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**REGULATION OF SULFUR ASSIMILATION IN
ONION (*ALLIUM CEPA* L.)**

A thesis presented in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Physiology and Biochemistry at Massey University, Palmerston North, New Zealand.

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ABSTRACT

Onion (*Allium cepa* L.) is an example of a species that accumulates very high levels of reduced sulfur (S)-containing compounds, particularly in the bulb as alk(en)yl-L-cysteine-sulfoxides (ACSOs) and it is these compounds, or their derivatives, that confers the distinct odour and pungent flavour.

In common with higher plants, the S assimilation pathway in onion begins with the activation of uptaken sulfate (SO_4^{2-}) to 5'-adenylylsulfate (APS), a reaction catalysed by ATP sulfurylase (ATPS; EC 2.7.7.4). Then, APS is reduced to sulfide (S^{2-}) in a two-step process catalysed by the enzymes APS reductase (APSR; EC 1.8.4.9) and sulfite reductase (SiR; EC 1.8.7.1). To complete the reductive assimilation pathway, S^{2-} is incorporated into the amino acid skeleton of *O*-acetylserine (*OAS*) to form cysteine, and this reaction is catalyzed by *OAS* (thiol)-lyase (*OAS*-TL; EC 4.2.99.8). While the regulation of the pathway is quite well defined in the plant model *Arabidopsis*, much less is known about its regulation in S accumulating species such as onion.

The primary aim of this thesis, therefore, was to characterise the enzymes of the S assimilation pathway in onion, with a particular emphasis on ATPS. As part of this characterisation two genotypes of onion were compared. These comprised a mild genotype, 'Texas Grano 438' (TG) with a lower level of S-containing compounds in the bulb tissues, and 'W202A' (W), a cultivar with a higher level of S containing compounds in the bulb tissues. As well, comparisons were made between seedlings (typically harvested at 7 weeks) and plants at a designated mature stage (at bulbing; typically after 4 months growth), and for plants grown in S-sufficient (S^+) media or S-deficient (S^-) media, as appropriate.

In terms of plant growth, S-deprivation generally had a negative influence for both genotypes, with significant reductions in total biomass (measured as fresh weight) for TG at both the seedlings and mature stages. ATPS activity and accumulation were shown to be present in all tissues examined (leaf, root, bulb) as well as the chloroplasts, with highest activity measured in the roots, particularly in seedlings. ATPS activity and accumulation were also compared between the two genotypes (TG and W) with ATPS activity and accumulation higher in W, particularly at the seedling stage.

In terms of the influence of S supply, in general higher ATPS activity was measured in chloroplast, leaf and root extracts from plants of both genotypes grown in the S- media, at the seedling stage. In roots of mature plants of both genotypes, a significant increase in activity was measured in response to S-deprivation, while in chloroplasts isolated from mature plants of both genotypes, highest activity was measured in those grown in the S+ media. Finally diurnal variations were observed in chloroplast, leaf and root extracts of both genotypes with a general trend of an increase in ATPS activity and accumulation a few hours after illumination and upon the onset of the dark period.

Although a single gene coding for ATPS is presumed to be present in onion, the enzyme was characterized as two electrophoretic forms using ID-PAGE during western analyses following fractionation of chloroplasts by anion exchange chromatography and also as an alignment of spots using 2D-PAGE. As protease inhibitors were routinely included in the extraction buffers, these forms suggest the occurrence of ATPS isoforms that may arise as a consequence of post-translational modifications.

The regulation of ATPS by one mechanism of post-translational modification, phosphorylation, was therefore investigated using several techniques including the detection of a shift in molecular mass, a change in enzyme activity or pI (as determined by 2D-PAGE) and the capability to bind to 14-3-3 proteins using affinity chromatography. Following treatments of chloroplast extracts to promote either the phosphorylation (P^+) or the dephosphorylation (P^-) of proteins, no molecular mass change or change in activity was observed. However, after fractionation by 2D-PAGE, differences in the spot alignment of ATPS were visualized, suggesting that ATPS is a phosphoprotein. The enzyme was detected in pull-downs after affinity chromatography, suggesting that ATPS may also interact with 14-3-3 proteins (although this needs to be confirmed unequivocally). A model is advanced, therefore, in which upon phosphorylation, no variation in ATPS activity occurs but a change in the surface charged and possibly a change in conformation of the protein does occur to make the enzyme competent to interact with 14-3-3 proteins.

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LIST OF ABBREVIATIONS

1D-PAGE	one dimensional polyacrylamide gel electrophoresis
2D-PAGE	two dimensional polyacrylamide gel electrophoresis
AAP	antartic alkaline phosphatase
Abs	absorbance
ACSO	alk(en)yl-L-cysteine-sulfoxide
Amp	ampicillin
ANOVA	analysis of variance
<i>Arabidopsis</i>	<i>Arabidopsis thaliana</i>
APS	5'-adenylylsulfate
APSR	5'-adenylylsulfate reductase
ATP	adenosine 5'-triphosphate
ATPS	ATP sulfurylase
APS	adosome 5'phosphosulfate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BMH1	14-3-3 protein from <i>S. cerevisiae</i> , isoform 1
BMH2	14-3-3 protein from <i>S. cerevisiae</i> , isoform 2
bp	base pair
BSA	bovine serum albumin
C	cross-linker
°C	degree Celsius
CBB	Coomassie Brilliant blue
cDNA	complementary DNA
CHAPS	3-[(3-cholamidopropyl)dimethyl-ammonio]l-propanesulfonate
CK2	casein kinase II
cm	centimetre
CNBr	cyanogen bromide
CS	cysteine synthase
D+x h	dark period after x hours of darkness
dH ₂ O	distilled water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol

EC	enzyme commission
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol-bis(β -aminoethyl ether) <i>N,N,N',N'</i> -tetraacetic acid
EtOH	ethanol
F2KP	fructose-2,6-biphosphatase
FAO STAT	Food and Agriculture Organization of the United Nations – statistic data
FPLC	fast protein liquid chromatography
g	g-force
g	gram
GS	glutamine synthase
GSH	glutathione
h	hours
IEF	isoelectric focusing
IMAC	ion metal affinity chromatography
IPG	immobilized pH gradient
IPTG	isopropyl- β -D-thioglucoopyranoside
kDa	kiloDaltons
kPa	kilo Pascal
L	litre
L+ x h	light after x hours of illumination
LB	Luria-Bertani
m	meter
M	mole L ⁻¹
meOH	methanol
meq	milliequivalent
mg	milligram
MgATP	magnesium ATP
min	minute
mL	millilitre
mm	millimeter
mM	millimolar
MW	molecular weight
Na	sodium

NAD ⁺	β-nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
Na ₄ P ₂ O ₇	sodium pyrophosphate
NaCl	sodium chloride
NaF	sodium fluoride
NBT	nitrotetrazolium blue chloride
NCBI	National Center for Biotechnology Information
nm	nanometer
NOA	National Union Association of the United States
NR	nitrate reductase
OAS	<i>O</i> -acetylserine
OAS-TL	<i>O</i> -acetylserine thiol lyase
OKA	okadaic acid
p	probability
P ⁺	treatment for protein phosphorylation
P ⁻	treatment for protein dephosphorylation
PAGE	polyacrylamide gel electrophoresis
PALP	pyridoxal phosphate dependent enzyme
PAPS	3'-phospho-5'-adenylylsulfate
PBS	phosphate buffered saline
PBST	PBS-Tween 20
PEG ₈₀₀₀	polyethylene glycol, MW 8000
pI	isoelectric point
pH	potential of hydrogen
PMSF	phenylmethyl sulphonyl fluoride
PP1	protein phosphatase, type 1
PP2A	protein phosphatase, type 2A
PP2B	protein phosphatase, type 2B
PP2C	protein phosphatase, type C
PPi	inorganic phosphate
Pro-Q DPS	ProQ Diamond phosphoprotein staining
PTPase	protein tyrosine phosphatase
PVDF	polyvinylidene difluoride

RO	reverse osmosis
rpm	rotation per minute
RuBisCO	ribulose-1,5-biphosphate carboxylase oxygenase
S	sulfur
S ⁺	high level of sulfur
S ⁻	low level of sulfur
SAT	serine acetyltransferase
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second
SiR	sulfite reductase
SO ₄ ²⁻	sulfate
SPS	sucrose phosphate synthase
T	total monomer
TCA	trichloroacetic acid
TEMED	<i>N,N,N',N'</i> -tetramethylethylenediamine
TG	'Texas Grano 438'
Tris	tris(hydroxymethyl)aminomethane
Tween-20	poly(oxyethylene) sorbitane-monolaurate
U	unit
μA	microampere
μg	microgramme
μL	microlitre
μmol	micromole
v	volume
V	volt
v/v	volume to volume ratio
Vhrs	volt hours
w	weight
W	'W202A'
w/v	weight by volume ratio
WHO	world health organization

ABBREVIATIONS FOR AMINO ACIDS

Amino acid	Three-letter abbreviation	One letter abbreviation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamic acid	Glu	E
Glutamine	Gln	Q
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

1. INTRODUCTION

Introduction to the biology of onion

Onion (*Allium cepa* L.) belongs to the *Allium* genus, in common with garlic (*Allium sativum*), leek (*Allium porrum*), chive (*Allium schoenoprasum*), and shallot (*Allium oschaninii*). Composed by more than 700 species (Jones *et al.*, 2004), the genus is distributed all over Europe, North America, Northern Africa and Asia (Fenwick and Hanley, 1985). All the economically important species are characterized by their pungency and flavours.

Apparently originating in central Asia, Iran or West Pakistan, onions have been cultivated for at least 5000 years, and are thought to have been widely used in the prehistoric diet (National Onion Association of the United States (NOA)). Onions are known to have been cultivated by the ancient Egyptians (NOA), and may indeed be one of the first vegetables domesticated by man. Today, onions are most often used to enhance flavours in a wide range of foods. In 2005, more than 64.1 million tons worldwide were produced (Statistical data from the database of the Food and Agriculture Organization of the United Nations (FAOSTAT)) and it is now the fourth most important horticultural crop worldwide after tomatoes (*Solanum lycopersicum*), watermelons (*Citrullus lanatus*) and cabbages (*Brassica oleracea*). Many different varieties exist, characterized by their bulb colour (red, yellow, white, and green) (McManus *et al.*, 2005) and composition of the volatiles responsible for the aroma and taste of onions.

The characteristic odour and taste of onion is derived from both environmental and genetic factors (Lin *et al.*, 1995; Simon, 1995; Wall *et al.*, 1996). The environmental influences include temperature (Platenius and Knott, 1941), irrigation (Freeman and Mossadeghi, 1973), the form of nitrogen available to the plants (Gamiely *et al.*, 1991) and the most important feature (Randle and Bussard, 1993), sulfur fertility of the soil (Balasubramonian *et al.*, 1978; Freeman and Mossadeghi, 1970; Hamilton *et al.*, 1997; Paterson, 1979; Randle, 1992; Randle and Bussard, 1993; Smittle, 1984;).

Flavour in onions, which accumulate comparatively large quantities of sulfur (Coolong and Randle, 2003; McManus *et al.*, 2005), is dominated by up to 80 unique sulfur compounds and several water-soluble carbohydrates (Lancaster and Boland, 1990). These flavour compounds result from the hydrolysis by the enzyme alliinase (EC 4.4.1.4) of the precursors, alk(en)yl-L-cysteine-sulfoxides (ACSOs) to produce volatile sulfur compounds and the by-products pyruvic acid and ammonia (Block, 1992; Lancaster and Boland, 1990; Lancaster and Collin, 1981). This reaction can only occur when plant cells are disrupted since the enzyme alliinase is located in the vacuole and the ACSOs in the cytoplasm (Lancaster and Boland, 1990; Lancaster and Collin, 1981). Four ACSOs have been identified in *Allium* and the flavour variation among species is due to differences in ACSO composition and concentration (Block, 1992; Lancaster and Boland, 1990; Lancaster and Kelley, 1983; Randle *et al.*, 1995). Onion, the most important edible *Allium* worldwide, has three main ACSOs. Trans-(+)-S-(1-propenyl)-L-cysteine sulfoxide (PrenCSO) is normally found in the highest concentration, while (+)-S-methyl-L-cysteine sulfoxide (MCSO) and (+)-S-propyl-L-cysteine sulfoxide (PCSO) are found in lower concentrations (Lancaster and Boland, 1990).

In addition to flavour, onions also have health promoting properties as they provide nutrients such as dietary fibre, folic acid, iron, calcium, potassium, vitamin C and vitamin B6 as well as phytochemicals (disulfides, trisulfides, cepaene, allicin) and flavonoids such as quercetin (Price and Rhodes, 1997). Antioxidants are compounds that can help delay or slow oxidative damage to cells and tissues of the body. For example, the antioxidant effect of quercetin is proposed to eliminate free radicals, inhibit lipoprotein oxidation, protect and regenerate vitamin E, and inactivate the harmful effects of chelating metal ions (Campos *et al.*, 2003; Chu *et al.*, 2002). Further, it is proposed that the phytochemicals contained in onions have anticancer and antimicrobial activities (Ankri and Mirelman, 1999), especially against the ulcer forming bacteria *Helicobacter pylori* (Sivam, 2001). In addition, these phytochemicals may reduce serum cholesterol (Bakhsh and Khan, 1990), and enhance antiplatelet activity (Ariga *et al.*, 1981; Block, 1992; Block *et al.*, 1984; Bordia *et al.*, 1996; Goldman *et al.*, 1995; Lawson *et al.*, 1992), which are important for cardiovascular health. This antiplatelet activity is influenced significantly by the environment interacting with the onion plant during development (Goldman *et al.*, 1996) and the presence of high sulfur has been shown to increase both the pungency and antiplatelet activity of the bulb. Sulfur

compounds provide some protection against tumour growth (Dorant *et al.*, 1996; Heber, 2004) and play an important role in carbohydrate metabolism, where sulfur is an indirect component of insulin (Stipanuk, 2004). Additionally, the World Health Organization (WHO) promotes the use of onions to prevent atherosclerosis. Finally, the most fundamental role of onions is their supply of sulfur, as cells of the body contain sulfur-containing compounds, and those of primary importance in nutrition include methionine (Met), cysteine (Cys), homocysteine, heparin, thiamine, biotin, coenzyme A, glutathione (GSH), and inorganic sulfate.

However, as important cereal grains and legume seeds in the human diet often do not provide the desired amounts of Cys and particularly, Met (Hell, 1997), it is important to understand sulfur assimilation in plants to be able to improve its content further in crop plants such as onions, a natural accumulator of sulfur. For other crop plants that do not accumulate S to higher levels, the nutritional improvement of the seeds is being attempted by modification of amino acid biosynthetic pathways (Sun *et al.*, 1992).

Sulfur: an essential nutrient in plants

Sulfur is an essential element in all living organisms. It represents only around 0.1% of dry matter, and it is the least abundant of the macro-elements found in plants when compared with carbon (45%) and nitrogen (15 %) (Leustek *et al.*, 2000). As sulfur is involved in the biosynthesis of primary and secondary compounds, the element plays an important role in plant metabolism. It is found in proteins in Met and Cys (Saito, 2000), and in oligopeptides (GSH and phytochelatins) that are essential human nutrients (Hell, 1997). Sulfur serves to form disulfide bonds between polypeptide chains that confer the three-dimensional structures to proteins (Berg *et al.*, 2002), and the element is an important compound of enzyme function, including Fe-S clusters (Kopriva *et al.*, 2002a). Sulfur is also incorporated into the vitamins biotin and thiamine (B1) (Hawkesford *et al.*, 2006; Saito, 2004), and plays a role in stress responses and disease resistance in plants (Bloem *et al.*, 2005; Cooper and Williams, 2004). Additionally, sulfur is also found in secondary compounds that include signalling molecules in the legume:*Rhizobia* symbiosis (Dénarié and Cullimore, 1993).

Sulfur assimilation and transport in plants

Unlike animals, plants assimilate inorganic sulfate (SO_4^{2-}) via both reductive and oxidative assimilative pathways. In the reductive pathway, SO_4^{2-} is reduced to Cys (Leustek *et al.*, 2000), the first sulfur compound that can be assimilated into proteins. Because of this reductive assimilation pathway, plants play a key role in the sulfur cycle in nature (Saito, 2004) and further provide reduced sulfur to herbivorous species, including humans. The assimilation pathway provides plants with Cys, which is used for protein synthesis and also as a source of reduced sulfur for the biosynthesis of Met, GSH, coenzymes, and many secondary compounds (Kopriva and Rennenberg, 2004).

For both reductive and oxidative assimilation, plants take up sulfur through the roots by high-affinity sulfate transporters (Hawkesford, 2003), in the form of SO_4^{2-} . Plasma membrane-localised $\text{H}^+/\text{SO}_4^{2-}$ co-transporters, which are dependent on the electrochemical gradient produced by a proton pump, mediate the uptake and long-distance transport of the element (Saito, 2000). In *Arabidopsis thaliana*, 14 genes have been identified that code for sulfur transport proteins (Shibagaki *et al.*, 2002; Takahashi *et al.*, 1997; Takahashi *et al.*, 1999; 2000; Vidmar *et al.*, 2000; Yoshimoto *et al.*, 2002). Within the cell, sulfate can be either stored in the vacuole, or enter the metabolic stream for further assimilation (Leustek *et al.*, 2000). The reductive assimilation pathway occurs in the chloroplasts of leaves (Hawkesford *et al.*, 2006; Hell, 1997; Saito, 2000), although evidence suggests that limited sulfur assimilation also occurs in the cytoplasm and in plastids of roots (Lunn *et al.*, 1990; Rotte and Leustek, 2000).

The overall rate of sulfur assimilation is regulated during development. Generally, the highest activity of sulfur assimilation enzymes occurs at the growing points of plants including the youngest leaves and the root tips, where there is a high demand for Cys and Met for protein synthesis (Saito, 2004).

The sulfur requirement strongly varies between species and it may strongly vary at different developmental stages of the same plant species, for example, during vegetative growth, or during seed production. At optimal growth conditions, the sulfur requirement of different crop species ranges from 2 to 10 $\mu\text{mol g}^{-1}$ plant fresh weight day^{-1} . In addition, sulfur uptake and assimilation is dependent upon adequate nitrogen and carbon availability (Kopriva *et al.*, 2002b; Koprivova *et al.*, 2000).

1.2.1.1. Reductive assimilation pathway

The sulfur assimilation pathway was first resolved in the enteric bacteria *Escherichia coli* and *Salmonella typhimurium* using mutants auxotrophic for different sulfur compounds (Jones-Mortimer, 1968; Kredich, 1971). In total, reduction of sulfate requires eight electrons and 14 molecules of ATP (Hawkesford *et al.*, 2006; Hell, 1997) (Reaction 1), and the reduction reaction occurs predominantly in green and non-green plastids (Hell, 1997) (Figure 1.1). As SO_4^{2-} has a very low oxidation/reduction potential, the primary step in both oxidative and reductive sulfate assimilation pathways involves the conversion (activation) of sulfate to 5'-adenylylsulfate (APS), a reaction catalysed by the magnesium-dependent ATP sulfurylase (ATPS; EC 2.7.7.4), which generates APS and pyrophosphate (PPi) (Renosto *et al.*, 1993). In the reductive assimilation pathway, APS reductase (APSR; EC 1.8.4.9) transfers two electrons to APS, which are derived from GSH (Bick *et al.*, 1998) to produce sulfite (SO_3^{2-}) (Bick and Leustek, 1998; Leustek *et al.*, 2000).



The next step consists of the reduction of SO_3^{2-} to sulfide (S^{2-}). This reaction is catalysed by sulfite reductase (SiR; EC 1.8.7.1) and requires six electrons from ferredoxin (Bork *et al.*, 1998; Nakayama *et al.*, 2000; Yonekura-Sakakibara *et al.*, 1998). To complete the reductive assimilation pathway, S^{2-} is incorporated into the amino acid skeleton of *O*-acetylserine (*OAS*; EC 4.2.99.8) to form Cys (Leustek *et al.*, 2000). *OAS* is synthesized by acetylation of serine with acetyl Co-A catalysed by serine acetyltransferase (SAT; EC 2.3.1.30). The incorporation of S^{2-} into *OAS* to form cysteine is catalysed by *OAS* (thiol)-lyase (*OAS*-TL, also called cysteine synthase; EC 2.5.1.47) (Leustek *et al.*, 2000). SAT and *OAS*-TL form a multi-enzyme complex (Hell *et al.*, 2001), and so, the availability of *OAS* is of central importance for regulating the flux through assimilatory sulfate reduction (Hawkesford *et al.*, 2006; Neuenschwander *et al.*, 1991; Smith *et al.*, 1997).

Once Cys is produced, it is rapidly exploited in chloroplasts (Hell, 1997) for the synthesis of Met, GSH, proteins (Leustek *et al.*, 2000) and other sulfur containing compounds including the ACSOs (Jones *et al.*, 2004). Met and Cys are the only sulfur containing amino acids. Cys is the only sulfide donor in the biosynthesis of all reduced

sulfur containing cell constituents in plants. Cys plays an essential role in both the structural and catalytic role of proteins and with iron is indispensable for electron transfer reactions in photosynthesis and respiration (Hell *et al.*, 2001). Cys also plays a role in the synthesis of phospholipids, taurine, carnitine and lecithin. GSH serves as storage and transported form of reduced sulfur and as a signal for regulation of sulfur assimilation (Noctor *et al.*, 1998). GSH also plays a role in transport and delivery of Cys to specific sites within cells and whole plants (Leustek *et al.*, 2000).

It should be noted too that an oxidative sulfur assimilation pathway, termed sulfation, exists in plants (Leustek *et al.*, 2000). This pathway begins with APS synthesis by ATPS in common with the reductive pathway. However, APS is then phosphorylated by APS kinase (EC 2.7.1.25) to generate 3'-phospho-5'-adenylylsulfate (PAPS) (Leyh, 1993) (Reaction 2). Then, PAPS is used by a variety of different sulfotransferases (EC 2.8.2.-) located in the cytosol (Varin *et al.*, 1997). These sulfotransferases add sulfate to an oxygen moiety forming a sulfate ester bond (Leustek, 2002) and thereby synthesize compounds such as alliins, thiosulfonates, brassinosteroids, flavonol and glucosinolates (Leustek *et al.*, 2000; Rouleau *et al.*, 1999; Varin *et al.*, 1997).



[2]

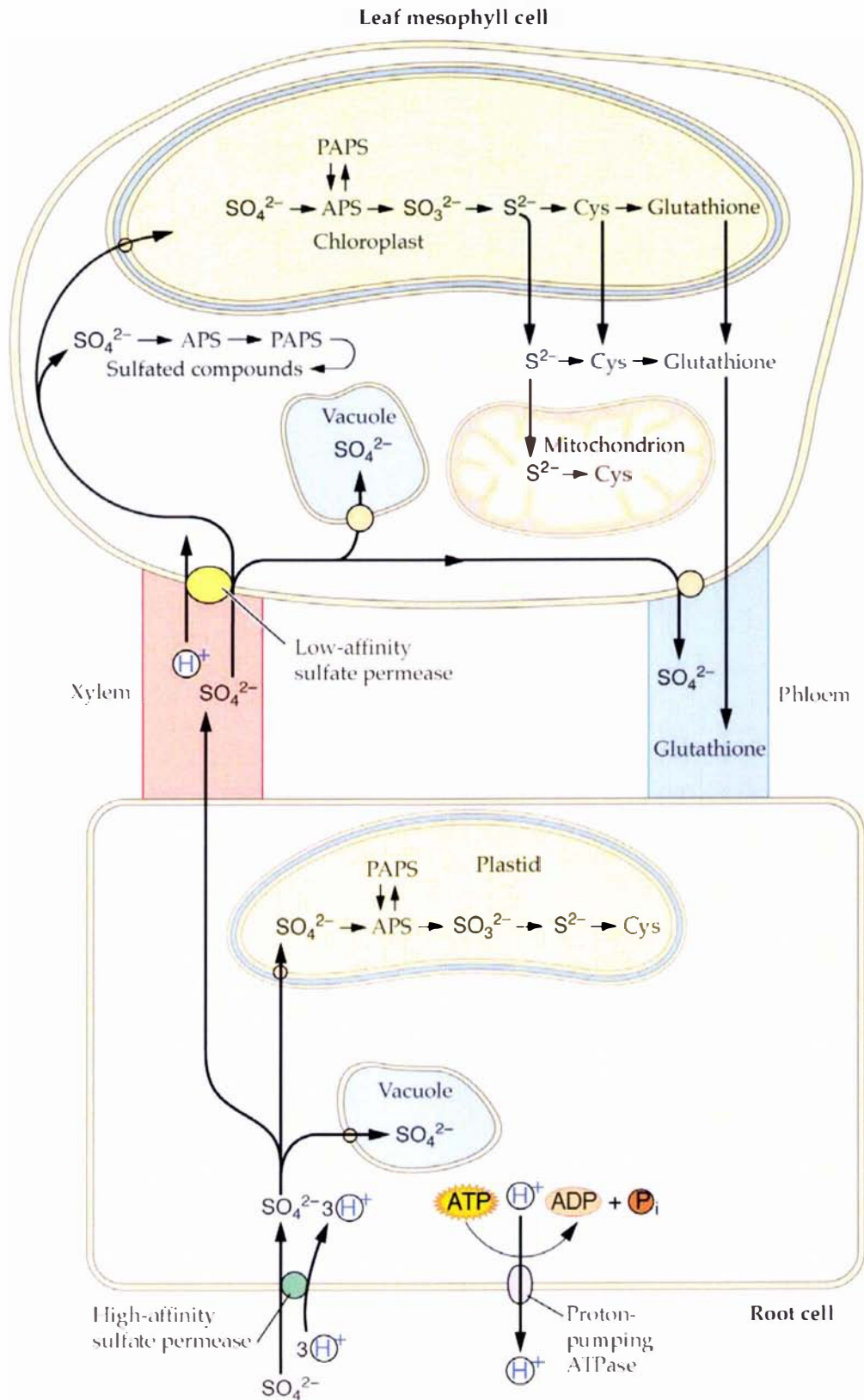


Figure 1.1. Overview of sulfur uptake, reduction, and transport in higher plants from Crawford *et al.* (2000).

1.2.1.2. ATP sulfurylase

Discovered by Robbins and Lipmann (1956) in fresh liver extracts of rabbit and lamb, and first demonstrated in spinach by Asahi in 1964, ATPS is the first enzyme involved in the sulfate assimilation pathway in plants. The enzyme hydrolyzes the bond between the α - and β -phosphates of ATP and then adds sulfate to the α -phosphate, resulting in the formation of APS and PPi (Reaction 3). The energy stored between the β - and α -phosphate bond is essential for the successful reduction of the inert sulfate. This activation step is necessary as sulfate is metabolically inert and the energy stored in the phosphoric acid-sulfuric anhydride bond of the reaction product, APS primes sulfate to undergo further reactions (Leustek, 2002). The reaction catalysed by ATPS depends on photosynthetically generated reductant (Yonekura-Sakakibara *et al.*, 2000).



In thermodynamics terms, the ATPS enzyme in all species carries out an unfavourable reaction, with a $K_{\text{eq}} \sim 10^{-8}$ (Farooqui, 1980; Murillo and Leustek, 1995). To move the reaction in the forward direction, the products APS and PPi must be efficiently removed (Seubert *et al.*, 1985). Since accumulation of APS or PPi inhibits the sulfate assimilation reaction, inorganic pyrophosphatase, APSR and/or APS kinase must be readily available to them.

ATPS is expressed both in roots and leaves of flowering plants, but the activity in roots of *Arabidopsis* is only about 30% of the activity in leaves (Lee, 1999). However, both organs play a significant role in sulfur assimilation.

The enzyme is localized in plastids, especially in chloroplasts, and the cytosol of cells (Lunn *et al.*, 1990; Renosto *et al.*, 1993; Rotte, 1998; Rotte and Leustek, 2000). Two isoforms of ATPS, localized in plastids and cytosol (Paynter and Anderson, 1974; Rotte and Leustek, 2000) have been demonstrated to exist in most plants (Leustek and Saito, 1999) and both enzymes have similar kinetic and structural properties. The plastid-localized ATPS, representing 70% to 95% of the total enzyme in leaves, is involved in assimilative sulfate reduction since the downstream enzymes APSR and SiR, at least in *Arabidopsis*, are localized *only* in plastids (Leustek, 2002). The cytosolic ATPS may be

responsible for other specialized functions, such as providing activated sulfate for the sulfation reactions in the cytosol (Rotte and Leustek, 2000).

Although it is proposed that most plants have two isoforms of ATPS, one in the cytosol and one in the plastids for example in rice (*Oryza sativa* L.) and potato (*Solanum tuberosum* L.) (Klonus *et al.*, 1994), genes encoding four different isoenzymes of ATPS, designated *AtAPS1*, *AtAPS2*, *AtAPS3* and *AtAPS4*, have been cloned in *Arabidopsis* (Hatzfeld *et al.*, 2000; Klonus *et al.*, 1995; Leustek, 2002; Leustek *et al.*, 1994; Murillo and Leustek, 1995). Even though all genes encode enzymes with a characteristic plastid transit peptide (Hatzfeld *et al.*, 2000; Leustek *et al.*, 1994; Murillo and Leustek, 1995), three of these are proposed to occur in the chloroplast and one in the cytosol (Hatzfeld *et al.*, 2000; Rotte, 1998). Hatzfeld *et al.* (2000) proposed that it might be encoded by *APS2*, since the alternative translation start point is proposed to result in an active *AtAPS2* without the transit peptide. However, only one gene coding for ATPS is suggested to occur in onion (Jackse, personal communication).

In common with the overall rate of sulfate assimilation, the gene expression and enzyme activity of ATPS is developmentally regulated. After a peak of activity in young leaves and actively growing regions of the plant, ATPS activity declines as leaves mature in soybean (*Glycine max*), lettuce (*Lactuca sativa*), tomato (Adams and Rinne, 1969), pea (*Pisum sativum*) (Adams and Rinne, 1969; von Arb and Brunold, 1985), *Macroptilium atropurpureum* (Bell *et al.*, 1995), *Arabidopsis* (Rotte and Leustek, 2000), and *Phaseolus vulgaris* (Schmutz and Brunold, 1982). However, subcellular fractionation experiments indicated that the cytosolic and chloroplastic isoforms are differentially regulated during development, where the chloroplastic form decreases as leaves mature, while the activity of the cytosolic form increases during leaf maturation (Rotte and Leustek, 2000). This increase in cytosolic activity and enzyme abundance (approximately five-fold) during the ageing of *Arabidopsis* leaves suggests that the cytosolic ATPS has a specialized function that is unrelated to sulfate reduction and assimilation (Rotte and Leustek, 2000).

ATPS is also regulated by the nutrient status of plants in *Lemna minor* and tobacco (*Nicotiana tabacum* L.) (Bell *et al.*, 1995; Brunold *et al.*, 1987; Hawkesford *et al.*, 2002), in particular by the sulfur and nitrate status (Brunold and Suter, 1984; Brunold,

1993; Reuveny *et al.*, 1980; Yamaguchi *et al.*, 1999). In canola (*Brassica napus*), maize (*Zea mays*) and *Arabidopsis*, ATPS mRNA levels decline after prolonged exposure to sulfur deprivation (Bolchi *et al.*, 1999; Lappartient and Touraine, 1996; Vauclare *et al.*, 2002). The mRNA levels of not only ATPS, but also APSR are also reduced under low nitrogen levels in a coordinated manner (Koprivova *et al.*, 2000).

ATPS activity has been shown to be increased by sulfur starvation treatments in *Macroptilium atropurpureum* (Bell *et al.*, 1995), *Lemna minor* (Brunold *et al.*, 1987), tomato (Hawkesford and Belcher, 1991), canola (Lappartient and Touraine, 1996), and barley (*Hordeum vulgare*) (Lee, 1982; 1993) and repressed when the sulfate levels are restored in *Brassica juncea* (Chen and Leustek, 1995), *Arabidopsis* (Logan *et al.*, 1996) and tobacco (Reuveny and Filner, 1977; Reuveny *et al.*, 1980). As members of the ATPS gene family have different response profiles to sulfate starvation, it is believed that the initial response to sulfate starvation is at the transcriptional level (Logan *et al.*, 1996). Finally, Cys (Bolchi *et al.*, 1999) and GSH (Lappartient *et al.*, 1999) have been suggested to repress expression of ATPS and sulfate uptake. In onion, the transcription of an ATPS gene decreased in response to S deprivation (McCallum *et al.*, 2001).

ATPS is down regulated by exposure to stress conditions in *Lemna minor* (Brunold, 1993) such as the presence of heavy metals (Barroso *et al.*, 1999; Heiss *et al.*, 1999; Lee and Leustek, 1999; Dominguez-Solis *et al.*, 2001). For example, Heiss *et al.* (1999) revealed that the exposure of *Brassica juncea* to cadmium, which chelates GSH and decreases the cellular concentration of GSH, induces expression of ATPS mRNA.

In addition, the activity of ATPS is enhanced by light energy (Passera *et al.*, 1989). Indeed, the enzyme activity increased with irradiation in oat (*Avena sativa*), barley and maize, and decreased when inhibitors of photosynthetic electron transport were added to the nutrient solution (Passera *et al.*, 1989). However, although ATPS activity may vary in response to environmental cues, no limitation in growth has been shown to be due to insufficient ATPS activity, suggesting that more active enzyme accumulates than is required for growth (Logan *et al.*, 1996).

1.2.1.3. APS reductase

The enzyme APSR, formerly named APS sulfotransferase (Bick and Leusteck, 1998; Wray *et al.*, 1998), catalyses the first reduction of activated sulfate (APS), by transferring two electrons from GSH to produce sulfite producing AMP and sulfite (Suter *et al.*, 2000) (Reaction 4). GSH is an efficient hydrogen donor for APSR *in vitro* ($K_m=0.6-3$ mM) (Bick *et al.*, 1998; Prior *et al.*, 1999; Schmidt, 1972). The reaction is stimulated by concentrations of sulfate higher than 0.6 M, and is inhibited by 5'-AMP (Brunold and Suter, 1990; Setya *et al.*, 1996).



This step became universally accepted as a possible sulfate reduction step after the isolation of three isoforms of APSR in 1996 (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996). Before this resolution of the APSR catalysing step, it was thought that APS sulfotransferase activity involved the transfer of a reduced sulfate to a thiol acceptor thereby forming a thiosulphate, such as GSH (Schmidt, 1975; Varin *et al.*, 1997). This hypothesis was refuted when it was shown that the cloned APS sulfotransferase from *Lemna minor* (Suter *et al.*, 2000) displayed very high identity to an APSR cloned from *Arabidopsis* (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996).

Three genes encoding APSR have been cloned from *Arabidopsis*, and are designated *AtAPR1*, *AtAPR2* and *AtAPR3* (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996). All contain a chloroplast transit peptide, a reductase domain and a thioredoxin-like domain (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996; Suter *et al.*, 2000). They have presumably specific functions, which are still unknown (Rotte and Leustek, 2000).

The molecular mass calculated from the deduced amino acid sequence of the APSR cDNAs from *Arabidopsis* and *Lemna minor* was 43 kDa (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996; Suter *et al.*, 2000). The molecular mass from *Lemna minor* APSR (Suter *et al.*, 2000) and recombinant APR2 from *Arabidopsis* (Weber and Kopriva, unpublished data) was estimated to be 91 kDa, suggesting that in higher plants the native enzyme was a dimer of two identical 43 kDa subunits.

Sulfate reduction was first demonstrated in isolated chloroplasts from spinach (*Spinacia oleracea* L.) (Schmidt and Trebst, 1969). Later, the examination of the full-length cDNA from all plants confirmed the presence of a chloroplast targeting peptide (Suter *et al.*, 2000). Using immunogold electron microscopy, APSR was detected in the chloroplasts of three *Flaveria* species, with 70% of detectable APSR localised near or associating with the thylakoid membrane (Koprivova *et al.*, 2001).

The mature APSR consists of two distinct domains, a PAPS reductase-like domain (N-terminal domain) and a thioredoxin-like one (C-terminal domain). The PAPS-reductase domain is very similar to the PAPS reductase from bacteria. However, the N-terminal domain from plants includes two additional cysteine pairs that are essential for the binding of the cofactor [4Fe-4S] (Kopriva *et al.*, 2001; 2002a). The C-terminal domain of APSR displays sequence identity with thioredoxin (EC 1.8.4.10), a class of thiol:disulphide oxidoreductase (Gutierrez-Marcos *et al.*, 1996; Setya *et al.*, 1996). However, it is now accepted in plants that this domain acts as a glutaredoxin (which uses reduced GSH) and not as a thioredoxin reductase (EC 1.8.1.9) (Bick *et al.*, 1998; Prior *et al.*, 1999).

The two domains of APSR cannot be expressed separately or without the addition of reduced thiols (Weber *et al.*, 2000), although they possess independent enzymatic activity and also seem to catalyse distinct steps in APSR reduction. The APSR reaction can be divided in three independent steps (Figure 1.2): (1) the transfer of sulfate from APS to an active cysteine residue, (2) the release of the sulfite by the C-terminal domain, probably by forming an intramolecular disulfide bridge, and (3) recovery of the active enzyme dimer by reaction with thiols. For the first step, which results in binding of sulfite to APSR, neither the thioredoxin-like domain nor an external electron source is required. The electrons necessary for the sulfite release are provided by the APSR protein.

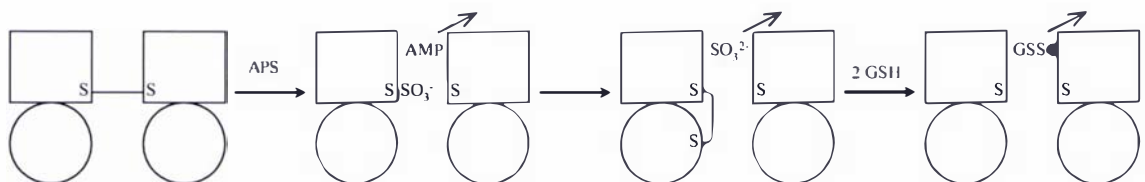


Figure 1.2. Reaction steps in APS reduction by APSR.

The squares and the circles represent the N-terminal and C-terminal domains, respectively. The active site cysteine residue, as well as the Cys possibly forming an intramolecular disulfide, are indicated by S. (Adapted from Kopriva and Koprivova, 2004)

APSR is considered to be the key enzyme of the assimilatory sulfate reduction (Brunold, 1990) because of its important role in controlling flux through the assimilatory sulfate reduction (Vauclare *et al.*, 2002). APSR changes its activity according to the need of the plant for reduced sulfur (Schmidt and Jäger, 1992; Smith *et al.*, 1997; Smith *et al.*, 2000; Takahashi *et al.*, 1997). APSR is regulated during plant development (Rotte and Leustek, 2000) with highest gene and mRNA expression occurring in the actively growing regions of the plant and also, in young leaves (Rotte and Leustek, 2000; Schmutz and Brunold, 1982).

When the external sulfur supply is limiting, the transcript level of APSR is observed to increase in spinach, *Arabidopsis* and *Chlamydomonas reinhardtii* to maximise sulfur uptake and reduction (Prosser *et al.*, 1997; Takahashi *et al.*, 1997; Yamaguchi *et al.*, 1999). However, under nitrogen deprivation, this response is reduced, suggesting that the sulfur deprivation signal is dependent on the nitrogen supply (Yamaguchi *et al.*, 1999). A reduction in APSR mRNA, protein levels and enzyme activity was observed under low nitrogen conditions, but the levels were fully restored with the addition of OAS to the media in *Arabidopsis* (Koprivova *et al.*, 2000), ammonia or nitrate in *Lemna minor* and tobacco (Brunold and Suter, 1984; Reuveny *et al.*, 1980). In *Arabidopsis*, the levels of APSR mRNA and enzyme activity were down-regulated when CO₂ was omitted, but levels were restored with the addition of sucrose (Kopriva *et al.*, 2002).

APSR is also regulated by the nutrient status and by different environmental factors such as heavy metal stress (Rüegsegger *et al.*, 1990) and chilling (Brunner *et al.*, 1995).

1.2.1.4. Sulfite reductase

SiR catalyses the reduction of SO₃²⁻ to S²⁻ and is localized in plastids (Leustek and Saito, 1999) of both photosynthetic and non-photosynthetic tissues (Saito, 2004). Investigations with pea and maize leaves proposed that the activity of SiR is directly linked to photosynthesis because the source of electrons for the reduction of SO₃²⁻ is ferredoxin (Fd) (Krueger and Siegel, 1982) (Reaction 5), which is reduced by the light reactions of photosynthesis (Leustek and Saito, 1999; Saito, 2004; Yonekura-Sakakibara *et al.*, 2000). In non-photosynthetic organs, like roots, it has been proposed that electrons from NADPH, provided from the oxidative pentose phosphate pathway, are donated to two Fd-dependent enzymes, nitrite reductase (NiR) and glutamate synthase (GOGAT),

and are then donated to SiR, *via* Fd-NADP⁺ reductase (FNR) (Bowsher *et al.*, 1989, 1992). In maize, SiR and Fd have been demonstrated to form a 1:1 protein-protein complex (Fd-SiR, EC 1.8.7.1), which is considered important for efficient electron transfer between the two proteins (Akashi *et al.*, 1999).



In non-photosynthetic tissues from maize and spinach, sulfite reduction is strongly influenced by the ratio of NADPH/NADP⁺ (Yonekura-Sakakibara *et al.*, 2000) but SiR can also utilize reduced methyl viologen as an electron donor (Krueger and Siegel, 1982). In addition, the activity of SiR is up regulated in response to long-term sulfate starvation in *Arabidopsis* (Hell *et al.*, 1997), and in chloroplasts, its expression is also determined by light and exogenous sulfur sources (Hell *et al.*, 1997). However, in *Arabidopsis*, the level of SiR mRNA and its expression does not show a circadian rhythm (Leustek, 2002).

Arabidopsis contains a single gene encoding SiR (Bork *et al.*, 1998; Leustek, 2002) whereas two copies of the SiR gene have been detected in tobacco (Yonekura-Sakakibara *et al.*, 1997). Poplars (*Populus trichocarpa*) possess two gene copies for SiR (Kopriva *et al.*, 2004), expressed in both non-photosynthetic and photosynthetic organs (Bork *et al.*, 1998; Yonekura-Sakakibara *et al.*, 1998). In spinach, the molecular mass of SiR is 69 or 63 kDa, depending on the subunit (Akashi *et al.*, 1999; Krueger and Siegel, 1982), whereas in pea (Sato *et al.*, 2001) and in *Arabidopsis* (Bruhl *et al.*, 1996), the mass of the enzyme has been predicted to be close to 70 kDa. In tobacco, the enzyme exists as a 69 kDa DNA-binding protein, abundantly found in the plastid nucleoids (Nemoto *et al.*, 1988; 1990).

In chloroplasts from higher plants, SiR also has a non-specific DNA-binding protein activity that has the ability to condense DNA into small particles (Sato *et al.*, 2001). It is possible that SiR protects the chloroplast nucleoid (complex of chloroplast DNA and various proteins), which is thought to be the functional unit of replication and gene expression (Kuroiwa, 1991; Sakai *et al.*, 1991; Sato *et al.*, 1999) from oxidative damage or from chemical modification by bisulfite (HSO₃⁻) ions. SiR has also been shown to repress the transcriptional activity of chloroplast nucleoids (Sekine *et al.*, 2002).

1.2.1.5. Cysteine synthase complex

The final step of sulfur assimilation is catalysed by two enzymes, SAT and *OAS*-TL, which associate in a bi-enzyme complex, the cysteine synthase complex (CS) (Hawkesford *et al.*, 2006). SAT that catalyses the formation of *OAS* from acetyl-CoA and L-serine (Hawkesford *et al.*, 2006; Kawashima *et al.*, 2005) (Reaction 5), and *OAS*-TL that subsequently catalyses the formation of L-cysteine by the incorporation of the reduced sulfide into *OAS* in a β -replacement reaction to synthesize cysteine and acetate (Hatzfeld *et al.*, 2000; Hawkesford *et al.*, 2006) (Reaction 6). One tetramer of SAT and two dimers of *OAS*-TL are presumed to form the hetero-oligomeric cysteine synthase complex (CS) that was first described in *E. coli* and *Salmonella typhimurium* (Kredich and Tomkins, 1966).



SAT and *OAS*-TL are located not only in chloroplasts but also in mitochondria and the cytosol (Lunn *et al.*, 1990; Rolland *et al.*, 1992; Kuske *et al.*, 1996; Ruffet *et al.*, 1995; Hoefgen *et al.*, 2001) and most plants have several nuclear genes that encode for different isoforms of both enzymes in each of these three compartments (Hell *et al.*, 2002), suggesting that the ability to form cysteine is essential for all compartments with the ability for protein biosynthesis (Hesse *et al.*, 2004). The *Arabidopsis* genome contains five SAT genes and six *OAS*-TL genes that characterize the different subcellular locations and developmental stages in plants (Hawkesford *et al.*, 2006; Hell *et al.*, 2002; Ruffet *et al.*, 1994). It is also proposed that this organization is similar in other species as well (Wirtz and Hell, 2006).

The molecular mass of SAT monomer sizes of SAT range between ~29 and 34 kDa in different species, whereas *OAS*-TL monomer molecular mass is between 68–75 kDa, giving rise to CS quaternary sizes of ~320 kDa (Wirtz and Hell, 2006). A recent structural study from Campanini *et al.* (2007) reported that two dimers of *OAS*-TL bind to one hexamer of SAT.

As predicted by Bogdanova and Hell (1997), SAT consists of two domains in plants. The N-terminus is dominated by an α -helix cluster and is involved in the SAT/SAT interaction, while the C-terminus consists largely of β -sheets and is responsible for SAT/OAS-TL interaction as well as catalysis (Bogdanova and Hell, 1997; Francois *et al.*, 2006; Kumaran and Jez, 2007; Mino *et al.*, 1999; Mino *et al.*, 2000; Wirtz *et al.*, 2001; Zhao *et al.*, 2006).

OAS-TL is a pyridoxal dependent enzyme and a lysine residue at the N-terminal region is involved in binding this cofactor (Bonner *et al.*, 2005; Hatzefeld *et al.*, 2000; Huang *et al.*, 2005; Saito *et al.*, 1993).

The interaction of SAT and OAS-TL coordinates sulfate assimilation and modulates Cys biosynthesis at the cellular level in plants in response to variations of sulfur availability (Droux *et al.*, 1998; Hell and Hillebrand, 2001; Berkowitz *et al.*, 2002). The importance of this final step of sulfate assimilation is comparable to the fixation of ammonia by glutamine synthetase in nitrate assimilation (Kredich, 1996; Saito, 2000; Leustek *et al.*, 2000).

Under conditions of sufficient sulfate supply, SAT associates with OAS-TL to form a complex. The OAS formed is released from the complex and reacts with sulfide through catalysis of free OAS-TL dimers (Berkowitz *et al.*, 2002; Droux *et al.*, 1998; Hell *et al.*, 2002; Kawashima *et al.*, 2005; Kredich *et al.*, 1969; Saito, 2004; Saito *et al.*, 1995; Wirtz and Droux, 2005). When sulfate and consequently sulfide becomes limiting the later reaction stops and OAS accumulates. Upon a certain threshold, the accumulated OAS dissociates the enzyme complex, thus reducing SAT activity and triggering the expression of genes coding for sulfate transporters, ATPS, OAS-TL, and SAT (Koprivova *et al.*, 2000; Hopkins *et al.*, 2005; Smith *et al.*, 1997).

Due to the increased affinity for sulfate uptake the process becomes reversible and this leads to increase sulfur uptake and reduction, and thus to reduce sulfide that reacts with OAS via free OAS-TL dimers (Droux *et al.*, 1998; Ruffet *et al.*, 1994). OAS concentrations decline until the complex can associate again and the genes of high and low affinity sulfate transporters become repressed, presumably triggered by increased levels of cytosolic sulfate, sulfide, Cys or GSH concentrations (Saito, 2004). Thus, SAT can resume its activity in the complex, the formation of which is adjusted to the availability of sulfide.

The equilibrium of complex association and dissociation would determine the rate of OAS and thus of cysteine formation. The cysteine synthase complex functions as a metabolic sensor and part of a control system of primary sulfur metabolism at the cellular level (Hell and Hillebrand, 2001). Activity assays of SAT and OAS-TL in bacteria (Kredich *et al.*, 1969) and plants (Hell *et al.*, 2002; Wirtz and Droux, 2005) generally revealed a flux limitation by SAT rather than OAS-TL.

1.2.2. Sulfur deprivation in plants

The phenotype of sulfate starvation typically consists of pale-green young leaves, while the mature leaves remain dark-green (Hawkesford *et al.*, 1993; Hell, 1997). Chlorophyll levels decreased under sulfur deficiency in wheat (Gilbert *et al.*, 1997), spinach (Warrilow and Hawkesford, 1998), canola (Blake-Kalff *et al.*, 1998; Lencioni *et al.*, 1997), sugar beet (*Beta vulgaris*) (Thomas *et al.*, 2000), and rice (Resurreccion *et al.*, 2002). This phenomenon is different from the symptoms of nitrogen and phosphate deficiency, where young leaves remain green due to nutrients mobilised from mature leaves (Hell, 1997). In addition, in *Arabidopsis*, insufficient levels of sulfate lead to growth retardation (Kutz *et al.*, 2002; Nikiforova *et al.*, 2003; 2004) and altered root morphology (Kutz *et al.*, 2002).

When the internal sulfate pools are reduced, soluble nitrogen pools are rising, leading to an N:S imbalance (Karmoker *et al.*, 1991; Prosser *et al.*, 1997; Warrilow and Hawkesford, 1998; Zhao *et al.*, 1996) and this has important consequences for crop quality (McGrath *et al.*, 1996; Zhao *et al.*, 1996). Limiting the availability of sulfur has been shown to favour the synthesis and accumulation of poor or low sulfur-containing storage proteins (Fullington *et al.*, 1987; Moss *et al.*, 1981; Wrigley *et al.*, 1984). Sulfur limitations can reduce the resistance to environmental stresses and pests. Thus the agronomic consequences of insufficient sulfur are decreased yields and decreased crop quality (McGrath *et al.*, 1996). These negative effects can be reversed with the addition of sulfate or Cys (Leustek *et al.*, 2000). On the other hand, excessive sulfur may cause physiological imbalances and negatively affect plant growth.

The sulfur assimilation pathway is highly regulated by the availability of sulfur nutrition. Under high sulfur conditions, the pathway is repressed, so the uptake rate fits the plant requirements (Clarkson and Lüttge, 1991; Imsande and Touraine, 1994), and

de-repressed when sulfate becomes limiting (Herschbach *et al.*, 2000; Kopriva and Rennenberg, 2004; Lappartient and Touraine, 1996; Lappartient *et al.*, 1999; Vauclare *et al.*, 2002). In response to low sulfate levels, plant metabolism (Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003) such as photosynthesis (Davies and Grossman, 1998; Gilbert *et al.*, 1997; Klapheck *et al.*, 1982) is inhibited, leading to retarded growth, cell division and a decline in most metabolic processes in the cell (Davies *et al.*, 1996). As the accumulation of key metabolites is blocked and pools become depleted, protein synthesis and the accumulation of amino acids is reduced. In addition, *OAS* and non-sulfur-containing amino acids such as glutamine, asparagine and serine accumulate (Migge *et al.*, 2000; Prosser *et al.*, 2001; Reuveny *et al.*, 1980). The S-assimilation pathway is also de-repressed during periods of high demand for growth and development (Hawkesford and De Kok, 2006; Saito, 2004).

Sulfur deficiency also leads to inhibition of both carbon and nitrate assimilation (Kopriva and Rennenberg, 2004), and this attenuates the responses to sulfur starvation in plants that are also nitrogen limited (Leustek *et al.*, 2000). Indeed, the sulfur assimilation pathway shows similarities with the nitrogen assimilation pathway and is well coordinated with nitrate assimilation so that a deficiency in one element represses the other pathway (Brunold, 1993; Brunold and Suter, 1984; Hesse *et al.*, 2004; Kim *et al.*, 1999; Koprivova *et al.*, 2000; Migge *et al.*, 2000; Neuenschwander *et al.*, 1991; Prosser *et al.*, 2001; Reuveny *et al.*, 1980; Smith, 1980; Takahashi and Saito, 1996).

Plants can adapt to short-term periods of low sulfate availability by modulating the transport of the oxyanion (Hawkesford *et al.*, 1993; Smith, 1975) by de-repressing the activity of the sulfate transporters when sulfur supply is low and repressing their activity when the supply is abundant (Hawkesford, 2000). Plants have also developed several other strategies such as induction of enzymes for the utilization of alternative sulfur sources (de Hostos *et al.*, 1988), or the synthesis of protein isoforms with a lower Met and Cys content (Takahashi *et al.*, 2001). The levels of mRNA and protein of ATPS, APSR, SiR and *OAS*-TL and chloroplastic SAT are also enhanced (Brunold *et al.*, 1987; Reuveny *et al.*, 1980; Smith, 1980; Takahashi *et al.*, 1997; Yamaguchi *et al.*, 1999) probably as an adaptive mechanism to increase the efficiency of the pathway (Kopriva *et al.*, 2004). Finally, the concentration of *OAS*, a direct precursor of cysteine biosynthesis, increases under sulfur deprivation (Awazuhara *et al.*, 2000; Kim *et al.*, 1997, 1999).

