RESEARCH PAPER

Adenosine 5'-phosphosulphate reductase is regulated differently in Allium cepa L. and Brassica oleracea L. upon exposure to H2S

Mark Durenkamp1,2,*, Luit J. De Kok1,3 and Stanislav Kopriva2

1 Laboratory of Plant Physiology, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands
2 Department of Metabolic Biology, John Innes Centre, Norwich Research Park, Colney, Norwich NR4 7UH, UK
3 School of Forest and Ecosystems Sciences, The University of Melbourne, Water Street, Creswick, Victoria 3363, Australia

Received 14 November 2006; Revised 30 January 2007; Accepted 31 January 2007

Abstract

The reduction of adenosine 5'-phosphosulphate (APS) by APS reductase (APR) is considered to be one of the rate-limiting steps in the assimilation of sulphur in plants. In order to identify the mechanisms of regulation of this enzyme, the impact of atmospheric H2S exposure on mRNA expression, protein level, and activity of APR was studied in two species (Allium cepa L. and Brassica oleracea L.) with different physiological responses to H2S exposure. As expected, H2S exposure resulted in a rapid increase in thiol compounds in the shoot of both species. There was a substantial increase in total sulphur content in shoots of A. cepa, whereas it was hardly affected or even slightly decreased in B. oleracea. Sulphate uptake was only marginally affected in A. cepa, whereas it was strongly decreased in B. oleracea upon H2S exposure. Furthermore, H2S exposure resulted in a down-regulation of APR activity in shoot and roots of both species, which was probably mediated by a transcriptional mechanism of regulation by thiols, since mRNA levels also decreased. However, in contrast to B. oleracea, APR protein level was not affected by H2S exposure in A. cepa. The reduction in APR activity in onion was therefore achieved by an additional as yet unknown post-translational regulation. These results demonstrate that not only the physiological response to H2S, but also the molecular mechanisms of regulation of APR differ in the two species.

Key words: APS reductase, curly kale, hydrogen sulphide, onion, regulation, sulphate reduction, sulphate uptake, sulphur metabolism, thiols.

Introduction

Plants meet their demand for sulphur generally by taking up sulphate via the roots. The uptake and distribution of sulphate are mediated by specific sulphate transporter proteins (Hawkesford, 2003). Although all enzymes are available in the roots, the reduction of sulphate for the largest part takes place in the shoot (Droux, 2004; Saito, 2004). Sulphate is activated to adenosine 5'-phosphosulphate (APS), a reaction catalysed by ATP sulphurylase, either in the cytosol or in the plastids. The subsequent reduction of APS occurs in two steps, which exclusively take place in the plastids. In the first step, APS is reduced by APS reductase (APR) to sulphite. In the second step, sulphite is further reduced to sulphide by sulphite reductase. Finally, sulphide is incorporated into cysteine, the precursor for most other organic sulphur compounds, by 3'-O-acetylserine(thiol)lyase (OAS-TL) using 3'-O-acetylserine (OAS) as a substrate. OAS is synthesized from serine and acetyl-CoA by serine acetyltransferase.

In addition to sulphate taken up by the roots, plants are able to take up atmospheric sulphur gases via the leaves (De Kok et al., 1998). Hydrogen sulphide (H2S) is a phytotoxic gas, which may negatively affect metabolism in a similar way to cyanide (De Kok et al., 2002b); however, at low atmospheric levels, it may be beneficial.
as a sulphur source for growth if the roots are sulphate deprived (De Kok et al., 2002a; Buchner et al., 2004). The foliar uptake of H$_2$S is largely dependent on its rate of metabolism into cysteine by OAS-TL and subsequent assimilation into other organic sulphur compounds (De Kok et al., 1998, 2002a; Stuiver and De Kok, 2001). There is an interaction between the atmospheric H$_2$S and pedospheric sulphur utilization, and H$_2$S exposure may affect the uptake of sulphate by the root, and its transfer to and reduction in the shoot (Westerman et al., 2000, 2001a, b). H$_2$S exposure generally results in an increased content of water-soluble non-protein thiol compounds [mainly glutathione (GSH) and cysteine] in the shoot, and in some species an increase in the sulphate content of the shoot was observed (De Kok et al., 2002b; Durenkamp and De Kok, 2004).

The uptake of sulphate and the reduction of APS by APR are controlled by the sulphur nutritional status of the plant and are considered to be rate-limiting steps in the assimilation of sulphur in plants (Vauclare et al., 2002; Hawkesford and De Kok, 2006). The majority of the genes encoding the sulphate transporter proteins and enzymes of sulphate assimilation, except sulphite reductase, are present in multiple isoforms in Arabidopsis. Three isoforms of APR are present in Arabidopsis, and their expression and activity are affected by the sulphur status, cysteine, GSH, OAS, nitrogen supply, sugars, and phytohormones (Kopriva and Koprivova, 2003).

