Investigation into the relationship between ethylene and sulfur assimilation in *Arabidopsis thaliana* and onion (*Allium cepa L.*)

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Abstract

The phytohormone ethylene (C_2H_4) mediates the adaptive responses of plants to various nutrient deficiencies including iron (Fe)-deficiency, phosphorus (P)-deficiency and potassium (K)-deficiency. However, evidence for the involvement this hormone in the sulfur (S) deficiency response is limited to date. In this study, the effect of C_2H_4 treatment on the accumulation of the S-assimilation enzymes ATP sulfurylase (ATPS), adenosine-5′-phosphosulfate-reductase (APR), *O*-acetylserine-(thiol)-lyase (*O*ASTL) and sulfite reductase (SiR) was examined in *A. thaliana* and onion (*A. cepa*). To complement this, the effect of short-term S-depletion on the expression of the 12-member gene family of the C_2H_4 biosynthetic enzyme, 1-amino-cyclopropane-1-carboxylic acid (ACC) synthase (ACS) from *A. thaliana*, designated *AtACS1-12*, was also examined.

Western analyses were used to show that plants of *A. thaliana* pre-treated with the C_2H_4-signalling inhibitor 1-MCP, had elevated levels of ATPS, APR and *O*ASTL protein in leaf tissue at all time points examined, suggesting that C_2H_4 has an inhibitory effect on the accumulation of these enzymes. However, SiR appeared to be under dual regulation by C_2H_4: under S-sufficient conditions C_2H_4 appears to prevent the unnecessary accumulation of SiR and conversely promote the fast accumulation of SiR under S-depleted conditions.

The changes in *AtACS1-12* expression in the root and leaf tissues of S-sufficient and S-depleted plants of *A. thaliana* were examined by RT-PCR using gene-specific, exon-spanning primers. The expression patterns of *AtACS2*, *AtACS6* and *AtACS7* were comparable regardless of S availability and may therefore be housekeeping genes. In contrast, the expression of *AtACS5* in leaf, and *AtACS8* and *AtACS9* in roots was repressed under S-depleted conditions, although the mechanism of this repression cannot be elucidated from this study. The protein products of these closely-related genes are believed to be phosphorylated and stabilised by a CDPK whose activity may be compromised by S-depletion. The inhibition of *AtACS5*, *AtACS8* and *AtACS9*

expression, and the decrease in AtACS5, AtACS8 and AtACS9 accumulation, and hence less C_2H_4 production, may be part of the plant adaptive response to S-depletion, as the C_2H_4-mediated repression of root growth is alleviated to allow the plant to better seek out the lacking nutrient. The expression of the MPK-stabilised genes *AtACS2* and *AtACS6* appeared to be similar regardless of S availability, although this may merely be a consequence of the scoring method used in this study, which cannot determine whether there was any difference in the level of expression of these genes. The expression of *AtACS10* and *AtACS12* was repressed in S-deficient plants. Although both AtACS10 and AtACS12 isozymes posses the hallmark seven conserved regions found in the ACSes of other plant species, they are also phylogenetically related to alanine and aspartate aminotransferases, and are known to encode aspartate (*AtACS10*) and aromatic amino acid transaminases (*AtACS12*). Therefore, the apparent downregulation of these genes suggests that the downregulation of amino acid metabolism may be part of the plant adaptive response to S-depletion.

The downregulation of several *AtACS* genes, and therefore possibly also C_2H_4 biosynthesis, in S-deficient plants was accompanied by an accumulation of APR protein. The increase in APR protein that also occurred in 1-MCP-treated plants indicates that C_2H_4 may be involved in the plant response to S-depletion, because in both cases the upregulation of the S-assimilation pathway, as manifested by the accumulation of APR protein, occurred when C_2H_4 biosynthesis and signalling was repressed. However, the possible role of other phytohormoes in the plant response to S-depletion cannot be excluded, as there is evidence for crosstalk between the C_2H_4 signalling pathway and those of auxin, abscisic acid (ABA), cytokinins and jasmonic acid (JA). Furthermore, because C_2H_4 has been implicated in the response of various plants to Fe-deficiency, P-deficiency, and K-deficiency, in addition to S-deficiency, it may be a regulator of the plant adaptive response to nutrient stresses in general.

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My mother.

Augusto.

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Abbreviations

^{O}C	degrees Celsius
-K	insufficient potassium supply, as determined for the specific study quoted
+N	sufficient nitrogen supply, as determined for the specific study quoted
-N	insufficient nitrogen, supply, as determined for the specific study quoted
+P	sufficient phosphorus supply, as determined for the specific study quoted
-P	insufficient phosphorus supply, as determined for the specific study quoted
1-MCP	1-methylcyclopropene
8L/16D	8 hours light/16 hours dark
A	amps
ABA	abscisic acid
ACC	1-amino-cyclopropane-1-carboxylic acid
ATPS	adenosine triphosphate (ATP) sulfurylase
APR	adenosine-5′-phosphosulfate (APS) reductase
AVG	aminoethoxyvinylglycine
bp	base pair(s)
BSA	bovine serum albumin
CBB	Coomassie® Brilliant Blue
CDPK	Ca^{2+} dependent protein kinase
CS	cysteine synthase complex
Cys	cysteine
DF	dilution factor
DTT	dithiothreitol
g	g force
GFP	green fluorescent protein
Gly	glycine
GUS	β-glucuronidase

hr	hours
IM	isolation medium
JA	jasmonic acid
kb	kilobase(s)
L	litre
M	molar concentration
Met	methionine
MPK6	mitogen-activated protein kinase 6
MPKK5	mitogen-activated protein kinase kinase 5
min	minute(s)
mRNA	messenger RNA
N	nitrogen
NaOAc	sodium acetate
*O*ASTL	*O*-acetylserine-thiol-lyase
P	phosphorus
PCR	polymerase chain reaction
PBS	phosphate buffered salt
ppm	parts per million
PVDF	polyvinylidene difluoride
PVP-40	polyvinylpyrollidone
re	refer
RT	reverse transcription
RT-PCR	reverse transcription-polymerase chain reaction
rpm	revolutions per minute
s	seconds
SAM	*S*-adenosylmethionine synthetase
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate – polyacrylamide gel electrophoresis
Ser	serine
SiR	sulfite reductase
U	unit(s)
V	volt(s)
Wt	wild type

1 Introduction

1.1 Sulfur (S) uptake in plants

Plants assimilate S mostly in its inorganic form of sulfate (SO_4^{2-}) from the soil, although atmospheric sulfur dioxide (SO_2) and hydrogen sulfide (H_2S) can also be taken up. It is an essential macronutrient for growth, but in the form of glucosinolates can also act as a defense compound against pathogen and herbivore attack [1].

Hydrogen/sulfate (H^+/SO_4^{2-}) symporters are proposed to uptake and transfer SO_4^{2-} from the roots to the xylem, from which it is unloaded into the leaf chloroplasts where the majority of S-assimilation occurs. In *A. thaliana* these symporters are encoded by a gene family of 14 members, which are classified into 5 groups based on protein sequence similarity [2]. Despite some functional redundancy, certain symporters have specialised roles: for example, AtSultr1;3 is localised to the sieve elements-companion cells of phloem and is responsible for S redistribution within the plant [3]. The symporters also differ in their S-responsiveness. For example, transient S deficiency upregulated root AtSultr2;1 expression in contrast to root AtSultr2;2 which was constitutively expressed [4].

1.2 Attributes of sulfur-deficient plants

In *A. thaliana*, short-term S deficiency ($\leq$ 2 days) causes no observable phenotypic effect [5]. However, plants subjected to long-term S deficiency ($\geq$7 days) exhibit an overall retarded growth, except for a more extensive root system when compared with S-sufficient plants. Hydroponically-grown S-deficient plants have a root:shoot mass ratio of 0.67, compared to a mass ratio of just 0.4 in S-sufficient plants [6]. There is also loss of chlorophyll and photosynthetic competence from the leaves, most dramatically seen as colour changes. The leaves go from green to yellow and finally reddish, before entire

plants starting from the petioles begin to turn purple. Light assimilation capability is reduced under S-deficient conditions, so normal light is perceived as light stress. In response, the plant accumulates high-light protective anthocyanins, which contribute to some of the observed colour changes [6].

The H^+/SO_4^{2-} symporters and S-assimilation enzymes are generally upregulated as the plant begins to acclimate to S-deficiency (section 1.4). However proteins involved in the auxin and C_2H_4 signalling pathways, photosynthetic electron transfer and in the detoxification of reactive oxygen species, among others, are also affected by S availability [5-7].

1.3 Overview of the S-assimilation pathway

Uptake of SO_4^{2-} and its translocation to the chloroplasts is followed by activation *via* adenylation, a reaction catalysed by ATP sulfurylase (ATPS, E.C. 2.7.7.4) to form adenosine-5′-phosphosulfate (5′-APS). An alternative mechanism operates in bacteria, yeast and fungi where 5′-APS is further phosphorylated to form adenosine 3′-phosphate 5′-phosphosulfate (PAPS) by APS kinase (E.C. 2.7.1.2.5). However, in higher plants, 5′-APS is largely reduced to sulfite (SO_3^-) by APS reductase (APR, E.C. 1.8.4.9), which catalyses a two-electron transfer from glutathione (GSH) to 5′-APS. In photosynthetic plastids, sulfite reductase (SiR, E.C. 1.8.7.1) further reduces SO_3^- to sulfide (S^{2-}) by catalysing the transfer of six electrons from ferredoxin. The non-photosynthetic plastids in petals and the meristematic cells of shoots, roots, embryos and endosperm are responsible for CO_2 fixation, manufacture of starch, fatty acids and pigments, and the synthesis of amino acids from inorganic nitrogen [reviewed in 8]. In these non-photosynthetic plastids, NADPH is the electron donor instead, and a root-localised ferredoxin and ferredoxin-$NADP^+$ reductase (FNR) is required for optimum SiR activity [9]. Ser acetyltransferase (SAT) and *O*-acetylserine-(thiol)-lyase (*O*ASTL), which form the dissociable complex Cys synthase, catalyses the two-step reaction that incorporates S^{2-} into Cys. Cys is then directly incorporated into proteins and peptides, or further metabolised to act as a S source for synthesis of Met and iron (Fe)-S clusters (Figure 1.1).

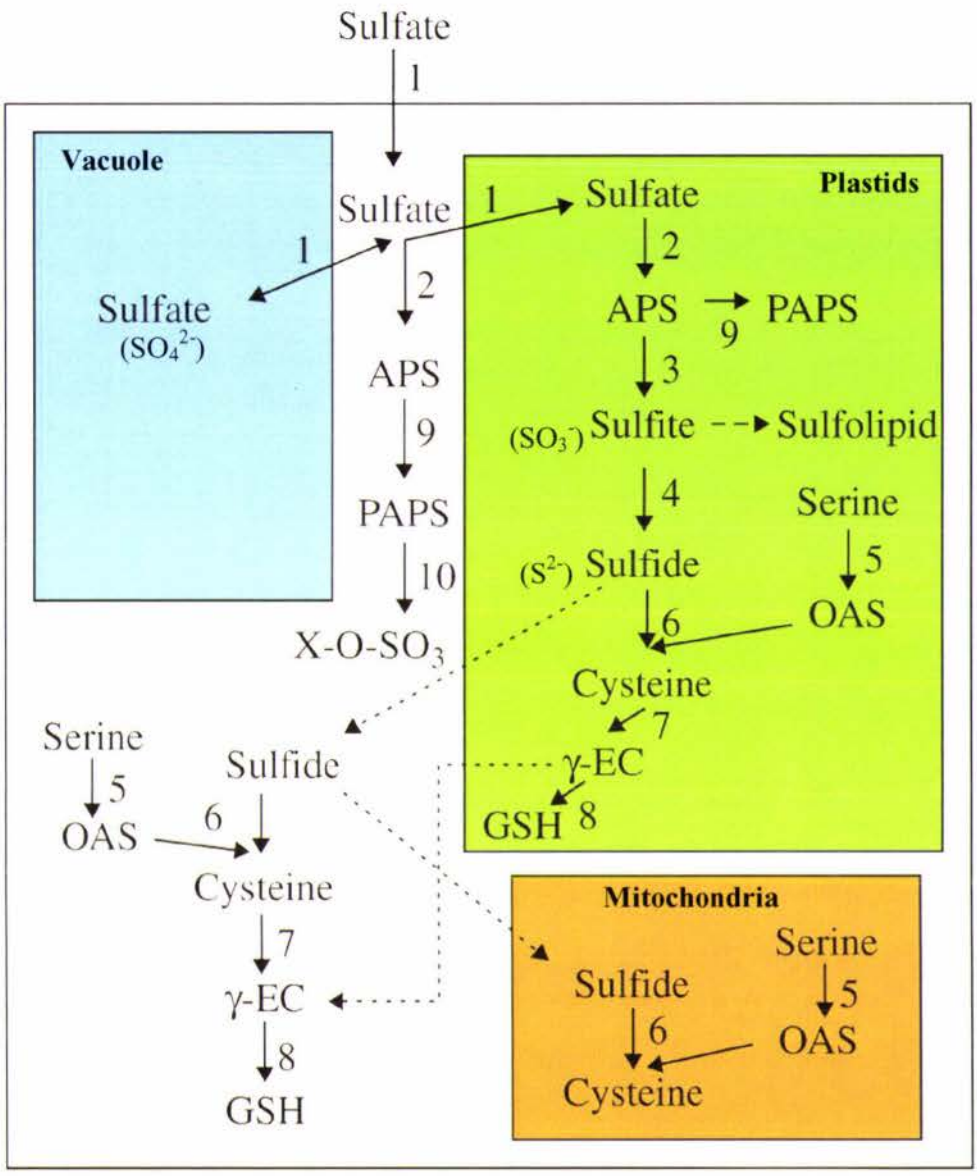

Figure 1.1 Outline of the plant S-assimilation pathway. Numbers represent enzymes as follows: 1, Hydrogen/sulfate (H^+/SO_4^{2-}) symporter; 2, ATP sulfurylase (ATPS); 3, APS reductase (APR); 4, sulfite reductase (SiR); 5, serine acetyltransferase (SAT); 6, *O*-acetylserine-(thiol)-lyase (*O*ASTL); 7, -glutamylcysteine synthetase; 8, GSH synthetase; 9, APS kinase; 10, sulfotransferase. Dashed lines represent multiple reaction steps; dotted lines indicate unconfirmed transport steps (from Kopriva, [10])

Multiple isoforms of most of the S-assimilation enzymes have been identified in *A. thaliana*, with the exception of SiR which exists as a single plastidic enzyme [11]. There are four plastidic ATPS isozymes, but an alternative translation initiation site of ATPS2 may result in a cytosolic isoform [12], and would explain the presence of ATPS activity in the cytosol [1]. Three APR isozymes have been identified by functional complementation of a PAPS reductase mutant strain of *Escherichia coli* [13, 14]. Differential centrifugation and the presence of marker enzyme activity showed that APR activity was confined to plastids, indicating that all three isozymes are plastidic [1]. There are five SAT isozymes: three are cytosolic and the remaining two are plastidic and mitochondrial, respectively [15, 16]. There are nine *O*ASTL genes, all nuclear-encoded, but are less clearly grouped compared with the other S-assimilation enzymes. The *O*ASTL-A isoform, which lacks a transit peptide, remains in the cytosol, whereas the *O*ASTL-B and *O*ASTL-C isoforms are imported into the plastids and mitochondria, respectively [17, 18]. The enzymes SAT and *O*ASTL are believed to form the cysteine synthase (CS) complex, whose association/dissociation is proposed to be determined by plant *O*AS levels (Figure 1.2) [reviewed in 2, 19]. The model of CS regulation by *O*AS is as follows: under S-deficient conditions, active, bound SAT catalyses *O*AS synthesis and accumulation. In contrast, S-sufficient conditions cause *O*AS to accumulate, resulting in complex dissociation and the inactivation of SAT to prevent further *O*AS synthesis. Meanwhile, *O*ASTL is now in the active, disassociated state and synthesises Cys from the available *O*AS and S^{2-}.

In *A. thaliana*, SO_4^{2-} is reduced and assimilated to completion only in plastids, the sole site where SiR and the APR isozymes are found. It is unclear what roles, if any, are played by the multiple isoforms of the other S-assimilation enzymes found in other subcellular compartments of the cell. One suggestion is that cytosolic ATPS may generate 5′-APS for SO_4^{2-} ester biosynthesis [1].

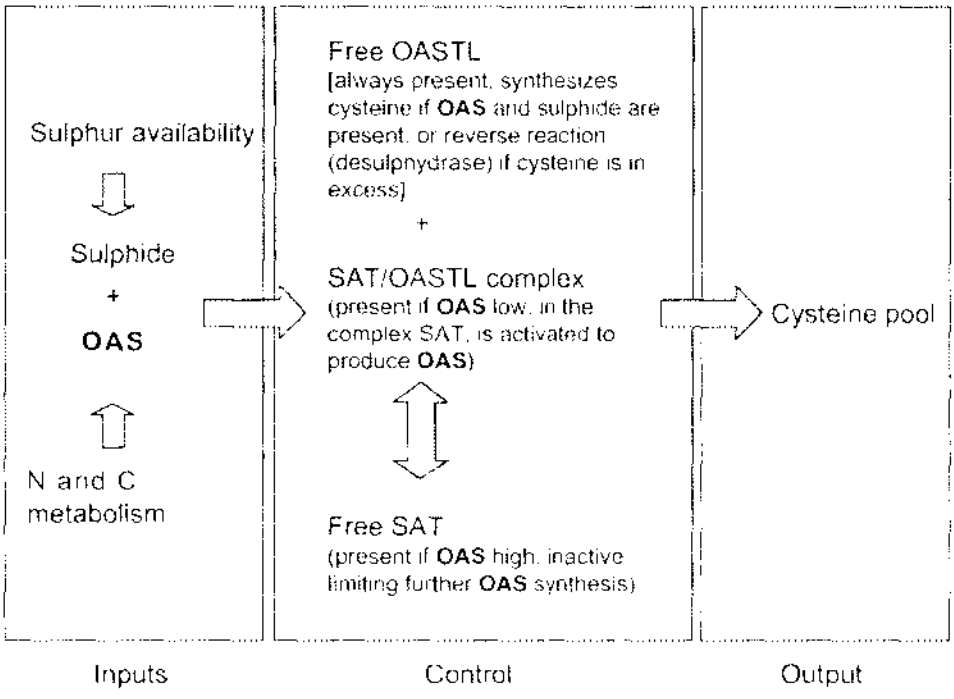

Figure 1.2 Formation of the CS complex is influenced by the level of *O*AS, which in turn depends on relative fluxes through the S-assimilation pathway and N and C metabolism. When the *O*AS level is high the complex is disrupted and SAT is inactivated to prevent further *O*AS accumulation; meanwhile, freed *O*ASTL synthesises Cys formation (from Hawkesford & Kok, [2])

1.4 Regulation of SO_4^{2-} uptake and S-assimilation

1.4.1 Developmental regulation of ATPS and APR

The enzymes ATPS and APR are both developmentally regulated in *A. thaliana*, as total activity and protein levels decline ~3-fold as the plant ages [1]. This decrease in APR activity and protein levels was also observed in leaves at different developmental stages, that is, it was highest in the youngest leaves but progressively declined in older leaves. This led the authors to propose that APR activity and protein levels were more closely linked with developmental leaf stage than plant age.

In contrast, ATPS activity is almost constant in leaves at different developmental stages. The plastidic and cytosolic isozymes are known to be reciprocally regulated: in the former, the ~2-fold decrease in activity in the developmentally older leaves is reciprocated by a ~3.5-fold increase in activity in the latter. This indicates that ATPS activity and protein levels correlate more closely with plant age rather than to developmental leaf stage.