Hence, *OAS* may be a positive regulator of sulfur assimilatory genes in response to sulfur availability in plants, in common with bacteria (Kredich, 1993).

1.3. Light and dark regulation

Light is one of the most important environmental factors for plants, as it provides the source of energy for plant life and controls many developmental processes. Plants have the ability to sense multiple parameters of ambient light signals, including light quantity (fluence), quality (wavelength), direction and duration. Plant responses to light occur in the context of multiple developmental processes, including seed germination, seedling photomorphogenesis, phototropism, gravitropism, chloroplast movement, shade avoidance, circadian rhythms and flower induction (Jiao *et al.*, 2007).

1.3.1. Photoperiodism

Photoperiodism (from Greek: photo=light and periodism=duration of light) is defined as the changes in plant growth and development in response to the daily duration of light and dark periods. Photoperiodism is an adaptation by organisms to adjust their physiology to environmental cues, and even to anticipate seasonal changes in the environment (Lakin-Thomas and Brody, 2004). Light, particularly its intensity and oscillation, is the most important factor influencing these rhythms (Kopriva *et al.*, 1999). For onions, long nights and short days favour vegetative growth whereas short nights and long days initiate bulb formation (Wickramasinghe *et al.*, 2000). In 1936, Bünning suggested that rhythmic processes, also called circadian rhythms, were involved in photoperiodism.

1.3.2. Circadian rhythms

Circadian rhythms (from Latin, circa = about and dies = day) are the regular pattern of physiological processes over a period of ca. 24 h that remain even when the organism is placed in continuous darkness (Lakin-Thomas and Brody, 2004). They were first measured in 1729 by the French biologist de Mairan, who noted that a heliotrope plant (probably *Mimosa*) sustained rhythms in leaf movement in continuous darkness (Barak *et al.*, 2000; Johnson *et al.*, 1995; McClung *et al.*, 2002; Pittendrigh, 1993). Circadian rhythms are observed in all the major groups of eukaryotes and in many prokaryotes (Harmer *et al.*, 2001; Johnson *et al.*, 1998; McClung *et al.*, 2002). They allow organisms

to anticipate reactions controlled by dawn and dusk (Engelmann and Johnson, 1998; Harmer *et al.*, 2000) by providing an internal estimation of the external time (Johnson *et al.*, 1998), and thus shorten the delay between a change in the environment and the appropriate alteration in physiology (Dodd *et al.*, 2005; Harmer *et al.*, 2000; Johnson, 2001; Ouyang *et al.*, 1998).

In order to remain synchronized with the environment, circadian clocks are reset or entrained by specific cues (Johnson *et al.*, 1998; Lakin-Thomas and Brody, 2004; Webb, 2003), called zeitgebers (= time givers in German) (Lakin-Thomas and Body, 2004) that relay information about the external time. In photosynthetic organisms, two major zeitgebers are found: the light/dark and temperature cycle (Devlin, 2002; Johnson *et al.*, 1998; Michael *et al.*, 2003; Millar, 2004), although rhythms in nutrient availability may also act as a resetting signal in some organisms (Harmer *et al.*, 2000; Roenneberg and Merrow, 1998). The complexity provides the clock with stability and protection against stochastic perturbations (Cheng *et al.*, 2001; Smolen *et al.*, 2001; Roenneberg and Merrow, 2003) and may provide the advantage of preventing the resetting of the clock by inappropriate signal, such as flashes of lightning (Devlin, 2002; Nelson and Takahashi, 1991).

Approximately 2-16% of transcripts encoded in the *Arabidopsis* genome have steady-state rhythms (Edwards *et al.*, 2006; Harmer *et al.*, 2000; Schaffer *et al.*, 2001). These include transcripts encoding proteins involved in flowering, flavonoid synthesis, lignin synthesis, cell elongation, nitrogen fixation, carbon metabolism, mineral assimilation and photosynthesis (Edwards *et al.*, 2006; Harmer *et al.*, 2000; Schaffer *et al.*, 2001). An important role of the circadian clock is to enhance the operation of the biochemical pathways leading to chlorophyll accumulation and carbon assimilation (Dodd *et al.*, 2005). In *Gonyaulax* (an alga), protein phosphorylation is proposed to play a role in the central clock mechanism or in the input pathway (Comolli *et al.*, 1994).

Light quality and intensity is an important element affecting the sulfur metabolism of plants (Ghisi *et al.*, 1987; Kopriva *et al.*, 1999). It has been revealed that the ATPS activity increased with irradiation in oat, barley and maize, and decreased when inhibitors of photosynthetic electron transport were added to the nutrient solution (Passera *et al.*, 1989). In *Arabidopsis*, the mRNA levels of SiR, OAS-TL and SAT were several times higher in green leaves than in etiolated tissues (Hell *et al.*, 1997). The

activity of APSR (Bick and Leustek, 1998; Wray *et al.*, 1998) is light induced in *Lemna minor*, and a diurnal rhythm with maximal activity during the light period has been observed in maize (Kocsy *et al.*, 1997) and in *Arabidopsis* (Kopriva *et al.*, 1999).

1.4. Phosphorylation - dephosphorylation

1.4.1. Introduction

Proteins can be altered in a diverse set of post-translational modifications, including acetylation, hydroxylation, methylation, sumoylation or ubiquitylation, among many others (Seet *et al.*, 2006), in response to changing conditions of the internal and external cell environments. However, it is thought that protein phosphorylation, which corresponds to the addition of a phosphate (PO₄) group by a protein kinase (Marré, 1961), is the most prevalent means of protein covalent modification (Cohen, 2002) (Figure 1.3). Phosphorylation is reversible by the activity of protein phosphatases in a process called dephosphorylation. It is estimated that as much as 30% of all proteins in eukaryotes may be phosphorylated at any time (Cohen, 2002; Hubbard and Cohen, 1993; Mann *et al.*, 2002; Zolnierowicz and Bollen, 2000).

Phosphorylation either activates or inactivates the substrate protein by direct induction of a new conformational state (Mann *et al.*, 2002). In eukaryotes, phosphorylation has been shown to play a central role in cell metabolism by regulating growth factor receptor activity, gene transcription, hormones, protein synthesis and biological activity (Andreeva and Kutuzov, 1999; Chernoff, 1999; Cohen, 2002; den Hertog, 1999; Iten *et al.*, 1999; Luan, 2000; Schillace and Scott, 1999). This mechanism also plays a role in facilitating or inhibiting movement between subcellular compartments (Cohen, 2002), such as inhibiting the movement of organelles along microtubules when synapsin I is phosphorylated (McGuinness *et al.*, 1989) or monitoring the movement of stomata in *Arabidopsis* (MacRobbie, 2002), or altering protein activity or targeting proteins for degradation (Peck, 2006). Phosphorylation often leads to the initiation or disruption of protein-protein interactions via interactions between the phosphorylated region and proteins containing specific phosphobinding domains (Cohen, 2002; Peck, 2006). This post-translational modification also plays a role in controlling plant defence mechanisms including cold stress and pathogen invasion, signal transduction, and metabolism (Bowler and Chua, 1994; Budde and Randall, 1990; De la Fuente *et al.*, 2006; Huber *et al.*, 1994a; McMichael, 1994; Stone and Walker, 1995), as well as triggering

fundamental cellular events such as mitosis and DNA synthesis (Fourest-Lieuvain *et al.*, 2006; Ottaviano and Gerace, 1985; Syljuåsen *et al.*, 2005).

Phosphorylation of eukaryotic proteins occurs predominantly (97%) on serine (Ser) and threonine (Thr) residues and to a lesser extent on tyrosine (Tyr) residues (Shenolikar, 1994). Nevertheless, Tyr phosphorylation is essential in many cell signalling pathways in mammalian cells in response to various stresses (Zhang and Klessig, 2001) and deregulation often contributes to oncogenesis (Blume-Jensen and Hunter, 2001). Histidine phosphorylation has also been reported in plants (Huber *et al.*, 1994), fungi (Ota and Varshavsky, 1993) and animals (Crovello *et al.*, 1995), but its relative contribution to the total phosphoamino acid content of eukaryotic cells is not known. An individual peptide can undergo phosphorylation on more than one residue, thereby producing many phosphoisoforms, each of which can be antagonist or additive in nature depending on the proteins (Cohen, 2002).

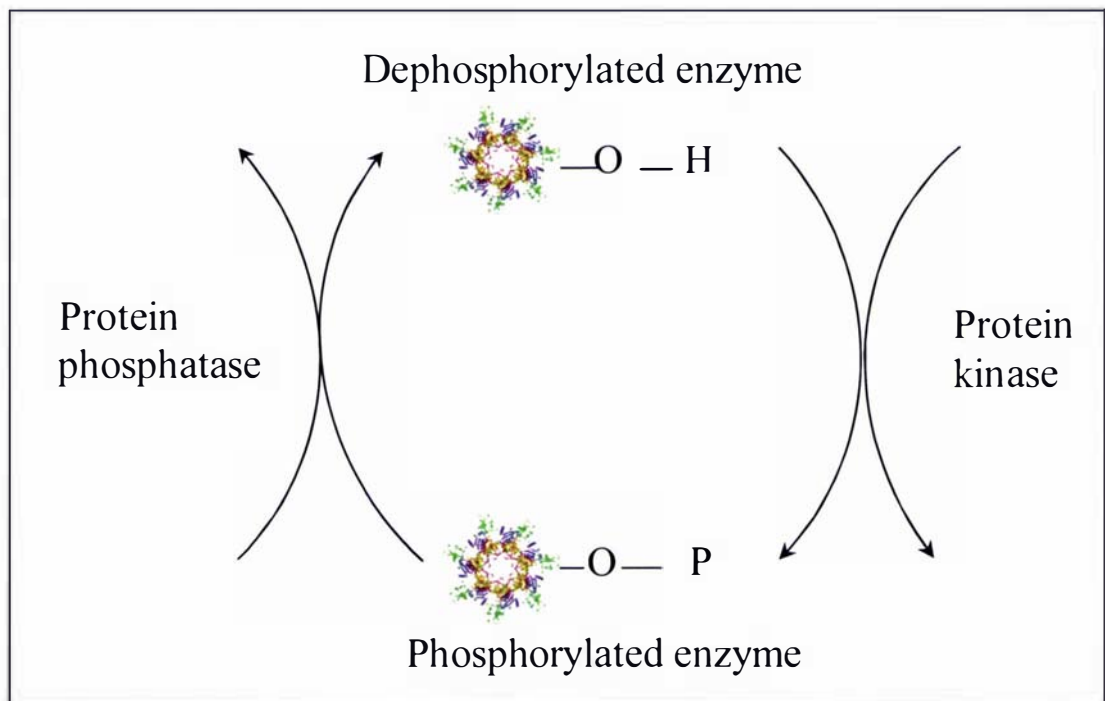


Figure 1.3. Phosphorylation/Dephosphorylation of enzymes.

1.4.2. Protein kinases

First observed in 1954 by Burnett and Kennedy, protein kinases represent one of the largest families in eukaryotes, with more than 1000 genes in *Arabidopsis* (Wang *et al.*, 2003). These kinases are divided into two families according to their phosphoryl amino acid substrate specificity: the Ser/Thr and the Tyr kinase family. Some kinases can act at both Ser/Thr residues and Tyr residues (“dual-specificity” kinases). All protein kinases share extensive similarities in their sequence and three-dimensional structures (Hanks and Hunter, 1995; Hardie and Hanks, 1995; Mohammadi *et al.*, 1997; Sicheri *et al.*, 1997) and can use ATP as the phosphoryl donor, with apparent K_m values for most protein kinases below 100 μM (Wang and Roach, 1992).

1.4.3. Protein phosphatases

Protein phosphatase activities have been reported in most plant subcellular compartments, including mitochondria, chloroplast, nuclei, and cytosol, and are associated with various membrane and particulate fractions (Huber *et al.*, 1994b; MacKintosh *et al.*, 1991). Like protein kinases, phosphatases represent a diverse family of enzymes of ~300 genes (Cohen, 1989; Neel and Tonks, 1997; Stone and Dixon, 1994) and can be grouped by substrate specificity into Ser/Thr, Tyr, and dual-specificity classes. Protein tyrosine phosphatases (PTPases) have a distinct evolutionary origin and catalytic mechanism from the Ser/Thr phosphatases. The Ser and Thr protein phosphatase activity in cells can be ascribed to four classes based on their unique substrate specificities and sensitivities to various inhibitors: type 1 (PP1), type 2A (PP2A), 2B (PP2B) and 2C (PP2C) (Cohen, 1989; Ingebritsen and Cohen, 1983). Neither PP1 nor PP2A is found in chloroplasts (MacKintosh *et al.*, 1991; Sun and Markwell, 1992) and no PP2B genes have been identified in plant genomes (Kerk *et al.*, 2002).

PP1 and PP2A are inhibited by okadaic acid (OKA), microcystin-LR and calyculin A (Carter *et al.*, 1990; Cicirelli, 1992; Huber and Huber, 1990; Kaiser and Huber, 1994; MacKintosh and MacKintosh, 1994; McNaughton, 1991). PP2B is Ca^{2+} and calmodulin dependent, and PP2C requires Mg^{2+} for activity. Most enzymes from these subgroups share high sequence similarities (Cohen, 1989; Walton and Dixon, 1993), but they are very different from PTPases.

The PTPases are categorized into three sub-groups: receptor-like PTPases, intracellular PTPases, and dual-specificity PTPases (Stone and Dixon, 1994). PTPases play important roles in a number of signal transduction pathways in animal and yeast systems (Neel and Tonks, 1997). The *Arabidopsis* genome encodes for 53 predicted tyrosine kinases. Most tyrosine phosphatases appear to have additional modular domains that function to regulate and/or target the enzyme to another molecule or compartment (Moorhead *et al.*, 2006).

1.4.4. Regulation by phosphorylation in plants

In plants, phosphorylation of key enzymes acts as an important control point of primary metabolism. Indeed, in leaves, the activities of several cytosolic enzymes are responsive to changes in photosynthesis. Thus, the light and dark cycle trigger the release of signals from the chloroplast to control the phosphorylation and activity states of nitrate reductase (NR), an enzyme involved in the nitrate assimilation, the control of sucrose-phosphate-synthase (SPS) that catalyses the synthesis of sucrose from glucose, and fructose, and phosphoenolpyruvate carboxylase (PEPc), which catalyses the formation of phosphoenolpyruvate in C4 plants (Huber *et al.*, 1992; 1994; Jiao *et al.*, 1991; Moorhead *et al.*, 1999; 2006). When photosynthesis is active, NR, SPS and PEPc are activated, but these three enzymes are not always regulated in parallel, because in leaves and other tissues, hormones, water stress, nitrogen and carbon supplies also modulate the phosphorylation and activity states of these enzymes. For examples, spinach SPS is activated in response to osmotic stress by a phosphorylation on Ser₄₂₄, distinct from the phosphorylation on Ser₁₅₈ which inactivates the enzymes (Toroser and Huber, 1997). In contrast, osmotic stress promotes inactivation of NR (Kaiser and Forster, 1989). Also in canola, light and dark transitions also influence the activity of the cytosolic glutamine synthase (GS), a key enzyme involved in ammonia assimilation in plants (Finnemann and Schjoerring, 2000). In plant metabolism, light/dark activation/des-activation is often a signature of phosphorylation/dephosphorylation of plant enzymes.

In sulfur metabolism, SAT from soybean has been found to be phosphorylated in response to oxidative stress, an event greatly stimulated by Ca²⁺. Analysis of the available plant SAT sequences shows that the phosphorylation site is conserved in several families of angiosperms and gymnosperms. However, the sequences of SAT from *Arabidopsis* and rice lack this site, suggesting that phosphorylation of SAT is not

universal (Liu *et al.*, 2006). Proteomic studies also revealed the phosphorylation of OAS-TL during seed filling in canola (Agrawal and Thelen, 2006) and in *Arabidopsis* by gel staining with the phosphospecific Pro-Q Diamond dye (Thelen, personal communication).

1.5. 14-3-3 proteins

First detected in bovine brain by Moore and Perez in 1967, the 14-3-3 proteins are binding proteins involved in almost every cellular process in all eukaryotic cells (Shaw, 2000) where they recognizing and bind to phosphorylated sites in target proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997; Tzivion and Avruch, 2002). Over the last 20 years, they have proven to be ubiquitous, being found in virtually every eukaryote organism and tissue (Ichimura *et al.*, 1987; Robinson *et al.*, 1994; Ferl, 1996). In any given organism, the 14-3-3 family usually consists of multiple genes and protein isoforms that are highly conserved (Robinson *et al.*, 1994). Localization of 14-3-3 family members inside organelles such as the chloroplast (Sehnke *et al.*, 2000), nucleus (Bihn *et al.*, 1997), and mitochondria (Sehnke and Ferl, 2000), in addition to the cytoplasm (Bihn *et al.*, 1997), further demonstrates both their global regulatory potential and their apparent need for diversity in expression and function. They function as homo- or heterodimers, with each monomer of ~30 kDa (Yaffe *et al.*, 1997, Ichimura *et al.*, 1988) and require divalent cations and the correct structural reorientation of the C-termini to become oxidized, and hence charged (Ferl, 2004; Lu *et al.*, 1994).

The 14-3-3 proteins play key functional roles in many physiological pathways that are regulated by phosphorylation. Their role is to complete the signal transduction process by binding to the phosphorylated target, and thereby ensuring that the precise protein conformation is achieved for enzyme activity (DeLille *et al.*, 2001). In plants, they exert a widespread influence on the primary metabolism (Bunney *et al.*, 2001) and cellular processes including controlling the cell cycle, modifying transcription in response to environmental cues (Bachmann *et al.*, 1996a, b; Moorhead *et al.*, 1996), controlling the shuttling of proteins between cellular compartments and regulating proteolytic degradation (Cohen, 2002). The binding of 14-3-3 proteins stabilizes enzymes, leading to enhanced post-translational modifications such as further phosphorylation. They play a key role in providing extra structural support to stabilize target enzymes when protein

phosphorylation is not enough to drive the conformational changes needed to alter enzyme activities.

Acting together, phosphorylation and 14-3-3 proteins play important roles in regulating complicated daily rhythms in co-ordinating sugar metabolism with photosynthesis, ATP production and nitrate assimilation (Szopa, 2002). For example, protein phosphorylation and the 14-3-3 proteins are shown to act as a regulatory mechanism of NR. NR is a cytosolic enzyme that catalyzes the reduction of nitrate to nitrite and requires phosphorylation for binding to 14-3-3 proteins (Athwal *et al.*, 1998). During the dark period, NR is phosphorylated and the enzyme complex is formed, and NR is inactivated. During the light period, NR dephosphorylates, the complex is interrupted and the enzyme is active again (Moorhead *et al.*, 1996).

The other enzymes that interact with 14-3-3 proteins include starch synthase (Sehnke *et al.*, 2001), Glu synthase, ascorbate peroxidase, and caffeoyl methyl transferase (Finnie *et al.*, 1999). The control of the plant's turgor pressure via regulation of at least one form of a plasma membrane H⁺-ATPase is accomplished by 14-3-3 proteins (Korthout and de Boer, 1994; Marra *et al.*, 1994; Oecking *et al.*, 1994). In addition, 14-3-3 binding partners include transcriptional machinery such as the G-box complex and core-transcription factors TBP, TFIIB, and EmBP (Chung *et al.*, 1999). Of relevance to sulfur assimilation, bio-informatic approaches have revealed that at least one phosphorylation site in two enzymes, ATPS and OAS-TL may be a binding site for 14-3-3 proteins (Ferl, personal communication).

1.6. Studies on sulfur assimilation in onion

Onions are valued as food and medicine primarily for their flavours and medicinal properties of their sulfur compounds. There is growing interest in optimising breeding and production to produce fresh or processed products with defined flavour and health characteristics (Griffiths *et al.*, 2002).

The flavour intensity of onions varies widely due to genetic, environmental and post-harvest factors (Randle and Lancaster, 2002). Glasshouse studies have demonstrated that sulfate supply is the major environmental factor determining pungency (Freeman and

Mossadeghi, 1970; Randle, 1992; Randle and Bussard, 1993). More recent studies have also shown a decrease in leaf and bulb fresh weights after hydroponic culture in sulfur deficient conditions (McCallum, personal communication). Sulfur deprivation is also linked to reduction in chlorophyll, sulfate and nitrate contents as well as Cys and GSH production in leaves (McCallum, personal communication). Levels of asparagine and glutamine were increased, while the flavour precursors PCSO and MCSO were also reduced under low sulfur supply suggesting the importance of sulfur nutrition for onion quality and flavours (McCallum, personal communication). However, onion genotypes respond differently to sulfur nutrition although this commonly shows little correlation between total sulfur content and pungency (Randle, 1992; Randle and Bussard, 1993). Thus, it is thought that mild varieties accumulate more sulfur and store it in the vacuole of cells, which excludes it from the incorporation into the flavour pathway (Randle *et al.*, 1999). This suggests that mild genotypes are less efficient at incorporating of the sulfur into thiols, and thus determine the genetic variations between mild and pungent genotypes. In addition, hydroponic studies have also shown that nitrogen supply can affect flavour precursor levels (Coolong and Randle, 2003; Randle, 2000).

Although onion and other *Allium* vegetables probably have high sulfur requirements, there is little agronomic data available (Aulakh, 2003), and there have been few studies examining the molecular basis for variation in *Allium* organosulfur content (McCallum *et al.*, 2002; Urano *et al.*, 2000).

The expression of ATPS gene has been shown to increase with high sulfur supply, and higher expression was also measured in the more pungent cultivars. Recently, a quantitative trait loci (QTL) for onion pungency has been mapped close to the location of the ATPS gene (McCallum *et al.*, 2006a). Therefore, it is thought that ATPS is an important candidate for the observed divergent sulfur metabolism between phenotypes (McCallum *et al.*, 2002). Also, ATPS could be a possible contender for pathway control because it catalyses the first step in the sulfur assimilation. Recent findings suggest that only one gene coding for ATPS may exist in onion (Jackse, personal communication), thus the mechanism for differential expression of the ATPS gene regulation is likely to be at the post-transcriptional and/or post-translational level.

In regards with APSR, one gene coding for the enzyme has been shown to be up regulated in response to sulfur starvation in roots of a pungent and a mild genotype (McCallum *et al.*, 2001). However, no association between APSR enzyme activity and higher pungency has been detected after hydroponic culture of Texas Grano and W202A genotypes (McCallum *et al.*, 2006b). Besides, investigations *in vitro* suggest the occurrence of a complex between ATPS and APSR, inducing up an regulation of ATPS activity (Cumming *et al.*, 2007).

The enzyme OAS-TL has been suggested to play a role in regulating sulfur assimilation flux in *Allium* (Urano *et al.*, 2000). Besides, Northern analyses have demonstrated that the expression of the *SAT1* gene is induced in leaf tissue in response to low sulfur supply (McManus *et al.*, 2005). Taken together, these studies suggest that differences occur in the control of the reductive sulfur assimilation pathway in onion when compared to the model plant species *Arabidopsis*. Thus the characterisation of enzymes in the pathway in onion is desirable to understand the basis of sulfur accumulation in onion and other *Allium*.

1.7. Aims of the project

While most of the investigations for the characterization of the enzymes involved in the sulfur assimilation pathway have been carried out using *Arabidopsis*, *Lemna minor*, *Physcomitrella patens*, or even poplar, much less is known about the regulation in sulfur accumulating species such as onion. Accordingly, the primary aim in this PhD project was to increase our knowledge on the regulation of the enzymes involved in the sulfur assimilation pathway, but with a particular focus on ATPS. This characterisation also extended to investigate the phosphorylation status of the enzymes, again with an emphasis on ATPS. As well, two genotypes of onion, ‘Texas Grano 438’ (TG) and ‘W202A’ (W), which accumulate differing levels of ACSOs (McCallum *et al.*, 2006a), were used to achieve specific objectives. To achieve the primary aim, the thesis was divided into a series of research objectives, as follows:

- I. To determine the occurrence of ATPS and APSR in onion tissues. To do this, proteins were isolated from chloroplasts, leaves, roots and bulbs and examined using western analysis and, where possible, activity assays.

- II. To identify the influence of S supply, genetic background and developmental status on ATPS activity and abundance and APSR abundance, using plants of both TG and W grown in either S-sufficient or S-deficient media.
- III. To monitor the abundance of both ATPS and APSR, and the activity of ATPS over a 24 h period from both TG and W to establish if any diurnal rhythm of activity exists.
- IV. To investigate the occurrence of ATPS and APSR isoforms that may occur in the chloroplasts of onions, using anion exchange chromatography and 2D-PAGE.
- V. To use bio-informatic tools, like NetPhos, to predict putative phosphorylation sites on all the enzymes involved in the sulfur assimilation pathway.
- VI. To examine the consequences of phosphorylation-dephosphorylation in terms of any changes of the molecular mass of ATPS, the activity of the enzyme or its charge (pI) using 2D-PAGE.
- VII. To use affinity chromatography to investigate the interaction between 14-3-3 proteins and the sulfur assimilation enzymes of onion.

2. MATERIAL AND METHODS

2.1. Propagation and harvesting methods

2.1.1. Plant material

Two genotypes of onion were used: 'Texas Grano 438' (TG; source: Crop & Food Research, Lincoln, New Zealand), and 'W202A' (W; source: Crop & Food Research, Lincoln, New Zealand). TG is classified as a mild line, whilst W is classified as a pungent line, accumulating higher levels of ACSOs (McCallum *et al.*, 2006a). In terms of sulfur content, W202A has significantly higher levels of the sulfoxides, *S*-methyl cysteine sulfoxide and *S*-propyl cysteine sulfoxide as well as cysteine (McCallum and McManus, unpublished).

2.1.2. Plant growth conditions

Plants were grown in growth chambers (TemperZone Ltd., Otahutu, New Zealand) at the Plant Growth Unit, Massey University, Palmerston North, New Zealand. As seedlings, plants were grown under 12 h light at 22°C, 12 h dark at 17°C and harvested at seven weeks (section 2.1.4). To promote bulbing (designated the mature stage), plants were grown under 16 h light at 22°C, 8 h dark at 17°C and harvested at four months (section 2.1.4). The relative humidity within the chamber was maintained at ~60% and the irradiance measured at 560 $\mu\text{mol sec}^{-1} \text{m}^{-2}$, for each growth stage.

2.1.3. Plant propagation

Seeds were germinated and rooted in vermiculite for three weeks in the growth chambers. The seedlings were then transferred to a hydroponic growth system in full strength Hoagland's solution (Hoagland and Arnon, 1950) modified to contain 4.0 (S-sufficient; S⁺) or 0.1 (S-deficient; S⁻) meq L⁻¹ of sulfur (Randle *et al.*, 1995; Lancaster *et al.*, 2000; McCallum *et al.*, 2002) in the growth chambers. This was achieved by varying the concentration of MgSO₄, Mg(NO₃)₂ and Ca(NO₃)₂ in the Hoagland's solution (Table 2.1).

The high level (4.0 meq L⁻¹) was selected as it resulted in pungency levels similar to those in field-grown onions fertilized with that level of S (Randle and Bussard, 1993). The low concentration was selected because growth of 'White Lisbon' onions was reduced by up to 50% with ~0.1 meq L⁻¹ of S (Freeman and Mossadeghi, 1970). The

pH of the solution was adjusted to 6.5 with NaOH. The liquid medium was replaced every four days and the volume of media in the pots was replenished with distilled water (dH₂O) to replace evaporated liquid throughout the growing period.

Table 2.1. Composition of the S-sufficient (S⁺) and S-deficient (S⁻) hydroponic media

	Components	S⁺ solution	S⁻ solution
Macro-elements	Ca(NO ₃) ₂ .4H ₂ O	0.925 g L ⁻¹	0.472 g L ⁻¹
	KNO ₃	0.594 g L ⁻¹	0.396 g L ⁻¹
	NH ₄ H ₂ PO ₄	0.113 g L ⁻¹	0.113 g L ⁻¹
	MgSO ₄ .7H ₂ O	0.482 g L ⁻¹	0.012 g L ⁻¹
	Mg(NO ₃) ₂ .6H ₂ O	0.000 g L ⁻¹	0.489 g L ⁻¹
	Iron-EDTA	0.0025 g L ⁻¹	0.0025 g L ⁻¹
Micro-elements	H ₃ BO ₃	2.803 mg L ⁻¹	2.803 mg L ⁻¹
	MnCl ₂ .4H ₂ O	1.774 mg L ⁻¹	1.774 mg L ⁻¹
	ZnCl ₂	0.102 mg L ⁻¹	0.102 mg L ⁻¹
	CuCl ₂ .2H ₂ O	0.053 mg L ⁻¹	0.053 mg L ⁻¹
	NaMoO ₄ .2H ₂ O	0.025 mg L ⁻¹	0.025 mg L ⁻¹

For plant growth, ten plants were cultivated together in a 1 L pot (14 cm long × 10 cm wide × 12 cm deep), while for the mature stage, five plants were grown in a pot, as shown in figure 2.1. For both seedlings and mature plants, there were secured in a polystyrene cover that acted as a float over the media. For the mature plants, the float supported the majority of the bulb above the liquid media. The lower part of the bulb and the roots were kept fully immersed in the medium below the polystyrene as shown in figure 2.2.

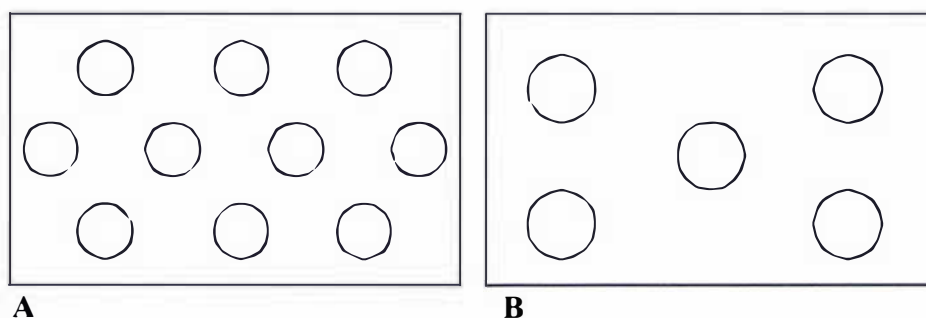


Figure 2.1. Arrangement of plants in pots in hydroponic culture.

Ten plants were cultivated together for the seedling stage harvest (A). Five plants were cultivated together for the mature stage harvest (B).

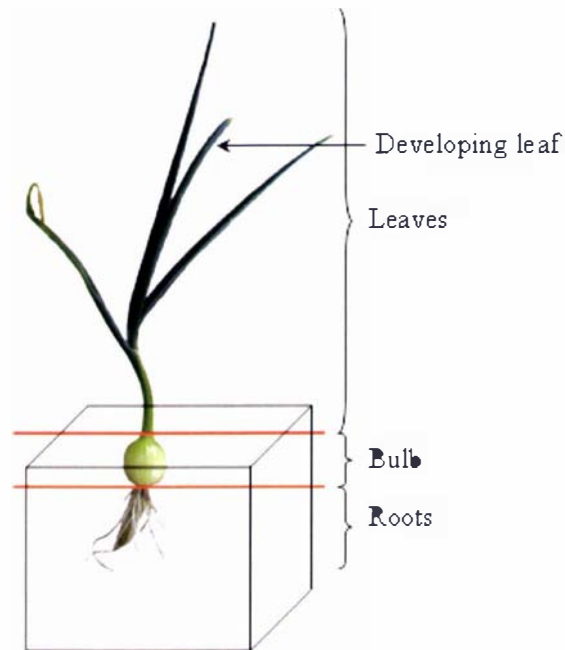


Figure 2.2. Plant position in a pot for hydroponic culture and indication of tissues used for plant growth analysis.

The brackets and red lines show the delineation of each tissue.

2.1.4. Plant harvest

At harvest (at seven weeks for seedlings and four months for mature plants), according to the experiment, plants were removed from the medium, wrapped in paper towels and placed on ice. Plant harvests were carried out under light, four hours into the light period (L+4 h) for all experiments, except for the light/dark regulation studies. For these light/dark studies, plant harvests were carried out every 3 h, over 24 h. Night harvests were carried out under a green safe light, emitting outside of the green pool of spectrum (490 nm to 560 nm) (Figure 2.3).

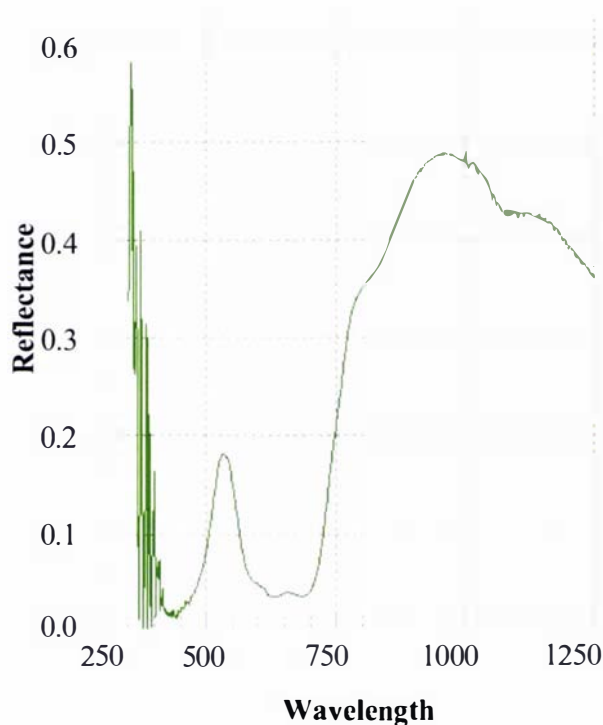


Figure 2.3. Emission spectrum of the green light used during dark harvest and chloroplast extractions in the dark.

Measured by M. Tuohy, Institute of Natural Resources, Massey University.

2.2. Physiological methods

2.2.1. Fresh weight measurement

For fresh weight determination, tissues were divided as shown in figure 2.2, blot dried and then weighed. Only the developing leaf (Figure 2.2, arrowed) was used immediately for chloroplast extractions (section 2.3.2.1). All other tissues were then wrapped in aluminium paper, frozen in liquid nitrogen, and stored at -80°C until use.

2.3. Biochemical methods

2.3.1. Chemicals

The chemicals used were analytical grade, obtained from either BDH Laboratory Supplies (Poole, Dorset, England), or Sigma-Aldrich Chemical Company (Saint Louis, Mo., USA), unless otherwise stated. The dH_2O was produced by reverse-osmosis (RO), followed by a three-step purification process (Milli-Q, Millipore Corporation, Bedford, MA, USA).

2.3.2. Extraction of proteins

2.3.2.1. Isolation of intact chloroplasts

2.3.2.1.1. Extraction under light

Percoll gradients were poured 24 h in advance of use and prepared by loading the heavy solution gently under the light solution in 1.5 mL microcentrifuge tubes (see table 2.2 for formulations), and then stored at 4°C until used.

To isolate chloroplasts, fresh leaf material was immersed in cold isolation medium (2 mM Na₂EDTA, pH 8.0, 1 mM MgCl₂, 2 mM Tricine-KOH, pH 7.9, 330 mM sorbitol) at a ratio of 1:5 respectively. Leaves were ground with a dispersing tool (T18N) attached to the IKA[®] Ultra-Turrax[®] T25 homogeniser (IKA[®] Werke GmbH & Co. KG, Staufen, Germany) for ~10 sec. The homogenate was then filtered through one layer of commercial nappy liner, previously boiled in dH₂O and further rinsed in cold dH₂O. The filtrate was centrifuged at 960 × g for 3 min at 4°C. The supernatant was removed and discarded and the pellet gently resuspended with 0.2 mL of isolation medium. This was loaded gently onto the top of the light solution of the Percoll gradient, and centrifuged at 2,150 × g for 10 min at 4°C. After centrifugation, intact chloroplasts sedimented to the light:heavy solution boundary and so the top (light solution) was removed. A further 0.5 mL of isolation medium was added to the Percoll solution and the layers were carefully mixed. After centrifugation at 2,650 × g for 10 min at 4°C, the diluted Percoll was removed to just about the intact chloroplast layer. A further 0.5 mL of isolation medium was added and the layers were mixed gently before centrifugation at 2,650 × g for 10 min at 4°C. The supernatant was discarded and the now pelleted chloroplasts were resuspended in 500 µL of isolation medium and stored at -20°C until further use. The supernatant was examined under a light microscope to verify the purity and intactness of the chloroplast extracts (Figure 2.4).

Table 2.2. Composition of the heavy and light solutions for the Percoll gradient.

Reagents	Heavy solution	Light solution
Percoll (v/v)	80%	40%
Na ₂ EDTA	0.04 mM	0.04 mM
MgCl ₂	0.02 mM	0.02 mM
Tricine-KOH, pH 7.9	2 mM	2 mM
Sorbitol	49.5 mM	49.5 mM

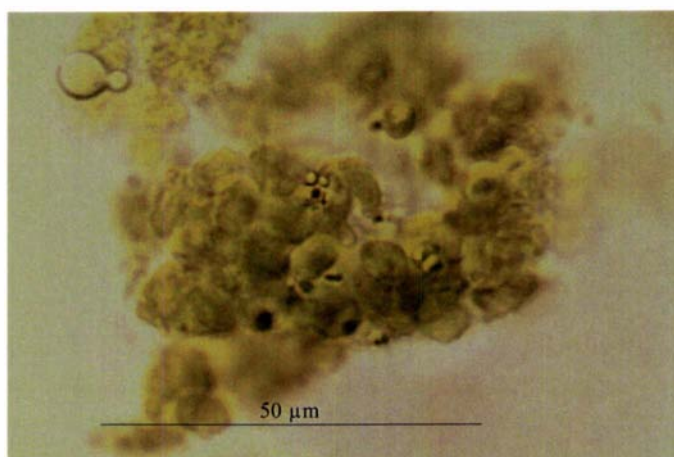


Figure 2.4. Intact chloroplast extracts visualized under light microscope.

2.3.2.1.2. Extraction in the dark

During extractions in the dark period, chloroplasts were isolated under green light. The wavelength of the light emitted was measured to confirm that it fell outside of the green pool of the spectrum (490 nm to 560 nm) (Figure 2.3).

2.3.2.1.3. Chloroplast lysis

Before use, chloroplasts were lysed by homogenisation in a mortar and pestle in three volumes of dH₂O containing 1 × complete protease inhibitor cocktail tablet (EDTA-free; Roche Applied Science, Mannheim, Germany). The homogenate was then centrifuged at 15,800 × g for 20 min at 4°C and the supernatant was immediately used for further analysis.

2.3.2.2. Preparation of tissue extracts

Whole leaf, root or bulb tissues were ground with mortar and pestle in liquid nitrogen to a fine powder and then immersed in three volumes of onion extraction buffer (50 mM potassium phosphate buffer, pH 7.5 containing 1 mM Na₂EDTA (pH 8.0), 1 mM DTT, 0.5 mM PMSF, 50 μM L-pyridoxal 5'-phosphate, and 1 × complete protease inhibitor cocktail tablet (EDTA-free; Roche). The extracts were vortexed for 1 min and cleared by centrifugation at 20,800 × g for 15 min at 4°C and used immediately.

2.3.2.3. Concentration of proteins by acetone precipitation

In some cases, proteins were precipitated using acetone prior to electrophoresis to concentrate them, and remove interfering substances for isoelectric focusing (IEF). To do this, protein extracts (section 2.3.2.1. and 2.3.2.2) were precipitated with at least 10 volumes of 100% (v/v) ice cold acetone, vortexed thoroughly and incubated overnight at -20°C . After the incubation period, the precipitated proteins were vortexed, centrifuged at $15,800 \times g$ for 10 min at 4°C and the acetone carefully removed. After air-drying for 5 min to remove excess acetone, the protein pellet was resuspended in 20 μL IEF buffer [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (w/v) IPG buffer (GE Healthcare, Bio-Sciences AB, Uppsala, Sweden), 40 mM DTT] using a pipette, followed by gently vortexing and centrifugation at $240 \times g$ for 1 min at 4°C . Proteins were stored at -20°C prior to IEF (section 2.3.7.2).

2.3.3. Protein purification by chromatography

2.3.3.1. Gel filtration chromatography using Sephadex G-25

Sephadex G-25 was used for desalting and removing thiols from protein extracts. This method is modified from that described by Neal and Florini (1973). Sephadex G-25 resin (Amersham, BioSciences AB, Uppsala, Sweden) was pre-swollen in dH_2O overnight at 4°C , poured into a syringe barrel plugged with two layers of glass fibre discs (Whatman International Ltd., Maidstone, England), allowed to settle and then pre-equilibrated with 50 mM Tris-HCl, pH 7.5. Chloroplast extracts (section 2.3.2.1) were chromatographed through the column and the eluted proteins collected as 0.5 mL fractions. The protein in each fraction was quantified (section 2.3.4) and fractions of interest pooled and used immediately for further purification.

2.3.3.2. Anion exchange chromatography connected to the Fast Protein Liquid Chromatography (FPLC) system

Ion exchange chromatography is based on the reversible interaction between a charged protein and an oppositely charged chromatographic medium. In the case of anion chromatography, the functional groups in the matrix carry negative charges. These interactions depend not only on the net charge of the protein but also on the ionic strength and pH of the buffer ions, the nature of these ions, and properties of the functional ligands. After pre-equilibration of the HiTrap-Q or Mono-Q (Amersham) column in 50 mM Tris-HCl, pH 7.5, proteins from chloroplast extracts previously

chromatographed through Sephadex G-25 (section 2.3.3.1; Amersham) were loaded onto the column. Proteins were eluted with an increasing linear gradient of 0 to 1 M of NaCl in 50 mM Tris-HCl, pH 7.5, over 50 min and a flow rate of 0.25 mL min⁻¹ and collected as 0.5 mL fractions using a FPLC system with the LCC-501 plus controller, two P-500 pumps and the Frac-100 fraction collector (Amersham Pharmacia Biotech, Uppsala, Sweden).

2.3.3.3. Ion metal affinity chromatography (IMAC) using Ni²⁺-nitrilotriacetic acid-agarose connected to FPLC system

Cells of *E. coli* (section 2.4.3) were harvested by centrifugation at 3,000 × *g* for 10 min at 4°C, resuspended in buffer A [50 mM sodium phosphate buffer, pH 8.0, containing 0.5 M NaCl and 20 mM imidazole (USB Corporation, Cleveland, USA)], and disrupted using the Virsonic 550 sonicator (VirTis, Gardiner, NY, USA) for 6 cycles of 30 sec of sonication and 30 sec of cooling. The sonicate was clarified by centrifugation at 3,000 × *g* for 15 min at 4°C and the supernatant applied to a Ni²⁺-nitrilotriacetic acid-agarose column (HisTrap, GE Healthcare) pre-equilibrated and washed in buffer A. Bound proteins were eluted with a linear gradient of 100% buffer A:0% buffer B (50 mM sodium phosphate buffer, pH 8.0, containing 0.5 M NaCl and 500 mM imidazole) to 0% buffer A:100% buffer B over 80 min at 0.5 mL min⁻¹, and 0.5 mL fractions were collected using a FPLC system with the LCC-501 plus controller, P-500 pumps and Frac-100 fraction collector (Amersham Pharmacia Biotech). The concentration of protein was then measured in each fraction as described in section 2.3.4. Fractions with the fusion proteins were then diluted with 150 µL of glycerol per 500 µL of collected proteins to limit protein denaturation and stabilize their tertiary structure and then stored at -20°C until used.

2.3.4. Quantification of proteins

The protein concentration of an extract was determined according to Bradford (1976). The absorbance of a 10 µL of sample, diluted in 70 µL of dH₂O and mixed with 20 µL of protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) was measured at 595 nm, after 5 min of incubation, using an Anthos Labtech HT2 plate reader (Anthos Labtech Instruments). The concentration of proteins in the sample was determined in comparison with a bovine albumin serum (BSA) protein standard curve (Zor and Selinger, 1996), as shown in figure 2.5.

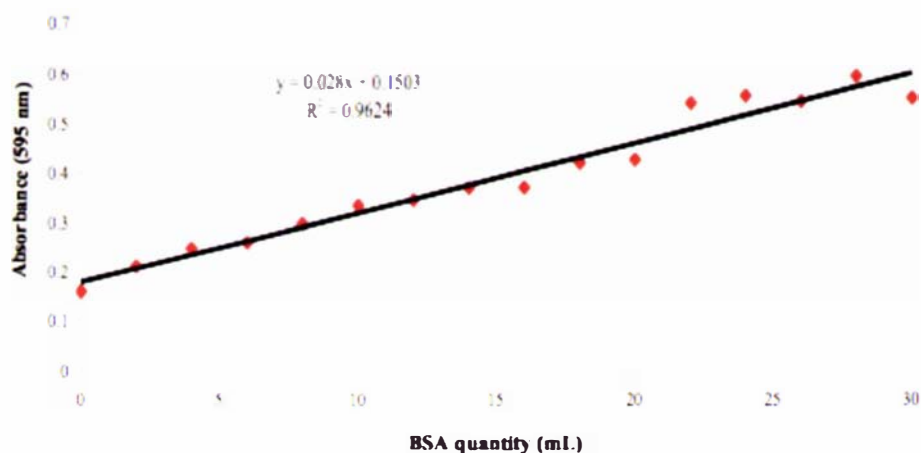


Figure 2.5. BSA standard curve.

BSA was dissolved in dH₂O to a final concentration of 0.1 $\mu\text{g } \mu\text{L}^{-1}$. A standard curve was then determined by measuring the absorbance of an increasing amount of BSA, as shown in a final volume of 80 μL .

2.3.5. ATPS analysis

2.3.5.1. Measurement of ATPS activity

ATPS activity was measured as the P_{Pi}-dependent conversion of APS to ATP using the method of Burnell (1984), as described by Lunn *et al.* (1990). ATPS activity was measured spectrophotometrically by coupling this reverse reaction catalyzed by ATPS, with hexokinase and glucose-6-phosphate dehydrogenase and measuring the APS- or P_{Pi}-dependent reduction of NADP as described in figure 2.6. Hexokinase catalyses the formation of glucose-6-phosphate from glucose, using the ATP produced by the reverse reaction of ATPS, which is dependent on the presence of APS and Mg²⁺. Then glucose-6-phosphate dehydrogenase catalyses the conversion of glucose-6-phosphate to 6-phosphoglucono- δ -lactone. This last reaction requires NAD⁺, which is reduced to NADH. The reaction was quantified by an increased accumulation of NADH, which is measured at an absorbance at 340 nm (Abs₃₄₀). The enzyme assay was initiated by adding sodium pyrophosphate (Na₄P₂O₇).

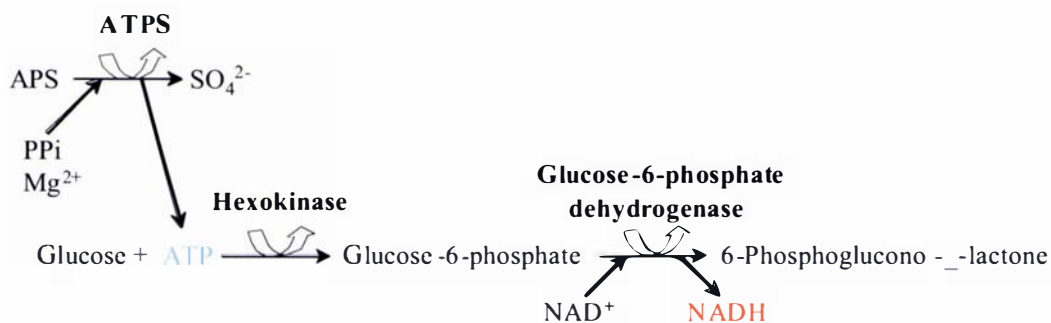


Figure 2.6. The coupled reaction for the measurement of the reverse reaction of ATPS.

ATPS activity was measured as the pyrophosphatase-dependent conversion of APS to ATP. Formation of ATP (in blue) is then utilized for the conversion of glucose to glucose-6-phosphate, which is subsequently converted into 6-phosphoglucono- δ -lactone. The enzymes necessary for reactions are shown in bold. NADH production is measured spectrophotometrically at an absorbance of 340 nm (in red).

ATPS activity was measured *in vitro* in a final concentration of 50 mM Tris-HCl, pH 8.0, 5 mM MgCl_2 , 5 mM glucose, 0.3 mM NAD^+ , 0.1 mM APS with 5 U of hexokinase (Roche), 5 U of glucose-6-phosphate dehydrogenase (Roche) in a microtiter plate (Greiner bio-one GmbH, Kremsmüster, Austria). The reaction was initiated by the addition of 1 mM $\text{Na}_4\text{P}_2\text{O}_7$. The activity measured at an absorbance of 340 nm every 5 min over 30 min using the Anthos Labtech HT2 plate reader (Anthos Labtech Instruments, Salzburg, Austria). Over this period, the reaction was performed at room temperature and in the absence of light.

The coupling enzymes hexokinase and glucose-6-phosphate dehydrogenase supplied as suspensions in ammonium sulfate were first pelleted by centrifuging at $20,800 \times g$ for 5 min at room temperature and dissolved in 1 M Tris-HCl, pH 8.0 before use.

2.3.5.2. ATPS activity determination

The reaction rate was expressed as $\mu\text{mol min}^{-1} \text{mg}^{-1}$, which was determined by the formula in figure 2.7. The ATPS activity was obtained by calculating the change in absorbance between point 0 and point 30 min per min per 0.2 mL reaction. This absorbance was multiplied by 5 to adjust the 200 μL microplate volume to 1 mL of reaction, and divided by 6.22, the millimolar extinction coefficient for NADH at 340 nm, to obtain the activity per μmol per minute per mL. The activity was multiplied by 10 to adjust the 100 μL of extracts used in the 1 mL of reaction volume to 1 mL of

extracts, and finally, the activity was divided by the protein concentration of the extract (mg mL^{-1}) to express the activity as per mg of protein.

$$\text{Formula for ATPS activity } (\mu\text{mol min}^{-1} \text{ mg}^{-1}) \\ = \frac{[(\text{Abs}_{340} \text{ 30 min} - \text{Abs}_{340} \text{ 0 min}) / 30 - 5] / 6.22 - 10}{\text{protein concentration } (\text{mg mL}^{-1})}$$

Figure 2.7. Formula to calculate the specific activity of ATPS.

2.3.6. Separation of proteins by one-dimensional polyacrylamide gel electrophoresis (1D-PAGE)

2.3.6.1. Sample preparation

Protein samples were heated for 10 min in boiling H_2O in an equal volume of SDS reducing buffer [100 mM Tris-HCl, pH 6.8, containing 20% (v/v) glycerol, 5% Ultrapure™ SDS (Invitrogen Corp., Carlsbad, CA, USA), 0.01% (w/v) bromophenol blue, 5% (v/v) 2-mercaptoethanol] and then centrifuged at $20,800 \times g$ for 2 min before loading on to the gel. A total of 10 μg and 35 μg protein were loaded per lane of one dimensional polyacrylamide gel electrophoresis (1D-PAGE) and gradient-PAGE, respectively. One lane was reserved for the pre-stained protein ladder (Bio-Rad or Fermentas International Inc., Burlington, Ontario, Canada).

2.3.6.2. Linear Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were separated according to their molecular mass by 1D-PAGE as described by Laemmli (1970). The resolving gels were prepared as 12% total monomer (T) and 2.6% cross-linker (C), pH 8.8. The stacking gels were prepared as 4% T and 2.6% C, pH 6.8. The gels were assembled using the Mini-Protean III® cell (Bio-Rad) apparatus. The resolving and stacking gel buffer concentrations were 0.375 M Tris-HCl, pH 8.8 and 0.125 M Tris-HCl, pH 6.8 respectively, and were prepared as shown in table 2.3. The resolving gel was allowed to polymerize for 1 h, and the stacking gel for 15 min, both at room temperature.

The running buffer concentration was 0.025 M Tris-HCl, 0.192 M glycine, pH 8.3. All gel and running buffers contained SDS to a final concentration of 0.1% (w/v).

Protein samples were prepared as described in section 2.3.6.1 and 10 μ g total protein was loaded per lane. Electrophoresis was conducted at 150 V at room temperature until the dye reached the bottom of the gel (usually 75 min).

Table 2.3. Composition of 12% Resolving and 4% Stacking gels for 1D-PAGE.

Reagents	12% Resolving gel (mL)	4% Stacking gel (mL)
dH ₂ O	2.25	1
4 × resolving buffer (0.75 M Tris-HCl, pH 8.8, 0.2% (w/v) Ultrapure™ SDS (Invitrogen))	1.25	-
2 × stacking buffer (0.25 M Tris-HCl, pH 6.8, 0.2% (w/v) Ultrapure™ SDS (Invitrogen))	-	1.25
40% (v/v) acrylamide stock solution (Bio-Rad)	1.5	0.25
10% (w/v) ammonium persulfate	0.05	0.025
TEMED	0.005	0.0025

2.3.6.3. Gradient-PAGE

Gradient-PAGE separates proteins according to their mass, but giving the highest resolution possible within a single dimension. A linear gradient-PAGE was produced by the gradual addition of a 8% light solution into a 15% heavy solution to provide a continuous gradient of 8-15% using a Hoefer™ SG 100 gradient-maker (GE Healthcare), which was emptied into the glass plate assembly through the 2120 Varioperpex® pump (LKB Bromma) (Table 2.4).