The greater part of current plant molecular research uses Arabidopsis as a model species. However, results obtained from Arabidopsis cannot be generalized, and the pattern of regulation of metabolic processes (e.g. sulphur metabolism) may differ between species (Kopriva, 2006) or even between cultivars (Westerman et al., 2001a). For instance, sulphur metabolism in Allium cepa L. and Brassica oleracea L. responded differently upon H$_2$S exposure (Durenkamp and De Kok, 2004, 2005b). Allium cepa (onion) is a member of the Alliaceae (garlic, leek) and is known for its capacity to synthesize relatively large amounts of secondary sulphur compounds (i.e. alliins and γ-glutamylpeptides). H$_2$S exposure resulted in an increased content of sulphate and organic (secondary) sulphur compounds, whereas sulphate uptake was not affected (Durenkamp and De Kok, 2004). However, in B. oleracea (curly kale), a member of the Brassicaceae and related to Arabidopsis, H$_2$S exposure hardly affected the total sulphur content, whereas sulphate uptake was partially down-regulated (Westerman et al., 2001a). In the present report, evidence is presented that not only does the physiological response to H$_2$S differ in the two species, but also the molecular mechanisms of regulation of APR. Whereas APR is transcriptionally regulated in B. oleracea, a novel post-translational regulation was observed in A. cepa.

### Materials and methods

#### Growth conditions

Seeds of onion (A. cepa L. cv. Nerato F1; Nickerson-Zwaan, Made, The Netherlands) and curly kale (B. oleracea L. cv. Arsis; Royal Sluis, Enkhuizen, The Netherlands) were germinated in vermiculite in a climate-controlled room. Day and night temperatures were 21°C and 17°C, respectively, relative humidity was 60–70%, and the photoperiod was 14 h at a photon fluence rate of 225±25 μmol m$^{-2}$ s$^{-1}$. Two-week-old and 10-d-old seedlings were transferred to 30.0 l tanks containing a 25% Hoagland nutrient solution, placed in 150.0 l cylindrical stainless steel cabinets with a polycarbonate top (Durenkamp and De Kok, 2002; Buchner et al., 2004). Day and night temperatures were 20°C and 16°C, respectively, relative humidity was 40–50%, and the photoperiod was 14 h at a photon fluence rate of 325±25 μmol m$^{-2}$ s$^{-1}$ at plant height. Plants were exposed to 0, 0.15, 0.3, 0.6, and 1.2 μl l$^{-1}$ H$_2$S, as described by Buchner et al. (2004), for 1 week. Allium cepa has a lower growth rate and a slower development compared with B. oleracea. Therefore, the two species had approximately the same developmental age at plant harvest.

#### Total sulphur

Analysis of the total sulphur content of the shoot was performed using a modification of the method described by Jones (1995). Samples were dried at 80°C for 18 h, pulverized by mortar and pestle, and 50–150 mg of the samples was weighed into porcelain ashing trays. Fifty percent Mg(NO$_3$)$_2$: 6 H$_2$O was added until the material was saturated, and it was dried in an oven at 100°C overnight. Subsequently, the samples were ashed in an oven at 650°C for 12 h. The residue was dissolved in 10 ml of 20% aqua regia (50 ml of HNO$_3$ and 150 ml of HCl in 1.0 l of distilled water) and the volume was adjusted to 100 ml with distilled water. One SulphaVer 4 Reagent Powder Pillow (HACH Permachem Reagents, Loveland, CO, USA) containing BaCl$_2$ was added to 25 ml of the mixture, and the turbidity was measured on a spectrophotometer (HACH DR/400V, Loveland) at 450 nm.

#### Water-soluble non-protein thiols

Water-soluble non-protein thiols in shoot and roots were extracted as described by Buchner et al. (2004) and their content was determined colorimetrically at 412 nm after reaction with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) according to De Kok et al. (1988).

#### Sulphate uptake

Sulphate uptake was determined over a 30 min period by transferring the plants to a 25% Hoagland nutrient solution containing 0.5 mM sulphate labelled with $^{35}$S-sulphate (4 MBq mmol$^{-1}$ sulphate). Subsequently, the roots were washed 3×20 s in cold non-labelled 25% Hoagland nutrient solution. Shoot and roots were separated and placed at 20°C in 10 ml of 1.2 M HCl for 1 week. Extracts were filtered over one layer of Miracloth, and an aliquot was taken and its radioactivity measured on a liquid scintillation counter (Tri-Carb 2000 CA Liquid Scintillation Analyzer, Perkin Elmer, Waltham, MA, USA).

#### RNA isolation and Northern analysis

Plants were harvested, and shoot and roots were separated, frozen in liquid N$_2$, and stored at −80°C. Total RNA was isolated from the...
Differential regulation of APS reductase in onion and curly kale upon H$_2$S exposure

plant material by phenol/chloroform/isoamylalcohol extraction and precipitation with LiCl. A 10 µg aliquot of RNA was separated on a 1% agarose gel in the presence of formaldehyde and transferred onto Hybond-N+ nylon membranes by capillary blotting. The membranes with RNA from *B. oleracea* and *A. cepa* were hybridized with a total cDNA fragment of APR2 from Arabidopsis thaliana and APR from *A. cepa* (provided by M McManus, Massey University, Palmerston North, New Zealand), respectively. The membranes were washed twice for 10 min and once for 30 min with 2× SSC in 0.1% SDS at 65 °C and exposed to an X-ray film (Kodak BioMax MS) for 4 h.

