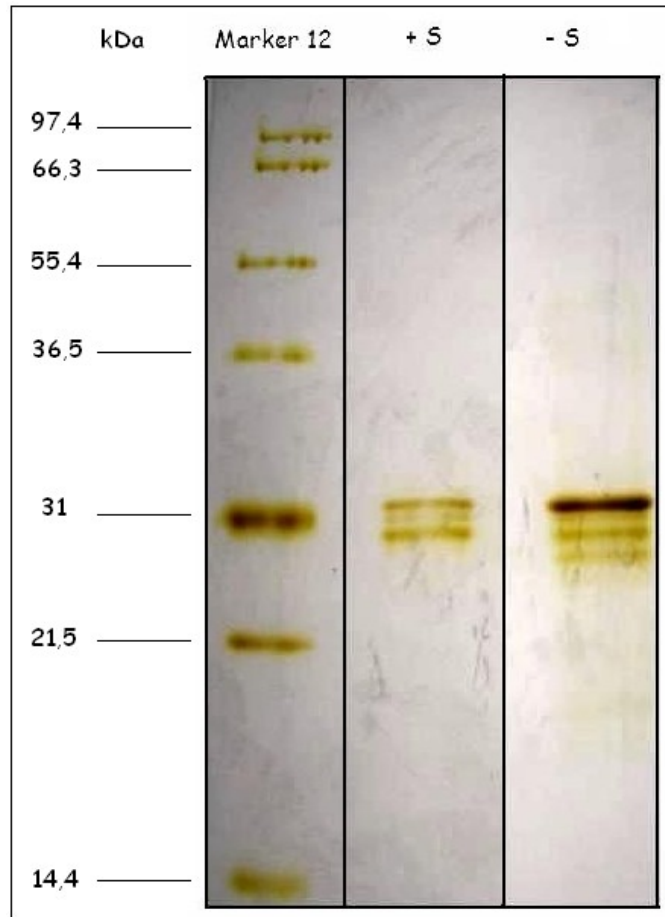
Figure 32: SDS-PAGE on purified OASTL extracts.

12,5% SDS-PAGE/Silver-stain on +S and –S OASTL purified extracts. 80 μg of proteins were loaded in each lane. Protein molecular-weight marker sizes are shown on the left (Mark12™ Unstained Standard, Invitrogen).

3.6 – OASTL isoforms in *Chlorella sorokiniana*

Western blot analysis of proteins from *Chlorella* (Figure 33), probed with three different antibodies against *Arabidopsis* OASTL isoforms (cytosolic (A), chloroplastic (B) and mitochondrial (C)) which cross-reacted with OASTL from *Chlorella sorokiniana,* showed a chloroplastic isoform in S-sufficient and S-deprived extract and the presence of a single cytosolic isoform in S-deprived cells.

Mitochondrial OASTL isoforms seems to be completely absent in both S-sufficient and S-starved *Chlorella sorokiniana* cells (Figure 33).
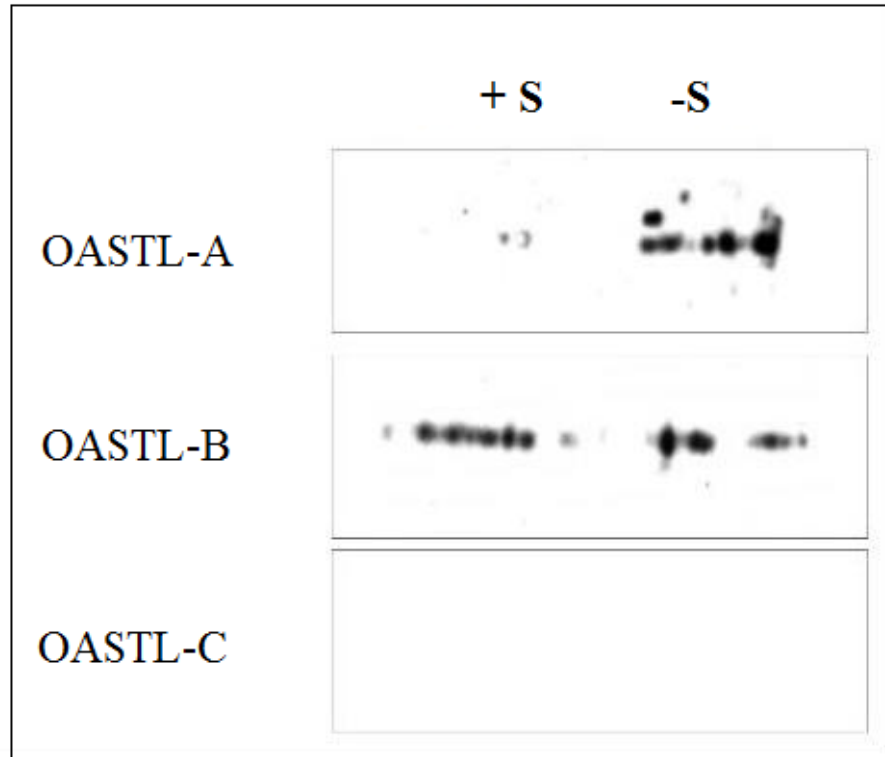
Figure 33: OASTL isoforms in *Chlorella sorokiniana*.

