Arabidopsis ATP Sulfurylase: Roles and Regulation of Individual Isoforms

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ABSTRACT

Sulfur is an indispensable macronutrient for all organisms. Plants take up sulfate from the soil and assimilate it into a range of essential compounds. ATP sulfurylase (ATPS) occupies a central position in plant sulfur metabolism as it catalyzes the first step of both primary and secondary sulfate assimilation. Despite its central position in the pathway, little is known about the contribution of ATPS to the control of sulfur metabolism. Four ATPS isoforms are present in *Arabidopsis thaliana*, yet their individual functions are unknown. The aim of this thesis was thus to assess the role of ATPS in regulation of sulfur metabolism and to contribute to better understanding of function of individual ATPS isoforms. Exploration of tissue specific expressions and responses of these four isoforms to environmental cues revealed extensive differences between the isoforms. The ATPS transcripts are targeted by a microRNA, miR395, which itself is induced by the SULFUR LIMITATION 1 (SLIM1) transcription factor in response to sulfur deficiency. Dissection of sulfur deficiency response exposed interplay between SLIM1 and miR395 in the modification of individual ATPS gene expression. Moreover, miR395 induction was shown to increase translocation of sulfate from root-to-shoot, ensuring sufficient sulfate supply where most needed for assimilation. A further role of miR395 in fine-tuning demand-driven regulation of ATPS under normal sulfur supply was revealed through systematic analysis of miR395 and its targets. Analysis of regulation of glucosinolate biosynthesis by two groups of R2R3-MYB transcription factors revealed that ATPS isoforms ATPS1 and ATPS3 are targets of these transcription factors. The two groups of MYB factors activated ATPS1 and ATPS3 differently, demonstrating that ATPS is an integral part of the glucosinolate biosynthesis regulatory network. The results presented in this thesis support the importance of the four individual ATPS isoforms, and increase our understanding of regulatory mechanisms for sulfate assimilation.

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Abbreviations

ABA	Abscisic acid
APK	APS kinase
APR	APS reductase
APS	Adenosine 5’ phosphosulfate
ATPS	ATP sulfurylase
BSO	Buthionine sulfoximine
ET	Ethylene
IAA	Indole-3-acetic acid
GA	Giberellic acid
γ-EC	γ-glutamylcysteine
GSH1 (γ-ECS)	Glutathione synthase (γ- glutamylcysteine synthetase)
GSH2	Glutathione synthetase
JA	Jasmonic acid
MeJa	Methyl jasmonate
miRNA	microRNA
OAS	*O*-acetylserine
OASTL	*O*-acetylserine (thiol) lyase
PAPS	3’-phosphoadenosine 5’-phosphosulfate
SA	Salicylic acid
SAM	*S*-adenosylmethionine
SAT	Serine acetyl transferase
SiR	Sulfite reductase
SLIM1	SULFUR LIMITATION 1
SMM	*S*-methylmethionine
SOT	Sulfotransferase
SULTR	Sulfate transporter

Chapter 1

General Introduction

1. GENERAL INTRODUCTION

1.1. Plant nutrition

Animals are heterotrophs that require the ingestion of other organisms to obtain their nutrients. Plants, on the other hand, are autotrophic and thus able to assimilate nutrients from the environment around them. The non-mineral nutrients - hydrogen, oxygen, and carbon - are converted into sugars during photosynthesis, but there are also thirteen mineral nutrients essential to plants, that must be acquired from the soil. Six of these essential minerals are necessary in larger quantities and hence considered macronutrients: nitrogen, potassium, calcium, magnesium, phosphorus, and last but not least sulfur. In a world in which demands on agriculture are constantly growing, changing, and diversifying, improved comprehension of plant nutrient assimilation systems undeniably impacts on human life. The output of plant nutrition research can often be applied directly to improve growing and breeding methods for crop plants. Alternatively, genetic engineering promises to deliver plants with increased nutrient assimilation capacity and better nutrient use efficiency, with the potential to reduce fertilizer and pesticide use, increase crop yield, and improve the crop nutritional value for the consumer. Not only could such traits increase food quality and crop yield, but also lower expenses for farmers and reduce environmental damage.

1.2. Sulfur nutrition

Sulfur is an essential element for all organisms. Animals have dietary requirements for organic sulfur compounds, such as methionine, which they obtain through the consumption of other organisms (Fukagawa, 2006). In contrast, plants, as well as most bacteria and fungi, use inorganic sulfate as their major sulfur source. In addition to sulfate, plants are able to supplement their sulfur supplies with reduced sulfur forms absorbed

from the atmosphere, such as sulfur dioxide and hydrogen sulfide (Leustek *et al.*, 2000; Durenkamp and De Kok, 2004). In plants, sulfur is found in reduced form in sulfur amino acids, peptides and proteins, iron-sulfur clusters, lipoic acid and other sulfur-containing metabolites and coenzymes/cofactors. It is also found in an oxidized state in many compounds, including sulfated proteins, sulfated hormones (e.g. phytosulfokine; PSK), polysaccharides and lipids. Sulfur is often central to the biological function of these compounds. Disulfide bonds created between the sulfur containing amino acids cysteine and methionine are vital for protein structure, stability, and regulation. Sulfur also plays a critical role in the catalytic or electrochemical functions of coenzymes and other biomolecules. Glutathione, a major product of the sulfate assimilation pathway, is a key component in the maintenance of cell redox homeostasis and the removal of reactive oxygen species (Leustek *et al.*, 2000; Hawkesford and De Kok, 2006; Kopriva, 2006). The reactivity of the nucleophilic thiol group makes cysteine, methionine, glutathione, coenzyme A and vitamins such as biotin, lipoic acid and thiamine very versatile molecules in intermediary metabolism (Hell *et al.*, 2002). Other sulfur containing compounds, such as the sulfur-rich secondary metabolites, the glucosinolates, function in defence against biotic stress caused by herbivore and pathogen attack (Halkier and Gershenzon, 2006). Glucosinolates in crops from the *Brassicales* order, and alkyl cysteine sulfoxides in *Allium* vegetables, are responsible for the distinctive smell and taste of these economically important species (McCallum *et al.*, 2005; Halkier and Gershenzon, 2006).

Sulfur deficiency has become an increasing problem for agricultural crops over the last few decades due to the combined effects of intensive farming methods, high yield crop varieties, decreased incidental application of sulfur in fertilisers, and declining atmospheric sulfur deposition (McGrath *et al.*, 1999). Available sulfur in the soils of agricultural land now lies well below the recommended levels for cereal and oilseed crops. McGrath *et al.* (1996) predicted deficiency problems for all crops, but particularly oilseed

rape, due to its higher sulfur requirement. Sulfur deficiency can cause reduced crop yield, quality, and nutritional value, and increased disease susceptibility. Sulfur limiting conditions during early plant development have been shown to have detrimental effects on carbon assimilation, partly due to reduced synthesis and activity of Rubisco (Gilbert *et al.*, 1997). Some degree of degradation of photosynthetic proteins has been recorded under such conditions as well, resulting in reduced chlorophyll and chlorosis of the leaves (Burke *et al.*, 1986). In addition, prolonged deprivation generally results in changes of shoot/root biomass partitioning in favor of root production. These effects can have significant consequences on the yield of many crops (Buchner *et al.*, 2004). Sulfur deficiency in wheat reduces the breadmaking quality due to a switch from the accumulation of sulfur-rich proteins to the synthesis and accumulation of sulfur-poor storage proteins (Zhao *et al.*, 1996; McGrath *et al.*, 1999). In addition, baked products using flour derived from sulfur-deprived wheat contain higher levels of toxic acrylamide (Muttucumaru *et al.*, 2006). In barley, not only the yield but also the malting quality of the grain is reduced by sulfur deficiency (Zhao *et al.*, 2006). Many sulfur containing phytochemicals have added nutritional value to the consumer, including isothiocyanates and glucosinolates thought to prevent cancer (Shapiro *et al.*, 2001; Verkerk *et al.*, 2009). Levels of these plant secondary metabolites are also reduced during sulfur deficiency (Hirai *et al.*, 2003; Nikiforova *et al.*, 2003). The availability of sulfur to plants is modified by interactions with other nutrients, mainly nitrogen and phosphorus. Thus, improved understanding of the mechanisms and control of sulfate assimilation is fundamental to improving sulfur nutrition of crop plants.

1.3. Sulfate assimilation in Arabidopsis

Assimilatory sulfate reduction occurs in various chemotrophic bacteria and fungi and in photosynthetic organisms but is missing in animals and most prokaryotic and eukaryotic

obligate parasites. Although the reactions of sulfate assimilation are essentially invariant throughout the organisms in which the pathway is present, high variability is seen among the structures of corresponding enzymes and the sizes of gene families in different organisms. This thesis is concerned with sulfate assimilation in the model plant species Arabidopsis thaliana.

Anionic sulfate (SO_4^{2-}), a chemically stable and therefore relatively unreactive sulfur-compound, is the major sulfur source utilized by plants. It is taken up by active transport into the roots, from which it can be redistributed to wherever it is required (Hawkesford, 2004). The first step of sulfate assimilation is the adenylation of sulfate to produce adenosine 5' phosphosulfate (APS), an activation reaction catalysed by ATP sulfurylase (ATPS; EC 2.7.7.4) in the presence of ATP (Figure 1.1). The pathway subsequently branches into primary and secondary metabolism. In primary assimilation, APS is reduced by APS reductase (APR; EC 1.8.4.9) to sulfite, which is further reduced to sulfide in a reaction catalyzed by sulfite reductase (SiR; EC 1.8.7.1). Sulfide is then incorporated into *0*-acetylserine (OAS) to form cysteine, a reaction catalyzed by *0*-acetylserine (thiol) lyase (OASTL; EC 2.5.1.47). Cysteine acts as a precursor or donor of reduced sulfur for many sulfur containing compounds, including methionine and glutathione. In the secondary assimilation pathway APS can be phosphorylated by APS kinase (APK; EC 2.7.1.25) to produce 3'-phosphoadenosine 5'-phosphosulfate (PAPS), a donor of activated sulfate for numerous sulfur secondary metabolites, such as glucosinolates. The sulfation of these secondary metabolites is catalyzed by a group of sulfotransferases (SOTs; EC 2.8.2.-; Leustek *et al.*, 2000; Kopriva, 2006). As a catalyst for the first step of both primary and secondary assimilation ATPS is a prime candidate for further investigation.

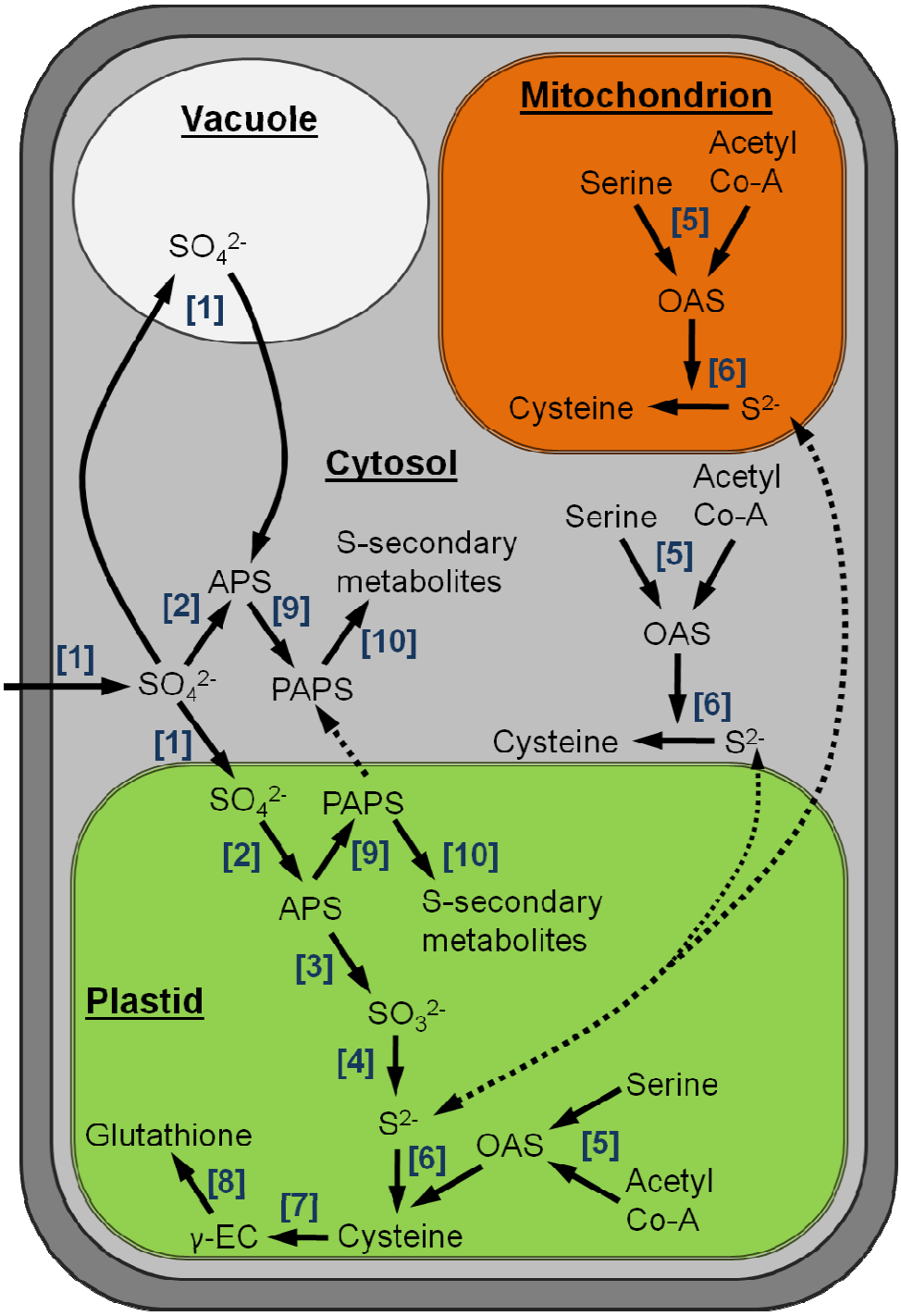


Figure 1.1 ***Scheme of the major steps of plant sulfate assimilation pathway.***
Numbers represent enzymes as follows: [1] sulfate transporter; [2] ATP sulfurylase; [3] APS reductase; [4] sulfite reductase; [5] serine acetyltransferase; [6] *O*-acetylserine (thiol) lyase; [7] γ-glutamylcysteine synthetase; [8] glutathione synthetase; [9] APS kinase; [10] sulfotransferase. Dotted lines represent hypothesized pathways.

Multi-gene families of most sulfate assimilation enzymes have been identified in many flowering plants (Table 1.1). The sequence conservation of these genes can vary substantially across species. The phylogenetic trees for the sulfate assimilation enzymes

are often very complex and the origins of the many plant genes, for example that of ATP sulfurylase, remain unclear (Patron *et al.*, 2008). In Arabidopsis, the exceptions to this are SiR and the glutathione synthesis enzymes for which only a single gene copy has been identified (Kopriva *et al.*, 2009). The biochemical characteristics of the sulfate assimilation proteins are well understood, and some of the gene families encoding these proteins have been systematically dissected. However, in many cases relatively little is known about the functions of the individual family members. The degree of specificity or functional redundancy of the individual isoforms remains to be deciphered. This is particularly true for the ATPS gene family.

	A. thaliana	***O. sativa***	***S. bicolor***	***P. trichocarpa***	***P. patens***	***S. moellendorffii***	***C. reinhardtii***	***T. pseudonana***	***E. huxleyi***
SULTR	14	13	9	15	7	7	5	2	5
ATPS	4	2	2	4	2	1	2	2	2
APK	4	3	3	3	4	4	1	2	2
APR	3	2	1	2	1 + 1[a]	1 + 1[a]	1	2[a]	1[a]
SiR	1	2	1	3	3	1	2	1	2
OASTL	9	9	6	10	3	4	3	3	11
SAT	5	5	3	5	5	3	2	3	3

Table 1.1 ***Genes of sulfate assimilation in sequenced plant and algal genomes***
The number of genes was determined in Kopriva et al. (2009).
[a] APR-B type of APS reductase

1.4. Sulfate transporters

Sulfate is actively transported from the soil into the cells of the roots. The genomes of plants, as well as of many algae, encode both high- and low-affinity sulfate transporter systems localized in plasma membranes which mediate H^+/SO_4^{2-} co-transport (Hawkesford, 2003; Buchner *et al.*, 2004; Takahashi, 2010). These transporters are responsible for uptake of sulfate from the rhizosphere and its translocation from source to sink tissues. The first sulfate transporter cDNAs were cloned from the tropical legume *Stylosanthes hamata* (Smith *et al.*, 1995). Multiple sulfate transporters have been identified

and characterised since (Cherest *et al.*, 1997; Smith *et al.*, 1997; Takahashi *et al.*, 2000; Vidmar *et al.*, 2000; Yoshimoto *et al.*, 2002; Yoshimoto *et al.*, 2003). Completion of the Arabidopsis genome sequencing project has enabled the identification of a large sulfate transporter gene family (The Arabidopsis Genome Initiative, 2000). This multi-gene family has fourteen members, which have been subdivided into four closely related groups as well as a fifth more diverse, but clearly related group (Table 1.2; Aravind and Koonin, 2000; Hawkesford, 2003). It has become apparent that the transporters within the individual groups are related not only by their sequences but also by their functions. Most knowledge about the function of these transporters has been obtained using Arabidopsis.

Name	AGI code	localisation	function
Group 1 (high-affinity)			
SULTR1;1	At4g08620	root hairs, epidermal and cortex cells, lateral root cap	S uptake from rhizosphere
SULTR1;2	At1g78000	root hairs, epidermal and cortex cells, guard cells	S uptake from rhizosphere
SULTR1;3	At1g22150	phloem companion cells in cotyledons and roots	Long-distance translocation?
Group 2 (low-affinity)			
SULTR2;1	At5g10180	xylem parenchyma cells, root pericycle, leaf phloem	endogenous translocation
SULTR2;2	At1g77990	root phloem and leaf vascular bundle sheath cells	endogenous translocation
Group 3 (low-affinity)			
SULTR3;1	At3g51900	-	endogenous translocation?
SULTR3;2	At4g02700	-	endogenous translocation?
SULTR3;3	At1g23090	-	endogenous translocation?
SULTR3;4	At3g15990	-	endogenous translocation?
SULTR3;5	At5g19600	xylem parenchyma and pericycle cells in roots	endogenous translocation?
Group 4 (low-affinity)			
SULTR4;1	At5g13550	tonoplast membrane	efflux from vacuole to cytoplasm
SULTR4;2	At3g12520	tonoplast membrane	efflux from vacuole to cytoplasm
Group 5 (unknown)			
SULTR5;1	At1g80310	-	
SULTR5;2	At2g25680		high-affinity molybdate transporter

Table 1.2 ***The sulfate transporter gene family - localisation and function*****.**

Primary uptake of sulfate into the roots is mediated by two Group one sulfate transporters, SULTR1;1 and SULTR1;2, which facilitate high-affinity sulfate uptake in micromolar concentrations. These two transporters are co-localized in the root tip and root endodermis, including root hairs and cells of the cortex (Takahashi *et al.*, 2000). A

third member of the Group one transporter family in Arabidopsis, SULTR1;3, is localized in the phloem companion cells of cotyledons and roots, where it is thought to mediate long-distance translocation (Yoshimoto *et al.*, 2003). Following initial uptake into the root epidermis, internal distribution of sulfate through the vasculature and cell-to-cell symplastic movement are required to ensure adequate sulfate supply to all organs and cell types. The endogenous translocation of sulfate from roots to leaves is facilitated in part by a pair of Group two low-affinity transporters: SULTR2;1 and SULTR2;2. SULTR2;1 is expressed in the xylem parenchyma of both roots and leaves, the root pericycle, and the leaf phloem. SULTR2;2 on the other hand is expressed in the root phloem and leaf vascular bundle sheath cells (Takahashi *et al.*, 2000). More recent evidence has revealed that SULTR2;1 may also be involved in the redistribution of sulfate from older to younger leaves (Liang *et al.*, 2010). Despite being the largest group, containing five members, the role of the Group three transporters is yet to be uncovered. Early experiments indicated that SULTR3;1 – 3;3 were leaf expressed (Takahashi *et al.*, 2000). However, Kataoka et al. (2004a) showed that one group member, SULTR3;5, is co-expressed with SULTR2;1 in the roots. Although sulfate uptake could not be detected with SULTR3;5 alone in a yeast expression system, cells co-expressing both SULTR3;5 and SULTR2;1 showed considerably higher sulfate uptake capacity that those expressing SULTR2;1 alone (Kataoka *et al.*, 2004a). Due to the co-expression with SULTR2;1, SULTR3;5 is hypothesized to be involved in root-to-shoot translocation. Surprisingly, Group three transporters have been identified as essential for efficient nitrogen fixation in *Lotus japonicas* root nodules (Krusell *et al.*, 2005). In the cells, a large sulfur reserve accumulates as sulfate in the vacuoles (Kaiser *et al.*, 1989). Two Group four transporters have been localized to the tonoplast membrane and shown to facilitate efflux of sulfate from the vacuole to the cytoplasm (Kataoka *et al.*, 2004b). Recently, a function for SULTR4;1 in determining the sulfate content of seeds was revealed (Zuber *et al.*, 2010). The role of the two Group five transporters remains to be

established, though recently SULTR5;2 was identified as having activity of a high-affinity molybdate transporter (Tomatsu *et al.*, 2007; Baxter *et al.*, 2008).

The sulfate transporters have a C-terminal extension designated as the STAS domain (Sulfate Transporter Anti-Sigma) that protrudes into the cytosol, and has significant similarity to bacterial anti-sigma factor antagonists (Aravind *et al.*, 2002; Shibagaki and Grossman, 2004, 2006). Investigation of the SULTR1;1 and SULTR1;2 STAS domains revealed they are essential for plasma membrane localization of the transporters, as well as affecting their stability and catalytic properties (Shibagaki and Grossman, 2004, 2006). More recently, these authors revealed that the STAS domains of SULTR1;1 and SULTR1;2 interact with OASTL. In the case of SULTR1;2, this interaction has a negative regulatory effect on the transporter activity (Shibagaki and Grossman, 2010).

1.5. Activation of sulfate by ATPS

Sulfate has a low oxidation/reduction potential compared to other cellular reductants. Therefore, the first step in sulfate assimilation requires the ATP-dependent activation of inorganic sulfate to APS. In APS sulfate is linked to a phosphate residue by a high energy anhydride bond, consuming ATP and releasing pyrophosphate:

$$SO_4^{2-} + MgATP \leftrightarrow APS + Mg\mathit{PPi}$$

This reaction is catalyzed by ATPS (ATP:sulfate adenylyl transferase; Schmidt and Jager, 1992; Leyh, 1993). As a catalyst for the first step of assimilation, and the only step common to both primary and secondary sulfate assimilation pathways, ATPS is a prime candidate for investigation of regulation of the pathway.

In plants, ATPS is a homotetramer of 52-54 kDa polypeptides (Murillo and Leustek, 1995). In comparison, bacterial ATPS consists of four heterodimers created from 35 kDa CysD and 53 kDa CysN subunits (Figure 1.2; Leyh *et al.*, 1988). The ATPS enzyme is not very efficient and the equilibrium for the forwards reaction is thermodynamically unfavorable. To keep the reaction moving in the forwards direction and prevent conversion of APS to sulfate requires the quick and efficient removal of products (Leyh, 1993). This is facilitated by the rapid conversion of APS into further intermediary metabolites of the assimilation pathway - sulfite and PAPS - and by removal of the pyrophosphate by inorganic pyrophosphatase. Different organisms achieved this by different mechanisms: Fungi, metazoa, and some bacteria contain a PAPS synthase protein that is a fusion of ATPS and APK domains for the direct activation of sulfate to PAPS (Figure 1.2; Kurima *et al.*, 1999; MacRae *et al.*, 2001; Gay *et al.*, 2009). In addition, it was shown that ATPS and APR from onion (*Allium cepa*) form protein-protein complexes *in vitro*, suggesting that substrate channelling might also occur in plants (Cumming *et al.*, 2007).

Recently, Patron et al. (2008) used phylogenetic analysis to dissect the origin of ATPS genes in photosynthetic organisms. There is high sequence identity amongst the plant ATPS genes. Surprisingly, plant ATPS genes are more closely related to ATPS from animals than to those in green algae, illustrating the complexity of the evolution of plant ATPS (Patron *et al.*, 2008). Interestingly, many eukaryotic microalgae, e.g. diatoms and haptophytes, possess both ATPS isoforms similar to plants and to green algae (Kopriva *et al.*, 2008; Patron *et al.*, 2008). The plant/animal-like isoform in *Thalassiosira pseudonana* and *Emiliania huxleyi* is fused to both an APK domain and a domain similar to inorganic pyrophosphatase (Figure 1.2). This fusion protein thus probably synthesizes APS much more efficiently, since the coupling of sulfate adenylation with pyrophosphate hydrolysis is physically linked (Kopriva *et al.*, 2009).

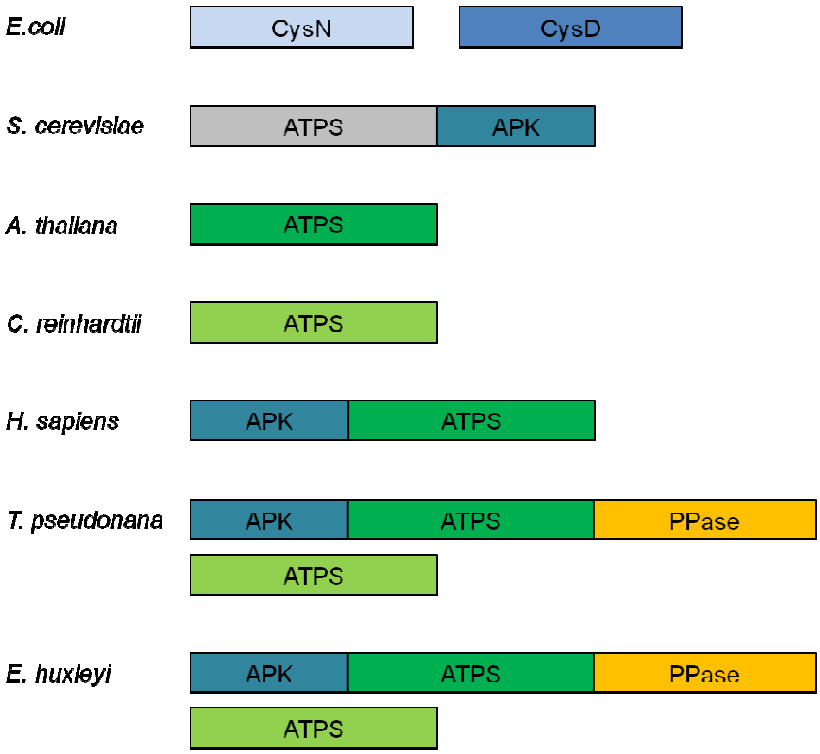


Figure 1.2 ***Isoforms and fusions of ATPS in different organisms***
The plant/animal-like ATPS isoform is represented in this figure by a dark green box, while the green algae-like ATPS isoform is represented by a lighter shade of green green.

In all organisms investigated so far, with the exception of *Selaginella moellendorffii*, at least two isoforms of ATPS have been found (Table 1.1). The *Arabidopsis thaliana* genome encodes a four-member family of highly similar ATPS genes, designated *ATPS1 - 4* (Table 1.3; Murillo and Leustek, 1995; Logan *et al.*, 1996; Hatzfeld *et al.*, 2000). Compared to the two genes identified in most other species this indicates a possibility of genetic redundancy within the family. However, while several sulfate transporters or SOT genes are adjacent to each other in the genome, indicating their recent origin by gene duplication, the ATPS genes, in contrast, are located on different chromosomes. Despite this, analysis of genome duplication indicates that *ATPS1* and *ATPS4* are located on duplicated segments of the genome (Kopriva *et al.*, 2009). Currently little is understood about the individual functions and regulation of the four Arabidopsis ATPS isoforms.

AGI code	Name	Alternative names	Gene length	Amino acid length	Reference
At3g22890	ATPS1	APS1	1898 bp	416 aa	Leustek et al., 1994
At1g19920	ATPS2	APS2/ASA1	1823 bp	420 aa	Logan et al., 1996
At4g14680	ATPS3	APS3	1860 bp	416 aa	Murillo and Leustek, 1995
At5g43780	ATPS4	APS4	1850 bp	429 aa	Hatzfeld et al., 2000

Table 1.3 ***The Arabidopsis ATPS gene family***

The presence of multiple ATPS isoforms in plants seems to be physiologically relevant, since ATPS activity is present in both the plastids and the cytosol (Lunn *et al.*, 1990; Rotte and Leustek, 2000). In both spinach (*Spinacia oleracea*) and potato (*Solanum tuberosum*) one plastidic isoform and one cytosolic isoform have been identified (Lunn *et al.*, 1990; Renosto *et al.*, 1993; Klonus *et al.*, 1994). In spinach, ~80% of total ATPS activity was found in the chloroplast fraction, suggesting this is the major isoform (Lunn *et al.*, 1990). However, in other species, the distinction between cytosolic and plastidic isoforms is less clear. Rice (*Oryza sativa*), poplar, and Arabidopsis ATPS genes encode proteins with putative plastid targeting peptides (Leustek *et al.*, 1994; Murillo and Leustek, 1995; Hatzfeld *et al.*, 2000). In these species, the identity of the corresponding cytosolic and plastidic isoforms is thus not known. Moreover, the function of the cytosolic activity remains to be discerned. In cell fractionation studies, Rotte and Leustek (2000) determined that ATPS was active in both cell compartments in Arabidopsis. However, only targeting of the full ATPS1 and ATPS4 proteins to the plastids has been experimentally confirmed (Leustek *et al.*, 1994; Hatzfeld *et al.*, 2000), and the isoform responsible for cytosolic activity remains elusive. Hatzfeld et al. (2000) hypothesized that a truncated ATPS2 protein could be synthesized, as the *ATPS2* gene encodes four methionine codons upstream of the enzyme's active site. Initiation of translation at two of these sites would result in a truncated protein, lacking the N-terminus transit peptide, and would therefore remain in the cytosol. One hypothesized mechanism for this truncation involved transcription initiation or RNA processing such that a shorter mRNA is produced. The stalling point of this theory was that only a single *ATPS2* mRNA species consistent with the

full length transcript had been identified (Logan *et al.*, 1996). An alternative mechanism would be through the initiation of translation at multiple initiation sites on a single transcript. Substantial experimental evidence to verify either of these theories is still to be presented. Although other sulfate assimilation enzymes have been shown to have cytosolic isoforms, the exclusively plastidic localization of APR and SiR activities indicates that sulfate reduction can only occur in this compartment. This raises the question of the role of cytosolic ATPS. A recent publication identified a cytosolic form of the APK enzyme, APK3, responsible for provision of PAPS for secondary metabolism (Mugford *et al.*, 2009). Hence, cytosolic ATPS activity might function in the provision of activated sulfate for PAPS synthesis. However, as Arabidopsis mutants lacking cytosolic APK are perfectly viable and do not show any phenotypic alterations, the true role of cytosolic ATPS activity remains to be determined.

1.6. Primary sulfate assimilation

In the reductive part of the pathway, APS is reduced to sulfide via the intermediate sulfite. This two-step reaction occurs exclusively in plastids. In plants, the first step is the two-electron glutathione-dependant reduction of APS to sulfite and AMP, which is catalyzed by APR (GutierrezMarcos *et al.*, 1996; Setya *et al.*, 1996; Bick and Leustek, 1998; Suter *et al.*, 2000; Kopriva and Koprivova, 2004). The plant APR genes encode proteins with three distinct domains: an N-terminal plastid targeting peptide, an APR domain, and a C-terminal thioredoxin domain (Bick *et al.*, 1998). The enzyme binds an $[4Fe\text{-}4S]^{2+}$ cluster as the cofactor (Kopriva *et al.*, 2001; Kopriva *et al.*, 2002). However, the genomes of several basal plants, *Physcomitrella patens*, *Marchantia polymorpha*, and *S. moellendorffii* all contain an APR isoform, designated APR-B, that resembles the bacterial PAPS reductase and is thus able to reduce PAPS (Kopriva *et al.*, 2007; Wiedemann *et al.*, 2007; Patron *et al.*, 2008).

The three Arabidopsis APR genes have homologous sequences and are generally regulated similarly (Kopriva and Koprivova, 2004). Therefore, the gene family may exhibit some degree of functional redundancy. This is particularly true of *APR1* and *APR3* which share the highest sequence similarities and have been co-regulated in all studies so far. On the other hand, *APR2* responds differently to these two genes in response to several hormone treatments (Koprivova *et al.*, 2008). Promoter analysis revealed a similar expression of all three APR isoforms in most tissues. However, distinct differences were obvious, suggesting that the individual APR isoforms may have specific functions (Kopriva *et al.*, 2009). Recently, mutants lacking *APR1* or *APR2* transcripts were reported to be viable and did not display any phenotypes in the absence of stress. While inactivation of *APR1* reduced total APR activity by only 20%, *APR2* inactivation reduced activity by 80%, showing that it is the major isoform (Loudet *et al.*, 2007; Kopriva *et al.*, 2009). Indeed, a QTL analysis identified *APR2* as responsible for differences in sulfate accumulation between the two Arabidopsis ecotypes Bay-0 and Shahdara. The difference between the ecotypes was mapped to a single nucleotide polymorphism in the thioredoxin active site leading to almost complete inactivation of the enzyme in Shahdara ecotype (Loudet *et al.*, 2007).

In the second reductive step of primary assimilation, catalyzed by SiR, a further six electrons are donated to sulfite, producing sulfide. In plants ferredoxin acts as the electron donor (Krueger and Siegel, 1982a). In Arabidopsis SiR is encoded by the only single-copy gene in primary sulfate assimilation, while rice, poplar, and *P. patens* genomes encode multiple copies (Kopriva *et al.*, 2009). Though only encoded by a single gene in the Arabidopsis genome, SiR does have a close paralogue in plants. Nitrite reductase (NiR), which catalyzes the equivalent reduction step in the nitrate assimilation pathway - reduction of nitrite to ammonium - has a similar structure, sequence and reaction

mechanism, and shares 19% identity at the amino acid level (Krueger and Siegel, 1982b; Crane *et al.*, 1995; Swamy *et al.*, 2005; Patron *et al.*, 2008). Indeed, SiR and NiR are both able to accept either sulfite or nitrite as a substrate, albeit with differing affinities (Schmidt and Jager, 1992; Nakayama *et al.*, 2000). Plant SiR is a plastid-localized enzyme formed from two 65 kDa subunits, and contains both a siroheme and $[4Fe\text{-}4S]^{2+}$ cluster as prosthetic groups (Krueger and Siegel, 1982a; Nakayama *et al.*, 2000). According to microarray data, it is expressed uniformly in nearly all tissue types (Khan *et al.*, 2010). Few studies have reported regulation of SiR, except induction at the mRNA level in response to OAS, and reduction in mRNA, but not activity, in response to SO_2 (Koprivova *et al.*, 2000b; Brychkova *et al.*, 2007). Nonetheless, in Arabidopsis SiR activity is essential for growth and development (Khan *et al.*, 2010). Thus, the hypothesis that activity is usually maintained in excess to ensure the further metabolism of toxic sulfite may well be true (Leustek *et al.*, 2000; Kopriva, 2006).

1.7. Cysteine synthesis

Following sulfate reduction, cysteine is formed by the incorporation of the sulfide into the amino acid precursor, OAS. This reaction is catalyzed by two enzymes. Serine acetyl transferase (SAT) catalyzes the formation of OAS by transferring the acetyl moiety from acetyl-Coenzyme-A to *L*-serine. Subsequently, the acetyl moiety is replaced with sulfide to form cysteine in a reaction catalyzed by OASTL (Hell *et al.*, 2002; Kopriva, 2006). The two enzymes form a cysteine synthase complex, composed of homohexameric SAT and homodimeric OASTL enzymes (Bogdanova and Hell, 1997; Wirtz *et al.*, 2001; Berkowitz *et al.*, 2002; Campanini *et al.*, 2005; Kumaran and Jez, 2007; Feldman-Salit *et al.*, 2009). The assembly of the subunits regulates their activity. SAT is activated in complex with OASTL, whereas OASTL is active only in the free form (Droux *et al.*, 1998; Berkowitz *et al.*, 2002). The ratio of OASTL to SAT in plastids is 300:1, so the majority of OASTL is in the free form.

The binding of the subunits in the complex is regulated by the substrate availabilities. The presence of sulfide promotes complex formation, while OAS excess disrupts it (Bogdanova and Hell, 1997; Droux *et al.*, 1998; Wirtz *et al.*, 2004). SAT is also susceptible to feedback inhibition by cysteine (Saito *et al.*, 1995). This mechanism enables rapid fine regulation of cysteine synthesis by the availability of the reaction substrates, complementary to transcriptional regulation.

In contrast to sulfate reduction which occurs exclusively in plastids, the final steps of cysteine synthesis take place in all three major compartments of the plant cell: cytosol, plastids and mitochondria (Kawashima *et al.*, 2005; Heeg *et al.*, 2008). In higher plants, both SAT and OASTL are encoded by small multi-gene families (Table 1.1; Kopriva *et al.*, 2009). In the Arabidopsis genome, SAT is encoded by five genes, all of which are expressed in the vascular tissues of both roots and leaves (Noji *et al.*, 1998; Howarth *et al.*, 2003; Kawashima *et al.*, 2005). Although the five SAT enzymes are targeted to different compartments and their enzymatic properties and sensitivity to cysteine inhibition differ substantially from each other, there appears to be functional redundancy within the gene family as each of the isoforms alone can support plant growth (Noji *et al.*, 1998; Kawashima *et al.*, 2005; Watanabe *et al.*, 2008b). However, the mitochondrial form seems to play the most important role in provision of OAS for cysteine synthesis (Haas *et al.*, 2008). OASTL belongs to a *β*-substituted alanine synthase (BSAS) family in the large pyridoxalphosphate-binding enzyme superfamily (Hatzfeld *et al.*, 2000; Watanabe *et al.*, 2008a). The Arabidopsis BSAS gene family contains nine members, five of which encode isoforms displaying OASTL activity and one which encodes a *β*-cyano-alanine synthase (CASase). The remaining three, less abundant, OASTL-like isoforms have received less attention, yet recently BSAS4;3 was demonstrated to have cysteine desulfhydrase activity, and was designated DES1 (Alvarez *et al.*, 2010). The cytosolic BSAS1;1/OASTL A1 isoform contributes the most to cysteine production in both leaves and roots, while mitochondrial

BSAS2;2/OASTL C contributes substantially to root cysteine production (Watanabe *et al.*, 2008a). However, despite obvious differences between the family members, functional redundancy is clearly present amongst the family, as null mutant lines were all viable (Heeg *et al.*, 2008; Watanabe *et al.*, 2008a).

Cysteine is the terminal metabolite of the sulfate assimilation pathway. However, cellular levels of cysteine are low and stable, due to its role as a mediator between primary assimilation and provision of reduced sulfur for further metabolism to methionine, glutathione and various other sulfur compounds (Hell *et al.*, 2002).

1.8. Methionine synthesis

Methionine is important in multiple processes including as a component of proteins, in the initiation of mRNA translation and as a substrate for synthesis of further methionine compounds (Hesse *et al.*, 2004). Humans are unable to synthesize methionine. Thus it is an essential amino acid requirement in the human diet. In plants methionine is synthesised from cysteine in three steps. Firstly, at the branching point of threonine and methionine synthesis cysteine and *O*-phosphohomoserine (OPH) are condensed to form cystathionine in a reaction catalysed by cystathionine γ-synthase (CgS). Subsequently, cystathione is converted to homocysteine and then to methionine by the enzymes CgS cystathionine β-lyase (CbL), and methionine synthase (MS), respectively (Hesse and Hoefgen, 2003). While methionine is essential for protein synthesis, the majority is further metabolised to methionine derivatives such as *S*-adenosylmethionine (SAM) and *S*-methylmethionine (SMM). SAM plays a vital role as a methyl-group donor and as a precursor for ethylene (ET), polyamines, vitamin B1 and the osmoprotectant 3-dimethylsulfoniopropionate (Amir *et al.*, 2002), whereas SMM is the major form in which reduced sulfur is transported in the phloem in some plant species (Bourgis *et al.*, 1999).

1.9. Glutathione synthesis

The tripeptide glutathione is an important thiol compound in many eukaryotic cells as well as in many bacteria, and is considered as the major product of the sulfate assimilation pathway. It is involved in the maintenance of cell redox homeostasis, especially in response to oxidative stress caused by reactive oxygen species (ROS), where it is a central component in the ascorbate/glutathione cycle (May *et al.*, 1998; Noctor and Foyer, 1998). Oxidative stress is caused in plants in response to multiple environmental stresses including high light intensity, salinity, drought, low temperature, or biotic stress such as pathogen attack (Rausch and Wachter, 2005; Kopriva, 2006). In addition, glutathione conjugates with secondary metabolites and xenobiotics via glutathione transferases (Marrs, 1996; Wagner *et al.*, 2002; Dixon *et al.*, 2010), and is the substrate for synthesis of phytochelatins which are involved in the detoxification of heavy metals (Cobbett and Goldsbrough, 2002). Glutathione is also important in the regulation of developmental processes in the root meristem and during flowering, probably due to its effect on redox homeostasis (SanchezFernandez *et al.*, 1997; Ogawa, 2004; Espunya *et al.*, 2006; Wang *et al.*, 2009; Koprivova *et al.*, 2010).

Biosynthesis of glutathione from cysteine takes place in two steps. Firstly, γ-glutamyl-cysteine synthetase (γ-ECS or GSH1) catalyzes the formation of γ-glutamylcysteine (γ-EC) from cysteine and glutamate. In the second step, glutathione is produced when glutathione synthetase (GSHS or GSH2) attaches glycine onto the C-terminal site of γ-EC. In Arabidopsis both enzymes are encoded by single genes, but whereas GSH1 is exclusively located in plastids, GSH2 is dually targeted to plastids and the cytosol from a single gene (Wachter *et al.*, 2005). In contrast, other plant species possess multiple copies of the glutathione synthesis genes and exhibit γ-ECS activity in both the cytosol and plastids (Hell and Bergmann, 1990; Kopriva, 2006). Although sulfate can be transported in its anionic form in both the xylem and phloem, glutathione and SMM are the major sulfur

compounds found in the phloem sap and are thus responsible for long-distance transport of reduced sulfur (Bourgis *et al.*, 1999). Furthermore, glutathione translocated in the phloem is thought to be a long-distance signal of sulfur status in vascular plants (Lappartient *et al.*, 1999).

1.10. Secondary sulfate assimilation

Following sulfate activation by ATPS there is a branching point in the assimilatory pathway. Instead of entering the reductive assimilation pathway APS can be phosphorylated to produce PAPS in a reaction catalyzed by APK. PAPS acts as a sulfur donor for numerous sulfation reactions. APK forms part of the primary sulfate assimilation in yeast, fungi, and many bacteria, since these organisms reduce PAPS instead of APS (Kopriva and Koprivova, 2004). Plants possess multi-gene APK families with high sequence conservation between species (Table 1.1; Kopriva *et al.*, 2008; Patron *et al.*, 2008). Despite the high sequence similarity, a clear separation of plastid and cytosol targeted isoforms is seen. In Arabidopsis, three of the four encoded APK isoforms, APK1, APK2, and APK4, are targeted to the plastids, while APK3 remains in the cytosol (Mugford *et al.*, 2009). Systematic analysis of single knock-out mutants revealed that loss of function of an individual APK gene has no effect on the plant, suggesting at least partial redundancy in function. However, in an *apk1 apk2* double mutant, the loss of these two isoforms has a marked effect on plant growth and ability to sulfate secondary metabolites (Mugford *et al.*, 2009). Thus it seems that APK1 and APK2 are the major isoforms, and that APK is essential for the synthesis of sulfated secondary compounds and plant growth. Furthermore, whilst APK1 alone was sufficient to maintain normal glucosinolate levels and growth, triple mutants in which only APK3 or APK4 remain functional revealed similar phenotypes to the *apk1 apk2* double mutant. Interestingly, an *apk1 apk3 apk4*

triple mutant in which only the APK2 isoform should be functional was not viable and the combined mutations are hypothesized to be pollen lethal (Mugford *et al.*, 2010).

Sulfation of numerous secondary metabolites, including glucosinolates and sulfated peptides, is catalyzed by SOT. The SOT enzymes require PAPS as the sulfur donor and a free hydroxyl group of a suitable acceptor compound. Higher eukaryotes have large SOT gene families due to the diversity of biological compounds undergoing sulfation. In Arabidopsis, 18 members have been identified to date (Klein and Papenbrock, 2004). Examples of compounds modified via sulfation are glucosinolates, brassinosteroids, flavanol and flavones, hydroxyjasmonates, choline, gallic acid glucoside, peptides, and extracellular polysaccharides. Thus, sulfation is important in regulation of plant growth and development and in stress defense. However, with the exception of a few individuals, substrate specificities of the majority of SOTs remain unknown (Klein and Papenbrock, 2004). Group seven SOTs are the best characterized group. This group encodes three cytosolic enzymes, SOT16 – 18, responsible for the sulfation of desulfoglucosinolates (Klein *et al.*, 2006).

1.11. Regulation of sulfate assimilation

Due to the wide range of sulfur compounds and the importance of their functions as well as the cytotoxicity of sulfite and sulfide, intermediates of sulfate assimilation, the metabolic pathways must be tightly regulated. Understanding the regulation of sulfur metabolism in plants will help to breed or engineer superior varieties with improved sulfur use efficiency. Therefore, substantial effort has been invested in characterizing the regulatory mechanisms, especially in the model organism Arabidopsis (Kopriva, 2006). As the first step of sulfate assimilation, conversion of sulfate to APS by ATPS was initially hypothesized as likely to be the control step of the pathway. However, briefly after its

discovery APR was identified as the key point of regulation of the primary sulfate assimilation pathway. Flux analysis in Arabidopsis root cultures indicated that between 70% and 90% of the total control of the assimilatory pathway was exerted by APR (Vauclare *et al.*, 2002). As a result, the importance of ATPS both in its own right, since it is the only metabolic step common to primary and secondary sulfur metabolism, and as a regulatory step is not reflected in the bias of published research and much remains to be discovered about this enzyme.

As nutrient availability is not uniform in the environment, plants must have robust physiological flexibility in their response to nutrient deficiency. Many physiological studies as well as microarray analyses in Arabidopsis have shown that sulfur limitation induces sulfate uptake and assimilation, whilst secondary processes, such as glucosinolate biosynthesis, are repressed (Hirai *et al.*, 2003; Nikiforova *et al.*, 2003). A considerable contribution to sulfur deficiency response is through the regulation of sulfate uptake and translocation by transporters, a process that is dependent upon the plant's sulfur requirements and the external availability of sulfate. Thus, the mRNA for high affinity transporter SULTR1;1 is strongly accumulated during sulfur limitation, while ubiquitously expressed SULTR1;2 and SULTR1;3 are moderately up-regulated in response to sulfur starvation (Smith *et al.*, 1997; Yoshimoto *et al.*, 2002; Yoshimoto *et al.*, 2003). The regulation of SULTR2;1 is more complex, as transcript levels decrease in the leaves but increase in the roots during sulfur limitation (Takahashi *et al.*, 2000). Recently, this regulation was attributed in part to a sulfate inducible microRNA species, miR395 (Jones-Rhoades and Bartel, 2004; Allen *et al.*, 2005; Kawashima *et al.*, 2009). SULTR2;2 is induced only following long term sulfur deprivation (Takahashi *et al.*, 2000). Both group four family members are responsive to sulfur limiting conditions increasing the efflux of vacuolar sulfate to enhance its availability for assimilation (Kataoka *et al.*, 2004b).