1.4.2 Regulation at the promoter level

The signal transduction pathway that exerts transcriptional regulation on SO_4^{2-} uptake and S-assimilation is not yet characterised, although recent work has begun to elucidate some of the mechanisms involved.

In terms of SO_4^{2-} uptake, Maruyama-Nakashita *et al.* [20] have identified a 16 bp, S-responsive element (SURE) in the H^+/SO_4^{2-} symporter AtSultr1;1 promoter. A luciferase reporter gene construct driven by the SURE was sufficient and necessary for induction by S deficiency, and inhibition by Cys and GSH at the transcriptional level (section 1.4.4.2). Subsequent microarray analysis revealed the presence of SUREs in the promoters of several, but not all, other S-responsive genes, including *AtSultr2;1* and *AtAPR3*.

The *A. thaliana* Snf1-related kinase 2.3 (SNRK2.3) has been implicated in the regulation of *AtSultr2;2* expression and *O*AS accumulation [21]. Recent work with the green alga *Chlamydomonas reinhardtii* is also suggestive of a role for SNRK2 in adaptive S-deficiency responses. This alga responds to S deficiency by accumulating aryl-sulfatase (ARS), an enzyme that releases SO_4^{2-} from esterified organic sulfates to meet immediate cellular needs. Conversely, the expression of genes encoding plastidic proteins declines, and SAC3, a putative Ser/Thr kinase of the SNRK2 subfamily, acts as a transcriptional regulator to mediate both events [22]. Run-on assays that measure the incorporation of [α-^{32}P]UTP into mRNA revealed that under S-deficient conditions, *sac3* mutants were unable to induce the expression of ARS, nor to repress the expression of plastidic proteins. Treatment of wild-type (wt) cells with the general protein kinase inhibitor 6-dimethylaminopurine (DMAP) mimicked the S-deficient *sac3* phenotype, further indicating SAC3's role in regulating transcription. As the SAC3 was required for the alga's adaptive response to S deficiency, it is feasible that SNRK2.3 is also involved in *A. thaliana*'s response to S deficiency, possibly exerting its influence *via O*AS accumulation. Plant SNRK2s also appear to be abscisic acid (ABA)-regulated, at least in rice [23], indicating that C_2H_4 may be just one signal implicated in the plant S deficiency response, and that other hormones are also involved.

The possibility of auxin-C_2H_4 crosstalk has been further supported by the identification of a conserved auxin response factor (ARF) binding site is nested within the SURE [20]. Li *et al.* [24] isolated auxin- and partially C_2H_4-resistant mutants of *A. thaliana* that lacked the functional transcriptional factors ARF7 and/or ARF19. The mutants did not exhibit stunted root growth as severe as wt plants in response to 1-amino-cyclopropane-1-carboxylic acid (ACC) feeding, yet when treated with C_2H_4 had comparable levels of C_2H_4-response factor (ERF) expression. This led the authors to propose that the ARF genes participate in desensitising the plant to C_2H_4 responses, and indicates a possible crosslink between C_2H_4, auxin, and S-deficiency in plants. Isolation of *wei2* and *wei7*, C_2H_4-insensitive *A. thaliana* mutants that also exhibit altered auxin biosynthesis prove the *in planta* interaction between C_2H_4 and auxin [25]. Furthermore, transcriptome analyses indicated that genes encoding auxin-

induced proteins are upregulated in response to S deficiency, possibly as a trigger for root growth [6].

1.4.3 S-mediated regulation of the H^+/SO_4^{2-} symporters and S-assimilation enzymes at the transcriptional level

Several H^+/SO_4^{2-} symporters and S-assimilation enzymes of *A. thaliana* were upregulated at the level of transcription in response to S deficiency. Plants grown under continuous S-deficient conditions accumulated high levels of AtSultr1;1 and AtSultr2;1 mRNA in roots, and AtSultr2;2 mRNA in leaves [4, 26]. More recently, the plant response to S deficiency was studied by transcriptome analyses, with similar results. The genes for the H^+/SO_4^{2-} symporters, namely AtSultr1;1, AtSultr1;2 and AtSultr2;1, were upregulated in response to short-term S deficiency [5, 7]. Similar work by Nikiforova *et al.* [6] showed that AtSultr2;1 and AtSultr4;2 were upregulated in response to long-term S deficiency.

The S-assimilation enzymes APR2 and ATPS were also upregulated in response to transient S deficiency [5, 7]. In addition, a C_2H_4-responsive element binding protein (EREBP)-like protein was also found to be upregulated, suggesting C_2H_4's involvement in the plant response to S deficiency. However, there have also been conflicting reports regarding in the increase in mRNA levels of ATPS [27], APR1 and plastidic SAT [26] in plants subjected to short-term S deficiency. The APR isozymes, especially APR1, may be indirectly upregulated by cellular redox status instead of S availability [28]. Under long-term S-deficient conditions, several auxin-related pathway genes were upregulated in addition to a SAM-like protein and C_2H_4-induced *myb* transcripton factors, supporting the integration of the auxin- and C_2H_4-signalling pathways in the responses to S deficiency observed in *A. thaliana* [6].

Expression of the soybean seed storage protein β-conglycinin is dependent on S levels. Earlier work on the plant response to S deficiency mostly involved the

expression of the β subunit of this protein transgenically in petunia and *A. thaliana*. Hirai *et al.* [29] expressed the β subunit of this protein, or the GUS gene fused to the β subunit gene promoter, in S-deficient *A. thaliana*. These authors observed that β subunit mRNA levels and GUS activity levels were 5-10-fold and 17-fold higher, respectively, in S-deficient plants. Likewise, GUS activity was 17-fold higher under S-deficient conditions. This effect was reversible upon resupply of S.

1.4.4 Metabolite-mediated regulation of the H^+/SO_4^{2-} symporters and S-assimilation enzymes

1.4.4.1 O-*acetylserine -mediated regulation*

O-acetylserine (*O*AS) links S metabolism to carbon (C) and nitrogen (N) metabolism, and may positively regulate the S-assimilation pathway by determining CS complex formation (Figure 1.2) [2, 30-32]. Under N-sufficient/S-deficient (+N/-S) conditions there is insufficient S^{2-} for *O*AS to react with to form Cys. Because of this, *O*AS accumulates and causes the CS complex to dissociate, thus inactivating SAT to prevent further *O*AS production. Meanwhile, the now free homodimers of *O*ASTL are activated to catalyse Cys synthesis from the abundant *O*AS supply and any S^{2-} available. Conversely, under S-sufficient conditions, *O*AS is depleted and the CS complex reforms to activate SAT and favour *O*AS synthesis.

Many studies have found that the elevated *O*AS levels under S-deficient conditions are paralleled by the upregulation of S-responsive genes. For example, Kim *et al.* [33] found that a construct of GUS fused to the β subunit promoter of β-conglycinin was upregulated when the S:N ratio was low, and vice-versa. Furthermore, *O*AS treatment mimicked the S-deficient response even in S-sufficient plants. Transcriptome and metabolome analyses have not only shown that this mimicry occurs in *A. thaliana* plants subjected to S-deficient conditions, but also that APR mRNA levels concomitantly increase when *O*AS accumulates in S-deficient plants [34]. Other work with GFP driven

by the AtSultr1;2 promoter revealed a dose-dependent increase in GFP fluorescence in response to *O*AS application [35]. In the aquatic plant *Lemna minor*, *O*AS treatment of plants in prolonged dark mimicked the light-induced upregulation of ASPR activity [36]. The induction occurred at the level of transcription and translation, as the effect of *O*AS was negated by the addition of cycloheximide, an inhibitor of protein translation, as well as 6-methylpurine, an inhibitor of transcription. Transcriptome data has also indicated that *O*AS is a regulator of many genes, not only those sensitive to S availability [5]. Recent identification of a thiol reductase that may control *O*AS levels in *A. thaliana* further supports its role as a regulator of the S-assimilation pathway [37]. Mutants lacking this thiol reductase had abnormally high levels of *O*AS, and constitutive activation of APR1 and AtSultr2;2.

However, the role of *O*AS as a regulator of SO_4^{2-} reduction has not been proven conclusively for all plant species. Hopkins *et al.* [38] suggested that *O*AS accumulation is a consequence of prolonged S depletion instead of being a genuine metabolic signal, at least in potato (*Solanum tuberosum*). Levels of *O*AS, SO_4^{2-} and total S were comparable in wt and SAT overexpressing plants, yet if *O*AS was a true signal of S availability, its levels should vary to alter the S-assimilation pathway.

There is also evidence to indicate that the S-assimilation pathway is controlled by N availability at the transcriptional level, and that *O*AS is a mediator of this regulation [39]. The downregulation of nitrate reductase expression by both S-deficient and N-deficient conditions further emphasises the link between the two pathways [34].

1.4.4.2 Reduced S compound-mediated regulation

In *A. thaliana* and canola (*Brassica napus*), there is evidence that GSH may regulate S uptake and assimilation. In these plants, ATPS mRNA and protein levels, and activity, as well as SO_4^{2-} uptake was negatively regulated by phloem-translocated GSH [40, 41]. Not surprisingly, microarray analysis has

also indicated that AtSultr1;2 expression was sensitive to inhibition by GSH at the transcriptional level [35]. The levels of GSH, but not those of other S compounds measured, most reflected S availability. Also, the inhibitory effect of Cys on ATPS was negated by buthionine sulphoximide (BSO), which prevents GSH synthesis [40, 41]. Similar work by Vauclare *et al.* [42] indicated that APR1, APR2 and APR3 in *A. thaliana* roots were similarly repressed, and were also more sensitive to inhibition by GSH than ATPS. In these experiments, APR mRNA and protein levels, and activity decreased within hours of GSH treatment. In contrast ATPS, SiR and SAT were unaffected by GSH application in the short-term.

Activity of APR also appears to be downregulated by H_2S in the shoots of curly kale (*Brassica oleracea*) [43]. This decrease in APR activity was accompanied by a rapid increase in thiol content, especially of Cys, and suggests that the inhibitory effect of H_2S may act *via* this reduced S metabolite. Activity of APR in the roots, and ATPS, SAT and *O*ASTL activity in both the shoots and roots were unaffected by H_2S application.

1.4.5 Cytokinin-mediated regulation

Cytokinin may be a general regulator of nutrient uptake and assimilation as it has also been implicated in the control of N and phosphorus (P) assimilation and uptake [reviewed in 10]. However, contradictory results have hindered the elucidation of the possible role of cytokinin as a regulator of the S-assimilation pathway.

In *A. thaliana*, mRNA levels of APR1 and AtSultr2;2, as well as a construct comprising GFP driven by the promoter of the β-conglycinin β subunit, accumulated in response to zeatin application [44]. A correlating increase in GFP fluorescence was also observed. Endogenous zeatin levels did not vary according to S availability, but did cause plant sucrose levels to increase, and sucrose is known to induce APR1 expression [45]. This led the authors to propose that instead of being a direct signal, cytokinins act *via* sucrose to

mediate the plant response to S deficiency by upregulating the expression of APR1 and AtSultr2;2.

More recently, however, work by Maruyama-Nakashita *et al.* [35] indicated that cytokinin is a negative regulator of AtSultr1;2, and to a lesser extent, AtSultr1;1. The authors expressed GFP fused to the AtSultr1;2 promoter in *A. thaliana*. When plants grown under S-deficient conditions were treated with the synthetic cytokinin benzyladenine, lower levels of GFP and AtSultr1;2 mRNA levels were measured, and a GFP fluorescence as low as 0.2-fold that of control plants was observed. A similar effect was seen in plants treated with zeatin. Nevertheless, these results still suggest that cytokinins may be involved in the plant response to S deficiency.

1.4.6 Light- and diurnal rhythm-mediated regulation

Light appears to be a positive regulator of the APR gene family, especially of *AtAPR2*, in *A. thaliana* [45]. A diurnal pattern of activity, transcript and protein levels of the *AtAPR2* gene family was seen in the roots and shoots of plants under a 10 hr light/14 hr dark (10L/14D) period. This rhythm was disrupted by an extended dark period, but subsequent re-illumination was sufficient for partial recovery of the diurnal pattern. Feeding experiments with $^{35}SO_4^{2-}$ revealed a concomitant increase in ^{35}S incorporation into reduced S compounds *in vivo*. Maize (*Zea mays*) APR also appears to be diurnally regulated [46]. Similar results were obtained for *L. minor* where prolonged dark decreased APR activity to 10% of control levels, as well as inhibiting $^{35}SO_4^{2-}$ uptake and incorporation into protein and GSH [36].

1.4.7 Iron (Fe)-mediated regulation

Iron (Fe) appears to regulate the S-assimilation pathway, as least in maize (*Zea mays*) [47]. Fe was found to increase the uptake and translocation of $^{35}SO_4^{2-}$ in both S-sufficient and S-deficient plants, albeit to a lesser extent in the latter. Under Fe-deficient conditions, the leaf free thiol content was found to increase

regardless of S availability, with a concomitant decrease in ATPS and *O*ASTL activity. Additionally, ATPS activity was enhanced under S-deficient conditions, but this induction was absent when Fe was omitted from the media. This effect was not seen for *O*ASTL however, possibly because that S^{2-} reassimilation occurred from an alternative source, perhaps from glucosinolate catabolism [7].

1.5 Ethylene (C_2H_4) as a plant stress hormone

Ethylene (C_2H_4) is a gaseous signal that diffuses from the site of synthesis to affect other tissues of the plant [48]. Its production is altered by various factors, such as other plant hormones including ABA, cytokinins and jasmonic acid (JA), and various stresses and environmental stimuli such as wounding, flooding, chilling, oxygen deficiency, ozone, pathogen attack, light, touch and gravity [reviewed in 49].

This phytohormone is involved in many aspects of the plant life cycle, including root development, senescence, abscission and fruit ripening. Not surprisingly, C_2H_4 treatment triggers genome-wide changes in gene transcription [reviewed in 50]. Various microarray studies also indicate that C_2H_4 regulates the expression of its own biosynthetic and signal transduction components, as well as genes involved in pathogen defense, primary and secondary metabolism, and the ubiquitin/26S proteasome pathway.

1.5.1 Overview of the C_2H_4 biosynthetic pathway

The C_2H_4 biosynthetic pathway is outlined in Figure 1.3. The enzyme *S*-adenosylmethionine (AdoMet) synthetase (SAM) converts approximately 80% of the Met pool in the cell into SAM at the expense of ATP [reviewed in 49]. The enzyme ACC synthase (ACS, E.C. 4.4.14) then catalyses the conversion of S-AdoMet into ACC. However, SAM is also a methyl donor and contributes to the methylation reactions of lipids, proteins and nucleic acids. The reaction catalysed by ACS, therefore, is the first committed step of the pathway and is

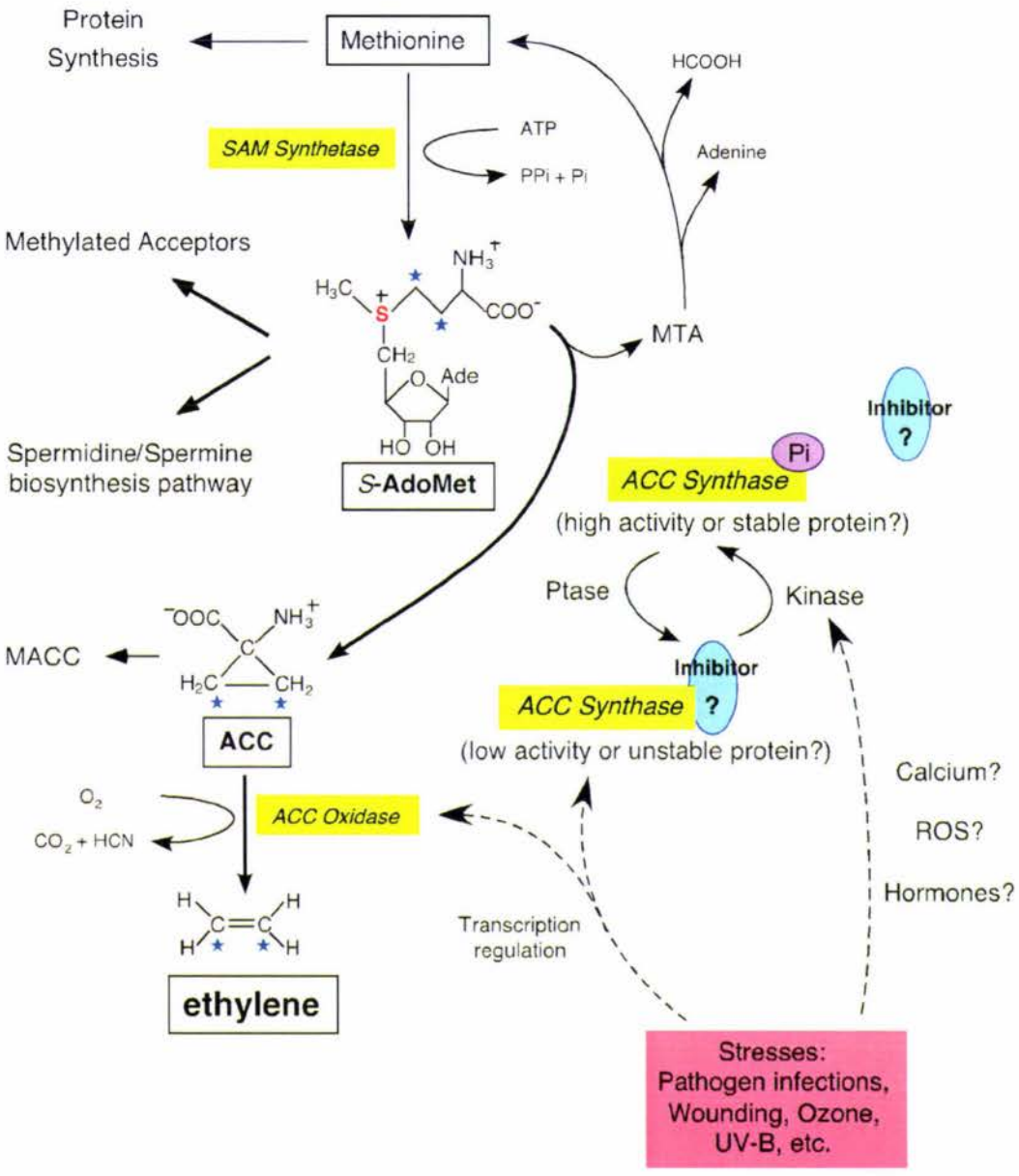

Figure 1.3 An overview of the C_2H_4 biosynthetic pathway. SAM synthetase catalyses S-AdoMet is formation from Met at the expense of ATP. The enzyme ACS converts S-AdoMet into ACC in a rate-limiting step. The by-product MTA is recycled back to Met to enable continuous C_2H_4 production. ACO catalyses the final step of C_2H_4 biosynthesis using ACC as substrate and generates carbon dioxide and cyanide. Transcriptional regulation of ACS and ACO is indicated by dashed arrows. Regulation of ACS by stress *via* reversible phosphorylation may occur as indicated (from Wang *et al.* [49]).

also proposed to be the rate-limiting step of the C_2H_4 biosynthetic pathway. At least 12 *AtACS* genes have been identified in *A. thaliana*, although not all are enzymatically active: *AtACS1* is inactive, *AtACS3* is a pseudogene, and *AtACS10* and *AtACS12* encode aminotransferases [51 and references therein]. The 5'-methylthioadenosine byproduct of the ACS-catalysed reaction is reconverted into Met by a salvage pathway, allowing for continuous C_2H_4 production without decreasing the pool of Met. Finally, ACC is oxidized by ACC oxidase (ACO, E.C. 1.14.17.4) to form C_2H_4, CO_2, and cyanide. Cyanide is detoxified to β-cyanoalanine in a reaction catalysed by β-cyanoalanine synthase (E.C. 4.4.1.9) in the mitochondria.