Western Blotting on +S and –S *Chlorella sorokiniana* crude extract. Specific antibodies toward cytoplasmatic (A), chloroplastic (B) and mitochondrial isoforms were used for the analysis. The blot was developed with ECL procedure.

3.7 - OASTL protein sequence

The protein sequence of chloroplastic OASTL isoform from *Chlorella sorokiniana* was determined in OASTL purified from S-sufficient cells.

Bands coming from SDS-PAGE of +S purified extract were excised, digested with trypsin and then analyzed by MALDI-TOF.

The obtained sequence had a length of 373 amino acids (Figure 34). It showed an high homology compared to the chloroplastic OASTL isoforms from higher plants and algae.

```
  1   MASIINNPFTSLCCNTNKCEPNRICSLRSQQSLVFDNVNRKVGFPSVVCK
 51   AVSVQTKSPTEIEGLNIAEDVTQLIGNTPMVYLNTIVKGCVANIAAKLEI
101   MEPCCSVKDRIGFSMISDAEEKGLISPGKTVLVEPTSGNTGIGLAFIAAS
151   RGYKLILTMPASMSLERRVILKAFGAELVLTDPAKGMKGAVSKAEEILNN
201   TPDAYILQQFDNPANPKIHYETTGPEIWEDTKGKIDILVAGIGTGGTISG
251   TGRYLKEKNPNIKIIGVEPTESNVLSGGKPGFIPGNLDQDVMDEVIEISS
301   DEAVETAKQALQEGLLVGISSGAAALAAIQVAKRPENAGKLIAVVFPSF
351   GERYLSSILFQSIREECEKMKPEL
```

Figure 34: OASTL protein sequence.

Protein sequence of OASTL from + S *Chlorella sorokiniana* cells. The sequence shows a length of 373 amino acids.

The protein sequence of OASTL chloroplastic isoform from *Chlorella sorokiniana* was aligned by ClustalW2 with the OASTL sequence of *Spinacia olearia* (Accession n° BAG 72098.1), *Zea Mays* (Accession n° NP 001140754.1), *Arabidopsis thaliana* (Accession n° NP 181903.1) and *Chlamydomonas reinhardtii* (Accession n° XP 001691935.1) (Figure 35).

In the Figure 35 is reported the multiple aligment, in red were reported the conserved residues, the green represents residues showing more than 60% identity. The region able to bind the cofactor PLP was showed as white letters on blue background.

Figure 35: OASTL multiple alignment by CLUSTALW2.

Multiple alignment of chloroplastic OASTL sequence from *Chlorella sorokiniana* with *Spinacia olearia* (Accession n° BAG 72098.1), *Zea Mays* (Accession n° NP 001140754.1), *Arabidopsis thaliana* (Accession n° NP 181903.1) and *Chlamydomonas reinhardtii* (Accession n° XP 001691935.1). Dashes indicate gaps in the alignment. Red: conserved residues; Green: residues showing more than 60% identity; White on yellow: substrate loop; White on blue: residues that interact with PLP; White on red: active site lysine.