Sulfur deficiency not only affects the sulfate transporters, but also other aspects of the sulfate assimilation pathway (Leustek *et al.*, 2000; Kopriva, 2006). While there have never been any doubts that APR activity and mRNA levels are strongly up-regulated by sulfur deficiency (Setya *et al.*, 1996), contrasting results concerning ATPS can be found in the literature. Initial experiments revealed that unlike in micro-organisms (Marzluf, 1997; Thomas and SurdinKerjan, 1997) two days of sulfur starvation did not cause changes in Arabidopsis ATPS gene expression (Logan *et al.*, 1996; Takahashi *et al.*, 1997; Hatzfeld *et al.*, 2000). In contrast, in tobacco cells ATPS activity was up-regulated following just four hours of sulfur starvation leading to a hypothesis of a post-transcriptional regulatory mechanism (Hatzfeld *et al.*, 1998). Further post-translational regulation has been suggested by Logan et al. (1996), who hypothesized a model involving structural interactions between sulfate transporters and ATPS. This would indicate not only that ATPS may be regulated by the rate of sulfate transport but also that it may regulate uptake rates. The situation became more complicated when the miRNA, miR395, was discovered which targets and cleaves the mRNAs of *ATPS1* and *ATPS4* and is inducible by sulfur starvation (Jones-Rhoades and Bartel, 2004; Kawashima *et al.*, 2009). miR395 also targets the low-affinity sulfate transporter, *SULTR2;1*, responsible for endogenous transport (Allen *et al.*, 2005; Kawashima *et al.*, 2009). The induction of miR395 by sulfur deficiency and the post-translational repression of the ATPS and SULTR gene products that would be expected to result are counterintuitive to the demand driven regulation of the pathway and conflict with previous reports of increased activity and transcript levels of the targets (Lappartient *et al.*, 1999; Takahashi *et al.*, 2000).

The coordinated regulation of the entire sulfur metabolism network by sulfur deficiency has been well characterized. However, only recently the first transcriptional regulator of the sulfur response was identified using a genetic screen (Maruyama-Nakashita *et al.*, 2006). A mutant was isolated with reduced sulfur response of the GFP reporter fused to

the *SULTR1;2* promoter and was accordingly designated *sulfur limitation1* (*slim1*). The mutation was mapped to a point mutation in the ETHYLENE-INSENSITIVE3-LIKE (EIL) family transcription factor EIL3 (SLIM1). Mutant *slim1* plants exhibited reduced internal sulfate levels even under sulfur-sufficient conditions and were compromised in their ability to maintain normal thiol levels, which was attributed to a de-regulation of multiple sulfate assimilation genes (Maruyama-Nakashita *et al.*, 2006). However, expression analysis revealed that a subset of genes responsive to sulfur starvation is not regulated by SLIM1, most notably the genes encoding APR isoforms. The SLIM1 transcription factor was also shown to be responsible for induction of miR395 under sulfur limiting conditions (Kawashima *et al.*, 2009).

Not only is the sulfate assimilation pathway regulated by sulfur deficiency but also more generally by the demand for reduced sulfur. Reduced forms of sulfur, such as glutathione, cysteine and H_2S exert feedback control on gene transcription and enzyme activities, limiting sulfate uptake and assimilation (Lappartient *et al.*, 1999; Westerman *et al.*, 2000). On the other hand, reduced levels of these compounds cause de-repression of the pathway. Again, sulfate uptake and APR are the components most highly regulated by these conditions, although some contribution of ATPS regulation has also been suggested (Lappartient *et al.*, 1999; Vauclare *et al.*, 2002). Feeding experiments with cysteine and glutathione indicated repression of both APR transcript and activity, while disruption of glutathione synthesis by buthionine sulfoximine (BSO), an inhibitor of GSH1, relieved the repression, indicating that regulation was most likely through the action of glutathione (Vauclare *et al.*, 2002).

Apart from thiols other metabolites are known to affect plant sulfate assimilation. The best known example is the precursor of cysteine, *O*-acetylserine. As well as being a limiting factor for cysteine synthesis and causing disassociation of the cysteine synthase complex,

OAS is a potent signal of sulfur status. OAS accumulates during sulfur deficiency as it cannot be further metabolized to cysteine. In Arabidopsis, microarray analysis revealed that exogenous OAS application triggers a response similar to the response to sulfur limitation (Hirai *et al.*, 2003). OAS dramatically increases flux through sulfate assimilation by induction of mRNA accumulation for many sulfate assimilation genes (Koprivova *et al.*, 2000b; Berkowitz *et al.*, 2002; Hirai *et al.*, 2003; Hirai *et al.*, 2005). Group one and two sulfate transporters as well as *ATPS3* and *APR2,* were among the genes shown to be tightly correlated to OAS levels. However, the role of OAS as a signal of sulfur deficiency is controversial as in some reports the increase in sulfate uptake preceded the accumulation of OAS (Hopkins *et al.*, 2005).

Sulfate assimilation is also regulated by various environmental conditions. It has long been known that cysteine synthesis is under regulation of light (Schmidt and Trebst, 1969). Plants grown in the light accumulated APK, SiR, OASTL and SAT mRNA to several times the levels in plants grown in the dark (Hell, 1997). In maize, both ATPS and APR activity undergo a diurnal rhythm (Kocsy *et al.*, 1997). The first systematic analysis of light regulation of sulfate assimilation was carried out by Kopriva et al. (1999), and reported on the light responsive diurnal fluctuations of APR activity and transcript levels. Feeding with sucrose produced similar responses in APR, revealing an interaction between sulfate assimilation and carbon metabolism (Kopriva *et al.*, 1999). Later Hesse et al. (2003) revealed that protein levels of ATPS, APR, and SiR all decreased following 38 hours of darkness while OASTL activity increased. The strongest decrease was detected in APR protein accumulation, which was also shown to be induced by glucose addition (Hesse *et al.*, 2003). CO_2 levels also affect sulfate assimilation, as APR activity is strongly diminished in a CO_2 free atmosphere (Kopriva *et al.*, 2002). Following more severe sulfur starvation, photosynthetic processes are also compromised, suggesting further interactions between the two assimilatory pathways (Reuveny *et al.*, 1980; Brunold and Suter, 1990; Gilbert *et*

al., 1997). Sulfate assimilation is also linked with nitrogen assimilation, as limitation of one element diminishes uptake and assimilation of the other (reviewed in Kopriva, 2006).

Indications of a substantial contribution of phytohormones to the control of sulfate assimilation have been emerging in the past decade. Glutathione levels increase upon treatment with abscisic acid (ABA) and salicylic acid (SA; Fodor *et al.*, 1997a; Jiang and Zhang, 2001). A few studies have linked indole-3-acetic acid (IAA), the most abundant auxin species, to sulfur deficiency response. Kutz et al. (2002) described the induction of nitrilase 3 (NIT3), a gene involved in IAA biosynthesis, by both sulfate starvation and OAS treatment. The role of the increased auxin production appears to be modulation of the root system architechture (Kutz *et al.*, 2002). Cytokinins may also play an important role in the sulfur deficiency response (Dan *et al.*, 2007). Previously, cytokinins have been shown to be involved in regulation of both nitrogen assimilation (Collier *et al.*, 2003; Maruyama-Nakashita *et al.*, 2004) and phosphate uptake (Martin *et al.*, 2000). Additionally, the expression of high-affinity sulfate transporters was repressed in response to cytokinin treatment, suggesting a more general regulatory role for cytokinins in nutrient uptake and assimilation (Maruyama-Nakashita *et al.*, 2004). Treatment of Arabidopsis with zeatin caused an increase in mRNA levels of *APR1* and the low-affinity sulfate transporter, *SULTR2;2*, thus promoting assimilation and transport of sulfate within the plants (Ohkama *et al.*, 2002). Recently, Koprivova et al. (2008) dissected the role of hormone signaling in the response of APR to salt stress. Increases in the transcript levels of all three Arabidopsis APR isoforms were detected following salt stress. The transcriptional response was not reliant upon signaling by ABA, SA, ET, jasmonic acid (JA), cytokinin or auxin signaling; however, changes in APR activity were. Gibberellic acid (GA) signaling was essential for the transcriptional response (Koprivova *et al.*, 2008). Jasmonate (JA and methyl jasmonate; MeJa) biosynthesis genes are amongst those induced by sulfur starvation (Hirai *et al.*, 2003; Jost *et al.*, 2005). Jasmonate signaling plays a role in regulation of both

primary assimilation and the biosynthesis of secondary metabolites, such as glucosinolates. While sulfate transport is not affected by jasmonate signaling, most genes of primary assimilation are jasmonate responsive, including all four ATPS genes (Jost *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005).

1.12. Open questions and aims of this thesis

Following the sequencing of the Arabidopsis genome all the sulfate assimilation genes could be identified leading to the discovery of multi-gene families for most steps of the pathway. Some of the gene families have since been systematically analyzed with interesting results, such as the differential compartmentalization of the pathway. However, ATPS has received relatively little attention despite its central position at the entry point of sulfate into the assimilation pathway. There are three major questions about ATPS which remain to be answered:

(1) Varying degrees of isoform specificity and functional redundancy have been reported in the multi-gene SULTR, APR, APK, SAT, and OASTL families of Arabidopsis, but the roles of the individual ATPS isoforms are yet to be characterized.

(2) Although regulation of the sulfate assimilation pathway has been extensively surveyed, regulation of APR has been the focus of many of these studies, and much remains to be discovered about the regulation of the ATPS genes, especially with respect to miR395.

(3) Recently, substantial progress has been made in understanding the mechanisms of compartmentalization and reasons for the separate localization of different processes. However, the identity and function of cytosolic ATPS activity still remains unknown.

In this thesis, I report the use of a range of analytical techniques to address these three outstanding questions.

Chapter 2

Characterisation of the four ATPS isoforms in *Arabidopsis thaliana*

2. CHARACTERISATION OF THE FOUR ATPS ISOFORMS IN *ARABIDOPSIS THALIANA*

2.1. INTRODUCTION

In recent years, large scale -omics-based approaches and the development of tools for analysis of their output have provided an effective method for investigation of molecular pathways in model systems. Large and comprehensive datasets from high-throughput microarray experiments have been published in online databases, such as NASCArrays (Craigon et al., 2004). Web-based bio-computational tools, such as Genevestigator, provide various possibilities for the analysis of such data. The ease with which this data can be analysed can facilitate rapid functional analysis of genes, and indicate directions for detailed biochemical analysis. This chapter describes the use of a selection of web-based tools, in combination with biochemical and molecular techniques, to determine what degree of specificity exists within the Arabidopsis ATPS gene family, and investigate potential functions and regulatory aspects of the four isoforms.

2.2. MATERIALS AND METHODS

2.2.1. Accession numbers

Sequence data for the Arabidopsis ATPS genes can be found in the GenBank database under the following AGI IDs: At3g22890 (*ATPS1*); At1g19920 (*ATPS2*); At4g14680 (*ATPS3*); At5g43780 (*ATPS4*).

2.2.2. Plant material and growth conditions

Arabidopsis thaliana ecotype Col-0 was used as wild-type for all experiments unless otherwise stated. Three homozygous ATPS T-DNA insertion mutation lines, *atps1* (GABI850C05), *atps2* (SAIL775D12), *atps3* (Salk037918), and an *atps4* RIKEN Ac/Ds transposon insertion mutant in the No-0 background were kindly provided by Naoko Yoshimoto, RIKEN institute, Japan (Figure 2.1). Seeds were planted on plates of media specific to the experiment, and, after 4 d at 4°C in the dark, plants were grown vertically in a controlled environment chamber at 22°C under 16-h-light/8-h-dark cycles.

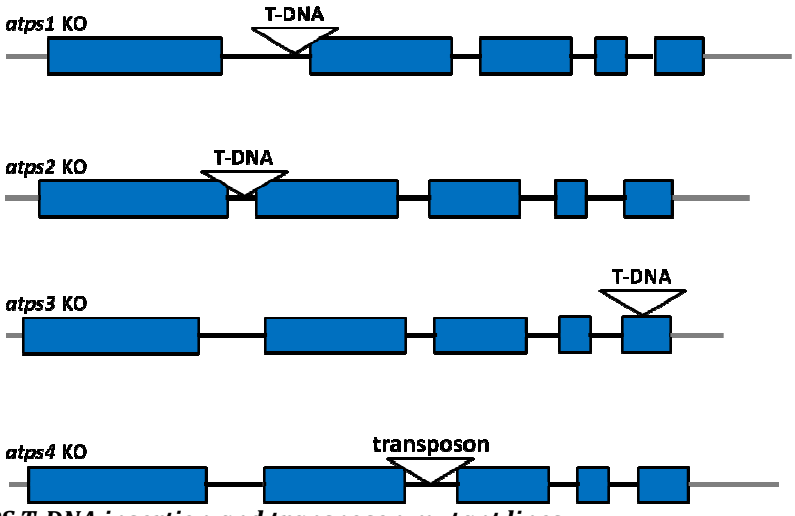


Figure 2.1 ***ATPS T-DNA insertion and transposon mutant lines***
Grey lines represent the untranslated region, while black lines and blue boxes represent introns and exons respectively. Arrow heads point to the site of the T-DNA (*atps1*, *atps2*, and *atps3*) or transposon (*atps4*) insertion.

2.2.3. eFP browser

The Electronic Fluorescent Pictographic (eFP) tool from the Bio-Array Resource for Plant Functional Genomics was used for visualisation of *Arabidopsis thaliana ATPS1 – 4* gene expression data from the Affymetrix ATH1 GeneChip (www.bar.utoronto.ca; Winter et al., 2007). For this study, the gene expression map of Arabidopsis development (Schmid et al., 2005), and the root spatiotemporal map (Brady et al., 2007) were searched using the AGI ID of the four ATPS genes. The data was visualised in absolute mode, with a signal threshold of maximum 1000.

2.2.4. Cloning of promoters of ATPS genes

Reporter constructs for expression analysis of ATPS promoters were created as follows: The promoter regions of the ATPS genes (taken as the intergenic region upstream of the ATG) were amplified using specific primers containing *attB* sites for Gateway® cloning (Hartley et al., 2000). PCR was carried out on genomic DNA prepared from *Arabidopsis thaliana* (ecotype Col-0), using the Easy-A High-Fidelity PCR Cloning Enzyme (Agilent). Subsequently, the PCR-amplified fragments were cloned into pCR-XL-TOPO (Invitrogen) and fully sequenced. For primer sequences and promoter lengths, see Table 2.1. The promoter fragments were then subcloned into the Gateway® pDONR207 vector using the Gateway® BP clonase enzyme mix (Invitrogen) and verified by sequencing. The entry clone and the binary plant transformation vector pKGWFS7 (kindly provided by Prof. Doonan, John Innes Centre; Karimi et al., 2002), were used for LR recombination to clone the promoters into the destination vector, resulting in translational fusions with the *GUS (beta-glucoronidase)* reporter gene (Figure 2.2). The resulting binary plasmids were transformed to *Agrobacterium tumefaciens* GV3101 (pMP90; Koncz and Schell, 1986) by the freeze-thaw method (Hoefgen and Willmitzer, 1988). Wild-type Arabidopsis plants were transformed using the floral dip method (Clough and Bent, 1998). Transgenic lines

were selected on plates of 0.8% agarose media containing 4.41 g l^{-1} Murashige and Skoog salts (Murashige and Skoog, 1962), and 3% sucrose, adjusted to pH 5.8 using NaOH, and supplemented with 50 mg l^{-1} kanamycin sulfate. To ensure the presence of a single insertion, T2 lines displaying a 3:1 segregation ratio in kanamycin resistance were self-polinated and homozygosity of the T3 generation was determined by 100% kanamycin resistance. For each construct, progeny from three independent lines were analyzed.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Promoter length
ATPS1	aaaaagcaggcctcggagaatcggcgtgac	agaaagctgggtctgttgaaggttttgttaggcta	5057 bp
ATPS2	aaaaagcaggctcccagcagaagctgtcatgtgt	agaaagctgggtcgaatcttgttgaagatagctac	2390 bp
ATPS3	aaaaagcaggctcctcctcattacacatagatacac	agaaagctgggtctgaagaaggagaatcgaaaaca	2393 bp
ATPS4	aaaaagcaggcgcatgtgtgtgtgtttaattgg	agaaagctgggtctgaaggcagattaatttgcttc	4878 bp

Table 2.1 ***Sequences of primers used for cloning of ATPS promoters.***
Forward and reverse primers contain the attB1 and attB2 sites (underlined), respectively, for Gateway cloning. The red text indicates the promoter template sequence.

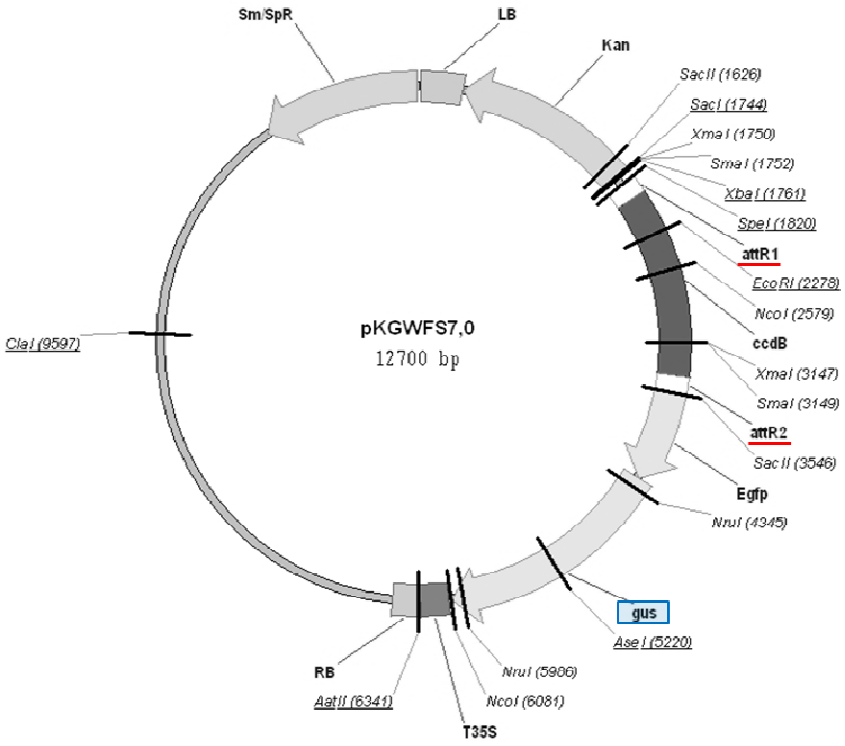


Figure 2.2 ***Gateway destination vector pKGWFS7***
The ATPS promoters were translationally fused with the *GUS* reporter gene (highlighted in blue) in the pKGWFS7 vector through recombination at the attR sites (underlined in red).

2.2.5. Histochemical staining of GUS

Transgenic lines expressing the four $ATPS_{PRO}$::GUS fusions were grown on agarose media in the absence of kanamycin. Plants were harvested after 4, 7, 14 and 21 days in the case of the *$ATPS1_{PRO}$::GUS*, *$ATPS3_{PRO}$::GUS* and *$ATPS4_{PRO}$::GUS* lines, and after 3, 7, 14 and 21 days in the case of *$ATPS2_{PRO}$::GUS* plants. GUS activity was visualised by staining in 500 µl of 1 mg ml^{-1} 5-Bromo-4-chloro-3-indolyl β-D-glucuronide sodium salt (X-GlucA; Sigma) in buffer containing 100 mM NaH_2PO_4 pH7.0, 10 mM EDTA, 3 mM potassium ferrocyanide (II), 0.5 mM potassium ferricyanide (III), and 0.1% (v/v) Triton X-100. The tissues were vacuum-infiltrated for 10 min, and staining reactions were allowed to proceed for 2 – 3 hrs at 37°C. Subsequently, the reaction was stopped and the chlorophyll removed by soaking in 70% ethanol.

2.2.6. Microscopy and imaging of GUS

Staining of GUS was visualised under an ECLIPSE E800 microscope (Nikon) coupled to a Pixera Pro 600ES camera.

2.2.7. HPLC analysis of low molecular weight thiols

The low molecular weight thiols, cysteine and glutathione, were analysed as described by Koprivova et al. (2008). Col-0, *atps1, atps2, atps3,* No (Ac), No (Ds), and *atps4* plants were grown vertically for 10 days on 0.8% agarose plates containing Murashige and Skoog medium supplemented with 1% sucrose, 0.55 mM myo-Inositol, 30 µM glycine, 4 µM nicotinic acid, 2.5 µM pyridoxine hydrochloride, 0.3 µM thiamine hydrochloride, adjusted to pH 5.7 with KOH. 20-30 mg of leaf material was ground in liquid nitrogen and extracted in 10-fold volume of 0.1 M HCl. To remove cell debris, the extract was centrifuged at 13,000 rpm for 10 min, and 25 µl of the supernatant neutralized by 25 µl of 0.1 M NaOH. To reduce disulfides, the neutralized extract was incubated for 15 min at 37°C with 1 µl of

100 mM dithiothreitol. Subsequently, 35 µl water, 10 µl 1 M Tris pH 8.0, and 5 µl of 100 mM monobromobimane (Thiolyte® MB, Calbiochem) were added and derivatization of thiols was allowed to proceed for 15 min at 37°C in the dark. The reaction was stopped and the conjugates stabilized by the addition of 100 µl of 9% acetic acid. Bimane conjugates were separated by HPLC (Spherisorb™ ODS2, 250 x 4.6 mm, 5 µm, Waters) using 10% (v/v) methanol, 0.25% (v/v) acetic acid (pH 9.3) as solvent A and 90% (v/v) methanol, 0.25% (v/v) acetic acid (pH 9.3) as solvent B. The elution protocol employed a linear gradient from 96 to 82% A in B within 20 min, with a constant flow rate of 1 ml min^{-1}. Bimane derivates were detected fluorimetrically (474 detector, Waters) with excitation at 390 nm and emission at 480 nm.

2.2.8. Determination of flux through sulfate assimilation

The flux through sulfate assimilation was measured as incorporation of ^{35}S from [^{35}S] sulfate to thiols and proteins essentially as described in Kopriva et al. (1999) and Vauclare et al. (2002). Plants were grown as above. 10-day old seedlings were transferred into 24-well plates containing 2 ml of nutrient solution (Table 2.2) adjusted to sulfate concentration of 0.2 mM and supplemented with 6.0 µCi [^{35}S]sulfate (Hartmann Analytic) to a specific activity of 994 kBq nmol $sulfate^{-1}$ for experiments with Col-0, *atps1*, *atps2*, and *atps3*, and with 5.7 µCi [^{35}S]sulfate to a specific activity of 907 kBq nmol $sulfate^{-1}$ for experiments with No-0 (Ac) and No-0 (Ds) lines. Subsequently, plants were incubated in the light for 4 hours. After incubation, the seedlings were washed 3 times with 2 ml of cold non-radioactive nutrient solution, carefully blotted with paper tissue, weighed, transferred into 1.5 ml tubes, and frozen in liquid nitrogen. The plant tissue was extracted 1:10 (w/V) in 0.1 M HCl. To determine sulfate uptake, 10 µl of the extract were added to 1 ml of Optiphase HiSafe3 scintillation cocktail (Perkin Elmer) and the radioactivity was measured in a scintillation counter (Beckmann).

To measure ^{35}S incorporation into proteins, total proteins were precipitated from 100 µl of the extract with 25 µl 100% trichloroacetic acid (TCA) as described in Kopriva *et al.* (1999). After 15 min on ice the precipitate was collected by centrifugation, washed once in 100 µl 1% TCA and once in 200 µl EtOH and dissolved in 100 µl 0.1 M NaOH. The radioactivity was determined after addition of 1 ml scintillation cocktail in scintillation counter (Beckmann).

Component	final concentration
Macro elements	
$Ca(NO_3)$ $4H_2O$	1.5mM
KNO_3	1mM
KH_2PO_4	0.75mM
Fe-EDTA	100uM
$MgSO_4$ $7H_2O$	0.2mM
$MgCl_2$	0.55mM
Micro elements	
$MnCl_2$ $4H_2O$	10 µM
H_3BO_3	50 µM
$ZnCl_2$	1.75 µM
$CuCl_2$ $2H_2O$	0.5 µM
Na_2MoO_4	0.8 µM
KI	1 µM
$CoCl_2$	0.1 µM

Table 2.2 ***Nutrient media composition for flux analysis***

To determine the radioactivity in thiols, 100 µl of the extract was mixed with 100 µl 0.1 M NaOH and 2 µl 0.1M DTT and incubated in the dark at 37°C for 15 min. Afterwards, 23 µl of 1M Tris pH 8.0 and 10 µl 100 mM monobromobimane was added, mixed and incubated in the dark at 37°C for 15 min. 22.5 µl 50% acetic acid was added, mixed and centrifuged for 15 min at maximum speed. 200 µl of the solution was transferred into HPLC vials. Standard thiol analysis was performed as described previously (Chapter 2.2.7) with an injection volume of 100 µl. The HPLC was connected to a fraction collector and fractions of

0.8 ml were collected in 6 ml scintillation vials. The radioactivity in these fractions was determined in a scintillation counter after addition of 2 ml scintillation solution.

2.2.9. ATPS expression responses in Genevestigator

ATPS gene expression heat maps were created using the Genevestigator V2 Meta-Analyzer tool (http://www.genevestigator.ethz.ch/at/; Zimmermann et al., 2005). The 'Stress Response' option was used to explore publicly available transcriptome data from the AtGenExpress global stress (Kilian et al., 2007) and hormone (Goda et al., 2008) data sets, using the default settings. The global stress experimental set-up provided measurements of transcript levels from shoot and root tissue from eighteen day old seedlings, at seven time-points within twenty-four hours of the stress treatment (Kilian et al., 2007). The experimental design for hormone treatments differs in that measurements were made in seven day-old seedlings at three time-points, 30 min, 1 hr, and 3 hr. Shoots and roots were not separated (Goda et al., 2008). Calculated averages of all data from a single treatment were compared and represented as a ratio of change in expression between the treatment and control (Zimmermann *et al.*, 2004; Zimmermann *et al.*, 2005). Thus, the heat map indicates a general up- or down-regulation.

2.2.10. Light treatment

Wild-type seeds were planted on 0.9% agarose media containing 4.3 g l^{-1} Murashige and Skoog salts (Murashige and Skoog, 1962), 1% sucrose, 0.5 g l^{-1} MES, 0.55 mM myo-Inositol, 4 µM nicotinic acid, 2.5 µM pyridoxine hydrochloride, 0.3 µM thiamine hydrochloride, adjusted to pH 5.7 using KOH. Following 4 days at 4°C in the dark, seedlings were grown for 10 days under standard light conditions. Control plants were maintained in darkness for a further hour. For the light treatment, seedlings were re-illuminated for an hour.

Whole seedlings were harvested in three biological replicates for transcript analysis, and immediately frozen in liquid nitrogen.

2.2.11. RNA extraction and expression analysis

For expression analysis of the four ATPS genes, total RNA was isolated from seedlings by phenol:chloroform:isoamylalcohol (25:24:1) extraction and LiCl precipitation (Sambrook et al., 1989) and re-purified using an RNeasy Plant Mini Kit (Qiagen, http://www.qiagen.com/) including the DNase treatment. Transcript levels were analysed by real-time quantitative RT-PCR (qPCR), using the fluorescent intercalating dye SYBR Green (Applied Biosystems) in a DNA engine OPTICON2 continuous fluorescence detector (Bio-Rad). The extracted RNA was reverse-transcribed into cDNA, using SuperScriptII Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. Subsequently, the cDNA was used as a template for qPCR with gene-specific primers (Table 2.3). The Arabidopsis *TIP41* gene was used as a standard for all measurements. Relative quantification of expression levels was performed using the comparative Ct method (manufacturer's instructions, bulletin 2, Applied Biosystems). At least three independent RNA preparations from independently grown plants were analyzed with three technical replicates for the qPCR.

Gene	AGI code	Forward primer (5'-3')	Reverse primer (5'-3')
TIP41	At4g34270	gtgaaaactgttggagagaagca	tcaactggataccctttcgc
ATPS1	At3g22890	cactcggaggtttcatgagag	agacgtagcgagttaaaatgaagag
ATPS2	At1g19920	gatcttgagtgggttcatgtgat	ctcatcttctctcatgaacccttt
ATPS3	At4g14680	tgggtttatgagggaatctgag	gacccatcatcgagattcaac
ATPS4	At5g43780	caaaggtttcatgagacagtcag	gagccggaacgagttaaaatg

Table 2.3 ***Gene specific primers for expression analysis***

2.2.12. Expression Angler

To discover which genes are co-expressed with the different ATPS isoforms, the Expression Angler tool from The Bio-Array Resource for Arabidopsis Functional Genomics was used (BAR; www.bar.utoronto.ca; Toufighi et al., 2005). Existing microarray data were compared to a chosen query gene, and Pearson correlation coefficients calculated to identify genes with similar expression and response patterns. Parameters were set to return hits with a correlation coefficient of 0.6 or higher from the AtGenExpress plus - extended tissue, global stress (Kilian et al., 2007), and hormone (Goda et al., 2008) expression data sets.

2.3. RESULTS

2.3.1. Tissue specific expression of ATPS

To determine whether the four Arabidopsis ATPS isoforms may have distinct functions, we investigated their tissue specific expression. If the individual ATPS isoforms have different mechanisms of regulation, they can be expected to exhibit different transcriptional expression patterns. To investigate the tissue-specific expression of the four *ATPS* genes *in silico*, we used the Arabidopsis electronic fluorescent pictographic (eFP) browser (Winter et al., 2007), from the Bio-Array Resource for Arabidopsis Functional Genomics (BAR) tool suite (http://bar.utoronto.ca/). This tool enables the user to explore the available microarray data, by producing a comprehensive visualisation of gene expression from Arabidopsis, as represented on the Affymetrix ATH1 GeneChip. Absolute expression mode was used to investigate expression of the four *ATPS* genes in the developmental series (Figure 2.3; (Schmid *et al.*, 2005). The output showed clearly differential expression patterning specific to each isoform.

Expression of *ATPS1* was detected in all tissues, and the signal level was considerably higher than that of the other three ATPS genes, indicating that ATPS1 might be the major ATPS isoform (Figure 2.3A). Its highest expression was observed across all developmental stages in the leaves, the main site of sulfate assimilation (Leustek et al., 2000). A comparatively lower level of expression was measured in the later stages of flower development. In the seeds, a peak in *ATPS1* expression was seen during the early stages of embryogenesis, followed by a drop then a gradual increase until the emergence of green cotyledons. In contrast to *ATPS1*, transcript levels of *ATPS2* were much lower, though expression was detected in most tissues and developmental stages (Figure 2.3B). *ATPS2* was consistently expressed in the shoot apex, throughout vegetative, transition, and inflorescence stages. In the leaves, *ATPS2* was expressed at most development stages, with

the exceptions of leaf two and senescent leaves. Expression was detected in the flowers, but decreased in later stages of floral development, when expression was retained only in the sepals and carpels. In seeds *ATPS2* expression was reduced to undetectable levels following the onset of embryogenesis. Similarly to *ATPS1*, expression of *ATPS3* was maximal in the vegetative tissue - especially the vegetative shoot apex, the hypocotyl and the leaves - and was scarcely detectable in either flowers or seeds (Figure 2.3C). Coinciding with the decreased *ATPS2* mRNA levels described previously, a peak in *ATPS3* expression was measured in leaf two. Subsequently, expression declined to a minimum in senescent leaves. *ATPS4* expression was restricted to a few specific developmental stages, during which transcript levels were comparable to those of *ATPS1* (Figure 2.3D). Particularly high expression was detected in cauline and senescent leaves, in mature flowers - with the exception of the pedicel and carpels, and during the heart stage of embryogenesis. In the rosette leaves, *ATPS4* was expressed at slightly higher levels through the mid-developmental stages, but then decreased again.

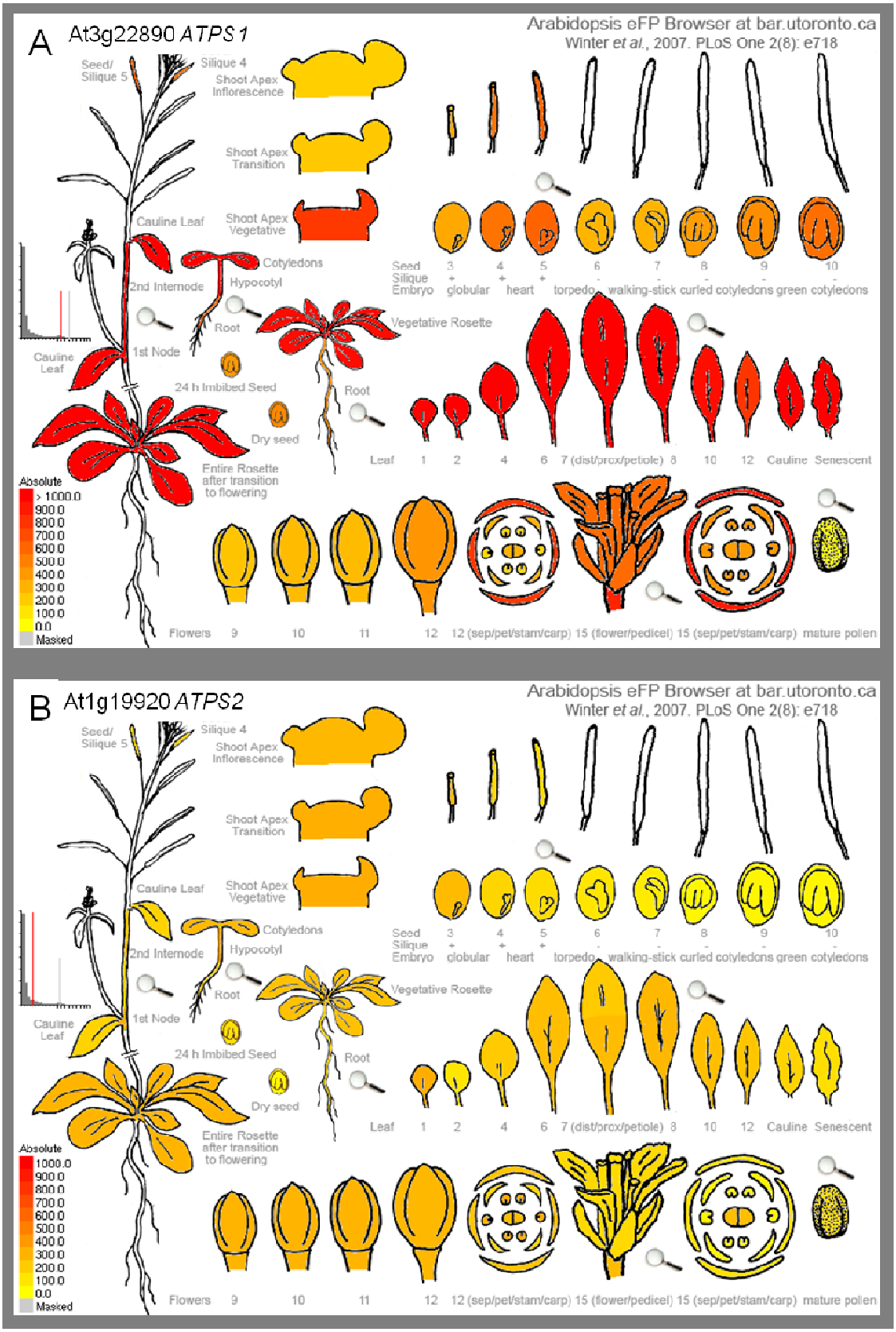


Figure 2.3 ***Arabidopsis eFP browser developmental series***
Images of *ATPS1* **(A)**, *ATPS2* **(B)**, *ATPS3* **(C)**, and *ATPS4* **(D)** transcript levels at different developmental stages produced using absolute mode in the eFP browser at the BAR (www.bar.utoronto.ca; Winter et al., 2007). The threshold maximum was set to 1000. The scale is displayed in the lower left hand corner of each image.

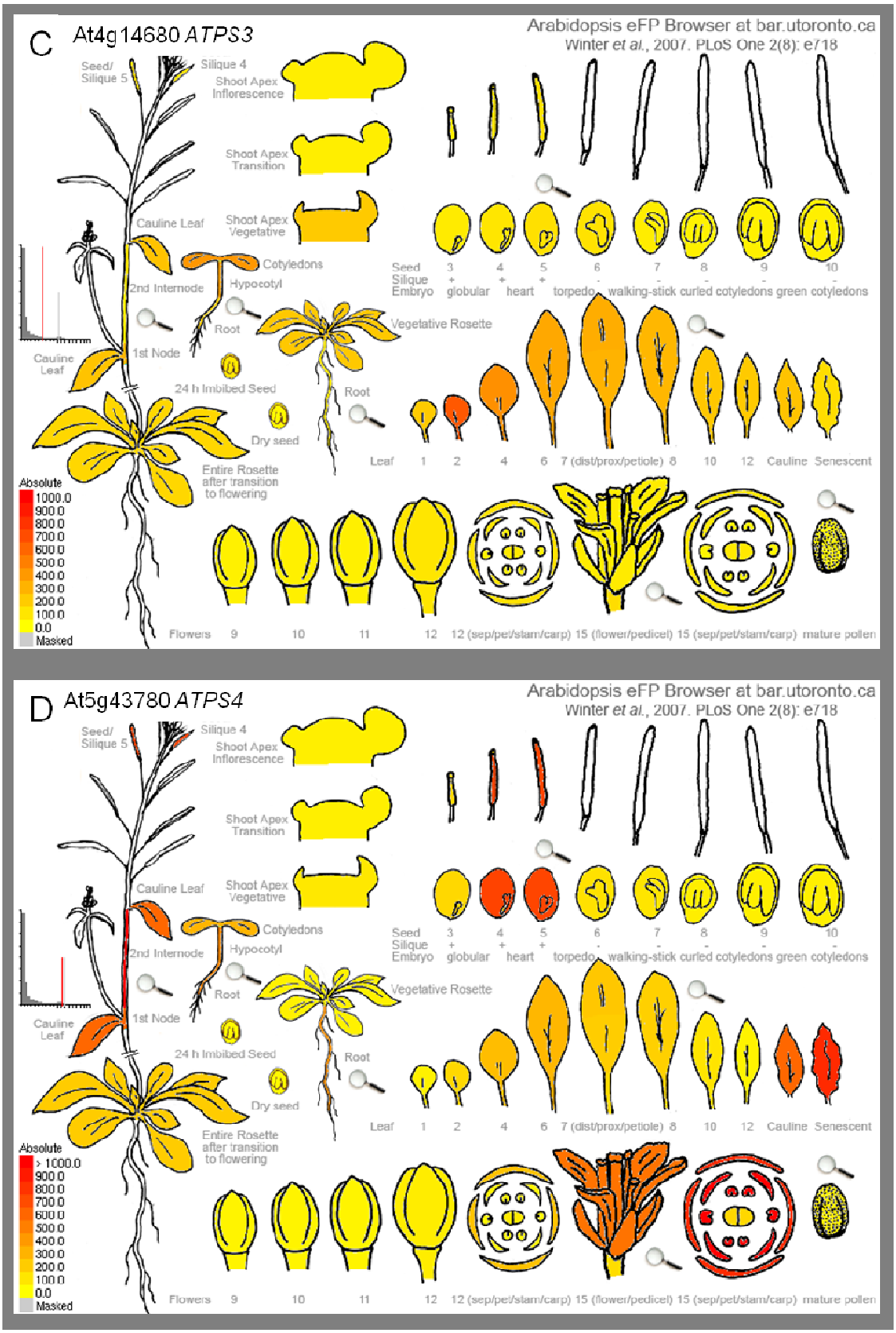


Figure 2.3 *continued*

As the site of sulfate entry to plants, roots are essential to sulfate assimilation. In the roots, the thiol glutathione participates in the regulation of cell division at the apical meristem, and is vital for root development (Sanchez-Fernandez *et al.*, 1997; Espunya *et al.*, 2006; Koprivova *et al.*, 2010). Therefore, the activation of sulfate by ATPS is also necessary in roots. A second function of the eFP browser tool allows the exploration of gene expression in roots, representing expression in a gene spatiotemporal map (Brady et al., 2007; Cartwright et al., 2009). We employed this function to visualise ATPS transcriptional expression patterning in the root (Figure 2.4).

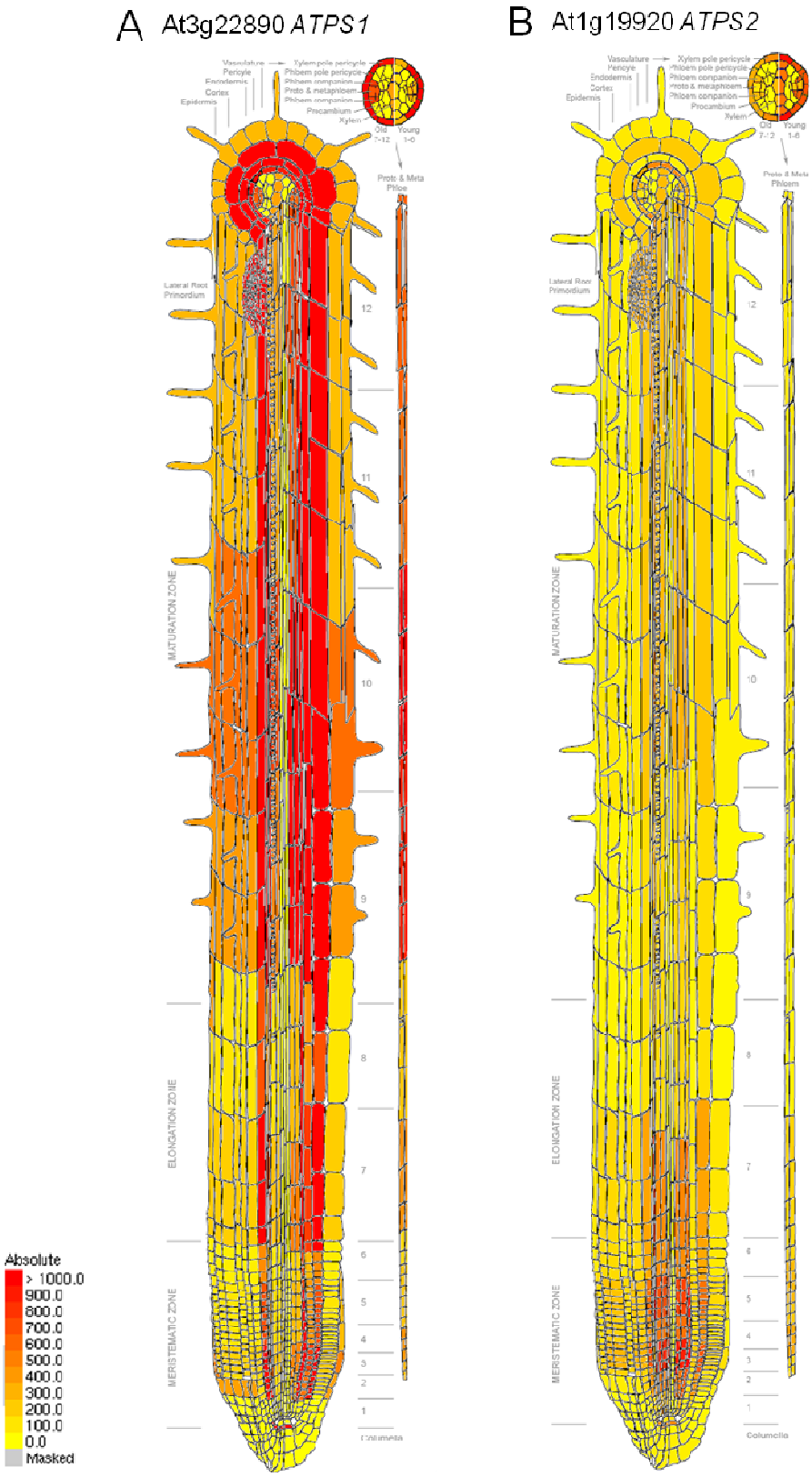


Figure 2.4 ***Arabidopsis eFP browser root spatiotemporal map***
Representation of *ATPS1* **(A)**, *ATPS2* **(B)**, *ATPS3* **(C)**, and *ATPS4* **(D)** transcript levels on a root spatiotemporal map produced using absolute mode in the eFP browser at the BAR (www.bar.utoronto.ca; Winter et al., 2007). The threshold maximum was set to 1000. The scale is displayed in the lower left hand corner.