Gradient makers consist of two containers as shown in figure 2.8, joined by a narrow connector at their bases with one container (A) having also an additional outlet in its base. As liquid is drained from (A) it is replaced from (B) due to the equilibration of the hydrodynamic pressure which keeps the levels in (A) and (B) equal. (A) is constantly stirred, which causes the solution draining from (A) to be progressively diluted with (B) until, when the gradient maker is emptied, the out-flowing material is essentially 100% B. For the production of gradient gels used in this thesis, chamber (A) comprised the 15% heavy solution, while chamber (B) comprised the 8% light solution. Once poured, the resolving gel was set aside for polymerization overnight at room temperature, while the stacking gel was allowed to polymerize for 1 h at room temperature.

The running buffer concentration was 0.025 M Tris, 0.192 M glycine, pH 8.3. All gel and running buffers contained SDS to a final concentration of 0.1% (w/v).

Protein samples were prepared as described in section 3.3.6.1, and 35 μ g total protein was loaded per lane. Electrophoresis was conducted at 150 V gel at room temperature until the dye reached the bottom of the gel (usually 6 h).

Table 2.4. Composition of resolving and stacking gels for gradient-PAGE (8-15%).

Reagents	Resolving gel		
	Heavy solution, 15% (mL)	Light solution, 8% (mL)	Stacking gel
Sucrose	3 g	-	-
dH ₂ O	6	11	4
4 × resolving buffer	5	5	-
2 × stacking buffer	-	-	5
40% (v/v) acrylamide stock solution	7.5	4	1
10% (w/v) ammonium persulfate	0.1	0.1	0.1
TEMED	0.003	0.003	0.001

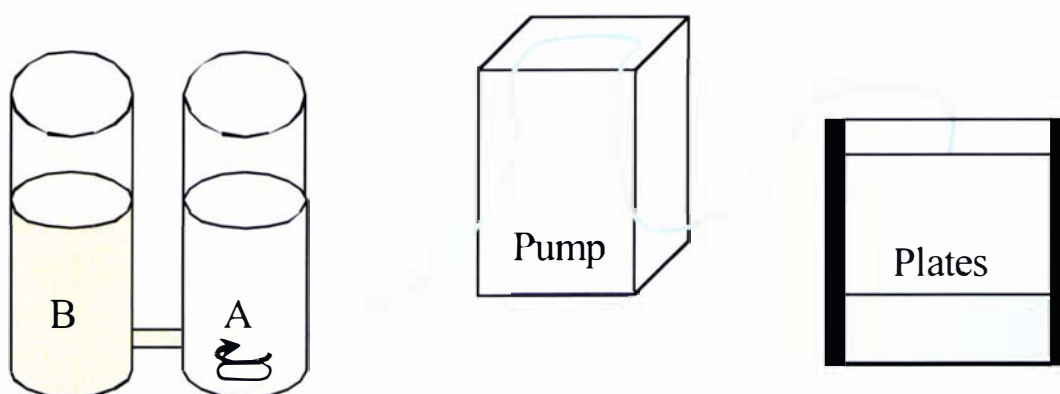


Figure 2.8. Diagrammatic representation of the pouring system of a linear gradient SDS-PAGE.

As the apparatus emptied, the composition of the out-flowing solution became progressively closer to the content of B.

2.3.7. Separation of proteins by two-dimensional electrophoresis (2D-PAGE)

2.3.7.1. Sample preparation

2.3.7.1.1. *Chloroplast extracts*

Chloroplast extracts (section 2.3.2.1) were thawed on ice and protein concentrations were determined by Bradford (section 2.3.4). An appropriate volume of sample for the detection method chosen (60 μg total protein for silver nitrate staining and 40 μg total protein for western analyses) was then diluted in IEF rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer (GE Healthcare), 0.002% (w/v) bromophenol blue] to a total volume of 125 μL , vortexed, and centrifuged at $425 \times g$ for 2 min at room temperature.

2.3.7.1.2. *Leaf extracts*

Frozen leaves were ground to a fine powder in liquid nitrogen and immediately suspended in 10 volumes of 10% (w/v) TCA in acetone with 0.5% (w/v) DTT added. The suspension was vortexed, and incubated overnight at -20°C . Following the incubation period, the suspension was centrifuged at $4,500 \times g$ for 15 min at room temperature and the pellet was then resuspended in 10 volumes of 80% (v/v) ice-cold acetone. The pellet was washed three more times in 10 volumes of 80% (v/v) ice-cold acetone to obtain of a firm pellet. Then, the pellet was allowed to air-dry for 5 min before that proteins were solubilized in 1.25 mL of solubilization buffer per g of fresh leaf [6 M urea, 4 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer (GE Healthcare), 0.2% (w/v) DTT]. Insoluble material was sedimented at $15,300 \times g$ for 15 min at 4°C , the supernatant was decanted and the protein concentration in the supernatant was determined (section 2.3.4). The volume of each sample (60 μg total protein for silver nitrate staining and 40 μg total protein for western analyses) was adjusted to 125 μL by the addition of rehydration buffer [7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 0.5% (v/v) IPG buffer, 0.002% (w/v) bromophenol blue]. The mixture was vortexed, and then centrifuged at $425 \times g$ for 2 min at room temperature before use in IEF (section 2.3.7.2).

2.3.7.2. Isoelectric focusing (IEF)

During the first dimension, proteins are separated on the basis of their pI, the pH at which a protein carries no net charge and will not migrate any further in an electrical field (O'Farrell, 1975; Klose, 1975, Görg *et al.*, 1988). IEF was performed using a precast immobilized pH gradient (IPG) polyacrylamide gel strip due to the difficulty of the IPG strips casting (Righetti, 1983) and to achieve high resolution and reproducible results. Dry polyacrylamide gel strips of 7 cm-length with an immobilized pH gradient of 4-7 or 6-11 were used depending of the enzyme to be analysed (Immobiline DryStrip, GE Healthcare). Once the rehydration buffer (125 μ L) containing the sample to be focused was loaded onto the ceramic strip holder, the IPG strip was gently positioned on top of it, avoiding trapping air bubbles. Each strip was covered with mineral oil to prevent evaporation, and allowed to passively rehydrate to their original thickness (0.5 mm) for 14 h at 20°C. The IEF protocol was conducted at 20°C using the Ettan IPGphor II (Amersham) with the running conditions described in table 2.5. At the end of the run, the strips were placed in a 15 mL capacity conical tube and stored at -20°C until processing for the second dimension by SDS-PAGE.

Table 2.5. Running conditions for protein focusing using IPG strips.

Step & voltage mode	Voltage (V)	Duration (h:min)
1: Step & Hold	500	0:30
2: Step & Hold	1000	0:30
3: Step & Hold	5000	2:00
Total	-	3:00

2.3.7.3. Equilibration

Prior to the second dimension run, IPG strips were conditioned for SDS-PAGE in a two step equilibration to reduce disulfide bonds and alkylate the resultant sulfhydryl groups of the cysteine residues. Concurrently, proteins are coated with SDS for separation on the basis of their mass. During the first step, strips were incubated with 1% (w/v) DTT in equilibration buffer [50 mM Tris-HCl, pH 8.8, containing 6M urea, 30% (v/v) glycerol, 2% (w/v) Ultrapure™ SDS (Invitrogen) and 0.002% (w/v) bromophenol blue] for 15 min on a rocker. The second equilibration step was carried out in a similar way, except that 2.5% (w/v) iodoacetamide was added in place of the DTT.

2.3.7.4. Second dimension by SDS-PAGE

In the second dimension, the proteins resolved in IPG strips were applied to a 12% SDS-PAGE (Laemmli, 1970) and separated by molecular mass. The gels were prepared in a similar way as described in section 2.3.6.2, but without stacking gel. After a brief rinse with SDS-PAGE running buffer (section 2.3.6.2), the strips were carefully loaded on top of the gels and overlaid with molten sealing agarose solution [0.5% (w/v) agarose and 0.002% (w/v) bromophenol blue prepared in SDS-PAGE buffer] to secure their position. Electrophoresis was conducted in a Mini-Protean III[®] cell (Bio-Rad) at 100 V at room temperature until the tracking dye reached the bottom of the gel (usually 2 h).

2.3.8. Detection of proteins by western analysis

2.3.8.1. Transfer of proteins from polyacrylamide gel to PVDF membranes

Proteins separated by 1D-, 2D- or gradient-PAGE were transferred to PVDF membranes (PolyScreen PVDF transfer membranes, PerkinElmer Life Sciences Inc., Boston, MA, USA) as described by Towbin *et al.* (1979) using a Trans-Blot[®] Electrophoretic Transfer Cell (Bio-Rad). First, the membrane was prepared for transfer by soaking in MeOH and then in transfer buffer (25 mM Tris, 190 mM glycine, pH 8.3, containing 10% (v/v) MeOH), chilled to 4°C. After electrophoresis, the gel was rinsed in transfer buffer, then, the membrane was carefully applied on the gel. Any air bubbles trapped between gel and membrane was removed by gently rolling a glass tube over the membrane. The transfer cassette holder with gel and membrane was protected between a layer of filter paper (3MM Chromatography paper, Whatman) and fibre pad and then assembled as shown in figure 2.9. Proteins were transferred in cold transfer buffer at 100 V for 1 h with constant stirring and the inclusion of a Bio-Ice[™] cooling unit (Bio-Rad).

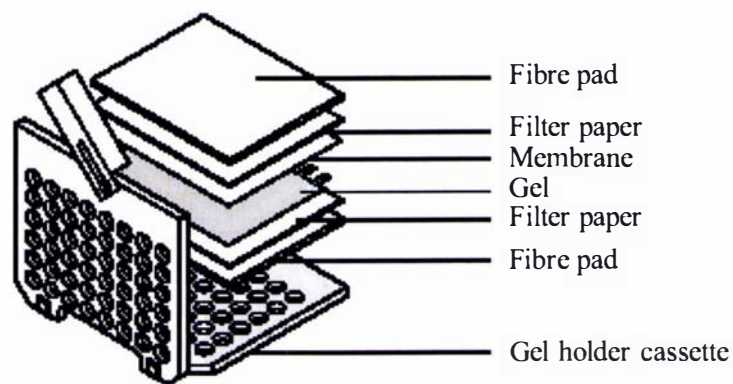


Figure 2.9. Gel and membrane assembly for the electrophoretic transfer¹.

2.3.8.2. Probing detection using antibodies on PVDF membranes

All steps were carried out with constant shaking at a speed of 50 rpm. Following electrophoresis transfer, the PVDF membranes were blocked for 1 h at ambient temperature, or overnight at 4°C in blocking solution [12% (w/v) skim milk powder (Pams, The Foodstuffs Co-operative Society Ltd, Wellington, New Zealand)] in phosphate buffered saline (PBS) (50 mM sodium phosphate, pH 7.4, containing 250 mM NaCl). The membranes were washed five times in 50 mL of PBST (PBS with 0.05% (v/v) Tween 20) for 5 min and incubated with the primary antibody diluted as described in table 2.6 in PBST for 1 h at 37°C. The membranes were washed five times in 50 mL of PBST for 5 min and incubated with the secondary antibody diluted as described in table 2.6 in PBS for 2 h at room temperature.

Primary polyclonal antibodies for the detection of the enzymes involved in the S assimilation pathway were raised separately in New Zealand white rabbits against purified recombinant proteins obtained by expressing cDNA in *E. coli* (S. Leung, personal communication). For the ATPS (AF212154, isolated by S. Leung), APSR (AAF18999, isolated by S. Leung) and OAS-TL (*OAS-TL1* isolated by M. Pither-Joyce, Crop & Food Research, Lincoln, New Zealand) antibodies, cDNAs were isolated from onion, while for SiR (CAA89154, gifted by S. Kopriva, John Innes Centre, Norwich, UK), the cDNA was from *Arabidopsis* (S. Leung, personal communication). The secondary antibody was an anti-rabbit antibody, raised in goat and conjugated either with alkaline phosphatase for chromogenic detection or with horseradish peroxidase for chemiluminescence (Promega Corp., Madison, WI, USA).

¹ Adapted from Mini Trans-Blot[®] electrophoretic transfer cell, Instruction manual (Bio-Rad Laboratories, Hercules, CA, USA).

For the detection of 14-3-3 proteins, an anti-14-3-3 antibody from spinach leaves (Moorhead *et al.*, 1999) raised in sheep was used as the primary antibody (gifted from Professor C. MacKintosh, Medical Research Council Protein Phosphorylation Unit, University of Dundee, UK). The secondary antibody was an anti-sheep developed in monkey, and conjugated with horseradish peroxidase.

Table 2.6. Concentrations of primary and secondary antibodies used for western analyses.

Detected enzyme	Chromogenic method		Chemiluminescent method	
	Primary antibody	Secondary antibody	Primary antibody	Secondary antibody
ATPS	1:1000	1:10,000	1:10,000	1:13,000
APSR	1:1000	1:10,000	1:10,000	1:13,000
SiR	1:1000	1:10,000	1:10,000	1:13,000
OAS-TL	1:1000	1:10,000	1:10,000	1:20,000
14-3-3	-	-	1:15,000	1:20,000

2.3.8.3. Immunodetection of proteins immobilised on PVDF membrane

2.3.8.3.1. Chromogenic detection

After the secondary antibody incubation, membranes were washed five times in 50 mL of PBST for 5 min and then briefly rinsed twice in substrate buffer (100 mM Tris-HCl, pH 9.6, containing 100 mM NaCl) followed by incubation in the dark with fresh developing buffer [100 mM Tris-HCl, pH 9.6, containing 100 mM NaCl, 0.01% (w/v) BCIP (Roche), 0.02% (w/v) NBT, 1% (v/v) DMSO and 8 mM MgCl₂] to visualise the protein band of interest. When sufficient colour had occurred, the reaction was stopped immediately by rinsing with RO H₂O and the membranes allowed to air-dry.

2.3.8.3.2. Chemiluminescence

After the secondary antibody incubation, membranes were washed five times in 50 mL of PBST for 5 min and then membranes were briefly rinsed twice in blot rinse [10 mM Tris-HCl, pH 7.4, containing 150 mM NaCl, 1 mM EDTA and 0.1% (v/v) Tween-20]. Equal volumes of both solutions in the commercial SuperSignal[®] West Pico Chemiluminescence Substrate (Pierce Biotechnology Inc., Rockford, IL, USA) kit were mixed together, applied onto membranes and incubated for 5 min at room temperature. The membranes were blot dried and wrapped between two layers of transparent plastic

film for protection and placed into a FujiFilm BAB cassette (Fujifilm USA Inc., Valhalla, NY, USA). One X-ray film (Kodak BioMax XAR Film, Kodak's Graphic Communications Grp, Rochester, NY, USA) was placed on top of the membranes and the cassette closed for exposure. Finally, the film was developed using the Automatic X-Ray Film processor 100 Plus (All Pro Imaging, Hicksville, New York, USA) for results.

2.3.9. Detection of proteins by gel staining

2.3.9.1. Coomassie Brilliant blue (CBB) staining

In order to control the protein loading and the quality of protein extracts, proteins separated by 1D-PAGE (section 2.3.6) proteins were detected by CBB staining in parallel to western blot analysis. To do this, gels were immersed in CBB staining solution [0.1% (w/v) Coomassie Brilliant blue, 40% (v/v) MeOH, 10% (v/v) acetic acid] with gentle shaking for 30 min at room temperature. The removal of excess stain was achieved by incubating the gel in CBB destaining solution [30% (v/v) EtOH] until a protein *versus* background ratio appropriate for visualisation was achieved. Images were acquired using a Gel-Doc XR system (Bio-Rad).

2.3.9.2. Pro-Q Diamond phosphoprotein staining (Pro-Q DPS)

For phosphoprotein analysis, 1D- or 2D-PAGE gels (sections 2.3.6 and 2.3.7, respectively) were stained using the fluorescent Pro-Q DPS (Molecular Probes Inc., Eugene, OR, USA) according to the method described in Agrawal and Thelen (2005). To minimize signal quenching, all steps after the fixing step were carried out with constant shaking at a speed of 50 rpm, at room temperature and in the dark.

The 1D- and 2D-PAGE gels were fixed overnight in 50 mL of 50% (v/v) MeOH and 10% (v/v) acetic acid. The following day, the gels were washed twice in 50 mL of dH₂O for 15 min and stained in 65 mL of three-fold diluted Pro-Q DPS in dH₂O for 2 h. Then, excess staining was removed by incubating the gels in 50 mL of 50 mM sodium acetate, pH 4.0, containing 20% (v/v) acetonitrile, for 30 min. This destaining step was repeated three more times. The gels were finally rinsed twice in 50 mL of dH₂O for 5 min.

Following the Pro-Q DPS staining procedure, the image was captured using a FLA 5000 laser scanner (Fujifilm) with 532 nm excitation. Data were collected as 200 μm , 8 bit TIFF files using the Image Gauge Analysis software (Fujifilm).

2.3.9.3. Silver staining

Following Pro-Q DPS (section 2.3.9.2) or 2D-PAGE (section 2.3.7), total proteins were stained by silver staining according to the method of Blum *et al.* (1987). All steps were carried out with constant shaking at a speed of 30 rpm, at room temperature, and in the light. Gels were first fixed overnight in 50% (v/v) MeOH and 10% (v/v) acetic acid. However, if Pro-Q DPS stained gels were used, this step was not required since gels had already been fixed and stained for the detection of phosphoproteins (section 2.3.9.2).

All gels were then washed in 30% (v/v) EtOH, twice, for 30 min each time and then once in dH₂O for 20 min. The gels were then sensitized in 0.02% (w/v) sodium thiosulfate reagent for 1 min, rinsed briefly three times in dH₂O and soaked in 0.3% (w/v) silver nitrate and 0.0148% (v/v) formaldehyde for 20 min. After three rinses in dH₂O, gels were developed with 0.3% (w/v) sodium carbonate, 0.037% (v/v) formaldehyde and 0.0005% (w/v) sodium thiosulfate for 3 to 5 min. The staining was stopped by soaking the gel for 5 min in 0.5% (w/v) glycine followed with further rinses with dH₂O. Images were acquired using a Gel-Doc XR system (Bio-Rad).

2.3.10. Phosphorylation and dephosphorylation treatments

2.3.10.1. Extraction

Chloroplasts and whole leaf extracts were extracted as described in section 2.3.2.1 and section 2.3.2.2 respectively.

2.3.10.2. Incubation to preserve or alter the phosphorylation state

After lysis of chloroplasts (section 2.3.2.1.3), MgATP (0.2 mM ATP, 5 mM MgCl₂, mixed fresh) as the phosphoryl donor (Wang and Roach, 1992) was routinely included in some protein extracts to maintain or possibly enhance phosphorylation (designated as P⁺). The Mg²⁺ ion is required for the activity of the protein (Ser/threonine) kinases (van der Geer *et al.*, 1993). As well, the activity of endogenous phosphatases was also limited by adding phosphatase inhibitors to the protein extracts as these enzymes can dephosphorylate residues. Since several classes of phosphatases (see chapter 1, section

1.4.3) are present in cell extracts, 5 μM OKA (Roche) or 50 mM NaF with 1 mM EDTA and 1 mM EGTA were used to provide a broad spectrum of inhibition.

In parallel, some extracts were incubated with purified PP2A from human red blood cells (Upstate, Lake Placid, NY, USA) or recombinant alkaline phosphatase (AAP) from an Antarctic bacterium (New England BioLabs Inc, Ipswich, MA, USA) to supplement the activity of endogenous plant protein phosphatases and reduce the phosphorylation of proteins in the extracts (designated as P⁻). One U of PP2A was used per 1000 μg of protein or 1 U of AAP per 4 μg of protein. Since Mg²⁺ enhanced ATPS activity, 5 mM MgCl₂ was also added to the P⁻ treatment. This ion is also required for the activity of the phosphatase PP2B (Cohen, 1989).

Once prepared (see Table 2.7 for full treatment preparation), the P⁺ and P⁻ reactions were vortexed and incubated for 15 min at 30°C. Considering previous studies (Alexander and Morris, 2006; González-Camacho and Medina, 2004; Röhrig *et al.*, 2006; Samaniego *et al.*, 2006), the incubations were extended to 30 min when using AAP. All incubations were terminated by incubation for 10 min on ice.

To control the efficiency of the treatments, recombinant albumin from chicken egg white was incubated instead of chloroplast extracts with either the P⁺ treatment, using OKA as the phosphatase inhibitor or the P⁻ treatment, using either PP2A or AAP as the phosphatase. One lane was reserved for the Peppermint Stick phosphoprotein ladder (Molecular Probes).

Table 2.7. Full composition of phosphorylation and dephosphorylation treatments.

	P ⁺	P ⁻
Chloroplast extracts	1000 μg	1000 μg
Tris-HCl, pH 7.5	25 mM	-
MgCl ₂	5 mM	5 mM
ATP	0.2 mM	-
OKA or	5 μM or	-

NaF	50 mM	-
EDTA	1 mM	-
EGTA	1 mM	-
PP2A or	-	1 U
AAP	-	250 U

2.3.10.3. Affinity chromatography of treated chloroplast extracts through BMH1- and BMH2-Sepharose

2.3.10.3.1. Coupling proteins to activated CNBr Sepharose

CNBr-Sepharose (Amersham) was extensively swollen, first with ice-cold 1 mM HCl and then coupling buffer (0.1 M NaHCO₃, pH 8.0 containing 0.5 M NaCl). The recombinant BMH1 and BMH2 14-3-3 isoforms from *Saccharomyces cerevisiae* were produced in transformed *E. coli* (section 2.4.1.2) and purified by IMAC (section 2.3.3.3). Then, the recombinant proteins were reacted (separately) overnight, with end-over-end mixing at 4°C with the CNBr-Sepharose slurry at a ratio of 2 mg protein to 1 ml CNBr-Sepharose. The following day, beads were collected by centrifugation at 800 × g for 1 min at 4°C, washed with cold coupling buffer to remove unbound ligands and incubated with 0.1 M Tris-HCl, pH 8.0 with end-over-end mixing for 1 h at room temperature. The Sepharose was extensively washed with five cycles of alternating low pH buffer (0.05 M sodium acetate, pH 4.0 containing 0.5 M NaCl) and high pH buffer (0.05 M Tris-HCl, pH 8.0 containing 0.5 M NaCl). The proteins coupled to CNBr-Sepharose were then combined and designated BMH1/BMH2-Sepharose and transferred to tubes and stored in 50 mM Tris-HCl, pH 8.0 with 20% (v/v) EtOH at 4°C. Parallel preparations using bovine serum albumin (BSA)-Sepharose were prepared using the same protocol.

2.3.10.3.2. Affinity chromatography of chloroplasts

Ten volumes of chloroplast extracts, either treated to favour phosphorylation or dephosphorylation of proteins (section 2.3.10.2), or untreated, were incubated overnight with end-over-end mixing at 4°C with one volume of BMH1/BMH2-Sepharose equilibrated in 50 mM Tris-HCl, pH 7.5. The beads were collected by centrifugation at 800 × g for 1 min at 4°C, and then washed four times in 15 column volumes of 50 mM Tris-HCl, pH 7.5 containing 0.5 M NaCl to remove unspecific binding. Proteins attached to the BMH1/BMH2-Sepharose were eluted by boiling the beads for 10 min in

SDS-PAGE sample buffer (section 2.3.6.1). The beads were pelleted by centrifugation at $20,800 \times g$ for 2 min at room temperature, and the supernatant was analysed by 1D-PAGE (section 2.3.6) and western analyses (section 2.3.8).

2.4. Molecular methods

2.4.1. Bacterial culture and recombinant protein production

2.4.1.1. Preparation of Luria-Bertani medium with ampicillin

To make LB^{Amp100} plates, bacteriological agar was added to Luria-Bertani (LB) (Bertani, 1951) medium [1% (w/v) bacto-tryptone, 0.5% (w/v) bacto-yeast extract, 1% NaCl (w/v), pH 7.5] at a final concentration of 1.5% (w/v) and the suspension autoclaved at 103 kPa for 20 min. The medium was allowed to cool-down to 40°C before adding the ampicillin (Amp) to a final concentration of 100 µg mL⁻¹ and then poured into Petri dishes under a laminar flow bench. When solidified, the plates were sealed with Parafilm (Pechiney Plastic Packaging Company, Neenah, WI, USA) and stored until required at 4°C. In the case of liquid culture, no agar was added and the medium was poured into flasks before autoclaving, as described in this section.

2.4.1.2. Transformation of *E. coli* competent cells

2.4.1.2.1. Preparation of competent cells

Cells of the *E. coli* strain DH-5α (Gibco BRL, Gaithersburg, MD, USA) were inoculated from glycerol stocks into 10 mL of liquid LB medium (section 2.4.1.1) and incubated with shaking at 180 rpm overnight at 37°C. The following day, 400 µl of medium was transferred to a fresh 40 mL of LB and incubated with shaking at 180 rpm at 37°C for 2 to 2.5 h or until the optical density at 600 nm reached 0.4. Then, the cell culture was transferred to a pre-cooled (4°C) centrifuge tube, and centrifuged at 4,000 × g for 5 min at 4°C. After discarding the supernatant, the pellet was gently resuspended in 10 mL of ice-cold 60 mM CaCl₂, kept for 30 min on ice, and centrifuged at 4,000 × g for 3 min at 4°C. Again, the supernatant was discarded and the pellet gently resuspended in 4 mL of 60 mM CaCl₂ containing 15% (w/v) glycerol. The competent cells were then stored as 300 µl aliquots at -80°C.

2.4.1.2.2. Transformation of *E.coli/DH5α* vector using the heat-shock method

An aliquot of competent cells (100 µL) (section 2.4.1.2.1) was thawed on ice for 5 min, and ca. 50 ng of plasmid (pTrcHisA, Invitrogen; Appendix I) DNA containing the gene coding for either BMH1 or BMH2 cloned from *S. cerevisiae* added (gifted from

Professor C. MacKintosh), the tube contents gently mixed, and incubated for 20 min on ice. After this time, the mixture was incubated for 45 sec at 42°C to heat-shock the *E. coli* cells, followed by incubation for 5 min on ice after which 500 µl of LB (section 2.4.1.1) was added. The cells were incubated with shaking at 180 rpm for 1 h at 37°C, and then, the putatively transformed cells were spread onto LB^{Amp100} plates (section 2.4.1.1) and incubated overnight at 37°C.

2.4.1.2.3. Preparation of glycerol stock

A single colony of transformed cells was inoculated into 10 mL of LB^{Amp100} (section 2.4.1.1) and grown with shaking at 180 rpm overnight at 37°C. The following day, an 850 µl aliquot of the bacterial cells was diluted with 150 µl of cold sterile glycerol and pipetted in a cryotube. After quick freezing in liquid nitrogen, the glycerol stock was stored at -80°C.

2.4.2. Sequencing of cloned DNA in *E. coli*

2.4.2.1. Purification of plasmid DNA for DNA sequencing

A 10 mL LB^{Amp100} broth, prepared as described in section 2.4.1.1, was inoculated with an aliquot (typically 5 µL) of transformed *E. coli* cells from a glycerol stock (section 2.4.1.2.3) and grown with shaking at 180 rpm overnight at 37°C. The following day, cells were harvested by centrifugation at $1,700 \times g$ for 10 min at room temperature and resuspended in cold sterile buffer A (25 mM Tris-HCl, pH 8.0 containing 50 mM glucose and 10 mM Na₂EDTA). Then, 300 µL of freshly prepared buffer B [0.2 M NaOH, 1% (w/v) Ultrapure™ SDS (Invitrogen)] was added and cells were incubated for 5 min on ice.

After the incubation, 300 µL of cold sterile buffer C (3 M potassium acetate, pH 4.8, 2 M acetic acid) was incorporated, the tube shaken vigorously, and cells were again incubated for 5 min on ice. The lysed cells were subsequently centrifuged at $20,800 \times g$ for 10 min at room temperature and the supernatant transferred to a fresh tube and incubated for 20 min at 37°C after the addition of 2.5 µL of 10 mg mL⁻¹ RNase A dissolved in 10 mM Tris-HCl, pH 8.5 (Roche).

After the incubation, 400 µL (v/v) of chloroform:iso-amyl alcohol (at a ratio of 24:1 respectively) was added, shaken and centrifuged at $20,800 \times g$ for 1 min at room temperature. Then, the top aqueous layer, containing the DNA, was transferred to a

fresh tube and another 400 μL (v/v) of chloroform:iso-amyl alcohol added to the aqueous, mixed and centrifuged at $20,800 \times g$ for 1 min at room temperature. The DNA was precipitated by adding 400 μL (v/v) of isopropanol and centrifuged at $20,800 \times g$ for 10 min at room temperature.

After discarding the supernatant, the DNA pellet was washed with 500 μL of 70 % (v/v) EtOH and centrifuged at $20,800 \times g$ for 2 min at room temperature. Again, the supernatant was discarded and the DNA pellet was vacuum dried for 5 min. The DNA pellet was subsequently resuspended with 32 μL of sterile dH_2O , and 8 μL of 4 M sterile NaCl was added to the solution. A further 40 μL of 13% (v/v) sterile PEG₈₀₀₀ was added, the mixture shaken and then incubated for 20 min on ice and subsequently centrifuged at $20,800 \times g$ for 20 min at 4°C .

Once the supernatant was discarded, the DNA pellet was washed with 500 μL of 70% EtOH and centrifuged at $20,800 \times g$ for 2 min at room temperature. The supernatant was again discarded and the DNA pellet air-dried for 5 min. Finally, the DNA was resuspended in 25 μL of sterile 10 mM Tris-HCl, pH 8.0, in preparation for sequencing.

2.4.2.2. Automated sequencing of DNA

DNA sequencing was carried out by the Allan Wilson Centre Genome Service, Palmerston North, New Zealand using the ABI PRISM™ 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). Primers shown in table 2.8 (Invitrogen) were designed from the pTrcHisA vector map (Appendix I) to amplify the relevant inserted DNA fragments.

Table 2.8. Sequences and melting points of the primers used for DNA sequencing.

Primers	Position in plasmid (in bp)	Sequence
Forward primer	367 to 393	AAAGAGGTATATATTAATGTATCGATT
Reverse primer	688 to 705	TTTCACTTCTGAGTTCGG

2.4.2.3. DNA sequence analysis

The DNA sequences (section 2.4.2.2) of BMH1 and BMH2 were aligned using the Sequencher™ version 4.7 programme (Genes Codes Corp., Ann Arbor, MI, USA).

They were then compared to the DNA sequences of BMH1 and BMH2 from *S. cerevisiae* by BLAST (www.ncbi.nlm.nih.gov/).

The sequences of the DNA fragments inserted in the pTrcHisA plasmid were translated to protein sequences using ExPaSy tools (<http://expasy.org/tools/dna.html>) and the molecular mass of these proteins was estimated using the same programme (http://expasy.org/tools/pi_tool.html).

2.4.3. Preparation of recombinant proteins

Under a laminar flow hood, an aliquot (typically 5 μ L) of transformed *E. coli* cells from a glycerol stock was spread over a LB^{Amp¹⁰⁰} plate (section 2.4.11), and incubated overnight at 37°C. The following day, a single colony was used to inoculate a 100 mL liquid LB^{Amp¹⁰⁰} broth and placed with shaking at 180 rpm overnight at 37°C. The following day, 20 mL of the bacterial culture was pipetted into two 500 mL flasks of LB^{Amp¹⁰⁰} and grown with shaking at 180 rpm at 37°C. Five hours later, the synthesis of recombinant proteins was induced with the addition of IPTG to a final concentration of 0.6 mM IPTG and the flasks were placed, with shaking at 180 rpm, overnight at 20°C. Finally, proteins were purified as described in section 2.3.3.3.

2.5. Statistical analyses

Statistical analyses were performed with MiniTab[®] 15 Statistical Software. Analysis of variance (ANOVA) and Student *t*-tests were used to assess statistical differences between samples in sections 3.1.1, 3.2.1, 3.3.1 and 4.1.7. A difference between means at 5% ($p \leq 0.05$) was considered significant, and is indicated in the results presented in chapters 3 and 4.

3. CHARACTERISATION OF THE S ASSIMILATION ENZYMES AND THEIR RESPONSE TO S SUPPLY

3.1. Influence of S starvation on onion growth and development

3.1.1. Changes in fresh weight of plants and tissues in response to S supply

To determine the influence of the S supply on onion growth and development and assess if any observed change had a genetic basis, differences in total biomass and tissue weights of freshly harvested seedlings and mature plants between the mild line, TG, and the pungent line, W, were measured. All plants were grown from seeds that were germinated and rooted in vermiculite for three weeks. The vermiculite was watered with either the S-sufficient (S^+) or the S-deficient (S^-) media. Then, the young plants were transferred to hydroponic S^+ or S^- media. In seedlings, 12 repetitions of 10 plants each were measured while in mature plants, four repetitions of 10 plants each were measured.

In seedlings, S withdrawal reduced plant biomass for both genotypes (Figure 3.1). In seedlings of TG only, the variations in total biomass between plants grown in the S^+ and the S^- media were significantly different with $p= 0.001$. In TG, the variations of leaf and root weights were also significantly different ($p < 0.001$ and $p= 0.036$, respectively). In W, total biomass and leaf weight were only slightly reduced with S withdrawal, while root fresh weight was not reduced at all.

For seedlings grown in either the S^+ or the S^- media, those of TG were heavier than W, but the difference in total biomass between the two genotypes was statistically significant only for those grown in the S^+ media ($p= 0.002$) (Figures 3.1 and 3.2). Leaf and root weights were also greater in seedlings of TG. Again, the difference in leaf and root weights between TG and W was statistically significant only for those grown in the S^+ media, with p values of 0.002 and 0.007, respectively.

In mature plants of TG, total biomass and leaf, root and bulb weights were reduced in those grown in the S^- media (Figure 3.2). The variations in total biomass, root and bulb fresh weights between the S^+ and the S^- media were significant at $p= 0.004$, $p= 0.001$ and $p= 0.016$, respectively. In mature plants of W, the S^- media induced minor increases

of total biomass and leaf, root and bulb weights, but these were not statistically significant. In mature plants grown in the S⁺ media only, total biomass and root and bulb weights were higher in TG than in W. These differences were not statistically significant. In mature plants grown in the S⁻ media, total biomass and leaf, root and bulb weights were greater in W, but again these differences were not statistically different.

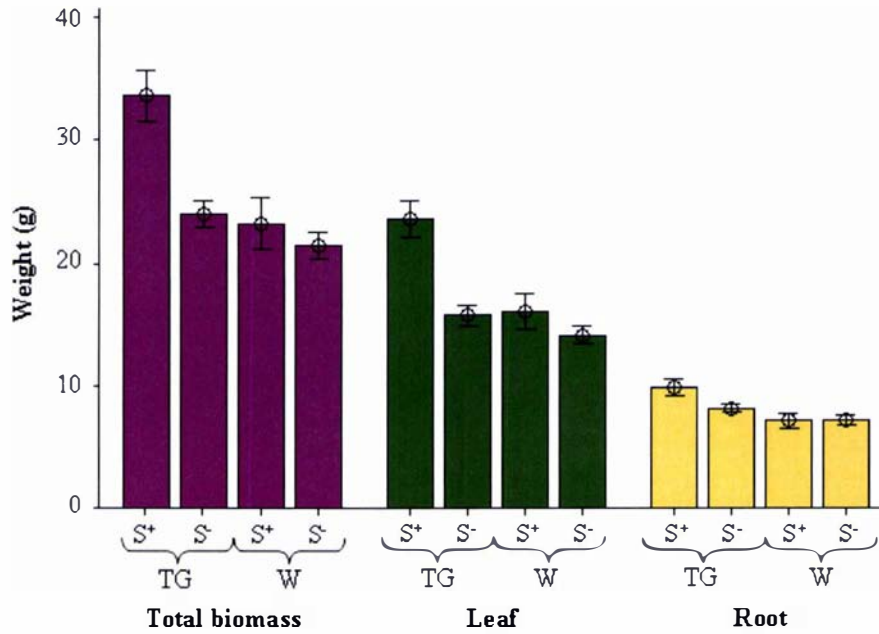


Figure 3.1. Influence of the S supply on the total biomass and tissue weights, as indicated, in seedlings (7-week old) of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Each bar represents the average of 12 repetitions; each consisting of 10 plants. Interval bars represent the standard error.

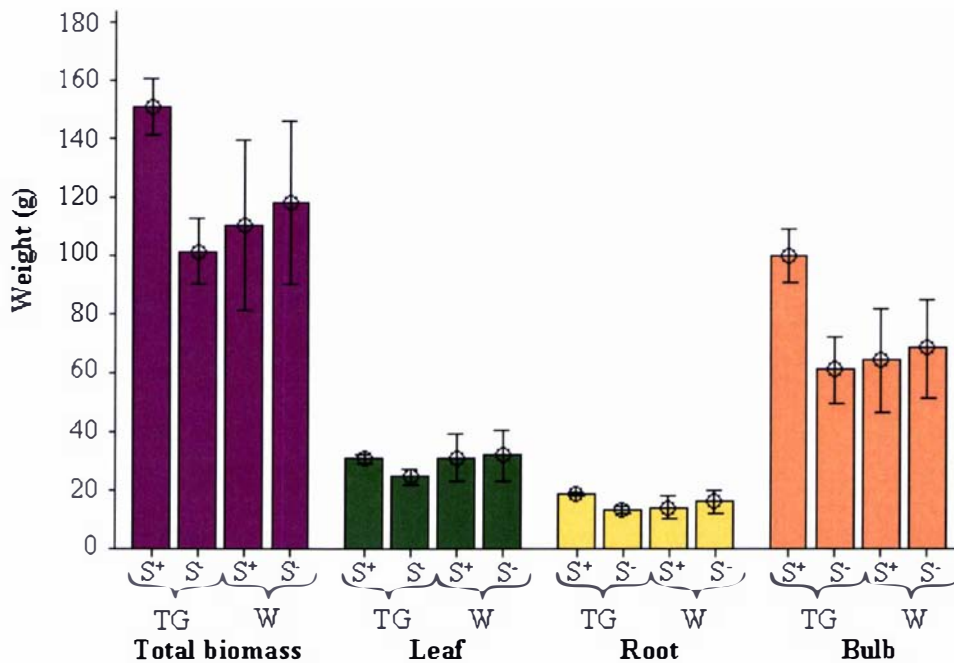


Figure 3.2. Influence of the S supply on the total biomass and tissue weights, as indicated, in mature plants (4-month old) of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Each bar represents the average of four repetitions; each consisting of 10 plants. Interval bars represent the standard error.

3.1.2. Morphological analysis

To examine further the influence of S depletion on plant growth and development as depicted by the changes in fresh weights of plants and tissues in section 3.1.1, the developmental differences between seedlings and mature plants of TG and W were observed, using the same plant material as referred to in section 3.1.1.

After seven weeks in hydroponic culture, seedlings of TG and W were normally developed with two fully expanded leaves and a third one emerging when grown in both levels of S supply (Figure 3.3). The root system was composed of several main roots with little evidence of the development of lateral roots. No obvious symptom of S starvation was observed in either seedlings of TG or W grown in the S^- media. However, differences were observed consistently between the two genotypes, with leaves of TG being more elongated and the presence of a larger root mass in the TG genotype. Further, growth progression was more rapid in seedlings of TG as indicated by a more elongated third leaf (Figure 3.3, arrows).

After four months of culture (the mature growth stage) in the S^+ media, four fully expanded leaves were developed with a fifth one emerging in both genotypes (Figure 3.3). In both genotypes, the first two leaves were senescing and roots were more elongated and denser than in the seedlings. In W, small secondary roots had developed which were not observed to the same extent in TG (Figure 3.4). A large bulb was already present. In the S^- media, plant growth was retarded, particularly the bulb formation and the growing point of leaves were pale-green in both genotypes. In all plants observed, roots were more elongated with the development of more lateral roots suggesting that root growth is also altered with S withdrawal. In the S^- media, some secondary roots developed in TG but these were more elongated in W (Figure 3.4). In terms of bulb size, bulbs were bigger in plants grown in the S^+ , and the difference was more obvious in TG.

Finally, differences between TG and W were also visualized. Leaves were longer, and darker in TG but more vigorous in W. Bulbs were of bigger size in TG than in W.

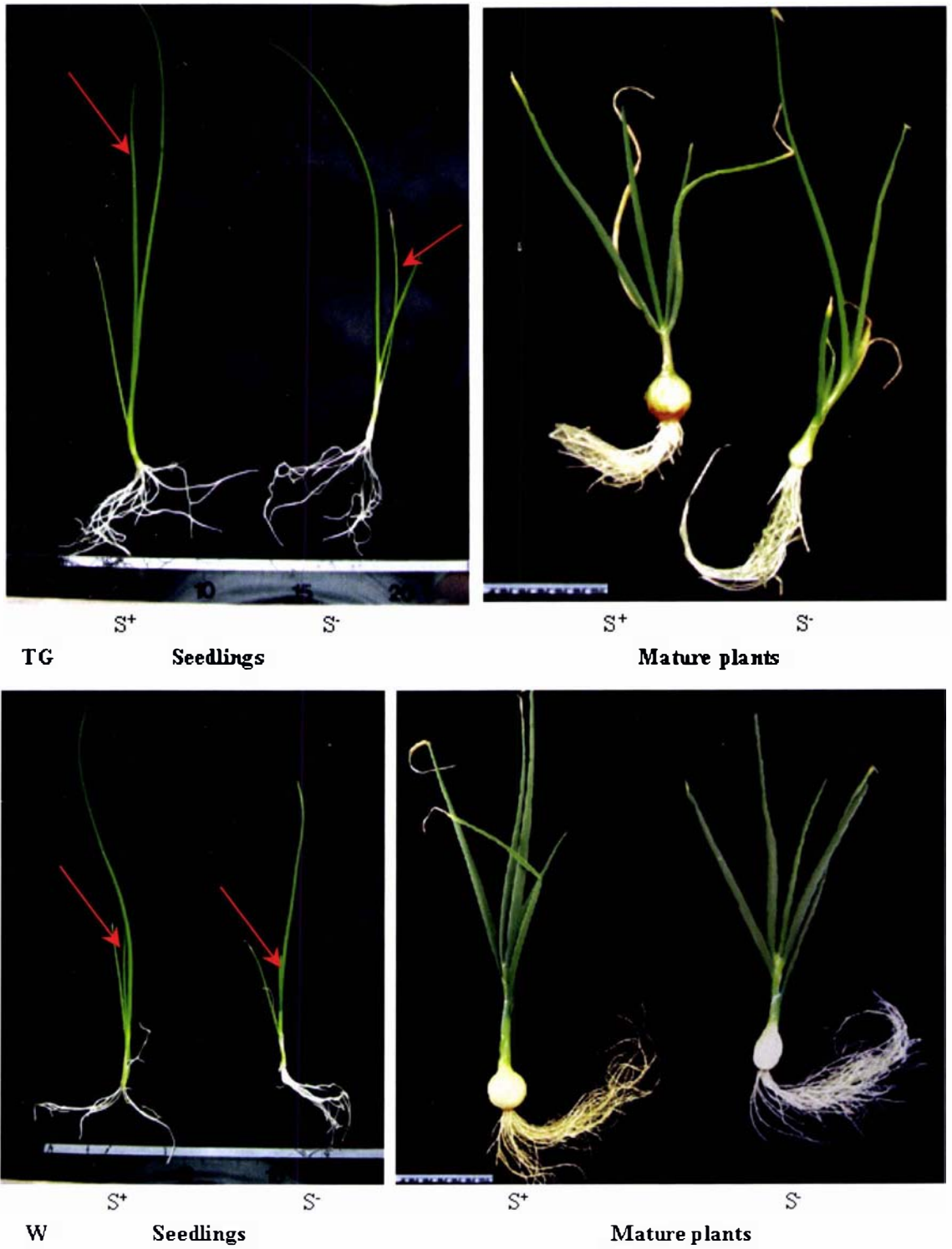


Figure 3.3. Influence of the S supply on the plant morphology of seedlings and mature plants, as indicated, of TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Arrows show the third emerging leaf.

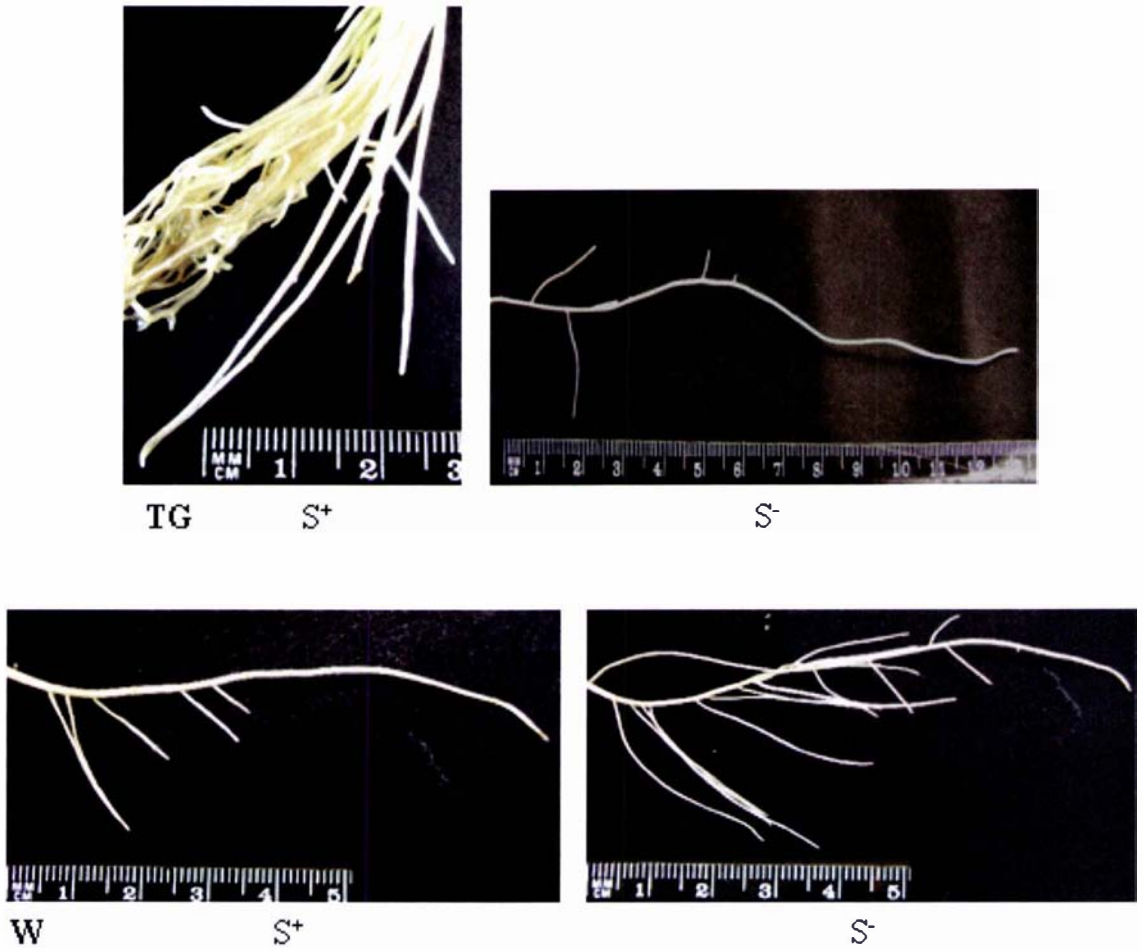


Figure 3.4. Influence of the S supply on the root morphology of mature plants of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

3.2. Regulation of the S assimilation enzymes in response to S supply

3.2.1. Variations in ATPS activity

To determine the regulation of ATPS activity by S supply, enzyme activity was measured in different tissues from seedlings and mature plants of both TG and W, using the same plant material as referred to in section 3.1. Although 10 plants were grouped to form one repetition and four repetitions were performed for each tissue and treatment, large variations in ATPS activity were measured between repetitions, as shown by the interval bars in figures 3.5 and 3.6. The variations of ATPS activity between repetitions were more pronounced in root extracts than in the other extracts and more particularly in mature plants.

In terms of S supply, in seedlings of TG, ATPS activity was slightly up regulated with S deprivation in chloroplast and leaf extracts (Figure 3.5). However, the variations in ATPS activity between plants grown in the S⁺ and the S⁻ media were not statistically significant. In roots, no difference in ATPS activity was measured. In seedlings of W, ATPS activity increased with S limitation in chloroplast and root extracts. However, these differences in ATPS activity were not statistically significant. In leaf extracts, the ATPS activity decreased with the S⁻ media, and this difference in activity was statistically significant (p= 0.048).

In terms of genotype differences in seedlings, ATPS activity was similar in chloroplast extracts from both TG and W grown in either the S⁺ or the S⁻ media (Figure 3.5). In leaf extracts from plants grown in the S⁺ media, the activity was significantly higher in W (p= 0.044) when compared to TG, but was slightly higher in TG in plants grown in the S⁻ media. In root extracts, the activity was similar between genotypes for plants grown in the S⁺ media, but was higher in plants of W grown in the S⁻ media, but this difference was not statistically significant.

In terms of S supply, in mature plants of TG, ATPS activity was up regulated with S deprivation in root and bulb extracts, but these differences were not statistically significant (Figure 3.6). However, no significant difference in enzyme activity was measured in chloroplast or leaf extracts. In mature plants of W grown in the S⁻ media, ATPS activity increased in leaf and root extracts, but these differences were not

statistically significant. In chloroplasts, ATPS activity decreased with S deprivation, but this difference was not statistically significant. In bulb extracts, the activity did not fluctuate with the S supply. In both genotypes, the up regulation in ATPS activity measured in roots was marked but the variations between repetitions were high also, as shown by the interval bars (Figure 3.6). However, in root extracts, all differences in ATPS activity measured between the S⁺ and the S⁻ media were not statistically significant in any tissues from both genotypes (TG, p=0.133; W, p=0.54).

In terms of genotype differences, in mature plants grown in the S⁺ media, ATPS activity was similar in TG and W in leaf and bulb extracts. ATPS activity was higher in W in chloroplast extracts and was higher in TG in root extracts, but these differences were not statistically significant (Figure 3.6). In mature plants grown in the S⁻ media, ATPS activity was higher in TG in all extracts, but not statistically different, with the exception in leaf extracts where it was higher in W (p= 0.071).

In terms of tissue differences in seedlings of TG and W grown in either the S⁺ or the S⁻ media, highest ATPS activity was recorded in root extracts. The differences in ATPS activity between the tissues were statistically significant in TG grown in the S⁺ and S⁻ media and in W grown in the S⁺ media, with p values of 0.028, 0.033 and 0.012, respectively. In mature plants of TG and W grown in the S⁺ media, highest activity was measured in leaf and chloroplast extracts, while in plants grown in the S⁻ media, maximum activity was in root extracts. In TG plants grown in the S⁻ media only, the differences in ATPS activity between the tissues were statistically significant (p= 0.045).

In terms of developmental differences in chloroplast extracts from TG and W, ATPS activity decreased with plant maturation for plants grown in the S⁺ or the S⁻ media, with the exception of plants of W grown in the S⁺ media where it stayed constant. In chloroplast extracts from TG only grown in the S⁻ media, the differences in ATPS activity induced with plant maturation were statistically significant (p= 0.031). In leaf extracts from TG, ATPS activity increased with plant maturation for plants grown in the S⁺ media, whereas it decreased for those grown in the S⁻ media. In leaf extracts from W, regulation was opposite with lower activity in mature plants grown in the S⁺ media and higher activity in mature plants grown in the S⁻ media. However, none of

these differences in leaf extracts were statistically significant. In roots extracts from TG and W, ATPS activity was down regulated with plant maturation, with the exception of plants of TG grown in the S^- media where it stayed constant. Only in plants grown in the S^+ media, these decreases were significant (TG, $p= 0.034$; W, $p= 0.019$).

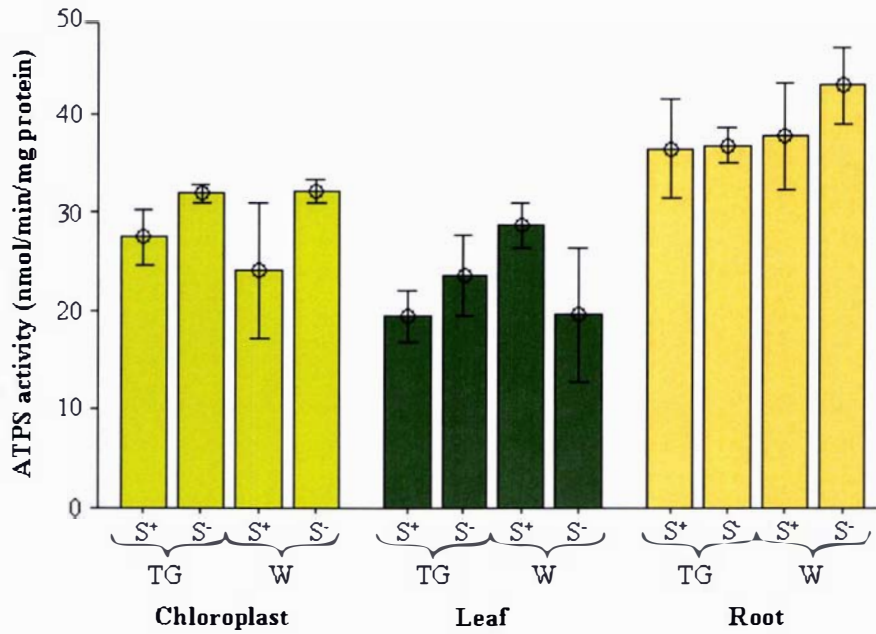


Figure 3.5. Influence of the S supply on the ATPS activity in chloroplasts or the tissues, as indicated, from seedlings of TG and W, as indicated, grown in S-sufficient (S⁺) or S-deficient (S⁻) media, as indicated.

