



RESEARCH PAPER

Effect of glucose on assimilatory sulphate reduction in *Arabidopsis thaliana* roots

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Received 13 December 2002; Accepted 24 March 2003

Abstract

With the aim of analysing the relative importance of sugar supply and nitrogen nutrition for the regulation of sulphate assimilation, the regulation of adenosine 5'-phosphosulphate reductase (APR), a key enzyme of sulphate reduction in plants, was studied. Glucose feeding experiments with *Arabidopsis thaliana* cultivated with and without a nitrogen source were performed. After a 38 h dark period, APR mRNA, protein, and enzymatic activity levels decreased dramatically in roots. The addition of 0.5% (w/v) glucose to the culture medium resulted in an increase of APR levels in roots (mRNA, protein and activity), comparable to those of plants kept under normal light conditions. Treatment of roots with D-sorbitol or D-mannitol did not increase APR activity, indicating that osmotic stress was not involved in APR regulation. The addition of O-acetyl-L-serine (OAS) also quickly and transiently increased APR levels (mRNA, protein, and activity). Feeding plants with a combination of glucose and OAS resulted in a more than additive induction of APR activity. Contrary to nitrate reductase, APR was also increased by glucose in N-deficient plants, indicating that this effect was independent of nitrate assimilation. [³⁵S]-sulphate feeding experiments showed that the addition of glucose to dark-treated roots resulted in an increased incorporation of [³⁵S] into thiols and proteins, which corresponded to the increased levels of APR activity. Under N-deficient conditions, glucose also increased thiol labelling, but did not increase the incorporation

of label into proteins. These results demonstrate that (i) exogenously supplied glucose can replace the function of photoassimilates in roots; (ii) APR is subject to co-ordinated metabolic control by carbon metabolism; (iii) positive sugar signalling overrides negative signalling from nitrate assimilation in APR regulation. Furthermore, signals originating from nitrogen and carbon metabolism regulate APR synergistically.

Key words: Adenosine-5'-phosphosulphate, APS reductase, O-acetyl-L-serine, sulphate assimilation.

Introduction

Reduced sulphur compounds produced by assimilatory sulphate reduction play an important role in many plant responses to various kinds of stress, including oxidative stress, chilling and exposure to heavy metals, and xenobiotics (Brunold and Rennenberg, 1997). Plants have to adapt to a rapidly changing demand for reduced sulphur by modulating the flux through sulphate assimilation (Brunold, 1993; Hawkesford and Wray, 2000; Leustek *et al.*, 2000; Bick *et al.*, 2001; Vauclare *et al.*, 2002). This pathway, consisting of succeeding reactions catalysed by ATP sulphurylase (ATPS), adenosine 5'-phosphosulphate (APS) reductase (APR), sulphite reductase (SiR), and O-acetyl-L-serine (thiol)lyase (OASTL), has been shown to be highly regulated (Brunold, 1990, 1993; Hawkesford and Wray, 2000; Leustek *et al.*, 2000). The first two enzymes of the pathway, ATPS and APR, are

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up-regulated at mRNA, protein and activity level by sulphur deficiency (Brunold *et al.*, 1987; Takahashi *et al.*, 1997; Bolchi *et al.*, 1999; Lappartient *et al.*, 1999). On the other hand, an increased demand for sulphur-containing compounds due to exposure to heavy metals, herbicide safeners or chilling temperatures also resulted in increased activity of APR (Farago and Brunold, 1990; Rüegeegger *et al.*, 1990; Brunner *et al.*, 1995). APR activity (Neuenschwander *et al.*, 1991; Koprivova *et al.*, 2000) and APR mRNA (Koprivova *et al.*, 2000) were also increased after the addition of *O*-acetyl-L-serine (OAS) to the culture medium. In plant metabolism, OAS is formed from acetate and L-serine via serine acetyltransferase (SAT). OAS is subsequently used for cysteine synthesis by OASTL (Leustek *et al.*, 2000). OAS thus links assimilatory sulphate reduction with carbohydrate and nitrogen metabolism and has been proposed as a signalling molecule coordinating these three pathways (Brunold, 1993). In various systems APR expression and activity was shown to be down-regulated by nitrogen starvation (Brunold and Suter, 1984; Haller *et al.*, 1986; Yamaguchi *et al.*, 1999; Koprivova *et al.*, 2000).

Many nuclear-encoded genes for plastidic localized enzymes in *Arabidopsis* are light-regulated (Kloppstech, 1997; Dijkwel *et al.*, 1997; Hesse *et al.*, 1999) or follow circadian rhythms, dependent on light/dark cycles (Brouquisse *et al.*, 1998; Harmer *et al.*, 2000). APR activity and flux through the sulphate assimilation pathway are also induced by light (Neuenschwander *et al.*, 1991; Kocsy *et al.*, 1997; Kopriva *et al.*, 1999). The light induction of APR could be mimicked by the addition of OAS to the nutrition solution in the dark (Neuenschwander *et al.*, 1991). The APR of maize and *Arabidopsis thaliana* follows a diurnal rhythm with maximum activity during the light period (Kocsy *et al.*, 1997; Kopriva *et al.*, 1999). In *Arabidopsis*, mRNA of APR starts to accumulate 4 h before light onset, indicating that the enzyme is not controlled by light alone, but also by an internal signal. Indeed, feeding of sucrose in the dark led to increased levels of APR mRNA, protein and enzyme activity in roots (Kopriva *et al.*, 1999). Interestingly, APR2 mRNA was more susceptible to regulation by light/dark cycles and sucrose feeding than APR1 and APR3 mRNA (Kopriva *et al.*, 1999).