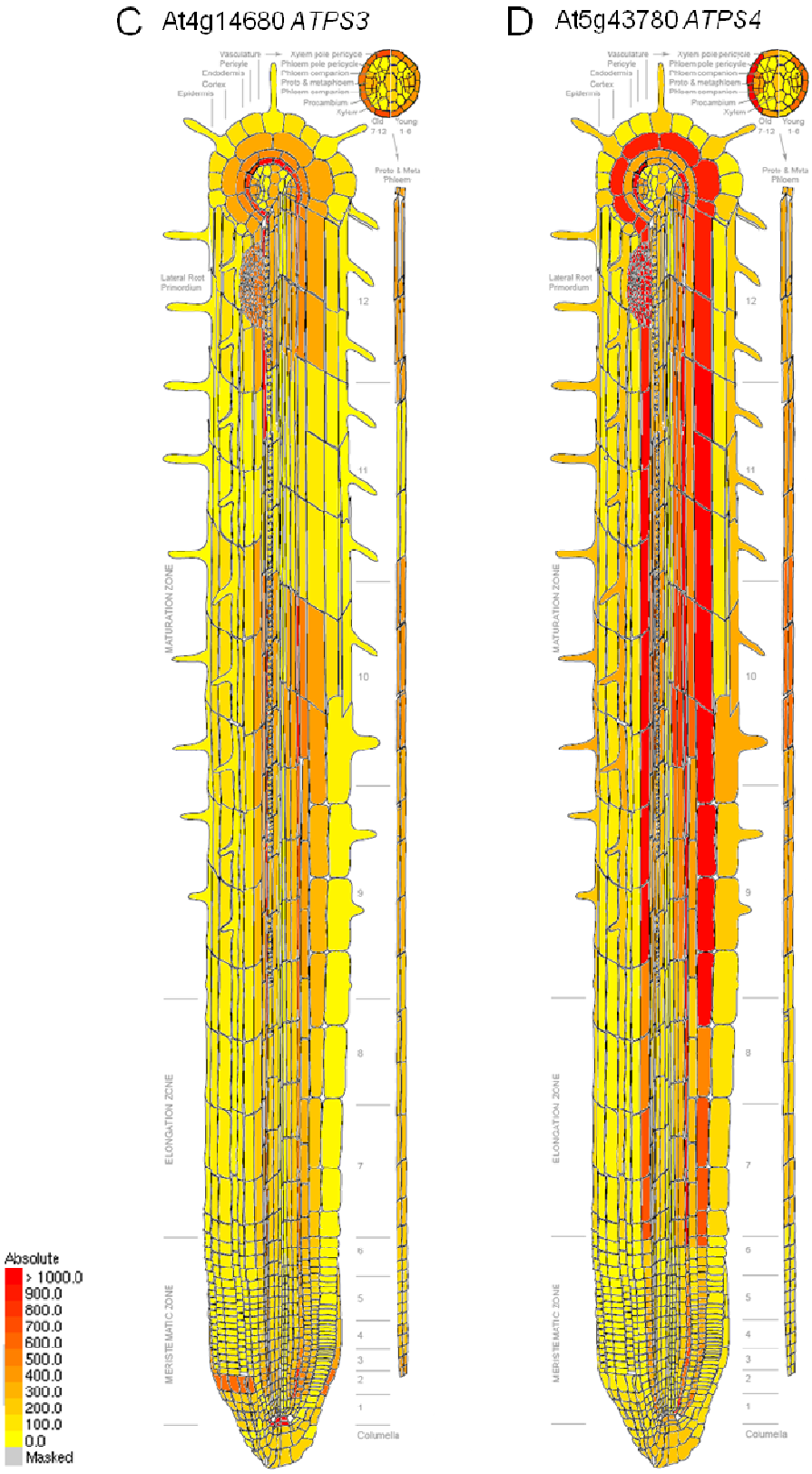


Figure 2.4 *continued*

The root gene-expression map revealed that both *ATPS1* and *ATPS4* were very highly expressed, suggesting they are the key players in root sulfate activation (Figure 2.4A + D). *ATPS2* and *ATPS3* were expressed at much lower levels (Figure 2.4B + C). *ATPS1* transcript levels were highest in the cortex, endodermis and pericycle, though expression was also detected in the epidermis, most intensely in the maturation zone. Phloem and xylem tissues also indicated relatively high levels of *ATPS1* expression, as did the columella initials. Though transcript levels were lower, the pattern of *ATPS3* expression in the roots was very similar to that of *ATPS1*. The highest *ATPS3* expression was detected in the pericycle, cells of the quiescent centre, and columella initials, with expression also detected in the cortex and endodermis. *ATPS2* was the lowest expressed ATPS isoform in the roots, and showed a very different pattern to the other three. Expression was concentrated to the vasculature of the root meristematic zone, expanding into the elongation zone. In young tissue, *ATPS2* was expressed in the cortex, pericycle, xylem, and phloem companion cells. This pattern was maintained in mature roots, but at a much reduced level of expression. *ATPS4* was also expressed in the cortex and phloem pole pericycle, and to a lesser degree in the endodermis, phloem and xylem cells. However, its expression increased with tissue age, opposite to that of *ATPS2*. As a result, *ATPS4* expression was at its highest levels in the maturation zone. Interestingly, higher *ATPS4* transcript levels were measured in the trichoblast cells of the roots than in atrichoblast cells, coinciding with a difference in glutathione content between these two cell types (Sanchez-Fernandez *et al.*, 1997; Meyer and Fricker, 2000).

To validate the microarray expression data using an independent biochemical approach and to further examine the expression of the *ATPS* genes, we generated transgenic lines expressing the *uidA* (*GUS*) reporter gene under control of Arabidopsis *ATPS1 – 4* promoters (*ATPS1*$_{PRO}$*::GUS*, *ATPS2*$_{PRO}$*::GUS*, *ATPS3*$_{PRO}$*::GUS*, and *ATPS4*$_{PRO}$*::GUS*, respectively). Accordingly, we cloned the promoter fragments into the binary plant

transformation vector pKGWFS7, resulting in a translational fusion with the *GUS* reporter gene. The constructs were introduced into wild-type Col-0 Arabidopsis plants, and homozygous lines were selected among the kanamycin resistant plants. Wild-type and transgenic plants were collected and stained at four developmental stages: 4, 7, 14 and 21 days in the case of $ATPS1_{PRO}$*::GUS*, $ATPS3_{PRO}$*::GUS*, and $ATPS4_{PRO}$*::GUS*, and at 3, 7, 14 and 21 days after germination for $ATPS2_{PRO}$*::GUS* lines. Three independent lines were grown and examined for each construct throughout development. Wild-type controls showed no staining (data not shown), and minimal differences were observed between the independent lines corresponding to the same construct, so that a single representative line from each fusion construct is shown in Figure 2.5– Figure 2.8.

Output from the eFP browser indicated that *ATPS1* expression was high in both shoot and root tissues. In $ATPS1_{PRO}$*::GUS* plants, activity of GUS driven by the *ATPS1* promoter was also detected in both shoots and roots, at all four developmental stages sampled (Figure 2.5). Staining of four-day old seedlings revealed high levels of GUS in the vegetative apical meristem and hypocotyl, consistent with data represented in the eFP browser. GUS expression remained in the vegetative meristem as the seedlings developed, but was almost undetectable in the hypocotyl after seven days. From day seven onward, staining of GUS was observed in the leaf vasculature, though after fourteen days expression in leaves one and two was reduced below detection. As leaf two samples used in the eFP developmental map were taken at a single time-point, this level of detail was not available. Root expression of *ATPS1* promoter driven GUS was observed throughout at four days, despite stronger staining in the root meristematic and elongation zones. Higher expression in the cortex and endodermis could not be detected, as seen in the eFP browser's depiction of *ATPS1* transcript levels. In the primary root, expression decreased to non-detectable levels over the first two weeks of development. In contrast, increasing expression in cortex and endodermis tissues was detected throughout the lateral roots.

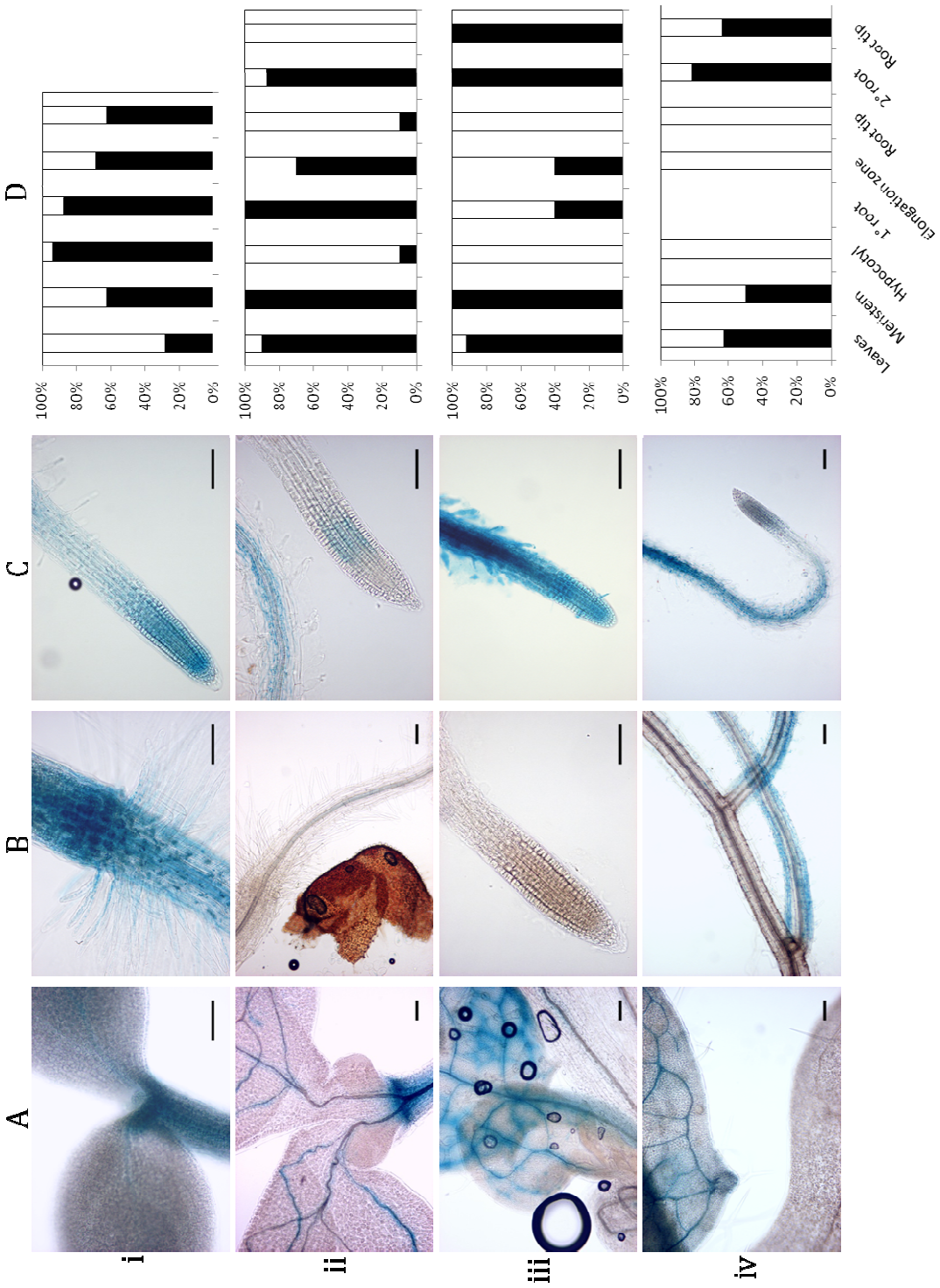


Figure 2.5 ***Histochemical staining of ATPS1$_{PRO}$::GUS seedlings***
ATPS1$_{PRO}$::GUS seedlings were stained to detect the localisation of GUS activity driven by the ATPS1 promoter at 4 days **(i)**, 7 days **(ii)**, 14 days **(iii)**, and 21 days **(iv)** post germination. **(A – C)** GUS expression is shown in leaves (Ai - iv), apical meristem (Ai - ii), hypocotyl (Bi - ii), primary root (Biii – iv and Ci - ii), and lateral roots (Biv and Ciii - iv). Scale bars = 200 µm. **(D)** Graphs indicate the frequency of expression in different tissues, where black indicated the presence of GUS staining as observed in a minimum of six plants from three independent lines.

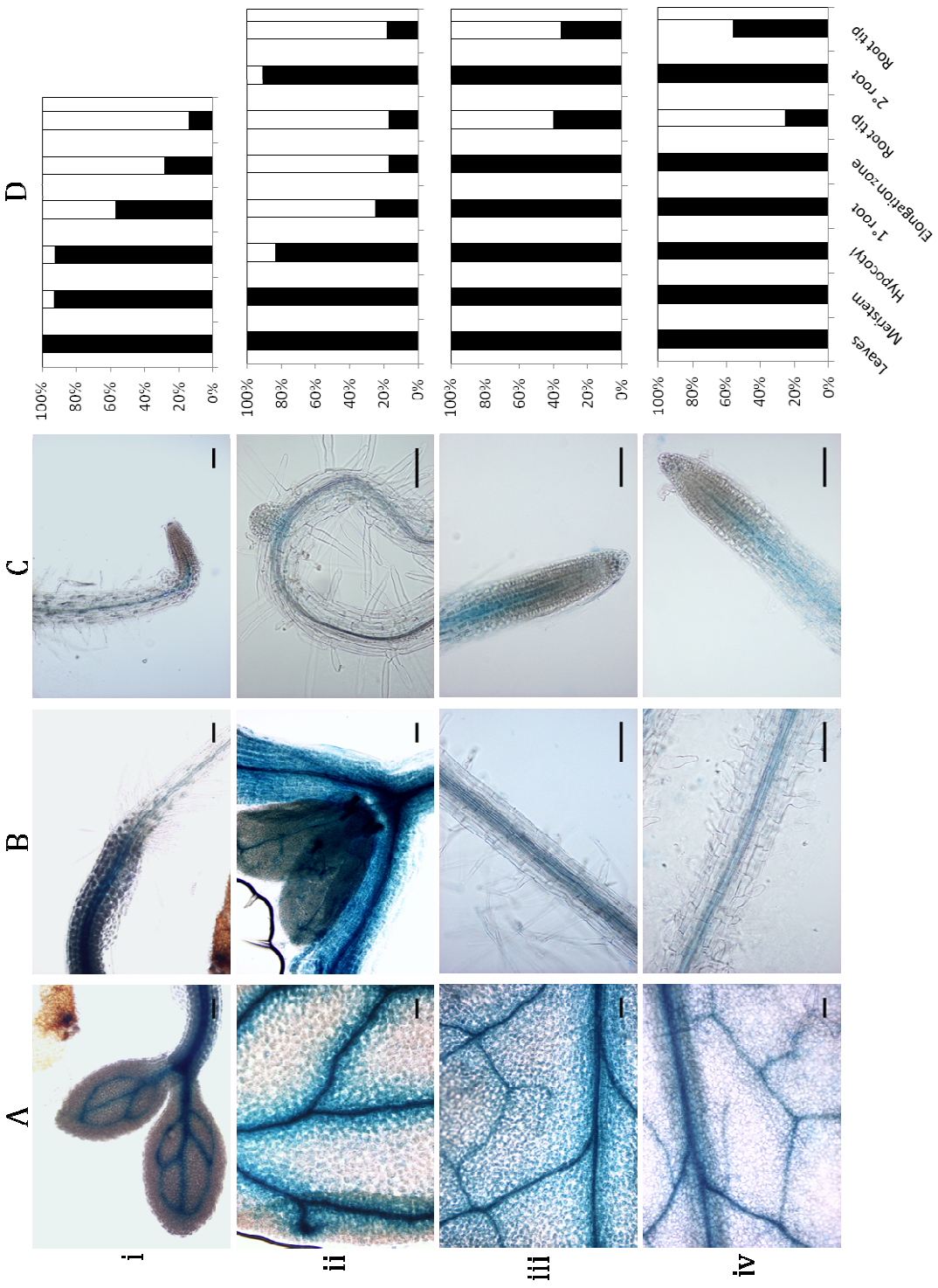


Figure 2.6 ***Histochemical staining of ATPS2$_{PRO}$::GUS seedlings***
ATPS2$_{PRO}$::GUS seedlings were stained to detect the localisation of GUS activity driven by the ATPS2 promoter at 3 days **(i)**, 7 days **(ii)**, 14 days **(iii)**, and 21 days **(iv)** post germination. **(A – C)** GUS expression is shown in leaves (Ai - iv), apical meristem (Ai and Bii), hypocotyl (Bi), and primary root (Biii - iv and Ci - iv). Scale bars = 200 µm. **(D)** Graphs indicate the frequency of expression in different tissues, where black indicated the presence of GUS staining as observed in a minimum of six plants from three independent lines.

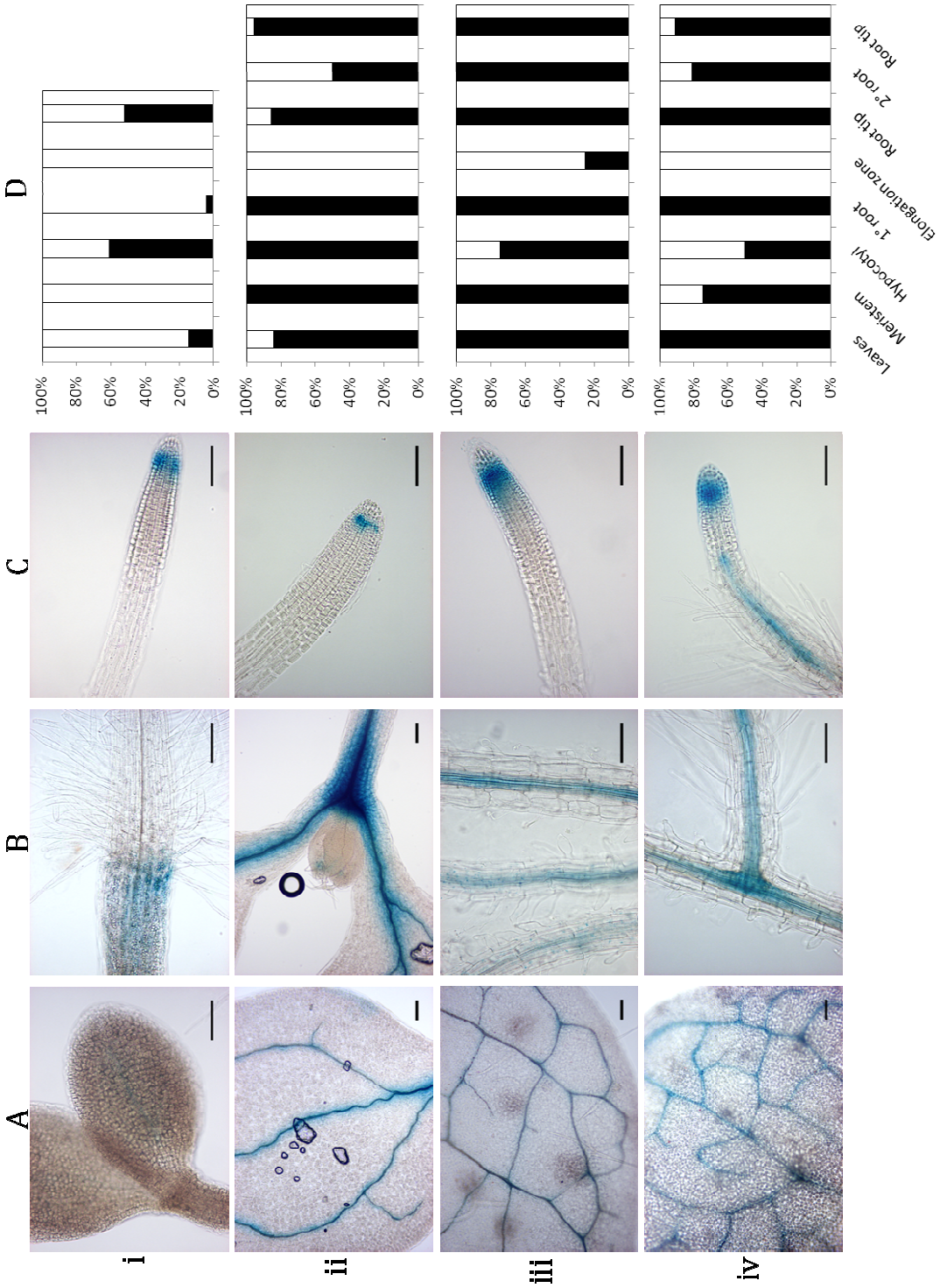


Figure 2.7 ***Histochemical staining of ATPS3$_{PRO}$::GUS seedlings***
ATPS3$_{PRO}$::GUS seedlings were stained to detect the localisation of GUS activity driven by the ATPS3 promoter at 4 days **(i)**, 7 days **(ii)**, 14 days **(iii)**, and 21 days **(iv)** post germination. **(A – C)** GUS expression is shown in leaves (Ai - iv), apical meristem (Ai and Bii), hypocotyl (Bi), primary root (Biii - iv and Ci - iii), and lateral roots (Biii - iv and Civ). Scale bars = 200 µm. **(D)** Graphs indicate the frequency of expression in different tissues, where black indicated the presence of GUS staining as observed in a minimum of six plants from three independent lines.

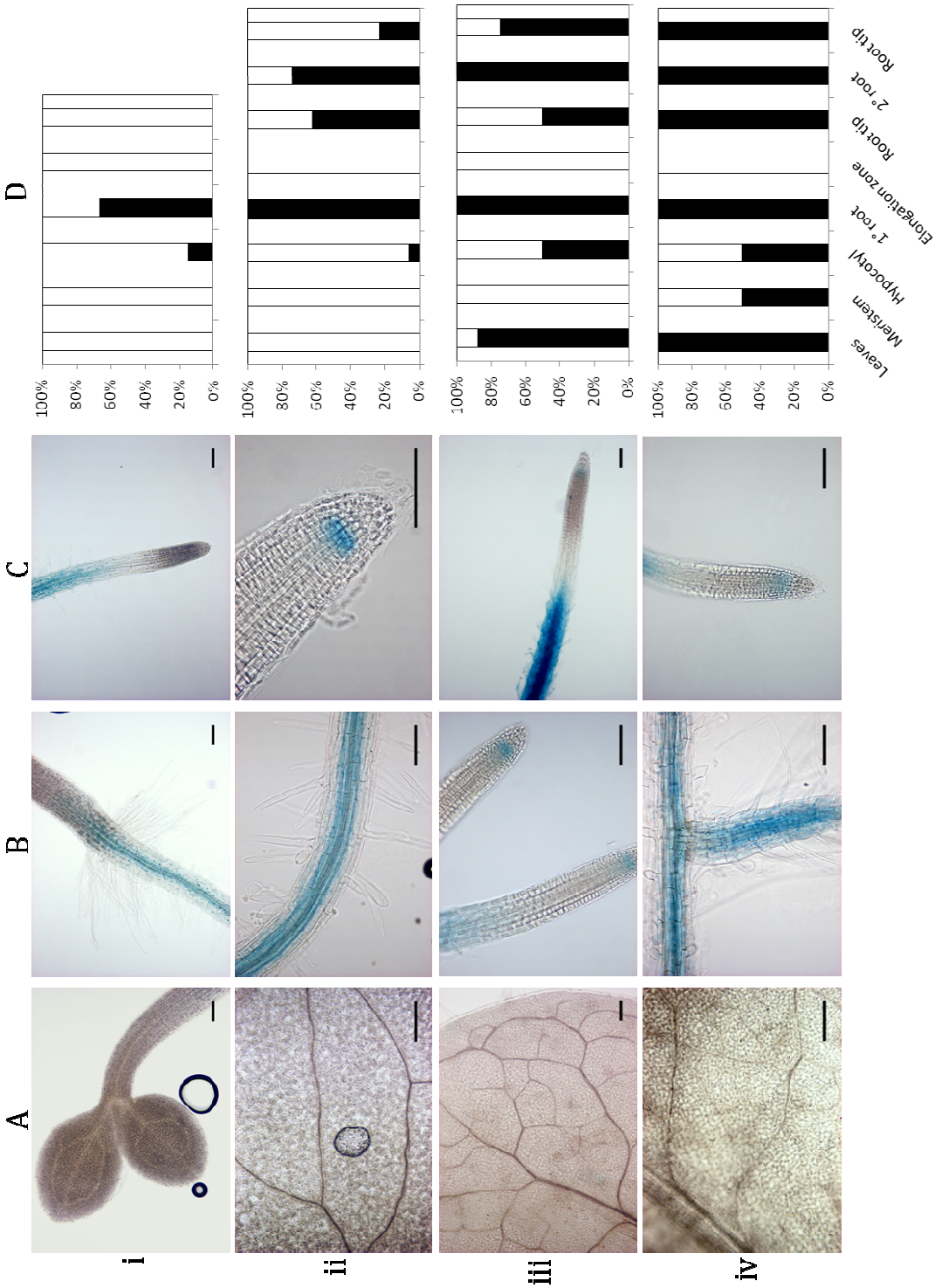


Figure 2.8 ***Histochemical staining of ATPS4$_{PRO}$::GUS seedlings***
ATPS4$_{PRO}$::GUS seedlings were stained to detect the localisation of GUS activity driven by the ATPS4 promoter at 4 days **(i)**, 7 days **(ii)**, 14 days **(iii)**, and 21 days **(iv)** post germination. **(A – C)** GUS expression is shown in leaves (Ai - iv), apical meristem (Ai), hypocotyl (Bi), primary root (Bii - iv and Ci - ii), and lateral roots (Biii - iv and Ciii-iv). Scale bars = 200 µm. **(D)** Graphs indicate the frequency of expression in different tissues, where black indicated the presence of GUS staining as observed in a minimum of six plants from three independent lines.

Contrary to *ATPS1*, the distribution of GUS activity under control of the *ATPS2* promoter maintained a similar pattern throughout the first twenty-one days of development (Figure 2.6). The staining indicated *ATPS2* promoter activity both in and surrounding the leaf vasculature, in the leaf apical meristem, and in the hypocotyl. Driven by the *ATPS2* promoter, GUS expression in the leaves indicated a similarly even distribution to that of *ATPS2* mRNA levels shown in the eFP browser. However, obvious discrepancies were seen between the root expression patterns from the two approaches. In the root, expression was again detected in the vasculature extending into the maturation and elongation zones.

GUS expression in $ATPS3_{PRO}$*::GUS* plants was detected in the leaf vasculature of seedlings at all developmental stages, consistent with the eFP expression pattern of *ATPS3* (Figure 2.7). Four-day old seedlings showed very weak expression in the early leaf vascular tissue and the hypocotyl, which did not extend into the root. However, three days later a strong expression of GUS emerged in and surrounding the vasculature of leaves one and two and the hypocotyl, extending into the root system. The shoot apex maintained a high level of expression throughout the experiment, corresponding to the vegetative meristem-specific expression in the eFP browser. Expression in the roots was initially restricted to the meristematic zone, with the exception of the root cap. However, seven-day and older seedlings also exhibited expression in the root vascular tissue, excluding the elongation zone, similar to the spatiotemporal representation of *ATPS3* expression in the eFP root map.

No GUS expression was observed in the leaves of $ATPS4_{PRO}$*::GUS* plants at any of the four developmental stages (Figure 2.8). The eFP browser provided evidence of leaf expression of *ATPS4*, but at very low levels, with the exception of cauline and senescent leaves. Strong expression was detected in the roots, expanding some way into the hypocotyl, in agreement with the microarray data represented in the eFP browser. The root-specific

expression appeared concentrated to the cortex, pericycle, stele, and xylem tissues of the maturation zone, as transcription levels previously indicated. After seven days of growth, quiescent centre-specific expression developed. However, this root tip expression became increasingly diffuse after two weeks.

Using two complementary methods, we were able to investigate the expression patterns of the four Arabidopsis *ATPS* genes. We showed that the four ATPS genes are differently expressed, suggesting individual and perhaps specific roles. *ATPS1* had the highest mRNA levels, present across all developmental stages, indicating it may be a pivotal player in primary sulfate assimilation. In comparison, *ATPS2* and *ATPS3* have considerably lower transcript levels, and most likely play a secondary role. On the other hand, *ATPS4* is expressed predominantly in the root, with possible additional functions in sulfate activation in the seeds, and during leaf senescence.

2.3.2. Analysis of ATPS mutant lines

In order to investigate the involvement of the individual ATPS isoforms in primary sulfate assimilation, we analysed levels of the thiols cysteine and glutathione in transgenic lines in which transcription of one of the four ATPS isoforms had been disrupted. Three T-DNA insertion mutation lines, *atps1, atps2*, and *atps3,* and a transposon insertion mutant line, *atps4*, were kindly provided by Dr. Yoshimoto, RIKEN institute, Japan. Wild-type and mutant lines were grown for ten days before thiol levels were analysed by HPLC in whole seedlings.

Cysteine and glutathione levels were not altered from wild-type levels in *atps1* or *atps4* seedlings, indicating that under favourable conditions (sufficient sulfate supply and absence of environmental stress), the remaining three isoforms are able to maintain

normal levels of reduced sulfur compounds (Figure 2.9). Surprisingly, *atps2* and *atps3* seedlings exhibited elevated levels of both cysteine and glutathione. This suggests that the loss of functionality of either of these isoforms results in reorganisation of the remaining isoforms, such that more sulfur is assimilated into primary metabolites. We show in Chapter 3 that this increased assimilation into reduced sulfur compounds is not detrimental to the accumulation of the secondary sulfur compounds, glucosinolates (Figure 3.8). Thus, it seems that an increase in the rate of sulfate activation is responsible for this increase, rather than redistribution of activated sulfur in the form of APS.

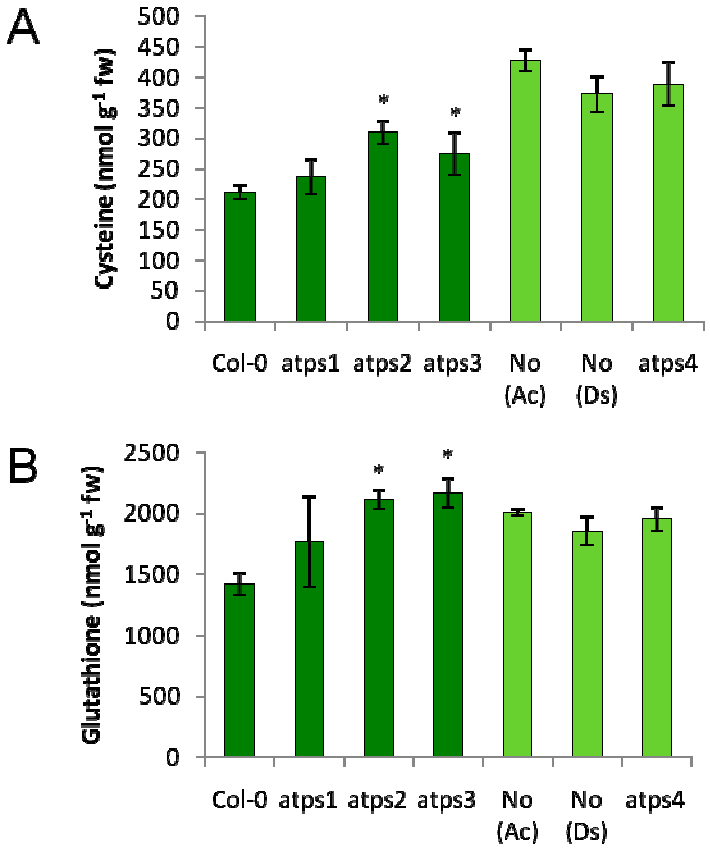


Figure 2.9 ***Analysis of thiol accumulation in ATPS loss-of-function mutants***
Cysteine **(A)** and glutathione **(B)** content was determined in ten-day old seedlings of all four single *atps* mutants and the appropriate parental lines. The data are presented as the mean ±SD from four biological replicates. Asterisks denote values significantly (Student's t-test; $P < 0.05$) different from the Col-0 wild-type for *atps1*, *atps2*, and *atps3*. For *atps4* 'a' and 'b' denote significant differences from the two parental lines, No (Ac) and No (Ds), respectively.

The thiol measurements provide an indication of the plants' ability to maintain metabolite levels. However, to further investigate the specific contributions of the ATPS isoforms to the rates of sulfate activation and reduction, we analysed the flux of [^{35}S]sulfate through the pathway in the mutant lines. Ten-day old seedlings from the four mutant lines were exposed to media containing [^{35}S]sulfate for four hours, following which ^{35}S uptake was measured. The amount of ^{35}S remaining in the sulfate pool, as well as the flux into the cysteine, glutathione and protein fractions were determined as percentages of ^{35}S taken up. In spite of the differences in thiol accumulation measured in two of the mutants, uptake of ^{35}S was not altered from the wild-type rates in any of the four mutant lines, indicating that the action of the sulfate transporters was not affected by demand for reduced sulfur due to the disruption of individual *ATPS* genes (Figure 2.10A). The only change detected was an increased uptake in the *atps4* transposon insertion mutant line, compared to the No (Ds) parental lines. However, the increase was not significant in comparison with the No (Ac) parental line.

Although changes in ^{35}S uptake were small, analysis of ^{35}S incorporation into reduced sulfur compounds did reveal differences between the mutant lines (Figure 2.10). Calculation of the relative values revealed that around 75% of the [^{35}S]sulfate taken up by the plants remained in the form of sulfate. Glutathione accounted for around 10% of assimilated ^{35}S, whilst cysteine and proteins accounted for 2% and 3% respectively. The remaining 10% of the radioactivity will have been incorporated into methionine and its derivatives, glucosinolates, and various other sulfur-containing compounds that were not measured in these experiments. However, in other experiments it was determined that approx. 1% of ^{35}S can be found in methionine and 5% in glucosinolates (B.-R. Lee, S. Kopriva, personal communication). Consistent with the findings of the thiol analysis, no differences were detected between the flux of sulfur in *atps1* and wild-type plants. Thus, the loss of ATPS1 function can essentially be compensated by the remaining three

isoforms despite the high expression levels and seemingly central role reported in section 2.3.1.

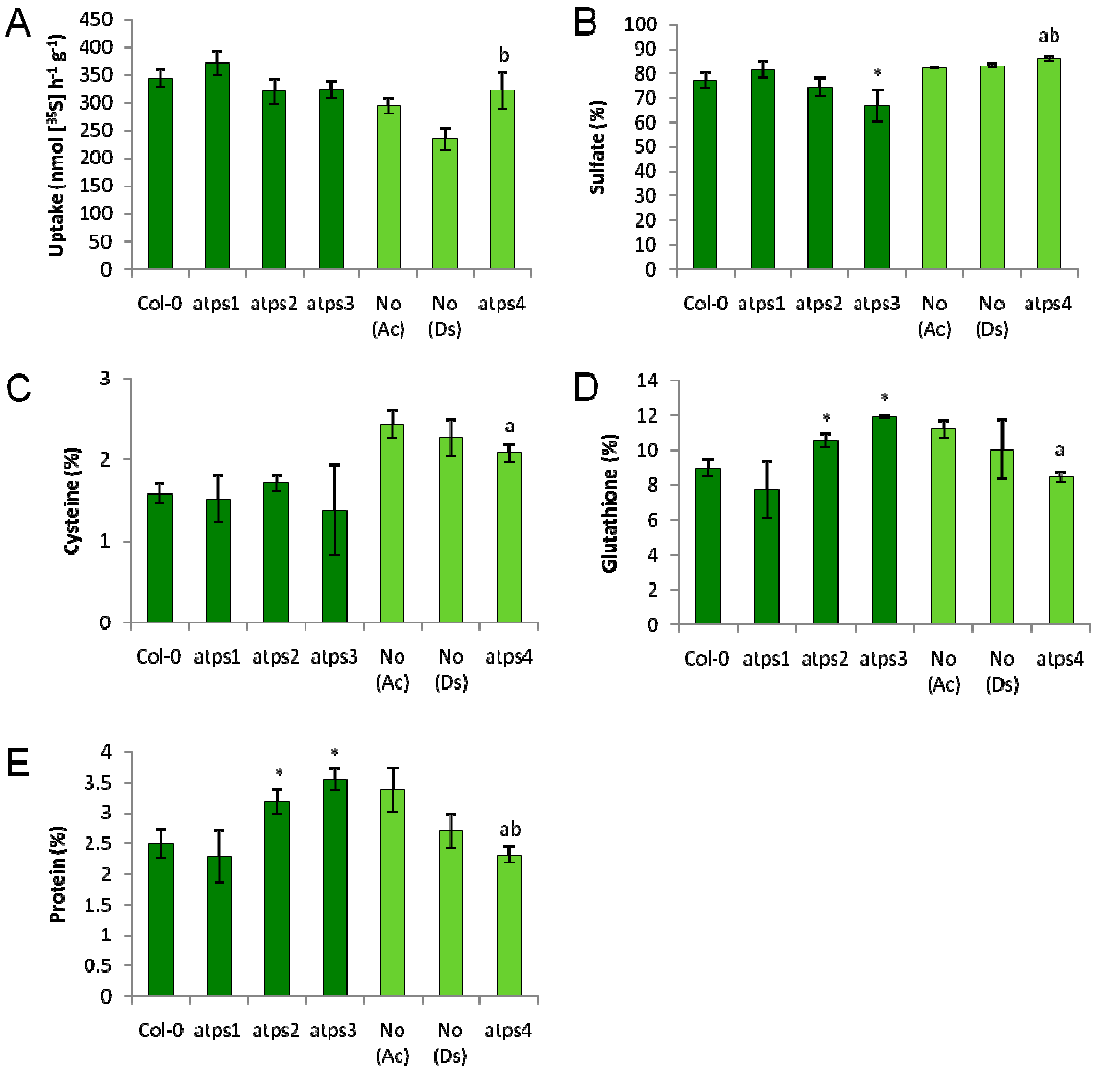


Figure 2.10 ***Analysis of flux through sulfate assimilation in ATPS loss-of-function mutants***
Ten-day old seedlings of all four single *atps* mutants and the appropriate parental lines were incubated with 0.2 mM [^{35}S]sulfate for four hours. Uptake of ^{35}S was measured (A), and the percentage remaining as sulfate (B) or incorporated into cysteine (C), glutathione (D) or proteins (E) was quantified. Data are presented as means ±SD from four biological replicates. Values for *atps1*, *atps2*, and *atps3* were compared to Col-0 wild-type and marked with asterisks where significantly (Student's t-test; $P < 0.05$) different. Values for *atps4* were compared independently to the two parental lines, No (Ac) and No (Ds), and marked with 'a' and 'b' respectively to denote significant differences.

In agreement with the accumulation of thiols, the analysis showed an increase in the flux through sulfate assimilation in *atps2* and *atps3* plants. Incorporation of ^{35}S into the glutathione and protein fractions was higher in the mutant lines than in wild-type. However, no change in incorporation into cysteine was detected. In *atps3* plants, the

increased rate of sulfate activation and reduction even caused a significant decrease in ^{35}S in the sulfate pool. Minimal differences were detected between *atps4* and its parental lines in the thiol levels and uptake rates. However, increased accumulation of [^{35}S]sulfate was detected in the *atps4* mutant, accompanied by attenuated incorporation of ^{35}S into cysteine, glutathione, and proteins. Hence, ATPS4 is essential for maintaining normal rates of sulfur flux through the primary assimilation pathway.

2.3.3. Regulation of ATPS transcript levels

Products of the sulfate assimilation pathway are known to be involved in defence against environmental stresses (reviewed in Rausch and Wachter, 2005). Glutathione is a major component of plant response to oxidative stress, heavy metal stress, and is important for detoxification of xenobiotics. The capacity for synthesis of glutathione is correlated with tolerance to various environmental stresses (Noctor *et al.*, 1998; Kocsy *et al.*, 2001; Ruiz and Blumwald, 2002; Mittova *et al.*, 2003; Kocsy *et al.*, 2004a; Kocsy *et al.*, 2004b). Glucosinolates, on the other hand, contribute considerably to defence against biotic stress, particularly herbivore and pathogen attack (Halkier and Gershenzon, 2006). To better understand whether the individual ATPS isoforms have specific roles during environmental stress, we investigated, *in silico*, the transcriptional responses to various stimuli. We interrogated the publicly available transcriptome data, using the Meta-Analyser in the Response Viewer tool from the Genevestigator V2 software suite (http://www.genevestigator.ethz.ch/at/; Zimmermann et al., 2005). This tool provided a heat map representation of changes in gene-expression of the four Arabidopsis *ATPS* genes in response to environmental stresses (

Figure 2.11).

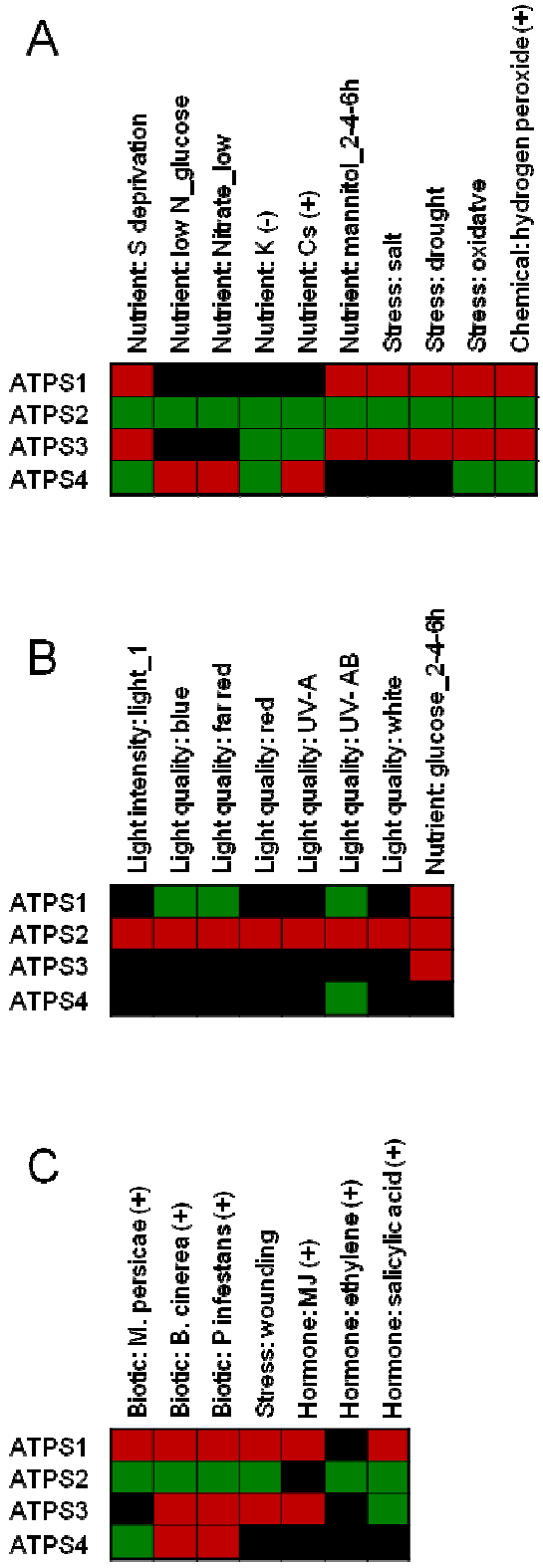


Figure 2.11 ***Genevestigator stress responses***
Heat map showing the response of the four Arabidopsis ATPS genes following a selection of stress treatments, produced using Genevestigator V2 (http://www.genevestigator.ethz.ch/at/; Zimmermann et al., 2005). Black indicates no change from the control group, whereas red and green denote up- and down-regulation, respectively. **(A)** abiotic stress, **(B)** light treatment, **(C)** biotic stress.

Sulfate assimilation is regulated in a demand driven manner, with increases in both ATPS and APR transcription and activity seen in response to sulfate deprivation (Lappartient and Touraine, 1996, 1997; Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003). Transcript levels of *ATPS1* and *ATPS3* depicted in the heat map follow this demand driven regulation, and are up-regulated during sulfate deprivation (

Figure 2.11A). In contrast, *ATPS2* and *ATPS4* expression is reduced. The assimilation of sulfur is also known to be co-ordinately regulated through interactions with the metabolism of other nutrients, such as nitrogen and carbon (Kopriva *et al.*, 1999; Yamaguchi *et al.*, 1999; Koprivova *et al.*, 2000a; Hesse *et al.*, 2003). For example, APR mRNA and activity decrease during periods of nitrogen deficiency (Koprivova *et al.*, 2000a). Two treatments that simulated nitrogen deprivation both resulted in decreased *ATPS2* expression, but increased *ATPS4* expression (

Figure 2.11A).

The toxic metal caesium (Cs) can be easily taken up by Arabidopsis roots in competition with potassium (K). Sahr et al. (2005) showed that K levels are negatively correlated to Cs levels, and implicated the *ATPS* genes in the response to Cs accumulation. Transcriptional responses from treatment with Cs, and from K-deprivation, were similar, with a decrease in *ATPS2* and *ATPS3* mRNA levels following both treatments, but different effects on *ATPS4* mRNA accumulation. *ATPS1* did not respond to either treatment (

Figure 2.11A). Accumulation of Cs interferes with internal K ions, influencing the osmotic balance in the plant (Ghosh et al., 1993). Thus, it is intuitive that osmotic stress also causes the same transcriptional response of the ATPS genes as Cs (

Figure 2.11A). The sulfate assimilation pathway is known to respond to treatments causing osmotic and oxidative stress (Bick *et al.*, 2001; Sahr *et al.*, 2005; Koprivova *et al.*, 2008; Queval *et al.*, 2009; Yoshida *et al.*, 2009). Treatments inducing osmotic stress

(drought, salt, mannitol), and those mimicking oxidative stress (oxidative, H_2O_2), caused similar responses in transcript levels of the ATPS genes. All of these treatments resulted in raised *ATPS1* and *ATPS3* transcript levels. The two oxidative stress treatments also caused a decrease in *ATPS4* transcription. Treatments directly increasing demand for glutathione, such as sulfur deprivation, osmotic, and oxidative stress, consistently induced *ATPS1* and *ATPS3* transcription, indicating they may be involved in the demand-driven regulation of sulfate assimilation. Interestingly, all nutrient, osmotic, and oxidative stress treatments resulted in decreased *ATPS2* levels.

Biotic stresses from fungal, bacterial, and insect pathogens prompt a multitude of defence responses in plants (Dixon, 2001). In Arabidopsis, these responses include accumulation and metabolism of glucosinolates, amongst others (Bednarek *et al.*, 2009; Clay *et al.*, 2009; Burow *et al.*, 2010). Hence, up-regulation of sulfate assimilation is vital to plant survival during biotic stress. Though expression of all four genes was regulated by the majority of biotic stress treatments represented in the Genevestigator data, the responses varied greatly (

Figure 2.11B). The fungal moulds *Botrytis cinerea* and *Phytophthora infestans* as well as the generalist aphid *Myzus persicae* are all known to induce a glucosinolate response in Arabidopsis (Zimmerli *et al.*, 2004; Mewis *et al.*, 2006; Kim and Jander, 2007; Rowe *et al.*, 2010). These four pathogens caused an increase in expression of either *ATPS1* or *ATPS3*, or both. Mechanical wounding and exogenous application of MeJa are also able to induce glucosinolate biosynthesis (Mikkelsen *et al.*, 2003; Jost *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005). Both treatments also elicited a rise in *ATPS1* and *ATPS3* transcript levels. SA and ET are also involved in regulation of glucosinolate biosynthesis (Mikkelsen et al., 2003), but, whilst SA increased *ATPS1* mRNA levels, ET application did not affect transcription of either *ATPS1* or *ATPS3*. Thus, these two isoforms appear to be central to pathogen and herbivore response, as well as glutathione synthesis as shown in Figure 2.3.9A. As

previously observed in response to nutrient, osmotic, and oxidative stresses, *ATPS2* levels were decreased in the majority of treatments. However, *ATPS4* transcript levels rose in response to infestation by half of the biotic stresses tested.

It has long been known that sulfate assimilation is stimulated by light (Schmidt and Trebst, 1969), and that ATPS and APR activities are light induced in maize and *Lemna minor* (Passera *et al.*, 1989; Neuenschwander *et al.*, 1991). The Genevestigator Response Viewer presents microarray data from a series of experiments in which seedlings were exposed to different light conditions following four days in the dark. Shoot material was used for the microarray analysis, explaining the lack of response seen in transcript levels of the predominantly root expressed *ATPS4*. However, leaf expressed *ATPS3* was not altered by any of the seven light conditions. Response to light was seen predominantly in the *ATPS1* and *ATPS2* genes. *ATPS1* transcript levels decreased following transfer of seedlings to blue, far red, and UV-AB light conditions. In contrast to the consistent down-regulation of *ATPS2* in response to abiotic and biotic stresses, re-illumination by any form of light resulted in an accumulation of *ATPS2* transcript, indicating that transcription of this gene is light-responsive. Glucose is also able to induce APR activity (Kopriva *et al.*, 2002; Hesse *et al.*, 2003). The Response Viewer indicated that *ATPS1*, *ATPS2*, and *ATPS3* transcripts also increase in response to glucose.