1.5.2 Overview of the mode of ethylene action and signal transduction

Etiolated *A. thaliana* seedlings treated with C_2H_4 exhibit the 'triple response': an exaggerated curvature of the apical hook, radial swelling of hypocotyls, and inhibition of root and hypocotyl growth (Figure 1.4) [reviewed in 50]. The 'triple response' is highly reproducible and specific, and enabled the isolation of mutants with impaired C_2H_4 responses. Studies done largely in the 1990's led to the characterisation of the genes disrupted in these mutants, and finally to elucidation of the C_2H_4 signaling pathway [52-54].

In *A. thaliana*, the C_2H_4 receptors are encoded by a five-member gene family (ETR1, ETR2, ERS1, ERS2 and EIN4), and are divided into two subfamilies based on sequence similarity and structural organization (Figure 1.5 a) [reviewed in 49, 50]. In the absence of C_2H_4, the function of the C_2H_4 receptors is to negatively to repress C_2H_4 responses (Figure 1.5 b). Immediately downstream is CTR1, which physically interacts with the C_2H_4 receptors at the endoplasmic reticulum membrane to negatively regulate the C_2H_4 signaling pathway. Downstream of CTR1 is EIN2, a positive regulator which is required for all C_2H_4 responses. Six members of the EIN3 family have been identified in *A. thaliana*, and all have characteristic features of transcription factors, suggesting a role as transcriptional regulators of the C_2H_4 response.

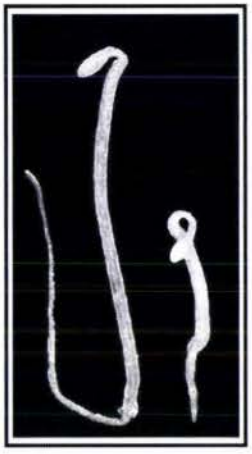

Figure 1.4 Phenotypes of etiolated seedlings of *A. thaliana* without (left) and with (right) application of 10 µM of the C_2H_4 precursor ACC. The plant of the right exhibits the characteristic 'triple response': an exaggerated curvature of the apical hook, radial swelling of the hypocotyl, and stunted root and hypocotyl growth (from Benavente & Alonso, [50]).

1.6 Ethylene (C_2H_4) as a signal for nutrient stress in plants

1.6.1 Ethylene (C_2H_4) as a signal for iron (Fe) deficiency

There is some evidence for C_2H_4-mediated regulation of Fe-deficiency responses in Strategy I plants [55, reviewed in, 56]. Strategy I plants are non-graminaceous monocotyledons and dicotyledons that uptake Fe^{2+} following its reduction from Fe^{3+}, while graminaceous monocotyledons that uptake Fe^{3+} directly comprise the Strategy II plants. In these experiments, an enhanced Fe^{3+}-reducing capacity, subapical root hair development and acidification of the rhizosphere paralleled the increase in C_2H_4 production. Other work showed that C_2H_4 production increased up to five-fold in Fe-deficient plants depending on species, and that inhibitors of C_2H_4 synthesis and action generally prevented the induction of the plant response to Fe-deficiency. For example, when *A. thaliana* plants were subjected to Fe-deficient conditions and treated with inhibitors of the C_2H_4 biosynthetic enzymes (cobalt, Co, inhibits ACO, and aminoethoxyvinylglycine, AVG, inhibits ACS), flavin secretion decreased to 2% and 25% of control levels, respectively. The Fe^{3+}-reducing capacity of

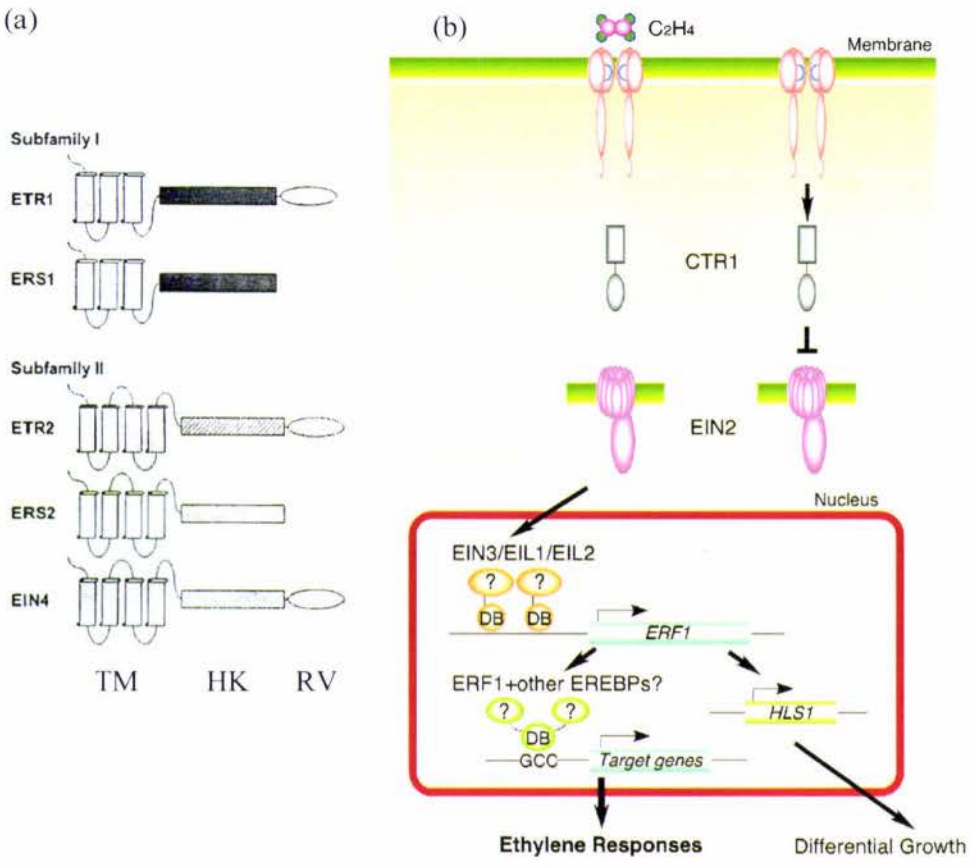

Figure 1.5 Overview of the C_2H_4 signal transduction pathway. (a) The C_2H_4 receptors ETR1 and ERS1 have three transmembrane domains (TM) and a conserved His kinase domain (HK), and function as homodimers. ETR2, EIN4, and ERS2 have four membrane-spanning regions (TM) and a degenerate histidine kinase domain. Only ETR1, ETR2, and EIN4 have receiver domains (RV) at their C termini (from Benavente & Alonso, [50]). (b) C_2H_4 binds at the N-terminal transmembrane domain of the receptors. In the absence of C_2H_4, the receptors activate CTR1, which negatively regulates downstream components (including EIN2). Conversely, C_2H_4 binding inactivates the receptors and therefore also CTR1, allowing EIN2 to act positively on the EIN3 family of transcription factors downstream. These transcription factors act on downstream C_2H_4-responsive genes, for example ERF1, to modulate the plant C_2H_4 response (from Wang *et al.* [49]).

these plants also declined to 23% and 29% of control levels, respectively. Conversely, ACC-fed plants had enhanced Fe^{3+}-reducing capacity and subapical root hair development. A Fe^{3+} reductase encoded by the *FRO2* gene may be subject to C_2H_4-mediated regulation and therefore responsible for the observed induction or repression of Fe^{3+}-reducing capacity [57].

However, the C_2H_4-mediated regulation of Fe-deficient responses has not been conclusively proven. For example, Fe-deficient plants do not necessarily exhibit all Fe-deficient responses. Furthermore, wt, C_2H_4-insensitive and *ctr1* mutants of *A. thaliana* all exhibit an enhanced Fe^{3+}-reducing capacity in response to Fe-deficiency, although the Fe^{3+}-reducing capacity of the C_2H_4-insensitive and *ctr1* mutants should have remained unchanged [58]. Also, the *ctr1* mutants, which display other C_2H_4 responses constitutively, do not have a concomitantly constitutive high Fe^{3+}-reducing capacity.

1.6.2 Ethylene (C_2H_4) as a signal for phosphorus (P) deficiency

In *A. thaliana*, C_2H_4 appears to have a dual effect on root architecture, depending on P availability [59]. Phosphorus-sufficient and phosphorus-deficient plants treated with the C_2H_4 signaling inhibitor 1-methylcyclopropene (1-MCP) displayed a dose-dependent increase and decrease, respectively, of root elongation rate (RER). The authors interpreted these observations to suggest that C_2H_4 acted to maintain RER under P-deficient conditions, and to limit RER under P-sufficient conditions. Similar work and results in bean (*Phaseolus vulgaris*) have also been obtained indicating a two-fold regulation of lateral root density by C_2H_4 [60]. This results in a shallower but wider root system, thus reducing spatial competition for P among roots of the same plant, and is ideal for nutrient foraging in surface soil, where P is most available [61]. More recently, microarray analysis in *A. thaliana* has shown that EIN3 expression was upregulated in response to P-deficiency, indicating C_2H_4's involvement in the plant response to P-deficiency [62]. Two CDPKs (CDPK, At1g49580; and CDPK9, At3g20410) were also upregulated, potentially phosphorylating and stabilising AtACS4, AtACS5, AtACS8 and AtACS9. In addition, MAPKK5, which acts upstream of MAPK6 [reviewed in 63], was

downregulated. This suggested that C_2H_4 biosynthesis was tightly regulated under P-deficient conditions.

1.6.3 Ethylene (C_2H_4) as a signal for S deficiency

There is limited evidence to indicate C_2H_4-mediated regulation of the plant response to S deficiency. Maruyama-Nakashita *et al.* [35] expressed a construct of GFP fused to the promoter of *AtSultr1;2* in S-deficient *A. thaliana*, and found that GFP fluorescence was 1.4-fold greater when the plants were also given the C_2H_4 precursor ACC. Transcriptome analysis has indicated that certain *myb* transcription factors were commonly induced by S deficiency as well C_2H_4 treatment [6]. Furthermore, a SAM-like protein was also upregulated in S-deficient plants, suggesting a possible link between C_2H_4 signaling and the plant response to S deficiency, as SAM cycling provides the necessary Met for C_2H_4 synthesis.

1.7 Thesis aims

This study, therefore, aims to be the first to determine directly whether a relationship exists between C_2H_4 and the control of S-assimilation. To do this, *A. thaliana*, a model plant species, and in *A. cepa*, a S-accumulating species, will be examined. Two independent approaches will be used:

- Plants of *A. thaliana* and *A. cepa* will be treated with C_2H_4 and the induction or repression of the S-assimilation enzymes monitored by the protein accumulation of the respective enzymes, and
- Plants of *A. thaliana* will be subjected to transient S deficiency and the induction or repression of different members of the *AtACS* gene family documented.

2 Materials and Methods

Unless otherwise noted, all chemicals used for experimental work in this thesis were analytical grade, and obtained either from BDH Laboratory Supplies (Dorset, England), Sigma Chemical Company (St. Louis, Mo., USA) or Bio-Rad (Hercules, CA, USA). Solutions were made using purified water produced by reverse-osmosis and microfiltration (Milli-Q water, Millipore Corp., Bedford, MA, USA), subsequently referred to as MQ water.

2.1 Propagation and harvest of *A. thaliana*

2.1.1 Plant material and growth conditions for *A. thaliana* in soil

Seeds of *A. thaliana* (Columbia) were sown on damp topsoil and grown until 5 weeks old in a growth cabinet (Plant Growth Unit, Massey University, Palmerston North), where the plants were under an 8L/16D cycle, and where temperatures of 18°C and 23°C during the day and night, respectively, were maintained.

2.1.2 Plant material and growth conditions for *A. cepa* in soil

Seeds of *A. cepa* were sown on damp topsoil and grown until 3 months old in a glasshouse, where the temperature ranges of 25 to 30°C and 15 to 20°C during the day and night, respectively, were maintained. Two cultivars were used: W202A, a pungent, high S compound storage line, and Texas Grano, a mild, low S compound storage line. Both cultivars were sourced from Crop & Food Research, Lincoln.

2.1.3 Plant material and hydroponic growth conditions for *A. thaliana*

- Flame-sealed and autoclaved 200 µL pipette tips
- Nutrient gel: 0.7% (w/v) agar and 50% (v/v) nutrient solution (Table 2.1)

- Surface-sterilised *A. thaliana* seeds: agitation in 70% (v/v) ethanol for 5 min followed by 0.5% (w/v) SDS for 15 min, then rinsed in MQ water
- Hydroponic tank apparatus: opaque plastic tanks with perforated styrofoam lids, and plastic pipe aerators attached to aquarium pumps

Plants were grown based on the method described by Norén *et al.* [64]. The tips were filled with nutrient gel which was allowed to set before 2-3 sterilised seeds were sown per tip. The tips were placed in tip boxes that were then sealed with parafilm and placed in the dark at 4°C to synchronise germination. After two days the boxes were transferred to a 8L/16D cycle at a light intensity of 85 µmol photons m^{-2} s^{-1} (white fluorescent light: Gro-Lux Sylvania, Germany; and Alto™ Philips), in a room where temperatures were maintained at 25°C and 23°C during the day and night, respectively. The seedlings were grown until the six-leaf stage prior to transfer to hydroponic media. To do this, the lower $^1/_3$ of the tips was excised and the remainder of the tip containing the seedlings were placed in the hydroponic tank apparatus (Figure 2.1 a, b). The media initially diffused through the nutrient gel to the growing seedlings until the plant roots eventually grew into the media. The nutrient solution was replenished as necessary.

2.1.4 Harvesting of plant material

2.1.4.1 Harvesting for chloroplast extracts – A. thaliana *and* A. cepa *in soil*

Leaves were excised and weighed, and the chloroplasts extracted from them immediately (section 2.2.1). All samples were kept on ice during processing.

2.1.4.2 Harvesting for whole leaf extracts – A. thaliana *in hydroponic culture*

Leaves were excised and weighed, immediately frozen in liquid N_2, and then stored at -80°C until subjected to crude protein extraction (section 2.2.2).

Table 2.1 Composition of the nutrient solution used in the nutrient gel and for hydroponic culture of *A. thaliana*.

Stock solutions	Concentration	100% S (mL stock)	2.5% (mL stock)
Macronutrients:			
$Ca(NO_3)_2.4H_2O$	1 M	20	1.25
KNO_3	1 M	30	30
$NH_4H_2PO_4$	1 M	5	5
$MgSO_4.7H_2O$	1 M	10	.25
$Mg(NO_3)_2.6H_2O$	1 M	-	9.75
Fe-EDTA	18.17 mM	2	2
Micronutrients:		5	5
H_3BO_3	46 mM		
$MnCl_2.4H_2O$	9.15 mM		
ZnCl	0.76 mM		
$CuCl_2.2H2O$	0.03 mM		
$Na_2MoO_4.2H_2O$	0.10 mM		
	MQ water to 5 L for both 100% and 2.5% S solution		

100% and 2.5% S media have a final S concentration of 10 mM and 0.25 mM, respectively.

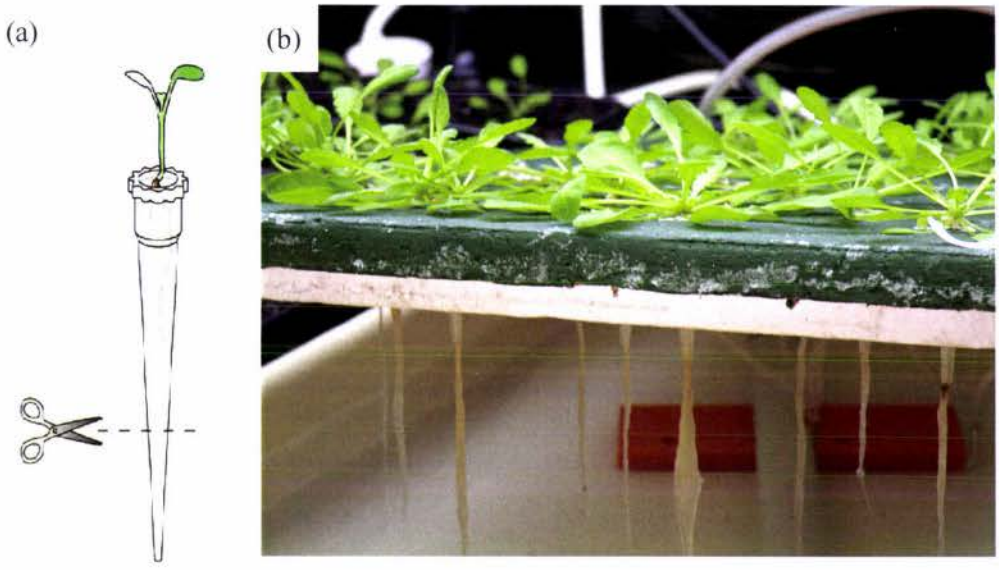

Figure 2.1 Hydroponic setup of *A. thaliana* used in S-deficiency experiments. (a) At the six-leaf stage, the lower $^{1}/_{3}$ of the tips were excised (from Norén *et al*, [64]), and (b) the remainder with the seedlings were placed in the hydroponic tank apparatus and grown in 100% S solution until harvesting.

2.1.4.3 Harvesting for RNA isolation – A. thaliana *in hydroponic culture*

Leaves and roots were excised and weighed, immediately frozen in liquid N_2, and then stored at -80°C until subjected to RNA extraction (section 2.3.1).

2.1.5 Treatment of plant material

2.1.5.1 1- methylcyclopropene (1-MCP)- and C_2H_4-treatment of soil-grown plants

§ 1-MCP: when using 3.3% or 0.14% formulation of 1-MCP, 22 mg or 520 mg respectively were dissolved in 20 or 30 mL of MQ water pre-warmed to 70°C in a water bath. The dissolved 1-MCP was placed immediately in the glass tank with the plants to be treated prior to sealing immediately

§ Glass tank (37 L-capacity-volume) made airtight with a Vaseline-sealed glass lid

to 70°C in a water bath. The dissolved 1-MCP was placed immediately in the glass tank with the plants to be treated prior to sealing immediately

- Glass tank (37 L-capacity-volume) made airtight with a Vaseline-sealed glass lid
- Ethylene (10 ppm in air)

Plants (5-week-old *A. thaliana* and 3-month-old *A. cepa*) were transferred into a temperature-controlled room (18°C and 23°C during the day and night, respectively), and the C_2H_4-treated plants (C) were left standing overnight, in air, in a glass tank. The next day, (C) plants were removed from the tank and watered until saturation. Meanwhile, the tank was made airtight and gassed with C_2H_4 for 40 min, at a flow rate of 1 L/min to provide a final concentration of 10 ppm. The (C) plants were returned to the tank and C_2H_4-treated for 0, 2, 4 or 6 hrs (Figure 2.2), and chloroplast extracts were made (section 2.2.1) at each of these time points. The tank was regassed for a further 10 min every time the seal was broken to remove plants for chloroplast extractions. The following day, control plants (M+C) were treated with 1-MCP in the same glass tank: once at noon, and again in the late afternoon, and then left in the tank overnight. The next day, (M+C) plants were likewise watered, C_2H_4-treated and then subjected to chloroplast extractions as described previously for the (C) plants.