3.8 – Changes of enzymatic DES activity during S-starvation

DES activity was 0.026 ± 0.009 U mg $^{-1}$ proteins in S-sufficient cells and appeared increased of 50% (Figure 36) in S-starved cells for 24 h. Considering that S-deprivation caused a reduction of proteins content (see above Figure 25), the activity was also expressed respect to PCV and total chlorophyll content (Figure 36, panel B) demonstrating that there wasn't a statistically significant difference between S-sufficient and S-starved cells.
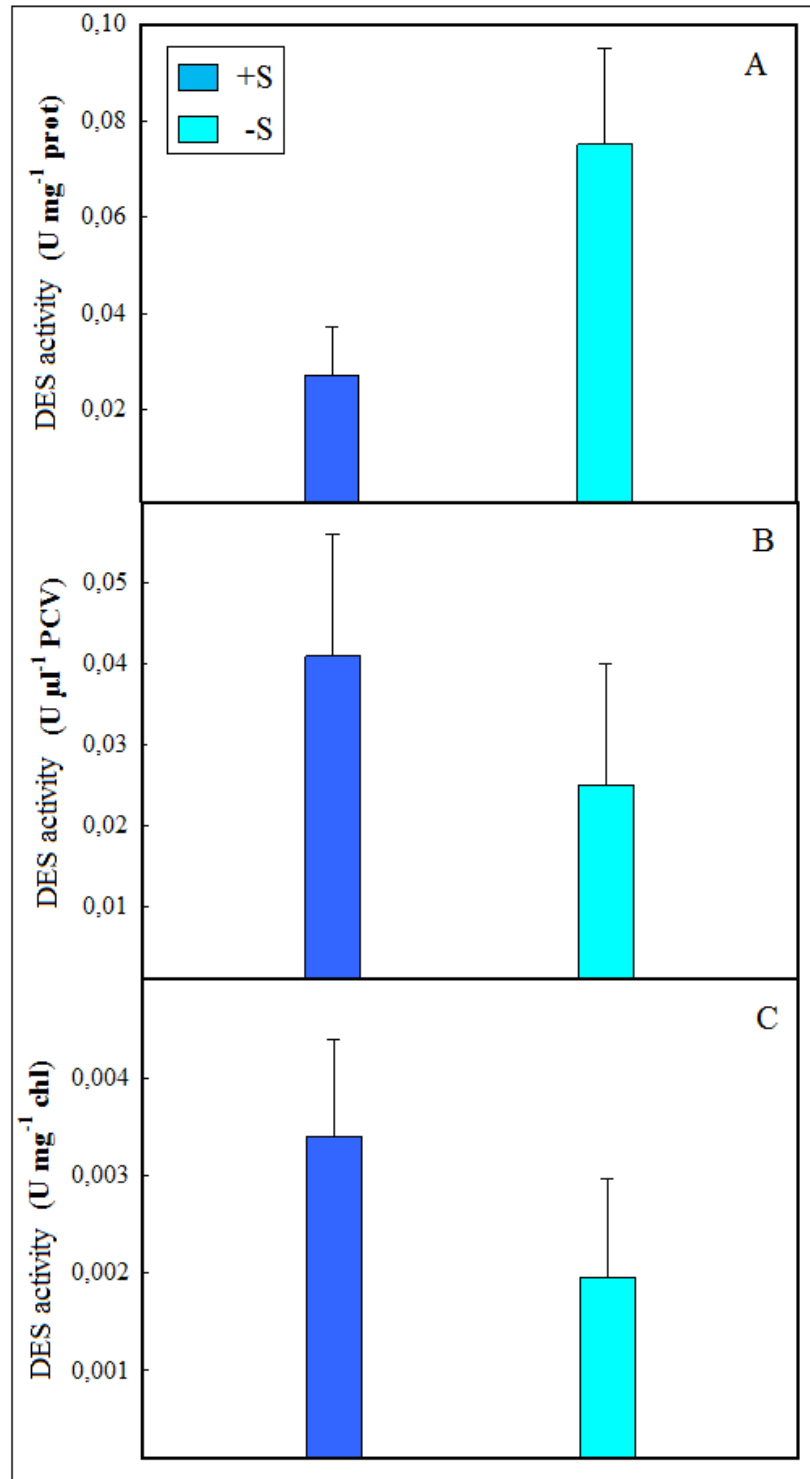
Figure 36: Changes of enzymatic DES activity during S-starvation.

DES enzymatic activity in +S (dark blue) and –S 24 h (light blue) *Chlorella sorokiniana* cells. Sulphur was given to the culture as $MgSO_4$ 1.2mM. The values were expressed as U mg^{-1} proteins (panel A), U μl^{-1} PCV (panel B), U mg^{-1} chl (panel C). Significant differences, using one-way ANOVA, were analyzed in S-starved cells with respect to S-sufficient cells. There was not a statistically significant difference: $P = 0.095$ (Panel A); $P = 0.358$ (Panel B); $P = 0.254$ (Panel C).