Together with experimental data in other species (Passera *et al.*, 1989; Neuenschwander *et al.*, 1991), the results described above from the Genevestigator and Expression Angler tools provide evidence for a regulation of ATPS transcription by light in Arabidopsis. The key point of regulation appears to be *ATPS2* transcription, with some regulation of *ATPS1* transcription. To test this hypothesis experimentally, we used qPCR to measure the steady state levels of *ATPS* transcripts following light induction treatment. Ten-day old Arabidopsis seedlings were kept in the dark for 38 hours, after which half of them

remained in the dark (control), and half were re-illuminated for 60 min. A clear increase was measured in *ATPS2* mRNA levels, whilst levels of *ATPS1*, *ATPS3* and *ATPS4* transcription remained the same (Figure 2.12). This confirms the activation of *ATPS2* transcription reported by Genevestigator in light conditions. It has to be noted that the same treatment results in 2-8-fold induction of APS reductase transcripts.

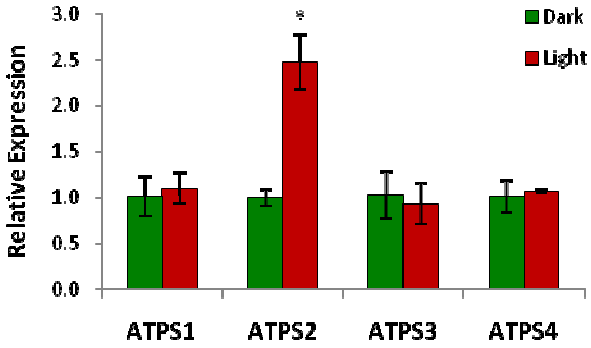
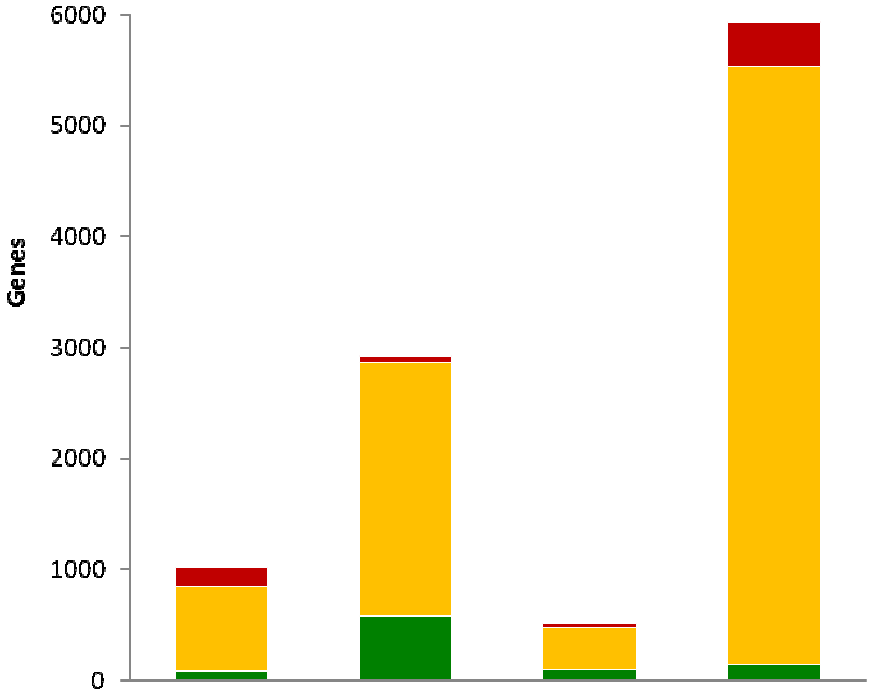


Figure 2.12 ***Transcript levels of ATPS isoforms following re-illumination***
Quantitative RT-PCR was carried out on RNA isolated from ten-day old seedlings that had been subjected to 38 hours in the dark, followed by a further hour dark for the control group (dark) or 1 hour of re-illumination for the treatment group (light). Primers specific to the four ATPS isoforms were used. The mRNA levels were compared to *TIP41* and levels in wild-type plants were set to 1. Results are presented as means ±SD from three independent RNA preparations. Significant (Student's t-test; $P < 0.05$) difference from the control group is denoted by an asterisk. Analysis done by Anna Koprivova, John Innes Centre, UK.

A second possibility to investigate potential functions of the ATPS isoforms *in silico* is through the exploration of genes co-expressed with the ATPS genes, for example using the web-based data-mining tool Expression Angler from BAR tool suite (www.bar.utoronto.ca; Toufighi *et al.*, 2005). This tool uses existing microarray data to calculate Pearson correlation coefficients and thus identifies genes with similar expression patterns, in response to different growth conditions, to a chosen query gene. Using this function, information was gathered on genes with expression profiles similar to those of the four Arabidopsis ATPS genes. Parameters were set to return hits with a Pearson correlation coefficient (R-value) between 0.6 and 1.0 (1.0 being a perfect correlation) from the AtGenExpress tissue PLUS, global stress and hormone response databases (Kilian *et al.*, 2007; Goda *et al.*, 2008). The highest numbers of expression-correlated genes were

returned from the stress database, in particular when using *ATPS2* and *ATPS4* as enquiry genes (Figure 2.13). In contrast, the tissue and hormone databases returned far fewer genes with similar profiles. Notably, in response to hormone treatments, *ATPS2* was co-expressed with the highest number of genes of the four ATPS genes. A formatted list of selected genes of interest, including any mentioned in the text can be found in Supplemental Tables 2.1 – 2.4.



	ATPS1	ATPS2	ATPS3	ATPS4
Tissue PLUS	175	56	35	399
Stress	763	2282	374	5396
Hormone	80	582	94	135

Figure 2.13 ***Gene totals from Expression Angler analysis***
Three databases were searched using the Expression Angler tool (www.bar.utoronto.ca; Toufighi *et al.*, 2005) for genes with expression patterns correlating (Pearsons correlation coefficient; R > 0.6) to the four ATPS genes. Total number of correlating genes returned from the Tissue PLUS (red), Stress (yellow), and Hormone (green) databases are represented in the graph, with the raw values indicated in the table below.

The expression angler analysis revealed that *ATPS1* and *ATPS3* expression patterns were highly correlated to each other in both the hormone and tissue PLUS databases (R-values were 0.741 and 0.660 respectively), confirming observations made of the Genevestigator Response Viewer results. *ATPS1* and *ATPS3* both showed a representation of key genes of

primary sulfate assimilation among the co-expressed genes, including *APR1* and *APR3*, consistent with the earlier hypothesis that both genes are central to primary assimilation. Surprisingly, *ATPS2* and *ATPS4* transcriptional responses did not correlate with many primary sulfate assimilation genes, with the exception of a few sulfate transporters and cysteine synthase genes, almost exclusively in the stress dataset. When interrogating the stress dataset, multiple genes involved in methionine and glutathione metabolism were identified, such as glutathione transferases, methionine and cysteine aminopeptidases, and genes involved in SAM cycling. Interestingly, the number of glutathione transferases was exceptionally high when using *ATPS4* as the query gene.

Sulfate and nitrate assimilation pathways are known to be coupled in their regulation (Koprivova *et al.*, 2000a); hence, it is no surprise that genes involved in nitrate assimilation were repeatedly observed to have correlating expression patterns to the *ATPS* genes. These included many of the key genes: nitrate transporters, nitrate and nitrite reductases, asparagine synthetases, glutamate synthases, glutamine synthetases, glutamate dehydrogenases, and PEP carboxylases. Co-expression of the ATPS genes with nitrate assimilation genes was primarily in response to stress, with the exception of *ATPS3*, the expression of which did not appear correlated with nitrate assimilation.

Stress responses of *ATPS1*, *ATPS2*, and especially *ATPS4* correlated to *SOT* genes involved in the sulfation of secondary sulfur compounds, such as glucosinolates, revealing potential roles for these genes in secondary metabolism. A large number of genes involved in glucosinolate biosynthesis were identified as sharing similar tissue expression patterns as *ATPS1*, consistent with links between *ATPS1* expression and glucosinolate biosynthesis drawn in the Genevestigator analysis. A range of glucosinolate genes shared hormone response patterns with *ATPS3*, and fewer with *ATPS1*, suggesting these two isoforms may function in glucosinolate response. Surprisingly, despite the previous implication of *ATPS3*

in glucosinolate biosynthesis, its tissue expression pattern only correlated to those of three glucosinolate genes. The Genevestigator representation of *ATPS2* expression indicated it was decreased in many stress treatments that might be expected to elicit a glucosinolate response. A contradiction is seen in the Expression Angler results, which calculated a strong correlation between *ATPS2* response patterns, and those of multiple glucosinolate network genes in response to both stress and hormone treatments, signifying a potential role for ATPS2 in the induction of these defence compounds. The discrepancy between outputs from the two tools may be due to averaging of responses in Genevestigator, which is not exercised in the Expression Angler calculations. Only under stress conditions were low numbers of glucosinolate biosynthesis genes found to correlate to *ATPS4* expression. Hence, the input of this root specific isoform into glucosinolate production is probably negligible. Hormone synthesis, regulation, and response factors were identified as co-expressed with *ATPS3* and *ATPS4* genes under stress conditions. The majority of these genes co-regulated with *ATPS3* were either JA or ET responsive elements, thus providing further evidence for a role of ATPS3 in hormone signalling for glucosinolate biosynthesis response. Hormone response genes co-regulated with *ATPS4* were primarily auxin response factors, suggesting alternative hormone regulation of this isoform compared with ATPS3.

Response heat maps viewed using Genevestigator showed that *ATPS1* and *ATPS2* expression is regulated by various light re-illumination treatments. Expression Angler co-expression data indicated that these two genes were regulated similarly to a remarkably large number of genes involved in photosynthesis. This implies that both *ATPS1* and *ATPS2* play a role in the regulation of primary sulfate assimilation by light, and may thus provide coordinated regulation of sulfate and carbon nutrient assimilation pathways.

Combining results from both the Genevestigator Response Viewer, and the BAR Expression Angler tools, a number of hypotheses can be drawn. Evidence suggests that *ATPS1* transcription is regulated by demand for either reduced sulfur compounds or for the pathogen defence compounds, glucosinolates. Though *ATPS3* expression patterns were very similar to those of *ATPS1*, and showed induction in connection with glucosinolate response, the main mode of regulation appears to be through JA and ET hormone signalling. Expression of *ATPS2* is down-regulated in response to multiple stresses, yet is co-expressed with a number of glucosinolate genes under stress conditions, suggesting it may play some part in the provision of activated sulfate for such sulfur containing secondary compounds. The links between ATPS and glucosinolates will be investigated in detail in chapter 3. In addition, ATPS2 appears to be the main point of control for light regulation.

2.4. DISCUSSION

2.4.1 The four ATPS isoforms are differentially expressed

Leaves are the main site of sulfate assimilation, and have the highest demand for reduced sulfur. In photosynthetic tissues, sulfate assimilation is intrinsically linked to carbon assimilation (Kopriva et al., 1999). We showed that three of the four ATPS isoforms, ATPS1 – 3, are expressed in cells surrounding the vasculature at most stages of leaf development, and that all four ATPS isoforms are expressed in the roots, predominantly also around the vasculature (Figure 2.3– 8). During sulfur assimilation sulfate is taken up into the roots and transported to various organs through the xylem stream for use or storage in the vacuole (Leustek and Saito, 1999). Stored sulfate and sulfur metabolites can be loaded into the phloem for remobilisation to sink organs (Yoshimoto *et al.*, 2003). Therefore, expression of ATPS around the vasculature may allow quick and efficient activation of sulfate at the site of transport from the xylem or phloem.

Coinciding with the expression of ATPS genes, APK, catalysing the first committed step of secondary sulfur assimilation, was also found to be expressed surrounding the vasculature in both leaves and roots (Mugford et al., 2009). As glucosinolates are found in and around the mid-vein and outer lamina (Shroff et al., 2008), and myrosinase is localized to special myrosin cells of the phloem parenchyma and in the guard cells (Andreasson *et al.*, 2001; Thangstad *et al.*, 2004; Barth and Jander, 2006), the expression of both ATPS and APK is at the site of glucosinolate accumulation and metabolism. A further similarity is seen in the tissue specific expression of *ATPS4* and *APK2* which are both present in the root quiescent centre, though the role of this specific expression is unknown. In situ RNA hybridization indicated that the tissue specific expression of APR in the roots of maize plants was predominantly in the exodermis of root tips, with significantly lower signal in the mature roots (Kopriva et al., 2001). In Arabidopsis, the three APR isoforms show different

expression patterns in the root tip: *APR1* is expressed throughout the root, similar to *ATPS1* expression in the primary root during early development and later in lateral roots. *APR2* is expressed in the mature root tissue but not the root tip, and *APR3* is expressed in a small cluster of cells in the root tip, similar to distribution of *ATPS3* and *ATPS4* (Kopriva et al., 2009).

Glutathione and glutaredoxin systems are involved in a variety of processes, including response to oxidative stress caused by adverse environmental conditions such as pathogen attack (Noctor *et al.*, 1998; Kocsy *et al.*, 2001; Ruiz and Blumwald, 2002; Mittova *et al.*, 2003; Kocsy *et al.*, 2004a; Kocsy *et al.*, 2004b; Rausch and Wachter, 2005; Wang *et al.*, 2009). Glutathione can directly reduce peroxides, but functions mainly in the reduction of dehydroascorbate which is vital for regeneration of the primary ROS scavenger ascorbate (Rouhier et al., 2008). Glutathione is essential for the maintenance of cellular redox status, and may be involved in the regulation of plant developmental processes, for example affecting root growth (Sanchez-Fernandez *et al.*, 1997; Espunya *et al.*, 2006; Koprivova *et al.*, 2010). Wang *et al*. (2009) describe the role of glutaredoxin in petal and anther initiation and differentiation and cell proliferation rates. A strong correlation has also been described between glutathione levels and the proliferative capacity of cells in the apical meristem of the Arabidopsis root (Sanchez-Fernandez et al., 1997). Root glutathione levels are highest in the outermost root cap, lateral root cap, and cells closer to the apical meristem (Fricker et al., 2000). We have shown that all four ATPS isoforms display specific patterns in the root tip, and thus may contribute to site specific production of glutathione in these tissues. *ATPS4* is predominantly expressed in the root system, and according to the eFP browser, *ATPS4* mRNA accumulates preferentially in the trichoblasts rather than atrichoblasts. A similar accumulation of glutathione in trichoblast cells has been reported (Meyer and Fricker, 2000), and may contribute to regulation of root hair establishment (Sanchez-Fernandez et al., 1997).

SOTs have been implicated in the regulation of hormones and flavonoids by sulfation, especially in flower and seed development (Rouleau *et al.*, 1999; Gidda and Varin, 2006). Expression of *ATPS1* and *ATPS4* in both flower and seed tissues may indicate they function in this regulation of development. Increased *ATPS1* and *ATPS4* expression during early seed development might boost glutathione biosynthesis in the embryo, as glutathione is essential for the successful maturation of Arabidopsis seeds (Cairns et al., 2006). Seeds are also known to be a sink for the sulfur-rich secondary metabolites, glucosinolates (Brown et al., 2003).

2.4.2 ATPS mutants have altered sulfur flux through primary assimilation

Differences between the expression patterns of the ATPS isoforms suggests that they might be differently regulated, and thus have individual functions. However, we observed no negative effects on thiol levels in knock-out mutants, in which a single ATPS gene has been disrupted, indicating potential for functional redundancy between the four genes. In addition, flux of sulfur through primary assimilation is negatively affected only in the *atps4* loss-of-function mutant. Despite high expression of *ATPS1* in all tissues, and its seemingly central role in response to environmental stresses (Figure 2.3, -4, and -11), the loss of ATPS1 function did not prove detrimental to primary sulfur metabolism in our analysis, suggesting some level of redundancy of this isoform (Figure 2.9– 10). However, as will be shown in next chapter, the *atps1* mutant shows a clear decrease in the accumulation of glucosinolates (Figure 3.8), indicating (1) that ATPS1 has low control over the flux through primary assimilation, but (2) it is important for the synthesis of secondary sulfur compounds. Analysis of sulfate accumulation in three-week old *atps1* plants revealed an increase in the foliar sulfate pool, though this accumulation was not as strong as that seen in *ATPS4-RNAi* lines (Liang et al., 2010). Since the flux in ten-day old *atps1* mutants was not different from WT plants, it seems that the ATPS genes are of varying importance at

different developmental stages. Interestingly, although we detected no changes in thiol levels in the *atps4* mutant, decreased flux of sulfur through primary assimilation reveals that this mutant does indeed have a reduced rate of sulfate activation, corroborated by accumulation of sulfate in *ATPS4-RNAi* lines (Liang et al., 2010). In contrast, the loss of *ATPS2* or *ATPS3* function actually increases thiol accumulation, and flux of sulfur through the primary assimilation pathway. One possible explanation for this is that a compensating increase of the remaining ATPS genes enables more efficient activation of sulfate for primary assimilation. As both *atps2* and *atps3* display a similar increase in primary assimilation, presumably the effect follows induction of either *ATPS1* or *ATPS4*. Surprisingly, however, the work of Liang *et al*. (2010) revealed that triple *atps1 atps3 ATPS4-RNAi* mutants can survive, indicating that ATPS2 alone can maintain adequate levels of sulfate assimilation for plant survival. In addition, sulfate accumulation in this triple mutant doesn't exceed that in the *ATPS4-RNAi* line (Liang et al., 2010), supporting our findings that ATPS4 has the biggest influence on sulfur flux through the primary assimilation pathway.

2.4.3 The ATPS genes are involved in coordinated regulation of nutrient assimilation

Many aspects of the sulfate assimilation pathway are regulated in a demand driven manner. In particular, transcript levels of all three APR genes are increased in response to sulfur starvation and some evidence exists for increased ATPS activity (Lappartient and Touraine, 1996, 1997; Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003). Information from Genevestigator indicated that demand driven regulation may extend to mRNA levels of *ATPS1* and *ATPS3*. However, *ATPS2* and *ATPS4* appear to be regulated in an opposite manner. As both *ATPS1* and *ATPS4* are targeted by sulfur starvation-induced miR395, the regulatory networks appear to be very complex. In

Chapter 4 we have dissected the regulation of ATPS in response to sulfur demand, and the role played by miR395 in this regulation.

Demand for sulfur is strongly influenced by both carbon and nitrogen assimilatory rates (reviewed in Kopriva and Rennenberg, 2004). We have shown that this coordinated regulation extends to the ATPS genes. The cellular components cysteine and glutathione originate from three of the most important metabolic pathways in plants: photosynthesis, nitrogen assimilation, and sulfur assimilation (Kopriva and Rennenberg, 2004). Whilst APR transcription and enzyme activity is strongly repressed during nitrogen deficiency (Yamaguchi *et al.*, 1999; Koprivova *et al.*, 2000a), the response of ATPS is somewhat less clear. Koprivova et al. (2000a) reported no changes in *ATPS1* transcript levels in response to nitrogen deficiency in Arabidopsis, consistent with the response to low N treatments displayed in Genevestigator (

Figure 2.11). However, repression of *ATPS3* transcript levels in Arabidopsis plants grown on medium lacking a nitrogen source indicated that this isoform is regulated in response to nitrogen status (Yamaguchi et al., 1999). The strongest evidence for coordination of ATPS regulation with nitrogen assimilation was shown in a correlation between ATPS activity and nitrate in cultured tobacco cells (Reuveny et al., 1980). Microarray analysis of nitrogen deficient plants showed that *ATPS2* transcription is indeed down-regulated in response to the decreased demand for reduced sulfur, but that *ATPS1* and *ATPS3* mRNA levels are not regulated (

Figure 2.11). Co-expression analysis of *ATPS2* revealed a high number of nitrogen assimilation genes shared similar stress and hormone responses (Supplemental Table 2.2). *ATPS1* expression was also correlated to nitrogen assimilation genes, especially under stress conditions, indicating this isoform may indeed have some level of coordinated regulation with nitrogen assimilation. Interestingly, *ATPS4* was up-regulated

in response to nitrogen deficiency treatments and shared expression with a number of nitrogen assimilation genes in response to stress. *ATPS4* is predominantly root expressed and as such may be involved in the provision of glutathione for root growth and morphological changes (Sanchez-Fernandez *et al.*, 1997; Espunya *et al.*, 2006; Koprivova *et al.*, 2010).

ATPS activity undergoes diurnal fluctuations in the leaves of C_4 maize, with increases in activity after the transition from dark to light (Ghisi *et al.*, 1987; Passera *et al.*, 1989; Kocsy *et al.*, 1997). ATPS activity has also been shown to be light responsive in *Lemna minor* (Neuenschwander et al., 1991). In Arabidopsis, a reduction in ATPS activity is seen following 38 hour incubation in the darkness, compared with plants maintained in light conditions (Hesse et al., 2003). However, studies of sulfate assimilation regulation by carbon status have focussed for the most part on APR. Activity of APR exhibits a diurnal rhythm and transcription of all three APR genes responds to light, glucose, and sucrose (Kopriva *et al.*, 1999; Hesse *et al.*, 2003). Analysis of microarray data showed that *ATPS2* is the isoform responsible for up-regulation of ATPS activity in response to light (

Figure 2.11). This finding was confirmed by qPCR analysis of seedlings re-illuminated after 38 hours darkness (Figure 2.12). In addition, *ATPS1* transcript levels were repressed by blue, far red, and UV light, but up-regulated by glucose. Therefore, it appears that *ATPS1* does respond to carbon assimilation, but is sensitive to specific light sources. Expression of *ATPS1* and *ATPS2* was correlated to an exceptionally high number of genes involved in light harvesting and carbon metabolism processes (Supplemental Tables 2.1 and 2.2), providing further evidence that *ATPS1* and *ATPS2* are the key isoforms responsible for light regulation of ATPS activity. While *ATPS3* transcription is not induced by light treatments, it increases following addition of glucose, possibly indicating some capability for response to carbon metabolism. However, *ATPS3* transcript levels do not respond directly to light stimuli. Nitrogen and carbon assimilation are both under circadian

regulation. Thus, the regulation of ATPS and APR by light synchronizes sulfate assimilation with these nutrient assimilation pathways. An additional link between sulfur metabolism and photosynthesis is provided by the role of glutathione in the protection of Photosystem II from light-induced damage (photoinhibition) by ROS (Ding et al., 2009; Yin et al., 2010).

Several genes for components of sulfate assimilation are known to be up-regulated in response to both osmotic and oxidative stresses, causing increases in glutathione contents (Bick *et al.*, 2001; Sahr *et al.*, 2005; Koprivova *et al.*, 2008; Queval *et al.*, 2009; Yoshida *et al.*, 2009). We showed that a variety of treatments causing both osmotic and oxidative stress regulate the ATPS genes, mostly resulting in increased transcription of *ATPS1* and *ATPS3*. Potassium deficiency also causes an alteration of the plant osmotic potential (Hafsi et al., 2010), producing a potential requirement for glutathione. In addition, potassium deficiency has recently been shown to result in the accumulation of glucosinolates in Arabidopsis (Troufflard et al., 2010). Hence, it is surprising to see a repression of *ATPS2 - 4* transcript levels in response to this nutrient stress. Site specific responses, not detected in the global measurements used in this analysis, may be the reason for the lack of response detected. By reducing the internal K levels Cs can also alter the plant osmotic potential and elicit osmotic stress responses (Sahr et al., 2005). Cs toxicity and K nutrition are intricately linked, as Cs acts as a competitive inhibitor of K-channels (White and Broadley, 2000; Zhu and Smolders, 2000). However, treatment with Cs caused an increase in *ATPS4* transcription. As Cs accumulation results in the production of ROS and the activation of anti-oxidative defence systems, including increased glutathione (Ghosh et al., 1993), the increase in *ATPS4* mRNA may be through a different signalling pathway. A common feature of many environmental stresses, including osmotic stress, is the generation of ROS. The main mechanism for defence against cell damage by ROS is the ascorbate-glutathione cycle (Noctor and Foyer, 1998). Therefore, glutathione synthesis is induced by a number of stress conditions (Noctor *et al.*, 1998; Ruiz and Blumwald, 2002; Mittova *et al.*, 2003;

Koprivova *et al.,* 2008). Up-regulation of *APR* enables the increase in glutathione levels in response to both salt stress and treatment with H_2O_2 (Koprivova et al., 2008). The Genevestigator results show that this induction of *APR* transcription is accompanied by increases in *ATPS1* and *ATPS3* mRNA levels.

2.4.4 Links between ATPS and glucosinolate synthesis

The data mining tools indicated a tight link of ATPS1 and ATPS3 with glucosinolate synthesis. This was confirmed by a detailed analysis described and discussed in the next chapter.

Supplemental Table 2.1 *ATPS1 Expression Angler gene list.*
A representative selection of genes with expression patterns correlating (Pearsons correlation coefficient; R > 0.6) to those of *ATPS1*, as calculated using the Expression Angler tool (www.bar.utoronto.ca; Toufighi et al., 2005).

AGI-ID	Name	activity	r-value to Bait	database
BAIT				
At3g22890	ATPS1	ATP sulfurylase	1.000	-
Primary sulfate assimilation (14)				
At5g10180	SULTR2;1	sulfate transporter	0.693	tissue
			0.665	stress
			0.617	hormone
At1g23090	SULTR3;3	sulfate transporter	0.607	stress
At5g13550	SULTR4;1	sulfate transporter	0.695	hormone
At4g14680	ATPS3	ATP sulfurylase	0.660	tissue
			0.741	hormone
At4g04610	APR1	APS reductase	0.731	hormone
At4g21990	APR3	APS reductase	0.649	hormone
At5g04590	SiR	sulfite reductase	0.751	tissue
At1g55920	SAT2;1	serine acetyl transferase	0.627	hormone
At4g14880	OASTL A1	*O*-acetylserine (thiol) lyase	0.612	tissue
At2g43750	OASTL B	*O*-acetylserine (thiol) lyase	0.653	stress
At3g61440	BSAS3;1/CYS C1	*O*-acetylserine (thiol) lyase	0.710	stress
			0.622	tissue
At5g28030	OASTL-like/DES1	Cysteine desulfhydrase	0.611	stress
At5g27380	GSHS/GSH2	glutathione synthetase	0.755	hormone
At3g01120	CgS	cystathione *γ*-synthase	0.631	tissue
Other sulfur metabolism (15)				
At2g03750	SOT11	sulfotransferase	0.624	stress
At5g66040	ST16	sulfurtransferase	0.739	tissue
At1g08490	CPNIFS	cysteine desulfurase	0.665	stress
At4g37040	MAP1D	methionine aminopeptidase	0.602	stress
At4g21830	MSRB7	methionine sulfoxide reductase	0.637	hormone
At4g39460	SAMT1	S-adenosylmethionine transmembrane transporter	0.621	stress
At3g25570	-	adenosylmethionine decarboxylase	0.670	stress
At3g54660	GR2	glutathione reductase	0.680	stress
At2g25080	GPX1	glutathione peroxidase	0.617	stress
At4g31870	GPX7	glutathione peroxidase	0.667	stress
At5g57040	-	lactoylglutathione lyase	0.672	tissue
At1g67280	-	lactoylglutathione lyase	0.627	tissue

At2g30870	GSTF10	glutathione S-transferase	0.602	hormone
At1g27130	GSTU13	glutathione S-transferase	0.745	hormone
At1g78370	GSTU20	glutathione S-transferase	0.705	stress
Glucosinolate biosynthesis (22)				
At4g39940	APK2	APS kinase	0.733	tissue
			0.631	stress
At4g39940	APK2	APS kinase	0.658	hormone
At2g20610	SUR1	C-S lyase	0.724	tissue
			0.601	stress
Aliphatic glucosinolate biosynthesis (16)				
At1g18590	SOT17	glucosinolate sulfotransferase	0.702	tissue
			0.604	hormone
At1g74090	SOT18	glucosinolate sulfotransferase	0.673	tissue
At5g07690	MYB29	R2R3-type MYB transcription factor	0.663	stress
At3g49680	BCAT3	branched-chain aminotransferase	0.674	stress
At3g19710	BCAT4	branched-chain aminotransferase	0.753	tissue
At5g23010	MAM1	methylthioalkylmalate synthase	0.787	tissue
			0.634	stress
At1g16400	CYP79F2	cytochrome P450	0.681	tissue
At4g13770	CYP83A1	cytochrome P450	0.763	tissue
At1g65860	FMO GS-OX1	flavin-monooxygenase glucosinolate s-oxygenase	0.668	tissue
			0.680	stress
At1g62560	FMO GS-OX3	flavin-monooxygenase glucosinolate s-oxygenase	0.619	tissue
			0.639	stress
At1g12140	FMO GS-OX5	flavin-monooxygenase glucosinolate s-oxygenase	0.605	tissue
At4g12030	BAT5	bile acid:sodium symporter	0.619	tissue
At3g58990	IPMI1	isopropylmalate isomerase	0.687	tissue
At2g43100	IPMI2	isopropylmalate isomerase	0.658	tissue
At4g13430	IIL1	methylthioalkylmalate isomerase	0.711	tissue
At1g31180	IPMDH1	3-isopropylmalate dehydrogenase	0.665	tissue
Indolic glucosinolate biosynthesis (3)				
At5g05730	ASA1	anthranilate synthase alpha subunit	0.663	hormone
At5g17990	TRP1	anthranilate phosphoribosyltransferase	0.718	hormone
At1g24100	UGT74B1	UDP-glycosyltransferase	0.652	tissue
Nitrogen assimilation (6)				
At2g26690	NTP2	nitrate transporter	0.655	tissue
At3g21670	NTP3	nitrate transporter	0.636	stress
At1g37130	NR2	nitrate reductase	0.671	tissue
At5g35630	GLN2	glutamine synthetase	0.648	stress

At1g23310	GGAT1	L-alanine:2-oxoglutarate aminotransferase	0.661	stress
At5g47970	-	nitrogen regulation family protein	0.635	stress
Photosynthesis (65)				
AtCg00350	PSAA	photosystem I subunit	0.630	stress
At4g02770	PSAD1	photosystem I subunit	0.661	stress
At1g31330	PSAF	photosystem I subunit	0.649	stress
At1g55670	PSAG	photosystem I subunit	0.641	stress
At3g16140	PSAH1	photosystem I subunit	0.655	stress
At1g52230	PSAH2	photosystem I subunit	0.665	stress
AtCg00510	PSAI	photosystem I subunit	0.612	stress
AtCg00630	PSAJ	photosystem I subunit	0.621	stress
At1g30380	PSAK	photosystem I subunit	0.656	stress
At4g12800	PSAL	photosystem I subunit	0.647	stress
At1g08380	PSAO	photosystem I subunit	0.659	stress
At2g46820	PSAP	photosystem I subunit	0.671	stress
AtCg00280	PSBC	photosystem II subunit	0.638	stress
AtCg00270	PSBD	photosystem II subunit	0.604	stress
AtCg00580	PSBE	photosystem II subunit	0.636	tissue
			0.618	stress
AtCg00570	PSBF	photosystem II subunit	0.642	stress
AtCg00710	PSBH	photosystem II subunit	0.624	stress
AtCg00080	PSBI	photosystem II subunit	0.604	stress
AtCg00550	PSBJ	photosystem II subunit	0.643	tissue
			0.624	stress
AtCg00070	PSBK	photosystem II subunit	0.645	stress
AtCg00560	PSBL	photosystem II subunit	0.623	stress
AtCg00700	PSBN	photosystem II subunit	0.616	stress
At5g66570	PSBO1	photosystem II subunit	0.601	tissue
			0.652	stress
At3g50820	PSBO2	photosystem II subunit	0.658	stress
At1g06680	PSBP1	photosystem II subunit	0.653	stress
At4g05180	PSBQ2	photosystem II subunit	0.642	stress
At1g79040	PSBR	photosystem II subunit	0.652	stress
At1g44575	PSBS	photosystem II subunit	0.706	stress
At3g21055	PSBTN	photosystem II subunit	0.618	stress
At2g30570	PSBW	photosystem II subunit	0.661	stress
At2g06520	PSBX	photosystem II subunit	0.653	stress
At4g28660	PSB28	photosystem II subunit	0.623	stress
At1g51400	-	photosystem II protein	0.647	stress
At1g03600	-	photosystem II protein	0.635	stress

At4g15510	-	photosystem II protein	0.602	stress
At3g54890	LHCA1	light harvesting complex/chlorophyll binding protein	0.656	stress
At3g61470	LHCA2	light harvesting complex/chlorophyll binding protein	0.638	stress
At1g61520	LHCA3	light harvesting complex/chlorophyll binding protein	0.668	stress
At3g47470	LHCA4	light harvesting complex/chlorophyll binding protein	0.662	stress
At1g45474	LHCA5	light harvesting complex/chlorophyll binding protein	0.634	stress
At1g19150	LHCA6	light harvesting complex/chlorophyll binding protein	0.617	stress
At1g29910	LHCB1.2	light harvesting complex/chlorophyll binding protein	0.667	stress
At2g34420	LHCB1.5	light harvesting complex/chlorophyll binding protein	0.654	stress
At2g05070	LHCB2.2	light harvesting complex/chlorophyll binding protein	0.669	stress
At3g27690	LHCB2.4	light harvesting complex/chlorophyll binding protein	0.668	stress
At5g54270	LHCB3	light harvesting complex/chlorophyll binding protein	0.679	stress
At5g01530	LHCB4.1	light harvesting complex/chlorophyll binding protein	0.665	stress
At3g08940	LHCB4.2	light harvesting complex/chlorophyll binding protein	0.672	stress
At4g10340	LHCB5	light harvesting complex/chlorophyll binding protein	0.668	stress
At1g15820	LHCB6	light harvesting complex/chlorophyll binding protein	0.673	stress
At1g76570	-	chlorophyll A-B binding	0.671	stress
At3g24430	HCF101	high chlorophyll fluorescence phenotype	0.640	stress
At3g17040	HCF107	high chlorophyll fluorescence phenotype	0.662	stress
At5g23120	HCF136	high chlorophyll fluorescence phenotype	0.634	stress
At4g37200	HCF164	high chlorophyll fluorescence phenotype	0.619	stress
At1g16720	HCF173	high chlorophyll fluorescence phenotype	0.673	stress
At4g08920	CRY1	cryptochrome	0.602	tissue
At5g52570	BCH2	beta-carotene hydroxylase	0.635	stress
At2g20570	GLK1	transcription factor	0.603	stress
At5g38660	APE1	acclimation of photosynthesis to environment	0.637	stress
			0.639	hormone
At5g46110	APE2	acclimation of photosynthesis to environment	0.606	tissue
			0.665	stress
At3g59060	PIF5	phytochrome interacting factor	0.624	tissue
			0.670	stress
			0.630	hormone
At4g03280	PETC	electron transporter	0.656	stress
At1g09340	CSP41B	chloroplast RNA binding	0.667	stress
At1g02910	LPA1	low PSII accumulation	0.604	stress

Hormone synthesis/signaling (5)

At3g44300	NIT2	indole-3-acetonitrile nitrilase	0.686	tissue
At5g22300	NIT4	indole-3-acetonitrile nitrilase	0.703	stress
At3g25760	AOC1	allene-oxide cyclase	0.698	hormone
At1g19640	JMT	jasmonate O-methyltransferase	0.662	stress

At1g04240	SHY2	transcription factor	0.665	tissue

Supplemental Table 2.2 *ATPS2 Expression Angler gene list.*
A representative selection of genes with expression patterns correlating (Pearsons correlation coefficient; R > 0.6) to those of *ATPS2*, as calculated using the Expression Angler tool (www.bar.utoronto.ca; Toufighi et al., 2005).

AGI-ID	Name	activity	r-value to Bait	database
BAIT				
At1g19920	ATPS2	ATP sulfurylase	1.000	-
Primary sulfate assimilation (8)				
At5g10180	SULTR2;1	sulfate transporter	0.660	stress
At1g77990	SULTR2;2	sulfate transporter	0.804	stress
At1g23090	SULTR3;3	sulfate transporter	0.682	stress
At2g43750	OASTL B	*O*-acetylserine (thiol) lyase	0.750	stress
At3g61440	BSAS3;1/CYS C1	*O*-acetylserine (thiol) lyase	0.744	stress
At5g28030	OASTL-like/DES1	Cysteine desulfhydrase	0.688	stress
At3g01120	CgS	cystathione *γ*-synthase	0.734	stress
At3g03780	MS2	methionine synthase	0.619	hormone
Other sulfur metabolism (14)				
At2g03750	SOT11	sulfotransferase	0.732	stress
At4g33030	SQD1	sulfotransferase	0.675	stress
At1g08490	CPNIFS	cysteine desulfurase	0.712	stress
At2g27420	-	cysteine proteinase	0.696	stress
At5g06290	2CPB	2-cysteine peroxiredoxin	0.704	stress
At4g37040	MAP1D	methionine aminopeptidase	0.838	stress
At4g39460	SAMT1	S-adenosylmethionine transmembrane transporter	0.669	stress
At3g54660	GR2	glutathione reductase	0.710	stress
At2g25080	GPX1	glutathione peroxidase	0.635	stress
At1g67280	-	lactoylglutathione lyase	0.675	stress
At1g10360	GSTU18	glutathione S-transferase	0.710	stress
At1g78370	GSTU20	glutathione S-transferase	0.785	stress
			0.717	hormone
At5g44000		glutathione S-transferase	0.686	stress
At1g31170	SRX	sulfiredoxin	0.698	stress
Glucosinolate biosynthesis (16)				
At4g03070	AOP1	2-oxoglutarate-dependent dioxygenase	0.702	
Aliphatic glucosinolate biosynthesis (14)				
At1g74090	SOT18	glucosinolate sulfotransferase	0.741	hormone
At5g61420	MYB28	R2R3-type MYB transcription factor	0.794	stress
			0.799	hormone

At5g07690	MYB29	R2R3-type MYB transcription factor	0.686	stress
At3g49680	BCAT3	branched-chain aminotransferase	0.810	stress
			0.738	hormone
At3g19710	BCAT4	branched-chain aminotransferase	0.712	hormone
At5g23010	MAM1	methylthioalkylmalate synthase	0.608	hormone
At5g23020	MAM3	methylthioalkylmalate synthase	0.703	hormone
At4g13770	CYP83A1	cytochrome P450	0.710	stress
			0.761	hormone
At1g65860	FMO GS-OX1	flavin-monooxygenase glucosinolate s-oxygenase	0.701	stress
At1g62560	FMO GS-OX3	flavin-monooxygenase glucosinolate s-oxygenase	0.727	stress
At4g12030	BAT5	bile acid:sodium symporter	0.754	hormone
At3g58990	IPMI1	isopropylmalate isomerase	0.707	hormone
At2g43100	IPMI2	isopropylmalate isomerase	0.731	hormone
At1g31180	IPMDH1	methylthioalkylmalate isomerase	0.734	hormone
Indolic glucosinolate biosynthesis (1)				
At4g24670	TAR2	tryptophan aminotransferase	0.640	stress
Nitrogen assimilation (9)				
At1g32450	NRT1.5	nitrate transporter	0.729	hormone
At3g21670	NTP3	nitrate transporter	0.674	stress
At1g64780	AMT1.2	ammonium transporter	0.678	hormone
At2g15620	NIR1	nitrite reductase	0.610	hormone
At5g04140	GLS1	ferredoxin dependant glutamate synthase	0.831	stress
			0.624	hormone
At5g35630	GLN2	glutamine synthetase	0.777	stress
At1g23310	GGAT1	L-alanine:2-oxoglutarate aminotransferase	0.759	stress
At5g65010	ASN2	asparagine synthetase	0.864	stress
			0.627	hormone
At5g47970	-	nitrogen regulation family protein	0.674	stress
Photosynthesis (51)				
At4g02770	PSAD1	photosystem I subunit	0.689	stress
			0.642	hormone
At2g20260	PSAE2	photosystem I subunit	0.604	stress
			0.609	hormone
At1g31330	PSAF	photosystem I subunit	0.689	stress
At1g55670	PSAG	photosystem I subunit	0.688	stress
At1g52230	PSAH	photosystem I subunit	0.700	stress
AtCg00510	PSAI	photosystem I subunit	0.643	stress
			0.622	hormone
AtCg00630	PSAJ	photosystem I subunit	0.685	stress

At1g30380	PSAK	photosystem I subunit	0.704	stress
At1g08380	PSAO	photosystem I subunit	0.684	stress
At2g46820	PSAP	photosystem I subunit	0.714	stress
AtCg00570	PSBF	photosystem II subunit	0.647	stress
AtCg00710	PSBH	photosystem II subunit	0.658	stress
			0.617	hormone
AtCg00080	PSBI	photosystem II subunit	0.669	stress
AtCg00550	PSBJ	photosystem II subunit	0.656	stress
AtCg00070	PSBK	photosystem II subunit	0.669	stress
AtCg00560	PSBL	photosystem II subunit	0.649	stress
			0.657	hormone
AtCg00220	PSBM	photosystem II subunit	0.680	stress
At5g66570	PSBO1	photosystem II subunit	0.685	stress
At3g50820	PSBO2	photosystem II subunit	0.735	stress
At1g06680	PSBP	photosystem II subunit	0.700	stress
At4g05180	PSBQ2	photosystem II subunit	0.669	stress
At2g30570	PSBW	photosystem II subunit	0.676	stress
At2g06520	PSBX	photosystem II subunit	0.710	stress
At4g28660	PSB28	photosystem II subunit	0.885	stress
			0.671	hormone
At1g03600	-	photosystem II protein	0.713	stress
At1g05385	-	photosystem II protein	0.603	stress
At1g51400	-	photosystem II protein	0.647	stress
At4g15510	-	photosystem II protein	0.772	hormone
At3g61470	LHCA2	light harvesting complex/chlorophyll binding protein	0.685	stress
At1g61520	LHCA3	light harvesting complex/chlorophyll binding protein	0.667	stress
At3g47470	LHCA4	light harvesting complex/chlorophyll binding protein	0.608	stress
At1g19150	LHCA6	light harvesting complex/chlorophyll binding protein	0.815	stress
At1g29910	LHCB1.2	light harvesting complex/chlorophyll binding protein	0.664	stress
At5g01530	LHCB4.1	light harvesting complex/chlorophyll binding protein	0.680	stress
At3g08940	LHCB4.2	light harvesting complex/chlorophyll binding protein	0.604	stress
At2g40100	LHCB4.3	light harvesting complex/chlorophyll binding protein	0.881	stress
At4g10340	LHCB5	light harvesting complex/chlorophyll binding protein	0.689	stress
At1g15820	LHCB6	light harvesting complex/chlorophyll binding protein	0.665	stress
At3g17040	HCF107	high chlorophyll fluorescence phenotype	0.685	stress
			0.657	hormone
At5g36170	HCF109	high chlorophyll fluorescence phenotype	0.751	stress
At3g09650	HCF152	high chlorophyll fluorescence phenotype	0.669	stress
At4g31560	HCF153	high chlorophyll fluorescence phenotype	0.705	stress
At4g37200	HCF164	high chlorophyll fluorescence phenotype	0.694	stress
At1g16720	HCF173	high chlorophyll fluorescence phenotype	0.757	stress

At2g18790	PHYB	phytochrome B	0.628	stress
At2g47590	PHR2	photolyase	0.813	stress
At3g45780	PHOT1	phototropin	0.615	stress
At2g20570	GLK1	transcription factor	0.691	stress
At2g42610	LSH10	light sensitive hypocotyls	0.612	stress
At1g02910	LPA1	low PSII accumulation	0.771	stress
At5g18660	PCB2	pale-green and chlorophyll B reduced	0.610	hormone
Hormone synthesis/signaling (1)				
At3g44300	NIT2	indole-3-acetonitrile nitrilase	0.675	stress

Supplemental Table 2.3 *ATPS3 Expression Angler gene list.*
A representative selection of genes with expression patterns correlating (Pearsons correlation coefficient; R > 0.6) to those of *ATPS3*, as calculated using the Expression Angler tool (www.bar.utoronto.ca; Toufighi et al., 2005).

AGI-ID	Name	activity	r-value to Bait	database
BAIT				
At4g14680	ATPS3	ATP sulfurylase	1.000	
Primary sulfate assimilation (6)				
At5g10180	SULTR2;1	sulfate transporter	0.612	tissue
At5g13550	SULTR4;1	sulfate transporter	0.792	hormone
At3g22890	ATPS1	ATP sulfurylase	0.660	tissue
			0.741	hormone
At4g04610	APR1	APS reductase	0.713	stress
			0.658	hormone
At4g21990	APR3	APS reductase	0.755	stress
At5g27380	GSHS/GSH2	glutathione synthetase	0.744	hormone
Other sulfur metabolism (11)				
At3g22740	HMT3	homocysteine S-methyltransferase	0.642	hormone
At4g21830	MSRB7	methionine sulfoxide reductase	0.798	hormone
At4g21850	MSRB9	methionine sulfoxide reductase	0.664	hormone
At4g11280	ACS6	SAM methylthioadenosine-lyase	0.691	stress
At1g47990	ACS8	SAM methylthioadenosine-lyase	0.615	stress
At4g37770	ACS8	SAM methylthioadenosine-lyase	0.673	stress
At2g29440	GSTU6	glutathione S-transferase	0.667	hormone
At1g69930	GSTU11	glutathione S-transferase	0.792	stress
At5g48850	SDI1	sulfur deficiency induced	0.786	stress
At3g49580	LSU1	response to low sulfur	0.719	stress
At5g24660	LSU2	response to low sulfur	0.698	stress
Glucosinolate biosynthesis (15)				
At2g14750	APK1	APS kinase	0.804	hormone
At4g39940	APK2	APS kinase	0.625	tissue
			0.880	hormone
At2g20610	SUR1	C-S lyase	0.727	hormone
Aliphatic glucosinolate biosynthesis (3)				
At1g18590	SOT17	glucosinolate sulfotransferase	0.712	hormone
At5g23010	MAM1	methylthioalkylmalate synthase	0.670	tissue
At1g65860	FMO GS-OX1	flavin-monooxygenase glucosinolate s-oxygenase	0.680	hormone
Indolic glucosinolate biosynthesis (9)				

At3g54640	TRP3	tryptophan synthase	0.601	hormone
At1g74100	SOT16	glucosinolate sulfotransferase	0.684	hormone
At1g32640	MYC2	transcription factor	0.839	stress
			0.685	hormone
At1g18570	MYB51	R2R3-type MYB transcription factor	0.679	stress
At5g05730	ASA1	anthranilate synthase alpha subunit	0.815	hormone
At4g39950	CYP79B2	cytochrome P450	0.700	hormone
At2g22330	CYP79B3	cytochrome P450	0.662	hormone
At4g31500	CYP83B1	cytochrome P450	0.626	hormone
At1g24100	UGT74B1	UDP-glycosyltransferase	0.782	hormone
Nitrogen assimilation (2)				
At1g37130	NR2	nitrate reductase	0.622	tissue
At5g65010	ASN2	asparagine synthetase	0.642	tissue
Hormone synthesis/signaling (25)				
At5g42650	AOS	allene oxide synthase (jasmonate biosynthesis)	0.755	hormone
At3g25760	AOC1	allene oxide cyclase (jasmonate biosynthesis)	0.748	hormone
At3g25780	AOC3	allene oxide cyclase (jasmonate biosynthesis)	0.682	stress
At2g24850	TAT3	tyrosine aminotransferase (jasmonate responsive)	0.726	hormone
At1g19180	JAZ1	jasmonate ZIM-domain protein	0.645	stress
			0.740	hormone
At1g74950	JAZ2	jasmonate ZIM-domain protein	0.771	stress
			0.690	hormone
At3g17860	JAZ3	jasmonate ZIM-domain protein	0.623	hormone
At1g17380	JAZ5	jasmonate ZIM-domain protein	0.884	stress
			0.642	hormone
At1g72450	JAZ6	jasmonate ZIM-domain protein	0.759	hormone
At1g30135	JAZ8	jasmonate ZIM-domain protein	0.624	hormone
At1g70700	JAZ9	jasmonate ZIM-domain protein	0.751	hormone
At5g13220	JAZ10	jasmonate ZIM-domain protein	0.796	stress
			0.734	hormone
At3g23240	ERF	ethylene responsive binding factor	0.781	stress
At4g17500	ERF	ethylene responsive binding factor	0.767	stress
At5g47220	ERF2	ethylene responsive binding factor	0.828	stress
At4g17490	ERF6	ethylene responsive binding factor	0.770	stress
At2g44840	ERF13	ethylene responsive binding factor	0.843	stress
At5g61600	ERF104	ethylene responsive binding factor	0.641	stress
At3g23230	-	ethylene responsive binding factor	0.856	stress
At3g15540	IAA19	indole-3-acetonitrile inducible transcription factor	0.709	stress
At3g62100	IAA30	indole-3-acetonitrile inducible transcription factor	0.764	stress

At1g04240	SHY2	indole-3-acetonitrile inducible transcription factor	0.659	tissue
At1g51760	IAR	indole-3-acetonitrile resistant	0.798	hormone
At2g46510	AIB	abscisic acid inducible	0.712	stress
			0.652	hormone
At3g50750	BEH1	brassinosteroid signalling positive regulator	0.608	tissue
			0.608	tissue

Supplemental Table 2.3 *ATPS4 Expression Angler gene list.*
A representative selection of genes with expression patterns correlating (Pearsons correlation coefficient; R > 0.6) to those of *ATPS4*, as calculated using the Expression Angler tool (www.bar.utoronto.ca; Toufighi et al., 2005).