**APR activity**

Plant extracts were prepared from 100 mg of plant tissue by homogenization in 1 ml of 50 mM Na/KPO$_4$ buffer (pH 8) supplemented with 30 mM Na$_2$SO$_3$, 0.5 mM 5'-AMP, and 10 mM dithioerythritol (DTE). APR activity was measured in extracts as the production of [35S]sulphite, assayed as acid volatile radioactivity formed in the presence of [35S]APS and DTE (Brunold and Suter, 1990). Protein concentration in the extracts was determined according to Bradford (1976) with bovine serum albumin as standard.

**Western analysis**

Aliquots of extracts from APR activity measurements representing 10 µg of protein were subjected to SDS-PAGE and electrotransferred to a nitrocellulose filter. The blots were analysed with antisera against recombinant APR2 (Kopriva et al., 1999) and purified transketolase (Teige et al., 1995), and developed with the SuperSignal Western Blotting System (Pierce). The analysis was performed on two independent extracts with the same results.

**Results**

**Impact of H$_2$S exposure on plant growth**

A 1 week exposure of up to 0.6 µl $^{-1}$ H$_2$S hardly affected growth of *A. cepa* and *B. oleracea* seedlings. In *B. oleracea*, plant growth rate was even slightly increased at 0.3 µl $^{-1}$ H$_2$S (Table 1). Plant growth rate was only negatively affected at concentrations of ≥0.6 µl $^{-1}$ and 1.2 µl $^{-1}$ H$_2$S for *A. cepa* and *B. oleracea*, respectively. Shoot to root ratio and dry matter content were not affected in *A. cepa*, but were both slightly increased at 1.2 µl $^{-1}$ H$_2$S in *B. oleracea* (results not shown).

**Impact of H$_2$S exposure on total sulphur and thiol content**

As expected, H$_2$S exposure resulted in a significant increase in total sulphur content in the shoot of *A. cepa*, which depended on the concentration and the duration of the exposure. After 1 week, an increase in total sulphur content of 73% was observed at 0.3 µl $^{-1}$ H$_2$S, up to a 4-fold increase at 1.2 µl $^{-1}$ H$_2$S (Table 1), which could partly be explained by an increase in sulphate content (data not shown). In contrast, in *B. oleracea*, a decrease in total sulphur content upon a 1 week H$_2$S exposure was observed, up to 27% at 0.6 µl $^{-1}$ H$_2$S (Table 1). At 1.2 µl $^{-1}$ H$_2$S, the total sulphur content did not differ from control levels, since the observed decrease at lower H$_2$S levels was counterbalanced by an accumulation of organic sulphur due to H$_2$S exposure (data not shown), in combination with a reduction in growth (Table 1).

H$_2$S exposure resulted in a rapid accumulation of water-soluble non-protein thiol compounds in shoots of *A. cepa* and *B. oleracea* (Fig. 1A, B). Thiol accumulation was already maximal after 1 d of exposure, though its level increased with the atmospheric H$_2$S concentration and remained relatively stable upon further exposure up to 1 week. The increase in thiol content upon a 1 week exposure to 0.3 µl $^{-1}$ H$_2$S was 113% and 86% for shoots of *A. cepa* and *B. oleracea*, respectively, whereas at 1.2 µl $^{-1}$ H$_2$S a 4-fold increase was observed in both species (Fig. 1A, B). Root thiol contents were hardly affected and gradually declined in the course of the experiment. Root thiol contents were significantly increased up to 56% and 30% after 1 week of exposure at concentrations of 1.2 µl $^{-1}$ and ≥0.6 µl $^{-1}$ H$_2$S for *A. cepa* and *B. oleracea*, respectively (Fig. 1C, D).

**Table 1. Impact of atmospheric hydrogen sulphide exposure on plant growth rate and shoot total sulphur content in Allium cepa and Brassica oleracea**

| Plant growth rate (g g$^{-1}$ d$^{-1}$) | *A. cepa* | *B. oleracea* | Total sulphur content (µmol g$^{-1}$ FW) | *A. cepa* | *B. oleracea* |
|---------------------------------------|-----------|---------------|----------------------------------------|-----------|---------------|
| 0 µl $^{-1}$ H$_2$S                   | 0.143±0.005 a | 0.237±0.002 b | 8.9±0.4 a                              | 47.3±0.9 a |
| 0.15 µl $^{-1}$ H$_2$S                | 0.139±0.009 a,b | 0.237±0.020 a,b | 12.1±1.3 b                             | 45.1±1.3 a |
| 0.30 µl $^{-1}$ H$_2$S                | 0.134±0.007 a,b | 0.253±0.002 a | 15.4±1.7 b                             | 38.3±2.3 b |
| 0.60 µl $^{-1}$ H$_2$S                | 0.121±0.009 b | 0.237±0.007 b | 22.0±1.0 c                             | 34.3±2.5 b |
| 1.20 µl $^{-1}$ H$_2$S                | 0.083±0.005 c | 0.199±0.003 c | 36.6±1.7 d                             | 48.8±2.1 a |
Impact of H$_2$S exposure on sulphate uptake