DES activity was also measured electrophoretically using a specific DES stain.

The native-PAGE showed different patterns from S-sufficient and S-starved crude extracts: two activity bands of different mobility were observed in the lane containing S-sufficient crude extract (Figure 37); and in the lane containing S-starved crude extract, five well-separated activity bands, with different charge and molecular mass, resulted (Figure 37).

Only one band (the second in order of migration) resulted common to the two lanes.
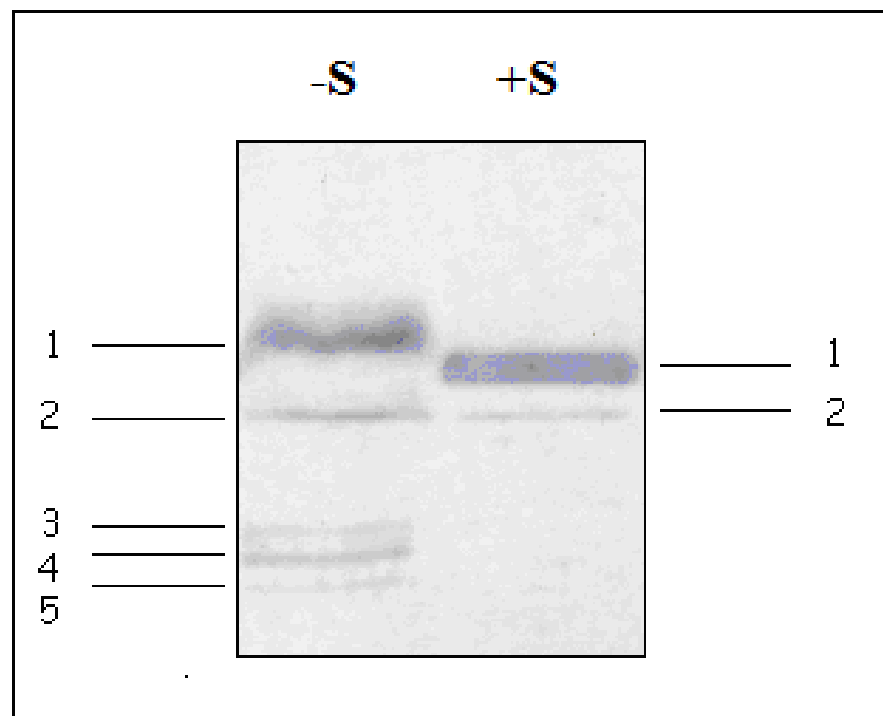
Figure 37: DES activity on native-gel.

Chlorella +S and −S crude extracts were used for the 12% Native-PAGE. The gel was stained with DES activity stain (other particulars in "Materials and methods").