AGI-ID	Name	activity	r-value to Bait	database
BAIT				
At5g43780	ATPS4	ATP sulfurylase	1	
Primary sulfate assimilation (4)				
At1g78000	SULTR1;2	sulfate transporter	0.827	stress
At5g56760	SAT1;1	serine acetyltransferase	0.754	stress
At3g59760	OASTL C	*O*-acetylserine (thiol) lyase	0.656	stress
At3g22460	OASTL A1	*O*-acetylserine (thiol) lyase	0.806	stress
Other sulfur metabolism (46)				
At5g43690	SOT1	sulfotransferase	0.819	stress
At3g45070	SOT5	sulfotransferase	0.702	stress
At1g13420	SOT8	brassinosteroid sulfotransferase	0.622	stress
At1g13430	SOT9	brassinosteroid sulfotransferase	0.748	stress
At5g66170	ST18	sulfurtransferase	0.704	stress
At5g46340	-	*O*-acetyltransferase-related	0.772	stress
At4g36880	CP1	cysteine proteinase	0.748	stress
At5g43060	-	cysteine proteinase	0.928	stress
At3g48350	-	cysteine proteinase	0.72	hormone
At5g05110	-	cysteine proteinase inhibitor	0.768	tissue
At4g35350	XCP1	xylem cysteine peptidase	0.818	stress
At5g63910	FCLY	farnesylcysteine lyase	0.674	stress
At1g67810	SUFE2	cysteine desulfurase activation	0.681	stress
At3g25900	HMT1	homocysteine S-methyltransferase	0.689	stress
At3g63250	HMT2	homocysteine S-methyltransferase	0.795	stress
At4g13940	SAHH1	S-adenosyl-L-homocysteine hydrolase	0.772	stress
At2g45240	MAP1A	methionine aminopeptidase	0.81	stress
At2g44180	MAP2A	methionine aminopeptidase	0.82	stress
At3g59990	MAP2B	methionine aminopeptidase	0.775	stress
At5g07460	MSRA2	methionine sulfoxide reductase	0.724	tissue
At5g07470	MSRA3	methionine sulfoxide reductase	0.728	stress
At4g04830	MSRB5	methionine sulfoxide reductase	0.673	stress
At4g29840	MTO2	methionine over-accumulator	0.62	stress
At1g02500	SAM1	S-adenosylmethionine synthetase	0.747	stress
At4g01850	SAM2	S-adenosylmethionine synthetase	0.837	stress
At5g38020	-	S-adenosyl-L-methionine:carboxyl methyltransferase	0.659	stress

At5g37990	-	S-adenosylmethionine-dependent methyltransferase	0.65	stress
At4g29210	GGT4	glutathione gamma-glutamylcysteinyltransferase	0.724	stress
At1g03980	PCS2	phytochelatin synthase	0.717	stress
At2g43350	GPX3	glutathione peroxidase	0.691	stress
At3g63080	GPX5	glutathione peroxidase	0.779	stress
At1g15380	-	lactoylglutathione lyase	0.896	stress
At1g02950	GSTF4	glutathione S-transferase	0.851	stress
At2g30870	GSTF10	glutathione S-transferase	0.642	stress
At5g17220	GSTF12	glutathione S-transferase	0.613	hormone
At1g49860	GSTF14	glutathione S-transferase	0.733	stress
At5g41220	GSTT3	glutathione S-transferase	0.754	stress
At2g29490	GSTU1	glutathione S-transferase	0.746	stress
At2g29420	GSTU7	glutathione S-transferase	0.779	stress
At3g09270	GSTU8	glutathione S-transferase	0.799	stress
			0.708	hormone
At1g27140	GSTU14	glutathione S-transferase	0.729	stress
At1g10370	GSTU17	glutathione S-transferase	0.72	hormone
At1g78360	GSTU21	glutathione S-transferase	0.782	stress
At1g78340	GSTU22	glutathione S-transferase	0.627	stress
At1g17190	GSTU26	glutathione S-transferase	0.745	stress
At2g02390	GSTZ1	glutathione S-transferase	0.848	stress
Glucosinolate biosynthesis (3)				
Aliphatic glucosinolate biosynthesis (3)				
At5g23020	MAM3	methylthioalkylmalate synthase	0.745	stress
Indolic glucosinolate biosynthesis (9)				
At5g60890	MYB34	R2R3-type MYB transcription factor	0.674	stress
At1g74080	MYB122	R2R3-type MYB transcription factor	0.641	stress
Nitrogen assimilation (9)				
At1g32450	NRT1.5	nitrate transporter	0.856	stress
At1g69850	NRT1.2	nitrate transporter	0.734	stress
At1g53310	PEPC1	phosphoenolpyruvate carboxylase	0.823	stress
At3g14940	PEPC3	phosphoenolpyruvate carboxylase	0.843	stress
At5g53460	GLT1	NADH dependant glutamate synthase	0.84	stress
At2g41220	GLU2	ferredoxin dependant glutamate synthase	0.909	stress
At5g18170	GDH1	glutamate dehydrogenase	0.812	stress
At5g07440	GDH2	glutamate dehydrogenase	0.843	stress
At1g62800	ASP4	aspartate aminotransferase	0.834	stress
Photosynthesis (1)				

At1g78815	LSH7	light sensitive hypocotyls	0.662	tissue
Hormone synthesis/signaling (35)				
At1g13280	AOC4	allene oxide cyclase (jasmonate biosynthesis)	0.653	stress
At3g44320	NIT3	indole-3-acetonitrile nitrilase	0.832	stress
At5g20960	AAO1	indole-3-acetaldehyde oxidase	0.924	stress
At3g02260	ASA1	polar auxin transport	0.741	stress
At5g01240	LAX1	auxin influx carrier	0.607	tissue
At4g27260	GH3.5	indole-3-acetonitrile-amido synthase	0.607	stress
At1g28130	GH3.17	indole-3-acetonitrile-amido synthase	0.696	hormone
At5g56650	ILL1	indole-3-acetonitrile-amino acid hydrolase	0.746	stress
At5g54140	ILL3	indole-3-acetonitrile-amino acid hydrolase	0.859	stress
At5g65670	IAA9	indole-3-acetonitrile inducible transcription factor	0.854	stress
At4g28640	IAA11	indole-3-acetonitrile inducible transcription factor	0.629	tissue
At2g33310	IAA13	indole-3-acetonitrile inducible transcription factor	0.76	tissue
			0.674	stress
At1g19220	IAA22	indole-3-acetonitrile inducible transcription factor	0.683	tissue
At3g16500	IAA26	indole-3-acetonitrile inducible transcription factor	0.68	tissue
At4g29080	IAA27	indole-3-acetonitrile inducible transcription factor	0.747	tissue
At5g25890	IAA28	indole-3-acetonitrile inducible transcription factor	0.646	tissue
			0.852	stress
At1g17350	-	indole-3-acetonitrile inducible transcription factor	0.785	stress
At1g19850	ARF5	indole-3-acetonitrile inducible transcription factor	0.641	stress
At5g20730	ARF7	indole-3-acetonitrile inducible transcription factor	0.659	stress
At4g23980	ARF9	indole-3-acetonitrile inducible transcription factor	0.656	stress
At4g30080	ARF16	indole-3-acetonitrile inducible transcription factor	0.843	stress
At1g19220	ARF	indole-3-acetonitrile inducible transcription factor	0.709	stress
At4g29080	ARF	indole-3-acetonitrile inducible transcription factor	0.707	stress
At5g47530	-	auxin-responsive protein	0.633	stress
At3g02875	ILR1	indole-3-acetonitrile-leucine resistant	0.705	stress
At2g38120	AUX1	auxin resisitant	0.619	tissue
At1g05180	AXR1	auxin resisitant	0.773	stress
At1g54990	AXR4	auxin resisitant	0.781	stress
At1g80680	SAR3	suppressor of auxin resistance	0.657	stress
At1g16540	ABA3	molybdopterin cofactor sulfurase (ABA biosynthesis)	0.793	stress
At1g49720	ABF1	abscisic acid responsive element	0.611	tissue
At5g48870	SAD1	abscisic acid sensitive	0.627	stress
At5g13680	ABO1	abscisic acid sensitive	0.615	stress
At1g55870	AHG2	abscisic acid sensitive	0.614	stress
At2g40940	ERS1	ethylene response sensor	0.815	stress

Chapter 3

ATPS Genes are Essential for Glucosinolate Biosynthesis

Parts of this chapter are published in Yatusevich et al., 2010

3. ATPS GENES ARE ESSENTIAL FOR GLUCOSINOLATE BIOSYNTHESIS

3.1. INTRODUCTION

Glucosinolates are sulfur-rich, amino-acid derived secondary metabolites involved in defence against biotic stress in plants of the order Brassicaceae (Fahey et al., 2001; Halkier and Gershenzon, 2006). The final step in the biosynthesis of glucosinolates, the sulfation of desulfo-glucosinolate precursors, is important for the function of glucosinolates (Ratzka et al., 2002). Biosynthesis of glucosinolates is induced in response to attack by a range of herbivores, and pathogens (Mewis *et al.*, 2006; Bednarek *et al.*, 2009; Clay *et al.*, 2009). This response is mediated at least in part through hormone responses. Therefore, a number of studies have investigated the effect of hormone treatments on glucosinolate levels, revealing that jasmonates, SA, and ET interact to regulate production of these plant defence compounds (Schenk *et al.*, 2000; Glazebrook *et al.*, 2003; Mikkelsen *et al.*, 2003; Mewis *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005). In particular, MeJa has been shown to regulate several processes in glucosinolate biosynthesis, including genes of primary sulfate assimilation (Jost et al., 2005). Glucosinolate synthesis is regulated by jasmonates through the action of a group of R2R3-MYB transcription factors (Gigolashvili *et al.*, 2007a; Gigolashvili *et al.*, 2007b; Gigolashvili *et al.*, 2008).

The efficiency of the glucosinolate response during herbivore or pathogen attack is dependent upon the coordinated up-regulation of the relevant steps of biosynthesis. Glucosinolate biosynthesis is regulated by a complex network of transcription factors (Celenza *et al.*, 2005; Levy *et al.*, 2005; Maruyama-Nakashita *et al.*, 2006; Skirycz *et al.*,

2006; Gigolashvili *et al.*, 2007a; Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007; Sønderby *et al.*, 2007; Gigolashvili *et al.*, 2008; Malitsky *et al.*, 2008). The best characterised of these belong to the R2R3-MYB family, and are responsible for the coordinated up-regulation of multiple glucosinolate genes (Celenza *et al.*, 2005; Hirai *et al.*, 2007; Sønderby *et al.*, 2007). The MYB factors can be divided into two groups, controlling biosynthesis of either aliphatic or indolic glucosinolates at multiple levels (Figure 3.1.1; Gigolashvili *et al.*, 2007a; Gigolashvili *et al.*, 2007b; Sønderby *et al.*, 2007; Gigolashvili *et al.*, 2008). The first group, *MYB28*, *MYB76*, and *MYB29*, alternatively named high aliphatic glucosinolate (HAG) 1-3, respectively, is involved in the control of aliphatic glucosinolate biosynthesis (Gigolashvili *et al.*, 2007b; Hirai *et al.*, 2007; Sønderby *et al.*, 2007; Gigolashvili *et al.*, 2008). The second group, consisting of *MYB51*, *MYB122*, and *MYB34*, otherwise known as high indolic glucosinolate (HIG)-1, -2, and -3 (also named ATR1), respectively, control the biosynthesis of indolic glucosinolates (Celenza *et al.*, 2005; Gigolashvili *et al.*, 2007a).

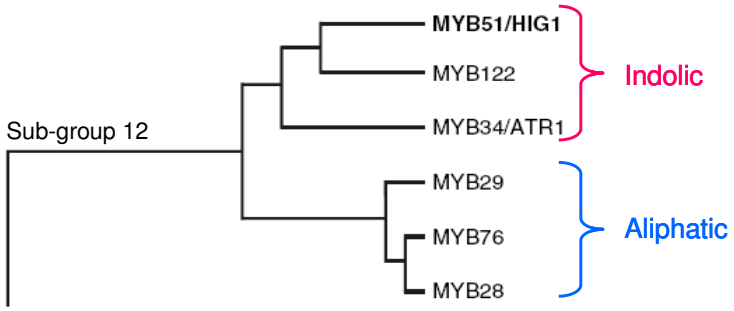


Figure 3.1 ***Phylogenetic tree of the six group 12 MYB factors***
The two clades are highlighted. Phylogenetic tree taken from Gigolashvili *et al.* (2007a)

During sulfur deficiency, a number of genes involved in glucosinolate biosynthesis are negatively regulated, whilst others, such as myrosinases, sulfate transporters, and nitrilases, involved in the remobilization of the sulfur stored in glucosinolates are up-regulated (Kutz *et al.*, 2002; Maruyama-Nakashita *et al.*, 2003; Hirai *et al.*, 2004; Nikiforova *et al.*, 2005; Falk *et al.*, 2007; Schonhof *et al.*, 2007). The SLIM1 transcription factor, a

central regulator in sulfur response, is at least partially responsible for these changes (Maruyama-Nakashita *et al.*, 2006), showing that regulation of sulfate assimilation and glucosinolate biosynthesis is tightly coordinated. Recently, the importance of this coordinated regulation was compounded through the discovery that two APK isoforms, APK1 and APK2, are essential for the production of sulfated secondary metabolites, including glucosinolates (Mugford et al., 2009). Large scale transcriptomics projects have shown that the glucosinolate MYB factors may regulate genes of primary sulfate assimilation including these two *APK* genes, and more surprisingly, *ATPS1* and *ATPS3* genes, (Sønderby *et al.*, 2007; Malitsky *et al.*, 2008). Further, analysis of public microarray data using the Expression Angler tool provided evidence for a link between the ATPS genes and glucosinolate biosynthesis (Chapter 2, discussed in this chapter). Therefore, we investigated the role of the ATPS genes in the provision of activated sulfur for glucosinolate sulfation reactions. We provide direct evidence that the ATPS genes are indeed regulated by the glucosinolate MYB factors, and are thus part of the glucosinolate biosynthesis network.

3.2. MATERIALS AND METHODS

3.2.1. Plant Material and Growth Conditions

A. thaliana ecotype Col-0 was used as the wild-type for all experiments unless otherwise stated. The MYB factor gain-of-function and loss-of-function mutants were kindly provided by Tamara Gigolashvili, University of Cologne, Germany (for list of mutants see Table 3.1). The construction and selection of the MYB factor transgenic plants was described previously (Gigolashvili *et al.*, 2007a; 2007b; 2008). For expression analysis by qPCR, biochemical analysis, and measurement of thiols and glucosinolates, wild-type Col-0 and the MYB transgenic plants were grown in soil in a controlled environment room under short-day conditions (8 h light, 16 h dark) at 22–25°C and 40% humidity. The MYB28-RNAi line used accumulated ca. 20% of WT transcript levels.

		Gain-of-function		Loss-of-function		
Gene	**AGI code**	**Line**	**Name**	**Line**	**Name**	**Reference**
MYB28 (HAG1)	At5g61420	*Pro35S:MYB28-15*	*MYB28_ox*	*MYB28-RNAi-10*	*MYB28-RNAi*	Gigolashvili *et al.*, 2007b
MYB76 (HAG2)	At5g07700	*Pro35S:MYB76-23*	*MYB76_ox*	SALK_N55242	*myb76 (hag2)*	Gigolashvili *et al.*, 2008
MYB29 (HAG3)	At5g07690	*Pro35S:MYB29-6*	*MYB29_ox*	GK_040H12	*myb29 (hag3)*	Gigolashvili *et al.*, 2008
MYB51 (HIG1)	At1g18570	*HIG1-1D*	*MYB51_ox*	GK_228B12	*myb51 (hig1-1)*	Gigolashvili *et al.*, 2007a
MYB122 (HIG2)	At1g74080	*Pro35S:MYB122-11*	*MYB122_ox*	SALK_039228	*myb122*	Gigolashvili *et al.*, 2007a; Gigolashvili unpublished
MYB34 (HIG3)	At5g60890	*Pro35S:ATR1-17*	*MYB34_ox*	WisDSLox424F3	*myb34*	Gigolashvili *et al.*, 2007a; Gigolashvili unpublished

Table 3.1 ***Transgenic MYB lines used in this study***

For glucosinolate analysis in ATPS loss-of-function mutants Col-0 and the three ATPS T-DNA insertion mutants, *atps1*, *atps2* and *atps3-1* were grown on soil in a controlled environment chamber under a 10-h-light/14-h-dark short-day cycle, at a constant temperature of 22°C, 60% relative humidity and light intensity of 160µmol m^{-2} s^{-1}.

3.2.2. Cloning of Promoters of *ATPS* Genes

To generate reporter constructs for the trans-activation assay, the promoter regions of the ATPS genes [5 kbp (*ATPS1* and *ATPS4*) and 2.5 kbp (*ATPS2* and *ATPS3*) upstream of the ATG] were amplified from genomic DNA of *A. thaliana* and cloned into the Gateway entry vector pDONR207 as described in Chapter 2.2.4. The promoter sequences were then subcloned into the binary plant transformation vector pGWB3i resulting in translational fusions with the *GUS* reporter gene. The fragments were fully sequenced to confirm their identity. As effectors, the constructs Pro35S:MYB28, Pro35S:MYB76, Pro35S:MYB29, Pro35S:MYB51, Pro35S:MYB122, and Pro35S:MYB34 were used as described previously (Gigolashvili *et al.*, 2007a; Gigolashvili *et al.*, 2007b; Gigolashvili *et al.*, 2008).

3.2.3. Trans-activation Assay Cell Culture

An Arabidopsis Col-0 root cell suspension culture was grown for the trans-activation assays in 50 ml *A. thaliana* (AT) medium (4.3 g l^{-1} MS basal salt media (Duchefa), 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D), 4 ml of vitamin B5 mixture (Sigma) and 30 g l^{-1} sucrose, pH 5.8). Suspension cell culture was diluted weekly to 1 : 4 or 1 : 5 with fresh AT media and gently agitated at 150 rpm in the dark at 22°C (Hartmann *et al.*, 1998).

3.2.4. Trans-activation Assays

The reporter and effector constructs were used to transform the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D (kindly provided by Dr Memelink, University of Leiden, Netherlands). For transient expression assays in the cell culture, *Agrobacteria* containing the effector constructs, the anti-silencing 19-K protein or one of the reporter constructs were taken from fresh yeast extract broth (YEB) plates, grown overnight, and resuspended in 1 ml AT medium. The *Agrobacteria* were mixed in a 1 : 1 : 1 ratio, and 75 µl of this suspension was added to 3 ml cultured *A. thaliana* root cells. The

transfected cell cultures were then grown for 3–5 or 7 days in the dark. To stain for GUS expression, cells were treated with 500 µl X-Glc staining solution (50 mM NaH_2PO_4, pH 7.0, 1 mM X-Gluc) for 1 hr to overnight at 37°C without shaking (Berger et al., 2007).

3.2.5. RNA Extraction and Expression Analysis

Expression of the four ATPS genes was analysed by real-time qPCR. Total RNA was isolated from 5-week old rosette leaves using TRIsure (Bioline, Luckenwalde, Germany), and cDNA produced by reverse transcription using the FirstStrand cDNA Synthesis SSII kit (Bioline) according to the manufacturer's instructions. Subsequently, the cDNA was used as a template for qPCR with gene-specific primers (for primer sequences see Table 3.2). The Arabidopsis *ACTIN2* gene was used as a reference. qPCR was performed using the SYBR Green master mix (Applied Biosystems), according to the manufacturer's instructions, in a GeneAmp 5700 sequence detection system (Applied Biosystems, Darmstadt, Germany). The Ct, defined as the PCR cycle at which a statistically significant increase of reporter fluorescence is detected, was used as a measure of the transcript level of the target gene. Relative quantification of expression levels was performed using the comparative Ct method (manufacturer's instructions, bulletin 2, Applied Biosystems). Three independent RNA preparations from independently grown plants were analyzed with two technical replicates for the qPCR.

Gene	AGI number	Forward primer (5'-3')	Reverse primer (5'-3')
ACTIN2	At3g18780	atggaagctgctggaatccac	ttgctcatacggtcagcgatg
ATPS1	At3g22890	cccgccagacggttttatgt	acttctggtagtctaccattaccgc
ATPS2	At1g19920	cagatcctcttacgtttctcacatca	ggttggtgaaagaggatggttttg
ATPS3	At4g14680	gagagaacccaccagatggattta	tccggttagtgtcaaactgtcgtag
ATPS4	At5g43780	ttcttcagcagcagccatcg	acgagaagcatgatggttatgga

Table 3.2 G*ene specific primers for quantitative RT-PCR analysis of ATPS*

3.2.6. ATPS Activity Assay

ATPS activity was measured in the reverse reaction as APS and pyrophosphate-dependent formation of ATP (Figure 3.2.1; Cumming *et al.*, 2007). Total protein was isolated from 5-week old rosette leaves by homogenization in 1:20 (w/v) of 50 mM Na/K phosphate buffer pH 8.0 supplemented with 30 mM Na_2SO_3, 0.5 mM 5'-AMP, and 10 mM DTE. The extract was centrifuged for 30 sec at 2,000 rpm to remove cell debris, and the protein concentration was determined by Bio-Rad protein assay with bovine serum albumin as a standard.

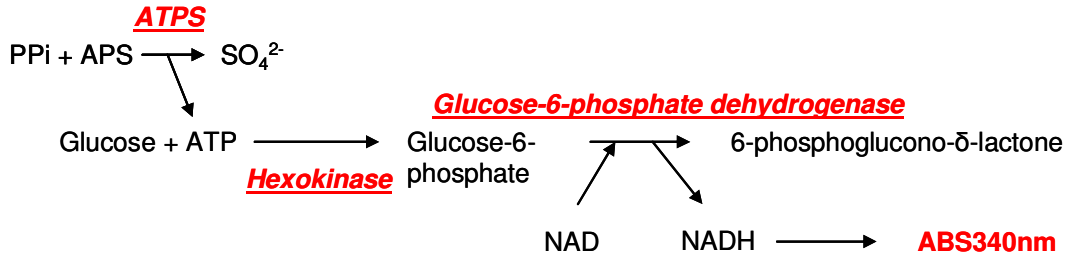


Figure 3.2 ***Reaction equation of the ATPS activity assay***

To measure activity, 40 µl of protein extract was mixed with 230 µl of reaction mix (for composition see Table 3.3) in a flat bottom 96 well polystyrene microplate (Greiner Bio-One).

Concentration	Component (abbr.)	Volume/230 µl
-	dH_2O	152.5 µl
1 M	2-amino-2-(hydroxymethyl)-1,3-propanediol (Tris) - HCl, pH 8.0	15 µl
100 mM	Magnesium chloride ($MgCl_2$)	15 µl
100 mM	D-Glucose	15 µl
6 mM	β-Nicotinamide adenine dinucleotide hydrate (NAD+)	15 µl
3.75 mM	Adenosine 5'-phosphosulfate sodium salt (APS)	7.5 µl
1 u $µl^{-1}$	Hexokinase from *Saccharomyces cerevisiae*, Type III (HK)	5 µl
1 u $µl^{-1}$	Glucose-6-phosphate dehydrogenase (G-6-PDH)	5 µl

Table 3.3 ***Composition of reaction mix for ATPS activity assay***

NADH production was measured at 340 nm using a SpectraMax 340PC^{384} microplate spectrophotometer. Background absorbance was measured for 3 min before the reaction was initiated by the addition of 30 µl 10 mM sodium pyrophosphate. Progress of the reaction was then measured for a further 3 min at 340 nm. Activity was calculated as nmol min^{-1} mg^{-1} from three biological replicates.

3.2.7. HPLC Analysis of Low Molecular Weight Thiols

The low molecular weight thiols, cysteine and glutathione, were extracted from 20-30 mg of leaf material and analysed as described in Chapter 2.2.7.

3.2.8. Screening of T-DNA Insertion Mutants

Homozygous *atps1* T-DNA insertion mutant seeds, line GABI850C05, were kindly donated by Naoko Yoshimoto, RIKEN institute, Japan (for further details see Chapter 2.2.2). An *atps2* T-DNA insertion mutant line identical to that described in Chapter 2.2.2 (SAIL775D12) and an individual *atps3* mutant line designated *atps3-1* (

Figure 3.3; SAIL312A08) were obtained from the NASC and homozygous lines were selected by PCR. All three mutant lines were in the Col-0 ecotype background of *Arabidopsis thaliana*.

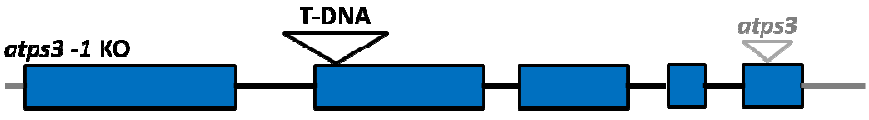


Figure 3.3 *atps3-1 T-DNA insertion line*
Grey lines represent the untranslated region, while black lines and blue boxes represent introns and exons respectively. The large black arrow head indicates the position of the *atps3-1* T-DNA insertion. The T-DNA insertion site of the *atps3* mutant used in Chapter 2 is depicted in grey.

The putative mutants were grown on soil in a standard glass house. Homozygous mutants were identified using the LBb1 primer and an appropriate gene-specific primer (Table 3.4). Crude DNA extractions were prepared from young rosette leaves by homogenisation in 400 µl extraction buffer (200 mM Tris-HCl, pH 7.5, 250 mM NaCl, 25mM EDTA and 0.5% SDS). Samples were vortexed for 10 sec and spun at 13,000 rpm for 5 min. The supernatant (300 µl) was mixed 1:1 with isopropanol in a fresh tube. Samples were vortexed briefly and spun at 13,000 rpm for 5 min. Pellets were washed in 70% ethanol, dried, and re-suspended in 100 µl sterile dH_2O. PCR amplification was carried out on 2.5 µl of crude extract in a 25 µl reaction using GoTaq Flexi DNA Polymerase (Promega) in accordance with the manufacturer's instructions.

Name	Sequence
LBb1	gcgtggaccgcttgctgcaact
ATPS3 Fw	ggttgggctagtcctcttcg
ATPS2 Fw	tctcaatttggtctataaacg

Table 3.4 ***Gene specific primers used in screening for homozygous mutants***

To verify the absence of ATPS transcripts in the T-DNA lines, total RNA was isolated from young leaves by phenol:chloroform:isoamylalcohol (25:24:1) extraction and LiCl precipitation (Chapter 2.2.12; Sambrook *et al.*, 1989). To produce the cDNA template, aliquots of 500 ng RNA were reverse transcribed using SuperScriptII Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. PCR was carried out using 1 µl cDNA template with GoTaq Flexi DNA Polymerase in a 25 ml reaction volume with primers specific to the individual genes and 34 amplification cycles.

3.2.8 Glucosinolate Analysis

Quantification of the glucosinolates was carried out according to the protocol described by Burow *et al.* (2006). Glucosinolates were extracted from 20-30 mg of ground freeze-dried leaf material with 1.5 ml hot 70% methanol (v:v) for 45 min at 70°C with occasional

mixing. 0.05 µmol of Sinigrin were added as an internal standard. After cooling, the extract was centrifuged at 4,000 rpm for 5 min to remove cell debris. 1 ml of the supernatant was loaded onto columns of DEAE Sephadex (Sigma) and the columns were washed twice with dH_2O and twice with 0.02 M sodium acetate. Bound intact glucosinolates were desulfated with 60 U of sulfatase (Sigma) overnight. After elution from the column with 1.25 ml dH_2O, desulfo-glucosinolates were separated by reverse-phase HPLC. Quantification was by UV absorption at 229 nm and was calculated as in Equation 1, relative to the internal standard and using response factors (Table 3.5) in accordance with the International Standard: Part 1 (1992). Identity of intact glucosinolates in the plant extracts was confirmed by liquid chromatography–mass spectrometry on a Bruker Esquire 6000 ion trap mass spectrometer (Bruker Daltonics).

$$\frac{A_g}{A_s} \times \frac{n}{m} \times K_g = \mu mol\ g^{-1}\ DW$$

Equation 1 Glucosinolate calculation: 'A_g' is the peak corresponding to the desulfo-glucosinolate; 'A_s' is the peak area corresponding to desulfosinigrin; 'K_g' is the response factor of the desulfo-glucosinolate; '*m*' is the mass in grams of the sample; and '*n*' is the quantity, in micromoles, of internal standard added.

	Glucosinolate	Response factor
Aliphatic	3MSOP	1.07
	4MSOB	1.07
	5MSOP	1.07
	6MSOH	1.00
	7MSOH	1.00
	8MSOO	1.00
	4MTB	1.00
Indolic	I3M	0.29
	4MOI3M	1.00
	1MOI3M	1.00
	4OHI3M	0.28

Table 3.5 ***Glucosinolate response factors***
The response factors used in this study were taken from the International Standard: Part 1 (1992). Where unknown, the response factor has been set at 1.00.

3.2.9 Determination of flux through sulfate assimilation

The flux through sulfate assimilation was measured as incorporation of ^{35}S from [^{35}S]sulfate to thiols and proteins essentially as described in Chapter 2.2.8. Col-0, *MYB51_ox*, and *myb51* plants were grown for 14 days on vertical MS-phytogel plates. The plants were transferred into 24-well plates containing 2 ml of MS nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with 5.6 µCi [^{35}S]sulfate (Hartmann Analytic) to specific activity of 1,860 kBq (nmol sulfate)$^{-1}$ and incubated in light for 4 hours. After incubation the seedlings were washed 3 times with 2 ml of non-radioactive nutrient solution, carefully blotted with paper tissue, weighed, transferred into 1.5 ml tubes, and frozen in liquid nitrogen. Plant tissue was extracted 1:10 (w/V) in 0.1 M HCl. Ten µl of the extract were added to 1 ml of Optiphase HiSafe3 scintillation cocktail (Perkin Elmer) and the radioactivity was measured in a scintillation counter (Beckmann) to determine sulfate uptake. The incorporation of ^{35}S into thiols and proteins was measured as described in Chapter 2.2.8.

3.3. 3.3 RESULTS

3.3.1. Regulation of ATPS by the glucosinolate MYB factors

To test the hypothesis that the glucosinolate MYB transcription factors have the potential to activate the ATPS genes in Arabidopsis, we used a co-transformation assay developed by Berger *et al*. (2007). Arabidopsis cell cultures were co-transformed with constructs expressing the *uidA* (GUS) reporter gene under control of the four ATPS promoters, and constructs expressing the six MYB transcription factors. Histochemical staining of GUS in the transformed cells revealed activation of the *ATPS1* and *ATPS3* promoters by all six MYB factors (Figure 3.4). However, the strength of the activation was not equal for all the MYB factors. The *ATPS1* promoter was more strongly activated by *MYB28*, *MYB76* and *MYB29*, the aliphatic glucosinolate transcription factors. The opposite was true for the *ATPS3* promoter, which was more strongly activated by *MYB51*, *MYB122* and *MYB34*, the indolic glucosinolate transcription factors. No GUS staining was detected in cells expressing the reporter gene under control of the *ATPS2* or *ATPS4* promoters, indicating that they are not activated by the MYB factors, or that activation is below the detection level of the assay.

The trans-activation assay showed that the six MYB factors are capable of activating the *ATPS1* and *ATPS3* promoters. In order to understand the biological significance of these interactions *in vivo*, we used qPCR to investigate the steady-state mRNA levels of the four ATPS genes in the leaves of transgenic plants constitutively overexpressing the MYB factors (see Table 3.1 for a list of transgenic lines; (Gigolashvili *et al.*, 2007a; 2007b; 2008). In comparison with the wild-type, qPCR analysis of these lines indicated 17x, 110x and 7x higher transcript accumulation of the overexpressed gene in the leaves of MYB28_ox, MYB76_ox, and MYB29_ox plants respectively. MYB51_ox, MYB122_ox, and MYB34_ox lines showed 40-, 8- and 26-fold increases in the relevant transcripts, respectively.

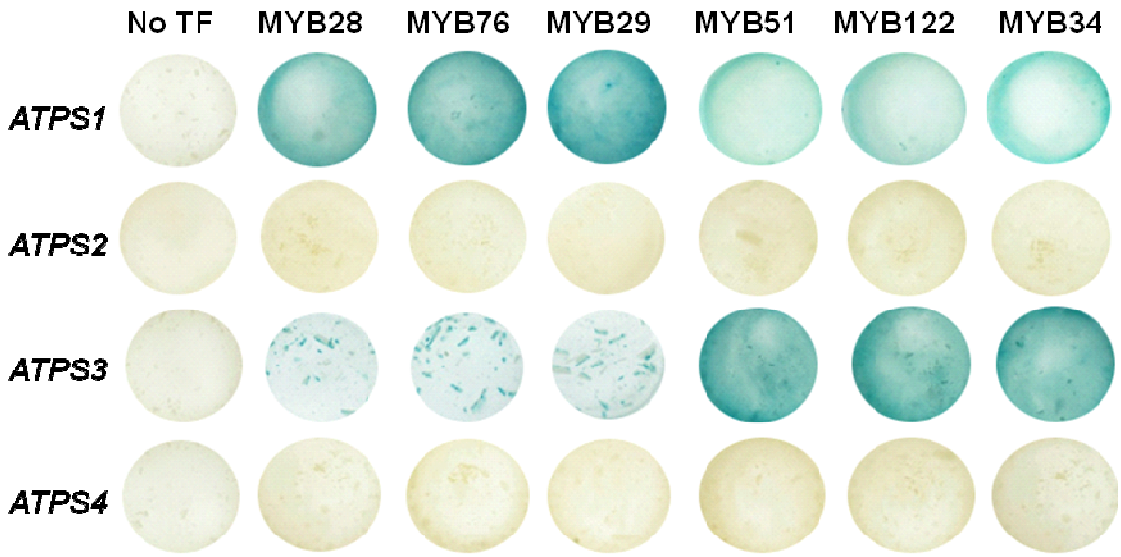


Figure 3.4 ***Transactivation assay of the ATPS promoters with MYB transcription factors.***
The promoters of all four ATPS isoforms were fused to the *uidA* (*GUS*) reporter gene. Cultured *A. thaliana* Col-0 cells were inoculated with the supervirulent *Agrobacterium* strain LBA4404.pBBR1MCS.virGN54D containing either only the reporter construct (No TF) or the reporter construct and, in addition, *Pro35S:MYB* effector constructs. GUS staining indicates *trans*-activation of a promoter by an effector. Constructs were provided to the Flügge laboratory, Cologne, Germany where Ruslan Yatusevich carried out the transactivation assay.

ATPS1 and *ATPS3* transcript levels increased substantially in all six MYB overexpressing lines (Figure 3.5 A + B). However, *ATPS1* transcripts accumulated to higher levels than those of *ATPS3* in transgenic lines overexpressing the aliphatic MYB factors. The opposite was true in lines overexpressing the indolic MYB factors, where a greater increase was seen in *ATPS3* transcript accumulation. This is in agreement with the results of the trans-activation assay, which showed higher activation of the *ATPS1* promoter by the aliphatic MYB factors and the *ATPS3* promoter by the indolic MYB factors. In addition to the accumulation of *ATPS1* and *ATPS3* mRNA, very slight increases were detected in *ATPS2* mRNA levels in MYB76_ox, MYB122_ox and MYB34_ox. A small increase in *ATPS4* transcript accumulation was also detected in the indolic MYB factor overexpression lines, MYB51_ox, MYB122_ox, and MYB34_ox.

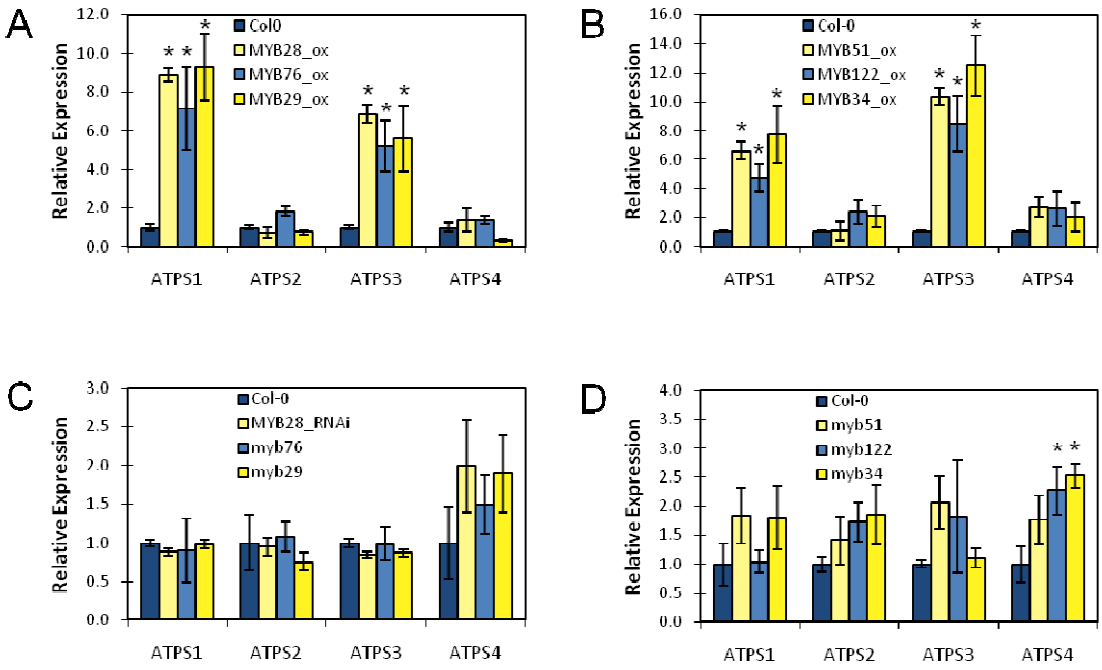


Figure 3.5 ***ATPS transcript levels in MYB transgenic plants.***
Transcript levels of ATPS isoforms in plants **(A)** overexpressing MYB factors involved in the regulation of aliphatic glucosinolates, **(B)** overexpressing MYB factors regulating indolic glucosinolates, **(C)** disrupted in genes of MYB factors involved in the control of aliphatic glucosinolates, and **(D)** disrupted in genes of MYB factors affecting indolic glucosinolates. RNA was isolated from mature leaves and subjected to qPCR with primers specific to the four ATPS isoforms. The Arabidopsis *ACTIN2* gene was used as a reference and the levels in wild-type plants are set to 1. Results are presented as means ± SD from three independent RNA preparations. Values marked with asterisks are significantly different from wild-type plants (Student's t-test; $p \leq 0.05$). qPCR was carried out by Ruslan Yatusevich, Cologne, Germany.

To test whether any of the MYB factors are essential for ATPS expression the ATPS transcript levels were analysed in the leaves of plants in which single MYB factor genes were disrupted (Figure 3.5 C + D). Five T-DNA insertion mutation lines were used: *myb76*, *myb29*, *myb51* (Gigolashvili *et al.*, 2007a; 2008), *myb122*, and *myb34* (Gigolashvili unpublished), and a MYB28-RNAi knock-down line (Gigolashvili *et al.*, 2007b); see Table 3.1 for a list of transgenic lines). No changes in *ATPS1* and *ATPS3* transcript levels were observed in any myb mutants. Similarly, no changes were seen in accumulation of *ATPS2* mRNA. Significant deviation from the wild-type levels was only detected in the levels of *ATPS4* transcript, which were slightly higher in *myb122* and *myb34* than in Col-0.

3.3.2. Effect of the MYB factors on sulfate assimilation

To investigate the effects of the observed changes in ATPS transcript levels on enzyme activity in the MYB factor overexpressing lines and mutants, we measured ATPS activity in the leaves of *MYB28* and *MYB51* gain-of-function and loss-of-function plants (Figure 3.6 A). No difference was detected in the ATPS activity in either MYB28_ox or *myb28* compared with wild-type. However, a significant increase in enzyme activity was detected in both MYB51_ox and *myb51* plants.

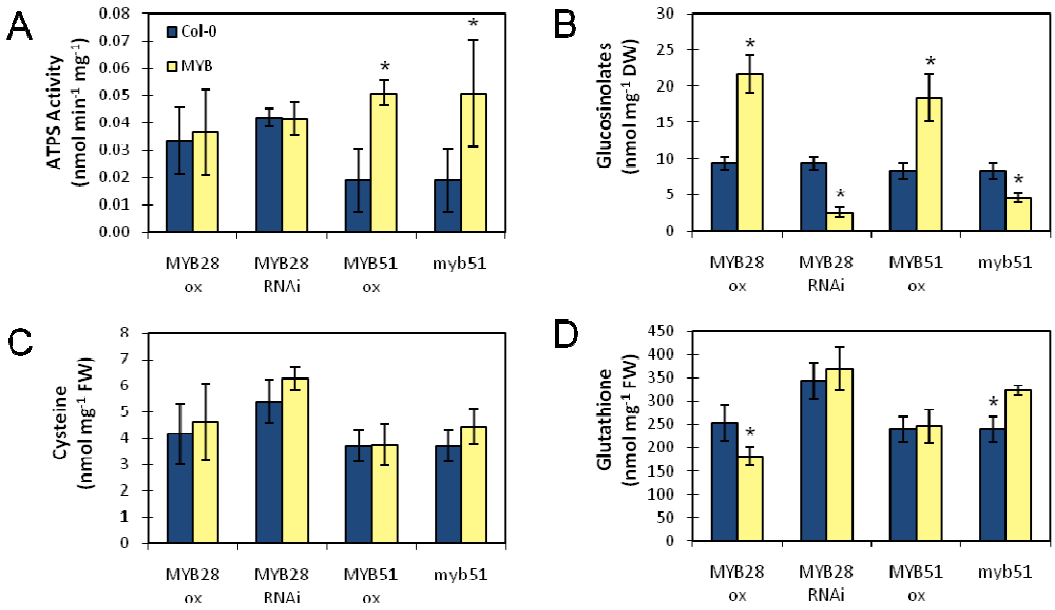


Figure 3.6 ***Enzyme and metabolite analysis in MYB transgenic lines.***
Regulation of **(A)** ATPS enzyme activity, **(B)** glucosinolate accumulation (as reported in Gigolashvili *et al.,* 2007a, b), **(C)** cysteine and **(D)** glutathione accumulation in *A. thaliana* plants overexpressing MYB28 and MYB51 or in plants with reduced expression of these effectors. Results are presented as means ± SD from three independent biological replicates. Values marked with asterisks are significantly (Student's t-test; $p \leq 0.05$) different from wild-type plants.

To understand the effect of these changes in ATPS activity on sulfate assimilation, we investigated thiol levels in the same plants. Gigolashvili *et al*. (2007a, b, 2008) showed that glucosinolate levels in the two overexpressing lines were twice as high as in the wild-type plants, whilst they were half the wild-type level in the mutants (Figure 3.6B). In contrast, the effects of modulation of MYB28 and MYB51 expression on primary assimilation were more subtle. No deviation from the wild-type was detected in cysteine levels in any of the

transgenic lines (Figure 3.6C). However, glutathione levels were altered in two of the lines (Figure 3.6D): In MYB28_ox plants, glutathione levels were decreased, whilst in *myb51* they were increased.

Plants overexpressing or disrupted in the *MYB51* gene showed alterations in ATPS transcript levels and activity. To assess the biological significance of these changes, we determined the flux through the sulfate assimilation pathway in both MYB51_ox and *myb51* plants, by feeding 14-day old seedlings with [^{35}S]sulfate. Uptake of [^{35}S]sulfate was increased in *myb51* plants (Figure 3.7A). The measurements of flux through primary sulfate assimilation identified a small, but significant, increase in the proportion of [^{35}S] sulfate that was incorporated into thiols in MYB51_ox plants. Additionally, a more substantial increase in incorporation into proteins was seen in the same plants (Figure Figure 3.7B + C). Despite the increased uptake in *myb51* plants, the relative incorporation of [^{35}S] into thiols and proteins did not vary significantly from the wild-type.

3.3.3. Disruption of *ATPS* genes affects glucosinolate biosynthesis

We have shown evidence that both the *ATPS1* and *ATPS3* genes are regulated by the six glucosinolate MYB transcription factors. Hence, we can hypothesise that *ATPS1* and *ATPS3* are important for glucosinolate biosynthesis. To test this hypothesis, we analysed the glucosinolate contents of rosette leaves of 5-week old *atps1* and *atps3-1* T-DNA insertion lines. As the trans-activation assay could not detect activation of the *ATPS2* promoter by the MYB factors, and only very moderate changes in transcript level were detected in the MYB mutant lines, we also measured glucosinolates in the *atps2* insertion mutation line for comparison. The analysis of glucosinolates in *atps1* plants revealed that there was indeed a decrease in the total glucosinolate content compared with wild-type plants (Figure 3.8A). This decrease was caused by the decrease in accumulation of aliphatic glucosinolates. Investigation of the individual glucosinolates revealed that three individual

aliphatic glucosinolates, 5-methylsulfinylpentyl-GL (5MSOP), 6-methylsulfinylhexyl-GL (6MSOH), and 4-methylthiobutyl-GL (4MTB), were decreased. However, levels of one indolic glucosinolate, 4-methoxyindol-3-ylmethyl-GL (4MOI3M), were also significantly decreased. In *atps2* plants, no changes were detected in the total levels of either aliphatic or indolic glucosinolates, nor in the levels of individual glucosinolates measured (Figure 3.8B). Surprisingly, no changes were detected in the glucosinolate levels in *atps3* plants either, with the exception of an increase in the indolic glucosinolate, 1-methoxyindol-3-ylmethyl glucosinolate (1MOI3M; Figure 3.8C).