H$_2$S exposure did not have a clear-cut effect on sulphate uptake by *Allium cepa* roots with the duration of the exposure (Fig. 2A). Sulphate uptake by *A. cepa* was significantly increased at 0.15 µl l$^{-1}$ and 0.6 µl l$^{-1}$ H$_2$S upon 1 d and 3 d of exposure, respectively, whereas at 1.2 µl l$^{-1}$ H$_2$S it was decreased by 47% upon 1 week of exposure. In contrast, H$_2$S exposure in *Brassica oleracea* resulted in a strongly decreased sulphate uptake from day 1 and day 3 onwards at 1.2 µl l$^{-1}$ and at 0.3 µl l$^{-1}$ and 0.6 µl l$^{-1}$ H$_2$S, respectively. In this species, sulphate uptake was already maximally reduced after 3 d of exposure, and upon a 1 week exposure to 0.3 µl l$^{-1}$ H$_2$S was reduced by 41% up to 78% at 1.2 µl l$^{-1}$ H$_2$S (Fig. 2B). Xylem loading of sulphate was hardly affected upon H$_2$S exposure in *A. cepa*, whereas it was slightly decreased in *B. oleracea* (data not shown). The decrease in sulphate uptake in *B. oleracea* correlated with the observed decrease in total sulphur content upon a 1 week exposure to 0.3 µl l$^{-1}$ and 0.6 µl l$^{-1}$ H$_2$S (Table 1).

**Impact of H$_2$S exposure on activity, expression, and protein levels of APR**

APR activity was determined in order to study the impact of H$_2$S exposure on sulphate reduction, since APR is considered to be the rate-limiting enzyme in the sulphate reduction pathway (Vauclare et al., 2002). APR activity in shoots of both *A. cepa* and *B. oleracea* was strongly...
reduced upon H₂S exposure, which was already apparent after 1 d and depended on the H₂S concentration. A 1 week exposure resulted in a decrease in shoot APR activity of 83% and 85% at 0.3 µl l⁻¹ and up to 98% and 96% at 1.2 µl l⁻¹ H₂S for A. cepa and B. oleracea, respectively (Fig. 3A, B). H₂S exposure also resulted in a decreased APR activity in roots of A. cepa and B. oleracea, although this was less pronounced when compared with the shoot. APR activity in A. cepa roots was significantly decreased at 0.3 µl l⁻¹ H₂S from day 1 to day 3, and at 0.6 µl l⁻¹ H₂S after 1 week of exposure (Fig. 3C). Brassica oleracea was not affected at 0.15 µl l⁻¹ H₂S, and only after 1 week at 0.3 µl l⁻¹ H₂S. A 1 week exposure to 0.3 µl l⁻¹ H₂S resulted in a decrease in root APR activity of 40% in both A. cepa and B. oleracea, and up to 96% and 75% at 1.2 µl l⁻¹ H₂S for A. cepa and B. oleracea, respectively (Fig. 3D). A gradual decrease in APR activity was observed in controls of A. cepa in the first days of the experiment, whereas controls in B. oleracea were relatively stable during the exposure period. The actual APR activity was higher in the shoot and lower in the roots of A. cepa compared with B. oleracea (Fig. 3).

In order to gain insight into the mechanism of regulation of APR, mRNA expression of APR and APR protein levels were determined in control plants and in plants exposed to 0.3 µl l⁻¹ H₂S. This concentration was selected since in both species APR activity was significantly reduced, whereas plant growth rate was not yet affected. Northern blots showed a decrease in expression of APR mRNA levels upon exposure to 0.3 µl l⁻¹ H₂S in shoot and roots of A. cepa and B. oleracea (Fig. 4), which closely matched with APR activity (Fig. 3).

For the assessment of APR protein levels, western blots were made with antibodies against onion APR and Arabidopsis APR2 for A. cepa and B. oleracea, respectively. These antibodies are considered to be non-specific and will determine all APR isoforms present. Surprisingly, APR protein levels in shoots and roots of A. cepa were hardly affected upon H₂S exposure (Fig. 5), which contrasted with the observed decrease in activity and expression of APR (Figs 3 and 4). However, in B. oleracea, H₂S exposure resulted in a rapid (within 24 h) and strong reduction in APR protein level in the shoot and from 3 d onwards in the root (Fig. 5). Here the decrease in APR protein level corresponded with the decrease in activity and expression of APR (Figs 3 and 4). There were no changes in the protein level of the used control transketolase (a Calvin cycle enzyme) upon H₂S exposure, which demonstrated that the observed effects on APR protein levels were exposure specific. From the present data, it is obvious that APR is down-regulated by H₂S at the transcriptional level in both A. cepa and B. oleracea, whereas the reduction in APR activity in A. cepa is achieved by an additional as yet unknown post-translational regulation.

**Fig. 3.** Impact of atmospheric hydrogen sulphide exposure on the activity of APS reductase (APR) in shoot (A, B) and roots (C, D) of Allium cepa (A, C) and Brassica oleracea (B, D). Three-week-old and 15-d-old seedlings, for A. cepa and B. oleracea, respectively, were grown on a 25% Hoagland nutrient solution and exposed to 0, 0.15, 0.3, 0.6, and 1.2 µl l⁻¹ H₂S for 1 week. APR activity was expressed as nmol g⁻¹ FW min⁻¹. Data represent the mean of three measurements with 12 and three plants each for A. cepa and B. oleracea, respectively.
Impact of atmospheric hydrogen sulphide exposure on growth and content of sulphur-containing compounds