2.1.5.2 Short-term S-deficiency of hydroponic plants of A. thaliana

Plants were grown under S-sufficient conditions until 8 weeks old before half of the plants were subjected to transient S deficiency. To do this, the roots were rinsed in 2.5% S solution, and then placed in fresh 2.5% S solution (+S/-S). The roots of the other half of the plants (the control plants) were rinsed in 100% S solution, and then replaced with fresh 100% S solution (+S/+S). Leaf and root material was sampled at various times following transfer, and ultimately subjected to total RNA extraction for RT-PCR.

Figure 2.2 Plants were treated with 10 ppm C_2H_4 in a glass tank made airtight with Vaseline. Both *A. cepa* (depicted) and *A. thaliana* plants were subjected to C_2H_4-treatment using the same apparatus.

2.2 Biochemical methods

2.2.1 Chloroplast extraction

- Isolation medium (IM): 50 mM Tricine-KOH (pH7.9) containing 2 mM Na_2EDTA (pH8.0), 1 mM $MgCl_2$, 0.33 M sorbitol
- Ultraturrex blender (Kanke and Kunkel GmBrl and Co, IKA-Labortechnick, Britain)
- Nappy liners: boiled in MQ water for 2-3 minutes, rinsed in RO water, rewet in MQ water and stored at 4°C until use
- Percoll gradient (Table 2.2)

Aliquots of leaf tissue (usually 5g) were weighed into 25 mL cold IM, ground by Ultraturrax blender at medium speed for 5-10 s, and the slurry filtered through one layer of nappy liner and the filtrate collected in 15 mL Oakridge tubes. The filtrate was centrifuged at 3,000 rpm for 3 min at 4°C. The resulting

supernatant was discarded, and the pellet carefully resuspended in 5 mL of IM. The resuspended pellet was loaded onto a 40%/80% Percoll gradient and then centrifuged at 4500 rpm for 10 min at 4°C. The upper band of broken chloroplasts and much of the upper 40% Percoll were discarded, leaving the intact chloroplasts at the 40%/80% interface. The remaining gradient was diluted by mixing with an additional 10 mL IM then centrifuged at 5,000 rpm for 10 min at 4°C. At this stage, the intact chloroplasts typically sat in the lower $^1/_3$ of the diluted gradient. Most of the upper diluted gradient was removed, and the remainder containing intact chloroplasts was diluted further, again by mixing with an additional 10 mL of IM. Following centrifugation at 5,000 rpm for 10 min at 4°C, the resulting pellet of intact chloroplasts was resuspended in 0.5 mL IM and stored at -20°C prior to further use.

Table 2.2 Composition of the 40%/80% Percoll gradient used in the extraction of intact chloroplasts by differential centrifugation.

	To make 40 mL of each	
Reagents	40%	80%
Percoll	16 mL	32 mL
Na_2EDTA pH8.0	160 µL	160 µL
$MgCl_2$	40 µL	40 µL
Tricine-KOH pH7.9	4 mL	4 mL
Sorbitol	2.4 g	2.4 g
Milli-Q water to	40 mL	40 mL

Gradients composed of 500 µL each of 40% and 80% Percoll, were made in microcentrifuge tubes and allowed to resolve overnight prior to use.

2.2.2 Crude protein extraction from whole leaf samples

- Whole leaves of *A. thaliana*
- Extraction buffer: 0.1 M K_2HPO_4 (pH7.5) containing 0.5 M Na_2EDTA (pH8.0), 1 mM DTT, 50 mM PMSF, 5 mM pyridoxal 5′ L-phosphate

Leaf samples were ground in liquid N_s, resuspended in extraction buffer (3 mL/g tissue) and then vortexed and centrifuged at 14,000 rpm for 5 min at 4°C. The supernatant was removed to fresh microcentrifuge tubes, the protein content determined (section 2.2.4), and the extract subsequently used in western analyses.

2.2.3 Sample preparation for SDS-PAGE and western analyses

- Whole chloroplasts suspended in IM
- Acid-washed sand
- Mortar and pestle

Prior to use, the chloroplasts were thawed and then ground with 3 volumes of Milli-Q water and some acid-washed sand. The slurry was transferred to 1.5 mL microcentrifuge tubes and centrifuged at 14,000 rpm for 10 min at 4°C. The protein concentration of the supernatant was determined (section 2.2.4), before subsequent use in western analyses.

2.2.4 Determination of protein concentration

- Bovine serum albumin (BSA)
- Plant tissue samples – chloroplast and whole leaf extracts
- Protein assay reagent
- Microtitre plate (Nunc, A/S, Roskdilde, Denmark)
- Anthos HT II plate reader (Anthos Labtech Instruments, Salzburg, Austria)

A series of BSA protein standards were prepared to concentrations of 0, 2, 4, 6, 8 and 10 mg/mL, and 5 and 10 µL aliquots were assayed in a microtitre plate with 20 µL protein assay reagent, and MQ water added to a final volume of 100 µL. The absorbance at 595 nm was measured using the plate reader, resulting in a standard curve. Aliquots of 2 to 20 µL of chloroplast, whole leaf, or whole root extracts were assayed with 20 µL protein assay reagent, and MQ water added to a final volume of 100 µL. The absorbance at 595 nm was

measured, and the protein concentration determined using the BSA standard curve.

2.2.5 Cold acetone method of protein concentration

- Ice-cold acetone
- Plant tissue samples

When necessary, protein samples were concentrated using acetone prior to SDS-PAGE and western analyses. A volume of sample was cooled on ice and then mixed with three volumes of ice-cold acetone, and the proteins were precipitated at -20°C for 3 hr. The proteins were pelleted by centrifugation at 14,000 rpm for 12 min at 4°C and the acetone removed. The pellet was air-dried for 5-10 min prior to resuspension in SDS-PAGE loading dye.

2.2.6 Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE)

- Mini-Protean® III apparatus (Bio-Rad)
- Power pack (Bio-Rad)
- Low range 27-107 kDa, pre-stained SDS-PAGE standards
- 40% (w/v) bis-acrylamide stock solution
- 10% (w/v) ammonium persulfate (APS) (Univar, Auburn, NSW, Australia)
- N,N,N',N-tetramethylethylenediamine (TEMED) (Riedelde haen ag seelze, Hanover, Germany)
- 1x SDS loading dye: 50 mM Tris (pH 6.8) containing 10% (v/v) glycerol, 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol (added just before use)
- 4x Resolving gel buffer: 1.5 M Tris-HCl (pH 8.8), 0.4% (w/v) SDS
- 2x Stacking gel buffer: 0.5 M Tris-HCl (pH 6.8), 0.4% (w/v) SDS
- SDS-PAGE running buffer (pH ~8.2): 25 mM Tris, 192 mM glycine, 0.1% (w/v) SDS

SDS-PAGE separates proteins by relative molecular mass. A 12% resolving gel (Table 2.3) was poured between glass plates assembled in a Mini-Protean apparatus until 1 cm below the level of the wells indicated by where the well-forming comb sits. The gel was overlaid with water and allowed to polymerise for 45 min. Once polymerised, the water was discarded and the gel rinsed with 1x stacking gel buffer. The stacking gel (Table 2.3) was poured and the well-forming combs inserted between the glass plates. The stacking gel was allowed to polymerise for 30 min before the gel assembly was transferred to the electrophoresis tank. The tank was filled with SDS-PAGE running buffer and the combs removed. Prior to loading, samples were prepared by boiling with one volume of SDS loading dye for 5 min, followed by centrifugation at 14,000 rpm for 5 min at room temperature. Ten µL of protein standards were loaded as required. Electrophoresis was run at 150 V for 1 to 1.5 hr.

Table 2.3 Composition of resolving and stacking gels used in SDS-PAGE.

Reagents[a]	Resolving gel (12%) (mL)	Stacking gel (mL)
Milli-Q water	4.5	3.25
4x resolving gel buffer	2.5	–
4x stacking gel buffer	–	1.25
40% (w/v) bis-acrylamide	3	0.5
10 % (w/v) APS	0.1	0.025
TEMED	0.01	0.0032

[a]Quantities shown to make two SDS-PAGE gels. Components were added in the order shown; APS and TEMED must be added just prior to pouring as they catalyse bis-acrylamide polymerisation.

2.2.7 Coomassie Brilliant Blue (CBB) staining

- CBB stain: 0.2% (w/v) CBB R-250 (Sigma), 40% (v/v) methanol, 10% (v/v) glacial acetic acid
- CBB destain: 30% (v/v) ethanol

CBB staining allows visualisation of proteins separated by SDS-PAGE. After completion of SDS-PAGE, gels were immersed in CBB stain with agitation for 30 min. The gels were then rinsed with several changes of CBB destain until the background was clear.

2.2.8 Western analyses

2.2.8.1 Protein transfer onto PVDF membrane

- Mini Trans-blot® apparatus (Bio-Rad)
- Transfer buffer: 25 mM Tris, 190 mM glycine, and 10% (v/v) methanol. The last component is added just before use.
- Methanol
- PVDF membrane: pore size 0.2 μm (Immobilin-P, Millipore Corporation, Bedford, USA)
- 3 mm Chromatography paper (Whatman)

The transfer cassette was assembled as shown in Figure 2.3. Prior to assembly, the PVDF membrane was first wet with methanol and then soaked in transfer buffer. The scotch pad and chromatography paper were also soaked in transfer buffer before assembly. The constructed cassette was then placed in the Mini Trans-blot® apparatus and submerged in transfer buffer. The transfer was performed at .25 A for 45 min-1 hr. Reversible staining with Ponceaus S staining solution was carried out as necessary prior to immunodetection (section 2.2.8.2).

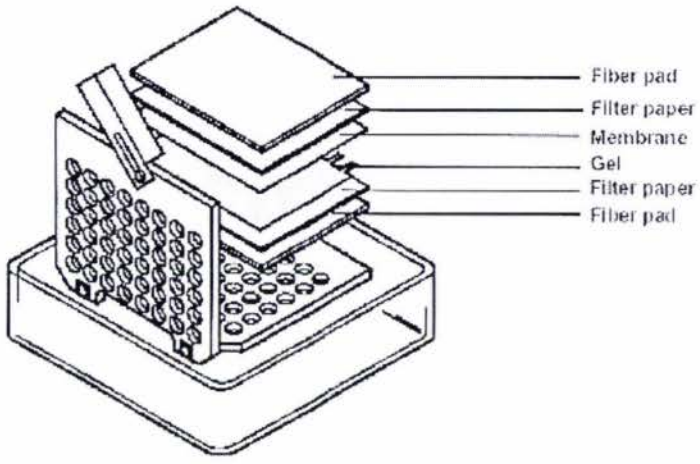

Figure 2.3 Construction of the transfer cassette for transfer of proteins to a PVDF membrane following SDS-PAGE.

2.2.8.2 Immunodetection of proteins on PVDF membrane – chromogenic method

- PBS: 50 mM sodium phosphate (pH 7.4) containing 250 mM NaCl
- Blocking solution: 12.5% (w/v) skim milk powder in PBS
- PBS-Tween: 0.2% (w/v) Tween-20 in PBS
- Primary antibody: 1:500 dilution in PBS. Antibodies were isolated from rabbits inoculated with recombinant enzyme proteins expressed in *E. coli* – anti-ATPS (α-ATPS) and α-*O*ASTL against onion enzymes (raised in rabbits at the Small Animal Unit, Massey University, Palmerston North, NZ), α-SiR and α-APR against *A. thaliana* enzymes (Dr Stanislav Kopriva, John Innes Centre, UK)
- Secondary antibody (anti-rabbit alkaline phosphatase-conjugated, Sigma): 1:10,000 dilution in PBS
- Substrate buffer: 100 mM Tris (pH9.6) containing 100 mM NaCl
- 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma) stock: 10 mg/mL in dimethyl formamide
- Nitro blue tetrazolium (NBT, Sigma) stock: 20 mg/mL in substrate buffer
- 1 M $MgCl_2$
- Developing buffer: 50 mL substrate buffer containing 10 mM $MgCl_2$, 0.2 mg/mL NBT stock, 0.1 mg/mL BCIP stock

After protein transfer, the PVDF membrane was submerged in blocking solution overnight at 4°C, or for 1.5 hr with gentle agitation at room temperature. The membrane was washed with 3 changes of PBS-Tween of 5 min each, before incubation with the primary antibody for 1 hr at 37°C, with agitation. The membrane was washed thrice as described previously prior to incubation with secondary antibody for 1 hr at room temperature, with agitation. The membrane was then rinsed with two changes of substrate buffer, and finally developed. The reaction was terminated by rinsing the membrane with RO water.

2.2.8.3 Immunodetection of proteins on PVDF membrane – chemiluminescent method

- PBS-Tween: 0.2% (w/v) Tween-20 in PBS
- Blocking solution: 3% (w/v) skim milk powder in PBS-Tween
- Primary antibody (α-APR against *A. thaliana* enzyme, from Dr Stanislav Kopriva, John Innes Centre, UK): 1:3,000 dilution in blocking solution
- Secondary antibody (horseradish peroxidase-conjugated, Sigma): 1:15,000 dilution in PBS-Tween
- Blot rinse: 10 mM Tris (pH 7.4) containing 0.1% (w/v) Tween-20, 1 mM Na_2EDTA, 150 mM NaCl
- SuperSignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, IL, USA)
- Photographic film (Kodak BioMax Light film)

After protein transfer, the PVDF membrane was submerged in blocking solution for 2 hr with agitation at room temperature. The membrane was then incubated with the primary antibody overnight at 4°C, with gentle agitation. The following day, the membrane was rinsed thrice and then washed with seven, 5 min changes of PBS-Tween. The membrane was incubated with secondary antibody for 2 hr at room temperature, before it was rinsed and washed again as described previously. The membrane was then rinsed and incubated for 7 min in blot rinse. The membrane was exposed to the

SuperSignal® substrate for 5 min, blotted dry and then exposed to photographic film for 3-5 s in the dark before developing.

2.2.9 Ponceaus S staining

- Ponceaus S staining solution (Sigma)
- 100 mM NaOH

Ponceaus S staining allows visualisation of proteins on a PVDF membrane, and was performed as described by the manufacturer. The membrane was submerged in Ponceaus S staining solution for 5 min, and then rinsed with MQ water until the background was clear. The protein bands were visualised, and then the stain was removed by submerging in NaOH. The membrane was further rinsed with MQ water for 2 to 3 min before proceeding with immunological detection.

2.3 Molecular methods

2.3.1 Total RNA extraction

- Glassware and mortars and pestles baked at 180°C overnight before use
- 15 mL Oakridge tubes were immersed in 0.2% (v/v) H_2O_2 overnight, rinsed in diethylpyrocarbonate (DEPC)-treated MQ water and autoclaved before use
- DEPC-treated MQ water: 1% (v/v) DEPC-treated, RNAse-free MQ water was used to avoid RNA degradation
- Extraction buffer: 200 mM sodium borate buffer (pH9.0) containing 30 mM EGTA, 1% (w/v) SDS, 1% (w/v) sodium deoxycholate, 10 mM DTT, 2% (w/v) PVP-40, 1% (w/v) NONIDET. The last three components were added just before use
- 20mg/mL proteinase K
- 2 M KCl
- 4 M LiCl

- 3 M NaOAc (pH 5.2)
- 24:1 (v/v) chloroform:isoamylalcohol
- Isopropanol: used at 1:1 (v/v) with aqueous phase
- Ice-cold 70% (v/v) ethanol

In a fume hood, tissue samples were ground in liquid N_2-cooled mortars and pestles, and then added to the extraction buffer (5 mL/g tissue) in Oakridge tubes. The extraction was vortexed for 30 s before addition of proteinase K (37.5 uL/g tissue), and was then incubated at 42°C for 1.5 hrs with agitation before 400 µL of KCl was added. The extraction mix was further incubated on ice for 30 min with agitation. The extraction mix was then centrifuged at 26,000 g for 20 min at 4°C to pellet the cellular debris and denatured proteins. The resulting supernatant (5 mL) was transferred to a fresh Oakridge tube and an equal volume of LiCl was added to precipitate the RNA overnight at 4°C. The RNA was collected by centrifugation at 26,000 g for 30 min at 4°C and was either resuspended in (i) 500 uL of DEPC-treated MQ water and 50 uL of NaOAc, or (ii) in DEPC-treated MQ water only (and immediately subjected to DNAseI treatment; section 2.4). The RNA resuspended with MQ water/NaOAc (i) was transferred to a sterile microcentrifuge tube, to which 500 uL of chloroform:isoamylalcohol was added. The aqueous and organic phases were vortexed for 30 s, and then separated by centrifugation at 14,000 rpm for 5 min at 4°C. A 400 uL aliquot of the (upper) aqueous phase was removed into another microcentrifuge tube and the organic phase re-extracted with 400 uL of DEPC-treated MQ water and 40 uL NaOAc. The aqueous and organic phases were again mixed by vortexing for 30 s, and separated by centrifugation at 14,000 rpm for 5 min at 4°C. Another 400 uL aliquot of the upper aqueous phase was removed into another microcentrifuge tube. An equal volume of isopropanol was added to both aqueous supernatants to precipitate the RNA for 1 hr on ice, and both were then pelleted by centrifugation at 14,000 rpm for 30 min at 4°C. The pellets were washed by inversion with 1 mL ice-cold 70% ethanol, followed by centrifugation at 14,000 rpm for 1 min at 4°C. The pellets were air-dried, both resuspended in DEPC-treated MQ water to give a total pooled volume of 25-50 uL of, and stored at -80°C until required.

2.3.2 RNA quantification

- Ultrospec 3000 UV/visible spectrophotometer (Pharmacia Biotech)

Each sample was diluted 100-fold and 50-fold in DEPC-treated MQ water, and the absorbance of these at 260 nm and 280 nm were measured using the Ultrospec 3000 UV/visible spectrophotometer. RNA purity was determined by the OD_{260}:OD_{280} (2.0 if pure), and the undiluted RNA concentrations were calculated as follows, before the values calculated from each dilution were averaged to obtain a final concentration:

RNA concentration (ug/uL) = A_{260nm} x 40 ug/mL ÷ 1,000 x dilution factor

2.4 DNase I treatment

- Block thermostat (BT1, Grant Instruments (Cambridge) Ltd, Cambridge, England)
- RNA sample
- Deoxyribonuclease I, amplification grade (Invitrogen):
  DNase I, Amp grade (1 U/μL)
  10x DNase I reaction buffer
  25 mM EDTA (pH 8.0)

The DNase I treatment of RNA samples was attempted using three different methods. In the first and second methods, total RNA extraction was carried out to completion (section 2.3.1) before DNase I treatment. DNase I treatment was performed as described by the manufacturer. The reaction was set up on ice as outlined in Table 2.4, incubated at room temperature for 15 min, and then the reaction was terminated by addition of 1 μL of 25 mM EDTA and incubation for 5 min at 65°C. In the first method, the RNA sample was used directly for RT-PCR. In the second, the RNA sample was further subjected to EtOH precipitation in an attempt to remove any residual DNase I (section 2.5).

For the third DNase I-treatment method, DNase I treatment was performed in the midst of RNA extraction, after RNA pellet resuspension in DEPC-treated water (section 2.3.1). The DNase I treatment reaction was set up on ice, in a microcentrifuge tube (Table 2.5), then incubated and inactivated as previously described. The RNA sample is then subjected to the remainder of the RNA extraction protocol to remove any residual DNase I (section 2.3.1).

Table 2.4 Setup of DNase I treatment of RNA samples after RNA extraction.

	Volume (µL)
RNA sample (1 µg)	*x*
10x DNase I reaction buffer	1
DNase I, Amp grade	1
DEPC-treated MQ water to 10 µL	

Table 2.5 Setup of DNase I treatment of RNA samples during RNA extraction.