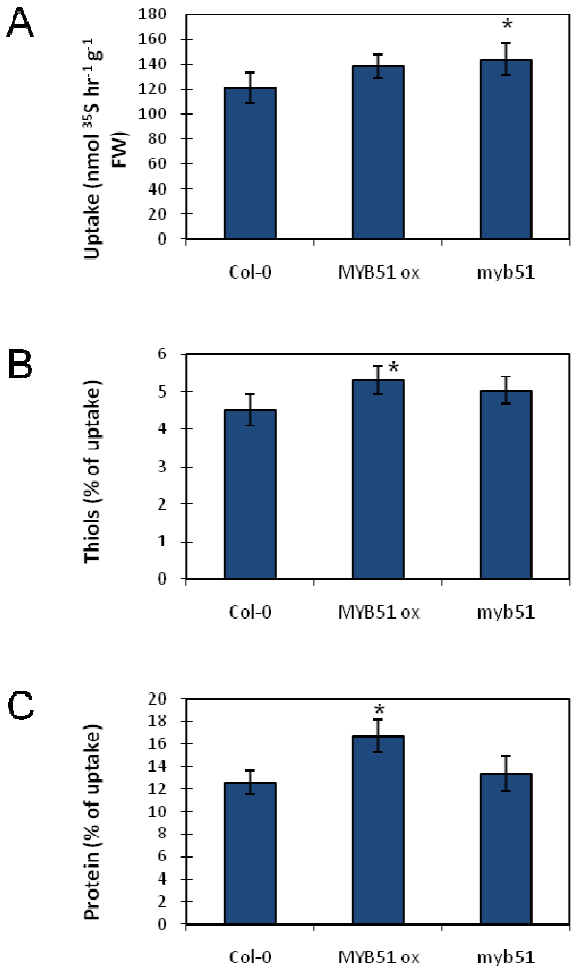


Figure 3.7 ***Flux through sulfate assimilation.***
2-week old seedlings of Col-0, *MYB51_ox*, and *myb51* were incubated for 4 h with 0.2 mM [^{35}S]sulfate. The rate of [^{35}S]sulfate uptake was measured **(A)**, and incorporation into thiols (glutathione and cysteine) **(B)**, and proteins **(C)** was quantified, and calculated as a percentage of the uptake. Data are presented as means ± SD from 3 biological replicates. Values marked with an asterisk are significantly different (Student's t-test; $p<0.05$).

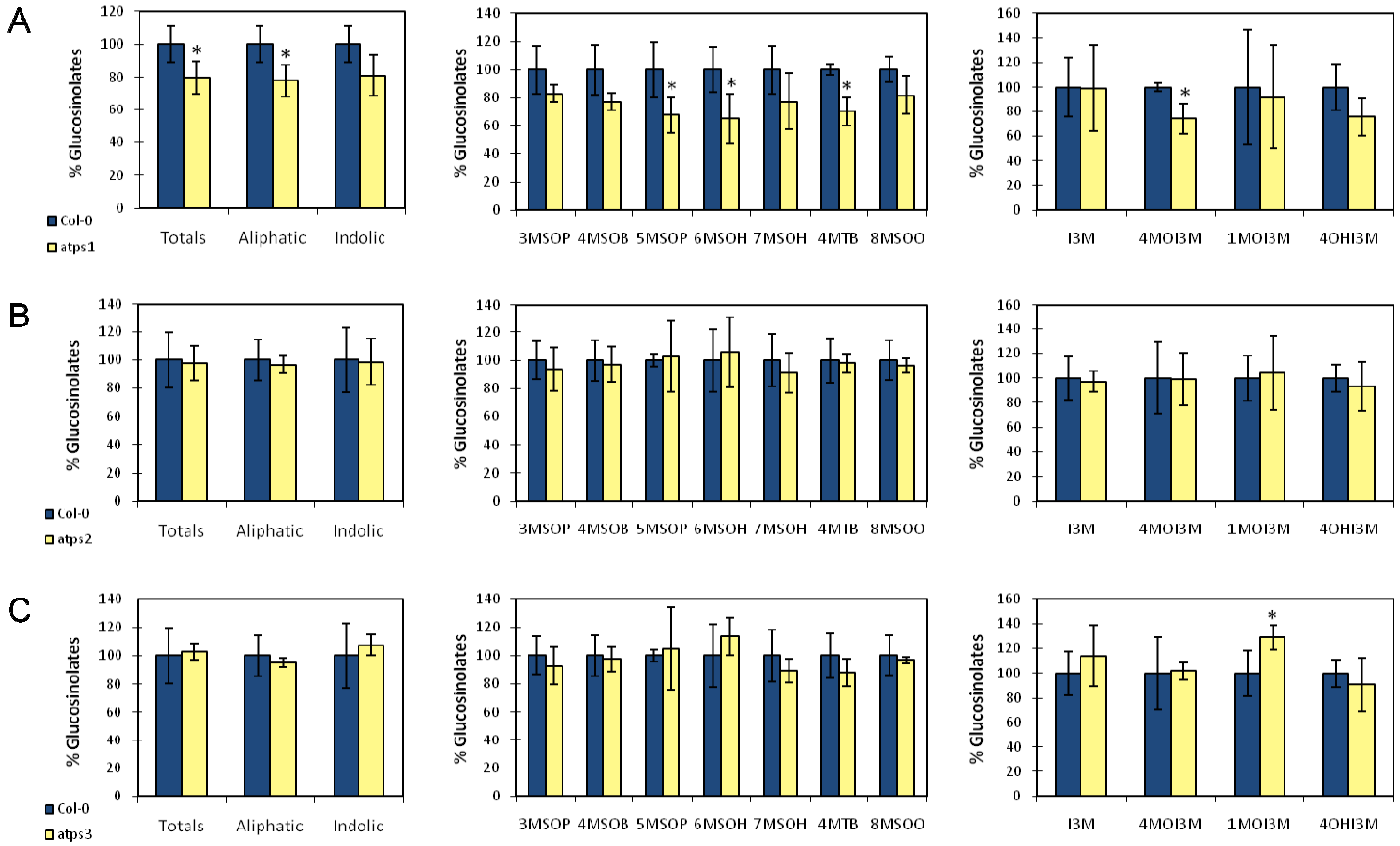


Figure 3.8. ***Glucosinolate measurements in atps1 (A), atps2 (B) and atps3-1 (C) knock-out mutants.***
Glucosinolates were measured in rosette leaves of 5-week old plants. Levels of total, aliphatic, and indolic GLs (left graph) are presented along with the levels of individual aliphatic (middle graph) and indolic GLs (right graph). 3MSOP, 3-methylsulfinylpropyl-GL; 4MSOB, 4-methylsulfinylbutyl-GL; 5MSOP, 5-methylsulfinylpentyl-GL; 6MSOH, 6-methylsulfinylhexyl-GL; 7MSOH, 7-methylsulfinylheptyl-GL; 4MTB, 4-methylthiobutyl-GL; 8MSOO, 8-methylsulfinyloctyl-GL; I3M, indol-3-ylmethyl-GL; 4MOI3M, 4-methoxyindol-3-ylmethyl-GL; 1MOI3M, 1-methoxyindol-3-ylmethyl-GL; 4OHI3M, 4-hydroxy-indol-3-ylmethyl-GL. Data are presented as mean ± SD for three biological replicates. Values marked with an asterisk are significantly different (Student's t-test; $p<0.05$).

3.4. DISCUSSION

3.4.1. Links between ATPS and glucosinolate synthesis

Analysis of available microarray data indicated a strong link between the ATPS genes and glucosinolate biosynthesis (Chapter 2). In this chapter, we revealed that *ATPS1* and *ATPS3* are regulated by the R2R3-type MYB factors controlling glucosinolate biosynthesis, and are thus part of the glucosinolate regulatory network. Co-expression analysis indicated that the expression patterns of the ATPS genes, especially *ATPS1*, *ATPS2*, and *ATPS3*, correlate to those of numerous glucosinolate biosynthesis genes. In particular, *ATPS1* and *ATPS2* are co-expressed with genes of the aliphatic glucosinolate biosynthesis pathway, whilst *ATPS3* expression correlates with multiple gene of the indolic glucosinolate biosynthesis pathway (Supplemental Tables 2.1-2.4). Thus, we hypothesised that the ATPS genes may be regulated together with the glucosinolate genes in response to herbivore and pathogen attack, perhaps with specificity towards either the aliphatic or indolic pathways. In response to various treatments known to affect glucosinolate biosynthesis, *ATPS2* transcript levels decreased, so the link between the response of this isoform and glucosinolate response does not seem to be very strong. In contrast, *ATPS1* and *ATPS3* transcript levels were increased by almost all treatments eliciting a glucosinolate response.

Glucosinolate response to both phloem-feeding and chewing herbivorous insects is well characterised (Mewis *et al.*, 2005; Mewis *et al.*, 2006; Kim and Jander, 2007; Kuśnierczyk *et al.*, 2007). Broad-spectrum anti-fungal defence was also recently attributed to the activation of the indolic glucosinolates by the myrosinase enzyme, PEN2 (Bednarek et al., 2009). The generalist, phloem feeding aphid, *Myzus persicae*, elicits a glucosinolate response that includes up-regulation of both aliphatic and indolic glucosinolates (Mewis *et al.*, 2005; Mewis *et al.*, 2006; Kim and Jander, 2007; Kuśnierczyk *et al.*, 2007). Microarray

analysis revealed an increase in *ATPS1* transcript levels in response to *M. persicae* infestation (

Figure 2.11). In agreement with these results, *ATPS1* and *ATPS3* transcript levels increased in response to mechanical wounding. Mechanical wounding of plant leaves, as a method of mimicking tissue damage caused by chewing insects, is known to cause increased accumulation of both aliphatic and indolic glucosinolates (Mikkelsen et al., 2003).

Infection of Arabidopsis with the oomycete *Phytophthora brassicae* increased 4-methyoxyindol-3-ylmethyl-GL (4MOI3M) whilst levels of its precursor, indole-3-ylmethyl glucosinolate (I3M), decreased (Bednarek et al., 2009; Schlaeppi et al., 2010). In contrast, the necrotrophic fungus, *Botrytis cinerea*, caused a decrease in aliphatic GL biosynthesis genes, including the three aliphatic MYB factors, *MYB28*, *MYB29*, and *MYB76* (Consonni et al., 2010). Thus, glucosinolate response to fungal pathogens most likely differs specific to the species. However, both *Phytophthora infestans*, and *B. cinerea* infection resulted in the same increase in transcript levels of *ATPS1* and *ATPS3*, but surprisingly also *ATPS4*. Although this increase in ATPS mRNA levels could be involved in the glucosinolate response to these pathogens, other defence mechanisms may also require up-regulation of ATPS. For example, the tripeptide, glutathione, is required for wound-induced resistance to *B. cinerea* (Chassot et al., 2008). Moreover, numerous glutathione-S-transferases are induced by this fungal pathogen (Consonni et al., 2010), and a role for glutathione conjugation in anti-fungal defence has been hypothesised (Bednarek et al., 2009). Thus, though the plant defence strategy may differ dependent upon the pathogen, both *P. infestans*, and *B. cinerea* increase sulfur requirement, which appears to be met, at least in part, by increased transcription of *ATPS1*, *ATPS3*, and *ATPS4* genes.

Plant defence is regulated by multiple signal transduction pathways, involving the hormones SA, ET, and jasmonates. Mikkelsen et al. (2003) have shown that the

involvement of these hormones in plant defence extends to the regulation of glucosinolate biosynthesis. The authors revealed that JA causes an up-regulation of glucosinolates, but that JA and ET act synergistically to direct this response to indolic glucosinolates under certain conditions. In contrast, SA plays a role in the inhibition of glucosinolate biosynthesis (Mikkelsen et al., 2003). Perhaps the best characterised hormone regulation of glucosinolate biosynthesis and sulfur metabolism is that of the jasmonate derivative, MeJa (Xiang and Oliver, 1998; Harada *et al.*, 2000; Mikkelsen *et al.*, 2003; Jost *et al.*, 2005; Sasaki-Sekimoto *et al.*, 2005; Gigolashvili *et al.*, 2007a; Gigolashvili *et al.*, 2007b; Gigolashvili *et al.*, 2008). Microarray analyses have also implicated the ATPS genes in the response to MeJa (Jost et al., 2005; Sasaki-Sekimoto et al., 2005). Indeed, we saw increases in transcript levels of *ATPS1* and *ATPS3* following MeJa treatment. The response of ATPS3 was particularly strong, further supporting a preferential involvement with indolic glucosinolate biosynthesis as these seem to be the group that are predominantly induced by MeJa (Jost et al., 2005; Sasaki-Sekimoto et al., 2005). No direct effect of ET alone on glucosinolate accumulation has been detected, although it acts in synergy with JA in the regulation of indolic glucosinolate response (Mikkelsen et al., 2003). In this context, the lack of response in *ATPS1* or *ATPS3* mRNA levels to ET treatment is in keeping with glucosinolate regulation. Considering the negative regulation of glucosinolate biosynthesis by SA, it is surprising to see an increase in *ATPS1* mRNA levels in response to this hormone. However, SA signalling also regulates other pathogen response mechanisms, including the production and metabolism of glutathione (Fodor *et al.*, 1997b; Mou *et al.*, 2003).

The glucosinolate MYB transcription factors were amongst the glucosinolate genes expressed in correlation with the ATPS genes. Moreover, the MYB factors are regulated by the stress treatments described above. Mechanical wounding induces all three aliphatic MYB factors, as well as the indolic regulator, *MYB51* (Gigolashvili *et al.*, 2007a; Gigolashvili

et al., 2007b; Gigolashvili *et al.*, 2008). MeJa, on the other hand, causes activation of two indolic factors, *MYB34* and *MYB122*, and one aliphatic glucosinolate, *MYB29*, and SA down-regulates *MYB28* and *MYB29* expression (Gigolashvili *et al.*, 2007a; Gigolashvili *et al.*, 2007b; Gigolashvili *et al.*, 2008). Thus, the response of the ATPS genes to these treatments may be affected by the MYB factors. Indeed, recent microarray analyses have implicated ATPS in the glucosinolate response, by revealing a potential for activation of some ATPS genes by the MYB factors regulating core glucosinolate biosynthesis (Sønderby *et al.*, 2007; Malitsky *et al.*, 2008).

3.4.2. ATPS is part of the glucosinolate biosynthesis regulatory network

Despite the importance of the sulfate group for glucosinolate function, interactions between glucosinolate biosynthesis and sulfate assimilation have not been investigated, with the exception of a couple of simple plant nutrition studies (Jost *et al.*, 2005; Falk *et al.*, 2007; Schonhof *et al.*, 2007). However, ATPS function has been linked to response to both abiotic and biotic stresses (Heiss et al., 1999; Rausch and Wachter, 2005). Following the exposure of further links between ATPS and glucosinolate biosynthesis from the analysis of available microarray data, we provided direct evidence that two *ATPS* genes, *ATPS1* and *ATPS3*, are regulated differentially by the glucosinolate MYB factors. Both trans-activation assays and the analysis of lines over-expressing the individual MYB factors indicated activation of both *ATPS1* and *ATPS3* by all six MYB factors (Figure 3.4– 5). However, *ATPS1* appeared to be more strongly activated by the aliphatic MYB factors, whilst the indolic factors activated *ATPS3* more strongly. These differences between the activation of ATPS1 and ATPS3 were more pronounced in the trans-activation assay than in transgenic plants, suggesting other unknown factors may modulate the binding of the MYB factors to the ATPS promoters *in planta*. Analysis of *ATPS2* and *ATPS4* transcript levels in the MYB transgenic lines showed minimal changes, and thus could not confirm the involvement of these two genes in glucosinolate biosynthesis. Moreover, the decrease in ATPS2 transcript

levels in response to various environmental stress treatments suggests that ATPS2 does not function in defence response (

Figure 2.11). Recently published work from our group revealed that the second step in the production of PAPS, catalysed by APK, is also up-regulated through the induction of *APK1* and *APK2* transcripts by the group 12 MYBs (Yatusevich et al., 2010). Interestingly, an increased transcription of the three *APR* genes in response to all six MYB factors was also revealed. Therefore, genes involved in PAPS synthesis as well as primary sulfate assimilation are part of the glucosinolate biosynthesis network.

3.4.3. The glucosinolate MYB factors affect primary sulfate assimilation

Activation of sulfate by ATPS is the only step common to both primary and secondary sulfur metabolism, such that APR and APK sit at a branching point in the assimilation pathway. We have shown that changes in the MYB factor transcript levels not only affect glucosinolate accumulation, but also sulfate uptake, reduction, and incorporation into products of primary assimilation (Figure 3.6– 7). Transgenic plants in which *MYB28* was over-expressed or silenced did not appear to be strongly affected in their ability to reduce sulfur, despite the induction of *ATPS* and *APR* transcripts (Figure 3.3.3; Yatusevich *et al.*, 2010). However, a slight decrease in glutathione levels in *35S:MYB28* plants indicates that over-expression of this gene does indeed compromise primary assimilation, and that *MYB28* preferentially channels sulfur toward synthesis of glucosinolates. This is perhaps due to the ability of MYB28 to activate the other two aliphatic glucosinolate MYB factors, *MYB29* and *MYB76* (Gigolashvili *et al.*, 2008), causing cascading activation of glucosinolate biosynthesis genes to the detriment of primary assimilation.

In contrast to *MYB28* transgenic lines, *MYB51* over-expressing plants and *myb51* mutants both showed increased ATPS activity compared with wild-type levels. Increases in APR

activity have also been detected in both transgenic lines (Yatusevich et al., 2010). We describe two mechanisms by which *MYB51* could affect primary sulfur assimilation: Direct regulation by *MYB51*, and indirect regulation due to increased requirement for alternative defence. Although *MYB51* over-expressing plants showed increased flux through sulfate assimilation, consistent with the up-regulation of both ATPS and APR activity, this did not translate into elevated thiol levels. Combined with the increased glucosinolate accumulation in the *MYB51* over-expressing plants, increased flux indicates that MYB51 may up-regulate both primary and secondary sulfur assimilation. Methionine, a product of primary sulfur assimilation, is a precursor for aliphatic glucosinolates (Fahey et al., 2001; Halkier and Gershenzon, 2006). In addition, glutathione is the source of sulfur for synthesis of the core glucosinolate structures. Hence, increased sulfur reduction is necessary for glucosinolate production.

MYB51 is the major MYB factor responsible for regulating the biosynthesis of indolic glucosinolates in rosette leaves (Gigolashvili *et al.*, 2009). A loss-of-function mutation in the *MYB51* gene results in a decrease of both aliphatic and indolic glucosinolates in rosette leaves (Gigolashvili *et al.*, 2007a). Thus, increased sulfate uptake and glutathione accumulation, as well as increased activity of ATPS and APR, in the m*yb51* mutant (Figure 3.3.3; Yatusevich *et al.*, 2010) may illustrate an up-regulation of alternative defence strategies. Indeed, *apk1 apk2* plants that are impaired in their ability to produce sulfated glucosinolates also accumulate glutathione (Mugford et al., 2009).

3.4.4. ATPS1 is important for glucosinolate biosynthesis

We have provided various forms of evidence that *ATPS1* and *ATPS3* can both be activated by the glucosinolate MYB factors and are thus part of the glucosinolate biosynthesis regulatory network. To better understand the contribution of these two to glucosinolate biosynthesis, we investigated the glucosinolate profiles of the *atps1* and *atps3* loss-of-

function mutants. This analysis revealed that *ATPS1* is important for the synthesis of sulfated aliphatic glucosinolates, as *atps1* mutant plants showed a 20% reduction in total glucosinolates. Consistent with the higher involvement of ATPS1 in biosynthesis of aliphatic glucosinolates, this reduction resulted predominantly from a similar decrease in aliphatic glucosinolate levels. As aliphatic glucosinolates are the major group in vegetative rosettes (Brown et al., 2003), this decrease could have a considerable effect on the mutants defence capacity. Interestingly, levels of the indolic glucosinolate 4MOI3M were also reduced in *atps1* plants, confirming that *ATPS1* also has some involvement in indolic glucosinolate production. In contrast to *ATPS1*, the loss of *ATPS3* function in *atps3* mutants surprisingly had no effect on glucosinolate accumulation, revealing a certain degree of functional redundancy. However, this compensation may not be sufficient under stress conditions. Despite co-expression with numerous glucosinolate genes, ATPS2 does not seem to contribute to the production of glucosinolates, a supposition conclusion that is compounded by a wild-type glucosinolate profile in the *atps2* mutant.

3.4.5. Conclusions

ATPS1 and ATPS3 are the main isoforms involved in the provision of activated sulfate for PAPS production, and are regulated by the glucosinolate MYB factors. However, this regulation is unequal revealing a principal role for ATPS1 in aliphatic and ATPS3 in indolic glucosinolate production. However, ATPS1 is the major ATPS isoform involved in glucosinolate biosynthesis and is able to maintain glucosinolate levels in the absence of ATPS3. Thus, ATPS is an integral part of the glucosinolate biosynthesis regulatory network. Moreover, we have shown that the MYB factors not only affect assimilation of sulfur into glucosinolates by up-regulating PAPS production, but also the primary assimilation pathway which provides substrates required in the synthesis of glucosinolates.

Chapter 4

Regulation of Sulfate Assimilation by miR395

4. REGULATION OF SULFATE ASSIMILATION BY MIR395

4.1. INTRODUCTION

Despite the careful study of physiological responses to sulfate starvation in plants, little is understood about the mechanisms of regulation during this environmental stress. Accumulation of OAS is thought to signal demand for sulfur during sulfate deprivation in Arabidopsis, while accumulation of cysteine and glutathione indicate a reduced demand (Kopriva, 2006). Thus far, however, the SLIM1 transcription factor is the only identified central regulator of sulfur response and metabolism, regulating expression of sulfate transporters from several families, as well as other genes involved in sulfate starvation. However, the induction of APR in response to sulfate starvation is not compromised in *slim1* mutants, so that this cannot be the only element involved in regulation of the sulfate assimilation pathway during sulfur limiting conditions (Maruyama-Nakashita *et al.*, 2006).

Recently, another player potentially involved in response to sulfur starvation has been identified, miR395. MiRNAs are a highly conserved class of small non-coding RNAs, involved in post-transcriptional regulation of gene expression (Carrington and Ambros, 2003; Bartel, 2004). MiRNAs negatively regulate transcripts by targeting them for cleavage or translational repression (Reinhart *et al.*, 2002; Carrington and Ambros, 2003; Bartel, 2004). In plants, miRNAs play a well documented role in developmental processes, through regulation of transcription factors. More recently, a number of plant miRNAs were shown to be involved in physiological responses to environmental stresses (Sunkar and Zhu, 2004; Bari *et al.*, 2006; Chiou *et al.*, 2006). Amongst the stress-induced miRNAs identified, a number were involved in the regulation of nutrient acquisition and metabolism: miR399, involved in phosphate deficiency response (Fujii et al., 2005);

miR398, involved in copper homeostasis (Yamasaki et al., 2007; 2009), nitrate-responsive miR393, involved in regulation of root system architecture (Vidal et al., 2010), and miR395, involved in sulfur response (Jones-Rhoades and Bartel, 2004; Kawashima et al., 2009). Computational analysis of the Arabidopsis genome predicted that miR395 targets both the low-affinity sulfate transporter, *SULTR2;1*, and three of the four ATPS genes (Jones-Rhoades and Bartel, 2004). 5' RACE experiments have confirmed that the mRNAs of *ATPS1* and ATPS*4*, and of *SULTR2;1* are indeed targeted for cleavage by miR395 (Jones-Rhoades and Bartel, 2004; Allen *et al.*, 2005; Kawashima *et al.*, 2009). Targeting of *ATPS3* mRNA by miR395 was also predicted, but in 5'-RACE experiments cleavage of *ATPS3* mRNA could not be confirmed in roots, and only in less than 50% of cases in the leaves (Kawashima et al., 2009). Hence, in this study we have considered ATPS3 not to be a target of miR395. The accumulation of miR395 is strongly induced by sulfate starvation, presenting additional evidence for its involvement in the sulfate assimilation regulatory network, or more specifically in regulation of the plants response to sulfur deficiency. However, this induction of miR395 seems counter-intuitive, as ATPS is the first enzyme in a pathway which is in fact up-regulated under sulfur starvation. Therefore, a detailed analysis of the regulation of miR395 and its targets is necessary to understand the contribution of the miRNA to the sulfate starvation response.

Recently, Kawashima et al. (2009) showed that the induction of miR395 following sulfur starvation is dependent upon the SLIM1 transcription factor. In addition, the authors described how the SULTR2;1 target is not simply down-regulated by miR395 in response to sulfur limiting conditions. Whilst transcript levels of *SULTR2;1* change very little in the leaves of sulfur starved plants, in the roots the mRNA accumulation actually increases despite the elevated miR395 levels. Because the cell-specific expression patterns of miR395 and *SULTR2;1* are not identical, the role of miR395 appears to be to limit expression of *SULTR2;1* to the xylem cells (Kawashima et al., 2009). Very little is known

about the role of miR395 in regulating its other targets, *ATPS1* and *ATPS4* and the consequences for ATPS enzyme activity. Here we describe the regulation of the ATPS targets by miR395, examining changes in both mRNA levels, and activity in response to sulfur starvation. We show that miR395 an integral part of the sulfur assimilation regulatory network, using a number of treatments to investigate demand-driven regulation. Using the *slim1-1* mutant, we investigate the interplay between SLIM1 and miR395 during sulfate deficiency.

4.2. MATERIALS AND METHODS

4.2.1. Plant material and growth conditions

In this study, wild-type (wt) *Arabidopsis thaliana* (ecotype Col-0), and the mutants *slim1-1* (Maruyama-Nakashita *et al.*, 2006) and *atps2* (see Chapter 3.2.8) were used. Unless otherwise stated, plants were grown for two weeks on vertical plates with GM-agarose medium (Fujiwara *et al.*, 1992; Table 4.2.1) at 22°C under 16-h-light/8-h-dark cycles. After two weeks, the seedlings were transferred to fresh GM plates (control) or treatment plates for further four days. Sulfur deficient media (S0) was made in accordance with Kawashima et al. (2009), using low EEO agarose with <0.15% sulfate ions (Sigma A5093), and by exchanging all sulfate salts for chlorides (Table 4.2). For the treatment conditions, plants were transferred to GM plates supplemented with 1mM OAS, 1.25mM BSO or 1mM cysteine.

Component	Final conc.
Agar	0.8 % (w/v)
MS Salts[a]	0.43 % (w/v)
MES hydrate	4 mM
Thiamine hydroxide	30 nM
Pyridoxine hydrochloride	40 nM
Nicotinic acid	40 nM

[a] Murashige and Skoog (1962)

Table 4.1 ***Composition of GM media***

4.2.2. HPLC analysis of low molecular weight thiols

See Chapter 2.2.7.

Component	Final conc.
Low EEO Agar	0.9 % (w/v)
MES hydrate	4 mM
Thiamine hydroxide	30 nM
Pyridoxine hydrochloride	40 nM
Nicotinic acid	40 nM
Substitution of MS salts	
Ammonium nitrate	20 mM
Potassium Nitrate	20 mM
Calcium chloride dihydrate	3 mM
Magnesium chloride 6-hydrate (replacing magnesium sulfate)	1.5 mM
Potassium phosphate anhydrous	1.25 mM
Boric acid	100 nM
Potassium iodide	5 nM
Cobalt chloride	0.2 nM
Copper chloride dihydrate (replacing copper sulfate pentahydrate)	0.1 nM
Manganese chloride tetrahydrate (replacing manganese sulfate monohydrate)	100 nM
Molybdic acid sodium salt dihydrate	1 nM
Zinc chloride (replacing zinc sulfate heptahydrate)	30 nM
Iron (III) chloride hexahydrate (replacing ferrous sulfate heptahydrate)	100 nM
EDTA disodium salt dihydrate	100 nM

Table 4.2 ***Nutrient components and concentrations for S0 media.***

4.2.3. RNA isolation and expression analysis

For analysis of expression of sulfate assimilation genes, and *GFP*, total RNA was isolated from leaves and roots, or whole seedlings, respectively, DNAse treated, and reverse-transcribed into cDNA, as described in Chapter 2.2.11. The expression of *ATPS1-4*, *APR1-3*, *SULTR2;1*, and *GFP* genes was analysed by qPCR, using gene-specific primers (for primer sequences see Table 4.3). The Arabidopsis *TIP41* gene was used as a standard for *ATPS1-4* and *SULTR2;1* measurements, whilst the *UBC* gene was used for *APR1-3* measurements. For *GFP* expression analysis, the *TIP41* gene was used as a standard, with the primers TIP41-A Fw and TIP41-A Rv. Relative quantification of expression levels was performed using the comparative Ct method (manufacturer's instructions, bulletin 2, Applied

Biosystems). At least three biologically independent RNA preparations were analyzed with three technical replicates.

Gene	AGI code	Forward primer (5'-3')	Reverse primer (5'-3')
TIP41	At4g34270	gtgaaaactgttggagagaagca	tcaactggataccctttcgc
UBC	At5g25760	ctgcgactcagggaatcttc	ttgtgccattgaattgaacc
ATPS1	At3g22890	cactcggaggtttcatgagag	agacgtagcgagttaaaatgaagag
ATPS2	At1g19920	gatcttgagtgggttcatgtgat	ctcatcttctctcatgaacccttt
ATPS3	At4g14680	tgggtttatgagggaatctgag	gacccatcatcgagattcaac
ATPS4	At5g43780	caaaggtttcatgagacagtcag	gagccggaacgagttaaaatg
SULTR2;1	At5g10180	cagagagttttgaatctctctcacatc	ccatctggatcatgtgtgttg
APR1	At4g04610	cgatcaagtatccgtcgtagaag	ggacaagattcaagaacgaagtc
APR2	At1g62180	aaaagagctccacgggctat	cgacatgagtgaatcaacatctc
APR3	At4g21990	ccaatcaagtatccatcagagaag	ccgaacaagattcaagaaagatg
sGFP	N/A	agtgcttcagccgctaccc	ccctcgaacttcacctcgg
TIP41-A	At4g34270	gaactggctgacaatggagtg	atcaactctcagccaaaatcg

Table 4.3 ***Primer sequences for qPCR expression analysis***

For the detection of miR395, total RNA was extracted from Arabidopsis shoot and root tissues using the TRIZOL reagent (Invitrogen). 15 – 20µg of total RNA was loaded per lane and resolved on a 15% denaturing polyacrylamide gel. The transfer and carbodiimide-mediated cross-linking of RNA to Hybond-NX was performed according to Pall et al. (2007). The membranes were labelled with a DNA oligonucleotide probe complementary to the miR395 sequence - GAGTTCCCCCAAACACTTCAG - that was end-labelled with γ-^{32}P-ATP using T4 polynucleotide kinase (Invitrogen). Blots were hybridised overnight at 37°C in ULTRAhyb-Oligo hybridisation buffer (Ambion), washed two times with 0.2x SSC and 0.1x SDS for 30 min at 37°C and the membranes were exposed to the phosphoimager. Membranes were then stripped in boiling water for 2 min, and exposed for 2 days to ensure all bands had been removed. Following this treatment, the membrane was re-probed with a U6 oligonucleotide: 5'-GCTAATCTTCTCTGTATCGTTCC-3'.

4.2.4. Protein extraction

Leaves and roots were separated and homogenized 1:20 (w/v) in 50 mM Na/K phosphate buffer pH 8 supplemented with 30 mM Na_2SO_3, 0.5 mM 5'-AMP, and 10 mM DTE and the extract was centrifuged for 30 sec at 2,000 rpm to remove cell debris. The protein concentration in the extracts was determined by Bio-Rad protein assay with bovine serum albumin as a standard.

4.2.5. Enzyme assays

APR activity was measured in the supernatants of the crude protein extracts as the production of [^{35}S]sulfite, assayed as acid volatile radioactivity formed from [^{35}S]APS and DTE (Brunold and Suter, 1990):

$$[^{35}S]APS + 2GSH \rightarrow AMP + [^{35}S]SO_3^{2-} + GSSG$$

The reaction assay (Table 4.4) was mixed in a lid-less 1.5 ml Eppendorf tube, and incubated at 37°C for 30 min. The reaction was stopped by the addition of 100 µl 1M Na_2SO_3, and the assay tubes were transferred to 20 ml scintillation vials containing 1 ml 1M triethanolamine (TEA). Scintillation vial lids were quickly closed following addition of 200 µl 1M H_2SO_4 to the assay tubes, which caused the production of radioactive SO_2 (typically 0.02 kBq per reaction), to be absorbed by TEA. This step was incubated overnight. Subsequently, the assay tubes were removed, and their bottoms washed with 200 µl H_2O into the scintillation vial. 3 ml of scintillation cocktail was added to the vials, well mixed, and radioactivity (cpm) was measured in scintillation counter. APR activity (nmol min^{-1} mg^{-1} protein) was calculated as in Equation 4.2.1.

Component	Amount
1 M Tris / HCl pH9	25 µl
2 M $MgSO_4$	100 µl
0.2 M DTE	10 µl
3.75 mM [^{35}S]APS (specific activity 1 kBq / 10 µl)	5 µl
H_2O	100 µl (85 µl)
Extract	10 µl (25 µl)

Table 4.4 ***APR reaction assay composition. Amounts in parentheses are for root samples.***

$$\text{APR activity (nmol min}^{-1}\text{ mg}^{-1}\text{ protein)} \;=\; \frac{37.5 \times cpm}{cpm_{APS} \times c_{prot} \times V_E \times 30}$$

Equation 4.2.1 *Calculation of APR activity. cpm_{APS} is the specific activity of APS, C_{prot} is the protein concentration in the extract (mg/ml), and V_E is the volume of extract used in the assay (ml).*

ATPS activity was determined by a coupled assay based on APS and pyrophosphate-dependent formation of ATP (Cumming et al., 2007), as described in Chapter 3.2.6.

4.2.6. 4.2.6 Creation of GFP constructs and transgenic plants

The chimeric gene construct of the *ATPS1* promoter and *sGFP* for plant transformation was created by Naoko Yoshimoto, RIKEN institute, Japan as follows: Oligonucleotide primers, ATPS1-prom-FXh and ATPS1-prom-RBm (Table 4.5) were used to amplify genomic DNA fragments from the 5'- promoter region, 3020 bp upstream of the translational initiation site, and terminated just after the translational initiation site of *ATPS1*. PCR was performed on genomic DNA prepared from Arabidopsis Col-0 ecotype using KOD plus DNA polymerase (Toyobo). The resultant PCR-amplified fragment was cloned into pCR-Blunt II-TOPO (Invitrogen) and fully sequenced. The XhoI-BamHI fragment of *ATPS1* promoter was inserted between SalI and BamHI site of the promoter-less GFP binary plasmid, pBI-GFP (Figure 4.2.1A; Mugford et al., 2009).

Primer Name	Primer sequence
ATPS1-prom-FXh	ctcgagtaaggatcatcgtaagatttagc
ATPS1-prom-RBm	ggatcccattgttgaaggttttgttag
ATPS1-prom-FBm	ggatcctaaggatcatcgtaagatttagc
ATPS1-CDSnostop-RNco	ccatggacaccggaaccacttctggtagtc

Table 4.5 ***Primer sequences for creation of GFP constructs***

For creation of the *ATPS1*::*ATPS1*:*sGFP* fusion gene construct, oligonucleotide primers ATPS1-prom-FBm and ATPS1-CDSnostop-RNco (Table 4.2.5) were used to amplify DNA fragments starting from the 5'-promoter region, 3020 bp upstream of the translational initiation site, and terminating just before the *ATPS1* translational stop site. PCR was performed on Col-0 genomic DNA using KOD plus DNA polymerase (Toyobo) and the PCR-amplified fragment was cloned into the pCR-Blunt II-TOPO vector (Invitrogen) and fully sequenced. The BamHI-NcoI fragment of *ATPS1* gene and the NcoI-EcoRI fragment of pTH2 (Chiu et al., 1996) containing the fusion gene cassette of the *sGFP(S65T)* and the nopaline synthase terminator (NOSter) were ligated (Figure 4.1A). The resultant BamHI-EcoRI fragment of the *ATPS1::ATPS1:GFP:NOSter* fusion cassette was placed into the position of β-glucuronidase gene and the NOSter in the binary plasmid, pBI101 (Figure 4.1B; Clontech). The resulting binary plasmids were transformed into *Agrobacterium tumefaciens* GV3101 (pMP90; Koncz and Schell, 1986) by the freeze-thaw method (Hoefgen and Willmitzer, 1988). Col-0 plants were transformed according to the floral dip method (Clough and Bent, 1998). Transgenic plants were selected on GM agar media (Valvekens et al., 1988) containing 50 mg L-1 kanamycin sulfate. Kanamycin-resistant T2 progenies were used for the analysis.

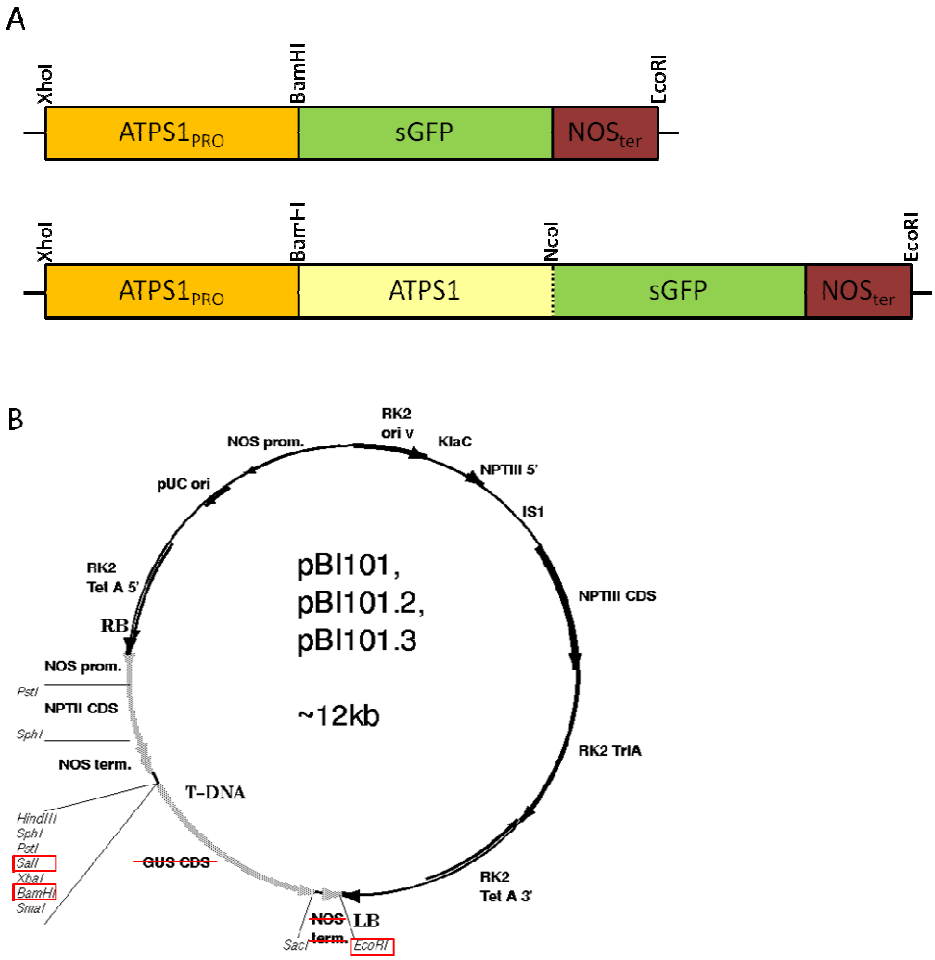


Figure 4.1 Vector constructs
(A) *T-DNA inserts for creation of ATPS$_{PRO}$::GFP (top) and ATPS$_{PRO}$::ATPS:GFP (bottom) plasmids. (B) pBI101 vector map. GUS and NOSterm. (struck through in red) were removed from the original plasmid, and replaced with the GFP fusions. Restriction sites used in the cloning process are highlighted in red.*

4.2.7. Microscopy and imaging of GFP

Fluorescence of ATPS1:GFP or GFP expressed under control of the *ATPS1* promoter was visualised using a Leica MZFLIII stereomicroscope coupled to a Nikon Coolpix 990 camera or a Zeiss LSM 510 confocal microscope with 488 nm/530 nm excitation/emission light for GFP. In preparation for confocal microscopy, seedlings were mounted in 10 µl/ml of propidium iodide to stain the cell walls.

4.2.8. Determination of flux through sulfate assimilation

The flux through sulfate assimilation was measured as incorporation of ^{35}S from [^{35}S] sulfate to thiols and proteins essentially as described in Chapter 2.2.8. Plants were grown on vertical GM plates for 10 days, before transfer to either GM plates (control) or S0 plates (treatment) for a further 4 days. Subsequently, the plants were transferred into 48-well plates, with the roots submerged in 1 ml of GM nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with 5.0 µCi [^{35}S]sulfate (Hartmann Analytic) to specific activity of 1,114 kBq nmol sulfate $^{-1}$ and incubated in light for 4 hours. After the incubation the seedlings were washed 3 times with 1 ml of cold nonradioactive nutrient solution and carefully blotted with paper tissue. Roots and shoots were separated, weighed, transferred into 1.5 ml tubes, and frozen in liquid nitrogen. The plant tissue was extracted 1:10 (w/V) in 0.1 M HCl, and the radioactivity in sulfate, cysteine, glutathione and protein was determined as in Chapter 2.2.8.

4.3. RESULTS

4.3.1. miR395 and ATPS during sulfate deficiency

To better understand the role of miR395 in the regulation of the sulfate assimilation pathway, we dissected the effects of sulfur starvation on the miRNA and its targets. Col-0 seedlings were grown on plates with normal (GM) media for two weeks and then transferred to either GM (control) or no sulfate (S0) media and grown for a further four days. The response of miR395 and its targets to this sulfate starvation treatment were analysed in roots and shoots separately (Figure 4.2).

In order to determine the effect of sulfate deficiency on sulfate assimilation, we carried out HPLC analysis of cysteine and glutathione, two major products of the assimilation pathway. As expected, sulfate deficiency caused significant decreases in both cysteine and glutathione, though this response was mostly limited to the shoots (Figure 4.2A+B). As expected, *APR* transcript levels increased in shoot material and to a lesser degree in root material (Figure 4.3.1C; Hirai *et al.*, 2003; Nikiforova *et al.*, 2003). In the shoots, increases in APR transcript levels did not translate to increases in APR enzyme activity; however, a large increase in activity was measured in the roots in response to sulfate deficiency (Figure 4.2D).

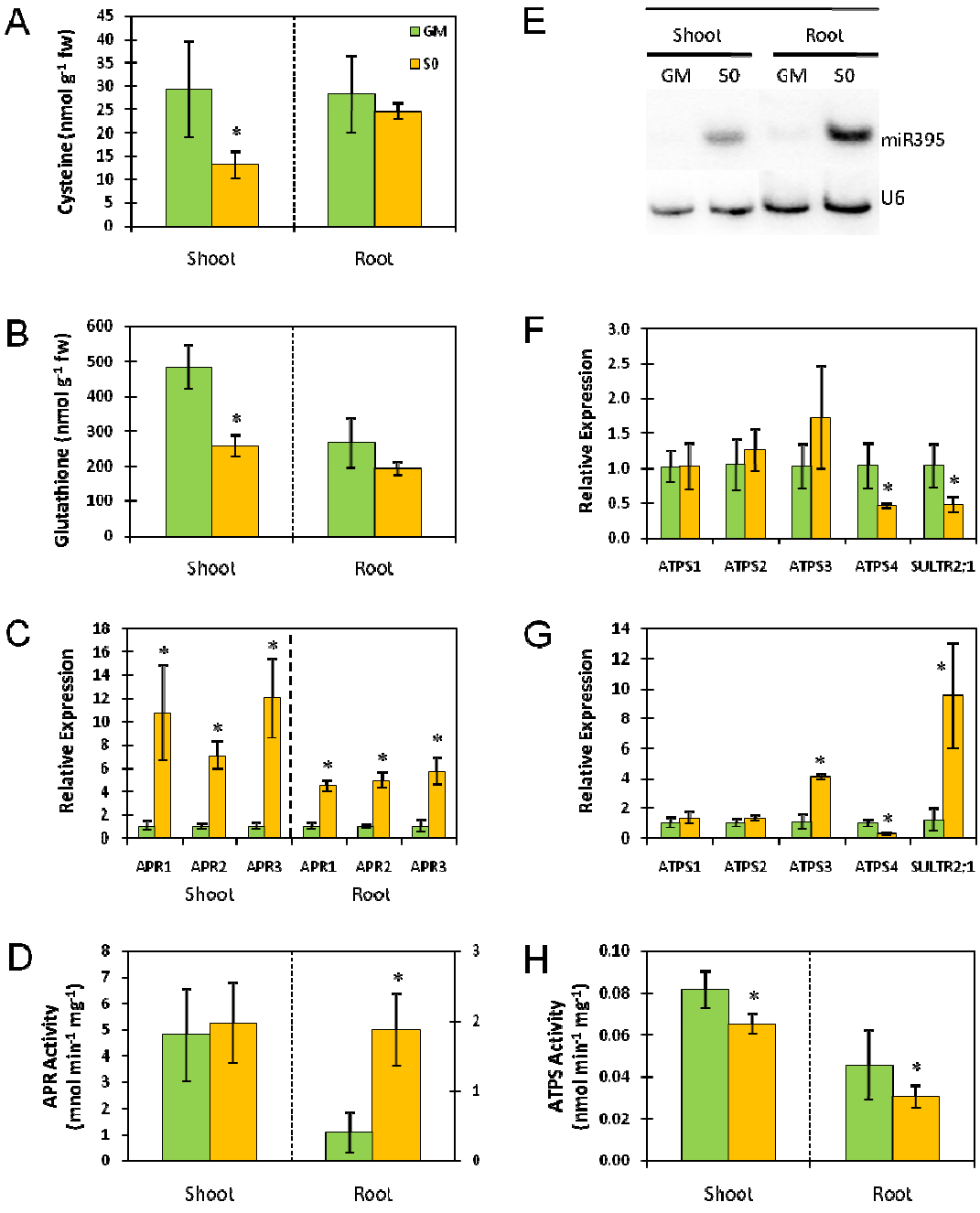


Figure 4.2 ***Effect of sulfate deficiency on miR395 and sulfate assimilation.***
Arabidopsis Col-0 plants were grown on growth media containing 1500 µM sulphate (GM) for two weeks and then transferred to either GM or media lacking a sulfur source (S0) and grown for a further four days. Shoot and root material was harvested separately. Results are presented as means ± SD from at least three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from control plants. **A** Cysteine and **B** glutathione levels were determined by HPLC. **C** RNA was isolated from roots and shoots separately and subjected to quantitative RT-PCR with primers specific to the three *APR* genes. The mRNA levels were compared to *UBC* and the levels in plants grown on GM are set to 1. **D** APR enzyme activity was measured in protein extracts from root and shoot tissue. **E** Northern blot analysis of miR395 carried out by Cintia Kawashima, University of East Anglia, UK. For control, the membranes were stripped and re-probed with a U6-specific probe. Quantitative RT-PCR with primers specific to the four *ATPS* genes and *SULTR2;1* was carried out on the RNA isolated from **F** shoot and **G** root, using T*IP41* as a reference gene. The levels in plants grown on GM are set to 1. **H** ATPS activity was measured in protein extracts from root and shoot tissue of six individual biological replicates.