Each bar represents the average of four repetitions; each consisting of 10 plants. Interval bars represent the standard error.

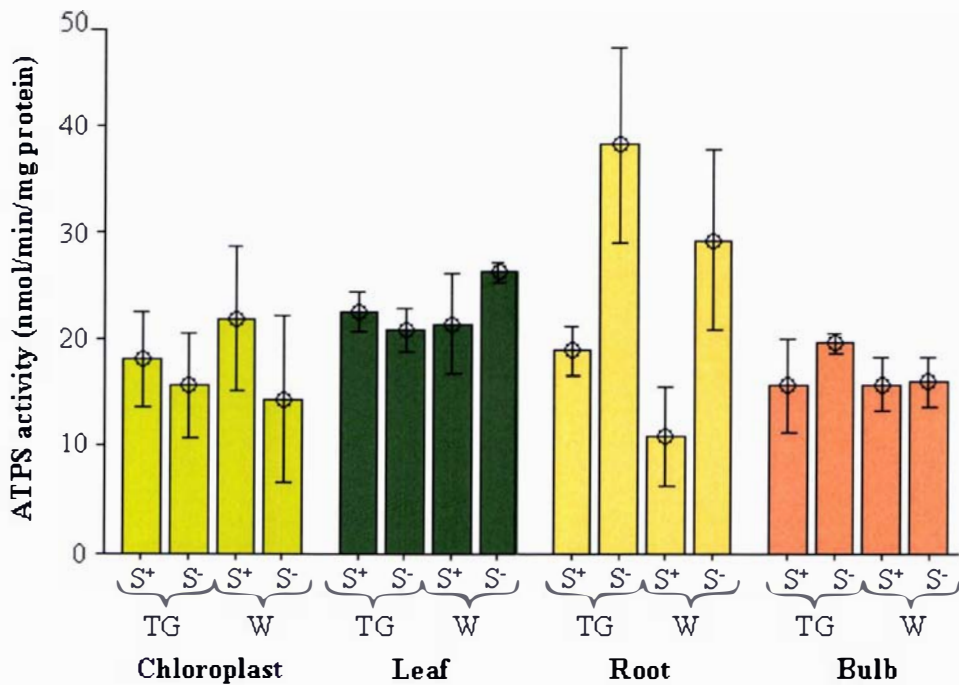


Figure 3.6. Influence of the S supply on the ATPS activity in chloroplasts or the tissues, as indicated, from mature plants of TG and W, as indicated, grown in S-sufficient (S⁺) or S-deficient (S⁻) media, as indicated.

Each bar represents the average of four repetitions; each consisting of 10 plants. Interval bars represent the standard error.

3.2.2. Abundance of the S assimilation enzymes and the influence of S supply

Abundance of S assimilation enzymes was detected using antibodies (see Chapter 2, section 2.3.8.2). Preliminary work in the McManus laboratory determined that ATPS and APSR were detected as ~50 kDa proteins in onion chloroplasts by western analyses after fractionation by 1D-PAGE. The anti-SiR antibody recognized a protein of ~60 kDa and the anti-OAS-TL antibody recognized a protein of ~30 kDa. All four enzymes accumulated in chloroplasts, leaves and roots from both seedlings and mature plants and also in bulbs from mature plants (Figure 3.7 for ATPS and APSR, data not shown for SiR and OAS-TL). In this section, 10 plants were grouped to form one repetition and four repetitions were performed for each tissue and treatment. Data for one representative repetition only are presented.

3.2.2.1. ATPS abundance

To further characterise the regulation of ATPS, the abundance of the enzyme was observed in different tissues extracted from seedlings and mature plants of TG and W grown in either the S⁺ or the S⁻ media, using the same plant material as referred to in section 3.1.

In terms of S depletion, in seedlings of both TG and W, ATPS abundance was increased with the S depletion in chloroplast extracts. In leaf extracts, the enzyme abundance increased with S depletion in TG while it decreased in W. In root extracts from both genotypes, the abundance was reduced with S depletion (Figure 3.7, ATPS). In mature plants of TG, ATPS abundance was reduced with the S⁻ media in chloroplast and leaf extracts (Figure 3.8, ATPS). ATPS abundance increased with S depletion in bulb extracts, but did not change in the roots. In mature plants of W, the abundance of ATPS increased in chloroplast and root extracts while it decreased in both leaf and bulb extracts.

In terms of comparison between genotypes, in mature plants, the abundance of ATPS in all extracts was higher in plants of W than TG, and this difference between genotypes was most marked in bulb extracts.

In terms of comparison between tissues, in seedlings of TG, ATPS abundance was higher in leaf extracts, while in W, it was less intense in chloroplast extracts than in leaf

and root extracts. In mature plants from both genotypes, ATPS abundance was greater in chloroplasts and leaves when compared with root and bulb extracts.

In terms of comparison between the two developmental stages, in chloroplast, leaf and root extracts, the abundance of the enzyme increased with maturation. However, comparisons between seedlings and mature plants were difficult because both the chromogenic and the chemiluminescence detection method were used for the visualization of ATPS.

In some repetitions of chloroplast extracts from seedlings and mature plants of W grown in either the S^+ or the S^- media and from seedlings of TG grown in the S^+ media, ATPS was detected as two closely related bands (Figures 3.7 and 3.8), which were more clearly observed when a shorter exposure was used (data not shown). In some repetitions, two ATPS bands were also visualized in leaf and root extracts in mature plants of TG and W. In the example shown, the exposure time is prolonged. However, the double bands are also visible when a shorter exposure is used (data not shown).

To complement the characterisation of ATPS abundance using 1D-PAGE, chloroplast extracts were also fractionated by 8-15% gradient-PAGE and the enzyme detected by western analysis.

In chloroplast extracts from seedlings of TG, ATPS abundance increased with the S depletion (Figure 3.9, ATPS). The enzyme was characterised by one band in extracts from plants grown in both the S^+ and the S^- media, and a mass shift of ATPS was suggested when the extracts from plants grown in the S^+ and the S^- media are compared. In seedlings of W, ATPS abundance decreased with the S depletion. Two bands were visible in chloroplast extracts from seedlings grown in the S^+ media, while only one band was detected in extracts from plants grown in the S^- media. The molecular mass of the single band detected in the S^- media was equivalent to the higher molecular mass band in the S^+ media.

In mature plants of TG and W, two bands of ATPS were visualized in extracts from plants grown in either the S^+ or the S^- media. In both genotypes, the abundance of the higher molecular mass band increased with the S depletion, while the abundance of the lower molecular mass band decreased with the S^- media. These differences were more

obvious than in figure 3.9 (ATPS), when the X-ray film was exposed to the blot for a shorter period (Appendix I).

In agreement with the previous immunological analyses (Figure 3.8, ATPS), ATPS abundance was greater in chloroplast extracts from W seedlings and in mature plants of both genotypes (Figure 3.9, ATPS). Thus the abundance of ATPS increased with plant maturation in both genotypes.

3.2.2.2. APSR abundance

Western analyses were also carried out to characterise the abundance of APSR in extracts from TG and W seedlings and mature plants, using the same plant material as in section 3.1. In chloroplast and leaf extracts from seedlings of TG and W, no variation in APSR abundance was detected between those grown in the S⁺ and the S⁻ media, using ID-PAGE (Figure 3.7, APSR). In root extracts, APSR abundance increased with the S depletion in both genotypes.

In mature plants of TG and W, APSR abundance slightly decreased with the S depletion in chloroplast and leaf extracts (Figure 3.8, APSR). In bulb extracts, APSR abundance increased with S withdrawal in TG, while it decreased in W. No APSR was detected in root extracts from both genotypes, possibly because of a low affinity of the antibody over the root form.

The abundance of APSR was similar in both genotypes, but it was higher in chloroplast and leaf extracts, when compared with root and bulb extracts. It was hardly detectable in root extracts, possibly because of the low antibody affinity for a distinct APSR isoform that may be present in these tissues, or through a lower abundance of the enzyme.

For APSR, two closely migrating bands were visualized in all extracts from both seedlings and mature plants. In mature plants, the two bands were more clearly visible than in figure 3.8, when the X-ray film was exposed to the blot for a shorter period (data not shown). In all samples, the higher molecular mass band was more strongly recognized than the other band. Both bands accumulated more in chloroplast and leaf extracts than in root and bulb extracts.

To further characterise the regulation of APSR, the abundance of the enzyme was also analysed by western after separation through 8-15% gradient-PAGE (Figure 3.9, APSR). In both seedlings and mature plants of TG and W, the abundance of APSR increased with the S depletion in chloroplasts. The increased abundance of the higher molecular mass band noticed with the S depletion was more prominent than for the lower molecular mass band. This difference was more clearly visible than the example shown in Figure 3.9 when the X-ray film was exposed to the blot for a shorter period (Appendix II). In terms of developmental state, the abundance of the enzyme decreased with maturation in both TG and W and in plants grown in both the S⁺ and the S⁻ media.

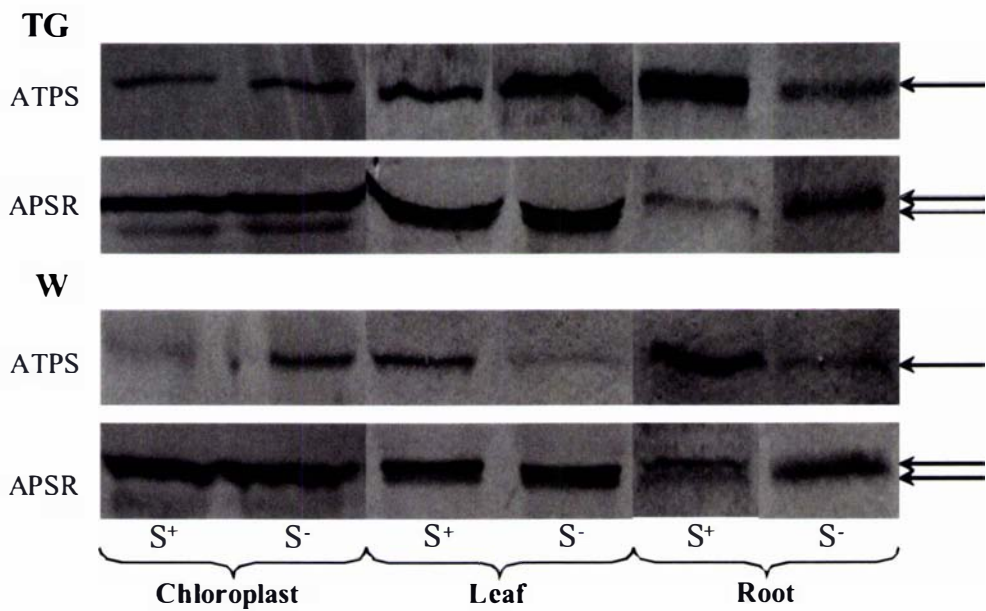


Figure 3.7. Influence of the S supply on the abundance of ATPS and APSR in chloroplasts or the tissues, as indicated, from seedlings of TG and W, as indicated, grown in either the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Protein extracts (10 µg) were separated by 1D-PAGE and the abundance of ATPS or APSR, as indicated, determined by western analyses using chromogenic detection. Arrows show the number of bands detected by the antibody.

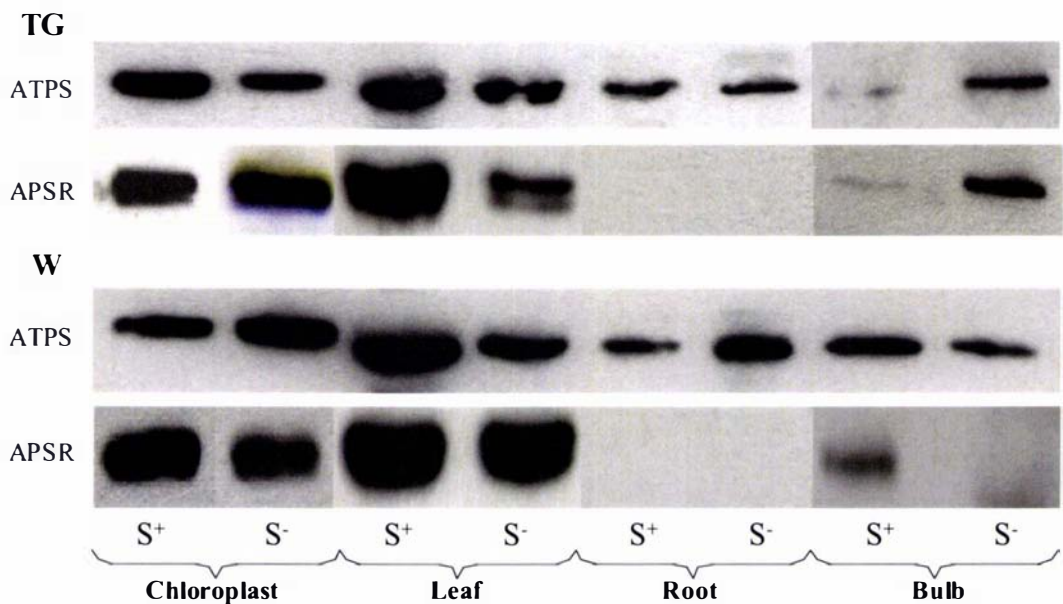


Figure 3.8. Influence of S supply on the abundance of ATPS and APSR in chloroplasts or the tissues, as indicated, from mature plants of TG and W, as indicated, grown in either the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Protein extracts (10 μg) were separated by 1D-PAGE and the abundance of ATPS or APSR, as indicated, determined by western analyses using chemiluminescence.

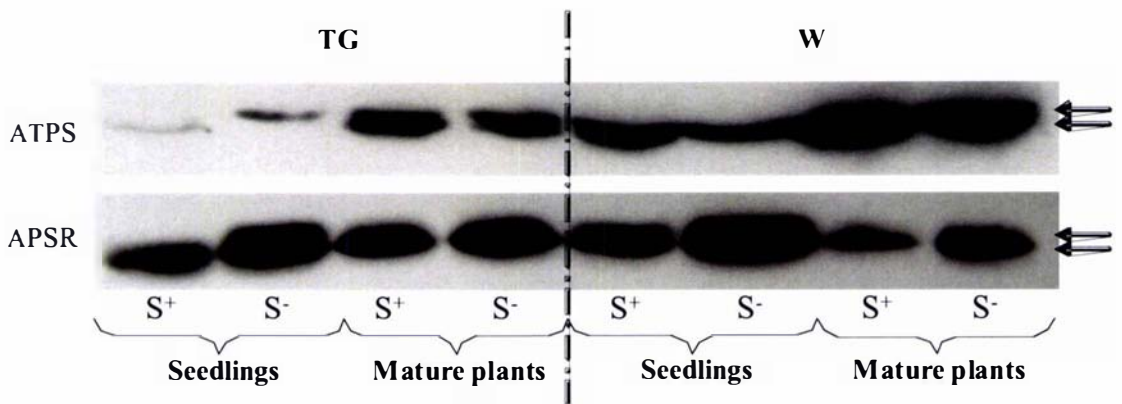


Figure 3.9. Influence of the S supply on the abundance of ATPS and APSR in chloroplasts from seedlings or mature plants, as indicated, of TG and W, as indicated, grown in either the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Protein extracts (35 μg) were separated by 8-15% gradient-PAGE and the abundance of ATPS or APSR, as indicated, determined by western analyses using chemiluminescence. Arrows show the number of bands detected by the antibody.

3.3. Light and dark regulation of the S assimilation enzymes

3.3.1. Changes in ATPS activity over a 24 h period

In order to further characterise the regulation of ATPS, the activity and abundance of the enzyme were measured every 3 h over a 24 h period in chloroplast, leaf, and root extracts from seedlings of TG and W grown in the S-sufficient media. Plant harvesting and chloroplast extraction were carried out under green light during the dark period.

Similar to section 3.2, 10 plants were grouped to form one repetition and four repetitions were performed for each tissue. In both cultivars, large variations in ATPS activity were measured between replicates, but they were less prominent in leaf extracts. So in this section, only the general trends in ATPS fluctuations over the 24 h period are described, although none of these were statistically significant, with the exception in leaf extracts, as indicated.

In chloroplast and leaf extracts from TG, maximum activity was measured at mid-day (L+10 h to L+13 h in chloroplasts and L+10 h in leaves) and during the dark period (D+6 h in chloroplasts and D+3 h in leaves) (Figure 3.10). In leaf extracts only, a 30% increase was measured between the onset of the light period (L+1 h) and L+10 h. In root extracts, the ATPS activity was maximal at L+7 h and minimal at D+3 h. Following this period of higher activity, as the dark period continued, a 33% decrease was observed between D+0 h and D+3 h.

In chloroplast, leaf and root extracts of W, maximum ATPS activity was recorded at L+4 h and at the onset of the night period (D+0 h) (Figure 3.10). In all extracts of W, the enzyme activity was minimal at illumination (L+1 h), mid-day (L+10 h in chloroplasts and roots; L+7 h in leaves) and during the dark period (D+3 h and D+6 h). Only in leaf extracts, the increase in ATPS activity between L+1 h and L+4 h was statistically significant ($p= 0.049$). Interestingly, the increase in ATPS activity began before the night onset in chloroplast, leaf and root extracts, but not before illumination.

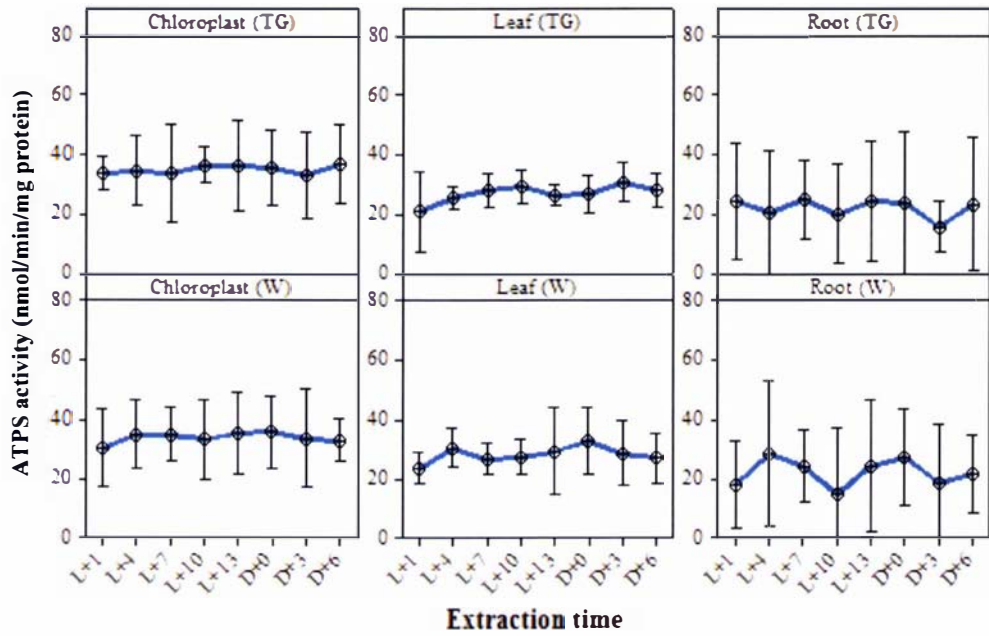


Figure 3.10. Influence of light and dark on the ATPS activity in chloroplasts or the tissues, as indicated, seedlings TG and W, as indicated, grown in the S-sufficient media.

The ATPS activity was measured in extracts over a 24 h period. Mean values of four repetitions are shown. Interval bars represent the standard error.

3.3.2. Changes in the abundance of the S assimilation enzymes

Western analyses were carried out to characterise the abundance of ATPS and APSR over the 24 h period in extracts from seedlings of both TG and W. Over the course of four repetitions per genotype, each of them consisting of 10 plants, variations in the abundance pattern were observed. Thus, only trends are described in this section.

3.3.2.1. ATPS abundance

In chloroplast and root extracts from seedlings of both TG and W, the abundance of ATPS increased slightly at L+4 h in two out of four repetitions for both genotypes (Appendix I, chloroplast and root extracts). The abundance then generally decreased in the middle of the light period (at L+10 h or L+13 h). A marked abundance of ATPS was visualized at the onset of the night period (D+0 h) in most samples. This was more prominent in chloroplast extracts of W, and persisted over the entire night period in chloroplasts only.

In leaf extracts of TG and W, the abundance of the enzyme generally increased between L+7 h and L+13 h (Appendix I, leaf extracts). In TG, another increase of ATPS abundance was visualized at D+3 h in three out of four repetitions, while in W, the increase occurred at D+6 h, in three out of four repetitions. In leaves of TG, ATPS was more abundant during the light period in three out of four repetitions.

In some western blots using chloroplast extracts, ATPS was characterized by two closely migrating bands, so, chloroplast extracts from TG and W were resolved on a gradient PAGE gel for better resolution and separation of the bands with one representative repetition. One representative repetition of leaf extracts from TG and W was also resolved on gradient gels.

The gradient gels allowed the visualization of the two closely migrating ATPS bands in chloroplast extracts from both genotypes (Figure 3.11). In TG, the abundance of the band with the higher molecular mass was higher at L+4 h and lower at the end of the dark period and at L+1 h. The band with the lower molecular mass was undetectable at D+6 h and L+1 h. The abundance of this band was low at L+4 h and increased massively at L+7 h.

In W, the abundance of the band with the higher molecular mass was very high throughout the 24 h period. However, the band was hardly detectable at L+4 h and was

reduced at D+6 h. The abundance of the lower molecular mass band was lower at L+1 h and higher at L+7 h.

In TG leaf extracts, ATPS abundance was high at the beginning of the light period and the two bands could not be distinguished. The abundance of the higher molecular mass was higher during the light period and decreased at L+10 h. The lower molecular mass band abundance was detected only from L+10 to D+0 h and at D+6 h. The abundance of this band was higher at D+6 h.

In W leaf extracts, the abundance of ATPS was very intense at illumination (L+1 h) and decreased constantly from L+4 h to L+7 h. The abundance increased at L+10 h and remained constant until the beginning of the dark period (D+0 h). The ATPS abundance gradually decreased throughout the dark period and finally increased back at D+6 h. Only at D+0 and D+3 h, two bands of ATPS were distinguished.

3.3.2.2. APSR abundance

In terms of APSR, in chloroplast, leaves and root extracts, a general trend toward higher abundance over the light period and lower at night was observed in most repetitions (Appendix II). In leaf extracts from W and root extracts from both TG and W, L+4 h generally correlated with an increased abundance of APSR. In leaf extracts from TG, APSR abundance was higher over the first half of the light period (from L+1 to L+7 h). In leaf extracts from W and in root extracts from both genotypes, APSR abundance increased at L+4 h in most of the repetitions. In chloroplast extracts from TG and W and in root extracts from TG only, a decrease in abundance was observed prior to dark onset (at L+10 and/or L+13 h). Finally, in two repetitions of roots from TG, a massive abundance at D+0 h was observed.

Two bands closely migrating with similar diurnal variations were visualized on the blots. In some repetitions of chloroplasts, up to three bands were distinguished with the middle one being the strongest. In general, APSR abundance was higher in chlorophyll containing tissues, but the detection was less marked in roots, probably because of the low specificity of the antibody over this form.

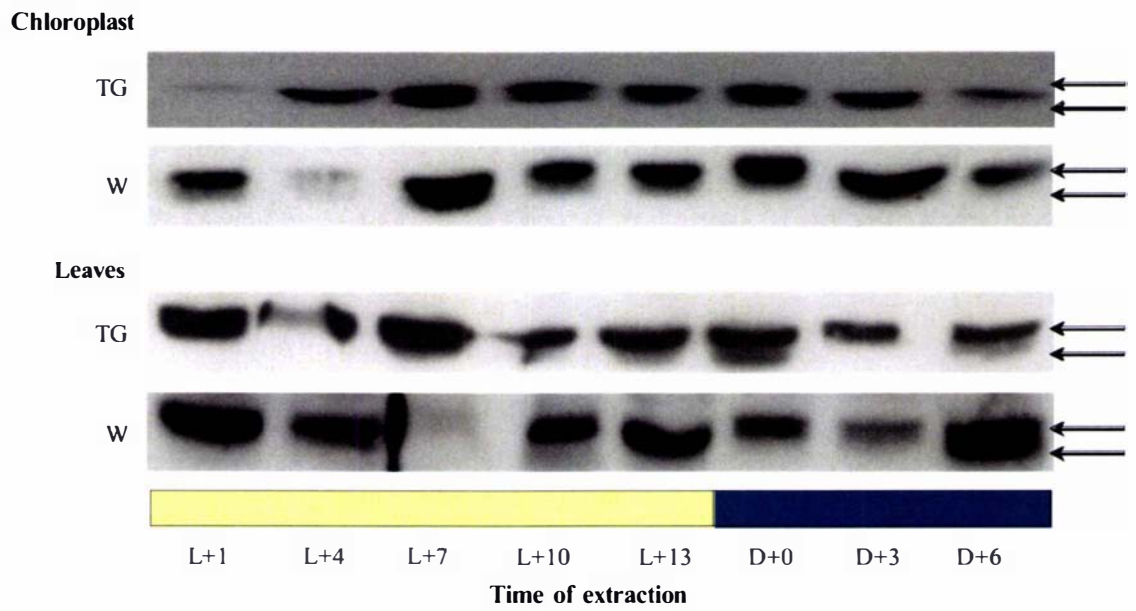


Figure 3.11. Influence of light and dark on the abundance of ATPS isoforms in chloroplast or leaf extracts, as indicated, in seedlings of TG or W, as indicated, grown in the S-sufficient media.

Proteins (35 μ g) extracted over a 24 h period from chloroplasts were separated by 8-15% gradient PAGE and the abundance of ATPS determined by western analyses and chemiluminescence. Arrows show the number of bands detected by the antibody.

3.4. Detection of ATPS isoforms and the influence of S supply

The analyses reported in section 3.2.2 revealed differences in ATPS activity and in the abundance of ATPS and APSR as a function of development, S supply and genetic origin. The separation of chloroplast extracts by gradient-PAGE and western blotting also revealed the occurrence of at least two electrophoretic forms of ATPS and APSR and confirmed the importance of the plant genetic, maturation and S supply. The identification of the different isoforms and their characterisation is necessary to understand the regulation of the S assimilation pathway.

3.4.1. Detection by anion exchange chromatography

Proteins from chloroplast extracts were fractionated using anion exchange chromatography to determine the occurrence of ATPS isoforms in onion chloroplasts. Previous results have demonstrated a genetic, developmental and S supply effect on ATPS regulation, so extracts from seedlings and mature plants from both TG and W grown in either the S-sufficient (S^+) or the S-deficient (S^-) conditions were chromatographed. Three repetitions (10 plants per repeat) were carried out per genotype and per S treatment for seedlings, whereas for mature plants, two repetitions (10 plants per repeat) with TG and one repetition (10 plants per repeat) with W were carried out per S supply. The general trend in ATPS and APSR detection is summarized in Table 3.1.

Proteins were purified by anion exchange chromatography using a HiTrap-Q column and eluted out of the column using a linear gradient of NaCl (0 to 1 M). The protein concentration and ATPS activity of every fraction was determined. Finally, the proteins were separated by 1D-PAGE and the S assimilation enzymes were visualized by western analyses.

3.4.1.1. Detection of protein in separated chloroplast extracts

In chloroplast extracts from seedlings, proteins eluted as two distinct phases (Figure 3.12). The first period of elution occurred between 0 and 0.08 M of NaCl, and corresponded to proteins not binding to the resin. In the second phase, proteins that did attach to the resin typically eluted between 0.2 and 0.6 M NaCl. However, the second phase of elution started with 0.12 M NaCl instead of 0.2 M during the purification of chloroplast extracts from seedlings of W (two repetitions) and from seedlings of TG

(one repetition), both grown in the S^+ media. In all samples purified with the exception of one repetition of TG grown in the S^+ media and one repetition of W grown in the S^- media, more protein bound to the column than passed through as the first phase, and the protein concentration of the collected fractions varied according to the amount of protein loaded onto the column. No consistent difference in the protein elution was detected between genotypes and S supply.

In chloroplast extracts from mature plants, the elution of proteins also occurred over two phases (Figure 3.13). The first phase, corresponding to proteins that did not bind to the resins, occurred between 0 and 0.08 M of NaCl. The second phase varied according to the genotype. In TG, proteins interacting with the column eluted between 0.16 and 0.56 M NaCl, whereas in W, they eluted between 0.16 M and 0.48 M NaCl. However, no difference in the protein elution pattern was observed between plants grown in either the S^+ or the S^- media for either genotype. Again, the magnitude of the peak of the bound proteins depended on the quantity of protein loaded.

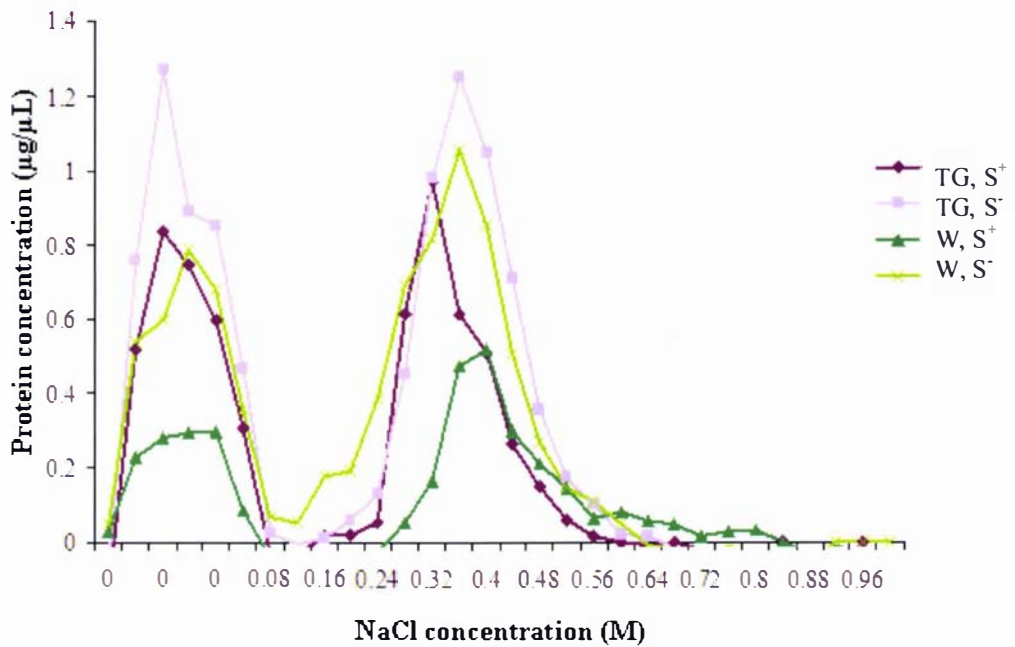


Figure 3.12. Comparison of elution patterns after separation of proteins from chloroplast extracts from seedlings of TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Protein concentration was measured in the fractions collected from the separation by anion exchange chromatography of chloroplast extracts. Four repetitions (10 plants per repeat) were carried out per genotype and S supply. Data represent the protein concentration of each fraction for one representative repetition.

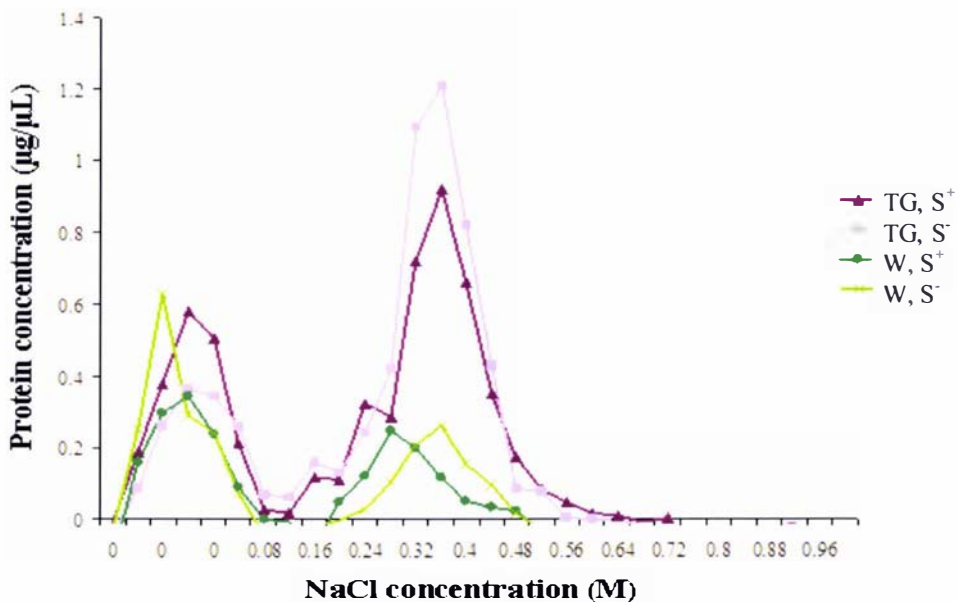


Figure 3.13. Comparison of elution patterns after separation of proteins from chloroplast extracts from mature plants of TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Protein concentration was measured in the fractions collected from the separation by anion exchange chromatography of chloroplast extracts. One repetition (10 plants per repeat) was carried out per genotype and S supply. Data represent the protein concentration of each fraction for this repetition.

3.4.1.2. Detection of ATPS activity in separated chloroplast extracts and the influence of S supply

In seedlings of TG grown in the S^+ media, ATPS activity was detected as eluting between 0.40 and 0.60 M NaCl (except for one repetition, between 0.36 and 0.68 M NaCl) while in seedlings grown in the S^- media, the activity was detected as eluting between 0.40 M and 0.64 M NaCl (except for one repetition, between 0.48 and 0.56 M NaCl) (Figure 3.14). In seedlings of W grown in the S^+ media, ATPS activity was measured as eluting between 0.44 and 0.60 M NaCl for all samples, while in seedlings grown in the S^- media, ATPS was detected as eluting between 0.40 and 0.60 M NaCl (except for one repetition, between 0.40 and 0.56 M NaCl).

In mature plants of TG grown in the S^+ media, ATPS activity was detected as eluting between 0.36 and 0.48 M NaCl, while in plants grown in the S^- media, ATPS was measured as eluting between 0.32 and 0.52 M NaCl (Figure 3.15). In mature plants of W grown in the S^+ media, the activity was detected as eluting between 0.36 M and 0.48 M NaCl, while when in the S^- media, the activity was measured as eluting between 0.40 and 0.48 M NaCl.

3.4.1.3. Detection of the abundance of the S assimilation enzymes in separated chloroplast extracts

3.4.1.3.1. ATPS abundance

In chloroplast extracts from seedlings of TG grown in the S^+ media, ATPS abundance was detected as eluting between 0.32 and 0.44 M NaCl, with the exception of one repetition (between 0.40 and 0.48 M NaCl) (Figure 3.14). In chloroplast extracts from seedlings of TG grown in the S^- media, ATPS abundance was detected as eluting between 0.36 and 0.48 M NaCl, except in one repetition where ATPS continued eluting until 0.52 M NaCl. In chloroplast extracts from seedlings of W, ATPS abundance was detected as eluting between 0.40 and 0.48 M NaCl when grown in either the S^+ or the S^- media, with the exception of one repetition for both S supplies where ATPS abundance started with 0.36 M NaCl.

No band was visualized in later fractions although activity was detected (Figure 3.14), possibly because of the low protein concentration in those fractions and/or the low sensitivity of the chromogenic method used in the western analyses. In seedlings from TG and W grown in the S⁺ media only, two bands were visualized. In TG, the two bands started eluting together, but the band with the lower molecular mass stopped eluting (0.40 M NaCl) before the other band (0.44 M NaCl). In W, the band with the lower molecular mass was detected as eluting between 0.36 and 0.44 M NaCl while the other band was visualized as eluting between 0.40 and 0.44 M NaCl (Figure 3.14, arrowed).

In chloroplast extracts from mature plants of TG, ATPS abundance was generally detected as eluting between 0.24 and 0.44 M NaCl when grown in the S⁺ media, and between 0.24 and 0.48 M NaCl when grown in the S⁻ media (Figure 3.15). In chloroplast extracts from mature plants of W, ATPS abundance was generally detected as eluting between 0.36 and 0.48 M NaCl when grown in either the S⁺ or the S⁻ media. In these mature plants, ATPS was also visualized as eluting as two closely migrating bands (Figure 3.15, arrowed). The abundance of the higher molecular mass band was generally greater than the lower molecular mass band. In plants grown in the S⁺ media only, this higher molecular mass band generally started eluting with a lower concentration of NaCl (at 0.24 M compared to 0.28 M NaCl in TG and at 0.36 mM compared to 0.40 M NaCl in W).

In both developmental stages, ATPS elution was similar between TG and W. In seedlings of TG, the onset of ATPS elution shifted during anion exchange chromatography from 0.32 M NaCl in plants grown in the S⁺ media to 0.36 M in plants grown in the S⁻ media. In mature plants of TG grown in the S⁻ media, ATPS eluted over a wider range of fractions when compared with mature plants grown in the S⁺ media. However, the enzyme elution was also influenced by the developmental stage in both genotypes, and occurred at a lower salt concentration in mature plants when compared with seedlings.

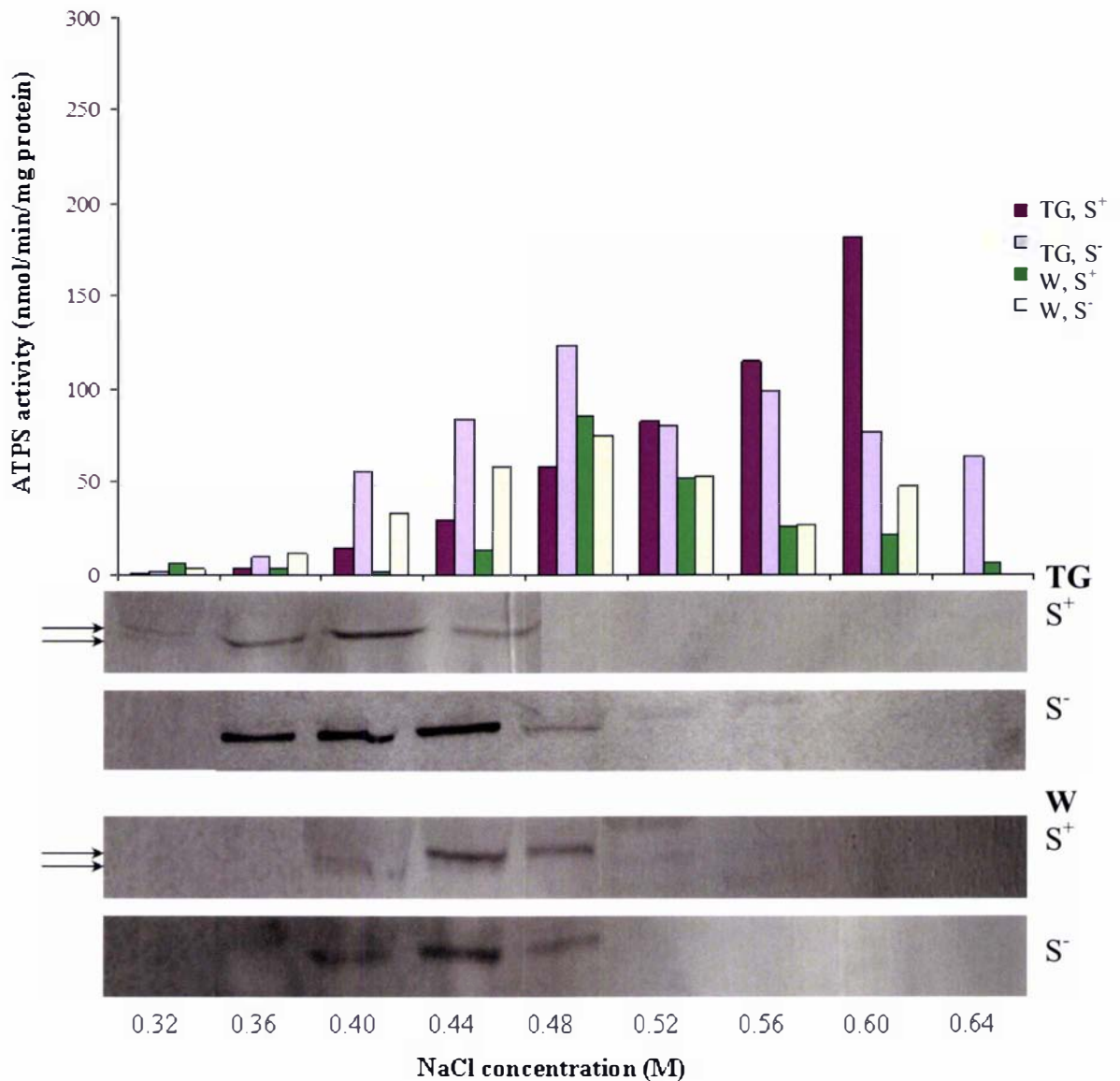


Figure 3.14. Comparison of ATPS activity and abundance after the separation by anion exchange chromatography of proteins from chloroplast extracts from seedlings of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Proteins in the collected fractions were separated by 1D-PAGE and the abundance of ATPS determined by western analyses using chromogenic detection. Four repetitions (10 plants per repeat) were carried out per genotype and S supply. Data represent ATPS activity and abundance for one representative repetition. Arrows show the number of bands detected by the antibody.

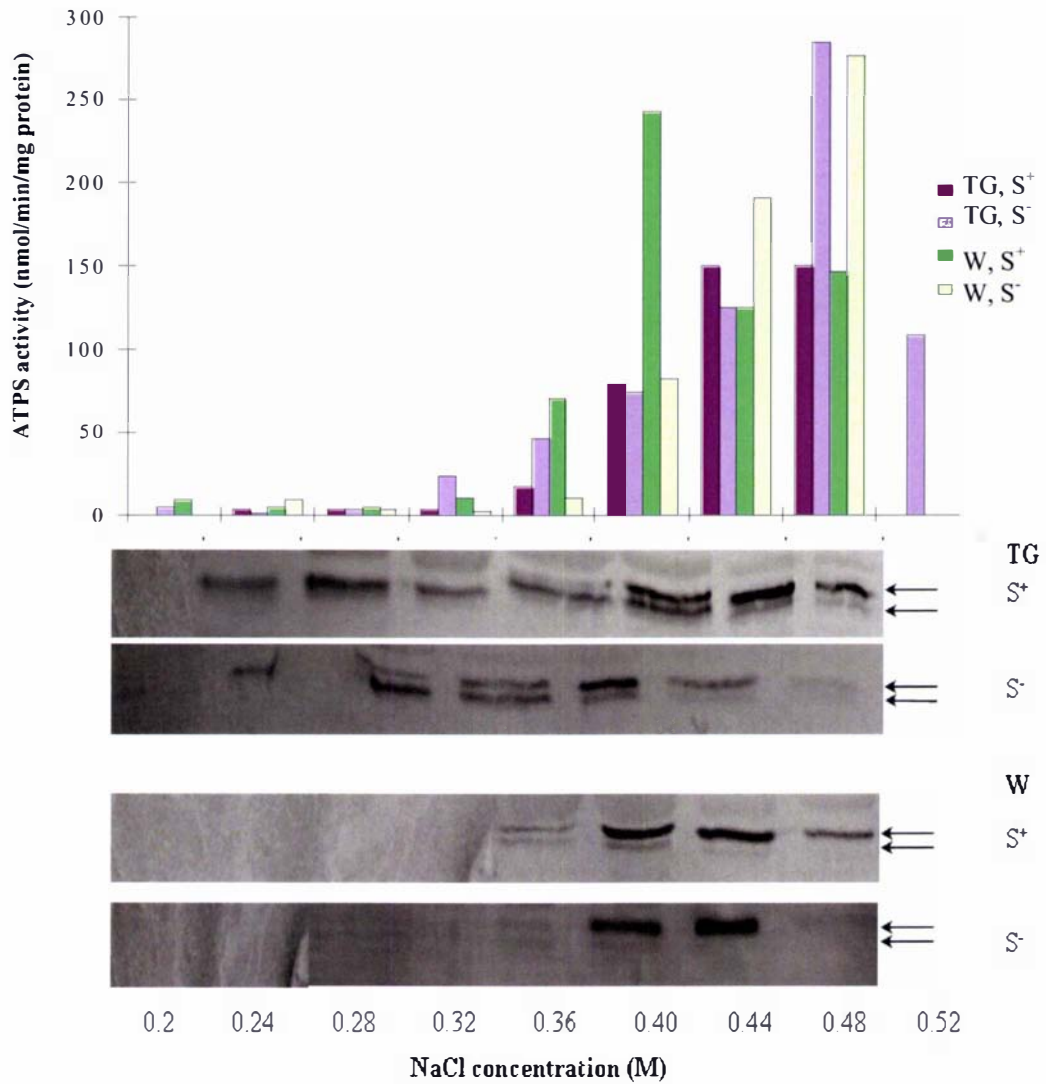


Figure 3.15. Comparison of ATPS activity and abundance after the separation by anion exchange chromatography of proteins from chloroplast extracts from mature plants of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Proteins in the collected fractions were separated by 1D-PAGE and the abundance of ATPS determined by western analyses using chromogenic detection. One repetition (10 plants per repeat) was carried out per genotype and S supply. Arrows show the number of bands detected by the antibody.

3.4.1.3.2. APSR abundance

In seedlings, the enzyme was detected as eluting during both phases of protein elutions in both genotypes and in extracts grown in either the S^+ or the S^- media. In seedlings of both TG and W, APSR eluted between 0 and 0.04 M NaCl in all samples with the unbound proteins (Figure 3.16). In seedlings of TG grown in the S^+ media, APSR was detected as eluting between 0.24 and 0.48 M NaCl, with the exception of one repetition (between 0.16 and 0.44 M NaCl). In seedlings of TG grown in the S^- media, APSR was visualized as eluting between 0.24 and 0.52 M NaCl, with the exception of one repetition where APSR stopped eluting with 0.48 M NaCl. In seedlings of W grown in the S^+ media, APSR was detected as eluting between 0.20 and 0.44 M NaCl. In seedlings of W grown in the S^- media, APSR was visualized as eluting between 0.24 and 0.52 M NaCl, with the exception of one repetition (between 0.28 to 0.56 M NaCl).

In seedlings of both TG and W grown in either the S^+ or the S^- media, APSR was visualized as a single band when eluting with the unbound proteins between 0 and 0.04 M NaCl. In seedlings of TG grown in either the S^+ or the S^- media, APSR was visualized as three bands in two repetitions out of three and as two bands in the last repetition. In samples of TG grown in the S^+ media, when two bands were visualized, the band with the higher molecular mass was detected as eluting between 0.28 and 0.32 M NaCl, while the band with the lower molecular mass was visualized as eluting between 0.24 and 0.32 M NaCl. In samples of TG grown in the S^- media, when two bands were visualized, the band with the higher molecular mass was detected as eluting between 0.28 and 0.48 M NaCl, while the band with the lower molecular mass was visualized as eluting between 0.24 and 0.32 M NaCl. In all samples of TG, when three bands were visualized, the two bands with the higher molecular weight started eluting with the same concentration of NaCl and before the other band(s). Also, the band with the higher molecular mass was detected as eluting over a wider range of NaCl concentrations than the other bands.

In seedlings of W grown in either the S^+ or the S^- media, APSR was detected as four bands in two out of three and as two bands in the other repetition, with the two higher molecular mass bands more intensely recognized. In all sample, the band with the

higher molecular mass was detected as eluting over a wider range of NaCl concentrations than the other bands. In most samples of TG and W, when three or four bands were visualized, the two bands with the higher molecular weight started eluting with the same concentration of NaCl and before the other ones.

In mature plants of both TG and W, the enzyme was only detected with elution of the bound proteins (Figure 3.17). In mature plants of TG, APSR was detected as eluting between 0.2 and 0.44 M NaCl when grown in the S⁺ media, while the enzyme was visualized as eluting between 0.20 and 0.48 M when grown in the S⁻ media. In mature plants of W grown in either the S⁺ or the S⁻ media, APSR was detected as eluting between 0.2 and 0.44 M NaCl.

In mature plants, APSR was detected as two closely migrating bands in all samples. The abundance of the higher molecular mass band was more marked than the second band. In all samples, the band with the higher molecular mass started eluting after the other band, with the exception of extracts from TG grown in the S⁻ media. In extracts from mature plants of TG grown in the S⁺ media, the elution of the higher molecular mass band started eluting with 0.20 M NaCl, while the lower molecular mass band began eluting with 0.24 M. In extracts from mature plants of W grown in the S⁺ media, the elution of the higher molecular mass band started eluting with 0.20 M NaCl, while the lower molecular mass band began eluting with 0.28 M.

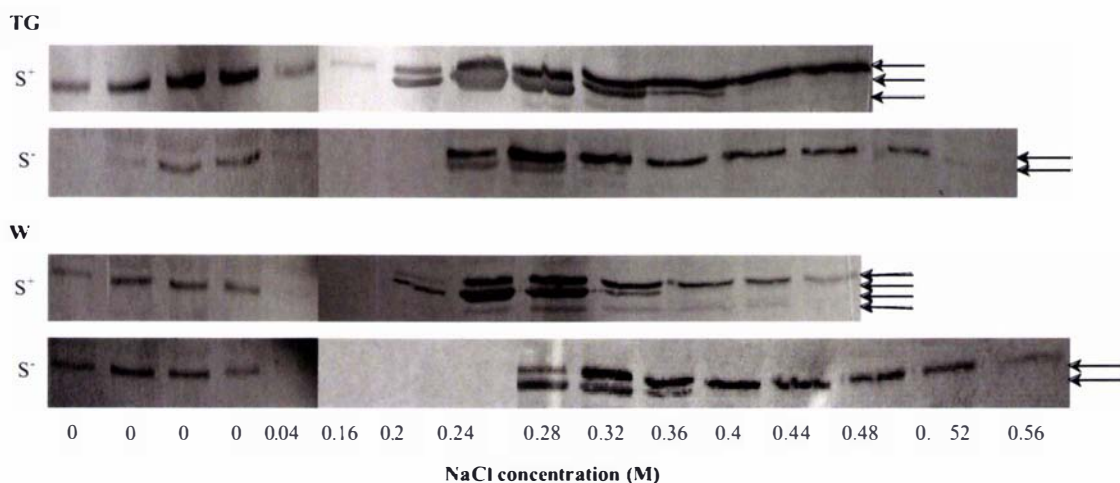


Figure 3.16. Detection of the abundance of APSR after the separation by anion exchange chromatography of proteins from chloroplast extracts of seedlings of TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Proteins in the collected fractions were separated by 1D-PAGE and the abundance of APSR determined by western analyses using chromogenic detection. Four repetitions (10 plants per repeat) were carried out per genotype and S supply. Data represent APSR abundance for one representative repetition. Arrows show the number of bands detected by the antibody.

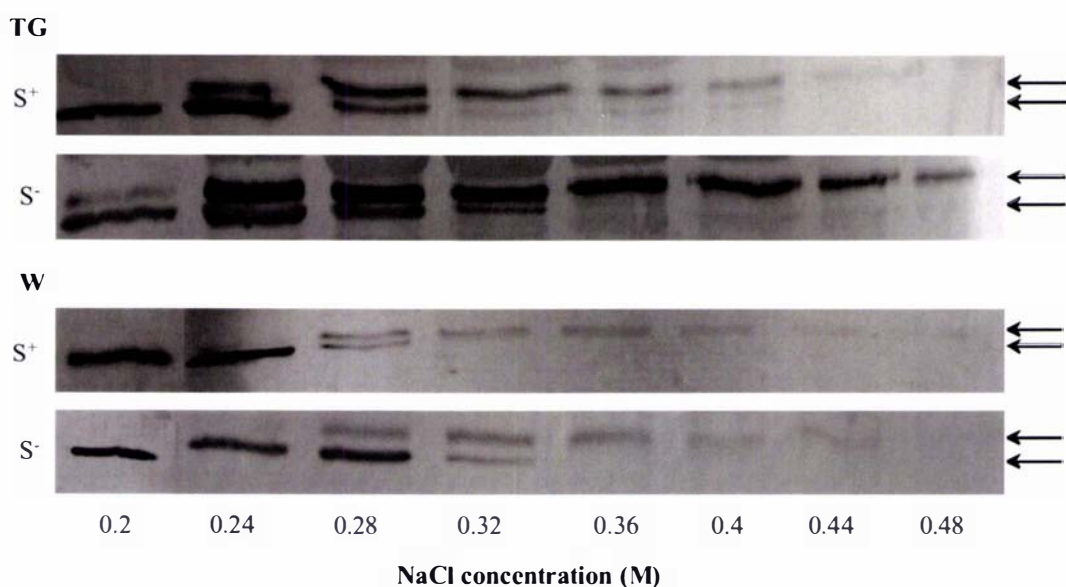


Figure 3.17. Detection of the abundance of APSR after the separation by anion exchange chromatography of proteins from chloroplast extracts of mature plants TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Proteins in the collected fractions were separated by 1D-PAGE and the abundance of APSR determined by western analyses using chromogenic detection. One repetition (10 plants per repeat) was carried out per genotype and S supply. Arrows show the number of bands detected by the antibody.