H₂S phytotoxicity generally depends on the concentration and duration of the exposure and differs greatly between species (De Kok et al., 1998; Durenkamp et al., 2005). *Allium cepa* and *Brassica oleracea* were not very susceptible to H₂S, and growth was only affected at relatively high atmospheric concentrations of ≥0.6 μl l⁻¹ and 1.2 μl l⁻¹ H₂S, respectively (Table 1). Growth reduction at these concentrations has to be taken into consideration when discussing changes in sulphate uptake, APR activity, and metabolite content. Therefore, a non-toxic concentration of 0.3 μl l⁻¹ H₂S was chosen for determination of APR mRNA and protein levels. The slight increase in plant growth rate at 0.3 μl l⁻¹ H₂S in *B. oleracea* could be explained by a positive effect of H₂S as sulphur source for growth or it may be attributed to an overcompensation upon disruption of plant homeostasis by low toxin doses, i.e. hormesis (Calabrese, 1999). A similar growth stimulation at low H₂S concentrations was observed in a number of species, although a satisfactory explanation was never found (Thompson and Kats, 1978; Durenkamp and De Kok, 2005a).

### Discussion

**Impact of H₂S exposure on growth and content of sulphur-containing compounds**

H₂S exposure generally results in a rapid increase in thiol compounds in the shoot and in some cases to a lesser extent in the roots (Fig. 1; De Kok et al., 2002b). GSH is the predominant thiol compound present in plants, even upon H₂S exposure. In addition to GSH, H₂S exposure generally results in strongly enhanced levels of cysteine (up to 30-fold) and, in the dark, high levels of γ-glutamylcysteine (up to 20-fold) in the shoot (De Kok et al., 1998, 2002b; Durenkamp and De Kok, 2004). The impact of H₂S exposure on total sulphur content differs between species. For example, from previous studies and the present study, it is evident that there was a substantial increase in total sulphur content in shoots of *A. cepa* upon H₂S exposure (Table 1; Durenkamp and De Kok, 2004), whereas that in *B. oleracea* was hardly affected or even slightly decreased (Table 1; Westerman et al., 2001a; Durenkamp and De Kok, 2004). Westerman et al. (2001a) showed that the total sulphur content was not affected by H₂S exposure in *B. oleracea*, since an increase in reduced sulphur compounds via incorporation of H₂S was compensated by a decrease in sulphate uptake and assimilation. The present study showed that the strong decrease in sulphate uptake may even result in a lower total sulphur content in *B. oleracea* upon H₂S exposure. This difference from previous results might be explained by differences in the developmental stage of the plants. Young seedlings as used in the present study have lower contents of sulphate. Sulphate might act as a buffer upon changes in the sulphur supply, and a lower content might result in more dramatic changes in sulphate uptake upon H₂S exposure.

Under normal conditions, the uptake and assimilation of sulphate will be in tune with the plant's sulphur demand for growth, which represents the plant's sulphur content times growth rate (Durenkamp and De Kok, 2004; Hawkesford and De Kok, 2006). On the basis of sulphur content and growth rate, the sulphur demand of *A. cepa* and *B. oleracea* was estimated to be ~1 μmol g⁻¹ plant d⁻¹ and 10 μmol g⁻¹ plant d⁻¹, respectively (Durenkamp and De Kok, 2004). These values are in close agreement with the extrapolated sulphate uptake measurements to a plant daily basis in this study. The sulphate uptake of *A. cepa* and *B. oleracea* was 1.1 μmol g⁻¹ plant d⁻¹ and 8.4 μmol g⁻¹ plant d⁻¹ for control plants at day 7, respectively (derived from Fig. 2). H₂S exposure resulted in a strong decrease in sulphate uptake and reduction in both species, which confirmed previous observations that H₂S absorbed by the shoot could replace sulphate taken up by the roots as a sulphur source for growth in these species (Figs 2 and 3; Westerman et al., 2000; Buchner et al., 2004; Durenkamp and De Kok, 2004).

**Regulation of shoot APR upon H₂S exposure**

APR is considered to be one of the main sites of regulation in the sulphate assimilation pathway and has gathered considerable interest during the last decade.
(Martin et al., 2005; Kopriva, 2006). APR activity is regulated by a range of compounds including thiol compounds (Lappartient et al., 1999; Vauclare et al., 2002; Hartmann et al., 2004), OAS (Koprivova et al., 2000), nitrogen supply (Koprivova et al., 2000), sugars (Kopriva et al., 1999; Hesse et al., 2003), and phytohormones (Ohkama et al., 2002). The impact of sulphate nutrition on APR transcripts and activity has been described in several species. Sulphate deprivation generally resulted in increased levels of mRNA and activity of APR, which showed that APR was mainly regulated at a transcriptional level. Transcriptional regulation of APR by various effectors has been shown in several species, including Arabidopsis (Kopriva et al., 1999, 2000; Vauclare et al., 2002), poplar (Hartmann et al., 2004; Kopriva et al., 2004), and maize (Kopriva et al., 2001; Hopkins et al., 2004). Specifically, APR activity and mRNA levels were feedback inhibited by thiols in Arabidopsis and poplar (Vauclare et al., 2002; Hartmann et al., 2004). In this study, mRNA levels, protein levels, and activity of APR in B. oleracea were significantly decreased within 1 d of exposure and depended on the concentration of H₂S, which indicated a regulation at the transcriptional level upon exposure to H₂S. Thiol compounds are the most likely candidates as regulators of APR at the transcriptional level, since the reduction in mRNA (protein level and activity) coincided with a strong increase in thiol compounds in the shoot (Figs 1, 3, 4, and 5). Although GSH has been indicated to act as a regulator of APR in most species, a role for cysteine cannot be excluded at this point. The strong reduction in APR protein level within 24 h indicated a quick turnover in B. oleracea, which seemed to be faster than that observed previously in Lemna minor and Arabidopsis, where a half-life of ~10 h was established (Brunold and Schmidt, 1978; Bick et al., 2001).