Northern blot analysis confirmed the induction of miR395 under sulfate deficiency, as has been previously reported (Figure 4.3.1E; Jones-Rhoades and Bartel, 2004; Kawashima *et al.*, 2009). This induction was considerably stronger in the roots than in the leaves. Analysis of ATPS mRNA levels by qPCR revealed a complex relationship between sulfur starvation and ATPS expression patterns. While activation of miR395 is weaker in shoot tissue than that in roots, there is a decrease in the transcript levels of two of the three targets in the shoots (Figure 4.2F): *ATPS4* and *SULTR2;1* transcript levels were reduced under sulfate deficient conditions, presumably in response to increased miR395 levels; however, no changes were detected in *ATPS1* mRNA accumulation. Transcript levels of the non-targeted *ATPS2* and *ATPS3* also remained unaltered. The picture in the roots is quite different (Figure 4.2 G). Transcript levels of *ATPS4* showed a strong down-regulation similar to that in shoots. However, *SULTR2;1* transcript levels increased around 10 fold as was described in Kawashima et al. (2009). As in shoots, *ATPS1* and *ATPS2* transcript levels remained unaltered in root tissue, but *ATPS3* mRNA levels were 4-fold increased. ATPS activity was slightly but significantly reduced in both sulfate starved leaves.

Despite strong induction of miR395, no change was seen in *ATPS1* mRNA levels. This may be due to separate compartmentalisation of the microRNA and its target, as has been shown to be the case for *SULTR2;1* (Kawashima et al., 2009). An alternative hypothesis is that an increase in ATPS transcription counters the cleavage directed by miR395. To test this second hypothesis, we exposed transgenic lines expressing GFP (*ATPS1*$_{PRO}$*::GFP*), or an ATPS1:GFP fusion (*ATPS1*$_{PRO}$*::ATPS1:GFP*), under the control of the *ATPS1* promoter, to sulfate starvation. These transgenic lines were grown, as previously, for two weeks on GM media, before transfer to fresh GM plates (control) or S0 plates for four days. This allowed *in vivo* investigation of *ATPS1* transcriptional regulation both with and without the influence of miR395, as *ATPS1*$_{PRO}$*::GFP* would only detect promoter regulation, but the ATPS1:GFP fusion would be targeted by miR395. At least three independent lines were

analysed by fluorescence microscopy, from both the control and sulfate-starved plants. Two lines from each construct are shown in Figure 4.3. No fluorescence was detected in wild-type plants. In sulfate-starved *ATPS1*$_{PRO}$*::GFP* plants, there was indeed a strong increase in GFP fluorescence, confirming an activation of the ATPS1 promoter under sulfate deficient conditions. However, lines expressing the miR395-targeted ATPS1:GFP fusion under control of the same promoter showed no increase in GFP fluorescence. qPCR analysis of *GFP* transcript levels confirmed that there was indeed a significant increase in GFP transcript in *ATPS1*$_{PRO}$*::GFP* plants; however, *ATPS1*$_{PRO}$*::ATPS1:GFP* plants showed a slight decrease of the *GFP* transcript (Figure 4.3K).

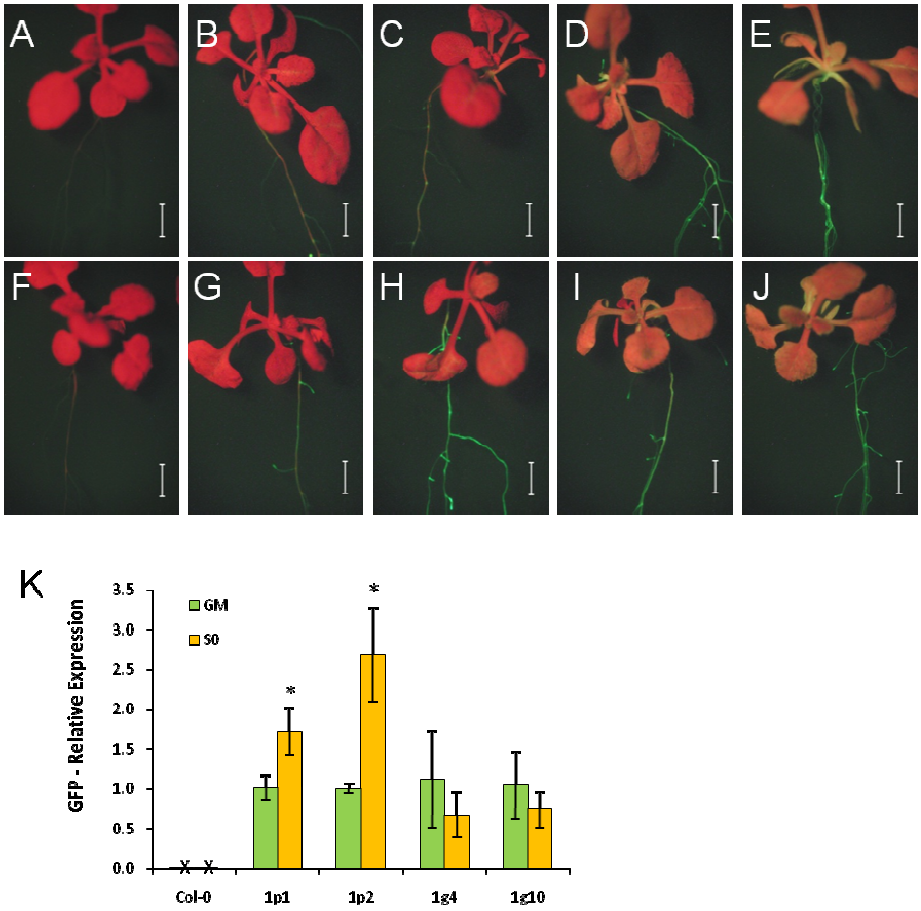


Figure 4.3 ***Sulfate deficiency up-regulates GFP expression driven by the ATPS1 promoter.*** Fluorescence microscopy images of seedlings grown on growth media containing 1500 µM sulfate (GM) for two weeks and then transferred to either GM **(A-E)** or S0 media lacking sulfate **(F-J)** and grown for a further four days. **A,F** Col-0; **B,G** ATPS1::GFP line 1p1; **C,H** ATPS1::GFP line 1p2; **D,I** ATPS1::ATPS1:GFP line 1g4; **E,J** ATPS1::ATPS1:GFP line 1g10. Scale bars = 2 mm **K** RNA was isolated from the transgenic seedlings and subjected to quantitative RT-PCR with primers specific to *GFP*. Transcript levels were compared to *TIP41* and the levels in plants grown on GM was set to 1. No *GFP* was detected in Col-0. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from GM controls.

Further investigation of the *ATPS1*$_{PRO}$*::ATPS1:GFP* lines by confocal microscopy revealed a change in the distribution of GFP in the root tip of the S-starved plants (Figure 4.4). Under control conditions, GFP fluorescence was localised predominantly to the quiescent centre, with some fluorescence extending into the columella and cortex cells. Following sulfate starvation, the localisation pattern was disrupted, as expression of ATPS1:GFP became more evenly distributed throughout all parts of the root tip and meristematic zone.

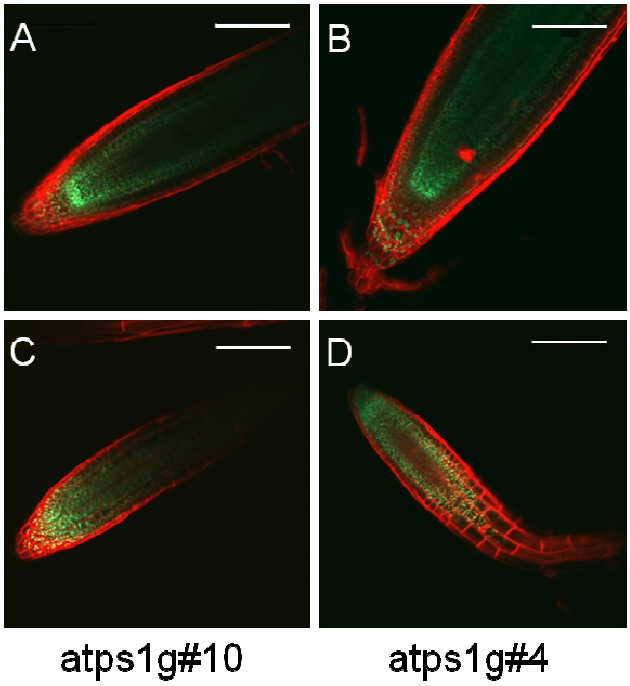


Figure 4.4 ***Sulfate deficiency alters ATPS1 expression patterns in the root tip.***
Confocal microscopy images of seedling root tips from two independent *ATPS1*$_{PRO}$*::ATPS1:GFP* lines grown on growth media containing 1500 µM sulfate (GM) for two weeks and then transferred to either GM **(A+B)** or S0 media lacking sulfate **(C+D)** and grown for a further four days. Scale bars = 100 µm.

ATPS2 is the only ATPS isoform not predicted to be targeted by miR395. As such, it may have a different role during sulfate deficiency and complement the effects of miR395 on the other isoforms. Therefore, we investigated the effect of the four day sulfate starvation on the *atps2* T-DNA insertion mutant line to see whether miR395 regulation of ATPS is affected by the loss of ATPS2 function. Although miR395 was still induced by sulfate starvation in *atps2*, the induction was less pronounced than in wild-type plants (Figure 4.5A). Under control conditions, ATPS activity was lower in the leaves of the *atps2* mutant

than in those of wild-type. However, despite the lower accumulation of miR395 under sulfate starvation, ATPS activity decreased in the leaves of sulfate-starved *atps2* to a similar extent as in WT (Figure 4.5B).

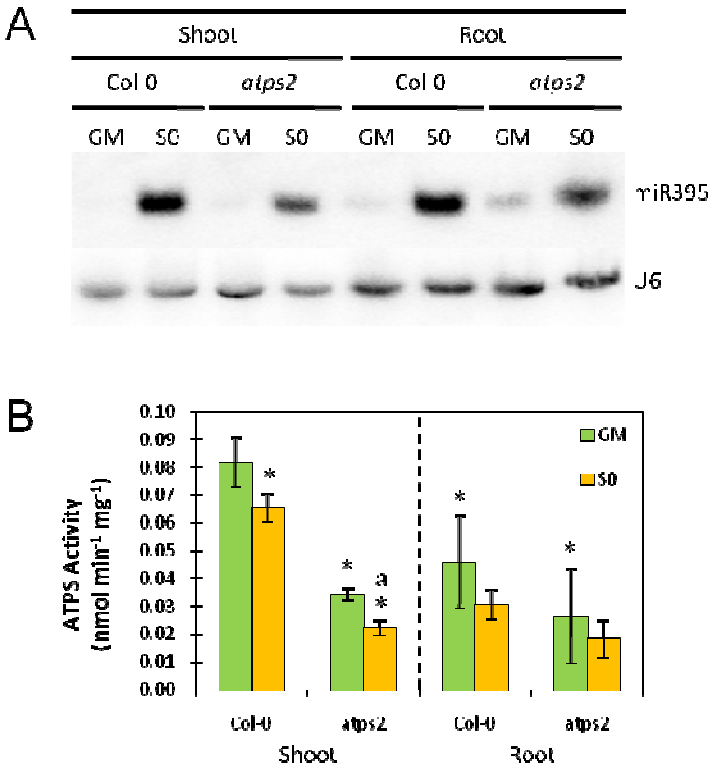


Figure 4.5 ***Analysis of miR395 in the aps2 T-DNA mutant line***
Arabidopsis Col-0 and *atps2* loss-of-function mutant plants were grown on growth media containing 1500 µM sulphate (GM) for two weeks and then transferred to either GM or media lacking a sulfur source (S0) and grown for a further four days. **A** Northern blot analysis of miR395 was carried out by Cintia Kawashima, University of East Anglia, UK on RNA extracted from roots and shoots. For control, the membranes were stripped and re-probed with a U6-specific probe. **B** ATPS activity was measured in protein extracts from root and shoot tissue. Results are presented as means ± SD of at least three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from control plants.

4.3.2. Regulation of miR395 by OAS

Since OAS treatment has been shown to mimic the effects of sulfate deficiency, we tested whether OAS may also be a signal for the up-regulation of miR395. Two week old seedlings were transferred to plates containing either no sulfate, or normal sulfate levels, supplemented with 1mM OAS. Northern blot analysis of miR395 levels indicated that after

two days of treatment, OAS indeed caused an increase in miR395 levels, though this induction was not as strong as that seen under sulfate deficiency (

Figure 4.6). The induction of miR395 by OAS was much stronger in roots than in leaves.

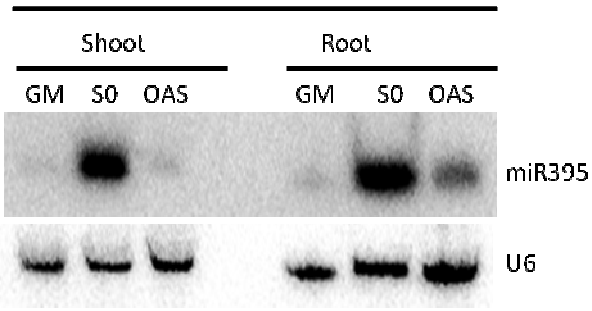


Figure 4.6 ***OAS regulation of miR395.***
Col-0 was grown for two weeks on growth media (GM) before transfer for two days on either standard GM, S0 media lacking sulfate, or GM supplemented with 1 mM *O*-acetylserine (OAS). Northern blot was carried out by Cintia Kawashima, University of East Anglia, UK. RNA was isolated from the roots and shoots, and subjected to northern blot analysis of miR395. Membranes were stripped and re-probed with a U6-specific probe as a control.

For further analysis of miR395 and its targets under the OAS treatment, root and shoot samples were collected after four days of OAS treatment. HPLC analysis of the thiols detected increases in both cysteine and glutathione in the roots, consistent with the up-regulation of the sulfate assimilation pathway by OAS (Figure 4.7A + B). However, no changes in thiol levels were detected in the leaves, suggesting the response is root specific. As expected, transcripts for all three APR isoforms were increased, though again this response was limited to the roots (Figure 4.7C). APR activity in the roots did not change significantly, but surprisingly, a decrease in activity was seen in the leaves following OAS treatment (Figure 4.7D).

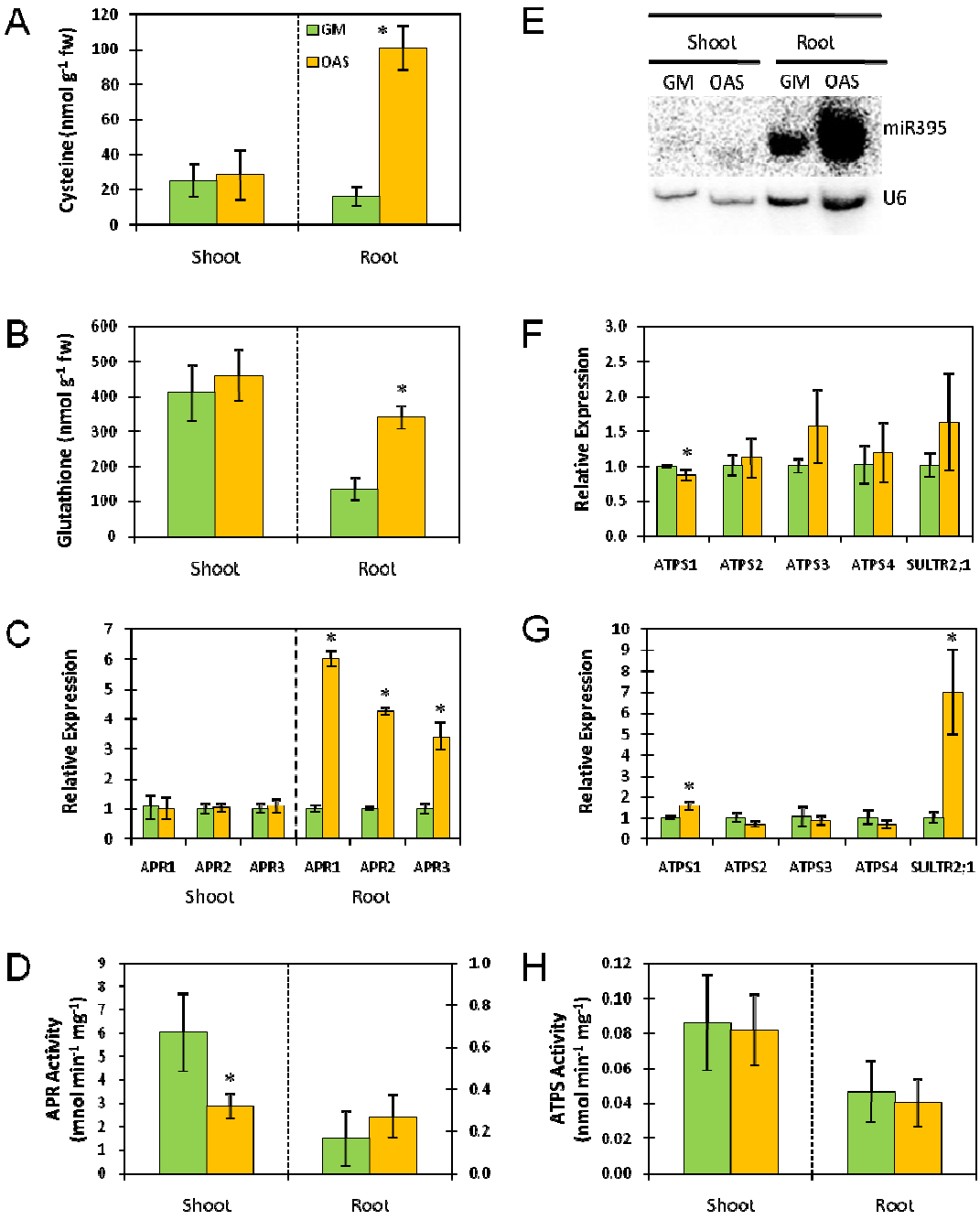


Figure 4.7 ***Analysis of miR395 and its targets following OAS feeding.***
Col-0 was grown for two weeks on growth media (GM) before transfer for four days on either standard GM or GM supplemented with 1 mM *O*-acetylserine (OAS). **A** Cysteine and **B** glutathione levels were determined by HPLC. **C** RNA was isolated from roots and shoots separately and subjected to quantitative RT-PCR with primers specific to the three *APR* genes. The mRNA levels were compared to *UBC* and the levels in plants grown on GM are set to 1. **D** APR enzyme activity was measured in protein extracts from root and shoot tissue. **E** Northern blot analysis of miR395 was carried out by Cintia Kawashima, University of East Anglia, UK. For control, the membranes were stripped and re-probed with a U6-specific probe. Quantitative RT-PCR with primers specific to the four *ATPS* genes and *SULTR2;1* was carried out on the RNA isolated from **F** shoot and **G** root, using *TIP41* as a reference gene. The levels in plants grown on GM are set to 1. **H** ATPS activity was measured in protein extracts from root and shoot tissue of six individual biological replicates. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from control plants.

Northern blot analysis confirmed that miR395 levels, induced by the increase in OAS levels, remained high after four days of treatment (Figure 4.7E). There were few changes in *ATPS* transcript levels in response to OAS in the shoot; except for a slight decrease in the mRNA levels of *ATPS1* (Figure 4.7F). No changes were detected in *SULTR2;1* mRNA levels in the leaves, but in root tissue they were greatly elevated, as under sulfate deficiency (Figure 4.7G). In contrast to shoot material, there was a small but significant increase in *ATPS1* transcript levels in roots, contrary to the expected decrease due to miR395 action. There were no other changes in *ATPS* transcript levels despite the substantial up-regulation of miR395. ATPS activity remained unaltered by the changes in miR395 levels and ATPS transcript levels (Figure 4.7H).

4.3.3. miR395 involvement in demand-driven regulation of sulfate assimilation

Accumulation of compounds containing reduced sulfur, such as glutathione and cysteine, is known to cause a strong decrease in sulfate uptake and assimilation. Glutathione negatively regulates multiple components of the assimilation pathway, including APR and ATPS (Brunold and Schmidt, 1978; Lappartient et al., 1999). On the other hand, low glutathione levels represent a demand for reduced sulfur and the pathway is up-regulated. For example, buthionine sulfoximine (BSO) inhibits the first enzyme of glutathione synthesis, GSH1, causing a depletion of glutathione, and hence a strong up-regulation of sulfate uptake and assimilation (Lappartient *et al.*, 1999; Hartmann *et al.*, 2004). To investigate the effect of glutathione depletion on miR395 and its targets, we exposed two week old seedlings to BSO for four days. Glutathione concentration was decreased both in shoots and roots upon BSO treatment, while cysteine accumulated in the leaves but was not affected in roots (Figure 4.8A + B). Despite significant decreases in glutathione levels, APR transcript accumulation only increased slightly in the roots, and not at all in the leaves (Figure 4.8C).

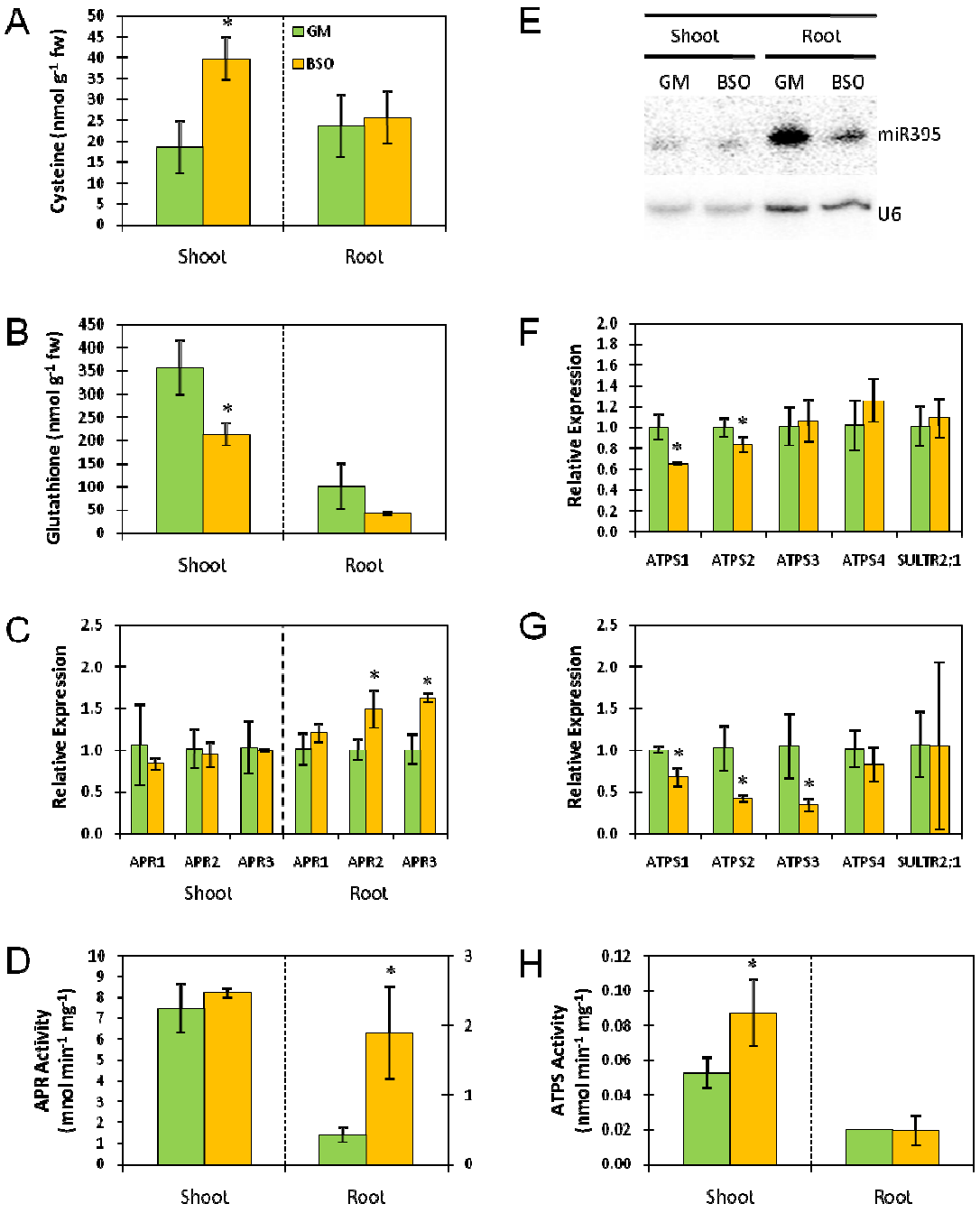


Figure 4.8 ***Analysis of miR395 and its targets following disruption of GSH production.***
After growing for two weeks on growth media (GM), Col-0 seedlings were transferred for a further four days on either standard GM or GM treated with 1.25 mM buthionine sulfoximine (BSO) to inhibit GSH production. **A** Cysteine and **B** glutathione levels were determined by HPLC. **C** RNA was isolated from roots and shoots separately and subjected to quantitative RT-PCR with primers specific to the three *APR* genes. The mRNA levels were compared to *UBC* and the levels in plants grown on GM are set to 1. **D** APR enzyme activity was measured in protein extracts from root and shoot tissue. **E** Northern blot analysis of miR395 was carried out by Cintia Kawashima, University of East Anglia, UK. For control, the membranes were stripped and re-probed with a U6-specific probe. Quantitative RT-PCR with primers specific to the four *ATPS* genes and *SULTR2;1* was carried out on the RNA isolated from **F** shoot and **G** root, using *TIP41* as a reference gene. The levels in plants grown on GM are set to 1. **H** ATPS activity was measured in protein extracts from root and shoot tissue of six individual biological replicates. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from control plants.

Nonetheless, the small increase in transcript levels in the roots led to a substantial increase in APR activity (Figure 4.8D). The BSO treatment did not affect APR activity in the leaves.

While northern blot analysis did not reveal any changes in miR395 levels in leaves in response to BSO treatment, its accumulation was decreased in the roots, consistent with the expected up-regulation of sulfate assimilation (Figure 4.8E). However, despite the decrease in miR395 levels, no corresponding increase in levels of any of the target mRNA was detected. On the contrary, *ATPS1* mRNA levels decreased in both roots and leaves, as did those of the non-targeted *ATPS2* (Figure 4.8F + G). A reduction was also seen in *ATPS3* transcript levels in the leaves. No change was observed in either *ATPS4* or *SULTR2;1* transcripts. Despite the decrease in levels of multiple ATPS transcripts, ATPS activity was higher in the roots of BSO treated plants (Figure 4.8H).

In contrast to the BSO treatment, cysteine represses the sulfate assimilation pathway. Four day exposure of seedlings to external cysteine indeed increased internal cysteine levels in both root and leaf tissue (Figure 4.9A). In addition, glutathione levels doubled in root tissue (Figure 4.9B). In response to these changes in thiols, transcripts of all three APR genes were decreased in both root and leaf (Figure 4.9C). The decreases in transcript levels, however, led to a reduction of APR activity in roots only (Figure 4.9D).

In accord with the reduction of miR395 levels following BSO treatment, northern blot analysis showed an induction of miR395 in both the leaves and roots of plants treated with cysteine (Figure 4.9E). Despite this accumulation of the microRNA, no changes were detected in the transcript levels of any of the targets, nor in the non-targeted *ATPS2*, in either root or shoot material (Figure 4.9F + G). ATPS activity showed no change in either shoot or root tissue of treated plants, in agreement with the lack of response of the mRNA levels (Figure 4.9H).

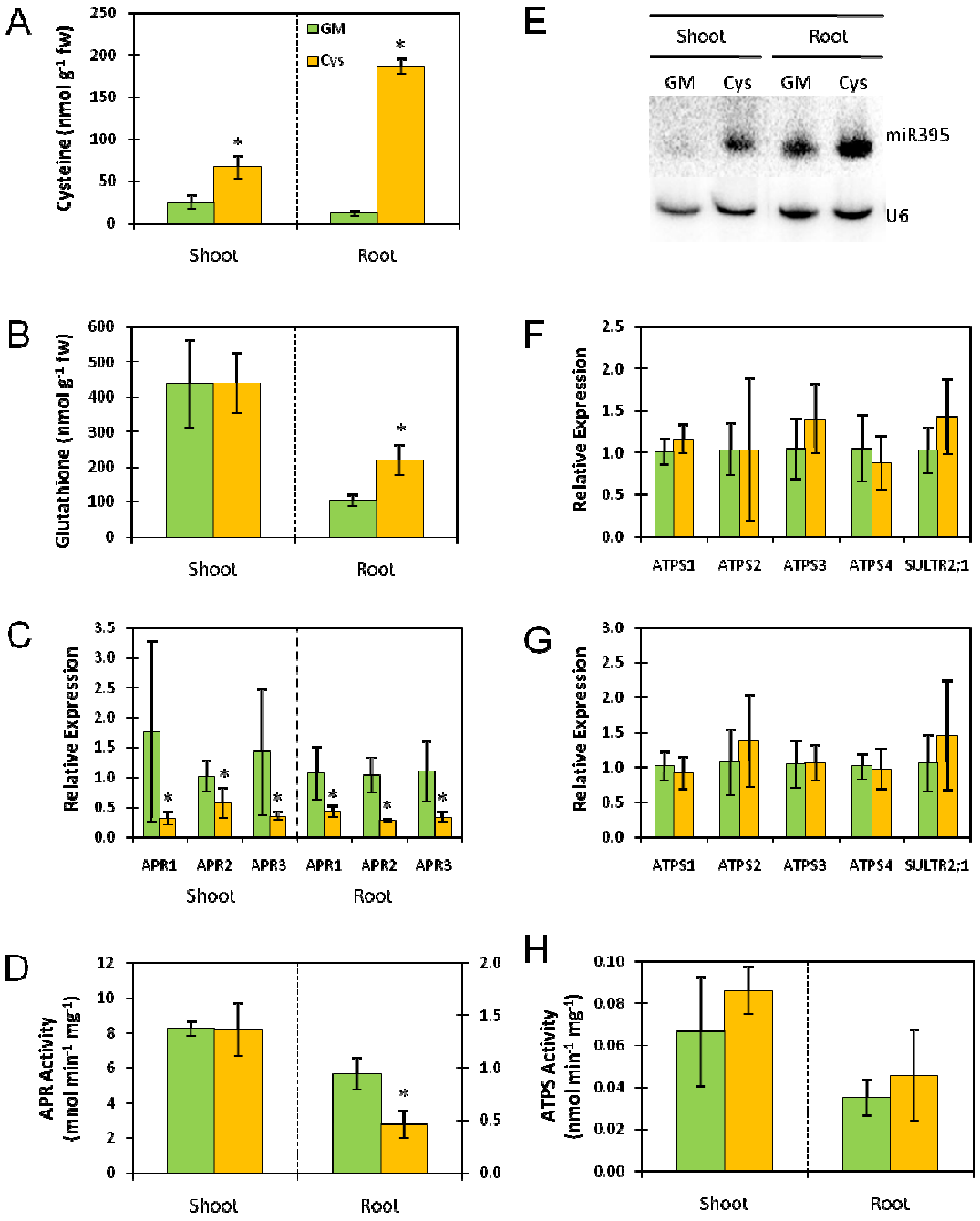


Figure 4.9 ***Analysis of miR395 and its targets following cysteine treatment***
Col-0 seedlings were grown on growth media (GM) for two weeks, before transfer to either GM or GM supplemented with 1mM cysteine, for a further 4 days. **A** Cysteine and **B** glutathione levels were determined by HPLC. **C** RNA was isolated from roots and shoots separately and subjected to quantitative RT-PCR with primers specific to the three *APR* genes. The mRNA levels were compared to *UBC* and the levels in plants grown on GM are set to 1. **D** APR enzyme activity was measured in protein extracts from root and shoot tissue. **E** Northern blot analysis of miR395 was carried out by Cintia Kawashima, University of East Anglia, UK. For control, the membranes were stripped and re-probed with a U6-specific probe. Quantitative RT-PCR with primers specific to the four *ATPS* genes and *SULTR2;1* was carried out on the RNA isolated from **F** shoot and **G** root, using *TIP41* as a reference gene. The levels in plants grown on GM are set to 1. **H** ATPS activity was measured in protein extracts from root and shoot tissue of six individual biological replicates. Results are presented as means ± SD from five independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from wild-type plants.

4.3.4. Contribution of SLIM1 to regulation of miR395 and its targets by sulfur deficiency

Kawashima et al., (2009) showed that the up-regulation of miR395 by sulfate deficiency is greatly diminished in *slim1-1* mutants, suggesting that the SLIM1 transcription factor regulates miR395 in sulfate starvation conditions. Thus, analysis of *slim1-1* mutants should help to reveal the role of miR395 in regulation of ATPS by sulfur starvation, as the miRNA contribution to such regulation will be negligible. Col-0 and *slim1-1* seedlings were exposed to four days of sulfate starvation, as previously described. Root and shoot tissue was collected separately. Northern blot analysis showed a strongly reduced response of miR395 to sulfate starvation in *slim1-1* in agreement with the results of Kawashima et al. (2009; Figure 4.3.9A).

APR transcript accumulation during sulfur starvation is regulated in a SLIM1 independent manner (Maruyama-Nakashita *et al.*, 2006). However, a detailed analysis revealed changes in the response of APR transcript levels and activity to sulfur starvation in *slim1-1* compared to the wild-type. In Col-0, sulfate starvation resulted in an up-regulation, predominantly in the root tissue, of all three APR transcripts. Constitutive levels of APR in the *slim1-1* mutant were higher that the wild-type levels, yet in both roots and leaves of *slim1-1* plants, the APR sulfur-starvation response was much stronger (Figure 4.10B). In the roots of *slim1-1* plants, the control levels of APR transcripts were slightly higher than those of the wild-type, and under sulfate starvation, the APR mRNA levels were up-regulated to higher levels than in the wild-type, though not to the same degree as in the leaves (Figure 4.10C). In agreement with the changes in transcript levels, APR activity in wild-type Col-0 remained the same in shoots, and increased in roots (Figure 4.10D). In contrast, in *slim1-1* APR activity increased four-fold in the leaves, but little change was seen in the roots. The APR activity in *slim1-1* was significantly lower than in the wild-type under control conditions.

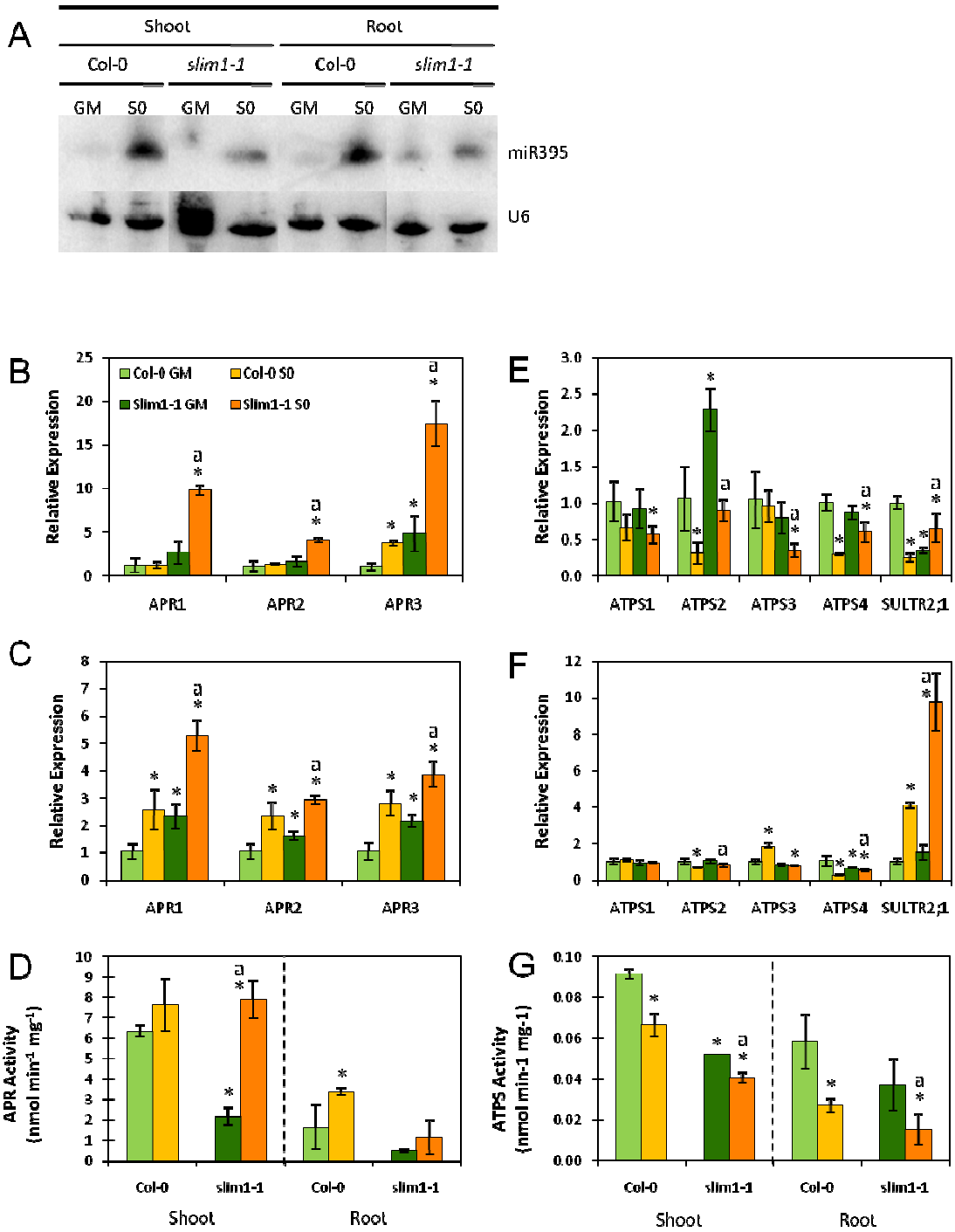


Figure 4.10 ***Regulation of miR395, ATPS, and APR by sulfate starvation in slim1-1 mutant.***
Col-0 and *slim1-1* were grown on growth media containing 1500 µM sulfate (GM) for two weeks and then transferred and grown for a further four days on either GM or media lacking a sulfur source (S0). **A** Northern blot analysis of miR395 was carried out by Siqi Huang, University of East Anglia, UK. The membranes were stripped and re-probed with a U6-specific probe as a control. Transcript levels were measured by quantitative RT-PCR with primers specific to the three *APR* isoforms in RNA isolated from **B** shoots and **C** roots. Transcript levels were compared to *UBC*, and levels in control plants grown on GM set to 1. **D** Regulation of APR enzyme activity in Col-0 and *slim1-1* protein extracts. Quantitative RT-PCR with primers specific to the four *ATPS* genes and *SULTR2;1* was carried out on the RNA isolated from **E** shoot and **F** root. **G** ATPS activity was measured in Col-0 and *slim1-1* protein extracts. Results are presented as means ± SD from three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from Col-0 plants grown on GM media. *Slim1-1* samples marked 'a' are significantly different between plants grown on GM and S0.

Previously it has been shown, that the usual sulfate-starvation transcriptional response of *SULTR2;1* is disrupted in the *slim1* mutant, most likely due to changes in miR395 expression (Kawashima et al., 2009). In agreement with this report, *SULTR2;1* transcript levels were increased in the leaves of sulfate-starved *slim1-1*, compared with the down-regulation observed in wild-type plants (Figure 4.10E). In roots, accumulation of *SULTR2;1* transcript in sulfate deficient *slim1-1* far exceeded that seen in the wild-type (Figure 4.10F). *ATPS1* transcript levels remained similar across both genotypes and treatments, though there was a trend of a slight decrease under sulfate starvation in both wild-type and the mutant. In contrast, regulation of transcript levels of *ATPS3* and *ATPS4* was disrupted in the *slim1-1* mutant. In leaves, sulfate starvation did not elicit a change in *ATPS3* transcription. However, in *slim1-1* a decrease in transcript level was measured. In the roots, the up-regulation of *ATPS3* mRNA levels was not observed in the mutant. While *ATPS4* transcript levels were greatly reduced in both leaves and roots of Col-0 under sulfate starvation, this reduction was strongly attenuated in *slim1-1*. Elevated accumulation of *ATPS2* mRNA was seen in *slim1-1*. Nonetheless, transcript levels decreased in both wild-type and *slim1-1* seedlings in response to sulfate starvation. The response of ATPS activity to sulfate starvation was the same in *slim1-1* as that seen in wild-type plants: A small decrease in activity was detected (Figure 4.10G). However, ATPS activity levels in both leaves and roots were significantly lower in the *slim1-1* mutant than in Col-0 under both control and sulfur-deficient conditions.

Mutants impaired in SLIM1 expression are altered in their response to sulfate starvation (Maruyama-Nakashita *et al.*, 2006). We investigated the levels of thiols in seedlings that had undergone sulfur starvation treatment for four days. Cysteine and glutathione levels were reduced by sulfur deficiency in roots or shoots of both Col-0 and *slim1-1* (Figure Figure 4.11). However, *slim1-1* plants had lower basal levels of cysteine in both the leaves and roots. Glutathione levels were lower in *slim1-1* leaves under normal conditions, but

were maintained at normal levels in the roots. These findings correspond to the results of Maruyama-Nakashita et al. (2006), despite differences in experimental set-up.

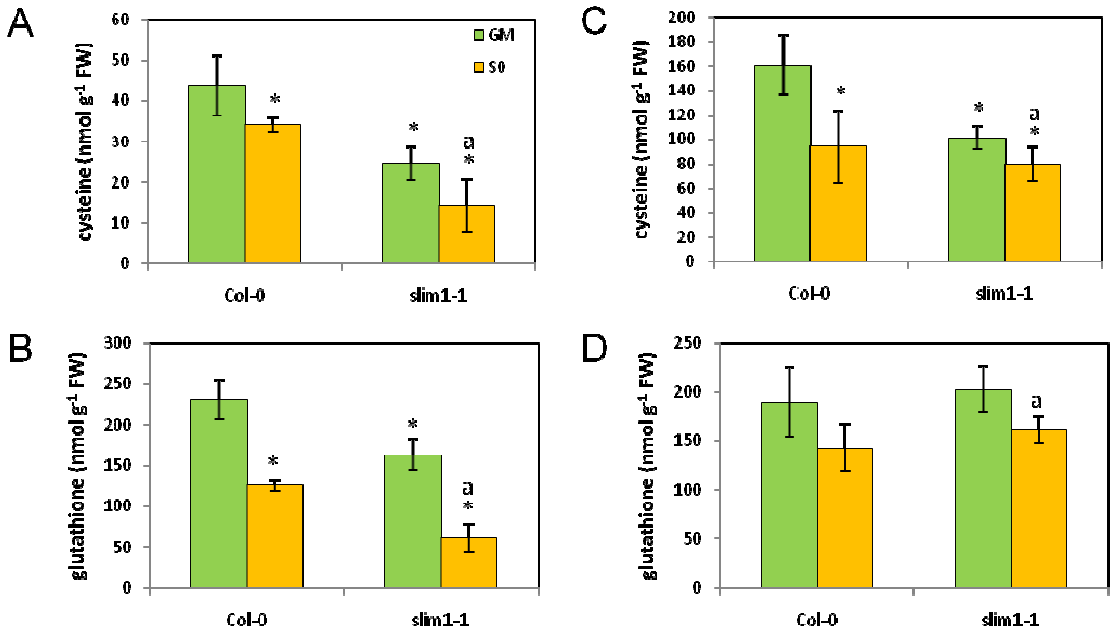


Figure 4.11 ***Levels of thiols in Col-0 and slim1-1 following sulfur deficiency***
Wild-type and *slim1-1* plants were grown on growth media with a sulfate concentration of 1500 µM (GM) for ten days before transfer for a further four days to either GM or sulfur-free media (S0). Cysteine and glutathione levels were determined in shoots **(A-B)** and roots **(C-D)**. The data are presented as means ± SD from three independent biological replicates. Asterisks denote values significantly (Student's t-test; $p \leq 0.05$) different from Col-0 plants grown on GM media. *Slim1-1* samples marked 'a' are significantly different between plants grown on GM and S0.

In order to gain a better insight into the roles that SLIM1 and miR395 play in the control of sulfate assimilation under sulfur limited conditions, we determined the flux of ^{35}S through the sulfate assimilation pathway. Two week old Col-0 and *slim1-1* seedlings underwent four days of sulfur starvation, following which their roots were exposed to [^{35}S]sulfate for four hours. First, we determined the rate of relocation of sulfate from root to shoot (Figure 4.12A). Whilst Col-0 plants exhibited a higher rate of relocation of sulfate to the leaves following sulfur starvation, *slim1-1* plants showed a decrease in sulfate accumulation in the leaves. Next we investigated the flux of ^{35}S through the primary sulfate assimilation pathway into reduced sulfur compounds (proteins, cysteine, and glutathione). In the

leaves of Col-0 plants, flux of sulfate through the pathway was increased by sulfate deficiency, whereas in *slim1-1* sulfur starvation caused no change (Figure 4.12B).

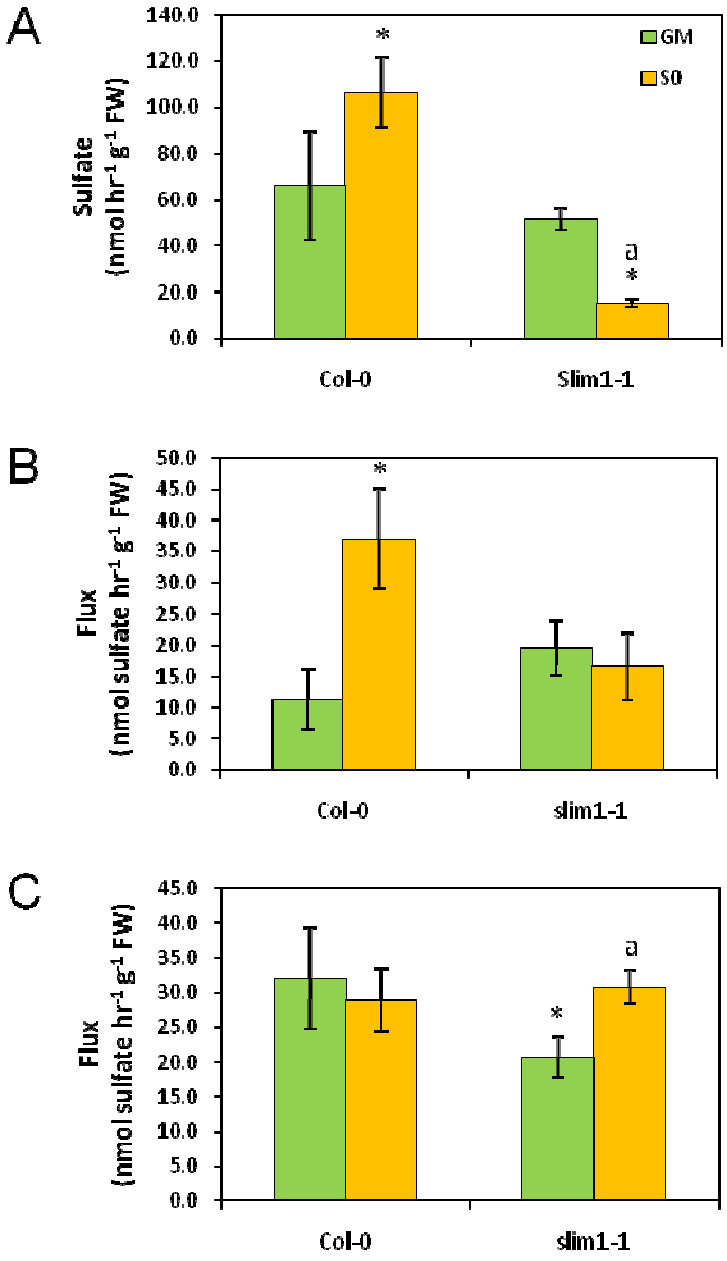


Figure 4.12 ***Measurement of sulfate flux in Col-0 and slim1-1***
Wild-type and *slim1-1* plants were grown on growth media with a sulfate concentration of 1500 µM (GM) for ten days before transfer for a further four days to either GM or sulfur-free media (S0). The seedlings were incubated for four hours with their roots submerged in nutrient solution adjusted to sulfate concentration of 0.2 mM and supplemented with 5.0 µCi [^{35}S]sulfate. Shoot and root material was harvested separately, and the flux was determined as incorporation of ^{35}S from [^{35}S] sulfate to thiols and proteins. **A** Translocation of ^{35}S from roots to shoots. **B** Flux through the pathway in the shoots. **C** Flux through the pathway in the roots. The data are presented as means ± SD from three independent biological replicates. Values marked with an asterisk are significantly (Student's t-test; $p \leq 0.05$) different from Col-0 plants grown on GM media. *Slim1-1* samples marked 'a' are significantly different between plants grown on GM and S0.