	Volume (µL)
Resuspended RNA sample	450
10x DNase I reaction buffer	50
DNase I, Amp grade (0.5:1 (w/v) of sample fresh weight:DNase I	*x*

2.5 Ethanol precipitation of RNA

- Sample RNA previously treated with DNase I
- 3 M NaOAc (pH 5.2)
- 100% (v/v) ethanol
- 80% (v/v) ethanol

The RNA sample that had already been subjected to DNase I treatment was additionally ethanol-precipitated in an attempt to remove any residual DNase I. A volume (0.1) of NaOAc and 3 volumes of ice-cold 100% EtOH was added to the RNA sample. The RNA was precipitated overnight at -20°C and then pelleted by centrifugation at 14,000 rpm for 15 min at 4°C. The pellet was rinsed with 80% EtOH and then centrifuged again at 14,000 rpm for 5 min at 4°C. The 80% EtOH was discarded and the pellet air-dried before resuspension in DEPC-treated MQ water, and used for RT-PCR.

2.6 Reverse transcriptase – polymerase chain reaction (RT-PCR)

2.6.1 cDNA synthesis/reverse transcriptase reaction

- Sample RNA
- DEPC-treated MQ water
- Block thermostat (BT1)
- Thermocycler (Biometra)
- Thermoscript™ RT-PCR System (Invitrogen):

  50 µM Oligo(dT)$_{20}$ primers

  10 mM dNTP mix

  5x cDNA synthesis buffer

  0.1 M DTT

  RNaseOUT™ (40 U/µL)

  ThermoScript™ reverse transcriptase (15 U/ µL)

  E. coli RNAse H (2 U/µL)

The reverse transcription reaction was performed as described by the manufacturer (Invitrogen). Each reaction tube, set up as described in Table 2.6 (i), was denatured at 65°C for 5 min and then placed on ice. A master reaction mix, sufficient for the total number of reaction tubes (re Table 2.6 (ii)), was prepared on ice and 8 µL added to each reaction tube. Each reaction was then incubated in a thermal cycler at 50°C for 45 min, followed by 85°C for 5 min to

terminate the reaction. RNAse H (1 μL) was added and the reaction tubes incubated at 37°C for a further 20 min before being used for PCR.

2.6.2 Polymerase chain reaction (PCR)

- RT reaction products
- DEPC-treated MQ water
- Gene-specific primer sets (*AtACS1-12*)
- Thermoscript™ RT-PCR system (Invitrogen):

  10x PCR buffer –Mg

  50 mM MgCl2

  10 mM dNTP mix

  Platinum® *Taq* DNA polymerase (5 U/μL)

PCR reactions were performed using gene-specific primer sets (Table 2.7, Table 2.8) and subjected to the following incubation: 94°C for 2 min, 1 cycle; 94°C for 15 s, 62°C for 15 s, and 72°C for 3 min, 35 cycles; and 72°C for 3 min, 1 cycle [51]. Reactions were performed in 25 μL volumes using the Thermoscript™ RT-PCR system (Invitrogen) (Table 2.9).

Table 2.6 Composition and volumes of RT-PCR reagents used

Component	Volume (μL)
(i) Reaction tube	
RNA sample (1-5 μg)	*x*
50 μM Oligo(dT)$_{20}$	1
10 mM dNTP mix	2
DEPC-treated MQ water	to 12 μL
(ii) Master reaction mix (for 1 reaction)	
5 x cDNA synthesis buffer	4
0.1 M DTT	1
RNaseOUT™ (40 U/μL)	1
DEPC-treated water	1
ThermoscriptTM reverse transcriptase (15 U/μL)	1

2.7 Agarose gel electrophoresis

- 25x TAE buffer: 1 M Tris (pH8.0) containing 0.5 M acetic acid, 25 mM EDTA
- 10x SUDS: 100 mM EDTA (pH 8.0) containing 50% (v/v) glycerol, 1% (w/v) SDS,
  0.025% (w/v) bromophenol blue
- Working solution of 1 kb ladder: 10 mM Tris-HCl (pH7.5) containing 1mM Na_2EDTA, 50 mM NaCl, 1 μg/μL 1 kb DNA ladder, 20% (v/v) 10x SUDS. Components were added in the order shown.
- Ethidium bromide (EtBr, 10 μg/mL)

Agarose gel electrophoresis was used to analyse PCR products. Agarose was dissolved in 1x TAE buffer, with heating, to give a 1% (w/v) solution. This was then poured into an
assembled horizontal gel apparatus containing a well-forming comb and allowed to set for 30 min before being immersed in 1x TAE buffer. The well-forming comb was removed prior to loading DNA samples. DNA samples were prepared by addition of 3-5μL 10x SUDS. A 1 kb DNA ladder was loaded as required. Electrophoresis was run at 100 V for 1 hr. Once complete, the gel was stained in EtBr for 10 min, and then destained in MQ water for 10 min. The gel was then visualised on a short wavelength (340 nm) UV Transilluminator (Gel Doc 2000^{TM}, Bio-Rad) and photographed.

Table 2.7 Sequences of gene-specific primer sets used for PCR amplification of *AtACS*1-12 (non-exon-spanning primers) from *A. thaliana* leaf and root material [51].

			Expected product size if cDNA was used as a template (bp)
AtACS1	F1:	TGTCTCAGGGTGCATGTGAGAATCAACTT	1,463
	R1:	AGCTCGAAGCAATGGTGAATGAGGAGACA	
AtACS2	F1:	GCGACTAACAATCAACACGGAG	1,417
	R1:	ACATTATCCCTGGAGACGAGAGAC	
AtACS4	F1:	CCAAGTCTCTTCGTATTTCCTT	1,351
	R1:	TAGTCGGAAAACCCAGTTAGAGAC	
AtACS5	F1:	CCAGCTATGTTTCGATCTAATCGAGTCATGGTTAAC	1,244
	R1:	TCCATGAAACCCGGAAAACCCAGTTAGAGACTGTC	
AtACS6	F1:	TGACGGTCACGGCGAGAATTCCTCTTATT	1,333
	R1:	CCTGAGGTTACTCTGCCAACACTTCTTCT	
AtACS7	F1:	AACAACAACAACGTCGAGCTTTCTCGAGT	1,191
	R1:	AGATCCCGGAGATATATTCAGGTTCAGCT	

Table 2.7 continued

AtACS8	F1:	CGATCTCATTGAGTCATGGCTTGCTAAGA	1,294
	R1:	ACGGTCCATCAACGAACCTCTTCAATCTA	
AtACS9	F1:	GGATGGGAAGAATACGAGAAGAACCC	1,247
	R1:	ATCACTCTTCTACTATCTGTTGACTC	
AtACS10	F1:	AGGGTTATTGTTCCGTTACAAGGTGTGGT	1,492
	R1:	TACGGAACCATCCTGGTTCGATACAGTGA	
AtACS11	F1:	AACCTCGGCTAACGAGACTCTAATGTTCT	826
	R1:	ATGACACGATGAGCCTGGAGAGATGTTAA	
AtACS12	F1:	TTCGTCGGCTCTCTCATTCCTTGTTGTCT	1,203
	R1:	ACCACCACTCTCAGCACATGGTATTCCTA	

Table 2.8 Sequences of gene-specific primer sets used for PCR amplification of *AtACS*1-12 (exon-spanning primers) from *A. thaliana* leaf and root material.

			Expected product size (bp)
AtACS1	F2:	TCTCGCCGAAAATCAACTTT	329
	R2:	ACCTTAAGTCTCTATCGAATGCAGC	
AtACS2	F2:	GGTCTTGCAGAGAATCAGCTTT	326
	R2:	AAGTCTCTATCAAATGCGGCA	
AtACS4	F2:	GTCTTGCGGAAAATCAGCTATG	328
	R2:	TTTAGATCCCTATCAAACCCTGG	
AtACS5	F2:	ATGCCTGAATTCAAAAAAGCTATG	515
	R2:	TCCGTGTCTTCGAGTTTCTTGT	
AtACS6	F2:	CTGAAAATCAGCTTTGTGGA	321
	R2:	TCAAATCTCTATCAAACCCTGG	
AtACS7	F2:	CGCTGAGAATCAGGTCTCG	328
	R2:	TCTCAAATCTCTATCGAATCCTGG	
AtACS8	F2:	GGGTCTAGCAGAAAATCAGTTGTCT	332
	R2:	ATTTCAAATCCCTATCAAATCCTGG	

Table 2.8 continued

AtACS9	F2:	GTCTTGCCGAAAATCAGCTATG	330
	R2:	ATTTCAAGTCTCTATCGAATCCTGG	
AtACS10	F2:	TTTAGCTCAAAACAACAAGTTGAGTTT	310
	R2:	AACATCCCTATCATATCCGGG	
AtACS11	F2:	CTGAAAACCAGCTTTCTTTTGAC	321
	R2:	TGAGATCTCTATCAAACCCTGGA	
AtACS12	F2:	TTCGTCGGCTCTCTCATTCCTTGTTGTCT	334
	R2:	ACCACCACTCTCAGCACATGGTATTCCTA	

Table 2.9 Composition of PCR reaction mix used to amplify *AtACS1-12* from *A. thaliana* leaf and root material.

Component	Volume per reaction (μL)
10x PCR buffer (-$MgCl_2$)	2.5
50 mM $MgCl_2$	.75
10 mM dNTP mix	.5
10 μM sense primer	.5
10 μM antisense primer	.5
Platinum® *Taq* DNA polymerase (5 U/μL)	.2
RT reaction product	2.5
	DEPC-treated MQ water to 25 μL

3 Results

3.1 The ability of antibodies to detect their target enzymes in both *A. thaliana* and *A. cepa* protein extracts

The α-ATPS, α-APR and α-*O*ASTL antibodies were raised against recombinant proteins accumulated in *E. coli* after expression of the onion (*A. cepa*) genes for each enzyme [65]. These primary antibodies were initially tested to determine their specificity for their target S-assimilation enzymes in various plant tissues, as outlined in Figure 3.1. It was crucial to ensure that the antibodies used would be able to detect the plastidic enzymes of the leaves in both species used in this study, as this is the site where reductive S-assimilation is carried out to completion.

The α-ATPS antibody detected a ~52 kDa band in the leaf and chloroplast extracts, but not the root extract, from onions. This may indicate that either the antibody is not sufficiently sensitive to detect any ATPS from the root extract, or a distinct isoform of ATPS accumulate in the onion root plastids and cytosol. The α-ATPS antibody, which was raised against the onion enzyme, was also able to detect the *A. thaliana* ATPS, as indicated by the ~52 kDa band observed from the western analyses of the chloroplast extract from *A. thaliana.*

In contrast, the α-APR antibody was only able to detect a ~51 kDa band in the leaf and root extracts from onion, and in the leaf extracts from *A. thaliana.* This is surprising since APR activity has been found in only plastids, including the chloroplasts, indicating that all three APR isozymes localise to the plastids but not to any other cellular compartment [1]. It is known that many chloroplasts are inadvertently broken during the isolation process, so the final protein content of the remaining intact chloroplast is usually fairly low. This may indicate that although the α-APR antibody raised against the onion APR was able to bind to the APR enzyme, it was not sensitive enough to produce a detectable signal, at least not by chromogenic methods. Because of this, an α-

Antibody	Approximate detected band size (kDa)	Tissue	Onion (*A. cepa*)		*A. thaliana*	
α-ATPS	52	Leaf		+	N/A	
		Root		-	N/A	
		Chloroplast		+		+
α-APR	51	Leaf		+		+
		Root		+		-
		Chloroplast		-		-
α-SiR	50	Leaf	N/A		N/A	
		Root	N/A		N/A	
		Chloroplast		+		+
α-*O*ASTL	35	Leaf		+		+
		Root		+		+
		Chloroplast		+		+

Figure 3.1 Antibody cross-reactivity summary. The antibodies α-ATPS, α-*O*ASTL and α-APR were raised against the onion enzymes, whereas anti-SiR was raised against the *A. thaliana* enzyme. The approximate band sizes detected by these antibodies are as indicated; + and – indicate ability of antibodies to detect their target enzymes; and N/A indicates untested plant tissue.

APR antibody raised against the *A. thaliana* isozymes was eventually used (courtesy of Dr. Stanislav Kopriva, John Innes Centre, UK), and the binding detected successfully by the more sensitive method of chemiluminescence (Figure 3.2, Figure 3.3, Figure 3.4).

The α-*O*ASTL antibody was able to detect a ~35 kDa band in the leaf, root and chloroplast extracts from both onion and *A. thaliana.* This meant that the antibody was sensitive enough to detect *O*ASTL in all three tested plant tissues, and in both species used in this study.

The α-SiR antibody was raised against the *A. thaliana* enzyme (courtesy of Dr Stanislav Kopriva, John Innes Centre, UK), but was able to detect a band of ~50 kDa in the onion chloroplast extracts.

3.2 Western analyses of chloroplast extracts from the leaves of whole plants of *A. thaliana* and *A. cepa* treated with C_2H_4

The first approach used to investigate the relationship between the plant hormone C_2H_4 and S-assimilation was to treat whole plants of *A. thaliana* or *A. cepa* with C_2H_4, or with 1-MCP, which blocks C_2H_4 action by binding irreversibly to the C_2H_4 receptors. The accumulation or reduction of several enzymes of the S-assimilation pathway in leaf chloroplasts, namely ATPS, APSR, SiR and *O*ASTL were then monitored using western analysis.

3.2.1 Western analyses of chloroplast extracts from the leaves of whole plants of *A. thaliana* treated with C_2H_4

The Ponceaus S stain was performed prior to chromogenic and chemiluminescent detection to show that an equal amount of sample total protein was loaded in each well (Figure 3.2). This enables any changes in enzyme protein accumulation to be compared visually. The equal loading of

sample total protein is most clearly seen as a ~50kDa protein. This is presumed to be Rubisco, which is very abundant in chloroplasts. Many faint bands of higher and lower masses were also detected although they were not distinct enough to be seen after photographing the gel image.

Western analyses of chloroplast extracts from C_2H_4-treated *A. thaliana* (Figure 3.2) show that protein levels of ATPS were higher at all sampling times in plants pretreated with 1-MCP prior to C_2H_4-treatment (M+C), and did not appear to fluctuate throughout the sampling period. In contrast, in plants treated with C_2H_4 alone (C), a peak of ATPS protein accumulation was observed at 4 hr before decreasing to almost undetectable levels.

Protein levels of APR were also higher at all sampling times in the chloroplast extracts from (M+C) plants, and their levels were constant throughout the sampling period. However, in (C) plants, the level of APR protein was highest at 0 hr, and was only detectable at fairly low levels at 2, 4 and 6 hr.

Protein levels of *O*ASTL were higher in (M+C) plants, and did not appear to fluctuate throughout the sampling period, whereas in (C) plants, a peak of *O*ASTL protein accumulation was observed at 4 hr. The *O*ASTL protein in the chloroplast extracts from both (M+C) and (C) plants were detected as doublets of ~35 kDa. Various factors such as alternative translation start and end sites, protein truncation, and most commonly phosphorylation will result in proteins of different masses that may still be recognisable by the same antibody. Any of these may be responsible for the doublets, although an attempt to isolate a phosphorylated and unphosphorylated form of *O*ASTL from chloroplast extracts of onion has so far been unsuccessful (Thomas, L.; *pers comm.* [66]). An unknown protein of ~30 kDa was also detected in (C) plants at 0 hr. It may be a truncated or degraded form of *O*ASTL, which was still recognised by the α-*O*ASTL antibody.

Protein levels of SiR were highest at 0 and 2 hr in chloroplast extracts from (M+C) plants, but then decreased and remained fairly low for the

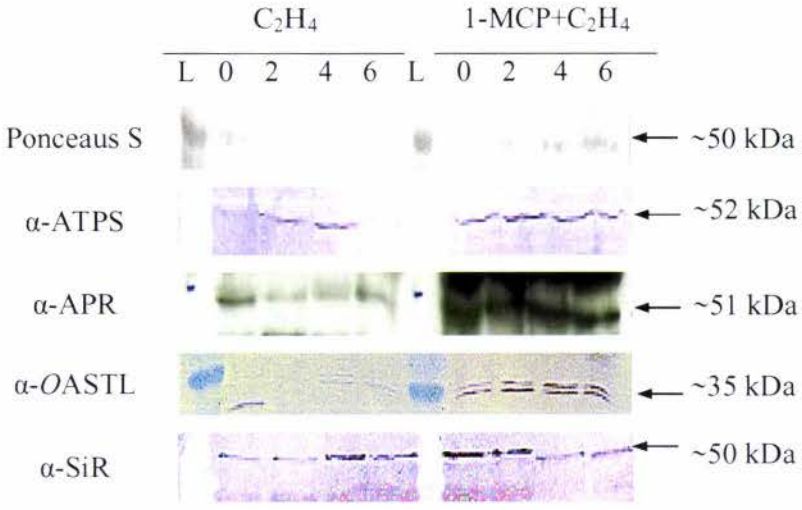

Figure 3.2 Western analyses of chloroplast extracts from C_2H_4-treated, whole plants of *A. thaliana*. The Ponceaus S stain indicates approximate equal loading to determine changes in accumulation of the S-assimilation enzymes. The antibodies used to probe for ATPS, APR, OASTL and SiR and their approximate sizes are as indicated. L, protein ladder; 0-6, sampling time in hr following commencement of C_2H_4 treatment.

3.2.2 Western analyses of chloroplast extracts from leaves of whole plants of *A. cepa* treated with C_2H_4

For the experiments with onion, two different cultivars were used. The first, W202A, is a pungent line that is marked by a higher accumulation of S-containing sulfoxides (per g of fresh weight). Conversely, the second line, Texas Grano, is a milder cultivar that accumulates less sulfoxides.

Unfortunately, the Ponceaus S stain indicated unequal loading of protein for the samples from the both onion cultivars, most noticeably the samples of (M+C) plants of the W202A cultivar, at 0 and 2 hr, and the (M+C) plants of the Texas Grano cultivar, at 4 hr (Figure 3.3). However, a visual comparison of the changes in enzyme protein accumulation could still be made as the protein levels of none of the enzymes were highest at the sampling times when the Ponceaus S stain suggested a higher protein loading.

In cultivar W202A onions, protein levels of ATPS were unchanged regardless of treatment (Figure 3.3). In contrast, APR protein levels were higher in (M+C) at 0 and 2 hr. In (C) plants, APR protein peaked at 4 hr before declining to undetectable levels, although the level of accumulation was much less than in the (M+C) plants. In common with ATPS, *O*ASTL protein levels appear unchanged regardless of treatment. In contrast, SiR protein levels decreased gradually to undetectable levels in (C) plants, but increased slightly to peak at 2 and 4 hr, before decreasing gradually at 6 hr, in (M+C) plants.