Sulphate transporters and APR are subjected to transcriptional regulation. However, changes in gene expression do not necessarily result in changes in protein content and/or enzyme activity, which indicates that post-transcriptional and/or post-translational regulation participates in modulating their activities (Hawkesford and De Kok, 2006). Under certain conditions, evidence for post-translational regulation of APR has been found, for instance during oxidative stress in plants (Kopriva et al., 1999; Bick et al., 2001) and in the green alga Chlamydomonas reinhardtii (Ravina et al., 2002). APR activity is probably modulated by thiols through a redox reaction, although the exact mechanism is still unsolved. It might involve a regulatory cysteine pair (Bick et al., 2001), or a change between an active dimer and an inactive monomer (Kopriva and Koprivova, 2004). The decrease in APR mRNA and activity in onion upon H₂S exposure (Figs 3 and 4) indicated a transcriptional mechanism of regulation as shown in B. oleracea (see above). However, the APR protein content was hardly affected, and consequently an additional post-translational mechanism should be required in A. cepa (Fig. 5). The absence of changes in APR protein level in A. cepa might be due to a very low turnover rate of the APR protein and/or inactivation of the protein upon H₂S exposure, which prevents its degradation. Post-translational regulation of APR in A. cepa may involve inactivation of the enzyme by thiols, since an increase in thiol compounds in the shoot (cysteine and GSH) is characteristic for H₂S exposure (Fig. 1; De Kok et al., 2002b; Durenkamp and De Kok, 2004). However, the subcellular site of thiol accumulation upon H₂S exposure remains to be investigated (De Kok et al., 2002b), although an increase in multiple subcellular compartments including the chloroplast, the site of APR, is most likely. It is proposed that APR is regulated transcriptionally in both species, whereas an additional post-translational regulation by thiols is required in A. cepa.

Shoot to root signalling: impact of H₂S exposure on root APR and sulphate uptake

Thiol compounds have been shown to regulate sulphate uptake and assimilation (Bolchi et al., 1999; Vauclare et al., 2002), which has been explained as a feedback control mechanism by reduced sulphur compounds, i.e. demand-driven control (Lappartient and Touraine, 1996). Sulphate uptake in B. oleracea and APR activity in the roots of both species were clearly down-regulated upon atmospheric H₂S exposure, which might indicate a shoot to root signalling (Figs 3 and 4). GSH is considered to be the most abundant form of reduced sulphur in the phloem and to be responsible for the transport of sulphur from the shoot to the root (Rennenberg et al., 1979; Lappartient and Touraine, 1996), although the significance of S-methylmethionine in phloem sulphur transport remains to be investigated further (Bourgis et al., 1999). A decrease in APR activity in the roots might result from transcriptional and post-translational regulation by thiols in B. oleracea and A. cepa, respectively, as was postulated for the shoot. However, H₂S exposure did not result in a significant increase in the thiol content of the roots at 0.3 μl l⁻¹H₂S. Many exceptions to the demand-driven control model can be found, and it is clear that GSH is not the only regulator of plant sulphur nutrition and mediator of feedback inhibition, which probably also differs between species (Westerman et al., 2000; Herschbach, 2003). For instance, in maize, cysteine and not GSH was shown to act as an inhibitor of sulphate uptake and assimilation (Bolchi et al., 1999). Either other compounds were involved in the regulation of APR activity or slight subcellular differences in thiol content at the site of APS reduction were responsible for the decrease in APR activity in the roots upon H₂S exposure.

In conclusion, sulphate uptake and APR activity are key regulators of sulphate assimilation in plants and are often
in tune with each other. From the present study it is evident that the uptake of sulphate and the activity of APR seemed to be in tune upon exposure of *B. oleracea* to H$_2$S and might be regulated by the same compounds. In *A. cepa*, APR activity was reduced by a post-translational mechanism in both shoot and root upon H$_2$S exposure, whereas sulphate uptake was hardly affected. A low APR protein turnover rate and/or protein stabilization by reduced sulphur compounds are likely to be responsible for the absence of changes in the protein level in *A. cepa*. The dissimilarities in APR protein turnover rate between *B. oleracea* and *A. cepa* might be explained by their differences in composition and partitioning of sulphur-containing metabolites. Allium species have a high capacity to incorporate sulphur into secondary sulphur compounds, which excludes the need to maintain a steady total sulphur content by down-regulation of sulphate uptake and assimilation. Furthermore, sulphate uptake might be necessary as a source of sulphate for sulphation reactions via the APS kinase pathway in *A. cepa*, whereas *B. oleracea* has large vacuolar sulphate reserves to fall back upon. The impact of a low APR protein turnover in *A. cepa* seemed to be over-ruled by a post-translational regulation upon H$_2$S exposure.

**Acknowledgements**

MD was supported by a grant from the NWO/British Council UK–Netherlands Partnership Programme in Science (PPS 829). SK is supported by the Biotechnology and Biological Science Research Council (BBSRC) of the UK.