No difference in sulfur flux was detected in the roots of sulfur starved Col-0 seedling. However, a slight increase in flux was measured in the roots of *slim1-1* plants, following sulfur starvation (Figure 4.12C). Thus, the relocation of sulfate from root to shoot under sulfur limited conditions appears disrupted in the *slim1-1* mutant, allowing increased assimilation in the roots only.

4.4. DISCUSSION

4.4.1. Induction of miR395 regulates ATPS during sulfur starvation

Jones-Rhoades and Bartel (2004) showed that a decrease in *ATPS1* transcript accompany the induction of miR395 levels following long-term sulfur deficiency. We have revealed that despite the accumulation of miR395 during sulfur starvation, the two ATPS targets are not regulated in the same manner as expected (Figure 4.2): *ATPS4* mRNA levels decrease, whilst levels of *ATPS1* stay the same. An increased accumulation of *ATPS3* transcript was detected, supporting the theory that it may not be targeted. Similar results were reported recently following ten days sulfur starvation experiments by Liang et al. (2010), who recorded a decrease in *ATPS4*, a small increase in *ATPS1*, and a larger increase in *ATPS3* transcript levels. Therefore, it seems that miR395 repression plays a role in various mechanisms to regulate the accumulation of its targets.

Transcript levels of *ATPS4* decrease in both root and shoot tissue during sulfur starvation, consistent with regulation by miR395. As *ATPS4* is predominantly root expressed (Chapter 2), this may act to reduce sulfate activation rates in roots in preference of transport of sulfate to the leaves (Figure 2.8). In contrast to previous studies in which decreases in *ATPS1* transcript levels were observed (Jones-Rhoades and Bartel, 2004; Liang et al., 2010), we did not detect any change in *ATPS1* transcript levels in either leaves or roots in response to sulfur deficiency. It is worth noting that both previous studies report on *ATPS1* response to induction of miR395 following long-term (10 – 14 days) sulfur deficiency treatment on low sulfur media, whereas we used a relatively short-term (4 day) treatment. The extended period of sulfur limitation may be the cause of the additional regulation of *ATPS1* transcript accumulation detected in these studies. In line with our results, down-regulation of *ATPS4* transcription, but not of *ATPS1* transcription, has consistently been shown in microarray analyses of sulfur starvation response (Hirai *et al.*,

2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003; 2006). Analysis of *ATPS1*$_{PRO}$*::GFP* lines revealed that increased promoter activity enhances *ATPS1* transcription, thus counteracting the cleavage by miR395 (Figure 4.3). The proposed antagonistic regulation of *ATPS1* seems over-complicated for simple maintenance of transcript levels. As transcript levels were measured in whole roots or shoots, we hypothesised that the role of this complex regulatory mechanism may be to alter the tissue- or cell-specific expression of *ATPS1*. Indeed, we saw differences in the root tip expression pattern of ATPS1:GFP under control of the *ATPS1* promoter (Figure 4.4). This is in keeping with the function of many characterised miRNAs which orchestrate specific alterations in target expression patterning (for examples see Kidner and Martienssen, 2004; Parry *et al.*, 2009; Marin *et al.*, 2010).

Due to lack of direct evidence confirming miR395-directed cleavage of *ATPS3* transcripts, we have considered this gene as non-targeted. During sulfur deficiency, *ATPS3* mRNA levels increase, positively correlating to the induction of miR395 contrary to the pattern that might be expected if ATPS3 were a target of miR395 (Figure 4.3.1; Liang *et al.*, 2010). However, similar positive temporal correlation of the target mRNA and miRNA levels has been observed previously, for example between miR171 and *SCARECROW* (Llave et al., 2002), between miR159 and *MYB33* (Achard et al., 2004), and between miR395 and *SULTR2;1* (Kawashima et al., 2009). Recent analysis revealed repression of *ATPS3* transcript accumulation in plants constitutively over-expressing miR395 under the 35S promoter (Liang et al., 2010). An almost complete spatial separation of *ATPS3* and miR395 expression could explain the low cleavage of the transcript detected (Kawashima et al., 2009). Constitutive over-expression of miR395 could cause the expression of the miRNA in the same tissue as *ATPS3*, with this contact leading to its repression by transcript cleavage. Thus, the status of *ATPS3* as a target of miR395 remains to be substantiated.

ATPS2 is the only ATPS isoform that was not predicted to be targeted by miR395. Consistent with this, transcript levels of *ATPS2* are not affected by sulfate deficiency in either roots or leaves in Figure 4.2. Thus, ATPS2 may function to maintain a basal level of ATPS activity during periods of sulfur deprivation. In support of this hypothesis, we observed nearly half the wild-type levels of ATPS activity in seedlings of the *atps2* loss-of-function mutant, suggesting that the contribution of ATPS2 to total activity is considerable (Figure 4.5). Therefore, preservation of ATPS2 levels might buffer the effect of changes in the other ATPS isoforms. Induction of miR395 was not as strong in *atps2* plants as in the wild-type, suggesting that there may be negative feedback regulation of miR395 (Figure 4.5). Both cysteine and glutathione are known to be involved in feedback regulation of sulfate assimilation, particularly affecting APR activity (Kopriva, 2006). However, accumulation of these thiols actually increased in *atps2* plants indicating that in this case a different mechanism must be involved (Chapter 2, Figure 2.9). As SLIM1 induces miR395 expression during sulfur deficiency (Kawashima et al., 2009), the reduced response of miR395 in *atps2* plants may be due to alterations in the level of this transcription factor.

Contrary to the demand-driven regulation of sulfate assimilation that has been previously reported (Reuveny *et al.*, 1980; Lappartient *et al.*, 1999), ATPS activity decreases slightly in both roots and leaves (Figure 4.2). The effect of substantial decreases in *ATPS4* mRNA levels are presumably somewhat compensated by the increased *ATPS3* mRNA levels. Plants respond to sulfur limitation by increasing sulfate uptake and reduction capacity through induced transcription of high affinity sulfate transporters and APR (Reuveny *et al.*, 1980; Smith, 1980; Takahashi *et al.*, 1997; Hirai *et al.*, 2003; Maruyama-Nakashita *et al.*, 2003; Nikiforova *et al.*, 2003; 2006). Therefore, as ATPS catalyses the first step of sulfate assimilation, down-regulation of ATPS would limit the potential assimilation rate. The evidence we have presented indicates that regulation of ATPS during sulfur limitation

functions to alter the distribution of the four ATPS isoforms and redistribute ATPS activity in a tissue-specific manner.

4.4.2. SLIM1 and miR395 have distinct functions in sulfur starvation response

Discovery of SLIM1 marked the first identified transcription factor in the regulation of plant sulfur response (Maruyama-Nakashita *et al.*, 2006). Its central role expands to the up-regulation of miR395 under sulfur limitation (Kawashima *et al.*, 2009; Figure 4.3.9). The analyses presented in this chapter illustrate that SLIM1 exerts two levels of regulation on the ATPS genes; by direct transcriptional regulation and through induction of miR395. In addition, we suggest that this SLIM1/miR395 mediated regulation is responsible for remobilisation of sulfate to the shoots during sulfur deficiency.

Microarray analysis of *slim1* mutants following sulfur starvation revealed a loss of *ATPS4* regulation (Maruyama-Nakashita *et al.*, 2006). We confirmed an attenuation of the *ATPS4* transcriptional response, attributed to reduced miR395 levels. The fact that *ATPS1* transcript levels were not altered by sulfur deficiency in the *slim1-1* mutants as in wild-type reveals that SLIM1 is the most likely candidate for activation of the *ATPS1* promoter in response to sulfur limitation. Regulation of miR395 is interrupted in the *slim1-1* mutant (Kawashima et al., 2009), allowing a de-repression of *ATPS1* which would result in raised accumulation of transcripts. However, *ATPS1* transcript levels were not increased in the mutant, conflicting with the promoter activation identified and leading us to hypothesise that SLIM1 itself is responsible for increased transcription. We also revealed that SLIM1 regulates *ATPS3* through comparison of *ATPS3* transcriptional response to sulfur deficiency in wild-type and *slim1-1* plants. The wild-type up-regulation of *ATPS3* transcript levels following sulfur deficiency is absent in *slim1-1* roots. Maruyama-Nakashita et al. (2006) showed that GFP expression under control of the *SLIM1* promoter is stronger in

Arabidopsis roots than in shoots, explaining the root specific response of *ATPS3* to sulfur deficiency. In Figure 4.10, the analysis of Col-0 under sulfur deficiency revealed a down-regulation of *ATPS2* transcript levels in both shoots and roots that was not detected in similar analyses displayed in Figure 4.2. This may be the result of an otherwise undetected stress to the plant or fluctuation in light conditions, as this isoform is light responsive (Chapter 2); however, all other responses were similar to the initial analysis and the effect appears limited to *ATPS2* alone. Interestingly, *ATPS2* transcript accumulation is considerably higher in *slim1-1* plants, indicating a de-repression of this gene. *ATPS2* mRNA is not targeted by miR395, thus in wild-type plants, SLIM1 itself may be responsible for the repression of ATPS2.

Despite regulation of multiple aspects of plant sulfur response by SLIM1, the key enzyme of primary assimilation, APR is not regulated by this transcription factor (Maruyama-Nakashita *et al.*, 2006). However, we detected higher accumulation of APR transcripts in the *slim1-1* mutant, as well as increased sensitivity to sulfur starvation. *Slim1* mutants are unable to accumulate wild-type levels of cysteine and glutathione, most likely due to a decrease in sulfate uptake (Figure 4.2.10; Maruyama-Nakashita *et al.*, 2006). Hence, the increase in *APR* transcript levels might be due to this decline in thiols rather than the direct action of SLIM1. APR activity, on the other hand, is greatly reduced in the *slim1-1* mutant, though in leaves activity is restored to wild-type levels during sulfur starvation. This uncoupling of APR transcript accumulation and activity is similar to that reported in response to salt and hormone treatments (Koprivova et al., 2008). Together with the analysis of ATPS regulation by miR395 and SLIM1, this SLIM1-independent regulation of APR indicates the involvement of further, as yet unidentified, factors in the regulation of sulfur starvation response.

Response to sulfur starvation is disrupted in the *slim1-1* mutant, including regulation of miR395, ATPS, and multiple other genes involved in sulfate assimilation (Figure 4.3.9; Maruyama-Nakashita *et al.*, 2006; Kawashima *et al.*, 2009). As SLIM1 has such a central role in sulfur-starvation response, the regulation of flux through sulfate assimilation might be altered in the *slim1-1* mutant (Figure 4.12). APR contributes a major portion of the control of flux in sulfate assimilation (Vauclare *et al.*, 2002; Scheerer *et al.*, 2010), but is not regulated by SLIM1 (Maruyama-Nakashita *et al.*, 2006). APR activity was induced by sulfur starvation in both Col-0 and *slim1-1*. Therefore, flux could be expected to increase in *slim1-1* plants also. Indeed, flux was increased in both genotypes, but interestingly in a tissue-specific manner. In Col-0 increased flux occurred in leaves, whereas in *slim1-1* mutants a smaller increase was in the roots. Complementary to the tissue specific changes in sulfate reduction rates during sulfur starvation, translocation of sulfate appears to be disrupted in the *slim1-1* mutant. At normal sulfur supply, the same amount of sulfate was transported from the roots to the shoots in both Col-0 and *slim1-1*. However, differences were seen following sulfur starvation, with a large increase in transport to the shoots in Col-0, but a significant decrease in *slim1-1*. Thus, it seems that SLIM1 is not only responsible for increased sulfate uptake in the roots of sulfur deficient plants, but also the internal re-distribution of sulfate from roots to shoots. *SULTR2;1* is hypothesised to be restricted to the xylem parenchyma by the action of miR395 following sulfur deficiency (Kawashima et al., 2009), which is consistent with a role in the translocation of sulfate to the leaves. Recently, further analysis of miR395 revealed that it regulates the translocation of sulfate between leaves by targeting *SULTR2;1* (Liang et al., 2010). Therefore, we propose that increased translocation of sulfate to the leaves during sulfur starvation is mediated by SLIM1 through the action of miR395. An alternative hypothesis might be that root-to-shoot transport is reduced in *slim1-1* mutants due to an inability to induce the vacuolar exporters, *SULTR4;1* and *SULTR4;2* (Kataoka *et al.*, 2004b; Maruyama-Nakashita *et al.*, 2006).

4.4.3. miR395 is involved in general regulation of sulfate assimilation

Our data clearly show that miR395 functions beyond sulfur starvation response, and is an integral part of the regulatory circuits of the sulfate assimilation pathway (Figure 4.7 – 10). Sulfur limitation is the most highly studied environmental condition regulating sulfur assimilation. However, the pathway is also regulated by other environmental stresses and sulfur metabolites (Davidian and Kopriva, 2010). Accumulation of the cysteine precursor, OAS, indicates a demand for reduced sulfur, and the pathway has been shown to be regulated accordingly. Consistent with the ability of OAS to mimic sulfur deficiency (Hirai *et al.*, 2003; 2005), the response of miR395 to OAS treatment was similar to that seen in sulfur-starved plants. miR395 accumulated in root tissues, although the induction was not as was not as strong as in response to sulfur deficiency (

Figure 4.6). In agreement with results from Hirai et al. (2003), treatment with OAS resulted in higher cysteine and glutathione levels in the roots only (Figure 4.7). The root specificity of the OAS response, including accumulation of APR transcripts, as well as induction of miR395, may indicate a limited ability of the plant to transport OAS to the shoots. Despite induction of miR395 by OAS treatment, regulation of the targets differed from that seen in response to sulfur starvation. As miR395 levels are not increased in the leaves, the lack of target response is intuitive. However, the increased miR395 in the roots fails to down-regulate *ATPS4* expression, whilst *ATPS1* and *SULTR2;1* mRNA levels both increased. These responses can be attributed to the lower accumulation of miR395 compared with the sulfur starvation response. Interestingly, *ATPS3* transcript levels are not up-regulated by OAS treatment, or any further treatments used in this study (Figure 4.7– 9), demonstrating that this is a sulfur starvation response, and is not dependent upon demand for reduced sulfur. In agreement with the reduced effect on transcript levels,

overall ATPS activity levels are not altered by OAS application. This does not exclude the possibility that the localisation of that activity is altered in response to OAS.

Inhibition of glutathione biosynthesis by BSO results in an up-regulation of sulfate uptake and assimilation due to the increased demand for reduced sulfur (Lappartient *et al.*, 1999; Hartmann *et al.*, 2004). Conversely, external application of glutathione provides negative regulation of ATPS activity (Lappartient et al., 1999). Feedback inhibition of APR activity was also observed following feeding with cysteine and glutathione (Vauclare et al., 2002). We dissected the effects of glutathione and cysteine on miR395 regulation to reveal that miR395 functions in this demand-driven regulation of sulfur assimilation (Figure 4.8– 9), except in response to sulfur deficiency. BSO and cysteine treatments caused repression and induction of miR395, respectively, in agreement with the concept of demand-driven regulation of sulfur assimilation. However, both treatments resulted in uncoupling of the ATPS target responses from the miR395 regulation: BSO caused decreases in mRNA levels of *ATPS1* and *ATPS3*, and the non-targeted *ATPS2*, whereas no transcriptional changes were observed following cysteine application. Surprisingly, despite the decreased transcript levels following BSO treatment, ATPS activity was increased, providing evidence of post-translational regulation of the ATPS enzyme. A similar uncoupling of APR activity and transcript levels has been described in response to salt stress (Koprivova et al., 2008).

Comparison of the changes in thiol accumulation and the response of miR395 identified a positive correlation between glutathione and miR395 levels in the four treatments investigated (Figure 4.13). Therefore, we propose that miR395 is part of the demand-driven regulation of sulfate assimilation, signalled by glutathione through an unknown mechanism. The response to sulfur starvation appears to be an exception, as decreases in glutathione coincide with induction of miR395. Presumably, SLIM1-regulation of miR395 is able to over-ride the glutathione-dependent regulation. The Arabidopsis genome

encodes six MIR395 loci, *MIR395a – f* producing two mature miR395 species (Kawashima et al., 2009). The expression patterns of the six genes differ in the degree and localisation of their activation by sulfur deprivation (Kawashima et al., 2009). Thus, variation in the expression response of these individual *MIR395* genes to glutathione and SLIM1 may result in the contrasting regulation revealed.

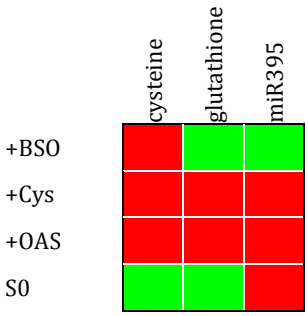


Figure 4.13 Heat map
Heat map showing regulation of the thiols, cysteine and glutathione, and miR395 regulation under the four treatments used. Treatments are listed on the left and the component measured is indicated across the top. Red indicates an increase in levels compared with no treatment controls. Green indicates a decrease.

4.4.4. MicroRNA regulation of plant nutrition

We have shown that miR395 is a central component in the regulation of sulfate assimilation, and plays a critical role in partitioning of sulfate between the shoots and roots. The initial targets identified for miRNAs were transcription factors involved in developmental processes (Llave et al., 2002; Rhoades et al., 2002); however, miRNA species involved in response to environmental stresses, including nutrient stress, were identified shortly after (Jones-Rhoades and Bartel, 2004; Sunkar and Zhu, 2004; Fujii *et al.*, 2005; Ruiz-Ferrer and Voinnet, 2009). Pant et al. (2008) identified as many as 20 miRNAs that respond to nitrogen or phosphate deficiency. To date, the two most notable examples of nutrient responsive miRNAs are miR399 which is induced by phosphate starvation (Fujii et al., 2005), and miR398 which acts during copper deficiency (Yamasaki et al., 2007; 2009). Many of the nutrient responsive miRNAs identified, including miR395, have been

detected in the phloem sap of *Brassica napus*, indicating a role in long distance signalling of nutrient status (Buhtz et al., 2010).

The phosphate starvation induced miR399 is the best characterised nutrient responsive miRNA (Fujii *et al.*, 2005; Aung *et al.*, 2006; Chiou *et al.*, 2006; Franco-Zorrilla *et al.*, 2007; Pant *et al.*, 2008). During phosphate deficiency, miR399 is induced by PHOSPHATE STARVATION RESPONSE 1 (PHR1), a MYB transcription factor responsible for regulating a subset of phosphate responsive genes (Rubio *et al.*, 2001; Franco-Zorrilla *et al.*, 2004; Bari *et al.*, 2006). miR399 represses the *PHO2* (*UBC24*) gene encoding an ubiquitin-conjugating E2 enzyme 24 (Aung et al., 2006), resulting in increased phosphate uptake and translocation to the shoots as well as remobilisation of phosphate between old and young leaves (Figure 4.4.2; Chiou *et al.*, 2006). Thus, miR399 functions as a long-distance signal of leaf phosphate status through its transport to root tissues in the phloem (Lin *et al.*, 2008; Pant *et al.*, 2008; Buhtz *et al.*, 2010). Similar to the up-regulation of miR399 by PHR1 during phosphate deficiency, miR395 is induced by the SLIM1 transcription factor in response to sulfate starvation (Figure 4.4.2; Kawashima *et al.*, 2009). During sulfur limitation, SLIM1 increases sulfate uptake, whilst APR is induced by an unknown SLIM1-independent mechanism (Maruyama-Nakashita *et al.*, 2006), resulting in increased flux through sulfate assimilation in the shoots. In addition, *SULTR2;1* expression is limited to the xylem parenchyma cells through the action of SLIM1 induced miR395, resulting in increased translocation of sulfate to the shoots. The synergistic actions of SLIM1 and miR395 maintain *ATPS* transcript levels at an optimal level to meet the increased sulfate reduction rate. Shoot-to-root transport in the phloem is essential for the function of miR399 (Pant *et al.*, 2008; Buhtz *et al.*, 2010), and accordingly, miR399 has only been identified in vascular plants (http://www.mirbase.org). Although miR395 has also been detected in the phloem sap of *Brassica napus* (Buhtz et al., 2010) the role of this phloem expression is not clear. Similarly, the origin of miR395 function in regulation of shoot-to-

root sulfate translocation is unclear considering miR395 is not only conserved in vascular plants, but also in the moss, *P. patens*, (Fattash et al., 2007). Therefore its original function may have been the regulation of sulfate assimilation, and a role in long-distance signalling may have evolved as a secondary function.

4.4.5. Conclusions

In this chapter we have discussed the regulation of ATPS at multiple levels – transcriptional regulation by SLIM1 and other unknown factors, post-transcriptional regulation by miR395, and possibly post-translational regulation. This complex regulation causing subtle changes in ATPS activity is consistent with the pivotal role of the enzyme. Sulfate acquired from soil through the roots is uploaded into the xylem, and then moves to apoplastic continuum and symplast, which brings sulfate to sink organs or tissues. Reduction occurs in plastids, or sulfate is stored in vacuoles (Buchner et al., 2004). As sulfate is the major form in which sulfur is transported around the plant, the site of its activation is critical for the provision of reduced sulfur compounds, and secondary sulfur metabolites, to the tissues that require them. We have demonstrated a role for miR395 and SLIM1 in limiting expression of ATPS and SULTR2;1 to specific tissues, and the importance of this regulation for remobilisation and allocation of sulfate. Therefore, I propose that specificity of tissue, cell, and potentially also compartmental expression of ATPS is not only important for its function, but is also fundamental to regulation of sulfate assimilation.

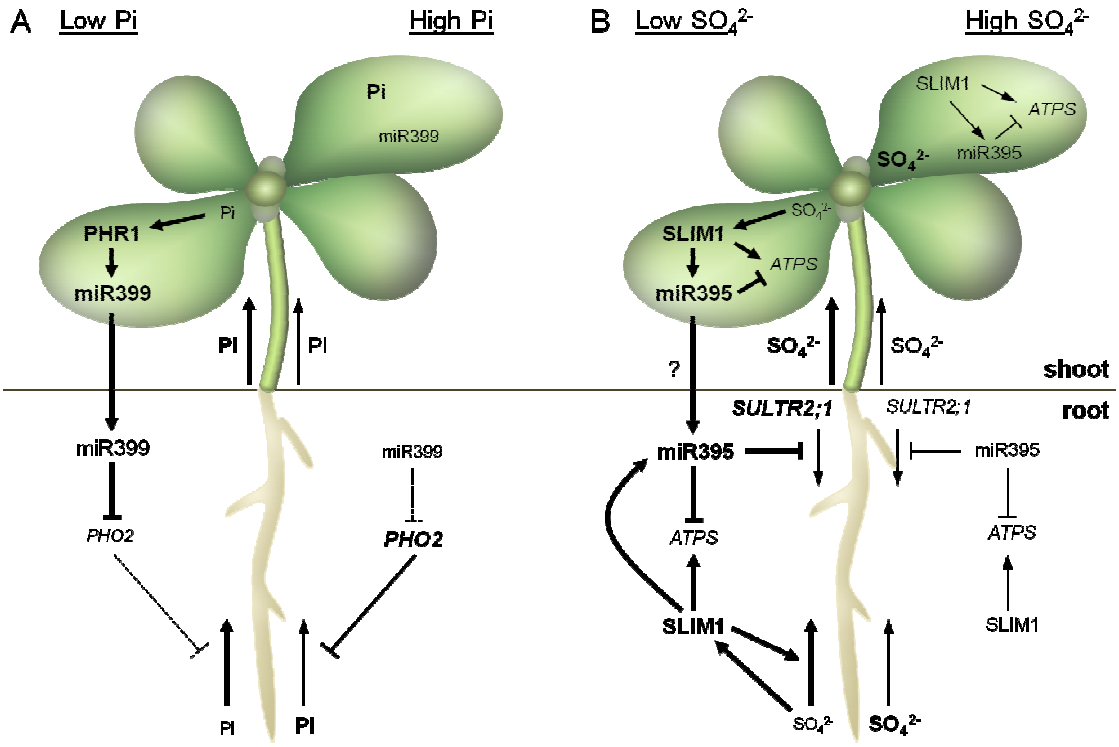


Figure 4.14 ***Comparison of miR395 and miR399 function***

A Under normal phosphate (Pi) supply PHO2 inhibits phosphate uptake. Phosphate deficiency in shoots activates PHR1 which induces miR399 expression. The miRNA is then transported to roots where it inhibits PHO2. This relieves the inhibition of phosphate uptake and phosphate is translocated to the shoots. **B** Under normal sulfate (SO_4^{2-}) supply SLIM1 and miR395 maintain levels of *ATPS1* and *ATPS4* transcripts for optimal assimilation of sulfate into organic sulfur-compounds. Sulfur limitation activates SLIM1 which directly induces root sulfate transporters to increase sulfate uptake. *SULTR2;1* is induced by sulfur limitation independently of SLIM1. SLIM1 induces accumulation of miR395 which limits expression of *SULTR2;1* to xylem parenchyma, thus enhancing sulfate translocation to the shoots and inhibiting shoot-to-root transport in phloem. miR395 inhibits *ATPS4* transcript levels and together with SLIM1 regulates ATPS to allow increased flux through the sulfate assimilation pathway achieved by SLIM1-independent induction of APR. The phloem transport of miR395 is indicated by arrow and its unknown function by a question mark.

Chapter 5

General Discussion

5. GENERAL DISCUSSION

The aim of the work presented in this thesis was to address key questions surrounding the roles and regulation of sulfate activation by ATPS in higher plants. Bioinformatic, biochemical, and molecular techniques were applied in the model species, Arabidopsis, to investigate isoform specificity and regulation of the four ATPS isoforms and to search for clues to the role of cytosolic ATPS activity. This section reviews to what extent these aims have been achieved and how the findings advance our understanding of sulfate assimilation in vascular plants.

5.1. Importance of cytosolic ATPS

The subcellular compartmentalization of the sulfate assimilation pathway is somewhat of an enigma (Figure 5.1). In Arabidopsis, while sulfate reduction occurs exclusively in plastids, recent efforts to understand the role of compartment-specific isoforms of SAT and OASTL have revealed that mitochondria are the major site of OAS synthesis (Haas et al., 2008), but that most cysteine is produced in the cytosol (Heeg et al., 2008). Similarly, while γ-EC is formed exclusively in plastids, glutathione can be produced in both plastids and the cytosol (Wachter et al., 2005). This separation of different steps of the pathway indicates a substantial amount of transport of intermediates between compartments. Adding a further level of complexity, PAPS can be produced in both the cytosol and plastids as ATPS and APK are present in both compartments (Rotte and Leustek, 2000; Mugford *et al.*, 2009). Although experimental evidence is still lacking to confirm which ATPS isoform is responsible for cytosolic activity, the ATPS2 isoform is favoured for the role (Rotte and Leustek, 2000). Cytosolic ATPS activity is considered to play a role in PAPS production for sulfation of secondary metabolism since APK is present in both plastids and

the cytosol (Mugford et al., 2009) and since sulfotransferases, e.g. SOT16, SOT17 and SOT18, which transfer sulfate from PAPS to desulfoglucosinolates, are restricted in their expression to the cytosol (Klein *et al.*, 2006). However, none of the analyses presented in this thesis uncovered a link between ATPS2 and secondary metabolism, suggesting that either ATPS2 is not dually-targeted as hypothesised or that the cytosolic ATPS has other unknown functions. Indeed, the importance of cytosolic PAPS production is questionable, since mutants lacking cytosolic APK were able to maintain wild-type levels of sulfated glucosinolates (Mugford et al., 2009). Therefore, PAPS must be readily transported from plastids to the cytosol. Consistent with these results, cytosolic ATPS activity is not essential in the absence of stress, considering none of the four ATPS loss-of-function mutants exhibited obvious biological phenotypes. As cytosolic PAPS production does not appear to be a crucial process in Arabidopsis and cytosolic ATPS activity is the result of dual targeting of one (or more) of four isoforms, it may prove difficult to dissect the role of cytosolic ATPS activity. It is, however, possible that redundancy of cytosolic sulfate activation in Arabidopsis is species specific and may not arise in plant species whose genomes encode just two ATPS isoforms, clearly targeted to the plastid and cytosol. Therefore, concentrating further efforts to understand the role of cytosolic ATPS activity on such species might help to provide some clarification.

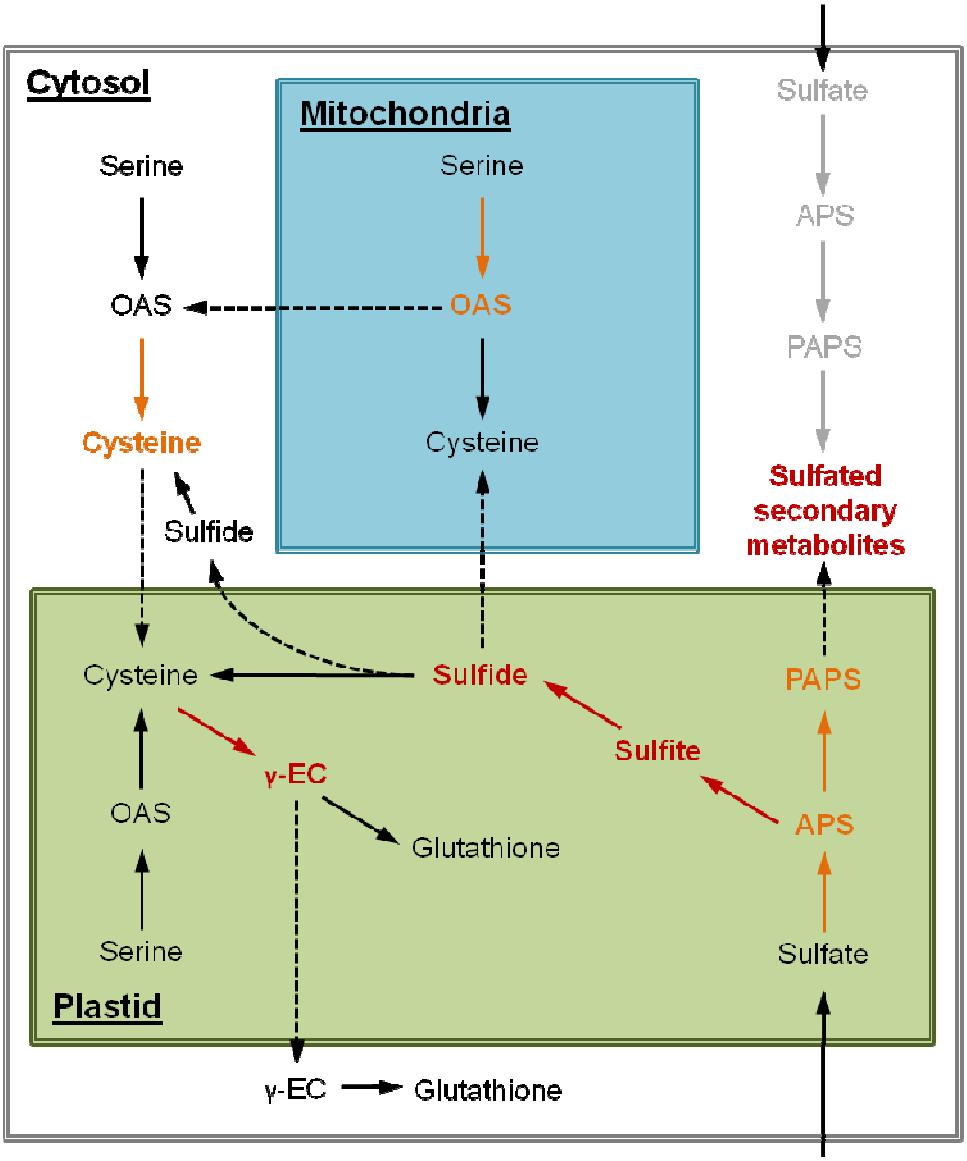


Figure 5.1 ***Subcellular compartmentalization of the sulfate assimilation pathway in Arabidopsis.***
Components in red are restricted to a single cellular compartment. Compounds highlighted in orange are produced principally in the compartment in which they are shown. Pathways in grey are potentially redundant. Broken arrows indicate hypothesized inter-compartment transport.

5.2. Regulation of the ATPS isoforms

ATPS is part of sulfate assimilation and is regulated by the demand-driven regulatory network, which still remains to be characterised (Reuveny *et al.*, 1980; Lappartient *et al.*, 1999; Takahashi *et al.*, 2000). However, during sulfur deficiency two of the four Arabidopsis ATPS mRNAs are targeted and cleaved by miR395, as well as the transcript for low affinity sulfate transporter *SULTR2;1* (Jones-Rhoades and Bartel, 2004; Allen *et al.*, 2005; Kawashima *et al.*, 2009). Chapter 4 described how the four ATPS genes respond differently to sulfur deficiency at the transcript level due to regulation by SLIM1 and miR395 (Figure 5.2). *ATPS2* and *ATPS3* transcripts are not targeted by miR395, but while the first appears to be suppressed by SLIM1, the latter is activated. Both *ATPS1* and *ATPS4* are targets of miR395, yet are oppositely regulated in response to sulfur starvation. *ATPS4* transcript levels decrease due to canonical regulation by miR395; however, the regulation of *ATPS1* is more complex. We ascertained that increased transcription of *ATPS1* mediated by SLIM1 acts in synergy with miR395 cleavage to maintain overall transcript levels. The result may be a modification of *ATPS1* tissue-specific expression. We showed that miR395 levels are correlated to glutathione content in the tissues and that miR395 functions beyond sulfur deficiency response as an integral part of the demand-driven regulation of the reductive sulfate assimilation pathway. Recent analysis in *Brassica napus* showed that miR395 is also induced by treatment with cadmium (Huang et al., 2010), revealing a potential new role for miR395 regulation of the ATPS genes in response to this heavy metal stress, but fitting in with the concept of general demand-driven regulation.

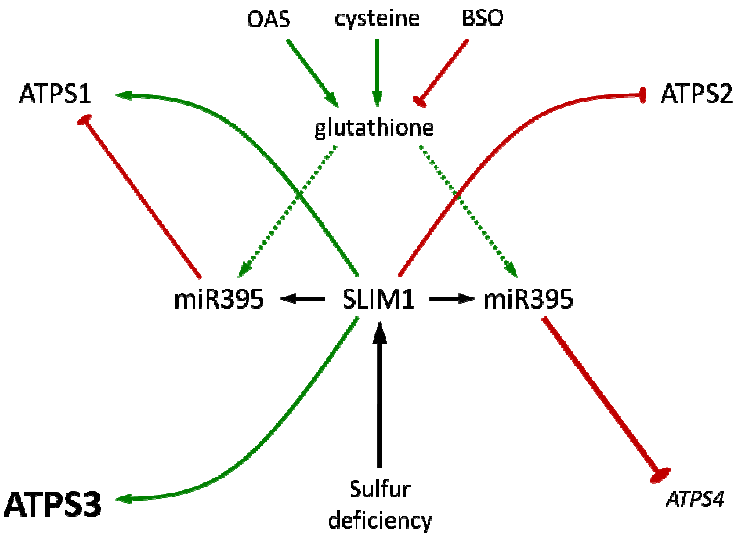


Figure 5.2 ***Regulation of the Arabidopsis ATPS genes during sulfur deficiency***
Green and red arrows indicate positive and negative regulation respectively, as described in the work presented in this thesis. Up-regulation of the ATPS genes by sulfur deficiency is represented by bold text and down-regulation by text in italics. Broken arrows indicate regulation by an unknown mechanism.

Our analysis revealed that miR395 induction leads to increased xylem translocation of sulfate to the shoots during sulfur deficiency. Recently, miR395-directed *SULTR2;1* cleavage was hypothesised to restrict *SULTR2;1* expression to the xylem parenchyma (Kawashima et al., 2009). Hence, miR395 regulation of SULTR2;1 appears to reduce uptake of sulfate into root tissues from the phloem, while up-regulation of SULTR2;1 in the xylem parenchyma increases xylem loading of sulfate, resulting in net increase in translocation from roots to shoots. Further to this root-to-shoot translocation, miR395 is proposed to enable redistribution of sulfate from older to younger leaves (Liang et al., 2010). In addition to the substantial evidence provided for a role of miR395 in regulation of long distance translocation of sulfate, miR395 has been identified in the phloem suggesting it may act as a long-distance signalling (Buhtz et al., 2010). However, the role of the phloem transport remains unclear. The conservation of miR395 throughout plant evolution is extensive, pointing towards an indispensable role for miR395. Target prediction of miR395 in the vascular plant species *Oryza sativa*, *Medicago truncatula*, *Solanum lycospersicum*, *Sorghum bicolour*, and *Brassica napus* revealed that both ATPS and a low affinity sulfate transporter are targeted, whereas in *Populus trichocarpa*, *Zea mays*,

and *Vitis vinifera* only ATPS genes are predicted to be targeted (Huang et al., 2010; Liang et al., 2010). A single miR395 species has also been identified in the moss *Physcomitrella patens* (Fattash et al., 2007). In contrast, the miR399, which regulates the response to phosphate deficiency and is dependent in its function on phloem transport (Pant et al., 2008) is not present in *Physcomitrella*. Therefore, the original function of miR395 must have been regulation of sulfate assimilation independent from the long-distance transport, which may have developed as a secondary role.

Glucosinolate biosynthesis is centrally regulated by six MYB factors. *MYB28*, *MYB29*, and *MYB76* control aliphatic glucosinolate biosynthesis, while *MYB34*, *MYB51*, and *MYB122* control the indolic biosynthesis pathway (Celenza *et al.*, 2005; Gigolashvili *et al.*, 2007a; 2007b; 2008; Malitsky *et al.*, 2008). ATPS activity is required in PAPS synthesis for the final step in the synthesis of glucosinolate core structure. Chapter 3 describes differential regulation of ATPS1 and ATPS3 by the MYB factors as summarised in Figure 5.3. These data were published recently together with research revealing that the APR and APK multi-gene families are regulated uniformly by the glucosinolate MYB factors (Yatusevich et al., 2010). Thus, the genes of primary assimilation are co-ordinately regulated with genes of glucosinolate biosynthesis. PAPS synthesis has been shown to be limiting for the synthesis of glucosinolates (Mugford et al., 2009). The regulation of ATPS1 and ATPS3 was distinct from that of APR and APK as the aliphatic and indolic MYB factors regulated the ATPS isoforms with a different strength. Therefore, ATPS provides a newly identified point of regulation for glucosinolate biosynthesis. Until now, the SLIM1 transcription factor was the only transcriptional regulator of sulfate assimilation to be identified (Maruyama-Nakashita *et al.*, 2006). Through our analysis, six novel transcription factors have been added to the sulfate assimilation regulatory network.

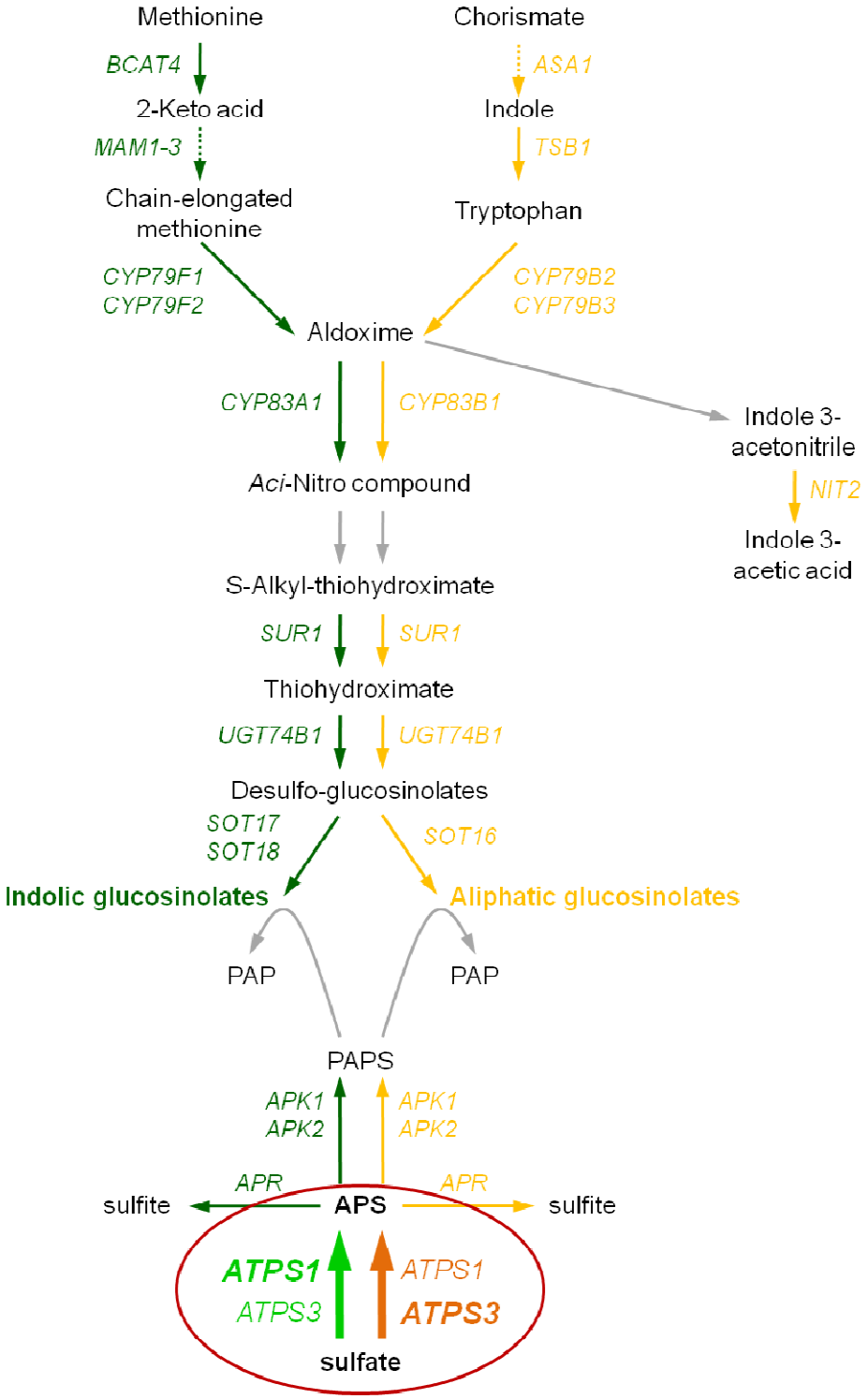


Figure 5.3 ***Glucosinolate biosynthetic network.***
The aliphatic and indolic glucosinolate biosynthesis from methionine and chorismate is outlined, together with PAPS synthesis. Steps representing regulation by the MYB factors are shown in green (MYB28/MYB29/MYB76) and yellow (MYB34/MYB51/MYB122). As described in Chapter 2, regulation of the ATPS genes by the MYB factors is emphasized, with the different extent of regulation of *ATPS1* and *ATPS3* indicated by the text size.

5.3. Future directions

We have revealed that functional specificity exists among the individual Arabidopsis ATPS genes. To build on this analysis of the ATPS mutant lines following environmental stress could further our understanding of the extent of this specificity. To clarify the degree of genetic redundancy within the Arabidopsis ATPS gene family, full systematic analysis of double and triple ATPS knock-out lines could be used as in reports on the SAT and APK gene families (Watanabe *et al.*, 2008b; Mugford *et al.*, 2009; Mugford *et al.*, 2010). Novel regulation of ATPS1 and ATPS3 by the glucosinolate MYB factors was revealed, but involvement of ATPS2 in PAPS production for glucosinolate sulfation was not confirmed. Glucosinolate analysis in *atps1 atps3* double mutants might reveal whether ATPS2 and ATPS4 are able to contribute to glucosinolate sulfation or whether this function is specific to ATPS1 and ATPS3 alone. A reduced rate of glucosinolate synthesis in *atps1* mutant plants (Bok-Rye Lee, personal communication) indeed points to as yet neglected strong control of glucosinolate synthesis by primary sulfate assimilation and particularly ATPS. Similarly, as the *atps1* and *atps4* single knock-out mutants are the most altered in secondary and primary sulfur assimilation respectively, analysis of *atps1 atps4* double mutants might reveal to what extent ATPS2 and ATPS3 support sulfate assimilation. As both ATPS1 and ATPS4 are targeted by miR395, further insights into the role of this regulation might be gained in these mutants. We disclosed a role of miR395 regulation in the shoot-to-root translocation of sulfate. However, the significance of miR395 phloem transport and the functional evolution of miR395 remain to be investigated. As miR395 is conserved in the moss *P. patens* (Fattash et al., 2007) the study of its targets, regulation and function in this species may shed some light on the original function of miR395. ATPS2 was not regulated by the MYB factors or miR395, nor was it proven to be responsible for cytosolic activity. Thus, the identity of the cytosolic ATPS isoform remains to be revealed. The characterisation of the light regulation of ATPS2 described in chapter 2

may aid understanding of the role of ATPS2 in sulfur assimilation. Moreover, such analysis will provide further insight into the mechanisms of ATPS regulation.

5.4. Conclusions

In conclusion, analysis of the four Arabidopsis ATPS isoforms revealed substantial differences in the regulation of individual ATPS genes, clearly pointing to specific functions. This has been confirmed by the differential regulation by MYB factors controlling glucosinolate synthesis. This analysis also firmly put ATPS and other genes of sulfate assimilation into the glucosinolate biosynthesis and regulatory network. ATPS isoforms are also differentially regulated by the miR395. Analysis of regulation of miR395 accumulation clearly identified this miRNA as an integral part of demand-driven regulation of sulfate assimilation. Whereas a final assignment of specific functions to individual ATPS isoforms is still not fully possible, this thesis contributes important data to enable this in the foreseeable future.

Chapter 6

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6. REFERENCES

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