Table 3.1. Summary of results of the detection of ATPS activity, and the abundance of ATPS and APSR after the separation by anion exchange chromatography of proteins from chloroplast extracts of seedlings or mature plants, as indicated, of TG or W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated. The values represent the molarity of NaCl used in the elution buffer.

Sample	Developmental stage	ATPS activity	Abundance	
			ATPS	APSR
TGC*S ⁺	Seedlings	0.40 to 0.60	0.32 to 0.44	0 to 0.04 0.24 to 0.48
	Mature plants	0.36 to 0.48	0.24 to 0.44	0.20 to 0.44
TGC*S ⁻	Seedlings	0.40 to 0.64	0.36 to 0.48	0 to 0.04 0.24 to 0.52
	Mature plants	0.32 to 0.52	0.24 to 0.48	0.20 to 0.48
WC*S ⁺	Seedlings	0.44 to 0.60	0.40 to 0.48	0 to 0.04 0.20 to 0.44
	Mature plants	0.36 to 0.48	0.36 to 0.48	0.20 to 0.44
WC*S ⁻	Seedlings	0.40 to 0.60	0.40 to 0.48	0 to 0.04 0.24 to 0.52
	Mature plants	0.40 to 0.48	0.36 to 0.48	0.20 to 0.44

3.4.2 Detection by 2D-PAGE

In order to further evaluate the genetic influence, and the influence of the developmental stage and S supply on the abundance of the ATPS isoforms suggested in sections 3.2.2.1 and 3.4.1, 2D-PAGE were carried out using chloroplast extracts from seedlings and mature plants of TG and W.

For the 2D-PAGE technique, the pI value of the amino acid sequence of ATPS was estimated between 6.27 and 8.7 depending on its phosphorylation status using Scansite¹. Preliminary gels were carried out to verify the presence of ATPS after focusing using IPG strips with a basic pH range. Several spots, ranging from pH 6.0 to pH 6.5, were clearly identified using the ATPS antibody in TG seedlings and these showed high reproducibility. Therefore, dry polyacrylamide gel strips of 7 cm-long with an immobilized pH 6-11 gradient were used for ATPS characterisation.

ATPS was visualized as a series of spots in all separations, except in seedlings of TG and W grown in the S⁻ media, confirming the presence of several putative isoforms (Figure 3.18). The number of spots and their abundance was greater in W when compared with TG, except in mature plants grown in the S⁻ media. In both genotypes and both S supply, spot numbers increased with maturation. In mature plants from TG and W grown in the S⁺ media, the distribution of ATPS spots shifted during IEF to a more basic pH compared with the protein pattern in seedlings, as determined by the alignment of the blots (Figure 3.18). While the abundance of each spot was comparable in seedlings, two of these on the basic side were stronger than the others in the mature plants of both TG and W.

The results in Chapter 3 suggest that ATPS is present as isoforms in onion chloroplasts that appear to be regulated on a genetic basis, as well as via developmental factors and S supply. To understand better the regulation of ATPS, and the possible significance of these isoforms, the biological significance of phosphorylation of ATPS activity and abundance was examined.

¹ <http://scansite.mit.edu/>

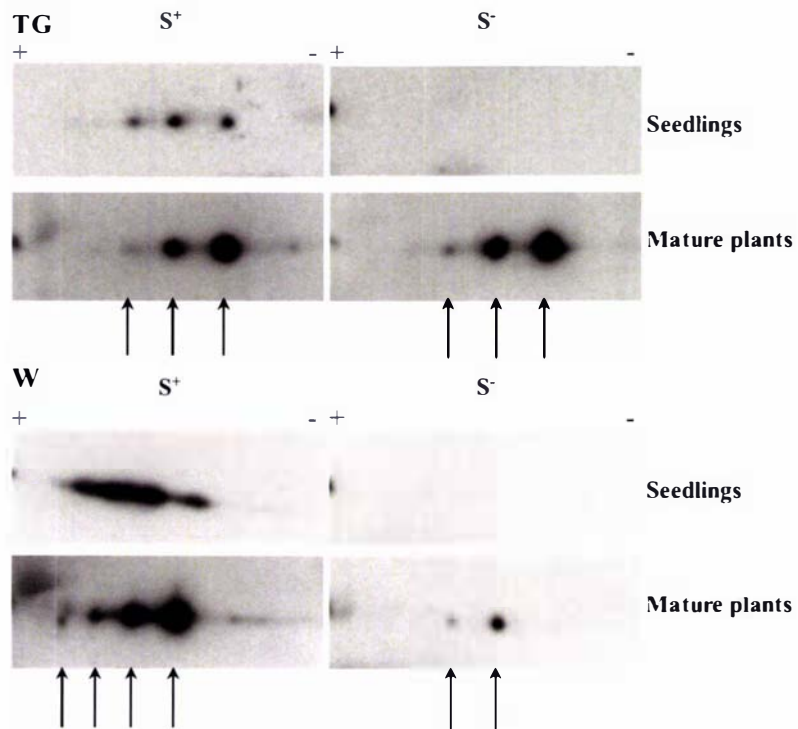


Figure 3.18. Comparison of the abundance of ATPS after separation by 2D-PAGE of proteins from chloroplasts of seedlings and mature plants, as indicated, of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

Proteins (40 μg) were separated by 2D-PAGE using IEF pH range 6-11 in the first dimension, 12% SDS-PAGE in the second dimension. The abundance of ATPS determined by western analyses using chemiluminescence. The positive (acidic end) and negative (basic end) polarity of the IEF are indicated by (+) and (-) respectively. Arrows show the number of spots detected by the antibody.

4. REGULATION OF ATPS ACTIVITY AND ABUNDANCE BY PHOSPHORYLATION

4.1. Detection of ATPS phosphorylation

4.1.1. Approaches for the detection of ATPS phosphorylation

In this study, several direct techniques, in addition to bio-informatic approaches, were used to detect the phosphorylation of ATPS, the consequences of this event on the enzyme activity, and any interaction with 14-3-3 proteins (Figure 4.1). For all experiments, chloroplast or leaf extracts were treated to promote phosphorylation, designated the P⁺ treatment, or dephosphorylation, designated the P⁻ treatment.

In the P⁺ treatment, MgATP was used as the phosphoryl donor in all incubations to promote kinase activity along with either NaF, EDTA and EGTA or OKA, EDTA and EGTA used to inhibit the activity of a broad range of phosphatases. In the P⁻ treatment, either PP2A or the Antarctic Alkaline Phosphatase (AAP) and Mg²⁺ were added to complement the activity of endogenous phosphatases. Firstly, the efficiency of the P⁺ and P⁻ treatments was assessed using the phosphostaining Pro-Q DPS with subsequent staining with silver nitrate to reveal all proteins (section 4.1.2) after separation of chloroplast and leaf extracts by 1D-PAGE. In addition, albumin, a phosphoprotein (Wei *et al.*, 1998) was stained with Pro-Q DPS after the P⁺ and the P⁻ treatments.

Protein phosphorylation corresponds to the addition of phosphate groups to proteins, which affect its molecular mass, pI and charges. Thus, several techniques were carried out to detect the putative phosphorylation of ATPS and a diagrammatic overview is presented as Figure 4.1.

For the detection of mass shift, chloroplast extracts were separated through 1D-PAGE and ATPS was visualized by western analyses. Then, for the detection of pI variation between the phosphorylated and dephosphorylated states, extracts were resolved by 2D-PAGE and ATPS detected by western analyses. Finally, the enzyme activity was assayed to detect any putative variation in ATPS activity after the P⁺ and P⁻ treatments and affinity chromatography was carried out for the identification of interactions with 14-3-3 proteins. The enzyme OAS-TL has been recently identified as a phosphoprotein

in canola (*Brassica napus* L.) during seed filling (Agrawal and Thelen, 2006) and in *Arabidopsis* (Thelen, personal communication), and thus this protein (with antibody staining) was used as a control. As well, as they were available, antibodies were used to assess any evidence of phosphorylation or dephosphorylation in the other S assimilation enzymes, APSR and SiR. These enzymes were examined by western analyses.

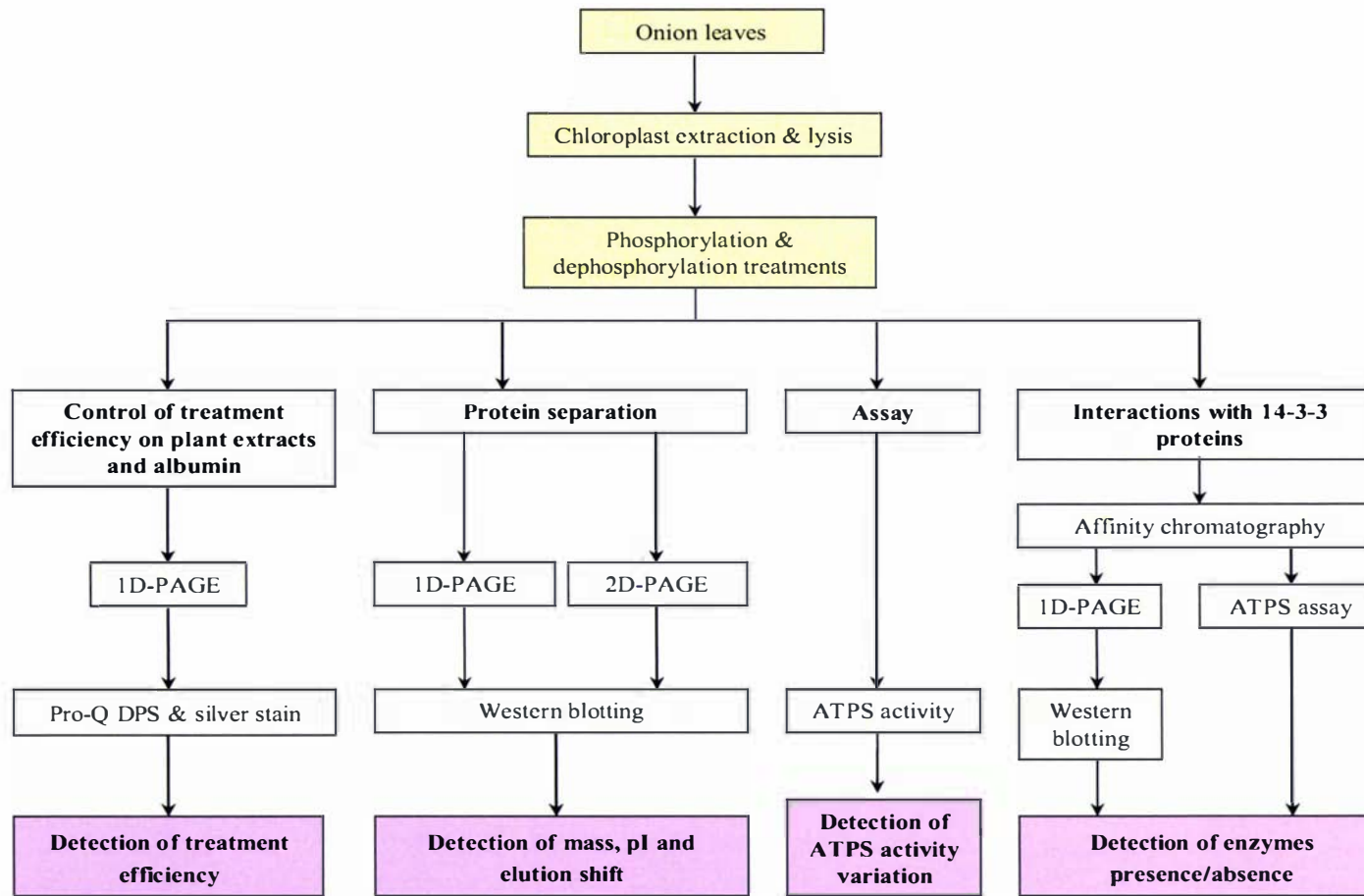


Figure 4.1. Schematic overview of sample preparation steps, analytical techniques and analysis employed for detection of phosphorylation and interactions with 14-3-3 proteins.

In yellow, the protein preparation and their treatments are designed to promote the phosphorylated or dephosphorylated state. In green, the analytical methods employed for the control of treatment efficiency, detection of phosphorylation and interactions with 14-3-3 proteins are given. In purple, the method used for the analysis of results are given.

4.1.2. Bio-informatic analysis of ATPS and OAS-TL sequences reveals putative phosphorylated sites

Several bio-informatic tools such as Scansite, NetPhos, KinasePhos, Phospho.ELM, or Phosphosite are publicly available to determine phosphorylation sites from the amino acid sequences of proteins. Examination of the two available ATPS sequences of onion revealed putative phosphorylation sites when using NetPhos¹ and Scansite². Several sites were predicted either on threonine or serine residues when using the NetPhos 2.0 programme, with many of these having a predictive score in excess of 0.5 (Appendix III). However, a score in excess of 0.95 is considered more likely to be phosphorylated (McManus, personal communication), and so Ser₂₂₁ (AAF18998) or Ser₂₂₄ (AAL61615) both with a score of 0.993 may be more promising as possible sites of phosphorylation. When analysing the available OAS-TL sequence from onion, three Ser sites, Ser₆₃, Ser₁₅₅, or Ser₂₅₇ with scores of 0.997, 0.975 and 0.996 respectively, emerged as likely to undergo the post-translational modification (Appendix IV).

Of the phosphoproteins examined thus far, the majority of phosphorylation occurs on serine and threonine residues, with phosphorylation of tyrosine residues accounting for less than 0.05% of the total, and these are usually involved in cell signalling pathways like the EGF receptor or MAP kinases (Gruhler and Jensen, 2006; Luan, 2000). Therefore in this assessment, only serine and threonine residues were considered.

However, current phosphorylation prediction programs are based mainly on mammalian data and so their predictions on plant proteins may have only limited value (Peck, 2006; Thelen, personal communication). This is because kinase specificity motifs generally consist of only a few residues, and so search results that reveal 20 to 30 putative sites predicted for any protein may be mostly sequence noise (Peck, 2006).

¹ www.cbs.dtu.dk/services/NetPhos

² <http://scansite.mit.edu/>

4.1.3. Fluorescence based detection of phosphoproteins using 1D-PAGE

To examine the effect of the P⁺ and the P⁻ treatments and their efficiency to either phosphorylate or dephosphorylate proteins, chloroplast and leaf extracts from mature plants of W only, grown in either the S sufficient (S⁺) or the S deficient (S⁻) media, were used initially. These extracts were either treated with MgATP and phosphatase inhibitors to maintain or possibly enhance phosphorylation (P⁺) or with AAP to supplement the activity of endogenous plant protein phosphatases and reduce protein phosphorylation (P⁻). Extracts were then separated by 1D-PAGE and stained with the phosphospecific Pro-Q DPS to detect phosphoproteins and subsequently with silver nitrate to reveal total proteins. Pro-Q DPS is now widely used as a tool to detect phosphoproteins prior to mass spectrometry analysis in proteomics studies (Agrawal and Thelen, 2005, 2006; Laugesen *et al.*, 2006; Martin *et al.*, 2003; Schulenberg *et al.*, 2003; Shin *et al.*, 2007; Su *et al.*, 2007) as it binds exclusively to the phosphate moiety of proteins with high sensitivity (Steinberg *et al.*, 2003).

Proteins stained with Pro-Q DPS were less abundant than in silver staining using both chloroplast and leaf extracts. The large subunit of ribulose-1,5-biphosphate carboxylase oxygenase (RuBisCO; EC 4.1.1.39), which has a mass of slightly above the 50 kDa marker (Figure 4.2, red arrow), is known as a phosphoprotein (Komatsu *et al.*, 1999; Shen and Huber, 2006; Schoonheim *et al.*, 2007) and was prominently stained by Pro-Q DPS (Figure 4.2).

Otherwise, no obvious difference in staining was observed, apart from the presence of AAP after the P⁻ treatment (Figure 4.2, black arrows). It should be noted that non-purified chloroplast and leaf extracts were loaded onto the wells, making comparison between actual band mass difficult. However, the results did reveal that the incubation for 30 mins at 30°C for the P⁺ and the P⁻ treatments did not result in major degradation of proteins.

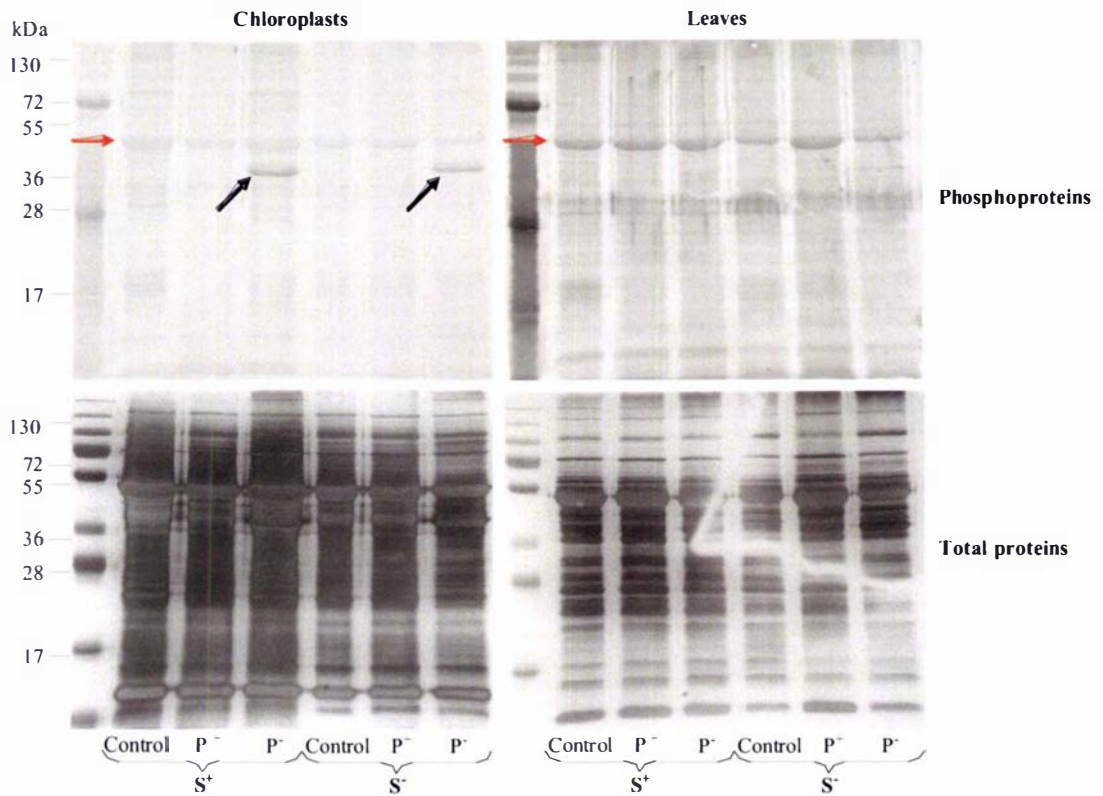


Figure 4.2. Influence of the phosphorylation (P^+) and the dephosphorylation (P^-) treatments, as indicated, on phosphoprotein abundance in chloroplast and leaf extracts, as indicated, from mature plants of *W* grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, separated by 1D-PAGE.

Proteins (10 μg) were treated with MgATP and NaF to promote protein phosphorylation (P^+) or with AAP to promote dephosphorylation (P^-) or untreated (control), and separated by 1D-PAGE. Gels were first stained with the phosphospecific dye Pro-Q DPS (designated phosphoproteins) and subsequently with silver nitrate general protein stain (designated total proteins). The red arrow shows the putative detection of RuBisCO; black arrows indicate the insertion of AAP after the P^- treatment.

4.1.4. Detection of phosphatase activity

In order to test the efficiency of the P^+ and P^- treatments, albumin, which is known to be a phosphoprotein (Wei *et al.*, 1998) was treated to induce the phosphorylation and the dephosphorylation states, respectively. The enzyme was treated with either PP2A or AAP and incubated for 15 and 30 min, respectively. Doubled time of incubation was also tested for each phosphatase. After the incubations, the protein was resolved by 1D-PAGE and stained with Pro-Q DPS to detect the phosphorylation status of albumin and to confirm the specific staining of the Pro-Q DPS and then with silver nitrate to verify the protein loading.

Albumin was detected as a phosphoprotein after both the P^+ and the P^- treatments. The gel observation after Pro-Q DPS (Figure 4.3, phosphoproteins) and the densitometry results (Figure 4.3, absorbance units) showed that the intensity of the Pro-Q DPS was reduced after both AAP and PP2A, but only for the batch 2 of PP2A. The reduction in albumin intensity was most intense after AAP treatment, although the protein signal was uniform after silver staining. Also, small differences between the two incubation periods (30 or 60 min) were detected for both PP2A (batch 2) and AAP, where longer periods induced greater dephosphorylation of albumin. Finally, the protein was more intensely stained after the silver staining than Pro-Q DPS.

The Peppermint stick molecular marker was also loaded onto the gel (Figure 4.3). The two phosphoproteins, ovalbumin and β -casein, were visualized after Pro-Q DPS confirming the specificity of the dye. All five proteins, namely β -galactosidase, bovine serum albumin, ovalbumin, β -casein, and avidin, constituting the ladder were visible after silver staining (Figure 4.3, total proteins).

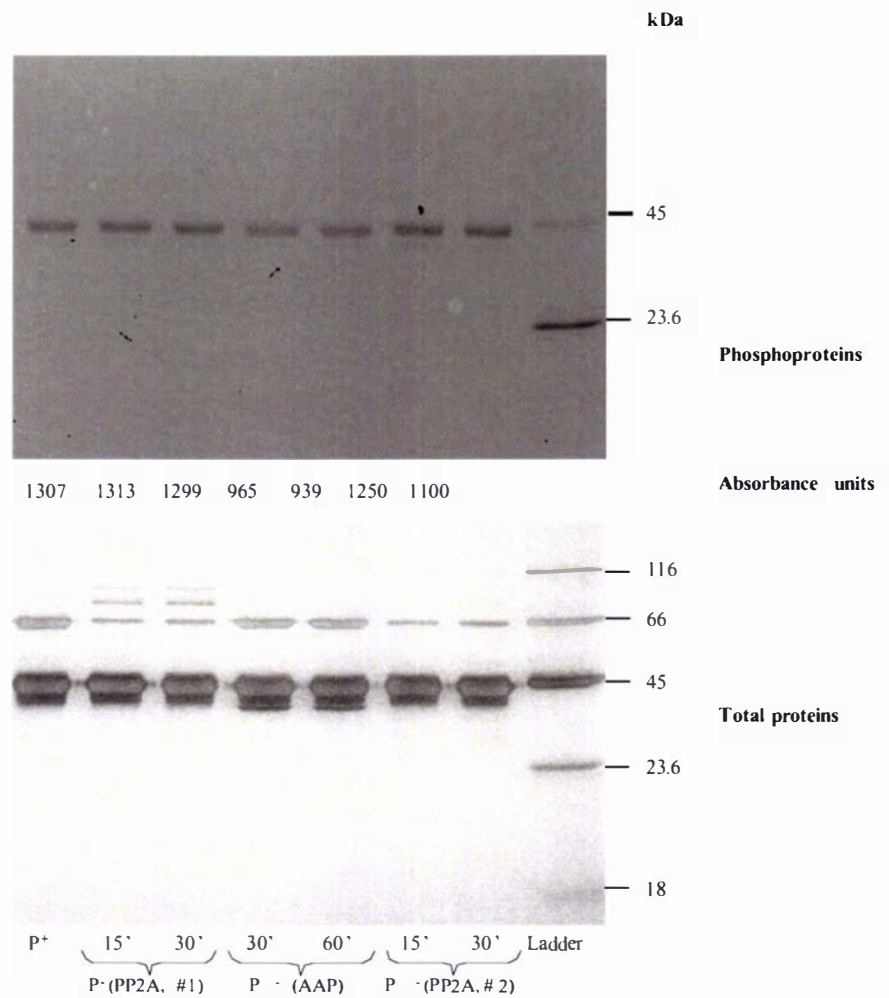


Figure 4.3. Influence of the phosphorylation (P⁺) and the dephosphorylation (P⁻) treatments, as indicated, on albumin, separated by 1D-PAGE.

Recombinant protein (1 μ g) was treated with OKA to promote protein phosphorylation (P⁺) or with PP2A or AAP, as indicated, to promote dephosphorylation (P⁻), and separated by 1D-PAGE. Gels were first stained with the phosphospecific dye Pro-Q DPS (designated phosphoproteins) and subsequently with silver nitrate general protein stain (designated total proteins).

4.1.5. Assessment of phosphorylation of the S assimilation enzymes by mobility shift

Evidence for protein phosphorylation can, in some cases, be gathered by observation of a mass shift in the protein. Although the mass increment is 80 Da (for each phosphate group added), much larger changes in mass can be observed (MacKintosh, personal communication) because of an altered mobility of the protein during 1D-PAGE induced via some conformational changes (MacKintosh, personal communication).

In order to detect any mass shift in the S assimilation enzymes, chloroplast extracts from TG and W were initially treated with MgATP (as the phosphoryl donor for protein kinase activity), and NaF, EDTA and EGTA (as phosphatase inhibitors) to promote the phosphorylation of proteins or with PP2A to promote their dephosphorylation. Because of lack of availability of PP2A (due to the expense of the enzyme), only extracts from mature plants were analyzed. After treatments, extracts were separated by 1D-PAGE. Untreated extracts were run in parallel as a control. After detection of the protein of interest by western analysis, the blot was carefully examined to monitor any putative changes in molecular mass.

Following the P^+ and the P^- treatments, no mass shift could be detected for ATPS and APSR in chloroplast extracts from TG and for ATPS, APSR, SiR and OAS-TL in chloroplasts from W (Figure 4.4). A mass shift was detected only for SiR in chloroplast extracts from mature plants of TG grown in the S^+ media and for OAS-TL in chloroplast extracts from plants grown in the S^- media (Figure 4.4, red squares). In both cases, proteins were migrating slightly slower after the P^+ treatment, suggesting an increase in molecular mass. However, this variation of mass was observed only once and could not be repeated in two further experiments (Appendix VIII).

PP2A is a specific enzyme that removes phosphate groups on serine and threonine residues only (MacKintosh, 1993). However in preliminary trials, there was some uncertainty about the activity of the batches of PP2A accessed as part of this study. In response, a second phosphatase was tested for protein dephosphorylation, AAP, which in preliminary assays had shown evidence of activity. This phosphatase was tested on chloroplast extracts from seedlings and mature plants of both TG and W grown in the S^+ and the S^- media. However, no mass shift could be observed after either the P^+ or the P^- treatment, under any of the conditions tested (Appendix IX).

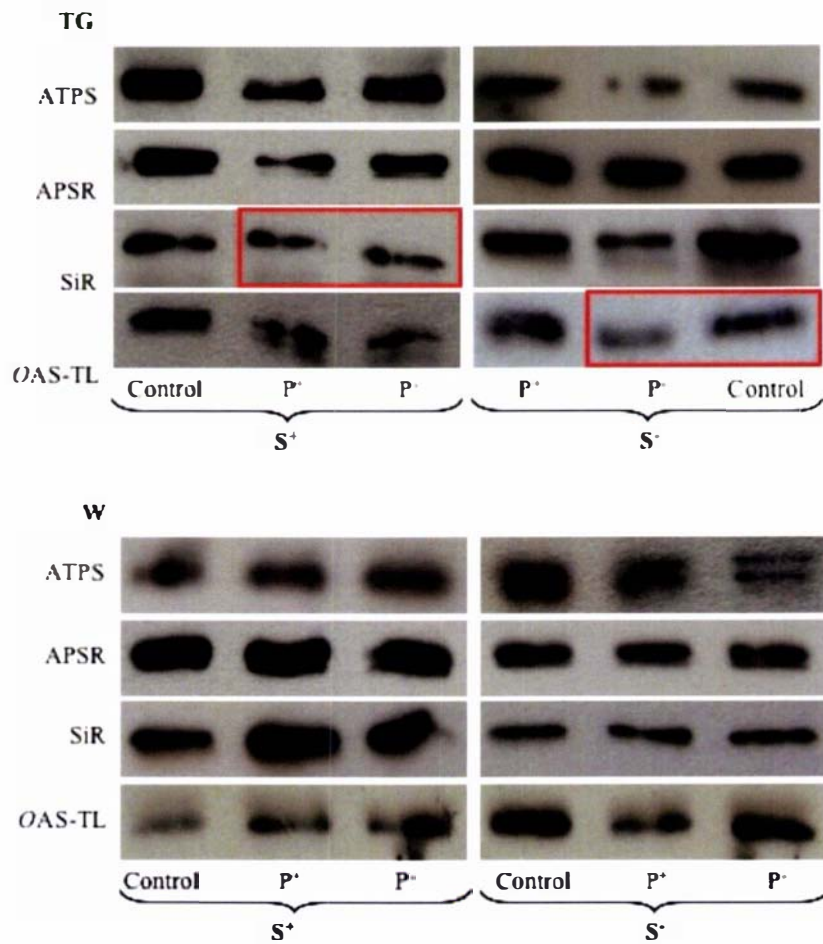


Figure 4.4. Influence of the phosphorylation (P⁺) and the dephosphorylation (P⁻) treatments, as indicated, on the molecular mass of ATPS, APSR, SiR or OAS-TL, as indicated, from chloroplast extracts of mature plants of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated, using PP2A as the phosphatase.

Proteins (10 μg) were treated with MgATP and NaF, EDTA and EGTA as phosphatase inhibitors to favour protein phosphorylation (P⁺) or with PP2A to promote dephosphorylation (P⁻) or untreated (control) and separated by 1D-PAGE. The enzyme ATPS, APSR, SiR or OAS-TL, as indicated, was detected by western analyses using chemiluminescence.

4.1.6. Identification of phosphorylation by changes in pI

It is possible to demonstrate protein phosphorylation by changes in the isoelectric point of the protein after 2D-PAGE. This is because an increase in phosphate content causes the protein to focus at a new, more acidic pI (Garrison and Wagner, 1982; Steinberg and Coffino, 1979). Therefore, if a protein moves to a more acidic pI following a stimulus, its net phosphate content has increased (Garrison and Wagner, 1982; Steinberg and Coffino, 1979). The magnitude of these charge shifts is typically about 0.3 pH units in the pH 6-7 region of the gel (Garrison and Wagner, 1982).

In order to further examine the putative phosphorylation of ATPS, comparative 2D-PAGE and immunological analyses were carried out using chloroplast extracts of mature plants of W. Chloroplast extracts from plants grown in either the S⁺ or the S⁻ media were studied to also assess the influence of the S supply on protein phosphorylation.

Parallel gels were run to control the effects of the P⁺ and the P⁻ treatments on protein phosphorylation using OAS-TL, as this enzyme has been shown to be a phosphoprotein during seed filling in canola (Agrawal and Thelen, 2006) and *Arabidopsis* (Thelen, personal communication). OAS-TL was detected in leaf extracts because of the low antibody affinity for the chloroplast form. In the analysis of OAS-TL, proteins extracted from mature plants of W grown in either the S⁺ or the S⁻ media were analysed.

In chloroplast extracts, the antibody recognized ATPS after both the P⁺ and the P⁻ treatments, but two spots from the acidic end of the pI range disappeared after the P⁻ treatment (Figure 4.5). This disappearance of spots after the P⁻ treatment was observed in extracts from plants grown in both the S⁺ and the S⁻ media, but was more obvious in the S⁺ media (Figure 4.5, arrowed).

In leaf extracts, OAS-TL was detected after both the P⁺ and the P⁻ treatments (Figure 4.6). In extracts from plants grown in the S⁺ media, OAS-TL spots distribution shifted during IEF to a more acidic pH after the P⁺ treatment when compared with the protein pattern after the P⁻ treatment (Figure 4.6, arrowed). No difference in spot number or abundance was visible between the two treatments. The abundance of OAS-TL was higher in extracts from plants grown in the S⁻ media. However, in extracts from plants

grown in the S^+ media, the visualisation of *OAS*-TL spots was more difficult after the P^+ treatment because of a poorer focusing (as determined by a lower accumulation of volt-hours).

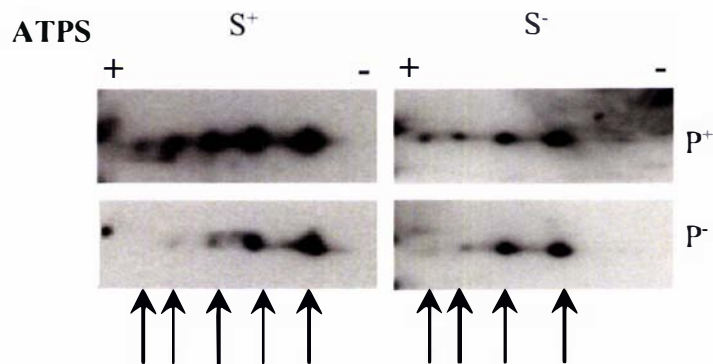


Figure 4.5. Influence of the phosphorylation (P^+) and the dephosphorylation (P^-) treatments, as indicated, on the isoelectric point of ATPS from chloroplast extracts from mature plants of *W* grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Proteins (40 μ g) were treated with MgATP and NaF to promote phosphorylation (P^+) or with AAP to promote dephosphorylation (P^-), as indicated, and separated by 2D-PAGE using IEF range 6-11. The abundance of ATPS was determined by western analyses using chemiluminescence. The positive (acidic end) and negative (basic end) polarity of the IEF are indicated by (+) and (-). Arrows show the number of spots detected by the antibody.

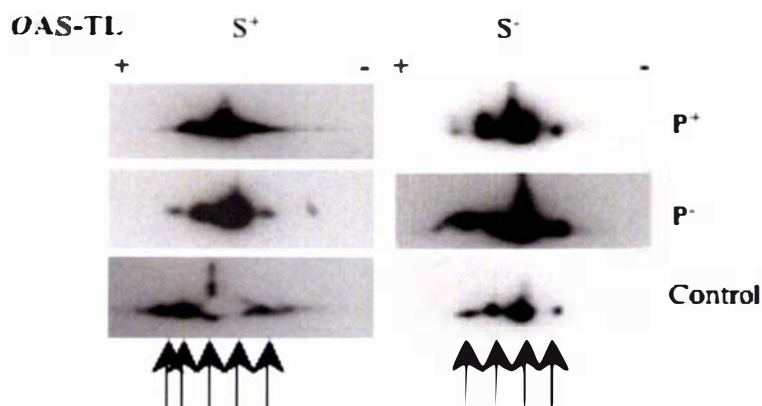


Figure 4.6. Influence of the phosphorylation (P^+) and the dephosphorylation (P^-) treatments, as indicated, on the isoelectric point of OAS-TL from leaf extracts from mature plants of *W* grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated.

Proteins (40 μ g) were treated with MgATP and NaF to promote phosphorylation (P^+) or with AAP to promote dephosphorylation (P^-), or kept untreated (control) as indicated, and separated by 2D-PAGE using IEF pH range 4-7 in the first dimension and 12% SDS-PAGE in the second dimension. The abundance of OAS-TL was determined by western analyses using chemiluminescence. The positive (acidic end) and negative (basic end) polarity of the IEF are indicated by (+) and (-). Arrows show the number of spots detected by the antibody.

4.1.7. Assessing the influence of phosphorylation and dephosphorylation on ATPS enzyme activity

To assess the biochemical significance of any change of phosphorylation status of ATPS, the enzyme activity was assayed after incubation of chloroplast extracts to promote either protein phosphorylation (P^+) or dephosphorylation (P^-).

Preliminary tests were carried out to verify the influence of the chemicals used during the P^+ and the P^- treatments on the ATPS activity. All tests were done using chloroplast extracts from seedlings of W grown in the S-sufficient media.

In the P^+ treatment only, ATP was added as the phosphoryl donor to a final concentration of 0.2 mM. ATPS activity increased from 19.44 to 26.24 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein with no ATPS added or the addition of ATP to 2.5 mM, respectively (Appendix X). Higher concentrations of ATP totally inhibited the enzyme activity. However, with ATP added to 0.25 mM, ATPS activity was slightly up regulated, from 19.44 to 19.52 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein.

These preliminary tests also showed that the addition of MgCl_2 to a final concentration of 5 mM slightly increased the enzyme activity, from 19.76 to 21.02 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein (Appendix X). The addition of MgCl_2 to higher concentrations (between 7.5 to 50 mM) had a minor inhibitory effect on the ATPS activity, decreasing the activity to around 18 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein. Considering this increase of activity upon the addition of MgCl_2 , it was also added to the P^- treatment to the same final concentration (5 mM). Thus, for the P^+ treatment, MgCl_2 to 5 mM and ATP to 0.2 mM were added to chloroplast extracts, and thus their stimulatory influence on enzyme activity kept minimal at these concentrations.

For the P^+ treatment, EDTA and EGTA were also used for their potency to limit phosphatase activity by chelating ions necessary for their activity. However, the presence of these compounds may also decrease ATPS activity which requires MgCl_2 . The addition of EDTA strongly inhibited ATPS activity when added to final concentrations greater than 1 mM (Appendix X). The addition to a final concentration of 1 mM slightly increased the activity, from 25.75 to 27.08 $\text{nmol min}^{-1} \text{mg}^{-1}$ protein.

The addition of EGTA to a final concentration between 5 and 50 mM caused only a mild reduction of ATPS activity when compared to EDTA (Appendix X). However, when added to a final concentration of 1 mM, EGTA induced a minor increase in activity, from 25.86 to 27.38 nmol min⁻¹ mg⁻¹ protein.

The effect of EDTA and EGTA on ATPS activity when added together to the chloroplast extracts was comparable to the influence of EDTA used alone (Appendix X). Considering these results, both chemicals were used at their minimal working (final) concentration for phosphatase inhibition (1 mM), without significant effect on the enzyme activity.

Finally, the influence of the incubation at 30°C on the enzyme activity was assessed. ATPS activity decreased from 19.44 to 17.15 nmol min⁻¹ mg⁻¹ protein after incubation of the chloroplast extracts for 15 min at 30°C (Appendix X). However, this was comparable to the decrease in activity after incubation for 15 min at 20°C (16.28 nmol min⁻¹ mg⁻¹ protein).

During the first attempt at determining the influence of dephosphorylation on ATPS, PP2A was used as the phosphatase and only chloroplast extracts from mature plants of both TG and W grown in either the S⁺ or the S⁻ media were used. In these assays, ATPS activity increased after both the P⁺ and the P⁻ treatments when compared to the activity in the untreated extracts (Figure 4.7). The difference in ATPS activity between P⁺ and P⁻ treated extracts and untreated extracts was statistically significant for mature plants of TG grown in the S⁺ media only ($p=0.008$). The increase in activity detected in extracts after both treatments might be due to the use of MgCl₂. In addition, the activity was slightly higher after the P⁺ than after the P⁻ treatment, but this difference was not statistically significant.

To further analyse the effect of the phosphorylation status on ATPS, similar incubations were carried out using AAP as the phosphatase instead of PP2A. Chloroplast extracts from seedlings and mature plants of both TG and W grown in either the S⁺ or the S⁻ media were tested (Figure 4.8). Unlike the first attempt, ATPS activity was similar between treated and untreated chloroplasts. In most instances, no variation of ATPS activity was measured between the P⁺ and the P⁻ treated extracts, particularly in seedlings. The exception was for seedlings of W grown in the S⁻ media, where the

dephosphorylation treatment increased ATPS activity, but this increase was not statistically significant.

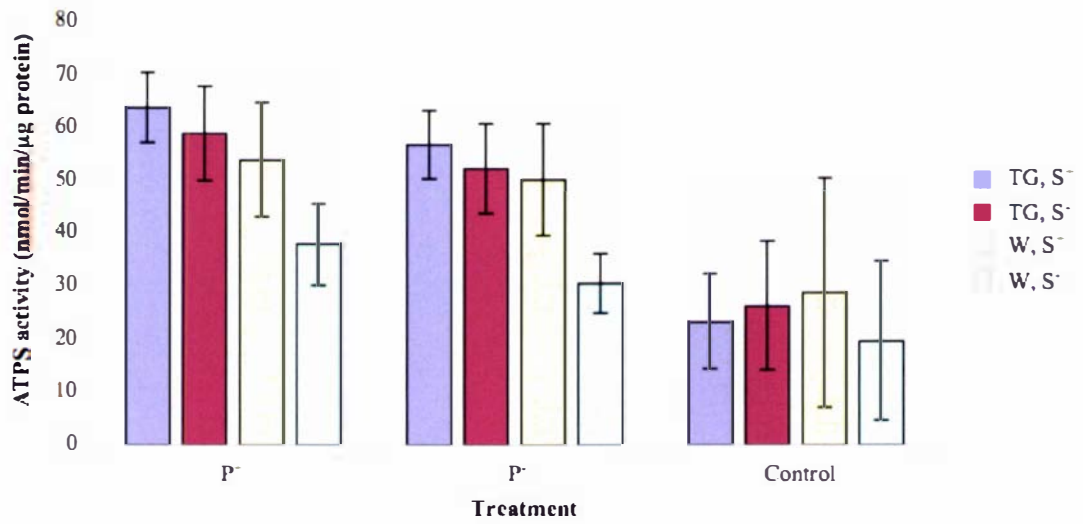
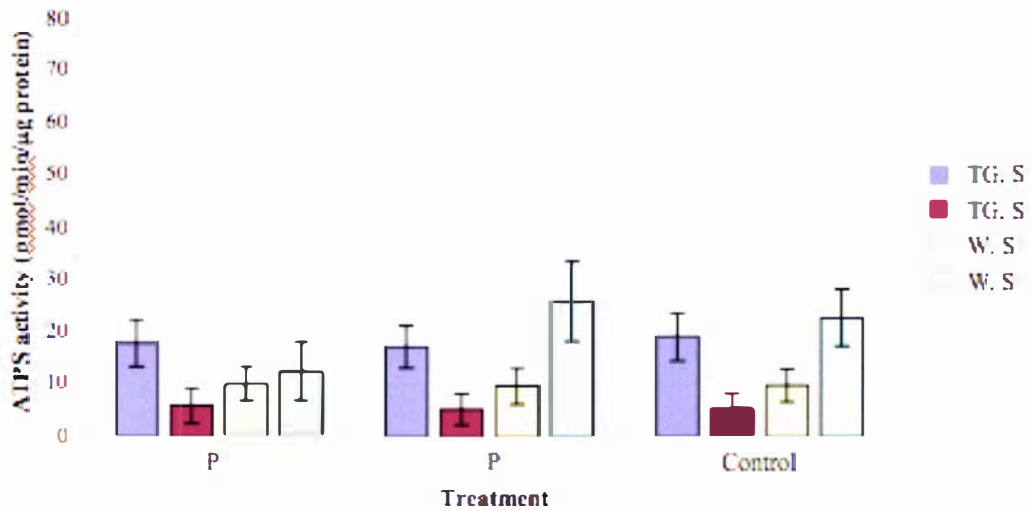


Figure 4.7. Influence of the phosphorylation (P⁺) and the dephosphorylation (P⁻) treatments, as indicated, on ATPS activity in chloroplast extracts from mature plants of TG and W, as indicated, grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated.

ATPS activity was measured in proteins previously treated with MgATP and NaF to promote phosphorylation (P⁺) or with PP2A to promote dephosphorylation (P⁻), as indicated. Each bar represents the average of three repetitions, each consisting of 10 plants. Interval bars represent the standard error.

Seedlings



Mature plants

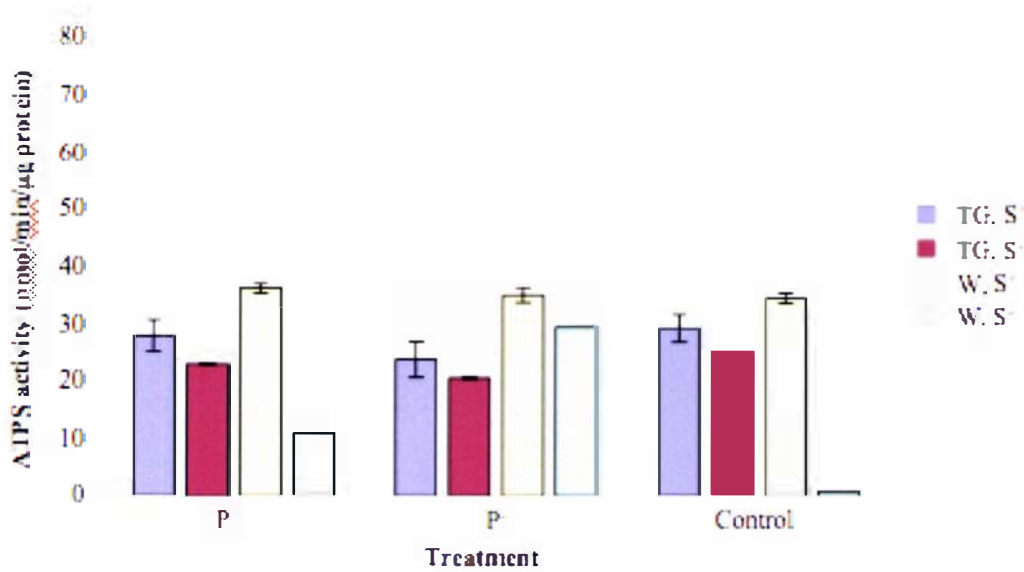


Figure 4.8. Influence of the phosphorylation (P^+) or the dephosphorylation (P^-) treatments, as indicated, on ATPS activity in chloroplast extracts from seedlings and mature plants, as indicated, of TG and W, as indicated grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated. ATPS activity was measured in proteins previously treated with MgATP and NaF to promote phosphorylation (P^+) or with AAP to promote their dephosphorylation (P^-), as indicated. Each bar represents the average of three repetitions, each consisting of 10 plants, with the exception of mature plants grown in the S^- media that shows only one repetition. Interval bars represent the standard error.

4.2. Detection of ATPS binding to 14-3-3 proteins

4.2.1. Analysis of ATPS and OAS-TL sequences reveal 14-3-3 binding motifs

According to Yaffe's Mode 1 and Mode 2 consensus sequence (Yaffe *et al.*, 1997), both ATPS onion sequences and the OAS-TL sequence display 14-3-3 protein binding motifs (Ferl, personal communication). Binding sites for 14-3-3 proteins were also detected on the *Arabidopsis* SiR sequence using the bio-informatic tool Scansite³ (Ser₅₆, Ser₁₃₀, Ser₃₃₆ and Thr₇₀) (Appendix VII). No 14-3-3 protein binding site were detected on the APSR onion sequence using Scansite.

This binding motif, RWS*LP (where * indicate the putative phosphorylation site), is conserved in several ATPS sequences from other plant species such as *Arabidopsis* (isoforms APS3 and APS4), *Brassica juncea*, *Camellia sinensis*, *Oryza sativa* and *Zea mays* (Figure 4.9). In other species such as *Arabidopsis* (isoform APS1, accession number NP_188929; isoform APS2, accession number NP_564099), *Brassica oleracea* (AAF13064), *Glycine max* (AAL74418), *Medicago truncatula* (ABE89666), *Solanum tuberosum* (CAA52953), *Homo sapiens* (AAC64583), the ATPS sequence still displays the 14-3-3 motif but with a single amino acid mutation at the serine phosphorylation site to aspartic acid for most species, or tyrosine for *Brassica oleracea*, or histidine for the human protein.

Searching for the Yaffe's Mode 1 and Mode 2 consensus sequence in these ATPS sequences from onion, a binding site is located around a putatively phosphorylated serine residue at position 288 for the sequence AAL61615 and 291 for the sequence AAF18998 (Appendix III). Using the NetPhos⁴ programme, this serine residue scored 0.973 in both ATPS sequences, but no known kinase was predicted to mediate phosphorylation using Scansite⁵.

According to the Scansite⁵ prediction on surface accessibility, this serine residue from the two ATPS sequences in onion scores over 1, which suggests they are highly accessible and are represented by one of the sharpest peaks of the plot (Figure 4.10).

³ <http://scansite.mit.edu/>

⁴ www.cbs.dtu.dk/services/NetPhos

Gene sequence	Serine residue number	RXS*XP
<i>Allium cepa</i> (AAL61615)	288	kmRWS*LPvd
<i>Allium cepa</i> (AAF18998)	290	kmRWS*LPvd
<i>Arabidopsis thaliana</i> (APS3)	296	kmRWS*LPvd
<i>Arabidopsis thaliana</i> (APS4)	298	kmRWS*LPvd
<i>Brassica juncea</i>	292	kmRWS*LPvd
<i>Brassica napus</i>	290	kmRWS*LPvd
<i>Camellia sinensis</i>	292	kmRWS*LPvd
<i>Oryza sativa</i>	307	kmRWS*LPvd
<i>Zea mays</i>	319	kmRWS*LPvd

Figure 4.9. Consensus 14-3-3 binding sites in the translated sequences of the ATPS gene sequences indicated.

The phosphoserine-containing binding sequence is present in ATPS sequences from several plant species listed in NCBI: ATPS from *Arabidopsis thaliana* (APS3, NP_193204 and APS4, NP_199191), ATPS from *Brassica juncea* (CAA11417), ATPS from *Brassica napus* (AAB53100), ATPS from *Camellia sinensis* (ABE01402), ATPS from *Oryza sativa* (plastidic form, BAA36274) and ATPS from *Zea mays* (AAB94542). The 14-3-3 proteins consensus binding domain is shown for comparison.

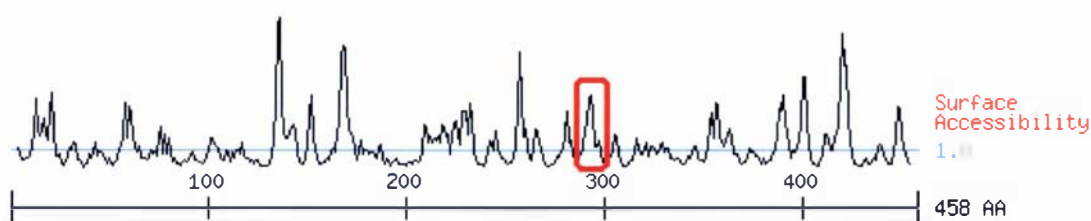


Figure 4.10. Plot of the predicted surface accessibility at each residue of the translated ATPS sequence of onion.

Using Scansite, the surface accessibility of each residue of the ATPS sequence (AAL61615) are predicted. The higher the peak, the more accessible the residue will be for other proteins. The ruler at the base of the figure denotes the residue number. The red square represents the putative 14-3-3 protein binding motif at Ser₂₈₈ (see Figure 4.9).

Using Scansite for the *OAS*-TL sequence, the domain SGGERYMS*TQLFNEV, located outside the already known domains of PALP (residues 27 to 317), pyridoxal redox (designated Pyr_redox_2; residues 194 to 226), methyltransferase (designated Methyltransf_12; residues 196 to 234) and Smg4_UPF3 (residues 208 to 220) (Figure 4.11, domains), was recognized as a putative 14-3-3 Mode 1 binding site (score 0.6081) when the serine residue at position 323 is phosphorylated (Figure 4.11, predicted sites: pST_bind). The calmodulin dependent kinase 2 (score 0.5266) and Akt kinase (score 0.6914) are predicted as the putative phosphorylating kinase(s) of this Ser₃₂₃ residue (Figure 4.11, predicted sites: Baso_ST_kin).

An analysis of surface accessibility of the *OAS*-TL sequence shows that this Ser₃₂₃ residue, with an accessibility score of 0.982, seems unlikely to be detected by protein kinases and then recognized by 14-3-3 proteins (Figure 4.11, red arrow). However, as kinases and 14-3-3 proteins recognize the surrounding motif sequence rather than the phosphorylated site itself, the Ser₃₂₃ surrounding sequences, scoring over 1.0, could be considered accessible (Figure 4.11, surface accessibility prediction, red bracket). This Ser₃₂₃ is likely to be first phosphorylated and then targeted by 14-3-3 proteins. However, using the NetPhos programme, this Ser₃₂₃ residue scored 0.112.

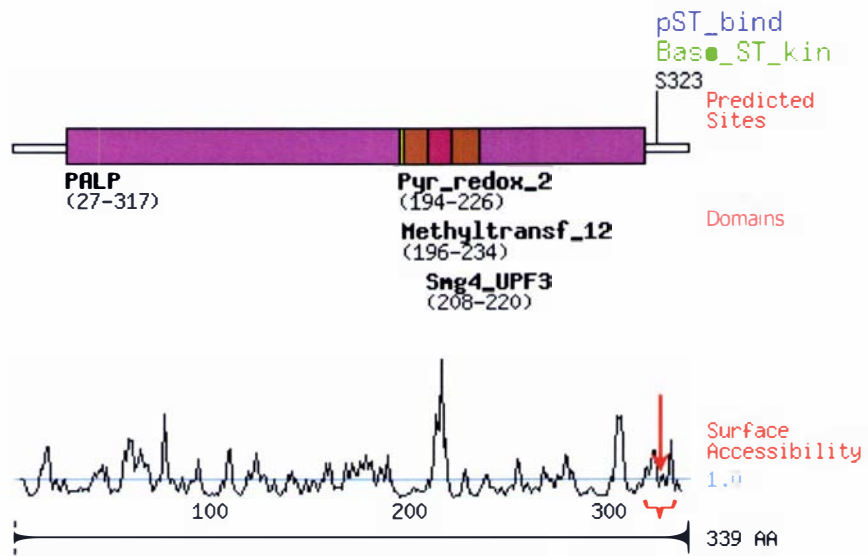


Figure 4.11. Plot of the known domains, the predicted 14-3-3 site and the predicted surface accessibility at each residue of the translated *OAS-TL* sequence from onion.