In the Texas Grano cultivar, ATPS protein levels peaked at 6 hr in the chloroplast extracts from (C) plants, but remained constant in (M+C) plants (Figure 3.3). Protein levels of APR were highest at 0 hr in (C) plants before declining to undetectable levels. Similarly, in (M+C) plants APR protein levels were highest at 2 hr before also becoming undetectable. Protein levels of *O*ASTL were higher in (M+C) plants at 0, 2 and 4 hr before declining slightly, but peaked at 2 hr in (C) plants before gradually declining. In (C) plants, levels

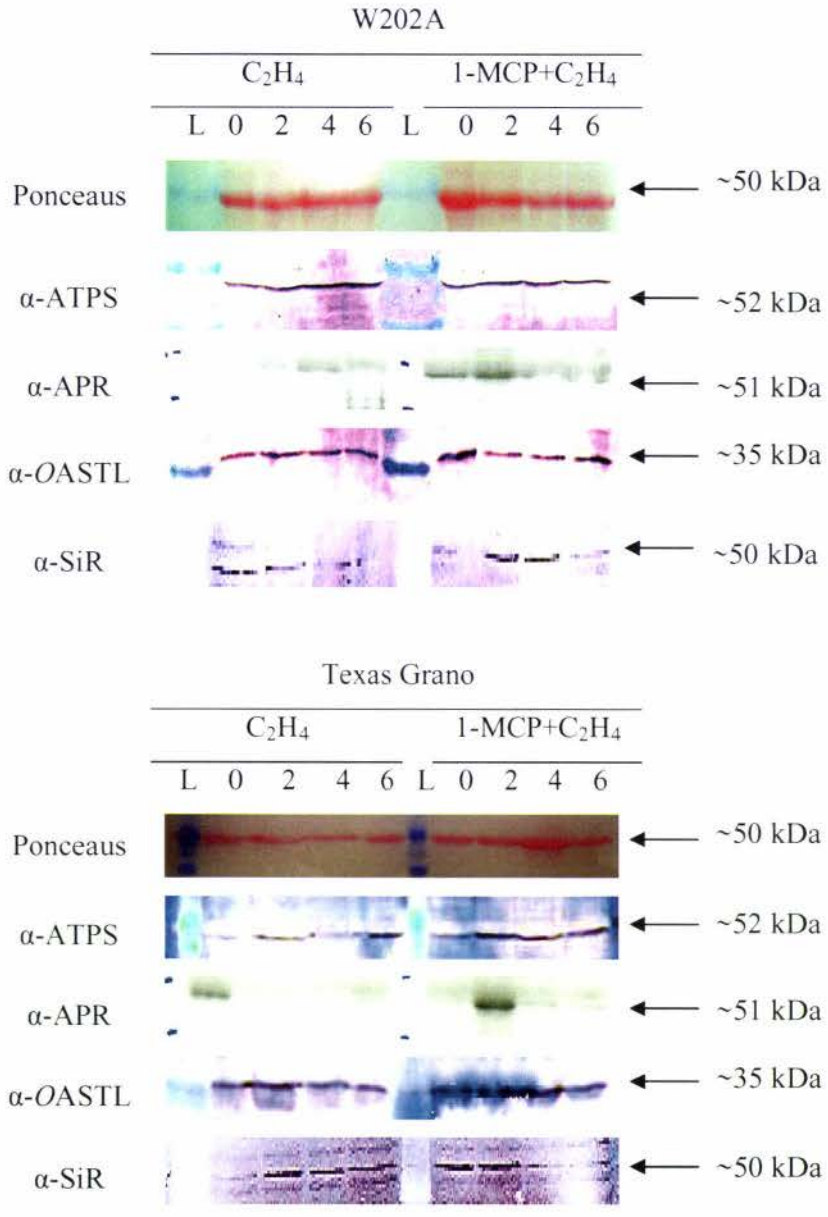

Figure 3.3 Western analyses of chloroplast extracts from C_2H_4-treated, whole plants of onions. The antibodies used to probe for ATPS, APR, *O*ASTL and SiR and their approximate sizes are as indicated. L, protein ladder; 0, 2, 4 and 6, sampling time in hr after commencement of C_2H_4 treatment.

of SiR protein peaked at 2 hr and remained constant thereafter, but declined to undetectable levels after 2 hr in (M+C) plants.

3.3 Expression of the members of the *AtACS* gene family in *A. thaliana* under short-term S-deficient conditions

The C_2H_4-treatment experiments appear to indicate that the S-assimilation enzymes, especially in *A. thaliana*, were negatively regulated by this phytohormone. This is because the protein levels of these enzymes were generally higher in the chloroplast extracts from plants that had been pre-treated with the C_2H_4-signaling inhibitor 1-MCP ((M+C) plants, Figure 3.2).To complement these results, additional *A. thaliana* plants were subjected to short-term S depletion, and the induction or repression of C_2H_4 biosynthesis monitored. To do this, changes in expression of the gene encoding the rate-determining step of the C_2H_4-biosynthetic pathway, ACC synthase (ACS), was examined. The model plant species, *A. thaliana*, was used for these experiments because its entire genome has been sequenced, and all 12 members of the *AtACS* gene family have been identified. In this study, primers were designed to amplify 11 members of the *AtACS* gene family (*AtACS3* was excluded because this gene codes for an inactive enzyme).

For all sampling times, the S-depleted plants were morphologically indistinguishable from the control S-sufficient plants (data not shown). The apparent lack of change in outward appearance agrees with previous findings [5], and would be expected over the very short treatment time.

3.3.1 Western analyses of APR accumulation of whole leaves from *A. thaliana* subjected to different S supply

To determine the time frame in which to detect any changes in *AtACS* expression, the accumulation of the key S-assimilation enzyme, APR, was examined in response to changes in S supply.

Previous findings show that APR most readily responds to changes in the plant's S status, as seen by an increase in APR mRNA levels [5, 26, 27, 67] in response to S-deficiency. Conversely a decrease in APR mRNA levels, protein accumulation and enzyme activity is observed in response to application of the thiols L-Cys and GSH, as well as the reduced S compound H_2S [42, 43]. The APR enzyme was therefore used as a marker for the onset of a response to S-depletion. For initial experiments, western analyses showed that APR levels peaked at 2 hr, before gradually becoming undetectable (Figure 3.4 a). This showed that the plant was beginning to respond to S-depletion (2.5% S) and indicated a time frame in which changes in the expression of the *AtACS* genes could be examined. That is, the activation or repression of C_2H_4 biosynthesis, *via AtACS* repression or induction, may be part of the plant response to S-depletion which is manifested as the aforementioned change in APR protein levels. In subsequent experiments, therefore, the shorter sampling times of 0, 30, 60 and 120 min were used, such that the 120 min sampling point signalled the end of the time course (Figure 3.4 b).

The Ponceaus S stain again indicated an unequal loading of protein, namely for the samples from the S-sufficient plants (100% S) at 120 min (Figure 3.4 b). The lower amount of APR protein detected at this sampling time reflects this unequal loading, and was therefore disregarded.

In these later experiments, levels of APR protein peaked at 0 min regardless of S availability. Under S-sufficient conditions, protein levels gradually declined after this initial peak. In contrast, APR protein levels in S-depleted plants remained high at 0 and 30 min, declined slightly at 60 min, then increased again at 120 min.

The peak in APR protein at 0 min regardless of S availability may be due to the oxidative stress incurred when the roots were exposed to air while being rinsed with media before transfer. The oxidation of a redox-responsive pair of Cys residues in the active site of APR1 results in the formation of a disulfide bond necessary for the activation of this isoenzyme [28]. It is possible this change in redox conditions also resulted in a greater accumulation of APR protein,

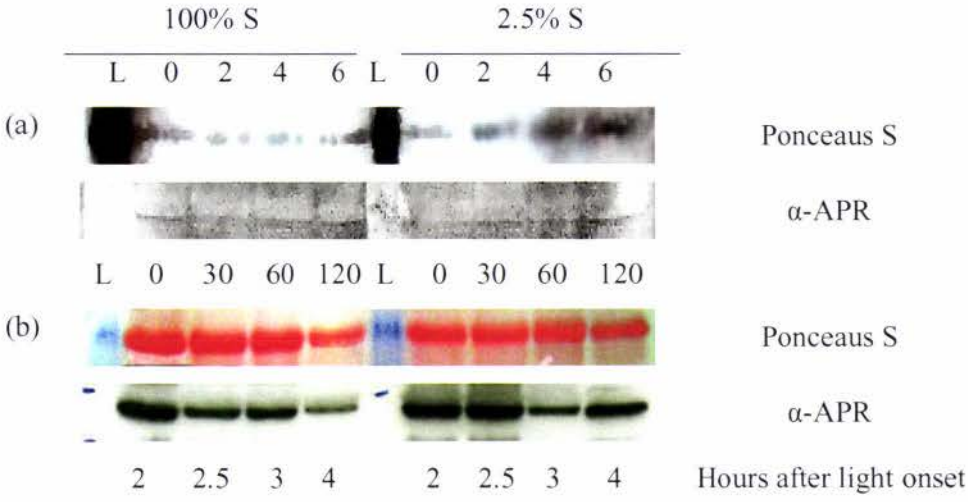

Figure 3.4 Western analyses of whole leaf extracts from S-deficient *A. thaliana* using (a) chromogenic and (b) chemiluminescent methods. The Ponceaus S stain indicated approximate equal loading of total protein, except for the sample taken at 120 min from the S-sufficient (100% S) plants. The APR protein detected at this sampling time was therefore disregarded from the final analysis of results. The hours after light onset and the primary antibody used are indicated. L, protein ladder; 0, 2, 4 and 6, and 0, 30, 60 and 120 are the sampling times in hr and min, respectively, after the plants were transferred to fresh 100% S or 2.5% S media.

although this is contrary to the observations of Bick *et al.* [28] that the elevated APR1 activity was not accompanied by an increase in the level of immunodetectable protein. This may be explained by the different growth conditions employed in this study, although measurements of APR activity would be necessary to help explain this discrepancy.

The pattern of APR protein accumulation may also be complicated by the inherent diurnal rhythm that regulates APR mRNA accumulation and enzyme activity in addition to protein levels [45]. These authors found that APR protein levels peaked 4 hr after light onset. Assuming that the plants did not recover from oxidative stress until 120 min, it appeared that oxidation and S-depletion had a synergistic effect on this diurnal rhythm, so there was a greater amount of APR protein in the roots from S-depleted plants than in S-sufficient plants at 30 min and perhaps also 120 min (Figure 3.4 b).

3.3.2 Examination of the expression of *AtACS* genes in *A. thaliana* under short-term S-deficient conditions by RT-PCR using a non-exon-spanning primer set

The expression of the *AtACS* gene family was initially monitored by RT-PCR using a non-exon-spanning primer set from Yamagami *et al.* [51]. The resulting band sizes were larger than the expected product size if cDNA was used as a template, and were instead consistent with the expected sizes of PCR products using genomic DNA as a template (Figure 3.5, Table 3.1). This showed that the RNA samples extracted from the S-depleted (2.5% S) plants were extensively contaminated with genomic DNA, and the PCR products could not therefore be used as an indicator of *AtACS* gene expression because genomic DNA is inherently present. Only mRNA levels, and the cDNA translated from these transcripts, reflect any up- or downregulation in gene expression.

However, there were also several bands of the expected size if cDNA was used as a template, and these were used as an early indicator of changes in the

expression of specific *AtACS* genes in response to short-term S-deficient conditions. These bands were scored as present (+) or absent (-) to indicate which *AtACS* genes were induced or repressed. Any difference in the level of *AtACS* gene expression, which would normally be represented by bands of different densities, was beyond the scope of this scoring system as the common RT-PCR amplification controls, actin and 18S RNA, could not be used. Actin and microtubules constitute the cytoskeleton that maintains cell shape, which is also influenced by C_2H_4 [reviewed in 68]. Because of this, any changes in *AtACS* gene expression, and presumably C_2H_4 biosynthesis, would also influence actin expression, negating its role as an indicator of equal RT-PCR amplification. The 18S RNA control could not be used because its ~300 bp RT-PCR product was too similarly-sized to the RT-PCR products of the *ATACS* genes if cDNA was the template used.

Using this scoring system, in the leaves, *AtACS1*, *AtACS4*, *AtACS6*, *AtACS8* and *AtACS9* appeared to be repressed under S-deficient conditions (Table 3.2 a). Similarly, in the roots, *AtACS1*, *AtACS5*, *AtACS9* and *AtACS11* seemed to be repressed under S-deficient conditions, whereas *AtACS10* and *AtACS12* appeared to be upregulated (Table 3.2 b). No expression of *AtACS4* and *AtACS2* was observed for either tissue in response to S supply.

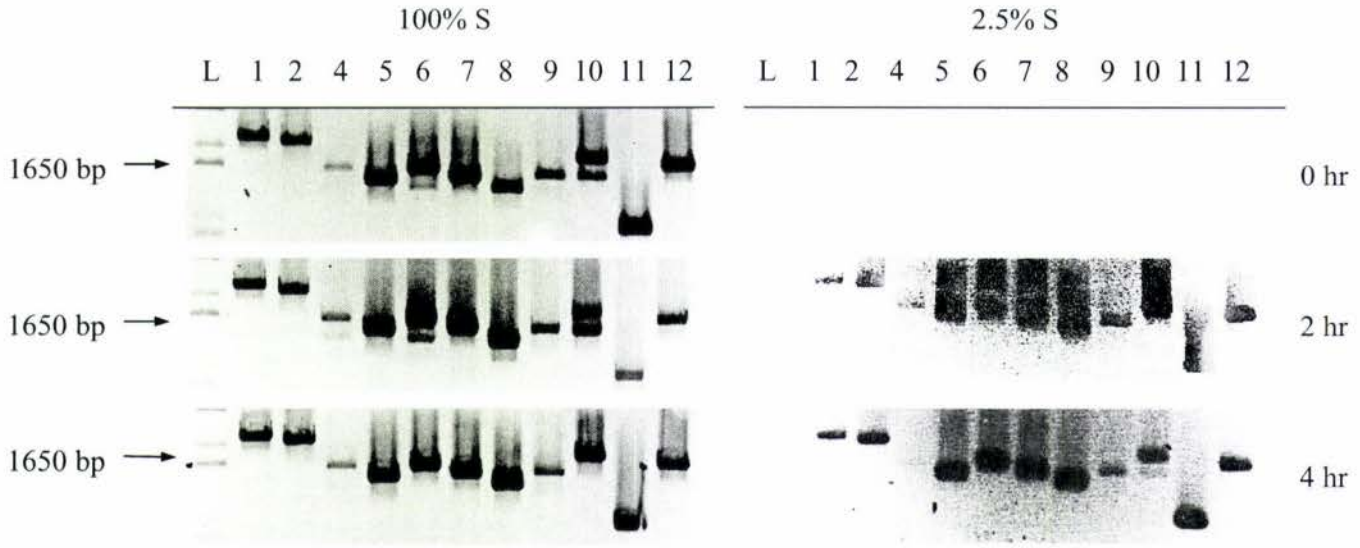

Figure 3.5 Typical results of RT-PCR amplification of *AtACS* without DNase I treatment. Results shown here are RT-PCR products of RNA samples extracted from leaves of S-sufficient (100% S) and S-depleted (2.5% S) *A. thaliana*, using a non-exon-spanning primer set for the *AtACS* gene family (Table 2.7). In lanes with double bands, the lower band is of the correct and expected size for a cDNA product (Table 3.1). Sampling times following S-depletion are indicated. L, 1 kb DNA ladder; 1-12, *AtACS1-12* respectively.

Table 3.1 The expected product sizes of mRNA or genomic DNA using the exon-spanning primer set in RT-PCR of RNA isolated from *A. thaliana* used in short-term S-deficiency experiments.

	Expected size of PCR product using cDNA as a template (bp)	Expected size of PCR product using genomic DNA as a template (bp)
AtACS1	1,463	2,232
AtACS2	1,417	2,116
AtACS4	1,351	1,608
AtACS5	1,244	1,434
AtACS6	1,333	1,600
AtACS7	1,191	1,487
AtACS8	1,294	1,326
AtACS9	1,247	1,506
AtACS10	1,492	1,777
AtACS11	826	919
AtACS12	1,203	1,658

Table 3.2 Initial indication of *AtACS* gene expression in S-sufficient (100% S) and S-depleted (2.5% S) *A. thaliana* plants.

		100% S			2.5% S		
		0	2	4	0	2	4
(a)	*AtACS1*	+	+	+		-	-
	AtACS2	-	+	-		-	-
	AtACS4	-	+	+		-	-
	AtACS5	-	-	-		-	-
	AtACS6	+	+	-		-	-
	AtACS7	-	-	-		-	-
	AtACS8	+	+	+		-	-
	AtACS9	+	+	+		-	-
	AtACS10	+	+	-		-	+
	AtACS11	-	-	-		-	-
	AtACS12	-	+	-		-	-
(b)	*AtACS1*	-	-	-		-	-
	AtACS2	+	+	+		-	-
	AtACS4	-	-	-		-	-
	AtACS5	-	-	-		-	-
	AtACS6	+	+	+		-	-
	AtACS7	+	+	+		-	+
	AtACS8	+	+	-		-	+
	AtACS9	-	-	-		-	-
	AtACS10	+	+	+		-	-
	AtACS11	-	-	-		-	+
	AtACS12	+	+	-		-	-

Summary of the presence (+) or absence (-) of a cDNA-sized RT-PCR product in RNA samples extracted from *A. thaliana* (a) leaves, and (b) roots. 0, 2 and 4 indicates sampling time in hr after transfer to fresh 100% S or 2.5% S media.

3.3.3 Attempts to remove genomic contamination from RNA samples extracted from *A. thaliana* by DNase I treatment

To obtain a clearer indication of the effect of S-supply on *AtACS* expression, RNA from leaves of *A. thaliana* plants was DNAseI-treated to remove genomic contamination (Figure 3.6). DNase I treatment was performed in three ways: either after completion of RNA extraction with or without EtOH precipitation (Figure 3.6 a), or during RNA extraction. However, all attempts were unsuccessful, as the RT-PCR products were again consistent with the expected sizes of PCR products using genomic DNA as a template, as indicated in Table 3.1 (data not shown). This meant that DNase I was either unsuccessfully inactivated or removed, as indicated by the absence of any bands (Figure 3.6 a, b), or resulted in a smear instead of a cDNA-sized band (Figure 3.6 c).

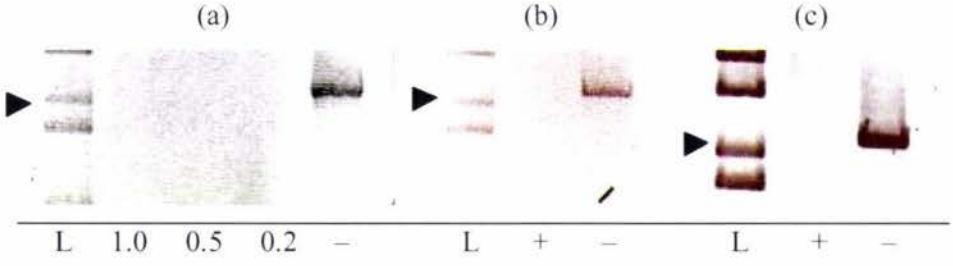

Figure 3.6 Attempts to remove genomic DNA contamination from RNA from *A. thaliana* leaves by DNase I treatment. RT-PCR was performed as described in section 2.6 using the non-exon-spanning primers for *AtACS1*. (a) DNAse I treatment as per the manufacturer's instructions, (b) DNAse I treatment as per the manufacturer's instructions followed by EtOH precipitation of the RNA, (c) DNase I treatment as per section 2.4. L, ladder; 1-0.2, volume of DNase I added in µL, +/–, absence/presence of DNase I; arrowhead, approximate position of 2000 bp band of DNA ladder.

As the DNaseI treatments were unsuccessful, exon-spanning primers were used (Table 2.8). These circumvented the problem of genomic contamination as they bind to and amplify cDNA only, and gave RT-PCR products of the expected sizes if cDNA was used as a template. These primers cannot bind to genomic

DNA as an intron intersperses the primer sequence. This is illustrated in Figure 3.7 and with *AtACS1* as an example.