The single activity bands, from +S an –S lanes, were excised and subjected to passive elution. The extracted proteins were then loaded into 12.5% SDS-PAGE (see "Materials and methods") (Figure 38).
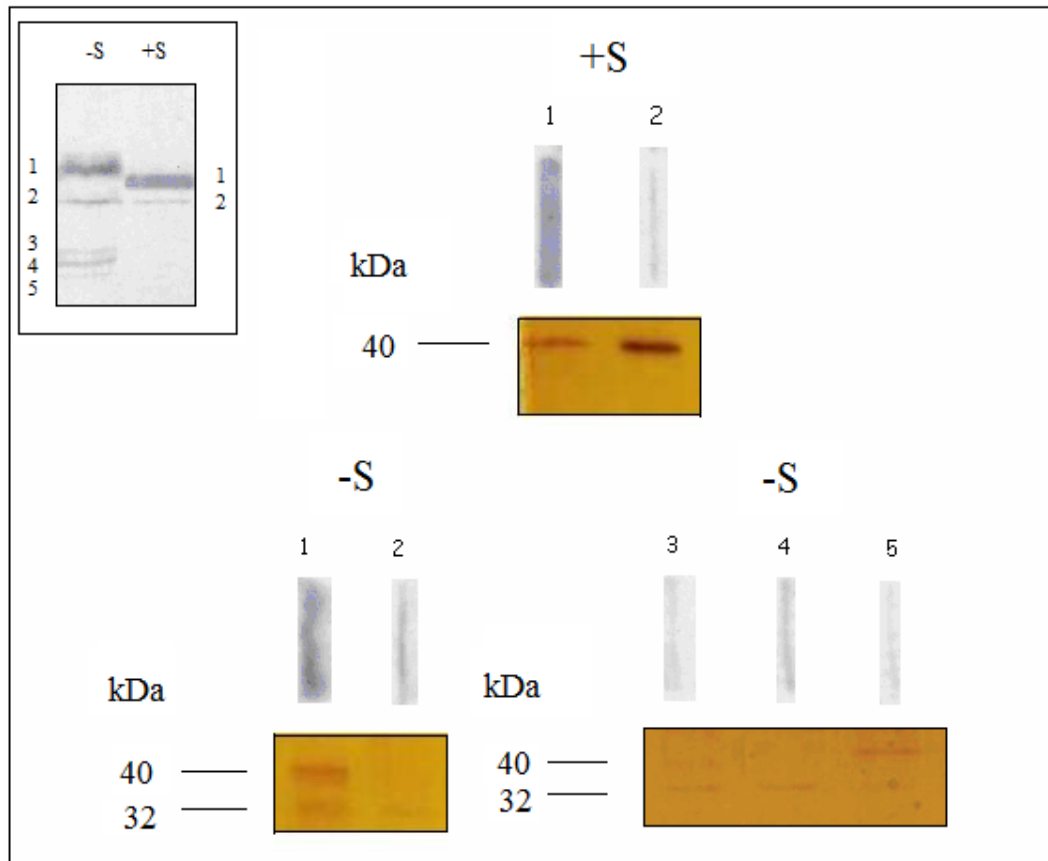
Figure 38: DES native bands reloaded in SDS-page.
12.5% SDS-PAGE/Silver stain. DES activity bands from native-gel were excised, the proteins eluted by passive elution and then loaded into SDS-PAGE. +S 1: band number 1 from native +S lane; +S2: band 2 from native +S lane; -S (1-5): bands number 1, 2,3,4,5 from native –S lane.

The SDS-PAGE/Silver-stain showed a different pattern between +S and –S re-charged-activity-bands.

DES activity bands from + S lane (Figure 37), gave on SDS-PAGE only one polypeptide having a molecular mass of 40 kDa.

DES activity bands from –S lane (Figure 37), gave not only a band of 40 kDa, but also a polypeptide of 32 kDa.

4 – DISCUSSION

Sulphur is a macronutrient essential for plant growth and development. The most abundant form of sulphur in nature and the source of sulphur for plants is sulphate. Sulphate is absorbed from the soil, reduced and then assimilated in the S-amino acid cysteine, which occupies a central role in plant metabolism not only as a constituent of proteins but also as a precursor-molecule of many essential and defence metabolites. Nevertheless, cysteine can be a very toxic molecule, because of the reactivity of its thiol moiety, which is why maintaining cysteine homeostasis in the cell is essential.

The plant organism used in this research was the unicellular green alga *Chlorella sorokiniana.* It showed to be a good model to study sulphur metabolism in the plant cell. This alga is very sensitive to S-nutritional stress, in fact previous research (Di Martino Rigano et al., 2000) demonstrated that, as a consequence of the sulphur starvation, the growth was limited and the photosynthesis was drastically reduced.

The aim of this PhD-research was to investigate effects of sulphate-deficiency on cysteine metabolism in the plant cell.

All experiments proposed in this thesis were carried out in the Plant Physiology Lab of Federico II University care off the Botanic Garden of Naples.

Two different cultures of *Chlorella sorokiniana* were prepared: the algae were grown in a medium containing all nutrients (S-sufficient cells) representing the control, or in a medium deprived of sulphate (S-starved cells).

After 24 hours of S-starvation, the organism showed important changes in the pool of S-containing and S-not-containing metabolites.

In S-starved cells, a strong decrease of soluble proteins was measured. We could assume that the S-starved cells, to satisfy their nutritional requirements, take sulphur from internal protein pools. Moreover, in the S-

starved cells the content of intracellular Met was quite poor, which is why the *ex novo* protein synthesis was almost completely compromised. In fact, Met represents the starter molecule of all polypeptide chains, corresponding to the triplet AUG.