Using Scansite, the domains of the *OAS-TL* sequence and the surface accessibility of each residue are predicted. The *OAS-TL* sequence, with four known domains, as indicated, displayed one putative 14-3-3 binding site, located outside the known domains. Also, this 14-3-3 binding site was detected if S₃₂₃ is phosphorylated, putatively by pST_bind or Baso_ST_kin protein kinases, as indicated. The ruler at the base of the figure denotes the residue number. The red square represents the putative 14-3-3 binding motif.

4.2.2. Production of the recombinant 14-3-3 proteins BMH1 and BMH2 from *Saccharomyces cerevisiae* in *E. coli*

The genes coding for the two isoforms of 14-3-3 proteins from *S. cerevisiae*, BMH1 and BMH2, were introduced into *E. coli* and used to produce large quantities of the 14-3-3 proteins that will be necessary for affinity purification steps (sections 4.2.5., 4.2.6. and 4.2.7.). The use of these two protein isoforms has already proven useful for the detection of several 14-3-3 protein targets in plants in previous studies (Moorhead *et al.*, 1999), and were thus used in the present thesis.

Prior to the production of recombinant BMH1 and BMH2 proteins, the BMH1 and BMH2 genes were sequenced to confirm their identity. The sequence coding for the recombinant BMH1 was 941 bp long and the sequence for the recombinant BMH2 was 840 bp (Appendix XI). The sequences started with the ATG start codon and terminated with the GAT stop codon. They also included six repeated CAT codons, corresponding to the (His)₆-tag.

BLAST analysis confirmed that the sequences matched to the BMH1 and BMH2 14-3-3 proteins from *S. cerevisiae* (Appendix XII). The recombinant BMH1 scored 99% in terms of identity, with a probability value of 0.0, to the BMH1 gene from *S. cerevisiae*. The recombinant BMH2 scored 100% in terms of identity, with a probability value of 0.0, to the BMH2 gene of *S. cerevisiae*. The BLAST results also showed that the total length of the genes from *S. cerevisiae* were 1284 bp for BMH1 and 1312 bp for BMH2, respectively.

4.2.3. Production and purification of recombinant BMH1 and BMH2

The genes coding for BMH1 and BMH2 from *S. cerevisiae*, were expressed in *E. coli*, and the recombinant proteins, tagged with (His)₆ were purified from other *E. coli* proteins by IMAC using the HisTrap column. Nickel ions were attached to this column and provided a mechanism by which the BMH1 and BMH2 proteins bound to the column through the (His)₆ tag, while other *E. coli* proteins eluted out without binding. Unspecific binding was limited by including 20 mM imidazole to the binding buffer. To ensure the efficiency of the purification, 1D-PAGE was conducted to separate proteins from the collected fractions (Figure 4.12). The CBB staining of the gels

revealed that the majority of *E. coli* proteins did not bind to the column as they eluted before the beginning of the gradient. Some proteins did bind but eluted before the recombinant proteins. In the example shown, BMH1 eluted out of the column between 132 and 180 mM imidazole (Figure 4.12, BMH1), and BMH2 between 180 and 212 mM (Figure 4.12, BMH2). The separated recombinant BMH1 and BMH2 proteins were thus purified with little contamination from other proteins.

Finally the theoretical molecular mass of the recombinant proteins was estimated after translation of the DNA sequence to the amino acid sequence. The molecular mass were estimated to be 37.57 kDa⁵ for BMH1 and 33.86 kDa⁵ for BMH2 and confirmed that the recombinant proteins detected after SDS-PAGE were the recombinant BMH1 and BMH2 (Figure 4.12).

⁵ExPaSy tools <http://expasy.org/tools/dna.html>

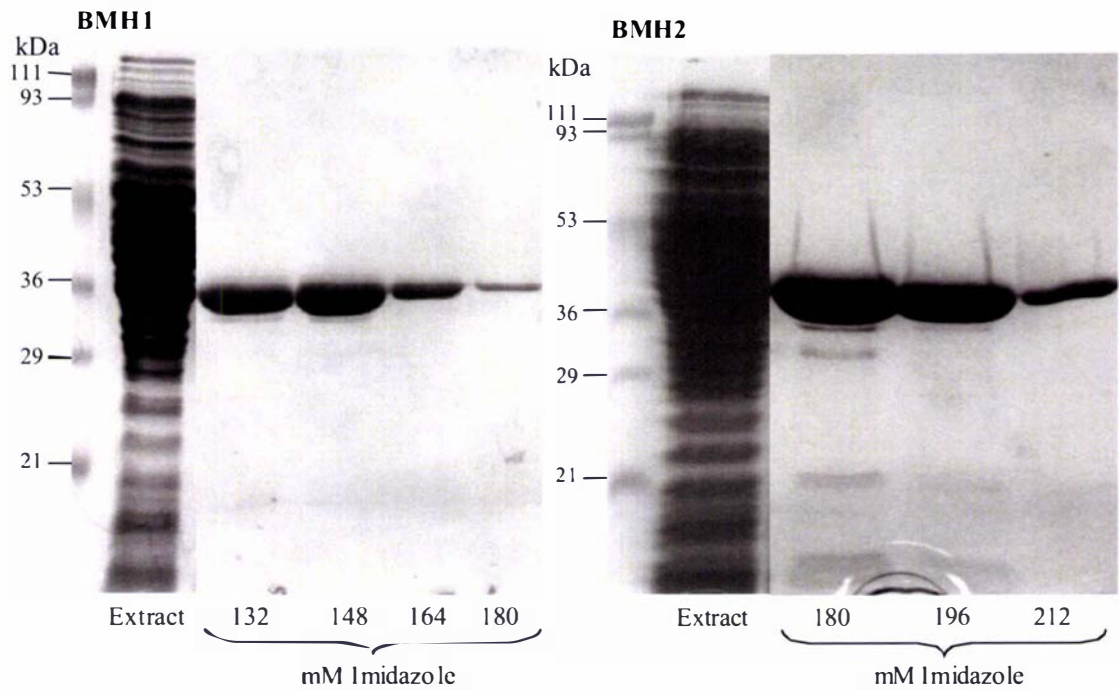


Figure 4.12. Detection of total proteins after IMAC purification of BMH1 and BMH2 recombinant proteins, as indicated, synthesized in *E.coli* using.

The recombinant BMH1 and BMH2, tagged with (His)₆, were synthesized in *E. coli* and purified using IMAC with Ni²⁺ ions. A linear gradient of 20 to 500 mM imidazole was applied over 80 min to elute proteins out of the column. BMH1 eluted between 132 and 180 mM imidazole and BMH2 eluted between 180 and 212 mM imidazole, as indicated. Detection of total protein by CBB. Extract: Total *E.coli* lysate prior to chromatography through the IMAC.

4.2.4. Detection of 14-3-3 proteins within onion extracts

To confirm that 14-3-3 proteins are present in onion tissues, western analyses were carried out using an anti-14-3-3 antibody raised against 14-3-3 proteins purified from spinach leaves (Moorhead *et al.*, 1999) (gift from Professor MacKintosh). The antibody is known to react with a variety of plant genera and recognizes several, or possibly all, plant 14-3-3 isoforms (Hartill *et al.*, 2006). One microgram of the purified recombinant BMH1 and BMH2 14-3-3 proteins of yeast was also loaded onto the gel as a control.

In the first experiment, the occurrence of 14-3-3 proteins in onion was assessed using chloroplast, leaf and root extracts from seedlings of TG and W grown in the S-sufficient media (Figure 4.13). The 14-3-3 proteins were clearly identified as ~30 kDa proteins in leaf and root extracts, but not in the chloroplast extracts. The recombinant BMH1 and BMH2 appeared as a single band at ~35 kDa, in accordance with the molecular mass predictions (section 4.2.3). In the tissues that were recognized, 14-3-3 abundance was stronger in roots than in other tissues. In root extracts, two closely migrating bands were detected suggesting the presence of isoforms.

Since no free (unbound) 14-3-3 proteins were identified in chloroplast extracts from onion seedlings, although they have been identified in this organelle in *Arabidopsis*, pea and tobacco (Sehnke *et al.*, 2000; Sehnke *et al.*, 2001; Riedel *et al.*, 2001), a second experiment was carried out using chloroplast, leaf, root and bulb extracts from mature plants of TG and W grown in either the S-sufficient (S⁺) or the S-deficient (S⁻) media. The western analyses confirmed the presence of the 14-3-3 proteins in leaf and root extracts and also revealed their occurrence in bulb extracts (Figure 4.14). However, again no 14-3-3 protein was visualized in chloroplast extracts.

In mature plants, 14-3-3 proteins were more abundant in extracts from W than from TG. No variation of signal intensity was detected in root extracts from both TG and W grown in either the S⁺ or the S⁻ media. However, in both genotypes, the abundance of 14-3-3 proteins decreased with the S⁻ media in leaf extracts, while their abundance increased in bulb extracts from plants grown in the S⁻ media.

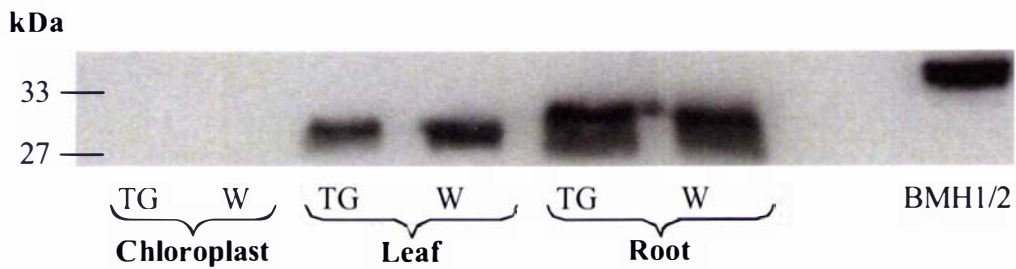


Figure 4.13. Detection of 14-3-3 proteins in extracts from seedlings of TG and W, as indicated, grown in S-sufficient media.

Protein (10 μ g) extracted from chloroplasts, leaves or roots, as indicated, from seedlings of TG or W, as indicated, grown in the S sufficient media were separated by 1D-PAGE and the abundance of 14-3-3 determined by western analyses using chemiluminescence. The recombinant BMH1 and BMH2 (1 μ g) were also included as a control.

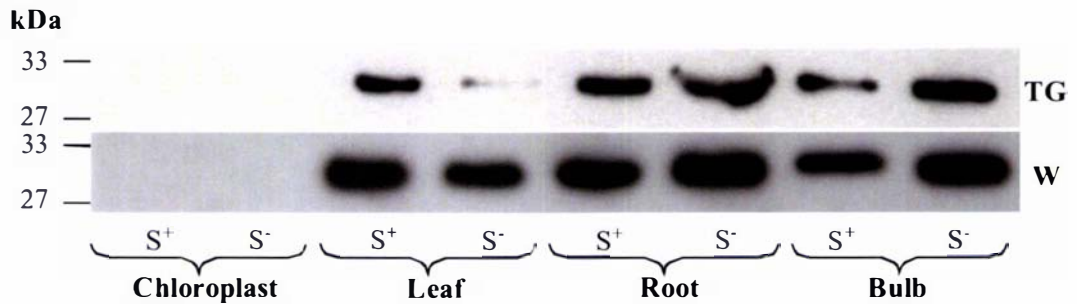


Figure 4.14. Detection of 14-3-3 proteins in extracts from mature plants of TG and W, as indicated, grown in S-sufficient (S⁺) or S-deficient (S⁻) media, as indicated.

Protein (10 μ g) extracted from chloroplasts, leaves, roots or bulbs, as indicated, were separated by 1D-PAGE and the abundance of 14-3-3 determined by western analyses using chemiluminescence.

4.2.5. Fluorescence-based detection of phosphoproteins from chloroplast extracts binding to 14-3-3 proteins

In order to verify the binding specificity of the 14-3-3 proteins to phosphoproteins, chloroplast extracts from mature plants of W only, grown in either the S-sufficient (S^+) or the S-deficient (S^-) media, were treated with MgATP and NaF to promote phosphorylation (P^+) and incubated with the recombinant BMH1 and BMH2 proteins coupled to Sepharose (designated 14-3-3-Sepharose) or BSA-Sepharose beads. After extensive washing, proteins that did bound to the 14-3-3-Sepharose (designated 14-3-3 pull-downs) or BSA-Sepharose (designated BSA pull-downs) beads were collected by centrifugation and resuspended in reducing buffer. Then, they were fractionated by 1D-PAGE, and stained with Pro-Q DPS (Figure 4.15, phosphoproteins) and subsequently with silver nitrate staining (Figure 4.15, total proteins).

The results showed that prior to the affinity chromatography, more phosphoproteins and total proteins were detected in extracts from plants grown in the S^- media. In the non-purified extracts, a protein, with a molecular mass of ~ 50 kDa, possibly representing RuBisCO, was detected with Pro-Q DPS (Figure 4.15, extract). The abundance of this protein was greater in chloroplast extracts from plants grown in the S^- media.

Following the purification of extracts with 14-3-3-Sepharose, numerous proteins were detected (Figure 4.15, total proteins) but only a portion of them were stained with Pro-Q DPS (Figure 4.15, phosphoproteins). Different proteins were visualized in chloroplast extracts from mature plants of W grown in the S^+ and the S^- media, suggesting the influence of the growing conditions on the binding to 14-3-3 proteins. More bands were stained in extracts from plants grown in the S^- media, but these were less abundant when compared with the fewer bands, but representing more abundant proteins, in extracts from plants grown in the S^+ media.

Following the purification of chloroplast extracts with BSA-Sepharose, some proteins were detected, but hardly any of these proteins were phosphorylated. BSA itself was detected at ~ 66 kDa after Pro-Q DPS. Following silver staining, more proteins were visualized in extracts from mature plants of W grown in the S^+ than in the S^- media.

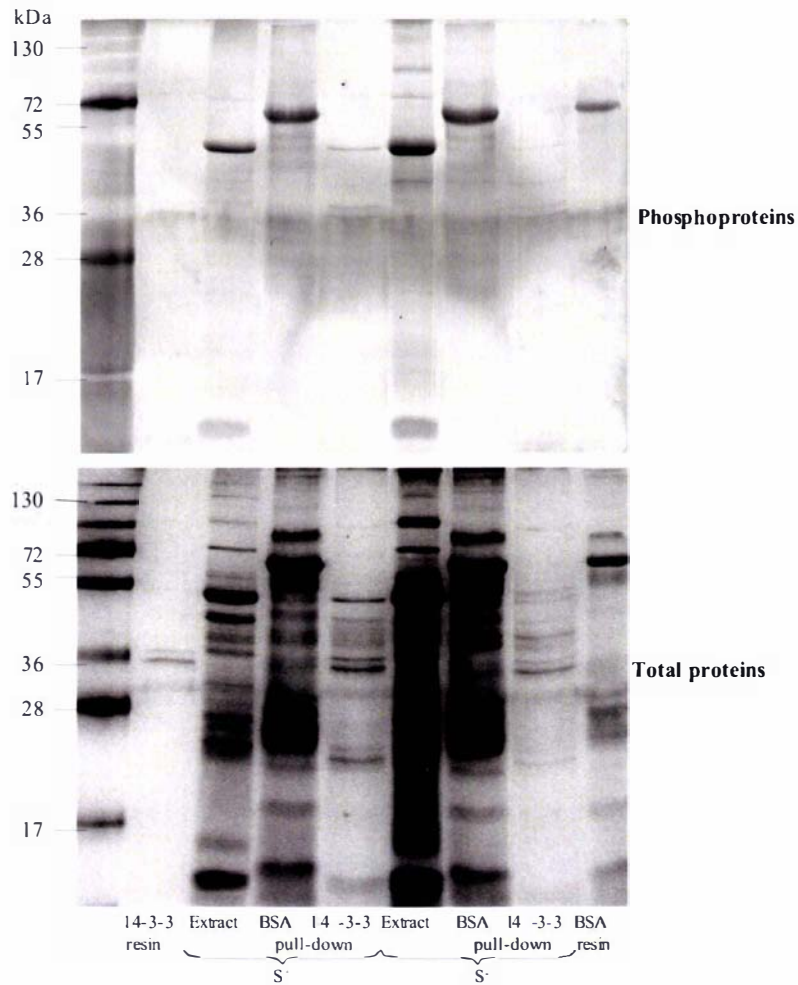


Figure 4.15. Detection of proteins after purification of chloroplast extracts from mature plants of *W* grown in the S-sufficient (S^+) or the S-deficient media (S^-), as indicated, with 14-3-3-Sepharose or BSA-Sepharose, as indicated.

Proteins previously treated with MgATP and NaF to promote protein phosphorylation were incubated with 14-3-3-Sepharose or BSA-Sepharose. After extensive washing, pull-down proteins were resuspended in reducing buffer and separated by 1D-PAGE. The gel was first stained with the phosphospecific dye Pro-Q DPS (designated phosphoproteins) and subsequently with silver nitrate general protein stain (designated total proteins). Extract: proteins before the binding to 14-3-3-Sepharose or BSA-Sepharose.

4.2.6. Detection of complex between S assimilation enzymes and 14-3-3 proteins by pull-down

In order to detect the formation of complexes between the S assimilation enzymes and 14-3-3 proteins, and to identify the conditions affecting the formation of these putative complexes, chloroplast extracts were purified by affinity chromatography with 14-3-3-Sepharose. Proteins that attached to 14-3-3-Sepharose were collected by pull-down centrifugation of the resin, resuspended in reducing buffer and separated by 1D-PAGE.

As an initial attempt, only proteins from seedlings of *W* grown in either the S^+ or the S^- media were used. Prior to their purification by affinity chromatography, the proteins were treated to promote phosphorylation (P^+) or dephosphorylation (P^-). During the P^+ treatment, proteins were incubated with MgATP, and the phosphatase inhibitors, OKA, EDTA and EGTA. To promote dephosphorylation, the phosphatase PP2A was added to the extracts. Both incubations were carried out for 15 min at 30°C. Untreated extracts, which were not incubated at 30°C, were also purified with 14-3-3-Sepharose.

Initially, ATPS was detected only after the purification of untreated proteins from plants grown in the S^+ or the S^- media (Figure 4.16). In plants grown in the S^+ media, ATPS abundance was similar after purification of untreated extracts previously incubated at 30°C or on ice. In untreated extracts from plants grown in the S^- media, ATPS abundance decreased with the 30°C incubation.

The abundance of APSR was visualized only after the purification of untreated extracts from plants grown in the S^+ or the S^- media. In plants grown in the S^+ media, the abundance of APSR was greater in extracts that were previously incubated at 30°C than on ice. On the contrary, in plants grown in the S^- media, the abundance of APSR decreased in untreated extracts that were incubated at 30°C.

SiR was also detected only after the purification of untreated extracts from plants grown in the S^+ or the S^- media. The abundance of the enzyme in purified extracts did not fluctuate with either the incubation at 30°C or on ice, in both plants grown in the S^+ or the S^- media.

The enzyme OAS-TL was not detected in any of the samples after affinity purification of chloroplast extracts with the 14-3-3 proteins.

The results of this initial attempt suggested that ATPS, APSR and SiR might be able to form a complex with 14-3-3 proteins. The results also suggested a negative influence of the incubation at 30°C, which may explain partially the lack of detection or the very low detection of the S assimilation enzymes after the P⁺ treatment. Previous studies (Athwal *et al.*, 2000; Shen and Huber, 2006) showed the importance of divalent cations in 14-3-3's complex formation, so it is also possible that EDTA and EGTA, used for their inhibitory role on phosphatases, limit the binding of the S assimilation enzymes to 14-3-3-Sepharose. Since 14-3-3 proteins bind almost exclusively to phosphorylated proteins (Muslin *et al.*, 1996; Yaffe *et al.*, 1997), the lack of detection of either enzyme from purified extracts previously treated to promote dephosphorylation may be expected. Further, there was also some uncertainty with the activity of batches of PP2A.

Thus in the second attempt, to clarify the results, the purification was carried out only with extracts previously treated to promote protein phosphorylation using NaF, a broad spectrum inhibitor of protein phosphatases. All extracts were incubated for 30 min on ice prior to the affinity purification. To control the specificity of the binding, parallel incubations were carried out with BSA-Sepharose instead of 14-3-3-Sepharose. This experiment was carried out on a large scale using chloroplast extracts from both seedlings and mature plants of both TG and W grown in either the S⁺ or the S⁻ media.

Following the purification with 14-3-3-Sepharose, the abundance of ATPS was visualized as a faint band, only in extracts from seedlings of TG and mature plants of W (Figure 4.17, designated 14-3-3). In TG, the abundance of ATPS was greater after the purification of extracts from seedlings grown in the S⁺ media. In these extracts, ATPS was visualized as two closely migrating bands. In W, the enzyme abundance was not influenced by the S supply and was detected as a single band. Following the purification with BSA-Sepharose of extracts from both TG and W, ATPS was also identified (Figure 4.17, designated BSA). The abundance of ATPS was similar in extracts purified using 14-3-3-Sepharose or BSA-Sepharose.

The enzyme APSR was detected after the purification with 14-3-3-Sepharose of extracts from both seedlings and mature plants of both TG and W (Figure 4.18, designated 14-3-3). In seedlings of TG and mature plants of W, the abundance of APSR was greater in extracts from plants grown in the S^+ media. On the contrary, in seedlings of W and mature plants of TG, the enzyme abundance was more marked in extracts from plants grown in the S^- media. The enzyme was also detected after the purification with BSA-Sepharose of extracts from both seedlings and mature plants of TG and W (Figure 4.18, designated BSA). In all extracts, the abundance of APSR was more intense in extracts purified with BSA-Sepharose than with 14-3-3-Sepharose, with the exception of extracts from seedlings of W grown in the S^- media. In all purifications, the enzyme was visualized as a single band.

In both TG and W, SiR was not detected after the purification of extracts with either 14-3-3-Sepharose or BSA-Sepharose (Figure 4.19). Extracts from mature plants of W were not tested due to the low availability of the extracts.

Finally, because of the low affinity of the *OAS-TL* antibody over the form located in chloroplasts, untreated leaf extracts from seedlings of W grown in the S^+ or the S^- media were purified with either 14-3-3-Sepharose or BSA-Sepharose to determine the occurrence of binding between *OAS-TL* and 14-3-3 proteins. Western analyses were also carried out to detect the other S assimilation enzymes after the purification of these leaf extracts.

The abundance of ATPS could not be detected after the purification of leaf extracts from seedlings of W grown in either the S^+ or the S^- media with 14-3-3-Sepharose or BSA-Sepharose (Figure 4.20, ATPS).

The enzyme APSR was present in the leaf extracts from seedlings of W grown in the S^+ or the S^- media that were purified with either 14-3-3-Sepharose or BSA-Sepharose (Figure 4.20, APSR).

Following the purification with 14-3-3-Sepharose, SiR was not detected in extracts from seedlings of W grown in either the S^+ or the S^- media (Figure 4.20, SiR, designated 14-3-3). Following the purification with BSA-Sepharose, SiR was visualized as a faint band in extracts from seedlings of W grown in either the S^+ or the S^- media (Figure 4.20, SiR, designated BSA). The abundance of the enzyme was greater in extracts from plants grown in the S^+ media.

Following the purification with 14-3-3-Sepharose, *OAS-TL* was detected in leaf extracts from seedlings of *W* grown in either the S^+ or the S^- media (Figure 4.20, *OAS-TL*, designated 14-3-3). However, the enzyme could not be detected following the purification with BSA-Sepharose of leaf extracts from seedlings of *W* grown in the S^+ or the S^- media (Figure 4.20, *OAS-TL*, designated BSA). The results suggested that *OAS-TL* may be able to form a complex with 14-3-3 proteins, and that the complex was not influenced by the S supply.

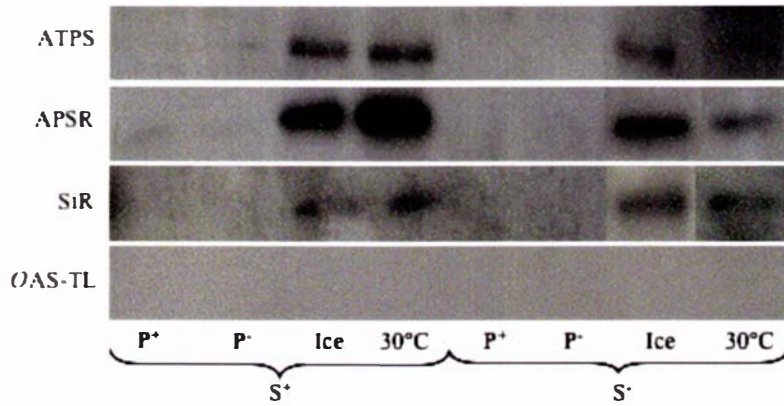


Figure 4.16. Detection of ATPS, APSR, SiR or OAS-TL, as indicated, in chloroplast extracts from seedlings of *W* grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, previously treated to promote phosphorylation (P^+) or dephosphorylation (P^-) and purified by affinity chromatography with 14-3-3-Sepharose.

Proteins were treated either with MgATP and OKA, EDTA and EGTA to promote phosphorylation (P^+) or with PP2A to promote dephosphorylation (P^-), as indicated, and purified by affinity chromatography. Parallel incubations were carried out with untreated proteins, previously incubated for 15 min at 30°C or conserved on ice. After extensive washing, pull-down proteins were resuspended in reducing buffer, separated by 1D-PAGE and the S assimilation enzymes detected by western analyses using chemiluminescence.

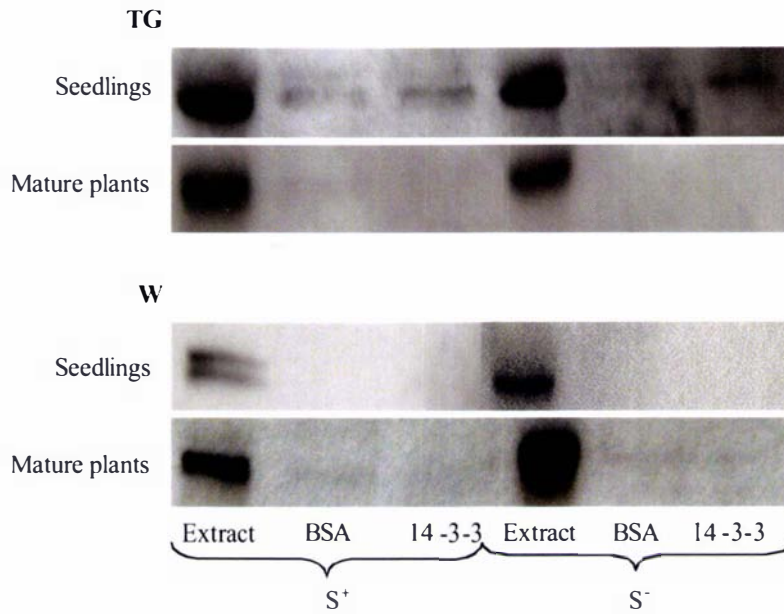


Figure 4.17. Detection of ATPS in chloroplast extracts from seedlings or mature plants, as indicated, of TG or W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, previously treated to promote phosphorylation and purified by affinity chromatography with 14-3-3-Sepharose or BSA-Sepharose, as indicated.

Proteins were treated with MgATP and NaF to promote phosphorylation (P^+) and purified by affinity chromatography. After extensive washing, pull-down proteins were resuspended in reducing buffer and separated by 1D-PAGE and ATPS detected by western analyses using chemiluminescence.

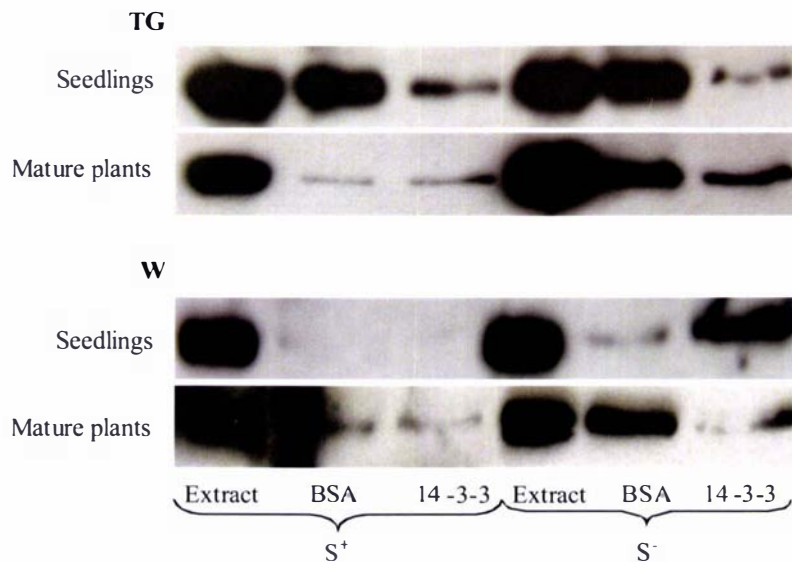


Figure 4.18. Detection of APSR in chloroplast extracts from seedlings or mature plants, as indicated, of TG or W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, previously treated to promote phosphorylation and purified by affinity chromatography with 14-3-3-Sepharose or BSA-Sepharose, as indicated.

Proteins were treated with MgATP and NaF to promote phosphorylation (P^+) and purified by affinity chromatography. After extensive washing, pull-down proteins were resuspended in reducing buffer and separated by 1D-PAGE and APSR detected by western analyses using chemiluminescence.

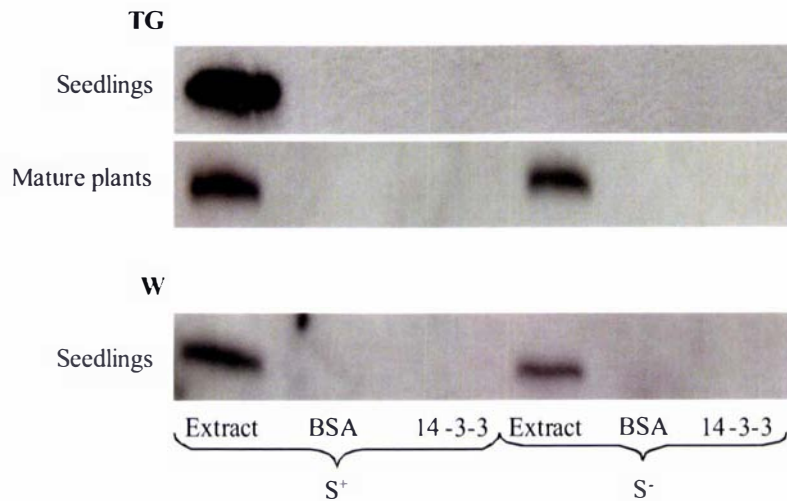


Figure 4.19. Detection of SiR in chloroplast extracts from seedlings or mature plants, as indicated, of TG or W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, previously treated to promote phosphorylation and purified by affinity chromatography with 14-3-3-Sepharose or BSA-Sepharose, as indicated.

Proteins were treated with MgATP and NaF to promote phosphorylation (P^+) and purified by affinity chromatography. After extensive washing, pull-down proteins were resuspended in reducing buffer and separated by 1D-PAGE and SiR detected by western analyses using chemiluminescence.

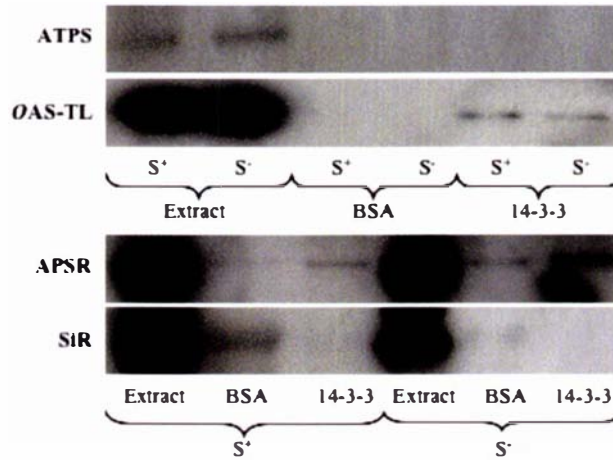


Figure 4.20. Detection of ATPS, OAS-TL, APSR or SiR in leaf extracts from seedlings of W grown in the S-sufficient (S⁺) or the S-deficient (S⁻) media, as indicated, previously treated to promote phosphorylation and purified by affinity chromatography with 14-3-3-Sepharose or BSA-Sepharose, as indicated.

Proteins were treated with MgATP and NaF to promote phosphorylation (P⁺) and purified by affinity chromatography. After extensive washing, pull-down proteins were resuspended in reducing buffer and separated by 1D-PAGE. Then, ATPS, APSR, SiR or OAS-TL, as indicated, were detected by western analyses using chemiluminescence.

5. DISCUSSION

While most studies to understand the regulatory mechanism of the S assimilation pathway have been carried out using the model plant *Arabidopsis*, and spinach, *Lemna minor*, *Physcomitrella patens*, or even poplars (reviewed in Kopriva, 2006), little is known about the mechanisms occurring in S accumulating plant species such as onion. In addition, most of the studies that have been carried out using model species have focussed on the molecular mechanisms of the regulation of the S assimilation, and more particularly on the enzyme APSR, which is thought to be a major regulatory point in the pathway. Some work has also been carried out to understand the formation of the cysteine synthase complex formed by the association of the SAT and OAS-TL enzymes.

In this thesis however, the primary focus was to investigate the regulation of ATPS in the S accumulating species onion, at the biochemical level where appropriate, and because antibodies were available, the abundance of APSR, SiR and OAS-TL were also studied in response to S supply.

For this, two genotypes, TG and W, were cultivated in the S-sufficient (S^+) or the S-deficient (S^-) liquid media. TG is deemed a mild line while W is deemed a pungent line, in terms of abundance of S containing compounds (McCallum *et al.*, 2006a). Plants were harvested at the seedling stage (7 week old) or the mature plant stage, when a bulb had already started to form (4 month old). Results of this investigation suggested the presence of ATPS and APSR as isoforms. Thus, chloroplast extracts were purified by anion exchange chromatography and 2D-PAGE to further characterize these proteins. Finally preliminary studies on the S assimilation enzymes, particularly ATPS, as phosphorylproteins were undertaken as well as determining any interactions with 14-3-3 proteins.

5.1. Influence of the S supply on onion growth and development

To determine the influence of the S supply on the onion growth and development, total biomass (as fresh weight), and leaf and root weights were compared in seedlings and mature plants grown in either the S^+ or the S^- media. In mature plants, bulb weight was also measured.

The results shown in section 3.1.1 revealed the negative influence of S deprivation with a significant decreased in total biomass (as fresh weights), and of the different organ fresh weights (Figures 3.1 and 3.2). Preliminary studies carried out in onion with TG and W grown in liquid media also revealed significant decreases of both leaf and bulb weights with S deprivation but only in mature plants (McCallum *et al.*, 2006b).

Similar decreases in fresh bulb weight with S deprivation were also measured in hydroponically grown onion ('Granex 33') in a temperature controlled greenhouse for five months (Coolong and Randle, 2003). For the high S treatment, the plants were grown in $125 \text{ mg L}^{-1} \text{ SO}_4^{2-}$ while in the low S treatment, only $5 \text{ mg L}^{-1} \text{ SO}_4^{2-}$ was included to the Hoagland media. These concentrations are similar to those used in this thesis. Previous studies also demonstrated the negative influence of S deprivation on *Arabidopsis* seedlings (ecotype 'Columbia') when grown for four weeks in a growth chamber on sterile agarose with either $915.05 \text{ } \mu\text{m L}^{-1} \text{ (S}^+)$ or $100 \text{ } \mu\text{m L}^{-1} \text{ (S}^-)$ in a half-full strength Hoagland media (Nikiforova *et al.*, 2003). In the same studies, *Arabidopsis* seedlings also displayed retarded growth, pale-green to pale-yellow leaves and excess development of the roots system when maintained in the S^- media. In this thesis, similar changes were observed in mature onion plants that displayed retarded plant growth, pale-green leaves, smaller bulbs, the elongation of roots and the development of more lateral roots (Nikiforova *et al.*, 2003) (Figures 3.3 and 3.4). Another study with *Arabidopsis* also revealed the influence of S withdrawal on the development of primary and lateral roots, thus, modifying the overall root architecture when cultured with no S supply (Kutz *et al.*, 2002).

In seedlings grown in the S^+ media, total biomass, leaf and root weights were significantly superior in TG than in W. Total biomass, root and bulb weights were also higher in plants grown in the S^+ media, but this was not significant. However, data from previous studies carried out with TG and W grown in liquid media did not reveal any difference in leaf and bulb weights between the two genotypes (McCallum *et al.*, 2006b).

The results of the physiological analysis revealed a negative influence of S deprivation on plant growth and development. The statistical analysis of the differences in total

biomass and tissues weights also showed that these effects were more marked in TG than in W.

5.2. Characterisation of ATPS in the two onion genotypes

To characterise the regulation of ATPS in onion, a S accumulating species, enzyme activity in both TG and W was compared as these cultivars accumulate different quantities of S compounds. Little is known about the regulation of the enzyme during long-term S starvation particularly in S accumulating species, as studies are generally carried out using *Arabidopsis* or spinach over short periods (up to four days) of S stress. In addition, in onion Southern blot analysis using a cDNA complementary to the ATPS gene sequence revealed only one band, suggesting that only one gene coding for the enzyme ATPS is present in onion (Jakse, personal communication). As well, a QTL for pungency has been mapped closely to the ATPS gene during genomic studies using a population from a cross between TG and W (McCallum *et al.*, 2006a). Thus attention to ATPS may reveal some insights into differences in pungency between TG and W.

The effects of plant development on ATPS activity and abundance

In onions, ATPS is located in all tissues examined as both enzyme activity and enzyme abundance (as determined by western analysis) were detected in chloroplast, leaf, root and bulb extracts from plants of TG and W. In accordance with previous studies, ATPS has been detected in plastids (i.e. in chloroplasts) in spinach (Lunn *et al.*, 1990; Renosto *et al.*, 1993) and *Arabidopsis* (Rotte, 1998; Rotte and Leustek, 2000). In roots, the activity was found in proplastids of pea (Brunold and Suter, 1989).

In roots from both TG and W, ATPS activity significantly reduced with maturation (Figures 3.5 and 3.6). In TG, a similar trend to lower activity in mature plants was observed in chloroplasts, while in leaves, the results showed a tendency towards higher ATPS activity with maturation. In chloroplasts and leaves from W, ATPS activity also decreased with plant maturation, but these differences were not significant. In common with these results, a previous experiment also measured a decrease in ATPS activity with plant maturation in chloroplasts from both TG and W grown in liquid media and a temperature controlled greenhouse (McCallum *et al.*, 2006b).

These results are also in agreement with previous studies in higher plants such as *Arabidopsis*, pea or soybean, showing that ATPS activity in leaves is developmentally regulated and declines with maturation (Adams and Rinne, 1969; Bell *et al.*, 1995; Rotte and Leustek, 2000; von Arb and Brunold, 1986). In *Arabidopsis*, subcellular fractionation experiments have shown that the chloroplastic ATPS isoforms decrease as leaves mature (Rotte and Leustek, 2000).

In seedlings of TG, higher ATPS activity was measured in chloroplasts when compared to leaves ($p=0.077$), while in mature plants, higher enzyme activity was measured in leaves when compared with chloroplasts (Figure 3.5 and 3.6). It is likely that the differences in ATPS activity between chloroplasts and leaves in seedlings of TG would become significant by increasing the number of repetitions. In seedlings and mature plants of W, similar ATPS activity was measured in both tissues. In TG, over the period from seedlings to mature plants, ATPS activity in chloroplasts declined 1.5-fold, while in leaves it increased 1.5-fold. Previous studies in *Arabidopsis* revealed that ATPS activity declines 2.0-fold in chloroplasts as plants matured, while the activity of the cytosolic form isolated by subcellular fractionation increases 3.5-fold with maturation (Rotte and Leustek, 2000). It is thus believed that the cytosolic isoform of ATPS has a specialized function unrelated to S assimilation (Rotte and Leustek, 2000).

Given that the activity measured in onion leaves would consist of both the cytosolic and chloroplastic ATPS activity, and as the chloroplastic activity in TG decreased with maturation, the results in this thesis suggest that the cytosolic activity might have increased with maturation, keeping the total leaf activity constant. This implies that the activity stays high in leaves of TG during maturation to promote the abundance of S secondary compounds like ACSOs. In seedlings of TG, ATPS activity was higher in chloroplasts than leaves, and decreased with maturation. The results also might suggest that the total enzyme activity in seedlings is primarily associated with the S assimilation pathway.

However, as the activity did not decrease with maturation in chloroplasts isolated from W, another level of regulation may occur in the pungent line, possibly underlining an association with ATPS activity in chloroplasts and onion pungency. This indicates that

the activity in chloroplasts from mature plants of W might be up regulated to increase the capacity in S assimilation and reduction.

Previous studies in spinach have suggested that higher ATPS activity occurred in photosynthetic tissues, particularly in chloroplasts, as the enzyme requires ATP for its activity and as chloroplasts are the site of ATP (and NADPH generation necessary for the next steps for the sulfur assimilation pathway) (Renosto *et al.*, 1993). In *Arabidopsis*, the activity in roots represents only around 30% of the activity measured in leaves (Lee, 1999). However in TG seedlings, ATPS activity was significantly higher in roots than in leaves ($p=0.024$). A similar trend was also observed in W, but this difference was not significant. In roots ATPS activity represented around 190% and 130% of the activity measured in leaves from TG and W, respectively. The enzyme activity declined in roots with maturation to 86% and 52% of the activity in leaves in TG and W, respectively.

The results revealed that ATPS activity in roots from onion was higher than in *Arabidopsis*, particularly in seedlings, suggesting the possibility of a different regulation in S accumulating species. In a review comparing the regulation of S assimilation in several plant species, Kopriva (2006) stated that knowledge gained from *Arabidopsis* is not always transferable to other species. This difference observed between onion and *Arabidopsis* suggests that ATPS activity may be up regulated in the entire onion plant, thus increasing the rate of S assimilation. Also, the results showed a significant decrease in ATPS activity in roots with plant maturation, which is similar to the trend towards down regulation measured in chloroplast, suggesting that ATPS activity in roots is probably involved in plant growth. However, in this study, ATPS activity was expressed per mg of protein, and variations in the ratio ATPS:total protein are likely between tissues, particularly as the abundant enzyme RuBisCO, involved in photosynthesis (Berg *et al.*, 2002), is not present in roots. Thus, it is likely that the ratio of root activity: total activity is lower than predicted in onions, as well as in *Arabidopsis*.

Fractionation of chloroplasts from TG by gradient-PAGE revealed an increase in ATPS abundance with plant maturation (Figure 3.9). This gel separation also indicated the possibility of two forms of ATPS in mature plants, based on differences in gel mobility,

while only one was visualized in seedlings. In W, ATPS abundance in chloroplasts also increased with plant maturation. However, in this case, two forms of the enzyme were observed in both seedlings and mature plants. As proteolysis was prevented, these two electrophoretic forms may indicate the presence of two ATPS isoforms in onion chloroplasts and the abundance of the form with higher molecular mass might be influenced by both the developmental stage and the plant genetic background. In chloroplasts from both TG and W, the regulation of ATPS abundance with plant maturation did not correlate with ATPS activity. This suggests that a large portion of the chloroplastic ATPS may be inactive in mature plants. In chloroplasts, therefore the difference in activity and abundance and the presence of ATPS as isoforms might reflect regulation by post-translational modifications.

Diurnal regulation of ATPS activity and abundance

To assess the effects of the light and dark cycle on the enzyme, ATPS activity and abundance were observed over the 24 h period. In leaves from W, ATPS activity increased significantly between L+1 h and L+4 h, a similar (but not significant) trend was also observed in chloroplasts and roots (Figure 3.10 and Appendix I). In leaves from TG, the enzyme activity was also up regulated at L+4 h, although this was not significant. These results provide some evidence to suggest that light influence positively ATPS activity. A similar up regulation of ATPS with illumination were previously observed in chloroplasts isolated from oat, barley and maize grown in liquid media (Passera *et al.*, 1989).

Even though ATPS activity significantly increased in leaves between L+1 h and L+4 h, no increase in the enzyme abundance was detected at the beginning of the light period. In some chloroplast and root samples from both TG and W, ATPS abundance increased at the beginning of the light period, but there was no clear correlation between enzyme activity and abundance.

In chloroplasts from both W and TG, ATPS abundance increased and remained high over the entire night period (Appendix I). In leaves from W, ATPS activity displayed a trend towards higher activity at D+0 h, which did not persist. In leaves from W, no correlation between higher activity at the beginning of the dark period and enzyme abundance was detected.

Even though the oscillations in ATPS variations over the 24 h period was much more marked in roots than in other tissues, particularly in W, the differences were not statistically significant in roots. In roots of TG and W, ATPS abundance correlated with the enzyme activity, as both displayed an increase upon dark onset (D+0 h), and declined at D+3 h.

The separation by gradient-PAGE of chloroplast and leaf extracts from both TG and W allowed the visualization of two forms of ATPS, and suggested a different time course in their diurnal regulation. One of the forms was up regulated few hours after illumination, while the abundance of the second one increased in the middle of the light period. However, further evaluation is necessary to confirm the different light and dark regulation of the putative ATPS isoforms. In *Arabidopsis*, the three mRNA APSR isoforms showed differences in their diurnal variations and the fluctuations in mRNA expression correlated with increase in APSR activity and abundance (Kopriva *et al.*, 1999). Thus, it is possible that the light and dark variations of ATPS might also be regulated at the transcriptional level.

In addition to APSR in which gene expression and enzyme activity are regulated by a diurnal rhythm, and the mRNA expression of SiR, OAS-TL and SAT mRNA is higher in green leaves than in etiolated tissues, the activity and abundance of ATPS exhibited a trend towards higher regulation with the light and dark transitions in onions, suggesting regulation by a diurnal rhythm. However, more replication and further experiments measuring ATPS activity and abundance over extended periods or time (either in continuous light or dark) are needed to confirm these conclusions.

Overview of genotype differences

One of the points of comparison made in this thesis is between two cultivars of onion that differs in terms of their contents of S containing compounds. In terms of differences between TG and W, ATPS activity was significantly higher ($p=0.044$) in leaves of W seedlings suggesting the enzyme activity might be up-regulated in the pungent line as early as the seedling stage to promote the accumulation of S compounds (Figures 3.5 and 3.6). In chloroplasts from mature plants, ATPS abundance also exhibited a trend towards higher activity in W than in TG (Figure 3.7 and 3.8). Also,

two electrophoretic forms (bands) were visualized in seedlings of W and in mature plants of both TG and W, suggesting the occurrence of ATPS as two isoforms in chloroplasts (Figure 3.9). However, the fractionation of chloroplasts by gradient-PAGE needs to be repeated to be more certain of these results.

Overall, the results revealed higher activity and abundance in W than in TG, especially at the seedling stage, possibly confirming the QTL analysis that has been shown between ATPS and pungency (McCallum *et al.*, 2006a).

The effects of S supply on ATPS activity and abundance

In terms of ATPS activity, in leaves from seedlings of W, the activity significantly decreased with S deprivation ($p=0.048$; Figure 3.5). On the contrary, in chloroplasts from seedlings of W, ATPS showed a tendency towards higher activity with S depletion (although this was not significant). A similar trend was also observed in leaves from mature plants, but none of these increases were significant. In leaves and chloroplasts from seedlings of TG, the enzyme exhibited a trend towards higher activity with the S⁻ media, but no difference was observed in mature plants.

In roots from seedlings of W, ATPS showed a tendency towards higher activity with S depletion, while no variation was measured in TG. In roots from mature plants of both TG and W, ATPS activity was strongly up-regulated with S depletion, but this was not significant ($p=0.133$ in TG; $p=0.054$ in W). However, it is likely that the differences would become significant by increasing the number of repetitions. In bulbs from mature plants of TG, ATPS activity showed a trend towards increased activity with the S⁻ media. However in W, no variation in ATPS activity was measured with S depletion in bulbs.

A similar trend was previously observed in a comparable study, where an up regulation in ATPS activity with S stress in chloroplasts from seedlings of TG, while in mature plants, the activity declined with low S supply (McCallum *et al.*, 2006b). In the same study, the results showed a trend towards down regulation with S deprivation measured in seedlings of W, and in agreement with this thesis, in mature plants of TG and W with low S supply (McCallum *et al.*, 2006b).

Although not significant, ATPS activity was increased with S deprivation 2-fold and 1.4-fold in roots from mature plants of TG and W, respectively, when the needs for S are high (Figures 3.6). A study performed on hydroponic culture of canola plants (Lappartient and Touraine, 1996) has shown a 3-fold up-regulation in ATPS activity in roots after three days of S starvation. This study also demonstrated that ATPS activity and S uptake rate are regulated by similar demand-driven processes involving the translocation of phloem-transported GSH (Lappartient and Touraine, 1996), to the roots that provides information concerning the nutritional status in the leaves. Thus, this demand-driven regulation of ATPS activity provides evidence to suggest that leaves, when sensing low S levels, might send a message to the roots, inducing a post-translational modification of ATPS and thus promote its activity in mature onions.

In higher plants, such as *Arabidopsis*, *Brassica napus*, or *Macroptilium atropurpureum*, ATPS activity and mRNA abundance have been shown to increase during short-term periods (ranging from one to four days) of S starvation (Brunold *et al.*, 1987; Bell *et al.*, 1995; Logan *et al.*, 1996). However, in *Arabidopsis* plants subjected to four weeks of S deficient conditions, symptoms of starvation such as retarded growth, pale-green leaves or extensive root system were observed, and ATPS mRNA transcripts decreased with S depression while the enzyme activity remained high (Nikiforova *et al.*, 2003). These studies suggested that ATPS activity is regulated at the transcriptional level during short-term starvation, while putative post-translational modifications might sustain the higher enzyme activity during long periods of S stress as ATPS mRNA transcripts declined.

In terms of ATPS abundance measured in this thesis, in chloroplasts and leaves from seedlings of TG, ATPS abundance increased with S deprivation, while it declined in roots (Figure 3.7 and 3.8). In chloroplasts from mature plants of TG, ATPS abundance increased with S depletion, while it decreased in leaves. No variation was observed in roots.

In chloroplasts from seedlings of W, ATPS abundance increased with the S⁻ media, while a reduction was observed in leaves (Figure 3.7 and 3.8). Similar to leaves, ATPS abundance decreased in roots with S depletion. In chloroplasts from mature plants of

W, ATPS abundance decreased with S stress, while it increased in leaves. Similar to leaves, ATPS abundance decreased in roots.

It is interesting to note that the enzyme abundance did not correlate with the up regulation in ATPS activity in roots observed in response to low S supply in both mature plants of TG and W (no variation in abundance was observed with S deprivation in any of the four repeats). However, in order to confirm the mode of regulation, it is desirable to measure the mRNA transcript levels in roots after S stress.

In a previous study, no influence of S deprivation on ATPS abundance was observed in chloroplasts from both TG and W (McCallum *et al.*, 2006b), suggesting variations between experiments. In the study by McCallum *et al.*, (2006b), plants were grown in a greenhouse with temperature control, but with natural lighting (an increase in daylength during the spring period as plants progress from seedlings to the adult stage). The differences in ATPS abundance, in common with ATPS activity between the two studies may suggest an influence of the environment on the pungent (W) line only.

Following fractionation of extracts by gradient-PAGE, ATPS was visualized as two bands in chloroplasts from seedlings of W and mature plants of both W and TG (Figure 3.9). These two bands probably represent two isoforms as precautions against protease activity were prevented to limit proteolysis of the proteins in the extracts. In seedlings of TG grown in either the S⁺ or the S⁻ media, only one form was visualized, and a mass shift was observed between the band detected in the S⁺ and the S⁻ media (Figure 3.9). In seedlings of W, two bands were visible when grown in the S⁺ media, while only one band appeared in the S⁻ media. In mature plants of both TG and W, two bands were distinguished, but some variations in their abundance was observed between the S⁺ and the S⁻ media. These results suggest an influence of both the S supply and the genetic background on the occurrence of these two isoforms. In seedlings of TG and W, the variations in mass and number of forms, respectively, detected between extracts from plants grown in either the S⁺ or the S⁻ media suggest the occurrence of a post-translational modification induced by S stress. However, repetitions are desirable to confirm this suggestion.

Dissection of the occurrence of ATPS isoforms

Extracts were purified by anion exchange chromatography to confirm the occurrence of ATPS isoforms in chloroplasts from onion. The purifications of extracts from seedlings of both TG and W grown in either the S⁺ or the S⁻ media did not reveal consistent differences between the S supply and genetic background on ATPS activity and abundance in terms of elution patterns with increasing NaCl concentration (Figures 3.14 and 3.15). In mature plants, ATPS abundance started eluting at a lower NaCl concentration and continued over a wider range of NaCl concentrations in TG than in W, but no influence of S supply in the detection of ATPS activity and abundance was observed.