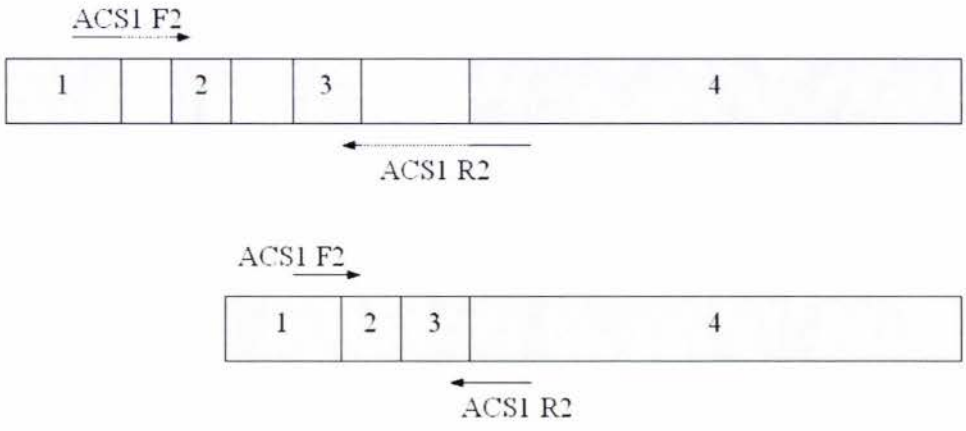

Figure 3.7 A schematic of genomic DNA (above) and cDNA (below) of *AtACS1*. The exon-spanning forward and reverse primers can only bind to and amplify cDNA, but not genomic DNA as an intron intersperses the primer sequence. ACS1 F2, *AtACS1* exon-spanning forward primer; ACS1 R2, *AtACS1* exon-spanning reverse primer; 1, 2, 3 and 4, *AtACS1* exons.

3.4 Examination of the expression of *AtACS* genes in *A. thaliana* under short-term S-deficient conditions by RT-PCR using an exon-spanning primer set

The use of exon-spanning primers allowed the identification and RT-PCR amplification of mRNA corresponding to *AtACS* genes. The same scoring system as previously described was used (section 3.3.2), and bands were scored as present (+) or absent (-) to indicate which *AtACS* genes were induced or repressed. All detected bands were of the expected size if cDNA was the template (Table 2.8).

In the leaves of S-sufficient (100% S) plants, a ~515 bp band was detected at 30 and 60 min, indicating that *AtACS5* was expressed at these times (Figure 3.8, Table 3.3). Similarly, a ~310 bp band was detected at all sampling times of 0, 30, 60 and 120 min, indicating that *AtACS10* was expressed at these times.

In contrast, no expression of any *AtACS* genes was detected at any time in the leaves of S-depleted (2.5% S) plants. Therefore it appears that *AtACS5* and *AtACS10* expression was repressed by S-depletion.

In the roots of S-sufficient plants, RT-PCR products representing *AtACS2* (~326 bp) and *AtACS5* (~515 bp) were detected at 0, 30 and 60 min (Figure 3.9, Table 3.4). Bands of the appropriate size, representing *AtACS6* (~321 bp), *AtACS7* (~328 bp), *AtACS10* (310 bp) and *AtACS12* (~334 bp) were also detected at all sampling times. Both *AtACS8* (~332 bp) and *AtACS9* (~330 bp) were expressed at 0 and 30 min. Several genes, namely *AtACS1*, *AtACS4* and *AtACS11* did not appear to be expressed as no RT-PCR products representing these genes were detected at any time. In contrast, in the roots of S-depleted plants, RT-PCR products representing *AtACS2* and *AtACS6* were expressed only at 60 and 120 min. The genes *AtACS7* and *AtACS10* were expressed at 0, 60 and 120 min but not at 30 min, and bands representing *AtACS8* and *AtACS11* were detected only at 0 min. The gene *AtACS5* was expressed at 30, 60 and 120 min in the roots of S-depleted plants. No bands representing *AtACS1, AtACS4, AtACS9* and *AtACS12* were detected at any time.

Certain patterns of *AtACS* expression can be elucidated from these observations. Several *AtACS* genes, namely *AtACS2*, *AtACS5*, *AtACS6* and *AtACS7*, were expressed regardless of S availability and appeared to have a similar pattern of expression. This pattern is most clearly seen for *AtACS7*: it was expressed in the roots of S-depleted plants at 0 min, became undetectable at 30 min, but was re-expressed at 60 and 120 min. At 0 min, *AtACS* gene expression should be the same regardless of S availability, but *AtACS2*, *AtACS5* and *AtACS6* were undetectable in the roots of S-depleted plants. For these genes, the transient decrease in expression may have been caused by the roots being rinsed before transfer, so by the time of harvesting only the re-expression was seen.

Another pattern of *AtACS* expression that could be seen was that the *AtACS* genes were generally repressed in the leaf and root tissues of S-depleted plants. Expression of *AtACS5* was detectable at 30 and 60 min in leaves of S-sufficient

but not in S-depleted plants, whereas *AtACS8* and *AtACS9* were both expressed at 0 and 30 min in the roots of S-sufficient but not S-depleted plants. Similarly, *AtACS10* was detectable in S-sufficient leaves and roots at all time points, but only at 0 min in leaves, and 0, 60 and 120 min in the roots of S-depleted plants. Expression of *AtACS12* was detected only in the roots of S-sufficient plants, and at all sampling times. Two genes, *AtACS1* and *AtACS4* were undetectable regardless of tissue type and S availability.

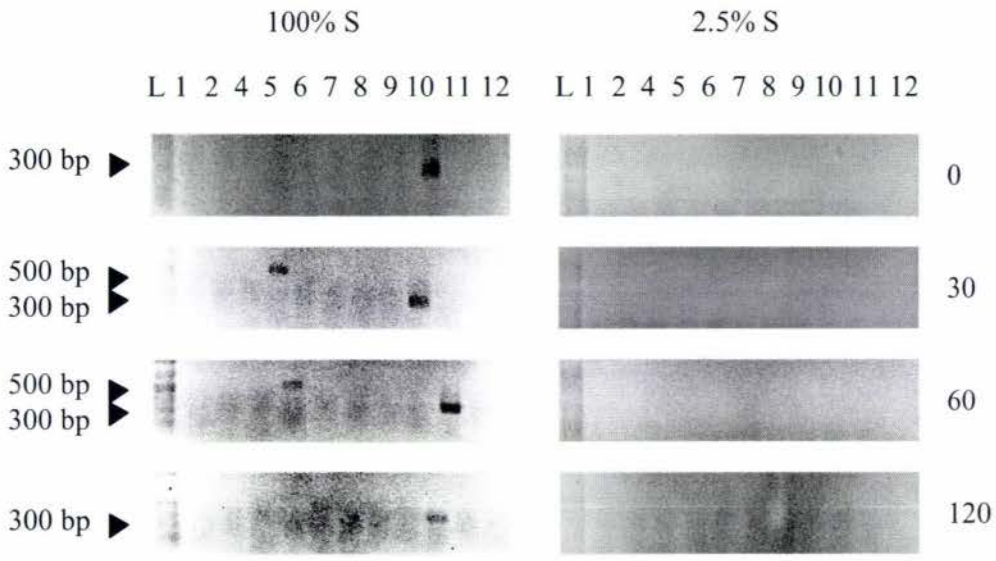

Figure 3.8 The expression the *AtACS* gene family in RNA extracted from the leaves of S-sufficient (100% S) and S-depleted (2.5% S) *A. thaliana* was examined by RT-PCR using exon-spanning primers. Exon-spanning primers for all *AtACS* gene family members (except *AtACS3*) are listed in Table 2.8. A summary of the presence and absence of cDNA-sized products is outlined in Table 3.3. Approximate product sizes and sampling times following S-depletion are indicated. L, 1 kb DNA ladder; 1-12, *AtACS 1-12* respectively. Sampling time in minutes in indicated on the right.

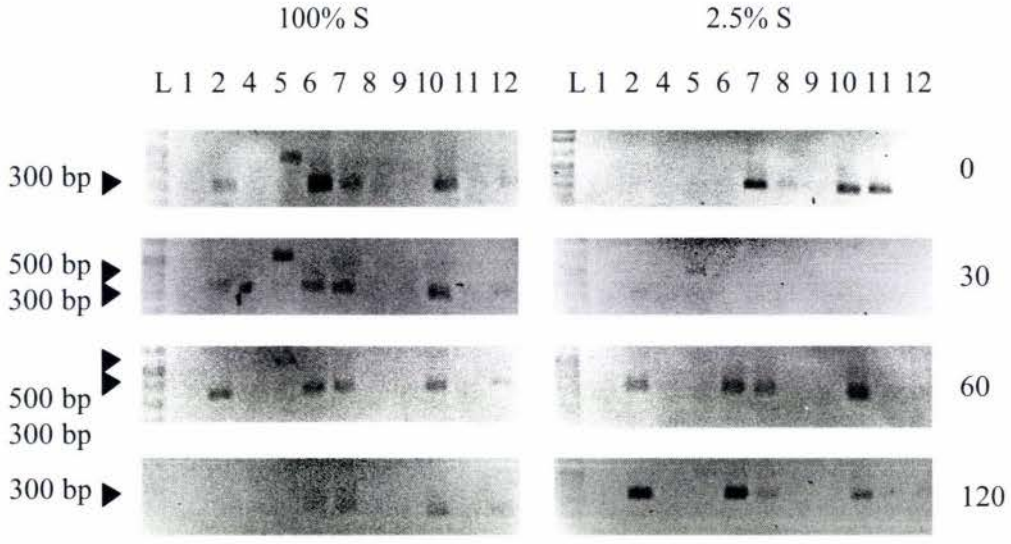

Figure 3.9 The expression the *AtACS* gene family in RNA extracted from the roots of S-sufficient (100% S) and S-depleted (2.5% S) *A. thaliana* was examined by RT-PCR using exon-spanning primers. Exon-spanning primers for all *AtACS* gene family members (except *AtACS3*) are listed in Table 2.8. A summary of the presence and absence of cDNA-sized products is outlined in Table 3.4. Approximate product sizes and sampling times following S-depletion are indicated. L, 1 kb DNA ladder; 1-12, *AtACS 1-12* respectively. Sampling time in minutes are indicated on the right.

Table 3.3 A summary of the RT-PCR analyses on the expression *AtACS* gene family from RNA extracted from the leaves of S-depleted *A. thaliana* using exon-spanning primers.

	100% S				2.5% S			
	0	30	60	120	0	30	60	120
*AtACS*1	-	-	-	-	-	-	-	-
*AtACS*2	-	-	-	-	-	-	-	-
*AtACS*4	-	-	-	-	-	-	-	-
*AtACS*5	-	+	+	-	-	-	-	-
*AtACS*6	-	-	-	-	-	-	-	-
*AtACS*7	-	-	-	-	-	-	-	-
*AtACS*8	-	-	-	-	-	-	-	-
*AtACS*9	-	-	-	-	-	-	-	-
*AtACS*10	+	+	+	+	-	-	-	-
*AtACS*11	-	-	-	-	-	-	-	-
*AtACS*12	-	-	-	-	-	-	-	-

Exon-spanning primers for all *AtACS* gene family members (except *AtACS*3) are listed in Table 2.8. + or – indicates the presence or absence of a cDNA-sized RT-PCR product respectively. 0, 30, 60 and 120 indicates the sampling time in min following S-depletion.

Table 3.4 A summary of the RT-PCR analyses on the expression *AtACS* gene family from RNA extracted from the roots of S-depleted *A. thaliana* using exon-spanning primers.

	100% S				2.5% S			
	0	30	60	120	0	30	60	120
*AtACS*1	-	-	-	-	-	-	-	-
*AtACS*2	+	+	+	-	-	-	+	+
*AtACS*4	-	-	-	-	-	-	-	-
*AtACS*5	+	+	+	-	-	+	+	+
*AtACS*6	+	+	+	+	-	-	+	+
*AtACS*7	+	+	+	+	+	-	+	+
*AtACS*8	+	+	-	-	+	-	-	-
*AtACS*9	+	+	-	-	-	-	-	-
*AtACS*10	+	+	+	+	+	-	+	+
*AtACS*11	-	-	-	-	+	-	-	-
*AtACS*12	+	+	+	+	-	-	-	-

Exon-spanning primers for all *AtACS* gene family members (except *AtACS*3) are listed in Table 2.8. + or – indicates the presence or absence of a cDNA-sized RT-PCR product respectively. 0, 30, 60 and 120 indicates the sampling time in min following S-depletion.

4 Discussion

4.1 Change in enzyme protein levels in response to C_2H_4 treatment

There is some evidence for the C_2H_4-mediated regulation of plant adaptive responses to various nutrient stresses. For example, in the response of Strategy I plants (non-graminaceous monocotyledons and dicotyledons that uptake Fe^{2+} following its reduction from Fe^{3+}) to Fe-deficiency [55-57], although this has not been conclusively proven [58]. There is also some evidence for the involvement of C_2H_4 in the responses of plants to P-deficiency [59, 60, 62] and S-deficiency [6, 35], although the latter has not been as extensively studied. Nevertheless it seemed plausible that C_2H_4 may mediate the plant response to S-deficiency, in terms of modulating the activity of the S-assimilation enzymes. To examine this, S-sufficient *A. thaliana* and onions were C_2H_4-treated, and western analyses performed to determine whether any change in enzyme protein levels were induced by this treatment.

4.1.1 Change in the protein levels of the S-assimilation enzymes in chloroplast extracts from the leaves of whole *A. thaliana* plants treated with C_2H_4

In general, C_2H_4 appears to act negatively on the S-assimilation enzymes (Figure 3.2). Protein levels of APR were highest at 0 hr in (C) plants, prior to C_2H_4 treatment, and were higher in (M+C) plants at all sampling times. This suggests that 1-MCP removes the repressive effect of C_2H_4 to allow APR to accumulate. By extrapolation, *O*ASTL and ATPS expression may be similarly repressed by C_2H_4. This contradicts previous findings that APR, and especially APR2, is most subject to regulation among the S-assimilation enzymes, whereas *O*ASTL and ATPS were either unresponsive [43, 67, 69] or responded relatively later [40, 42]. Additionally, because the α-APR antibody used in this

study recognises all three APR isozymes, it cannot be determined which isonzyme(s) was responsible for the observed accumulation of APR protein. It may also be possible that different APR isozymes were up- and downregulated at different sampling times, although the total amount of detectable APR protein remained fairly constant.

Despite the apparent repression by C_2H_4, as determined by the accumulation of ATPS protein at 0 hr in the (M+C) plants, ATPS protein levels peaked at 4 hr in C plants. This may be due to the differential regulation of the four ATPS isozymes. In other gene studies, the expression of *AtATPS1* and *AtATPS4* appear to be unresponsive to C_2H_4; in contrast *AtATPS2* and *AtATPS3* mRNA levels peaked after an hour of 10 ppm C_2H_4 treatment before declining to basal levels (Leung, S. and McManus, M. T.; *pers comm.* [70]).

The enzyme SiR was unusual because it appeared to be under dual regulation by C_2H_4. Furthermore, previous studies have found SiR to be generally unresponsive to short-term S-deficiency [27], steady-state S-deficiency [67], or application of the thiols L-Cys and GSH [42]. It should be noted that C_2H_4 acts positively or negatively on root elongation rate depending on P availability in *A. thaliana* [59]. Under P-deficient conditions, C_2H_4 maintains root elongation, but limits root elongation when P is sufficient. Ethylene (C_2H_4) may also have a dual effect when mediating the plant S response: under S-sufficient conditions the unnecessary accumulation of SiR is limited, and conversely a fast accumulation of protein occurs during S-depletion. In this study, all plants subjected to C_2H_4 treatment were S-sufficient, so 1-MCP treatment blocked C_2H_4 action and removed the limit normally placed on enzyme accumulation to result in more SiR at 0 and 2 hours. Because C_2H_4 regulates the expression of the genes encoding its own biosynthetic enzymes, namely *AtACS2*, *AtACS6* and *AtACO* [reviewed in 49], as well as the expression of the genes coding for the receptors ERS1 and ERS2 [71], the subsequent decrease in SiR observed at 4 and 6 hrs in (M+C) plants may reflect the waning effects of 1-MCP as C_2H_4 signalling is restored through the induction of new receptors. However, this proposed model of SiR regulation by C_2H_4 cannot explain why SiR protein levels remain low at 0 and 2 hr, but then accumulate at 4 and 6 hr in (C) plants.

For this proposed model of C_2H_4 repression of SiR under S-sufficient conditions to fully qualify, SiR protein levels should have also remained low at these later sampling points.

The effect of C_2H_4 on the S-assimilation enzymes may be complicated by endogenous rhythms that govern their expression. For example, APR in *Z. mays* has a diurnal pattern of expression, with activity peaks and troughs at subjective midday and at the end of the dark period, respectively [46]. In *A. thaliana*, APR activity and protein levels also show a similar diurnal rhythm, and the mRNA levels of 5'APS kinase, SiR, *O*ASTL and SAT were highest in green leaves [45, and reference therein]. In *L. minor*, APR is also light-induced, as prolonged darkness caused a significant decline in APR activity as well as SO_4^{2-} uptake and incorporation [36].

Furthermore, the apparent negative effect of C_2H_4 on the S-assimilation enzymes, especially SiR, may actually be a side effect of photosynthetic inhibition [72, 73]. Wullschleger *et al.* [73] treated soybean plants (*Glycine max*) with the same concentration of C_2H_4 used in this study (10 ppm), and found that C_2H_4 interfered with photosynthetic electron transport. SiR may therefore be adversely affected as it requires electrons from ferredoxin I and II in the leaves, and ferredoxin III in the roots, to function ([9], Figure 4.1). Recent microarray analysis data may support this proposed a link between C_2H_4 and S depletion, as they both have a negative effect on photosynthesis [6]. S depletion was found to downregulate the genes encoding accessory proteins of electron transport, possibly due to quantitative alterations of Fe-S centres of PSI proteins and/or changes in thylakoid membrane lipid compositions.

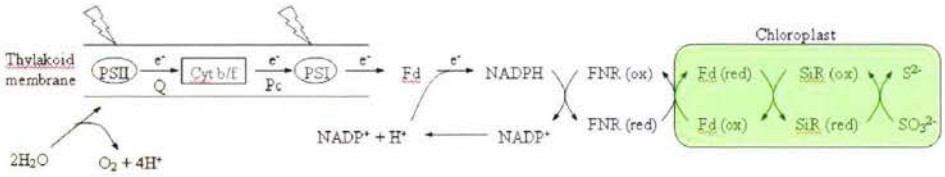

Figure 4.1 Ethylene (C_2H_4) interferes with photosynthetic electron transport, which may be linked to the S-assimilation pathway as these two processes both utilise ferredoxin as an electron donor. , light; PSII/I, photosystem II/I; Cyt b/f, cytochrome b/f; e^-, electrons; Q, quinone; Pc, plastocyanin; Fd, ferredoxin; FNR, Fd-$NADP^+$ reductase; SiR, sulfite reductase; SO_3^{2-}, sulfite; S^{2-}, sulphide (adapted from Yonekura-Sakakibara *et al.* [9]).

4.1.2 Change in the protein levels of the S-assimilation enzymes in chloroplast extracts from leaves of whole plants of *A. cepa* treated with C_2H_4

4.1.2.1 Change in the protein levels of the S-assimilation enzymes in chloroplast extracts from leaves of whole onions of the W202A cultivar treated with C_2H_4

In contrast to *A. thaliana*, in cultivar W202A onions, the enzymes ATPS and *O*ASTL do not appear to be regulated by C_2H_4 as the protein levels of these enzymes were comparable between treatments at all sampling times (Figure 3.3).

In contrast, APR appears to be negatively regulated by C_2H_4, as protein levels were higher in (M+C) plants at 0 and 2 hr before declining to undetectable levels. Although APR protein levels peaked at 4 hr in (C) plants, the level of accumulation was always lower than in (M+C) plants.