Lewandowska and Sirko (2008) have previously shown that in tobacco plants prolonged sulphur starvation influences the expression of numerous genes, including those involved in protein degradation. But, our results in S-starved cells suggest that the decrease of proteins could be due to a dilution effect that is a consequence of the cellular growth together with a reduction of the *ex novo* protein synthesis.

Not as a consequence of the enhanced protease activity but presumably because the lack of sulphate in the medium compromised the cysteine synthesis, *Chlorella* accumulated during S-deprivation high intracellular levels of Ser and OAS, the nitrogen precursors of cysteine. Nitrogen metabolism and S-assimilation are reciprocally influenced (Carfagna et al., 2010); the OAS molecule represents at this regard the bridge between the two assimilation pathways. The accumulation of OAS in S-deprived cells is also relevant for other reasons: during S-deprivation, amino acid metabolism appears to be strongly influenced, in fact the decrease in sulphur content reduced the amounts of total protein and unbalanced nitrogen assimilation, compromising the overall metabolism and the growth of the algae.

It is well documented (Wirtz et al., 2001) that *in vitro*, OAS dissociates the cysteine synthase complex (CSC) formed *in vivo* by SAT and OASTL enzymes (see the introduction chapter). It has been suggested that the rate of cysteine is regulated by the grade of association/dissociation of the CSC (Wirtz et al. 2001). Furthermore, under sulphur starvation, the low level of sulphide/thiol compounds and elevated OAS concentrations induce an expression of high-affinity sulphate transporters (Koprivova et al., 2000; Maruyama-Nakashita et al., 2004).

Activation of S-assimilation, beginning with sulphate uptake and subsequent, increases cellular sulphide levels and resets the conditions favoring formation of CSC (Hopkins et al., 2005; Yi et al., 2010).

In *Chlorella sorokiniana* cells grown under sulphate deficiency, a drastic decrease of intracellular cysteine content as well as a decrease of glutathione content was observed. GSH is the first S-containing compound that absolves a function of S-reserve in the plant cell. The degradation of GSH under S-deficiency allows the cell to maintain the S-status necessary for its survival. During exogenous sulphate deficit, the intracellular GSH, through its demotion, could contribute in maintaining cysteine homeostasis in the cells. *Chlorella* cells have very little vacuoles and this explains the low levels of intracellular GSH in respect to that of higher plants cells.

The key enzyme involved in the cysteine biosynthesis is the OASTL. In recent years many researchers purified and characterized OASTL from higher plants, algae and bacteria such as: *Arabidopsis thaliana* (Hesse and Altmann, 1995), *Chlamydomonas reinhardtii* (Ravina et al., 1999), *Escherichia coli* (Zhao et al., 2006).

Two to four isoforms of the OASTL enzyme are isolated in higher plants (Kuske et al., 1996; Warrilow and Hawkesford, 1998; Nakamura et al., 1999; Jost et al., 2000).

The OASTL isoforms are located in three different cellular compartments: cytosol, chloroplast/plastid and mitochondria.

By contrast, in *Chlamydomonas reinhardtii*, only one chloroplastic OASTL isoform has been found, therefore cysteine seems to be synthesized only in this organelle (Ravina et al., 2002).

In *Chlorella*, the synthesis of cysteine happens in the chloroplast and also in the cytosol. Denaturing electrophoresis carried out on OASTL purified extracts and Western blotting analysis reveal the presence of at least two OASTL isoforms localized in the cytosol and in the chloroplast, even if the

cytosolic isoform is only manifested in S-deprived cells. In *Chlorella* cells, S-deprivation induced a time-dependent increase of OASTL specific activity. After 24 h of S-starvation, the whole OASTL activity in *Chlorella* strongly increased (20-fold). This increase in OASTL activity should be attributed to an enhancement of the cytosolic OASTL isoform.

The purified OASTL from *Chlorella sorokiniana* had a subunit size of 32-34 kDa, like *Chlamydomonas* (Ravina et al., 1999). However, the subunit size of cytosolic and chloroplastic isoforms is probably slightly different. Native OASTL molecular weight was estimated to be about 66 kDa, indicating that, in *Chlorella* cells like in other organisms, the OASTL is enzymatically active in the dimeric form (Droux et al., 1998; Kumaran et al., 2009).

The amino acid sequence of *Chlorella sorokiniana* OASTL shows significant homology to those of other plant species and bacteria. *Chlorella* OASTL contains the conserved region PXXSVKDR, characteristic of cysteine synthase, which has demonstrated the ability to bind the cofactor PLP (Williams et al., 2009).