**References**

Bick JA, Setterdahl AT, Knaff DB, Chen YC, Pitcher LH, Zilinskas BA, Leustek T. 2001. Regulation of the plant-type 5′-adenylate sulphate reductase by oxidative stress. *Biochemistry* 40, 9040–9048.

Bolchi A, Petrucco S, Tenca PL, Foroni C, Ottonello S. 1999. Coordinate modulation of maize sulfate permease and ATP sulfurylase mRNAs in response to variations in sulfur nutritional status: stereospecific down-regulation by L-cysteine. *Plant Molecular Biology* 39, 527–537.

Bourgis F, Roje S, Nuccio ML, et al. 1990. Adenosine 5′-phosphosulfate sulfo-transferase. In: Lea P, ed. *Methods in plant biochemistry.* London: Academic Press, 339–343.

Buchner P, Stuiver CEE, Westerman S, Wirtz M, Hell R, Hawkesford MJ, De Kok LJ. 2004. Regulation of sulfate uptake and expression of sulfate transporter genes in *Brassica oleracea* as affected by atmospheric H$_2$S and pedospheric sulfate nutrition. *Plant Physiology* 136, 3396–3408.

Calabrese EJ. 1999. Evidence that hormesis represents an ‘over-compensation’ response to a disruption in homeostasis. *Ecotoxicology and Environmental Safety* 42, 135–137.

De Kok LJ, Buwalda F, Bosma B. 1988. Determination of cysteine and its accumulation in spinach leaf tissue upon exposure to excess sulfur. *Journal of Plant Physiology* 133, 502–505.

De Kok LJ, Castro A, Durenkamp M, Stuiver CEE, Westerman S, Yang L, Stulen I. 2002a. Sulphur in plant physiology. *Proceedings No. 300.* York: International Fertiliser Society, 1–26.

De Kok LJ, Stuiver CEE, Stulen I. 1998. Impact of atmospheric H$_2$S on plants. In: De Kok LJ, Stulen I, eds. *Responses of plant metabolism to air pollution and global change.* Leiden: Backhuys Publishers, 51–63.

De Kok LJ, Stuiver CEE, Westerman S, Stulen I. 2002b. Elevated levels of hydrogen sulfide in the plant environment: nutrient or toxin. In: Omasa K, Saji H, Youssefian S, Kondo N, eds. *Air pollution and plant biotechnology. Prospects for phytomonitoring and phytoremediation.* Tokyo: Springer-Verlag, 201–219.

Droux M. 2004. Sulfur assimilation and the role of sulfur in plant metabolism: a survey. *Photosynthesis Research* 79, 331–348.

Durenkamp M, De Kok LJ. 2002. The impact of atmospheric H$_2$S on growth and sulfur metabolism of *Allium cepa* L. *Phyton* 42 (3), 55–63.

Durenkamp M, De Kok LJ. 2004. Impact of pedospheric and atmospheric sulphur nutrition on sulphur metabolism of *Allium cepa* L., a species with a potential sink capacity for secondary sulphur compounds. *Journal of Experimental Botany* 55, 1821–1830.

Durenkamp M, De Kok LJ. 2005a. Low levels of H$_2$S may replace sulfate as sulfur source in sulfate-deprived onion. *Phyton* 45, 69–77.

Durenkamp M, De Kok LJ. 2005b. Sulfur metabolism in onion (*Allium cepa* L.): accumulation of sulphur compounds upon exposure to H$_2$S. In: Saito K, De Kok LJ, Stulen I, Hawkesford MJ, Schmug E, Sirko A, Rennenberg H, eds. *Sulfur transport and assimilation in plants in the post genomic era.* Leiden: Backhuys Publishers, 127–131.

Durenkamp M, Posthumus FS, Stuiver CEE, De Kok LJ. 2005. Metabolism of atmospheric sulfur gases in onion. In: Omasa K, Nouchi I, De Kok LJ, eds. *Plant responses to air pollution and global change.* Tokyo: Springer-Verlag, 3–11.

Hartmann T, Hönike P, Wirtz M, Hell R, Rennenberg H, Koprina S. 2004. Regulation of sulphate assimilation by glutathione in poplars (*Populus tremula*×*P. alba*) of wild type and overexpressing γ-glutamylcysteine synthetase in the cytosol. *Journal of Experimental Botany* 55, 837–845.

Hawkesford MJ. 2003. Transporter gene families in plants: the sulphate transporter gene family—redundancy or specialization? *Physiologia Plantarum* 117, 155–163.

Hawkesford MJ, De Kok LJ. 2006. Managing sulphur metabolism in plants. *Plant, Cell and Environment* 29, 382–395.

Herschbach C. 2003. Whole plant regulation of sulfur nutrition of deciduous trees—influences of the environment. *Plant Biology* 5, 233–244.

Hesse H, Trachsel N, Suter M, Koprina S, von Ballmoos P, Rennenberg H, Brunold C. 2003. Effect of glucose on assimilatory sulphate reduction in *Arabidopsis thaliana* roots. *Journal of Experimental Botany* 54, 1701–1709.
Differential regulation of APS reductase in onion and curly kale upon H₂S exposure

Hopkins L, Parmar S, Bouranis DL, Howarth JR, Hawkesford MJ. 2004. Coordinated expression of sulfate uptake and components of the sulfate assimilatory pathway in maize. Plant Biology 6, 408–414.