The SiR enzyme appeared to be again under dual regulation by C_2H_4, as a similar pattern of protein accumulation was also observed in *A. thaliana*

(section 4.1.1). In (C) plants, SiR protein levels were highest at 0 hr but then gradually decreased to undetectable levels, and may reflect the repression of SiR by C_2H_4 under S-sufficient conditions. Conversely, in the (M+C) plants, C_2H_4 signalling is blocked by 1-MCP, so SiR protein accumulated at 2 and 4 hr before declining at 6 hr, when the effect of 1-MCP presumably began to wane. However, this proposed model of SiR regulation by C_2H_4 does not explain why the level of SiR protein was not also high at 0 hr in the (M+C) plants.

4.1.2.2 ***Change in the protein levels of the S-assimilation enzymes in chloroplast extracts from leaves of whole onions of the Texas Grano cultivar treated with C_2H_4***

In the onions of the Texas Grano cultivar, ATPS seemed to be negatively regulated by C_2H_4, as the proteins levels of these enzymes were higher at 0, 2 and 4 hr in (M+C) plants (Figure 3.3). However, the ATPS protein accumulation at 6 hr was comparable between treatments. This was an unexpected result, because if C_2H_4 genuinely repressed ATPS protein accumulation, protein levels should have remained low for all sampling times. The APR enzyme also initially appeared to be negatively regulated by C_2H_4, as protein levels were highest at 0 hr in C plants before declining to undetectable levels. However, APR protein was only detected at 2 hr in (M+C) plants. If this enzyme was truly repressed by C_2H_4 it should also have been detected at 0 hr when 1-MCP action was strongest. The pattern of ATPS and APR protein accumulation was unexpected. Presumably, ATPS and APR in the Texas Grano cultivar is under regulation by other phytohormones, or errors have occurred while performing the western analyses for this enzyme.

In contrast, *O*ASTL appeared to be truly under negative regulation by C_2H_4. This is because in (C) plants, *O*ASTL protein levels were not only lower than in (M+C) plants at all time points, but also gradually declined after 2 hr. Additionally, in (M+C) plants, *O*ASTL protein levels were elevated at 0, 2 and 4 hr, before declining slightly at 6 hr, presumably due to the waning effects of

1-MCP. To confirm this, these experiments could be repeated with continuous 1-MCP treatment to ensure that C_2H_4 could not induce new receptors.

The pattern of SiR protein accumulation in onions of the Texas Grano cultivar was similar to that observed for SiR in *A. thaliana*, and therefore suggests a dual mode of regulation by C_2H_4 (section 4.1.1). In the proposed model of dual regulation by C_2H_4, SiR protein accumulation is prevented by C_2H_4 under S-sufficient conditions, and conversely promoted when the plant is S-depleted. All onion plants treated with C_2H_4 were S-sufficient, so 1-MCP effectively removes the repressive effect of C_2H_4 on SiR, resulting in the elevated levels of SiR protein at 0 and 2 hr. At the later sampling times of 4 and 6 hr, SiR protein levels drop as the effect of 1-MCP wanes. However, this model fails to explain why SiR protein begins to accumulate at the later sampling times of 2, 4 and 6 hr in (C) plants.

4.2 The response of *A. thaliana* to short-term sulfur (S) deficiency

To complement the experiments that examined if C_2H_4 had any effect on the S-assimilation enzymes, the effect of S deficiency on the C_2H_4 biosynthesis was examined in *A. thaliana*. Because ACS catalyses the rate-limiting step of C_2H_4 biosynthesis, changes in *AtACS* gene expression were investigated by RT-PCR amplification, and were used as an indicator of any changes in the level of C_2H_4 production. At least 12 *AtACS* genes have been identified in *A. thaliana*, although not all were expected to respond to the S-depletion treatment, as not all 12 *AtACS* genes are enzymatically active. For example, *AtACS3* is a pseudogene, and *AtACS10* and *AtACS12* code for aminotransferases. Additionally, because C_2H_4 is involved in so many aspects of the plant life cycle [reviewed in 50], several *AtACS* genes were expected to be housekeeping genes that would also not respond to the S-depletion treatment.

However, if C_2H_4 does mediates the plant response to S-depletion, a change in the expression of at least some of the *AtACS* genes was expected to occur. Leaf and root material were harvested from S-sufficient and S-depleted plants grown using a hydroponic system adapted from the method of Norén *et al.* [64] and subjected to RNA isolation followed by RT-PCR. Initially the primer sets from Yamagami *et al.* [51] were used, although exon-spanning primers were eventually employed to circumvent the problem of genomic contamination. Because genomic DNA is inherently present, the RT-PCR products of contaminated RNA samples amplified by non-exon-spanning primers cannot be used as an indicator of *AtACS* gene expression. Only mRNA levels, and the cDNA translated from these transcripts, reflect any up- or downregulation in gene expression. Levels of APR protein accumulation in whole leaf extracts were also examined as a marker to show that the plant was S-depleted, and to indicate a time-frame in which the plant would respond to S-depletion.

4.2.1 Change in APR protein levels in the leaves of S-depleted and S-sufficient *A. thaliana* plants

The enzyme APR is known to readily respond to changes in the plant's S status, as indicated by the observed increase in APR mRNA levels [5, 26, 27, 67] in response to S-deficiency, and conversely, a decrease in APR mRNA levels, protein accumulation and enzyme activity in response to application of the thiols L-Cys and GSH, and to H_2S [42, 43]. Because of this, APR was employed as a marker for the onset of the plant response to S-depletion.

Western analyses showed that APR levels peaked at 0 min regardless of S availability, although this may be discounted as it was likely that the observed accumulation of APR protein was due to oxidative stress incurred when the roots were rinsed prior to transfer to fresh media (Figure 3.4 b). Levels of APR protein progressively declined in S-sufficient leaves but remained high at 0 and 30 min before decreasing in the leaves of S-depleted plants. However, at 120 min, when the plant has presumably recovered from this perturbation, levels of APR were still higher in the leaves of S-depleted plants. The accumulation of

APR protein is among the earliest plant responses to S-depletion, along with upregulation of the H^+/SO_4^{2-} symporters [26, 39, 41, 42]. This indicated that the *A. thaliana* plants were becoming S-depleted and were responding to the treatment imposed.

Expression of APR in *A. thaliana* is under diurnal regulation, where a peak in protein levels was observed 4 hr after light onset [45]. Therefore the patterns of APR protein accumulation in this study may have been influenced by this endogenous rhythm in addition to S availability. However, a direct comparison cannot be made with certainty despite the fact a peak in APR protein accumulation was observed 4 hr after light onset in the leaves of S-depleted plants in this study. This is because Kopriva *et al.* [45] used younger plants (4.5-weeks-old instead of 8-weeks-old) grown at a higher light intensity (115-160 instead of 85 μmol photons $m^{-2}s^{-1}$) and under a different light/dark regimen (10L/14D instead of 8L/16D). However, the result could alternatively mean that S-depletion does accentuate the diurnal response, but again a direct comparison is difficult since Ponceaus S staining indicates that the 120 min (4 hr after light onset) is underloaded (Figure 3.4 b).

4.2.2 Change in *AtACS1-12* gene expression in S-sufficient and S-depleted *A. thaliana* plants

In general, *AtACS* gene expression appeared to be repressed in the leaf and root tissues of S-stressed plants (Figure 3.8, Figure 3.9, Table 3.3, Table 3.4). Expression of *AtACS5* was detected at 30 and 60 min in leaves of S-sufficient but not in S-depleted plants, whereas *AtACS8* and *AtACS9* were both expressed at 0 and 30 min in the roots of S-sufficient but not S-depleted plants. Expression of *AtACS10* was detected in S-sufficient leaves and roots at all time points, but only at 0 min in leaves, and 0, 60 and 120 min in the roots of S-depleted plants. Expression of *AtACS12* was detected only in the roots of S-sufficient plants, and at all sampling times.

Several genes, namely *AtACS4*, *AtACS5*, *AtACS8* and *AtACS9*, are closely related and the protein products of these genes are believed to be phosphorylated and stabilised by a CDPK [74]. Therefore is it perhaps not surprising that the expression of *AtACS5* in leaf, and *AtACS8* and *AtACS9* appeared to be repressed in common, in the roots of plants of *A. thaliana*, under S-depleted conditions (Figure 3.8, Figure 3.9, Figure 4.2, Table 3.3, Table 3.4). However, the mechanism of repression of these genes is unknown and beyond the scope of this study. It is also possible that S-depletion promote CDPK activity, thus increasing not only *AtACS5*, *AtACS8* and *AtACS9* expression, but also the accumulation of the protein products of these genes. Again, whether or not S-availability-mediated regulation of CDPK activity occurs is beyond the scope of this study, and there is no evidence for this to date. Nevertheless, the repression of *AtACS5*, *AtACS8* and *AtACS9* expression may be an adaptive response to S-depletion: the apparent decline in the expression of these genes would alleviate the C_2H_4-mediated repression of root growth, which is a characteristic feature of the 'triple response.' Auxin biosynthetic genes and several auxin-responsive genes have been reported to be upregulated in S-deficient plants, and alleviation of C_2H_4-mediated repression may act together with auxin to trigger root proliferation [6]. Root proliferation, among other nutrient stresses including S deficiency [6, 55, 59, 60].

The expression of *AtACS2* and *AtACS6* in the leaves and roots were similar regardless of S availability, in that they were both expressed in S-sufficient as well as S-depleted plants (Figure 3.8, Figure 3.9, Figure 4.2, Table 3.3, Table 3.4). However, although the level of expression of these genes may differ, this cannot be determined by the scoring system used in this study (section 3.3.2). [75]. Because *AtACS2* and *AtACS6* expression in both the leaves and roots were comparable regardless of S availability, it is possible that unlike *AtACS5* in leaf, and *AtACS8* and *AtACS9* in roots, which appear to be under transcriptional regulation by an unknown factor, as well as post-translational regulation by CDPK [74], *AtACS2* and *AtACS6* may be regulated solely at the post-translational level by MPK6. However, whether or not MPK6 is regulated somehow by S availability has not been determined. The protein products of *AtACS2* and *AtACS6* are both phosphorylated and stabilised by a mitogen-

activated protein kinase 6 (MPK6), which is an ortholog of the tobacco (*Nicotiana tabacum*) stress-induced protein kinase (SIPK) [75, 76]. Not surprisingly, SIPK also induces C_2H_4 biosynthesis, as was shown in a conditional gain-of-function transgenic system by Liu *et al.* [75]. There is some evidence that elevated CDPK signalling, which is responsible for stabilising *AtACS5*, *AtACS8* and *AtACS9* under S-sufficient conditions, also compromises MPK activation, as was found recently in tobacco [77].

The genes *AtACS10* and *AtACS12* were also repressed in the leaves and roots, respectively, of S-depleted plants (Figure 3.8, Figure 3.9, Table 3.3, Table 3.4). Although both isozymes posses the characteristic seven conserved regions also found in the ACSes of other plant species, they also phylogenetically resemble alanine and aspartate aminotransferases [51]. Complementation experiments confirmed that these genes encode aspartate (*AtACS10*) and aromatic amino acid transaminases (*AtACS12*) [51]. Therefore, the apparent downregulation of these genes suggests that the downregulation of amino acid metabolism may be part of the plant adaptive response to S-depletion. As S is an integral part of proteins, sulfolipids and other S-containing compounds, a possible explanation is that when S is in short supply the maintenance of protein integrity and the prevention of an unnecessary recycling of amino acids is a priority.

In the roots, other *AtACS* genes, in addition to *AtACS2* and *AtACS6*, namely *AtACS5* and *AtACS7*, had a similar pattern of expression regardless of S availability (Figure 3.8, Figure 3.9, Table 3.3, Table 3.4). This was most clearly illustrated by *AtACS7*: in the roots of S-depleted plants, it was expressed at 0 min, became undetectable at 30 min, but was expressed again at 60 and 120 min. At 0 min, *AtACS* gene expression should be the same regardless of S availability, but *AtACS2*, *AtACS5* and *AtACS6* expression was undetectable in the roots of S-depleted plants at 0 min. For these genes, the transient decrease in expression may have occurred when the roots were rinsed prior to transfer, so by the time samples were harvested only the subsequent re-expression is observed. This suggests that C_2H_4 production is slowed transiently, until the plant response to S-depletion was sufficiently activated for the plant to support C_2H_4 biosynthesis. These genes were also expressed in the

roots of S-sufficient plants, indicating that they were affected as a consequence of S-depletion, but are not part of the direct response. They may instead be involved in other aspects of plant growth and development.

Several genes, namely *AtACS1* and *AtACS4* were not expressed in either the leaves or the roots, regardless of S availability (Figure 3.8, Figure 3.9, Table 3.3, Table 3.4). Similarly, *AtACS11* expression was not detected regardless of plant tissue or S supply, except at 0 min in the roots of S-depleted plants, although this is likely due to the mechanical stress incurred when the roots were rinsed with media prior to transfer. It is not known why these genes were not expressed, but a possible explanation is that these genes may only be expressed very early or very late in plant development, or not in any or the tissues used in this study.

4.3 Proposed role of ethylene (C_2H_4) in the plant sulfur (S) stress response

The observed downregulation of *AtACS5* and *AtACS10* in the leaves, and of *AtACS8*, *AtACS9*, and *AtACS12* in the roots in S-depleted plants (Figure 3.8, Figure 3.9, Table 3.3, Table 3.4) was also accompanied by an accumulation of APR protein (Figure 3.4 b). The increase in APR protein that also occurred in (M+C) plants (Figure 3.2) suggests that C_2H_4 may be involved in the plant response to S-depletion. By inference, *O*ASTL and ATPS, which were also elevated in (M+C) plants, may also be regulated in the same way, as illustrated in Figure 4.2. However, this cannot be conclusively determined as the protein levels of these enzymes in S-depleted plants were not examined.

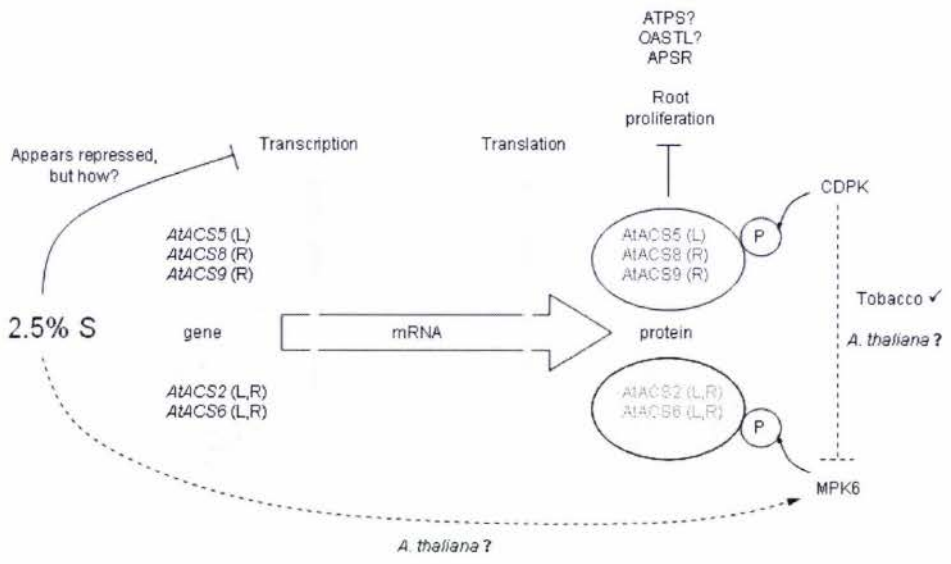

Figure 4.2 Proposed model of S depletion-induced regulation of *AtACS* and S-assimilation enzyme expression. Dashed lines indicate possible but unproven regulatory interactions that may occur in plants *A. thaliana* under S-depleted conditions. L and R indicate leaf and root tissue, respectively; CDPK, Ca^{2+}-dependent protein kinase; MPK6, mitogen-activated protein kinase 6; P, phosphate group.

Furthermore, the possible role of other phytohormoes in the plant response to S-depletion cannot be excluded. There is evidence for crosstalk between the C_2H_4 signalling pathway and those of auxin [6, 20, 24, 25, 62], ABA [23, 62], cytokinins [35, 44, 78, 79] and JA [5, 6]. As the results in this study appear to indicate that the C_2H_4 may be involved in mediating the plant response to S-depletion, it is perhaps not surprising that others have observed that S-depletion also causes several JA biosynthetic genes to be upregulated in the leaves and roots, and in entire seedlings [5-7]. Also, at least one S-assimilation enzyme, namely APR1, is upregulated by JA [reviewed in 34].

The involvement of C_2H_4 in the plant response to various nutrient stresses, including S-deficiency [5, 6, 35], Fe-deficiency [55-57], P-deficiency [59, 60, 62], and K-deficiency [80], suggests that it is a general regulator of the plant adaptive response. In fact, different nutrient stresses appear to regulate the same signalling pathways. For example, an EREBP-like protein was

upregulated in *A. thaliana* plants subjected to transient S-deficiency [5]. In response to prolonged S-deficiency, a SAM-like protein and C_2H_4-induced *myb* transcripton factors were also upregulated, in addition to several auxin-related pathway genes [6]. Under P-depleted conditions, the expression of EIN3 and two CDPKs, and MPKK5 were up- and downregulated, respectively, in *A. thaliana* [62]. In addition, a *myb* transcription factor (myb59, At5g59780) induced under S-deficient conditions was similarly induced in P-deficient plants. An auxin-regulated protein, as well as an ABA-responsive element-binding factor, was also upregulated under P-deficient conditions. The fact that most *AtACS* genes responded to S-depletion, i.e. were downregulated, also suggests that C_2H_4 is a general regulator of the plant response to nutrient stress.

From this, it may be concluded that although C_2H_4 appears to be involved in *A. thaliana*'s adaptive response to S-depletion. However, it is unlikely to be the only hormone involved in mediating the plant response to S-depletion. Furthermore, C_2H_4 is more likely to act as a general rather than a specific nutrient stress signal.

4.4 Future studies

Some suggestions for future studies are:

- To perform all Western analyses using the more sensitive chemiluminescent method instead of the chromogenic method, as the former shows changes in protein accumulation more accurately.

- Hydroponic *A. thaliana* needs to be grown on a larger scale to provide sufficient plant material to obtain chloroplast extracts so that the changes, if any, in protein levels of other S-assimilation enzymes, such as ATPS, *O*ASTL and SiR, and not just APR, can be observed. This would help determine if these enzymes are similarly regulated by C_2H_4 as APR.

- The expression of genes encoding the different isozymes of ATPS, APR and *O*ASTL may be examined in plants subjected to C_2H_4 treatment and S-depletion, by the use of isoenzyme-specific primers. Because the antibodies to these enzymes cannot distinguish between isoforms, RT-PCR analyses using isoenzyme-specific primers would help identify which isoform(s) was responsible for the changes in protein accumulation observed.

- Hydroponic onions may be grown under S-sufficient and S-deficient conditions, and the changes, if any, in *ACS* expression determined once the onion *ACS* genes are characterised and primers can be designed for use in RT-PCR. The results from the current study appear to indicate that C_2H_4 may negatively regulate *O*ASTL, and mediate dual regulation of SiR in onions. Therefore, an examination of the expression of the onion *ACS* genes would complement the enzyme accumulation data, as *ACS* catalyses the rate-determining step of the C_2H_4-biosynthetic pathway, and would reflect the level of C_2H_4 production in these plants.

- It may be interesting to examine the effects of C_2H_4 on the S-assimilation enzymes at different concentrations of N, as several studies have indicated sufficient N is necessary for the upregulation of these enzymes in response to S-depletion

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