In plants, cysteine synthesis is regulated differently in the cellular compartments both by distinct CSC properties and also by the uneven distribution of SAT and OASTL activities in the organelles (Burandt et al., 2001; Droux, 2004). The data here presented on *Chlorella* indicate that activities of different OASTL isoforms are regulated by the sulphur status of the algae; namely the cytosolic isoform appears more susceptible to the changes of sulphur, OAS and cysteine.

Cytosol is the major site of cysteine synthesis (Haas et al., 2008; Heeg et al., 2008). Krueger and co-workers (2009) demonstrating that the cysteine concentrations in the cytosol is higher than the other cell compartments.

Cysteine concentrations in the cytosol are estimated to be over 300 μM, whereas the other cell compartments contain below 10 μM (Krueger et al.,

2009). High levels of free cysteine generate ROS through the Fenton reaction catalyzed by iron (Alvarez et al., 2010). For these reasons, cysteine homeostasis should be accurately maintained in the cell especially in cytosol, the main site where cysteine biosynthesis occurs.

The synthesis of the cysteine and its degradation should be well coordinated.

In the plant cell a specific enzyme is able to catalyze the cysteine desulphuration. According to Alvarez and co-workers (2010), *L*-cysteine desulphydrase (DES) is the enzyme that has the ability to degrade *L*-cysteine in sulphide, pyruvate and ammonia. DES, in contrast to OASTL, has a poor or absent affinity toward the enzyme SAT.

This enzyme was purified and characterized in bacteria (Yano et al., 2009; Marciano et al., 2010) and in higher plants (Bloem et al., 2004; Alvarez et al., 2010). However, in algae the DES enzyme is still little researched. In *Arabidopsis* the intracellular localization of DES is the cytosol, which also represents the location where the major synthesis of cysteine occurs (Alvarez et al., 2010).

During this research the activity of the DES enzyme was measured in *Chlorella sorokiniana* cell growth in S-sufficient and S-deficiency conditions.

Results showed that the enzyme didn't significantly change it's activity during S-nutritional stress. On the other hand, the activity stain on native page shows the presence of multiple enzymes having DES activity more evident in S-starved crude extracts. The SDS-PAGE of these proteins revealed that OASTL enzymes in S-starved condition cooperate through their side-reaction with the DES enzyme, to catabolize cysteine *in vivo*. In general, OASTL proteins are supposed to be involved in cysteine synthesis (Burandt et al., 2001; Lopez-Martin et al., 2008) but under S-starvation, they could perform also cysteine degradation through their side-reaction.

In literature it is mentioned that affinity for cysteine is higher for the OASTL enzyme (Km 0.4-1.4mM) (Poortinga et al., 1997; Graham et al., 1998) than for DES (Km 2.6-3.7mM) (Yangsheng et al., 2007; Marciano et al., 2010). The compartmentalization in cells leads to specific physiological conditions inside the compartments. The activity of different isoenzymes is strongly dependent on the chemical environment of organelles. Even small changes in cysteine or H_2S concentrations in the cytoplasm might influence the side-reaction of the OASTL. In S-starved cells the enhancement of the OASTL cytosolic isoform could contribute to the demotion of cysteine to liberate H_2S. Being a gas, H_2S can easily diffuse through the chloroplast envelope (Mathai et al., 2009) to be organicated by the chloroplastic OASTL isoform.

The increase of OASTL activity in S-starved cells could represent a way to recycle cysteine and H_2S between cytosol and chloroplast. In the chloroplast H_2S could promote the synthesis of S-containing proteins to preserve the photosynthetic metabolism.

Taken together, these results indicate that *Chlorella* cells reveal suited mechanism to maintain viability under sulphur starvation. As expected, the lack of sulphate, provoked an accumulation of the nitrogen precursors of cysteine (Ser and OAS) and decreased the soluble proteins. For the first time in a unicellular alga, it was shown that the OASTL enzyme has two isoforms located respectively in the cytosol and in the chloroplast, which are differently regulated by the S status of the cells.

The OAS located in the cytosol or in the chloroplast may in turn regulate OASTL isoforms, resulting in enhanced activation of the enzymes. In particular, cytosolic isoform appears up-regulated by the S-deficiency and able to catabolize the cysteine together with the DES enzyme.

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