In terms of differences between the developmental stages, ATPS activity and abundance were detected as eluting over a wider range of NaCl concentrations in mature plants. These results suggest that ATPS properties might be influenced by the plant development in parallel with genetic background. However, more repetitions of purifications of chloroplasts from mature plants are desirable to confirm these influences. Interestingly, ATPS was visualized as two electrophoretic forms (bands) in all anion exchange purifications with the exception of seedlings of TG and W grown in the S⁻ media. As the activity of proteases was inhibited, the two bands may suggest that ATPS is occurring as two isoforms in onion chloroplasts, the expression of which seems to be influenced by S supply and the plant maturation state (Figure 3.14 and 3.15).

The effects of plant maturation and genetic background may have been confirmed by the separation of chloroplast extracts by 2D-PAGE. This revealed an increase in ATPS abundance with plant maturation (Figure 3.18). In addition, more spots were resolved in W than in TG revealing that more isoforms might occur in the pungent line. Also in agreement with the chloroplast purifications by anion exchange chromatography, 2D-PAGE allowed the visualization of several spots in both TG and W, the abundance and number of which was also influenced by the S supply. However, repetitions are desirable to confirm these findings, especially as no spot could be detected in seedlings grown in the S⁻ media. As only one gene is proposed to code for ATPS in onion (Jackse, personal communication), these isoforms are likely to be products of post-

transcriptional or post-translational modifications occurring in response to internal or external cues, such as plant maturation, plant genetic background or S supply.

Previous investigations on cytosolic GS from *Medicago trunculata* or tobacco and CK2 from onion already visualized an alignment of spots following fractionation of extracts by 2D-PAGE (Lima *et al.*, 2006; Riedel *et al.*, 2001; Samaniego *et al.*, 2006). The slight difference in their charges was induced by different phosphorylation states. As putative sites of phosphorylation are detected on the onion ATPS sequences (section 4.1.2), the alignment of spots might demonstrate the occurrence of ATPS phosphorylation in chloroplasts (Figure 3.18). The putative phosphorylation state might be influenced by the stage of plant development as the number of forms and their abundance increased in mature plants. The putative phosphorylation of ATPS is investigated in the second part of this thesis. It should be noted too, that in some cases, these putative modifications can also be result of degraded urea in the sample buffer causing carbamylation (Herbert *et al.*, 2001).

5.3. Regulation of APSR abundance by S supply

In chloroplasts and leaves from seedlings of both TG and W, S depletion did not influence APSR abundance (Figure 3.7 and 3.8). However, in roots from seedlings, APSR abundance increased with S stress. In chloroplasts and leaves from mature plants of both TG and W, APSR abundance declined with S deprivation. On the contrary, APSR abundance increased in roots from mature plants. In bulbs, APSR abundance increased in TG while it decreased in W. In roots from seedlings and in bulbs from mature plants of TG, the enzyme abundance increased with S stress. On the contrary, in roots from seedlings and bulbs from mature plants of W, a decrease in APSR abundance was visualized with S stress. The enzyme APSR accumulated in similar levels in both genotypes in the different tissues observed.

The fractionation of chloroplast extracts by gradient-PAGE also revealed a decrease in APSR abundance with plant maturation in both genotypes. In contrast to the results obtained after 1D-PAGE (Figure 3.7 and 3.8), APSR abundance was detected as increasing with S deprivation. Taken together, the results suggest an influence of the S supply on APSR abundance in chloroplasts. However, it is desirable to repeat the

gradient-PAGE to conclude an influence of S supply with any certainty. The results also showed that APSR abundance might not be influenced by the genetic background. In mature plants, the down-regulation observed in leaves from TG and W with S starvation (Figure 3.7 and 3.8) is also in contradiction with previous studies with mature plants of TG and W that showed significant increases in APSR activity and also mRNA expression following long-term S starvation (McCallum *et al.*, 2006b). however, the results obtained using gradient-PAGE (Figure 3.9) are in agreement with the findings of McCallum *et al.* (2006b).

In *Arabidopsis* (ecotype 'Columbia'), one isoform of APSR mRNA from roots was up regulated 5.0-fold following two days of S withdrawal (Yamaguchi *et al.*, 1999). Takahashi *et al.* (1997) also revealed increased mRNA transcripts following two days of S deprivation in both leaves and roots, but the increase was more marked in roots (5.5-fold) than in leaves (2-fold). In *Lemna minor*, S depletion was also correlated with a 50 to 100% increase in APSR activity, and this up regulation was more intense than in ATPS (20%) (Brunold *et al.*, 1987). In poplars, mRNA levels were increased in roots only after three days of S depletion. During long-term S deprivation, APSR mRNA was also induced, probably as an adaptation to the reduced availability of sulfur (Kopriva *et al.*, 2004).

Thus, the results in this study might suggest that other environmental factors may influence the APSR enzyme. Brunold (1990) stated that in *Arabidospis*, APSR is strongly regulated by various environmental factors, and the influence of light on enzyme activity and mRNA levels has been demonstrated (Kopriva *et al.*, 1999). Thus it might be necessary to repeat the experiment from this thesis and also to measure the APSR activity as well as the mRNA expression to more fully understand regulation of the enzyme in a S accumulating species during long-term starvation, particularly at the post-transcriptional and post-translational level.

In the separated chloroplast extracts using anion exchange chromatography, antibodies raised to APSR were used to determine if any putative APSR isoforms occurred in onion. In seedlings, APSR abundance was detected as eluting with the unbound proteins and also the bound proteins (Figure 3.16). When eluting with unbound proteins, APSR was visualized as a single band in both TG and W. When eluting with

bound proteins, two bands were distinguished in TG, while three to four bands were observed in W. In mature plants, APSR abundance was detected as two forms, eluting only with bound proteins (Figure 3.17).

In seedlings, the purifications of extracts from both TG and W grown in either the S⁺ or the S⁻ media did not reveal consistent differences between the S supplies and genetic background on APSR abundance in terms of elution patterns with increasing NaCl concentrations. Similar patterns of elutions were also detected following separation of chloroplast extracts from TG and W grown in either the S⁺ or the S⁻ media. As well, no consistent differences were detected in the detection of APSR elution between the two developmental stages.

The results suggest the occurrence of probably three forms of APSR in seedlings of TG, and at least four forms in seedlings of W, while only two forms may exist in mature plants in both TG and W. The results suggest the influence of the plant developmental stage on the abundance and expression of APSR isoforms. However, no influence of S supply or the plant genetic was observed on the elution patterns of APSR abundance.

5.4. Phosphorylation and binding to 14-3-3 proteins

Proteins can be altered in a diverse set of post-translational modifications, including methylation, acetylation, ubiquitylation, sumoylation, hydroxylation, among many others. Protein phosphorylation of the amino acids Tyr, Ser, and Thr is the most prevalent post-translational modification and may alter multiple characteristics of proteins, including the enzymatic activity, subcellular localization, protein-protein interaction networks and protein half-life (Hubbard and Cohen, 1993; Peck, 2006). As much as 30% of all proteins in an eukaryotic cell may be phosphorylated at any one time (Cohen, 2002; Hubbard and Cohen, 1993; Mann *et al.*, 2002; Zolnierowicz and Bollen, 2000). The results in the first part of the thesis suggested that ATPS (and possibly APSR) may undergo some form of post-translational modifications either in response to maturation signals or through S-supply. Therefore, evidence of phosphorylation is examined as the second part of this thesis.

In addition, phosphorylation alone is not always sufficient to complete the transduction of the regulatory signal, and often leads to the formation of new protein complexes via interactions between the phosphorylated region and proteins containing specific phosphobinding domains (Peck, 2006). An example of this are the 14-3-3 proteins, which recognize and bind to phosphopeptide motifs (Muslin *et al.*, 1996; Yaffe *et al.*, 1997). They generally function as adapters, chaperones, activators and repressors (Palmgren *et al.*, 1998). Therefore, phosphorylation and subsequent binding to 14-3-3 proteins was also investigated.

Little is known about regulation of ATPS and the other S assimilation enzymes by post-translational modifications. Bio-informatic tools predict putative phosphorylation sites on ATPS as well as the other S enzymes (Appendices III, IV, V and VI). Also, 14-3-3 binding motifs have been detected on ATPS and OAS-TL, which are likely to depend on a previous phosphorylation event.

Methodology used to detect the phosphorylated state of proteins

In this thesis, chloroplast or leaf extracts from onions were analysed following treatment to promote either phosphorylation (P^+) or dephosphorylation (P^-). Upon chloroplast lysis and protein extraction, protease inhibitors were included in the buffers to minimize proteolysis of kinases and phosphatases, particularly, and thus conserve

their activity as well as protecting the integrity of the S assimilation enzyme and other cellular proteins (Wang and Roach, 1992).

In the P⁺ treatment, ATP, as a complex with Mg²⁺ was added to the extracts, as the phosphoryl donor for the activity of endogenous protein kinases (Wang and Roach, 1992). The phosphatase inhibitors NaF or OKA along with EDTA and EGTA were also used to inhibit the activity of endogenous enzymes to dephosphorylate residues. NaF is a general protein phosphatase inhibitor that is potent against the PP2A, PP2B, PP2C phosphatases (Hardie *et al.*, 1992; Wang and Roach, 1992), and has been used successfully to demonstrate the phosphorylation of F2KP from *Arabidopsis* (Furumoto *et al.*, 2001) or sucrose synthase in maize (Duncan and Huber, 2007). OKA specifically inhibits PP1 and PP2A, and also decreases PP2B activity (Haystead *et al.*, 1989). EDTA and EGTA specifically inhibit PP2B and PP2C by chelating ions necessary for their activity (Hardie *et al.*, 1992). NaF, which has been used successfully in other studies (MacKintosh, 1993; Waegemann and Soll, 1996), was used in most of the experiments as an alternative to the expensive and more hazardous OKA. Microcystin-LR, to a final concentration of 0.5 µM, is used in many studies as it is an extremely potent inhibitor of PP1 and PP2A (Hardie *et al.*, 1992; MacKintosh *et al.*, 1990). However, this product was not used because of environmental protection measures in New Zealand.

Protein kinases were not added in the P⁺ treatment as they are generally used during investigations of phosphorylation *in vitro* of recombinant proteins (Lima *et al.*, 2006). The activity of protein tyrosine phosphatases was not taken into account as their action is limited to signalling pathways and regulation (Gruhler and Jensen, 2006; Luan, 2000), whereas the enzymes investigated in this thesis are involved in the S assimilation pathway.

In the P⁻ treatment, the activity of endogenous phosphatases was enhanced by the addition of PP2A or AAP. The ions Mg²⁺ were also added for PP2C activity (Cohen, 1989). PP2A was used in the first experiments, but was then replaced by AAP, a form of alkaline phosphatase, which is able to dephosphorylate proteins (Anguenot *et al.*, 2001; Brini *et al.*, 2007; Furumoto *et al.*, 2001) for availability and expense reasons. Both the P⁺ and the P⁻ treatments were incubated for 15 min at 30°C when PP2A was

used as the phosphatase, and extended to 30 min when using AAP. Finally, all reactions were stopped by cooling down on ice.

Efficiency of the P⁺ and the P⁻ treatments

In order to assess the efficiency of the treatments, albumin, which is known to be a phosphoprotein (Wei *et al.*, 1998) was treated with either the P⁺ or the P⁻ treatment to induce the phosphorylation and the dephosphorylation states of the protein, respectively (Figure 4.3). For the P⁻ treatment, both PP2A and AAP were tested. The protein was incubated for 15 and 30 min when using PP2A, and 30 and 60 min when using AAP. The Peppermint-Stick phosphoprotein ladder was also used to control the efficiency of Pro-Q DPS over phosphoproteins. This protein dye permits rapid and direct detection of phosphoproteins by binding exclusively to the phosphate groups (Steinberg *et al.*, 2003; Schulenberg *et al.*, 2003). Following Pro-Q DPS, only ovalbumin and β -casein, the two phosphoproteins from the Peppermint-Stick phosphoprotein ladder were visualized, while all proteins included in this ladder were stained with silver nitrate total protein stain. This result confirms the specificity of the Pro-Q DPS for phosphoproteins.

Albumin was visualized by Pro-Q DPS following the P⁺ as well as all the P⁻ treatments, and the intensity of the band declined after the treatment with AAP. The densitometry scanning of the bands confirmed that the abundance of albumin decreased after the P⁻ treatment using AAP as the phosphatase. The albumin exhibited a trend towards lower intensity after the 60 min incubation when compared to the 30 min incubation. However, only a small decrease in albumin intensity was observed following the P⁻ treatment using PP2A as the phosphatase, and with only one of the two batches tested. The results suggest that at least one of the batches of PP2A was possibly inactive, even after extended incubation, at least under the conditions used to assess activity in this thesis. Once stained with silver nitrate, a similar abundance of albumin was detected between the P⁺ and all the P⁻ treatments, suggesting that the differences visualized following Pro-Q DPS were specific to loss of phosphate groups.

Techniques used for the detection of phosphorylation in this thesis

Protein phosphorylation induces a mass increment of 80 Da for each phosphate group added. However, much larger changes in mass can be observed because of an altered mobility of the protein during 1D-PAGE *via* some conformational changes

(MacKintosh, personal communication). Demonstration of protein phosphorylation by mobility shift between the phosphorylated and the dephosphorylated states has already been mentioned (Furumoto *et al.*, 2001; Brini *et al.*, 2007). Thus proteins were resolved on 1D-PAGE for the detection of a mobility shift between the phosphorylation and the dephosphorylation states. This technique is one way among many to unravel protein phosphorylation, and not all proteins do show a mass change (Hartill, personal communication). Therefore, other techniques were also carried out for the identification of phosphorylation of ATPS and the other S enzymes.

Protein phosphorylation can also be demonstrated by changes in the position of the protein in the IEF separation of a 2D-PAGE because an increase in phosphate content causes the protein to focus at a new, more acidic pI (Steinberg and Coffino, 1979; Garrison and Wagner, 1982; Hardie *et al.*, 1992). The magnitude of these charge shifts is about 0.3 pH units in the pH 6-7 region of the gel (Garrison and Wagner, 1982; Steinberg and Coffino, 1979). Following the P⁺ and the P⁻ treatments, ATPS and OAS-TL were detected to observe change in the spot position and alignment. Previous studies have demonstrated phosphorylation of proteins, such as DHN-5 from *Triticum durum*, NopA100 and AcMFP1 from *Allium cepa* or CDeT11-24 from *Craterostigma plantagineum* by comparative 2D-PAGE immunoblot analyses (Brini *et al.*, 2007; González-Camacho *et al.*, 2004; Röhrig *et al.*, 2006; Samaniego *et al.*, 2006).

Phosphorylation of proteins, such as cytosolic GS or the plant plasma membrane H⁺-ATPase, has also been shown to modulate their activity by changing their structure (Finnemann and Schjoerring, 2000). Currently, SAT is the only one enzyme involved in the S assimilation pathway which has been shown to be regulated by phosphorylation. Indeed, studies in *Glycine max* cell suspension cultures revealed that SAT phosphorylation induces up-regulation of the enzyme activity by relief of feedback inhibition of cysteine (Liu *et al.*, 2006). Thus, ATPS activity was measured in chloroplast extracts to detect the influence of the P⁺ and the P⁻ treatments, and so begin to understand the biochemical significance of the putative ATPS phosphorylation.

Finally, as the ATPS and OAS-TL onion sequences (Ferl, personal communication) and also the *Arabidopsis* SiR sequence (Scansite prediction) display 14-3-3 binding motifs, and as 14-3-3 proteins target phosphorylated proteins (Ferl, 1996), complex formation

between the S assimilation enzymes and 14-3-3 proteins was assessed by affinity chromatography as a way to unravel phosphorylation of the S assimilation enzymes. This technique has already been used to uncover the phosphorylation and subsequent binding to 14-3-3 proteins for NR, GS, and SPS enzymes, among others (Moorhead *et al.*, 1996; Moorhead *et al.*, 1999). The same studies revealed similar capacity for the yeast, mammalian and purified spinach 14-3-3 proteins to bind to the phosphorylated form of NR from spinach (Moorhead *et al.*, 1996). Thus in this study, the recombinant BMH1 and BMH2 14-3-3 proteins from *S. cerevisiae* were used for the detection of 14-3-3 interactions with the S enzymes.

In this thesis, proteins bound to the 14-3-3 proteins were detected by western analyses after pull-downs of beads. To date, more than 30 plant proteins binding to 14-3-3 proteins have been identified using this affinity column method (Moorhead *et al.*, 1999).

Additional methods to detect protein phosphorylation

Before discussing the results obtained in this thesis, it is also worthwhile to briefly discuss other methods used to detect protein phosphorylation. In the P⁺ treatment, ³²P-labelled ATP may be added to the P⁺ incubation to monitor the incorporation of phosphate into the proteins by autoradiography following fractionation by 1D-PAGE of the plant extracts. This technique has been used for the identification of phosphorylation of GS from *Brassica napus* (Finnemann and Schjoerring, 2000), phosphoenolpyruvate carboxylase from *Zea mays* (Jiao *et al.*, 1991) or SAT from *Glycine max* (Liu *et al.*, 2006) following purification of these enzymes from crude extracts.

In the P⁻ treatment, even though no MgATP was added to promote their activity, protein kinase inhibitors can be added to stop the activity of endogenous kinases. However, in the P⁻ treatment used in this thesis, EDTA to a final concentration of 5 mM might have been sufficient to chelate all the divalent cations and prevent protein kinase action (Hardie *et al.*, 1992).

Previous work has revealed that incubation with phosphatase might be extended at 30°C to ~12 hrs to ensure completion of dephosphorylation (Tonks *et al.*, 1988).

However, in this study, the incubation period was limited to 15 or 30 min to conserve the ATPS enzyme active for further studies. Also, previous studies showed that it is possible to terminate the reactions by the addition of 7 - 25% TCA (Gonzalez-Camacho *et al.*, 2004), to precipitate proteins while free inorganic phosphate and nucleoside triphosphate remain in solution (Wang and Roach, 1992). For most of the procedures used in this thesis, maintenance of the dephosphorylated protein in the aqueous buffer was necessary for further procedures.

In order to unambiguously compare the influence of the P⁺ and the P⁻ treatments using 2D-PAGE, extracts can be labelled by difference gel electrophoresis (DIGE) with cyanine dyes. This technique allows multiplexed resolution of up to three samples per IEF strip, and thus makes possible the comparison of protein patterns and abundance between samples (Alban *et al.*, 2003; Gharbi *et al.*, 2002; Zhou *et al.*, 2002).

Investigations on the phosphorylation of ATPS

In terms of mass shift, following protein fractionation by 1D-PAGE, no change in molecular mass was observed between the P⁺ and the P⁻ treatments for ATPS in any of the three repetitions per genotype per S supply. This may be because of the limited resolving power of 1D-PAGE when compared to 2D-PAGE (Garrels, 1979; Anderson and Anderson 1978 a, b; O'Farrell, 1975) (Figure 4.4; Appendices 9 and 10). Thus, chloroplast extracts were fractionated by 2D-PAGE for the detection of phosphorylation.

In terms of change in pI, after fractionation of chloroplast extracts by 2D-PAGE, ATPS was visualized as a series of horizontal spots with same molecular mass in chloroplast extracts from mature plants of W grown in either the S⁺ or the S⁻ media (Figure 4.5). The number of spots representing the enzyme diminished after the P⁻ treatment, on the acidic side of the IPG strip. Also, the spot intensity was lower in extracts from plants grown in the S⁻ media. It is known that the phosphorylated sites in proteins might vary, implying that any given phosphoprotein is heterogeneous (i.e. it exists in several different phosphorylated forms) (Mann *et al.*, 2002). Thus the differences of spot numbers showed that ATPS might be present as a mixture of isoforms in chloroplast extracts, some of which can be dephosphorylated during the P⁻ treatment. This suggests that ATPS might be a phosphoprotein. It further may also suggest that only some of

ATPS isoforms are able to be phosphorylated. Although the results from the 2D-PAGE analysis suggested that ATPS might be phosphoprotein, mass spectrometry analysis of the different forms (i.e. spots) of both proteins is desirable to confirm the occurrence of the modification and to establish the exact number of phosphorylation sites (Hardie *et al.*, 1992).

In terms of enzyme activity, when using PP2A as the phosphatase, the results showed that the activity was significantly increased after both the P⁺ and the P⁻ treatment, in comparison with untreated extracts, suggesting that one chemical used in both treatments (but not in untreated), possibly Mg²⁺, may have promoted enzyme activity (Figure 4.7). However, as visualized by Pro-Q DPS, the phosphatase PP2A may not have efficiently dephosphorylated albumin, and so no change in ATPS activity between the P⁺ and the P⁻ treatments may be due to the PP2A not being active. However, when AAP was used as the phosphatase, no increase in ATPS activity was again observed between the P⁺ and the P⁻ treatments and also between the treatments and the control (Figure 4.8).

During the characterisation of ATPS regulation, an influence of the light and dark cycle on ATPS activity and abundance was observed, as for other enzymes that have been demonstrated to be phosphorylated. In case of the NR protein phosphorylation, the event has been showed to occur within minutes into the darkness and can be reversed with even just flashes of light (Huber, personal communication), thus making difficult the investigations on the phosphorylation state of this enzyme. However in this thesis, all the experiments for the detection of ATPS phosphorylation and dephosphorylation were carried out using extracts isolated at L+4 h. It is thus necessary to investigate the phosphorylation event using extracts harvested at other time points, especially upon light and dark onset. The variations in ATPS spot alignment observed during 2D-PAGE between the P⁺ and the P⁻ treatments suggest changes in the surface charge of the proteins, but not its activity levels. Any phosphorylation event of ATPS might be necessary, therefore, for further post-translational modification including mediating protein-protein interactions.

Investigations on the other S assimilation enzymes

In terms of mass shift, following fractionation of proteins from chloroplast extracts by 1D-PAGE, a mobility shift between the P⁺ and the P⁻ treatments was visualized for SiR and OAS-TL only. These shifts were detected in extracts from TG grown in the S⁺ media and from TG grown in the S⁻ media for SiR and OAS-TL, respectively. Mobility shift is one way among many others to unravel protein phosphorylation, but not all proteins show a mobility shift (Hartill, personal communication). This can be because of the limited resolving power of 1D-PAGE when compared to 2D-PAGE (Garrels, 1979; Anderson and Anderson, 1978a, b; O'Farrell, 1975) and this might explain why the mobility shift for SiR or OAS-TL was observed only once out of three repetitions. Thus the results suggest that SiR and OAS-TL might be phosphoproteins, although more analyses are required to confirm this finding. In regards to OAS-TL, the mobility shift agrees with proteomic studies in canola (Agrawal and Thelen, 2006), and *Arabidopsis* (Thelen, personal communication) suggesting that this enzyme is a phosphoprotein during seed filling.

As OAS-TL has been demonstrated to be a phosphoprotein in canola and *Arabidopsis*, further analyses by 2D-PAGE was carried out with leaf extracts from mature plants of W grown in either the S⁺ or the S⁻ media to detect any change in pl. In common with ATPS, OAS-TL was visualized as a series of horizontal spots with an identical molecular mass (Figure 4.6.), suggesting the presence of OAS-TL as isoforms in onion leaves. The number of spots and abundance decreased with S deprivation, suggesting an influence of S stress on the regulation of the different isoforms. Previous studies demonstrated that alignment of spots visualized after 2D-PAGE suggests the occurrence of post-translational modifications (Lima *et al.*, 2006; Riedel *et al.*, 2001; Samaniego *et al.*, 2006). Thus, leaf extracts were separated after the P⁺ and the P⁻ treatments.

In leaf extracts, the distribution of OAS-TL spots on IEF shifted to a more acidic pH after the P⁺ treatment (Figure 4.6.), suggesting that one or more of the OAS-TL isoforms that exist in onion leaf may be phosphorylated. Previous studies have demonstrated phosphorylation of proteins by comparative 2D-PAGE immunoblot analyses (Brini *et al.*, 2007; González-Camacho *et al.*, 2004; Samaniego *et al.*, 2006; Röhrig *et al.*, 2006). However, repetitions are necessary to confirm the differences

between the P^+ and the P^- treatments and the influence of the S supply on *OAS-TL* abundance and expression. As well, nothing is known about the size of the *OAS-TL* gene family in onion.

Investigations of interactions between the S assimilation enzymes and 14-3-3 proteins

Following affinity chromatography, ATPS, APSR and SiR from onion chloroplast extracts and also *OAS-TL* from onion leaf extracts were detected as binding to the immobilised BMH1 and BMH2 14-3-3 proteins (Figures 4.16, 4.17, 4.18, 4.19, 4.20). Binding of SiR was possibly non-specific as it was observed in only one out of three experiments. Binding of APSR may also be non-specific as binding to BSA was also observed.

Although some non-specific binding of ATPS to BSA could also be detected, the results suggest that this enzyme may form a complex with 14-3-3 proteins. Binding of ATPS to 14-3-3 proteins seems to be dependent on the presence of divalent cations, since treatment of chloroplasts with EDTA and EGTA weakened the binding. This is in correlation with previous studies that have determined complexes between 14-3-3 proteins and nitrate reductase (Bachman *et al.*, 1996c) and the plasma membrane H^+ -ATPase (Fullone *et al.*, 1998). In these studies, binding occurs only in the presence of Mg^{2+} as divalent cations induce a structural reorientation of the C-termini (Ferl, 2004; Lu *et al.*, 1994). Following affinity chromatography, ATPS was visualized as two bands but only in extracts from plants grown in the S^+ media. As protease activity was inhibited, the results suggest that the binding was not isoform dependent, but this binding was negatively influenced by S deprivation.

The results of affinity chromatography using untreated leaf extracts also showed interaction between *OAS-TL* and 14-3-3 proteins, suggesting that the two proteins might form a complex (Figure 4.20). Further, no interaction with the BSA-Sepharose was observed. The binding between *OAS-TL* and 14-3-3 proteins did not appear influenced by S deprivation. As 14-3-3 proteins almost exclusively bind to phosphorylated protein targets (Ferl, 1996), *OAS-TL* abundance was not optimal because phosphorylation was not promoted specifically. It is thus necessary to repeat

the experiment using leaf extracts treated with ATP to confirm the complex between 14-3-3 proteins and the requirement of *OAS-TL* to be phosphorylated.

These interactions described between the S assimilation enzymes and 14-3-3 proteins need confirmation by repeating the affinity chromatography with both chloroplast and leaf extracts that have been previously treated to promote protein phosphorylation. As well, the question of the use of control proteins (other than BSA) needs to be examined in more details as this protein (BSA) may be particularly prone to form on-specific protein-protein interactions. Methods other than affinity chromatography might be also tested such as 14-3-3 overlays, which have already proven useful for the detection of 14-3-3 target proteins from spinach (Shen and Huber, 2006) or surface plasmon resonance, which measures complex formation by monitoring changes in the resonance angle of light impinging on a gold surface. Surface plasmon resonance has been used to elucidate the regulatory mechanism of the *OAS-TL* complex from *Arabidopsis* (Berkowitz *et al.*, 2002) and to detect 14-3-3 isoform specificity in terms of binding to NR from barley (Sinnige *et al.*, 2005). To confirm the occurrence of protein-protein interactions *in vivo* and obtain information about the conditions required for the complex formation, techniques like blue-native PAGE, or far-western blotting with non-denaturing gels might be employed. Blue-native PAGE has been readily used to array and identify components from the respiratory chain of mitochondria (Jänsch *et al.*, 1996; Heazlewood *et al.*, 2003a) and the photosystem complexes of the chloroplast (Heinemeyer *et al.*, 2004). This technique has also been used to identify phosphorylation of protein complexes (Bykova *et al.*, 2003).

A proposed model for the regulation of ATPS by phosphorylation

The variations in spot alignment between the P⁺ and the P⁻ treatments observed by 2D-PAGE showed the influence of these treatments on ATPS charges, suggesting that ATPS might be a phosphoprotein. The phosphorylation event is probably likely to influence the surface charge of the enzyme, but with no influence in ATPS activity. This, in association with any conformation change, might trigger interaction with 14-3-3 proteins. In this thesis, the characterization of ATPS showed the influence of the plant development, genetic background and S supply on ATPS activity and abundance.

The results from ATPS characterisation showed the influence of the light and dark transitions on both ATPS activity and abundance, which might represent changes in phosphorylation states. Previous studies also exposed the occurrence of post-translational modification of NR and SPS in response to light and dark transitions (Huber *et al.*, 1996; Huber *et al.*, 1989a; Huber *et al.*, 1992b; Kaiser and Huber, 2001).

However further studies are necessary to confirm the results and understand the mechanisms involved that induced phosphorylation and subsequent interaction with 14-3-3 proteins. Further investigations should be carried out using proteins extracted during the dark period to determine the influence of the light and dark on the phosphorylation state.

The results also revealed a tendency for ATPS activity to increase at the beginning of the light period and upon dark onset, which might involve post-translational modifications. Diurnal regulation of phosphorylation and subsequent binding to 14-3-3 proteins has already been demonstrated for other enzymes involved in plant metabolism such as NR (Bachmann *et al.*, 1996a, b; Lillo *et al.*, 1997; Moorhead *et al.*, 1996) and F2KP (Furumoto *et al.*, 2001; Kulma *et al.*, 2004). Thus the results suggest that diurnal variations might influence the ATPS phosphorylation state and then interactions with 14-3-3 proteins.

Recent findings revealed the formation of a complex between recombinant ATPS and APSR (Cumming *et al.*, 2007). Previous studies already showed that 14-3-3 proteins can induce conformational change of target proteins (Tzivion *et al.*, 1998; Yaffe, 2002). Thus binding to 14-3-3 proteins might affect the ATPS three-dimensional structure and then allow interaction between ATPS and APSR (Figure 5.1). This hypothesis can also explain the detection of APSR in 14-3-3 pull-downs. However, further experiments are necessary to validate this hypothetical multi-enzyme complex, to determine the factors influencing the complex formation and finally to determine its biological significance.

The enzyme *OAS-TL* is already known to form a bi-enzyme complex with SAT for the control of cysteine synthesis (Bogdanova and Hell, 1997). *OAS-TL* is only active as the free enzyme and has also been recognized as a phosphoprotein in proteomic studies in canola (Agrawal and Thelen, 2006) and *Arabidopsis* (Thelen, personal

communication). Thus the results in this thesis, with current knowledge, suggest that OAS-TL might be regulated by phosphorylation and subsequent 14-3-3 binding, which might increase its activity and occur only when the enzyme is not interacting with SAT.

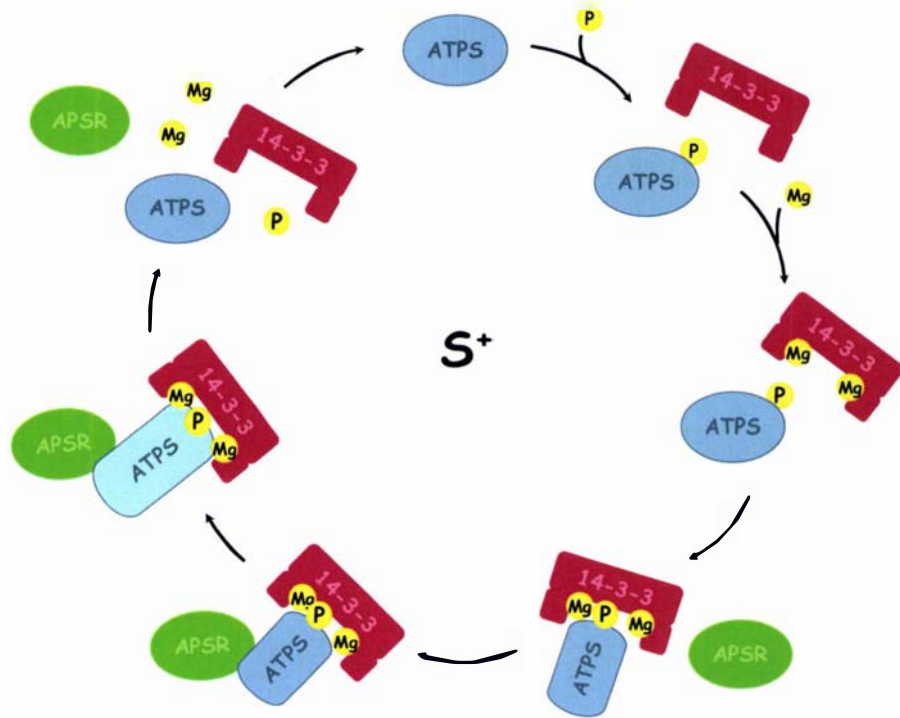


Figure 5.1. Regulation of ATPS by phosphorylation and subsequent 14-3-3 protein binding in chloroplasts from onion grown in the S-sufficient media.

In plants grown in the S^+ media, phosphorylation of ATPS triggers binding to 14-3-3 proteins, but only in the presence of Mg^{2+} . This interaction leads to a conformational change of ATPS, thus allowing the interaction with APSR, which induce an increase in ATPS activity. However, in plants grown in the S-deficient media, ATPS phosphorylation and thus binding to 14-3-3 proteins can not occur, thus preventing interaction to APSR.

5.5. Future work

The results in this thesis revealed that ATPS isoforms are regulated by several internal and external factors, namely genetic background, plant maturation state, S supply, and light and dark transitions. The investigations on ATPS regulation in onions also revealed that the enzyme may be present as isoforms in chloroplasts, which probably are products of post-transcriptional or post-translational modifications as only one gene coding for ATPS is proposed to be present in the onion genome. However, the detailed characterisation of these isoforms was not a definite finding of this thesis. To confirm unequivocally that isoforms of ATPS do exist, other separation methods (i.e. reverse phase) in addition to anion exchange chromatography need to be employed. Sequencing of these different forms of ATPS is desirable to identify their characteristics and differences. This would also contribute to understanding the variations in ATPS activity between the two genotypes used in this thesis.

In addition to isoform sequencing, isoform-specific antibodies can be raised and used to further characterize their specificity and regulation through changes in S supply, plant maturation or in the different genetic backgrounds. This technique has been used to characterize the three SUS isoforms present in maize (Duncan *et al.*, 2006). This isoform characterization could possibly allow the confirmation of the link between onion pungency and ATPS that has been revealed by genetic mapping (McCallum *et al.*, 2006a).

Further investigations propose that ATPS is a phosphoprotein and may interact with 14-3-3 proteins. In order to confirm the occurrence of phosphorylation, ATPS could be purified from plant extracts following the P^+ treatment using phospho-specific antibodies or IMAC coupled with Fe^{3+} or Ga^{3+} ions. Then, the recovered proteins could be analysed by mass spectrometry, which enables site mapping and quantification of chemical modifications on proteins on the basis of a characteristic mass shift owing to the loss of phosphate (Witze *et al.*, 2007).

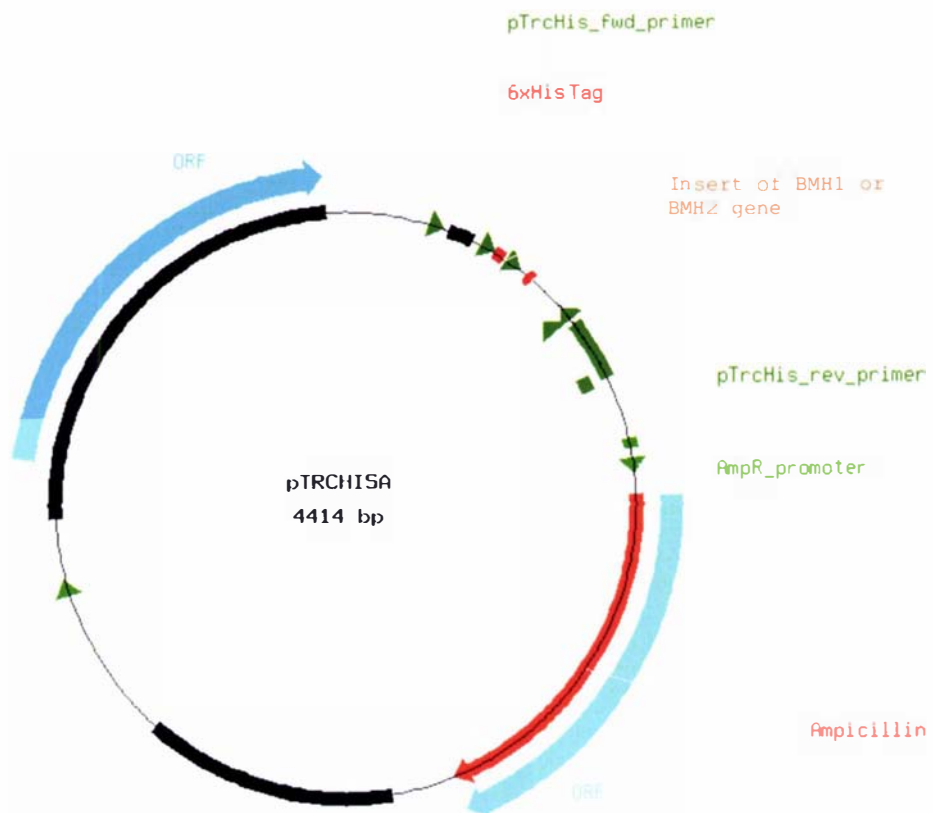
In addition, site-directed mutagenesis of the Ser₂₈₈ and Ser₂₉₀ on ATPS onion sequences or Ser₃₂₃ on OAS-TL onion sequence in the 14-3-3 binding motif would indicate whether this amino acid substitution would affect phosphorylation and thus the

subsequent interaction to 14-3-3 proteins. This technique has already proven useful for the characterisation of the complex between NR and 14-3-3 proteins (Sinnige *et al.*, 2005).

The results from this thesis suggest that ATPS undergo phosphorylation, and that this event at least induces a change in the surface charge, as suggested by the variations in the horizontal spot alignment following fractionation by 2D-PAGE. However, ATPS activity results following the P⁺ and the P⁻ treatments suggested that phosphorylation does not regulate the enzyme activity, but may mediate interactions with 14-3-3 proteins, as ATPS was detected following affinity chromatography of chloroplast extracts.

Appendix I

Map of the pTrcHisA plasmid¹.

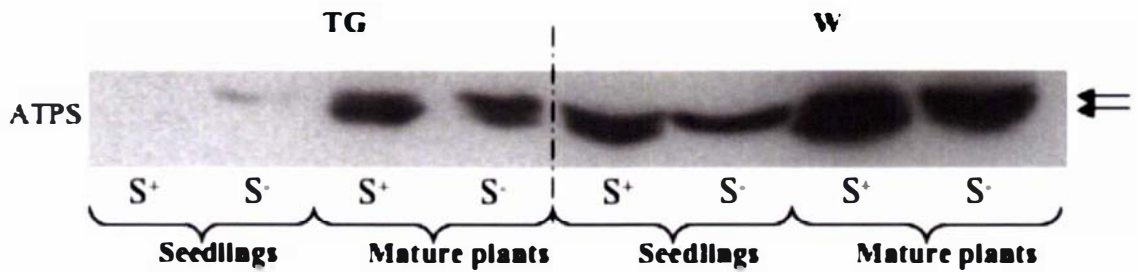


¹ Adapted from Addgene (www.addgene.org/)

Appendix II

Influence of the S supply on the abundance of ATPS in chloroplasts from seedlings or mature plants, as indicated, of TG and W, as indicated, grown in either the S-sufficient (S^+) or the S-deficient (S^-), as indicated.

Protein extracts (35 ug) were separated by 8-15% gradient-PAGE and the abundance of ATPS determined by western analyses using chemiluminescence. This western blot is the same as Figure 3.9 in the thesis, but the X-ray film was exposed for a shorter period of time.

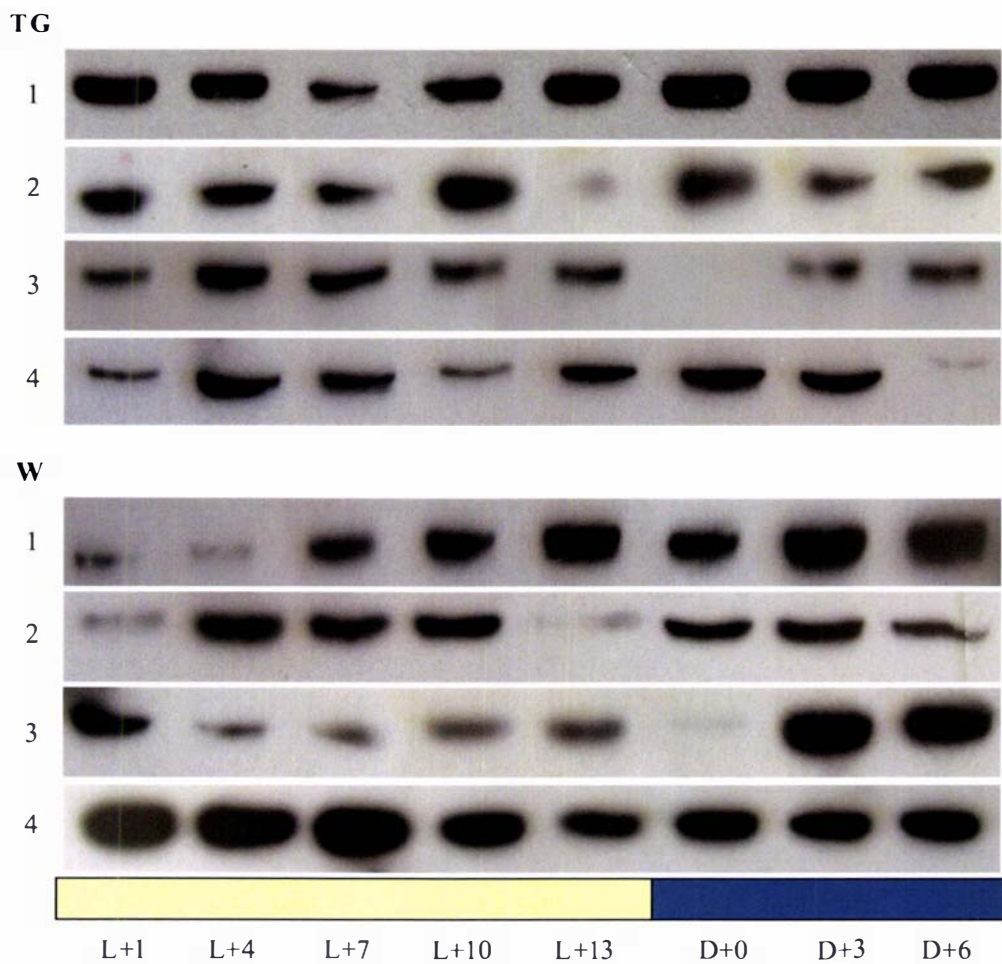


Appendix III

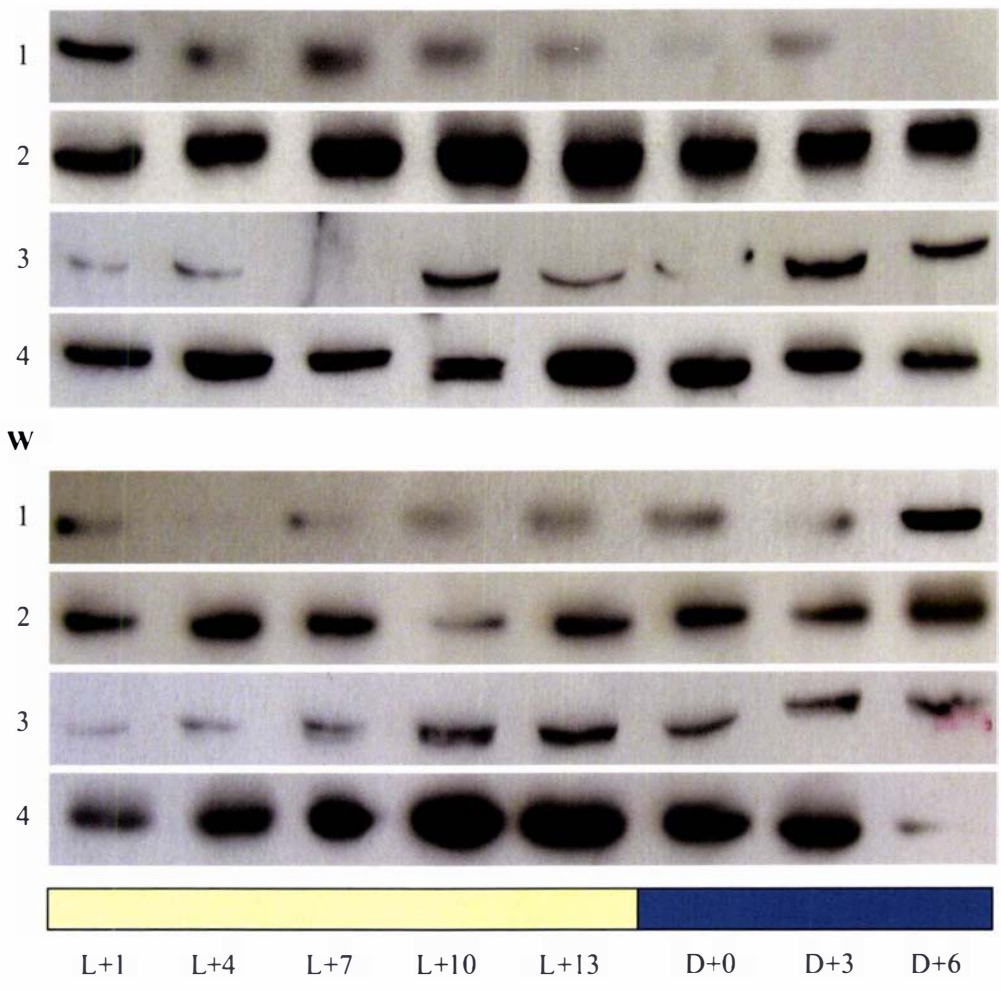
Influence of light and dark on the abundance of ATPS in chloroplast, leaf or root extracts, as indicated, from seedlings of TG or W, as indicated, grown in the S-sufficient media.

Proteins (15 μ g) extracted over a 24 h period were separated by 1D-PAGE and the abundance of ATPS determined by western analyses using chemiluminescence. Numbers represent the repetition.

Chloroplast extracts

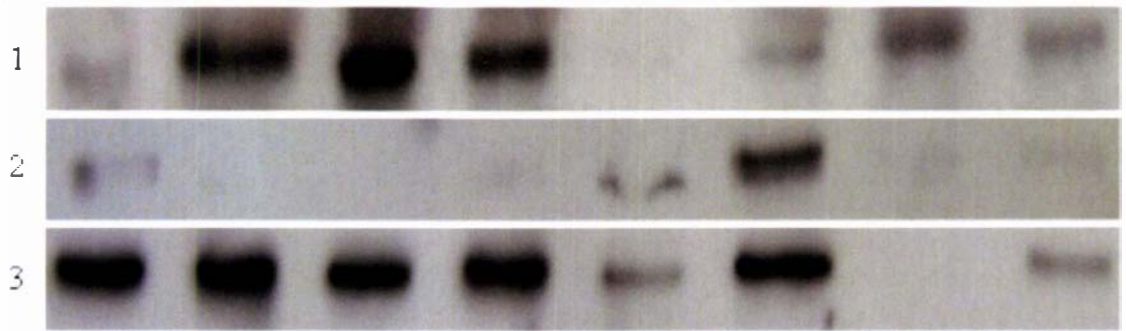


Leaf extracts

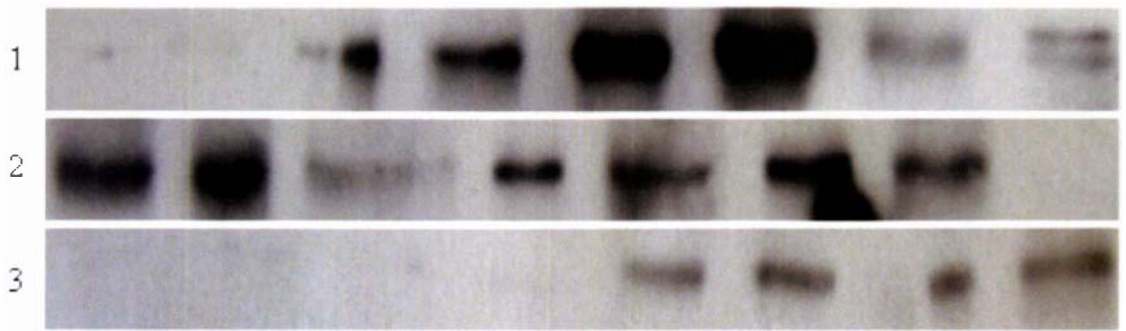


Root extracts

TG



W

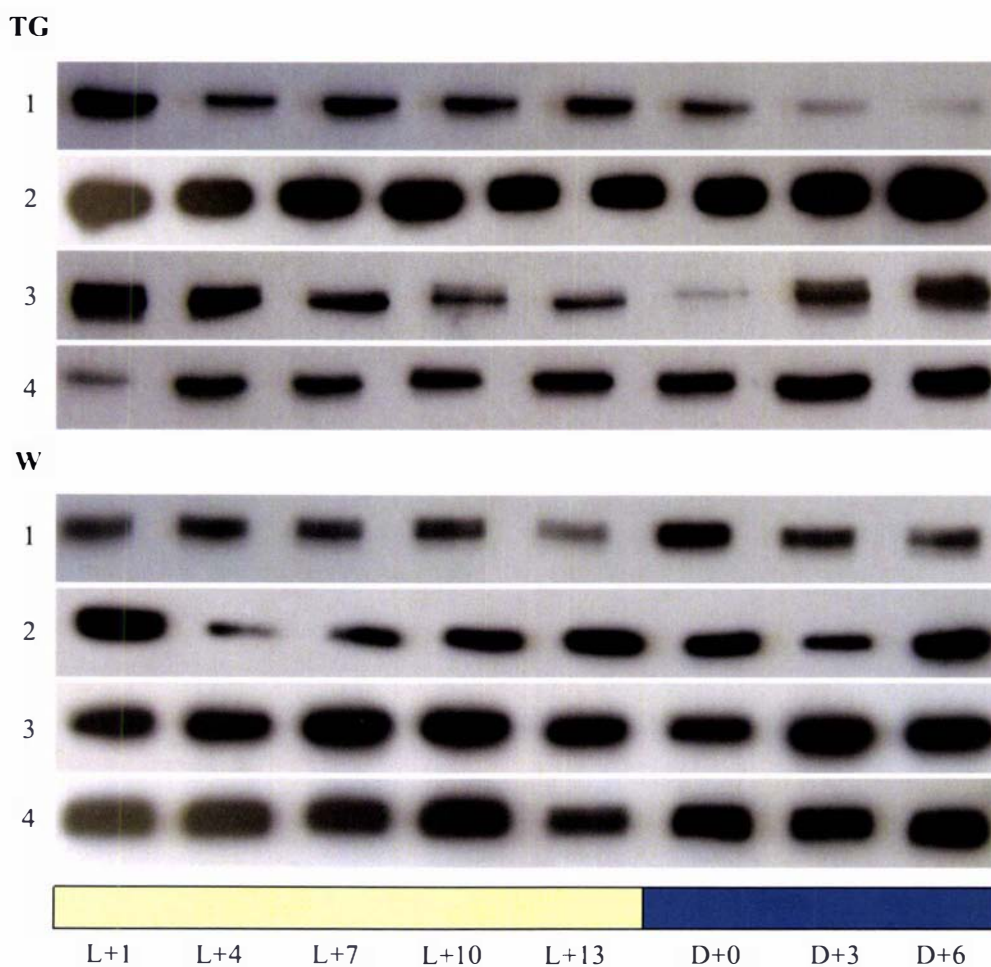


Appendix IV

Influence of light and dark on the abundance of APSR in chloroplast, leaf or root extracts, as indicated, from seedlings of TG or W, as indicated, grown in the S-sufficient media.

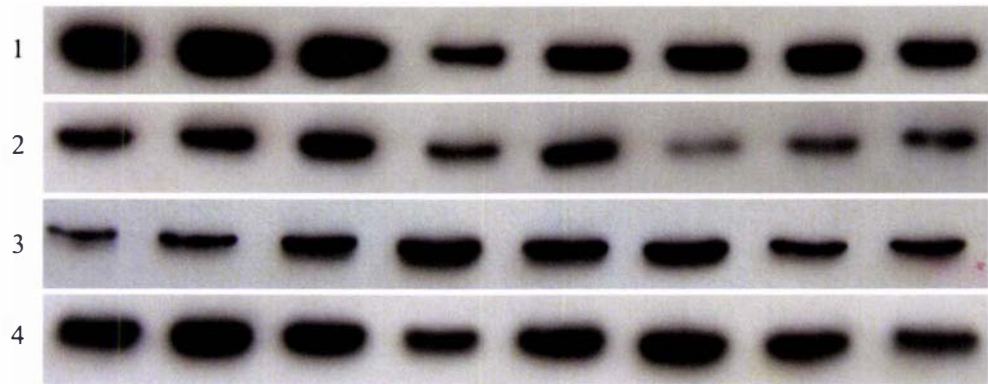
Proteins (15 μ g) extracted over a 24 h period were separated by 1D-PAGE and the abundance of APSR determined by western analyses using chemiluminescence. Numbers represent the repetition.