Hunt R. 1982. Plant growth curves. The functional approach to plant growth analysis. London: E. Arnold Publishers, 5–33.

Jones JB, Jr. 1995. Determining total sulphur in plant tissue using the HACH kit spectrophotometer technique. Sulphur in Agriculture 19, 58–62.

Kopriva S. 2006. Regulation of sulfate assimilation in Arabidopsis and beyond. Annals of Botany 97, 479–495.

Kopriva S, Hartmann T, Massaro G, Hönicke P, Rennenberg H. 2004. Regulation of sulfate assimilation by nitrogen and sulfur nutrition in poplar trees. Trees 18, 320–326.

Kopriva S, Jones S, Koprivova A, Suter M, von Ballmoos P, Brander K, Flückiger J, Brunold C. 2001. Influence of chilling stress on the intercellular distribution of assimilatory sulfate reduction and thiols in Zea mays. Plant Biology 3, 24–31.

Kopriva S, Koprivova A. 2003. Sulphate assimilation: a pathway which likes to surprise. In: Abrol YP, Ahmad A, eds. Sulphur in higher plants. Dordrecht: Kluwer Academic Publishers, 87–112.

Kopriva S, Koprivova A. 2004. Plant adenosine 5′-phosphosulphate reductase: the past, the present, and the future. Journal of Experimental Botany 55, 1775–1783.

Kopriva S, Muheim R, Koprivova A, Trachsel N, Catalano C, Suter M, Brunold C. 1999. Light regulation of assimilatory sulphate reduction in Arabidopsis thaliana. The Plant Journal 20, 37–44.

Kopriva S, Suter M, Op den Camp R, Brunold C, Kopriva S. 2000. Regulation of sulfate assimilation by nitrogen in Arabidopsis. Plant Physiology 122, 737–746.

Lappartient AG, Touraine B. 1996. Demand-driven control of root ATP sulfurylase activity and SO₄²⁻ uptake in intact canola. The role of phloem-translocated glutathione. Plant Physiology 111, 147–157.

Lappartient AG, Vidmar JJ, Leustek T, Glass ADM, Touraine B. 1999. Inter-organ signaling in plants: regulation of ATP sulfurylase and sulfate transporter genes expression in roots mediated by phloem-translocated compound. The Plant Journal 18, 89–95.

Martin MN, Tarczynski MC, Shen B, Leustek T. 2005. The role of 5′-adenylylsulfate reductase in controlling sulfate reduction in plants. Photosynthesis Research 86, 309–323.

Okkama N, Takei K, Sakakibara H, Hayashi H, Yoneyama T, Fujitwara T. 2002. Regulation of sulfur-responsive gene expression by exogenously applied cytokinins in Arabidopsis thaliana. Plant and Cell Physiology 43, 1493–1501.

Ravina CG, Chang CI, Tsakraklides GP, McDermott JP, Vega JM, Leustek T, Gotor C, Davies JP. 2002. The sac mutants of Chlamydomonas reinhardtii reveal transcriptional and posttranscriptional control of cysteine biosynthesis. Plant Physiology 130, 2076–2084.

Rennenberg H, Schnitzk K, Bergmann L. 1979. Long-distance transport of sulfur in Nicotiana tabacum. Planta 147, 57–62.

Saito K. 2004. Sulfur assimilatory metabolism. The long and smelling road. Plant Physiology 136, 2443–2450.

Stuiver CEE, De Kok LJ. 2001. Atmospheric H₂S as sulfur source for Brassica oleracea: kinetics of H₂S uptake and activity of O-acetylsereine (thiol)lyase as affected by sulfur nutrition. Environmental and Experimental Botany 46, 29–36.

Teige M, Kopriva S, Bauwe H, Suss KH. 1995. Chloroplast pentose-5-phosphate 3-epimerase from potato: cloning, cDNA sequence, and tissue-specific enzyme accumulation. FEBS Letters 377, 349–352.

Thompson CR, Kats G. 1978. Effects of continuous H₂S fumigation on crop and forest plants. Environmental Science and Technology 12, 550–553.

Vauclare P, Kopriva S, Fell D, Suter M, Sticher L, von Ballmoos P, Krähenbühl U, Op den Camp R, Brunold C. 2002. Flux control of sulphate assimilation in Arabidopsis thaliana: adenosine 5′-phosphosulphate reductase is more susceptible than ATP sulphurylase to negative control by thiols. The Plant Journal 31, 729–740.

Westerman S, Blake-Kalff MMA, De Kok LJ, Stulen I. 2001a. Sulfate uptake and utilization by two varieties of Brassica oleracea with different sulfur need as affected by atmospheric H₂S. Phyton 41, 49–61.

Westerman S, De Kok LJ, Stuiver CEE, Stulen I. 2000. Interaction between metabolism of atmospheric H₂S in the shoot and sulfate uptake by the roots of curly kale (Brassica oleracea). Physiologia Plantarum 109, 443–449.

Westerman S, Stulen I, Suter M, Brunold C, De Kok LJ. 2001b. Atmospheric H₂S as sulphur source for Brassica oleracea: consequences for the activity of the enzymes of the assimilatory sulphate reduction pathway. Plant Physiology and Biochemistry 39, 425–432.