Chloroplast extracts

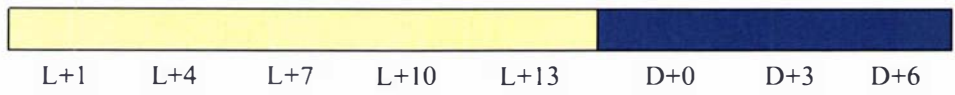
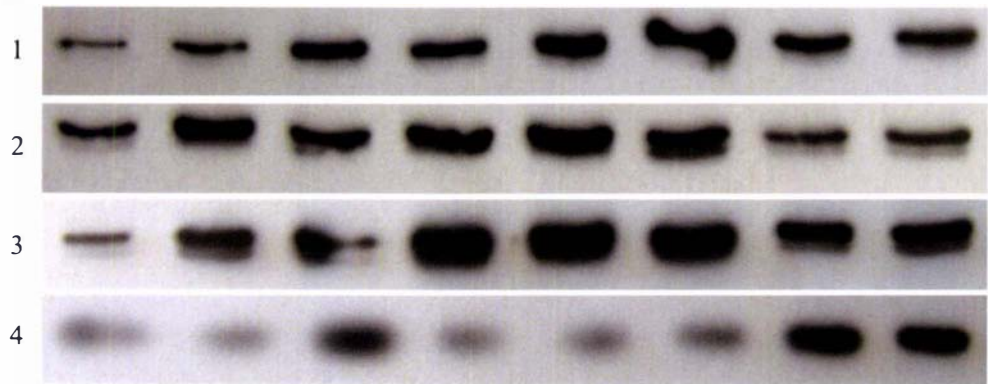


Leaf extracts

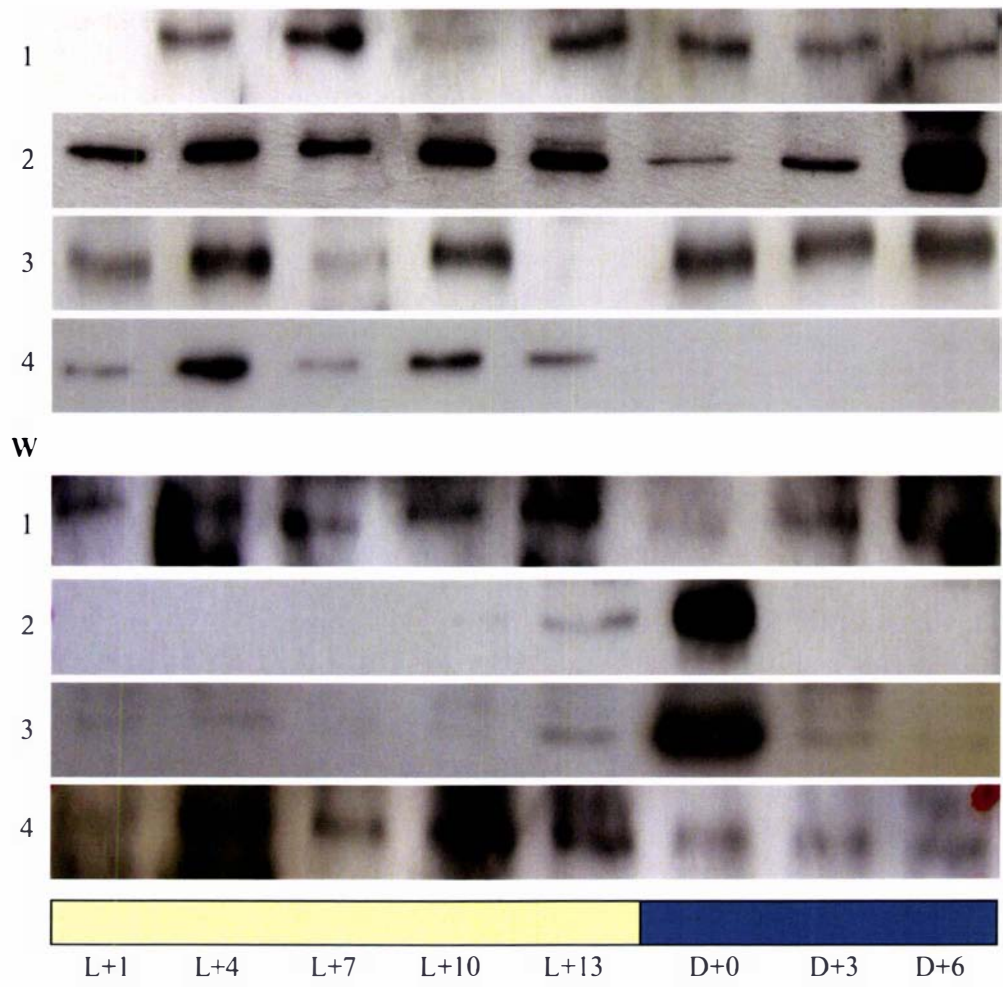
TG



W



Root extracts



Appendix V

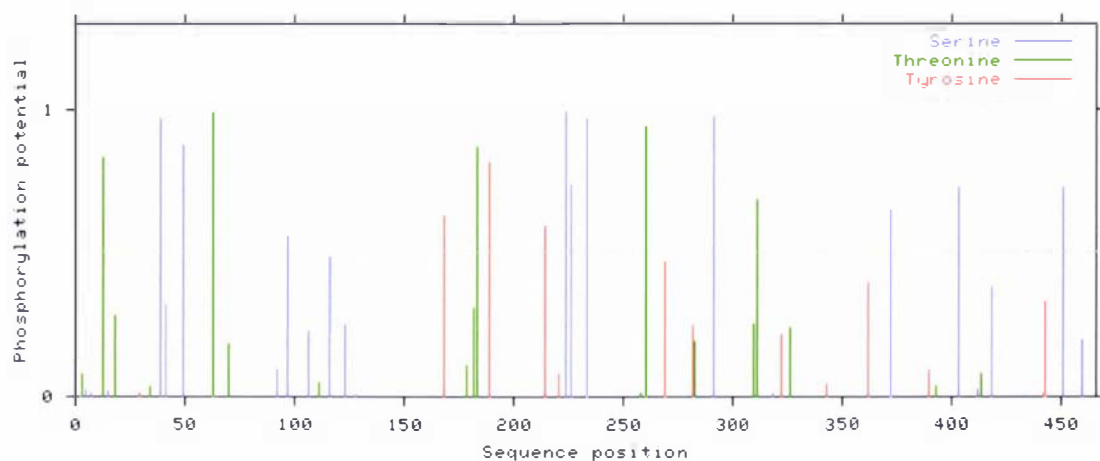
Putative phosphorylation sites and putative 14-3-3 binding site in ATPS sequences from onion, as determined by NetPhos 2.0²

Putative phosphorylated serine sites are highlighted in yellow, and putative phosphorylated threonine sites in green. The putative 14-3-3 binding site is highlighted in blue.

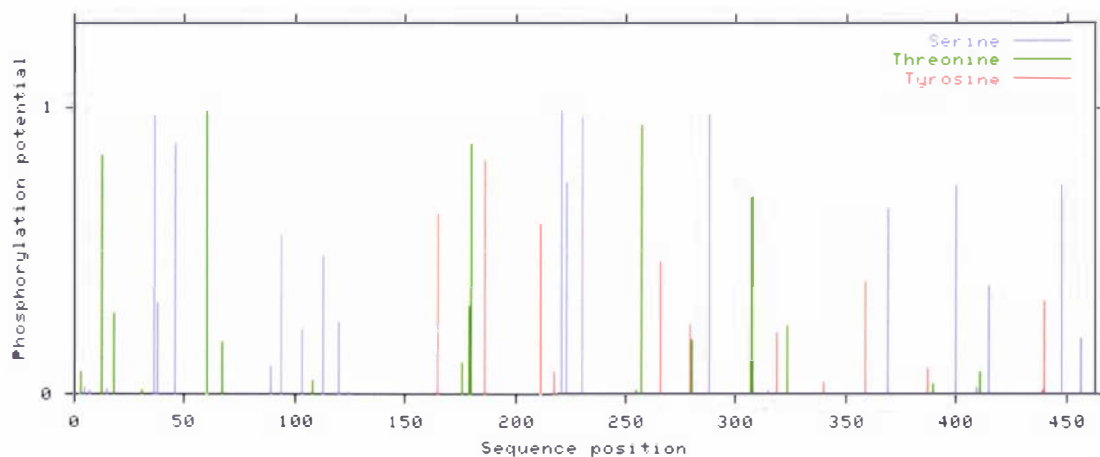
AAL61615	(1)	MSTLSNSFLA	PN T PSKLTQK	FQNRLILKCP	---THHRI SC	SLIEPDGG SL
AAF18998	(1)	MSTLSNSFLA	PN T PSKLTQK	FQNRLILKYP	KCP THHRI SC	SLIEPDGG SL
AAL61615	(51)	KNLVVPAGPA	RD T TRVKEAAT	AGQALPRVRL	KRVDLEWVHV	LSEGWASPLG
AAF18998	(51)	KNLVVPAGPA	RD T TRVKEAAT	AGQALRRVRL	KRVDLEWVHV	LSEGWASPLG
AAL61615	(101)	GFMRESEFLQ	TLHFNSIRLD	DGSFVNMSVP	IVLAIDDEKK	NEIGERKRVL
AAF18998	(101)	GFMRESEFLQ	TLHFNSIRLD	DGSFVNMSVP	IVLAIDDEKK	NEIGERKRVL
AAL61615	(151)	LVDQNDKAVA	FLNDIEIYKH	NKEERIARTW	GT T ARGLPYV	EEAIINAGNW
AAF18998	(151)	LVDQNDKAVA	FLNDIEIYKH	NKEERIARTW	GT T ARGLPYV	EEAIINAGNW
AAL61615	(201)	LVGGDLEVIE	PIKYNDGLDQ	YRL S PSQLRD	EF S RRNADAV	FAFQLRNPVH
AAF18998	(201)	LVGGDLEVIE	PIKYNDGLDQ	YRL S PSQLRD	EF S RRNADAV	FAFQLRNPVH
AAL61615	(251)	NGHALLMTD T	RRRLLEMGYK	NPILLHPLG	GYTKADDV PL	SW RMKQHEKV
AAF18998	(251)	NGHALLMTD T	RRRLLEMGYK	NPILLHPLG	GYTKADDV PL	SW RMKQHEKV
AAL61615	(301)	LEEGVLPET	TVVAIFPSPM	HYAGPTEVQW	HAKARINAGA	NFYIVGRDPA
AAF18998	(301)	LEEGVLPET	TVVAIFPSPM	HYAGPTEVQW	HAKARINAGA	NFYIVGRDPA
AAL61615	(351)	GMGHPIEKRD	LYDADHGKKV	LSMAPGLEKL	NILPFRVAAY	DKTQGMDF
AAF18998	(351)	GMGHPIEKRD	LYDADHGKKV	LSMAPGLEKL	NILPFRVAAY	DKTQGMDF
AAL61615	(401)	DQSRPEDFVF	ISGTMRSLSA	KNKENPPDGF	MCPGGWKVLV	EYYEGIAAVQ
AAF18998	(401)	DQSRPEDFVF	ISGTMRSLSA	KNKENPPDGF	MCPGGWKVLV	EYYEGIAAVQ
AAL61615	(451)	SKKKIGVPAS	V			
AAF18998	(451)	SKKKIGVPAS	V			

² www.cbs.dtu.dk/services/NetPhos

Phosphorylation prediction score for every amino acid on sequence AAL61615.



Phosphorylation prediction score for every amino acid on sequence AAF18998.



Summary of high scoring sites predicted for serine and threonine residues in ATPS sequences (AAF18998 and AAL61615).

Name of amino acid	Position		Score	
	AAL61615	AAF18998	AAL61615	AAF18998
Serine	39	36	0.967	0.967
	49	46	0.879	0.879
	224	221	0.993	0.993
	233	230	0.971	0.971
	291	288	0.973	0.973
Threonine	13	13	0.835	0.835
	63	60	0.986	0.986
	183	180	0.858	0.858
	260	257	0.937	0.937

Appendix VI

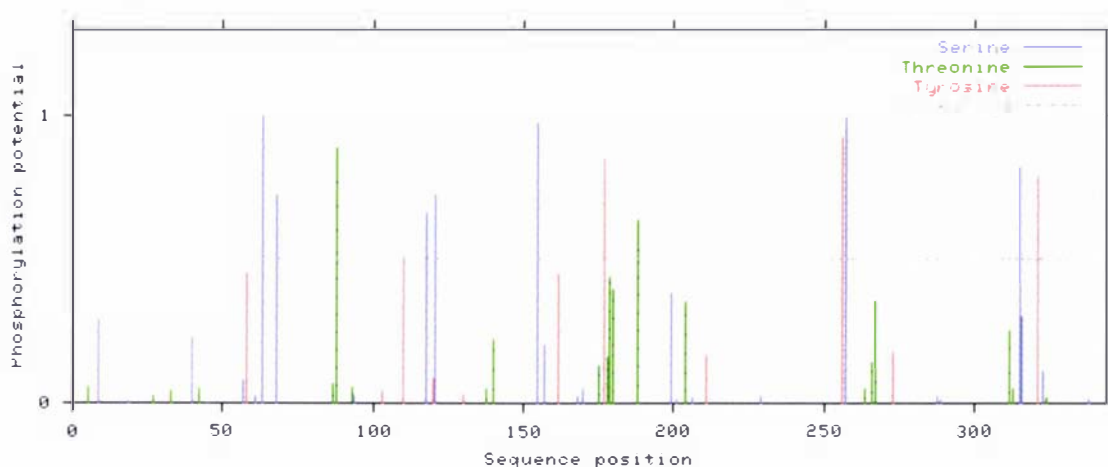
Putative phosphorylation sites in *OAS-TL* sequence from onion, as determined by NetPhos 2.0³

Putative phosphorylated serine sites are highlighted in yellow, and putative phosphorylated threonine sites in green. The putative 14-3-3 binding site is highlighted in blue.

```

Onion   (1)  MGERTGLASF IAAHND DASE NIVQDITQLV GWTPLIEIKS ITKADNV DAR
Onion   (51) IIAKLESYQP SNSVKDRSAL RMIEEAEEKG LIKPGVTILL EPTSGNLGIA
Onion   (101) LAYIGLKKGY KFIGVMPSHY SIERRMLLKY LGADIAITDT KLGFKGVLDK
Onion   (151) VGELSASDPN FYVLNQFSNS ANPGTHYTTT GPEIWKDTGG KVDIFVVGSG
Onion   (201) SGGTVSGAGK YLKEKNPNVK VICVEPAESA VIAGGPPGPH GIQGFPGPFV
Onion   (251) PDNDVYSVID EFLT VTTQEA LAYARRIAKE EGMLMGISSG ANLVACLKVG
Onion   (301) ARPENK GKMI VTFSSGGER YMSTQLFNEV REECVNMSF
  
```

Phosphorylation prediction score for every amino acid on sequence AAF18998.



Summary of high scoring sites predicted for serine and threonine residues on *OAS-TL* onion sequence.

Name of amino acid	Position		Score	
	Onion	Arabidopsis	Onion	Arabidopsis
Serine	63	46	0.997	0.995
	155	104	0.975	0.952
	257	171	0.996	0.875
		306		0.995
	315	312	0.820	0.834
Threonine	88	163	0.888	0.848
		256		0.790

³ www.cbs.dtu.dk/services/NetPhos

Appendix VII

Putative phosphorylation sites in APSR sequence from onion, as determined by NetPhos 2.0⁴

Putative phosphorylated serine sites are highlighted in yellow, and putative phosphorylated threonine sites in green.

```
AF212155 (1) MASATSTAAI SGSISAHGLL HHDVKVAQIG NLGIQLKNCN INTSLINKKR
AF212155 (51) WSVHAVDPAK ATAGAVSDQF VEKKNLNDIN DETVEDFDKL AADLENASPL
AF212155 (101) EIMDRALAKF GGDIAIAFSG AEDVALIEYA RLTFGFDLVEK HYNIHIEYMF
AF212155 (151) PDAVEVQGLV RNKGLFSFYE DGHQECCRVK KVRPLRRALK GLKAWITGQR
AF212155 (201) KDQSPGTRAH IPVVQVDPVF EGLEGGVGSV VKWNPLANVE GNNVWNFLRT
AF212155 (251) MDVPVNSLHT KGYVSI GCEP CTRPVLPGQH EREGRWWWED AKAKECGLHK
AF212155 (301) GNIASADSKD STTGGLTPAT ERISDLFKSG SIVDLTRPGM ENLLKIENKQ
AF212155 (351) DAWIVVLYAP WCQFCQGMES SYVELAEKLS GSGVKVGKFR ADGDQKEFAQ
AF212155 (401) QKLQLQTFPT VLFFPKFAAG PVKYPSEKRD VDSLLAFVNA LR
```

Phosphorylation prediction score for every amino acid on APSR onion sequence.

⁴ www.cbs.dtu.dk/services/NetPhos

Summary of high scoring sites predicted for serine and threonine residues in APSR sequence.

Name of Amino Acid	Position	Score
Serine	52	0.971
	98	0.923
	119	0.926
	167	0.831
	265	0.979
	308	0.988
	311	0.981
	324	0.717
	331	0.775
	426	0.997
Threonine	133	0.934

Appendix VIII

Putative phosphorylation sites in SiR partial sequence from onion, as determined by NetPhos 2.0⁵

Putative phosphorylated serine sites are highlighted in yellow, and putative phosphorylated threonine sites in green.

```
AY753557S1 (1) RSYSEMLRTK NPCGKVPNEL YIAMDTLTLADE FGIGTLRLTTT RQTFQLHGIL
AY753557S1 (51)KKNLKTVMST I IHNMGSTLG ACGDLNRNVL APAAPYTKKE YVFAQETADN
AY753557S1 (101) IAALLTPQSG FYYDMWVDGE KIMTSEPPEV TKARNDNSHG TNFPDSPEPI
AY753557S1 (151) HGTQFLPRKF KIAVTVPTDN SVDILTNDIG LVVISDSNGE PQGFNLYAGG
AY753557S1 (201) GMGRAHRTDT TFPRLAEHLG YVPKEDILYA IKAIVVTQRE
```

Phosphorylation prediction score for every amino acid on APSR onion sequence.

Summary of high scoring sites predicted for serine and threonine residues in SiR partial sequence.

Name of Amino Acid	Position	Score
Serine	146	0.997
	185	0.815
Threonine	26	0.832
	35	0.955
	39	0.957
	87	0.893
	124	0.715
	210	0.977
	237	0.768

⁵ www.cbs.dtu.dk/services/NetPhos

Appendix IX

Putative phosphorylated sites and putative 14-3-3 protein binding sites in SiR sequence from *Arabidopsis* (CAA89154), as determined by Scansite⁶

Putative phosphorylated serine sites are highlighted in yellow, and putative phosphorylated threonine sites in green. Putative 14-3-3 binding sites are highlighted in blue.

```
(1)  MSSTFRAPAG AATVFTADQK IRLGRLDALR SSSVFLGRY GRGGVPVPPS
(51)  ASSSSSPIQ AVSTPAKPEI ATKRSKVEII KEKSNFIRYP LNEELLTEAP
(101) NVNESAVQLI KFHGSYQQYN REERGGRSYS FMLRKNPSG KVPNQLYLTM
(151) DDLADEFGIG TLRLTTRQTF QLHGVLKQNL KTMSSIIKN MGSTLGACGD
(251) LNRNVLAPAA PYVKKDYLFQ QETADNIAAL LSPQSGFYD MWVDGEQFMT
(301) AEPPEVVKAR NDNSHGTFV DSPEPIYGTQ FLPRKFKVAV TVPTDNSVDL
(351) LTNDIGVVVV SDENGEPQGF NIYVGGGMGR THRMESTFAR LAEPIGYVPK
(401) EDILYAVKAI VVTQREHGRR DDRKYSRMKY LISSWGIEKF RDVVEQYYGK
(451) KFEPSRELPE WEFKSYLGWH EQGDGTWFCG LHVDSGRVGG IMKKTLEVI
(501) EKYKIDVRIT PNQNIIVLCDI KTEWKRPIIT VLAQAGLLQP EFVDPLNQTA
(551) MACPAFPLCP LAITEAERGI PSILKRVRAM FEKVGLDYDE SVVIRVTGCP
(601) NGCARPYMAE LGLVGDGPNS YQVWLGTPN LTQIARFMD KVKVHDLEKV
(651) CEPLFYHWKL ERQTKESFGE YTTRMGFEKL KELIDTYKGV SQ
```

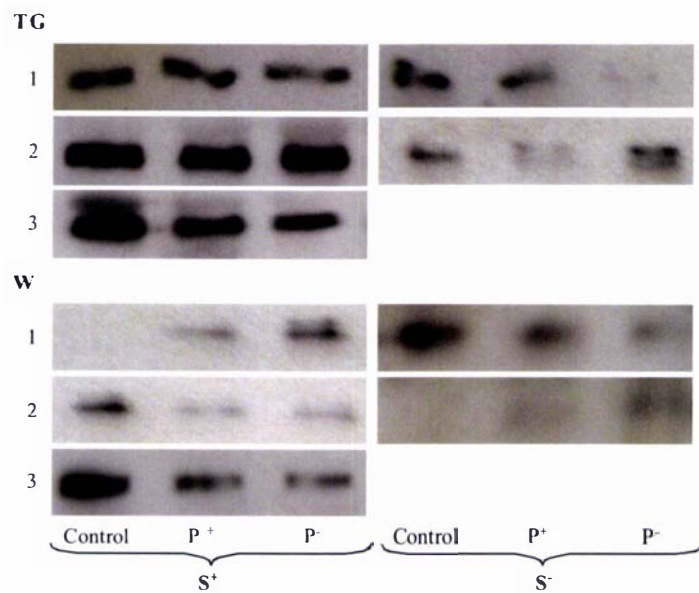
⁶ <http://scansite.mit.edu/>

Appendix X

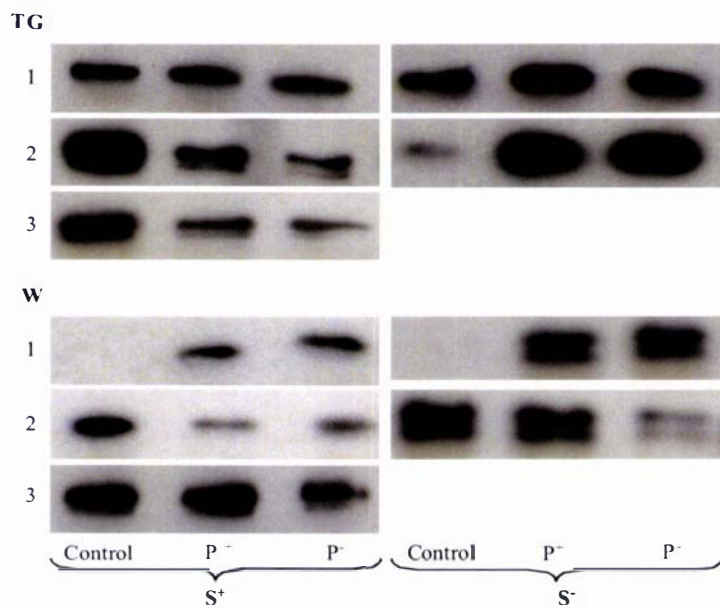
Influence of the phosphorylation (P^+) and the dephosphorylation (P^-) treatments, as indicated, on the molecular mass of ATPS, APSR, SiR, or *OAS*-TL, as indicated, from chloroplast extracts of mature plants of TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, using PP2A as the phosphatase.

Proteins (10 μ g) were treated with MgATP and NaF, EDTA and EGTA as phosphatase inhibitors to favour protein phosphorylation (P^+) or with PP2A to promote dephosphorylation (P^-) or untreated (control), and separated by 1D-PAGE. The enzyme ATPS was detected by western analyses using chemiluminescence. Numbers represent the repetitions.

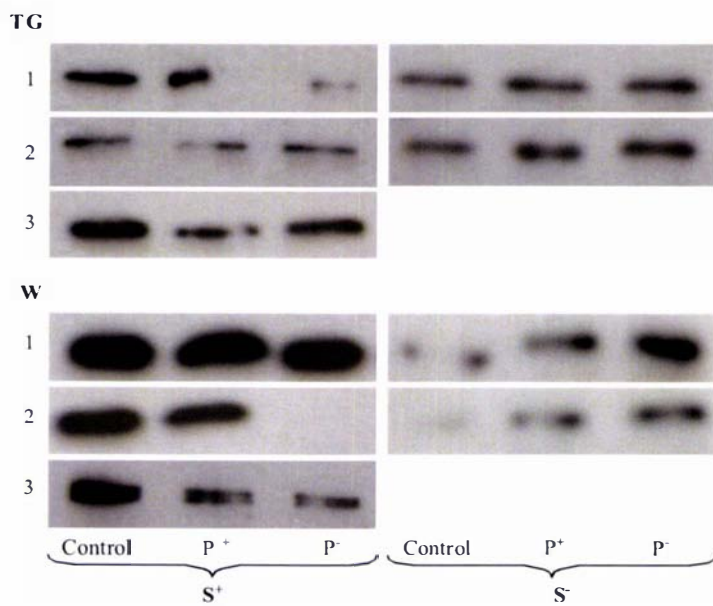
ATPS



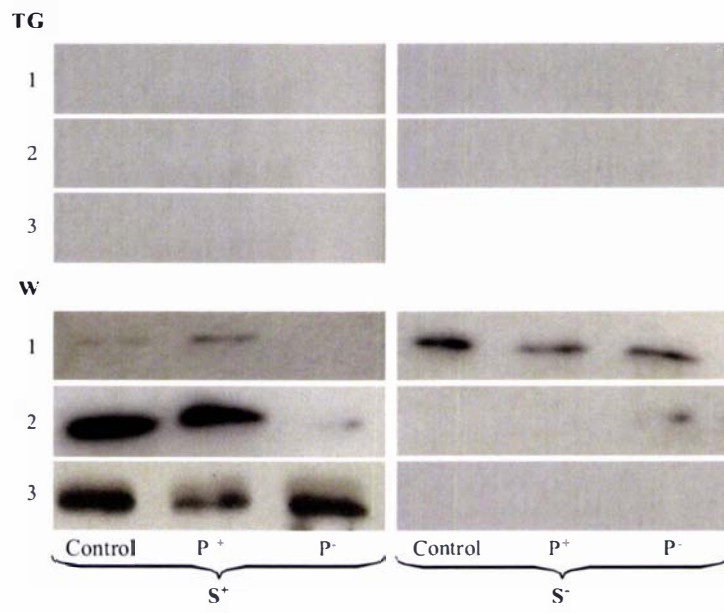
APSR



SiR



OAS-TL



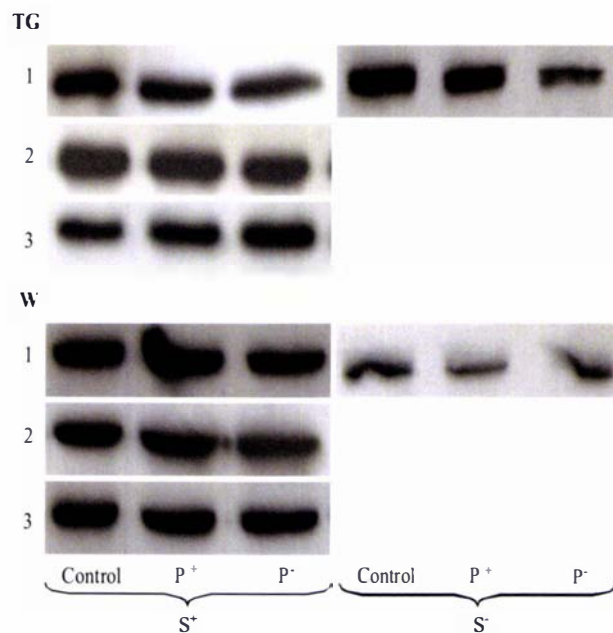
Appendix XI

Influence of the phosphorylation (P^+) and the dephosphorylation (P^-) treatments, as indicated, on the molecular mass of ATPS, APSR, SiR, or OAS-TL, as indicated, from chloroplast extracts of seedlings or mature plants, as indicated, of TG and W, as indicated, grown in the S-sufficient (S^+) or the S-deficient (S^-) media, as indicated, using AAP as the phosphatase.

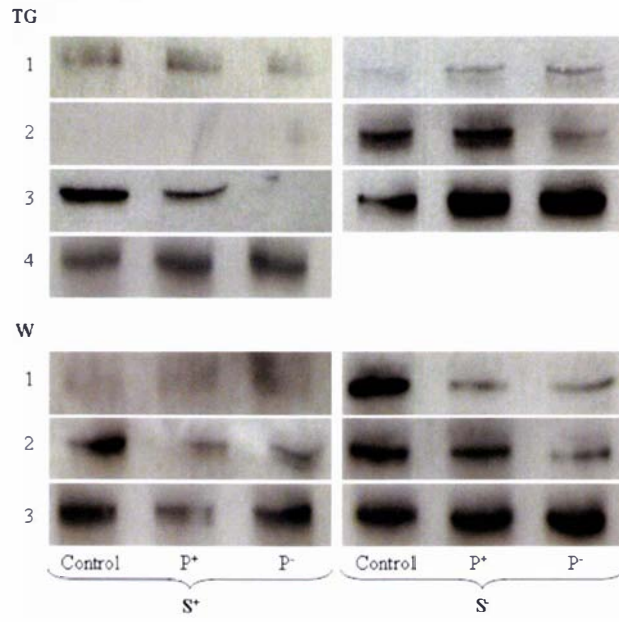
Proteins (10 μ g) were treated with MgATP and NaF, EDTA and EGTA as phosphatase inhibitors to favour protein phosphorylation (P^+) or with AAP to promote dephosphorylation (P^-) or untreated (control), and separated by 1D-PAGE. The enzyme ATPS was detected by western analyses using chemiluminescence. Numbers represent the repetitions.

ATPS

Seedlings

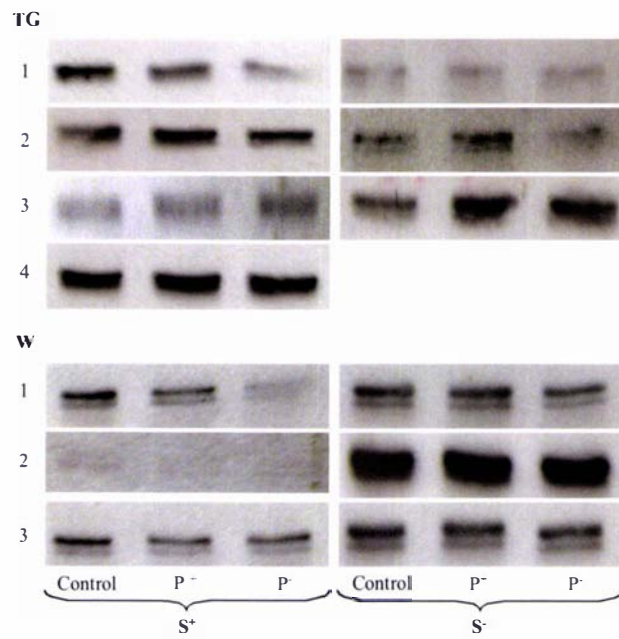


Mature plants

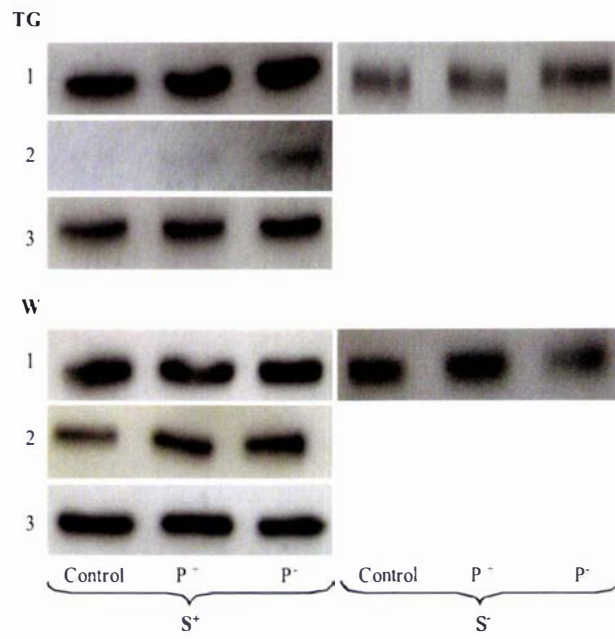


APSR

Seedlings

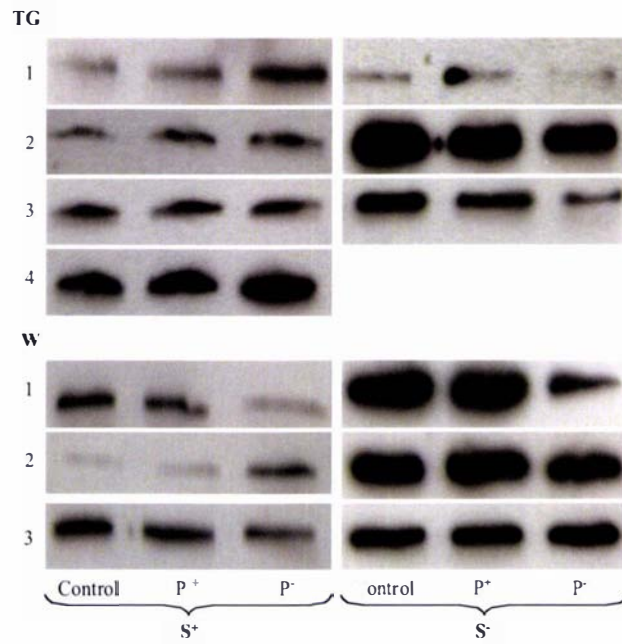


Mature plants



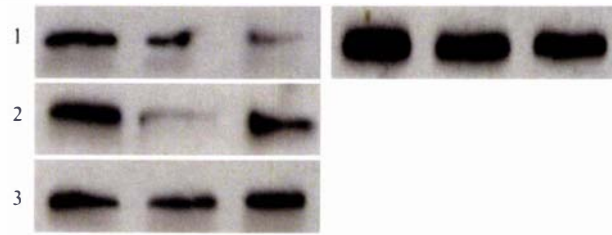
SiR

Seedlings

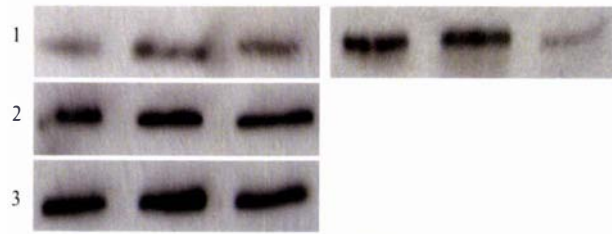


Mature plants

TG



W



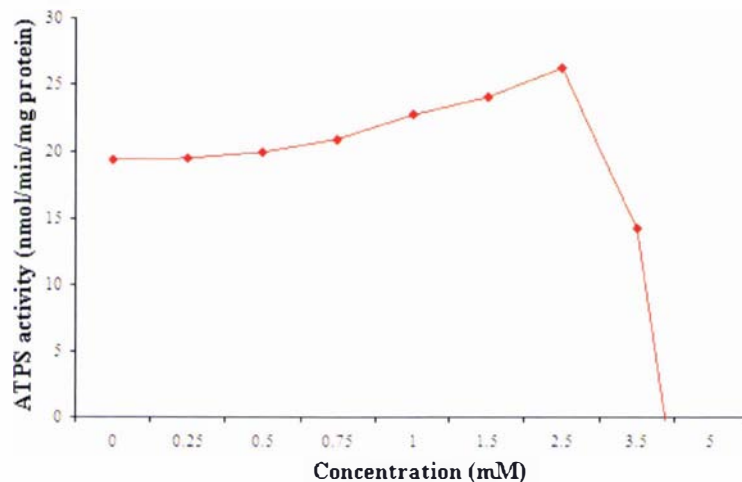
Control P⁻ P⁻ Control P⁻ P⁻
S⁺ S⁻

Appendix XII

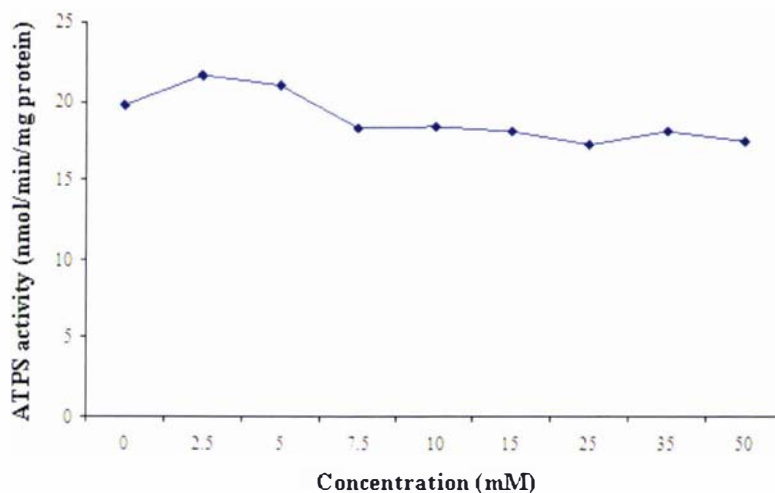
Influence of chemical treatments on ATPS activity using chloroplast extracts from *W* grown in the S-sufficient media.

ATPS activity was measured in chloroplast extracts after the addition of increasing concentrations of the chemical, as indicated, into the assay. Data represent the value for one repetition.

Effect of increasing concentrations of ATP

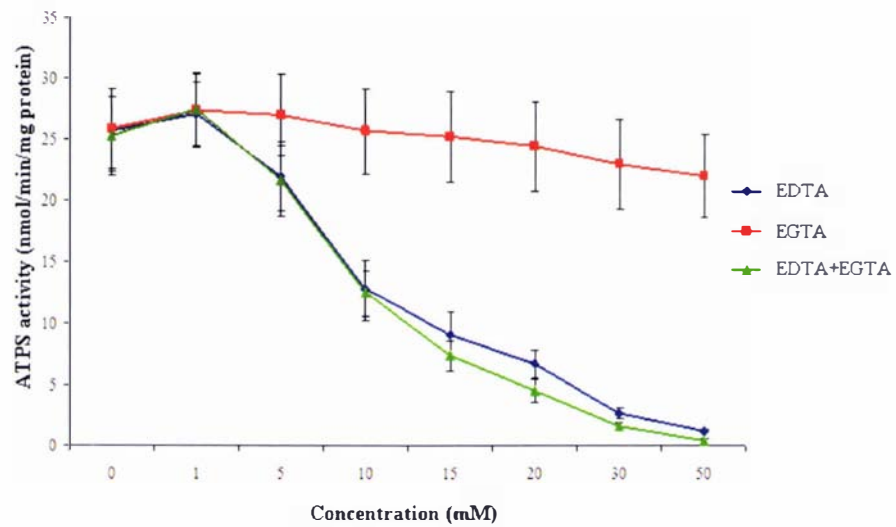


Effect of increasing concentrations of $MgCl_2$

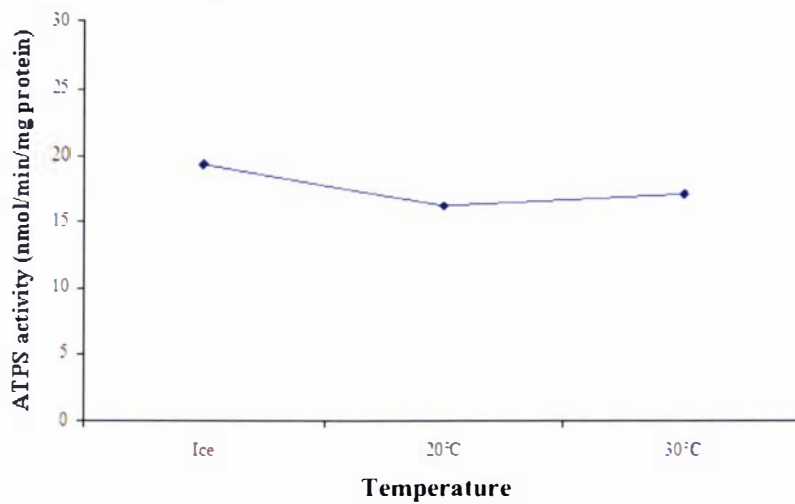


Effect of increasing concentrations of EDTA or EGTA or EDTA and EGTA

Each point represent the average of three repetitions. Interval bars represent the standard error.



Effect of the incubation temperature



Appendix XIII

DNA sequence of the BMH1 and BMH2 recombinant proteins.

The start and stop codon are highlighted in green, and the His-tag in red.

BMH1

```
BMH1 (1)      ATGGGGGGT CTCATCATCA TCATCATCAT GGTATGGCTA GCATGACTGS
BMH1 (51)     TGGACAGCAA ATGGGTCGGG ATCTGTACGA CGATGACGAT AAGGATCGAT
BMH1 (101)    GGGGATCCAT GTCAACCAGT CGTGAAGATT CTGTGTACCT AGCCAAGTTG
BMH1 (151)    GCTGAACAGG CCGAACGTTA TGAAGAAATG GTCGAAAACA TGAAGACTGT
BMH1 (201)    TGCCTCCTCT GGCCAAGAGT TGTCGGTCGA AGAGCGTAAT TTGTTGTCTG
BMH1 (251)    TTGCTTATAA GAACGTTATT GGTGCTCGTC GTGCCTCTTG GAGAATTGTT
BMH1 (301)    TCTTCTATTG AGCAAAAGGA GGAGTCCAAG GAGAAGTCCG AACACCAGGT
BMH1 (351)    CGAGTTGATT TGTTCGTACC GTTCGAAGAT TGAGACCGAA CTAACTAAGA
BMH1 (401)    TCTCCGACGA TATTTTGTCC GTGCTAGACT CCCACTTAAT TCCATCAGCC
BMH1 (451)    ACCACTGGCG AGTCCAAGGT TTTCTACTAT AAGATGAAGG GTGACTACCA
BMH1 (501)    CCGTTATTTG GCTGAATTTT CTAGTGGCGA TGCTAGAGAA AAGGCCACAA
BMH1 (551)    ACGCCTCTTT AGAAGCATAC AAGACCGCTT CTGAAATTGC CACCACAGAG
BMH1 (601)    TTACCCCAA CTCACCCAAT CCGTCTAGGT TTGGCTCTTA ACTTCTCTGT
BMH1 (651)    CTTCTATTAT GAAATTCAAA ACTCTCCAGA CAAAGCCTGC CATTGGCCA
BMH1 (701)    AGCAAGCTTT TGACGACGCT ATTGCTGAGT TGGACACTCT GTCTGAAGAA
BMH1 (751)    TCATACAAAG ATAGCACACT TATCATGCAA CTGCTAAGGG ACAATTTAAC
BMH1 (801)    CTTATGGACT TCAGACATGT CCGAGTCCGG TCAAGCTGAA GACCAACAAC
BMH1 (851)    AACACAACA ACATCAGCAA CAGCAGCCAC CTGCTGCCGC CGAAGGTGA.
BMH1 (901)    GCACCAAAGT AAGAATCGA AGCTTGGCTG TTT.GGCGGA T
```

BMH2

BMH2 (1) ATG GGGGGTT CT CATCATCA [REDACTED] GGTATGGCTA GCATGACTGS
BMH2 (51) YYKWCAGCAA ATGGGTCGGG ATCTGTACGA CGATGACGAT AAGGATCGAT
BMH2 (101) GGGGATCCAT GTCCCA AACT CGTGAAGATT CTGTTTACCT AGCTAAATTA
BMH2 (151) GCTGAACAAG CCGAACGTTA TGAAGAAATG GTCGAAAACA TGAAGGCCGT
BMH2 (201) TGCTTCATCA GGTCAAGAGT TATCTGTCGA AGAACGGAAT CTATTGTCGG
BMH2 (251) TTGCTTACAA GAACGTCATC GGTGCTCGCC GTGCTTCATG GAGAATAGTT
BMH2 (301) TCTTCGATCG AACAAAAAGA AGAATCAAAG GAGAAATCTG AACATCAAGT
BMH2 (351) TGAATTAATC CGTTCCTACC GTTCTAAAAT TGAAACTGAA TTGACCAAAA
BMH2 (401) TCTCTGACGA CATTTTATCT GTGTTAGATT CTCATTTAAT CCCTTCTGCT
BMH2 (451) ACTACTGGTG AGTCTAAAGT ATTTTACTAT AAGATGAAGG GTGACTACCA
BMH2 (501) CCGTTATTTA GCTGAATTTT CCAGCGGAGA TGCAAGAGAA AAGGCAACCA
BMH2 (551) ACTCCTCTTT GGAGGCTTAT AAAACCGCTT CCGAAATCGC CACA ACTGAA
BMH2 (601) TTGCCTCCAA CTCACCCAAT TCGTTTAGGT CTAGCTTTGA ATTTCTCCGT
BMH2 (651) CTTCTATTAC GAAATTCAAA ACTCTCCTGA TAAGGCTTGC CACTTGGCCA
BMH2 (701) AACAAAGCCTT TGATGATGCT ATTGCTGAGT TAGATACTTT ATCTGAAGAA
BMH2 (751) TCATACAAGG ATAGCACTTT GATCATGCAA TTATTAAGGG ACAACTTGAC
BMH2 (801) CTTATGGACC TCTGATATTT CTGAATCTGG TCAAGAA GAT

Appendix XIV

BLAST results using DNA sequences of BMH1 and BMH2 inserted into pTrcHisA.

The query represents the DNA sequence of the sequenced BMH, and the subject the DNA sequence of *S. cerevisiae*. The sign – represents unknown nucleotides.

S. cerevisiae BMH1 gene

Length=1284 bp

Score = 1347 bits (729), Expect = 0.0

Identities = 734/736 (99%), Gaps = 2/736 (0%)

Strand=Plus/Plus

```
Query 11 ATGTCAACCAGTCGTGAAGATTCTGTGTACCTAGCCAAGTTGGCTGAACAGGCCGAACGT 70
      |||
Subject 79 ATGTCAACCAGTCGTGAAGATTCTGTGTACCTAGCCAAGTTGGCTGAACAGGCCGAACGT 138

Query 71 TATGAAGAAATGGTCGAAAACATGAAGACTGTTGCCTCCTCTGGCCAAGAGTTGTTCGGTC 130
      |||
Subject 139 TATGAAGAAATGGTCGAAAACATGAAGACTGTTGCCTCCTCTGGCCAAGAGTTGTTCGGTC 198

Query 131 GAAGAGCGTAATTTGTTGTCTGTTGCTTATAAGAACGTTATTGGTGCTCGTCGTGCCTCT 190
      |||
Subject 199 GAAGAGCGTAATTTGTTGTCTGTTGCTTATAAGAACGTTATTGGTGCTCGTCGTGCCTCT 258

Query 191 TGGAGAATTGTTTCTTCTATTGAGCAAAAGGAGGAGTCCAAGGAGAAGTCCGAACACCAG 250
      |||
Subject 259 TGGAGAATTGTTTCTTCTATTGAGCAAAAGGAGGAGTCCAAGGAGAAGTCCGAACACCAG 318

Query 251 GTCGAGTTGATTTGTTTCGTACCGTTCGAAGATTGAGACCGAACTAACTAAGATCTCCGAC 310
      |||
Subject 319 GTCGAGTTGATTTGTTTCGTACCGTTCGAAGATTGAGACCGAACTAACTAAGATCTCCGAC 378

Query 311 GATATTTTGTCCGTGCTAGACTCCCCTTAATTCATCAGCCACCCTGGCGAGTCCAAG 370
      |||
Subject 379 GATATTTTGTCCGTGCTAGACTCCCCTTAATTCATCAGCCACCCTGGCGAGTCCAAG 438

Query 371 GTTTTCTACTATAAGATGAAGGGTGACTACCACCGTTATTTGGCTGAATTTTCTAGTGGC 430
      |||
Subject 439 GTTTTCTACTATAAGATGAAGGGTGACTACCACCGTTATTTGGCTGAATTTTCTAGTGGC 498

Query 431 GATGCTAGAGAAAAGGCCACAAAACGCCTCTTTAGAAGCATAACAAGACCCTTCTGAAATT 490
      |||
Subject 499 GATGCTAGAGAAAAGGCCACAAAACGCCTCTTTAGAAGCATAACAAGACCCTTCTGAAATT 558

Query 491 GCCACCACAGAGTTACCCCCAACTACCCAATCCGTCTAGGTTTGGCTCTTAACTTCTCT 550
      |||
Subject 559 GCCACCACAGAGTTACCCCCAACTACCCAATCCGTCTAGGTTTGGCTCTTAACTTCTCT 618

Query 551 GTCTTCTATTATGAAATTCAAAACCTCTCCAGACAAAGCCTGCCATTTG-GCCAAGCAAGC 609
      |||
Subject 619 GTCTTCTATTATGAAATTCAAAACCTCTCCAGACAAAGCCTGCCATTTGCGC-AAGCAAGC 677

Query 610 TTTTGACGACGCTATTGCTGAGTTGGACACTCTGTCTGAAGAATCATACAAAGATAGCAC 669
      |||
Subject 678 TTTTGACGACGCTATTGCTGAGTTGGACACTCTGTCTGAAGAATCATACAAAGATAGCAC 737

Query 670 ACTTATCATGCAACTGCTAAGGGACAATTTAACCTTATGGACTTCAGACATGTCCGAGTC 729
      |||
Subject 738 ACTTATCATGCAACTGCTAAGGGACAATTTAACCTTATGGACTTCAGACATGTCCGAGTC 797

Query 730 CGGTCAAGCTGAAGAC 745
      |||
Subject 798 CGGTCAAGCTGAAGAC 813
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***S. cerevisiae* 14-3-3-like protein (BMH2) gene, complete cds**

Length=1214 bp

Score = 1312 bits (710), Expect = 0.0

Identities = 710/710 (100%), Gaps = 0/710 (0%)

Strand=Plus/Plus

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Query 1 GTCCCAAAC TCGTGAAGATTCTGTTTACCTAGCTAAATTAGCTGAACAAGCCGAACGTTA 60
      |||
Subject 285 GTCCCAAAC TCGTGAAGATTCTGTTTACCTAGCTAAATTAGCTGAACAAGCCGAACGTTA 344

Query 61 TGAAGAAATGGTCGAAAACATGAAGGCCGTTGCTTCATCAGGTCAAGAGTTATCTGTCTGA 120
      |||
Subject 345 TGAAGAAATGGTCGAAAACATGAAGGCCGTTGCTTCATCAGGTCAAGAGTTATCTGTCTGA 404

Query 121 AGAACGGAATCTATTGTCGGTTGCTTACAAGAACGTCATCGGTGCTCGCCGTGCTTCATG 180
      |||
Subject 405 AGAACGGAATCTATTGTCGGTTGCTTACAAGAACGTCATCGGTGCTCGCCGTGCTTCATG 464

Query 181 GAGAATAGTTTCTTCGATCGAACAAAAAGAAGAATCAAAGGAGAAATCTGAACATCAAGT 240
      |||
Subject 465 GAGAATAGTTTCTTCGATCGAACAAAAAGAAGAATCAAAGGAGAAATCTGAACATCAAGT 524

Query 241 TGAATTAATCCGTTCTTACCGTTCTAAAATTGAAACTGAATTGACCAAATCTCTGACGA 300
      |||
Subject 525 TGAATTAATCCGTTCTTACCGTTCTAAAATTGAAACTGAATTGACCAAATCTCTGACGA 584

Query 301 CATTTTATCTGTGTTAGATTCTCATTTAATCCCTTCTGCTACTACTGGTGAGTCTAAAGT 360
      |||
Subject 585 CATTTTATCTGTGTTAGATTCTCATTTAATCCCTTCTGCTACTACTGGTGAGTCTAAAGT 644

Query 361 ATTTTACTATAAGATGAAGGGTGACTACCACCGTTATTTAGCTGAATTTCCAGCGGAGA 420
      |||
Subject 645 ATTTTACTATAAGATGAAGGGTGACTACCACCGTTATTTAGCTGAATTTCCAGCGGAGA 704

Query 421 TGCAAGAGAAAAGGCAACCAACTCCTCTTTGGAGGCTTATAAAAACCGCTTCCGAAATCGC 480
      |||
Subject 705 TGCAAGAGAAAAGGCAACCAACTCCTCTTTGGAGGCTTATAAAAACCGCTTCCGAAATCGC 764

Query 481 CACAAC TGAATTGCCTCCAAC TACCCAATT CGTTTAGGTCTAGCTTTGAATTTCTCCGT 540
      |||
Subject 765 CACAAC TGAATTGCCTCCAAC TACCCAATT CGTTTAGGTCTAGCTTTGAATTTCTCCGT 824

Query 541 CTTCTATTACGAAATTCAAAAC TCTCCTGATAAGGCTTGCCACTTGGCCAAACAAGCCTT 600
      |||
Subject 825 CTTCTATTACGAAATTCAAAAC TCTCCTGATAAGGCTTGCCACTTGGCCAAACAAGCCTT 884

Query 601 TGATGATGCTATTGCTGAGTTAGATACTTTATCTGAAGAATCATACAAGGATAGCACTTT 660
      |||
Subject 885 TGATGATGCTATTGCTGAGTTAGATACTTTATCTGAAGAATCATACAAGGATAGCACTTT 944

Query 661 GATCATGCAATTATTAAGGGACAAC TTAGACCTTATGGACCTCTGATATTT 710
      |||
Subject 945 GATCATGCAATTATTAAGGGACAAC TTAGACCTTATGGACCTCTGATATTT 994
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