The expression of many genes is altered by changes in carbohydrate levels (Koch, 1996; Smeekens, 2000). Which plant processes are affected by sugars, how plants sense the level of various sugars or sugar metabolites, and how environmental-response pathways are involved have yet to be determined. Several genes (including the small subunit of Rubisco, chlorophyll *alb* binding proteins and α -amylase) are repressed when carbohydrate levels are heightened (Krapp *et al.*, 1993; Umemura *et al.*, 1998), a phenomenon known as glucose repression (Halford *et al.*, 1999). Nitrate reductase expression, however, was induced

by sugars (Cheng *et al.*, 1992; Morcuende *et al.*, 1998). The exact mechanisms by which plants sense sugar and initiate signal transduction are still unknown (Smeekens and Rook, 1997; Gibson, 2000; Koch *et al.*, 2000).

In the present paper the effect of glucose feeding on APR2 mRNA levels, protein accumulation and activity in roots of *Arabidopsis thaliana* cultivated with and without a nitrogen source was analysed after a prolonged dark period. APR2 mRNA was estimated because it was more susceptible to regulation as indicated above (Kopriva *et al.*, 1999). To assess the effect of glucose on the flux through sulphate assimilation, *in vivo* incorporation of [³⁵S]-sulphate into cysteine, glutathione and protein was measured.

Materials and methods

Plant material and growth

Arabidopsis thaliana (var. Columbia) plants were grown in pots filled with moistened balls (2–6 mm) of burned clay (Migros, Switzerland) in trays containing Hentschel nutrition solution (Hentschel, 1970). Plants were grown under a 10/14 h light/dark cycle and a temperature of 25±2 °C. All experiments were performed with 4–5-week-old plants, which were preincubated in the dark in a fresh nutrient solution for 38 h. At the beginning of the experiments, 0.5% (w/v) glucose, mannitol or sorbitol or 0.5 mM OAS were added to the nutrient solution and the plants were further incubated in the dark. For experiments with nitrogen-free nutrition, plants were placed for 72 h before beginning the glucose treatment on a modified Hentschel nutrition solution substituting all nitrogen-containing components by the corresponding chlorides.

Enzyme assays and ATP measurement

Roots were extracted 1:10 (w/v) in 100 mM TRIS-HCl buffer (pH 8) supplemented with 30 mM Na₂SO₃, 0.5 mM 5'-AMP, and 10 mM DTE (Imhof, 1994), using a glass homogenizer. The crude extracts were filtered through two layers of Miracloth (Migros, Bern, Switzerland). APR activity in extracts was measured as the production of [³⁵S]-sulphite, assayed as acid volatile radioactivity formed in the presence of [³⁵S]-APS and DTE (Brunold and Suter, 1990). OASTL activity was determined by measuring the cysteine produced from OAS and S²⁻ as described in Pieniazek *et al.* (1973). ATPS activity was determined in the same extracts, diluted one-fifth with extraction buffer, by measurement of ATP production from APS and inorganic pyrophosphate using an ATP meter (Schmutz and Brunold, 1982). This method was also used for the determination of cellular ATP levels. SiR activity was determined according to von Arb and Brunold (1983) by measuring the cysteine formed from sulphide, the product of the enzyme, and added OAS using acid ninhydrin reagent (Pieniazek *et al.*, 1973). The protein concentrations of the root extracts were determined according to Bradford (1976) with bovine serum albumin as standard (BioRad Protein Assay, BioRad Laboratories, Munich, Germany).

Isolation of total RNA and northern blotting

Roots were homogenized with a mortar and pestle in liquid nitrogen and the RNA was isolated by phenol extraction and selective precipitation with LiCl. Total RNA was separated on an agarose-formaldehyde-gel. The RNA was transferred onto Hybond-N nylon membranes (Amersham) and hybridized with a [³²P]-labelled cDNA probe for APR2 (Setya *et al.*, 1996). The membranes were washed four times at different concentrations of SSC in 0.1% SDS for

20 min, the final washing step being $0.5\times$ SSC, 0.1% SDS at 65 °C, and exposed to an X-ray film (Fuji medical RX) at -80 °C for 3–8 d.

Western blot analysis

The protein extracts used for APR measurements were diluted with an equal volume of $2\times$ SDS-PAGE loading buffer and heated for 5 min at 90 °C (Laemmli, 1970). Aliquots representing 10 μ g protein were subjected to SDS-PAGE and electrotransferred to nitrocellulose filter (0.2 μ m pore size; Schleicher and Schuell, Dassel, Germany). The blots were analysed with an antiserum directed against APR and developed with the SuperSignal Western Blotting System according to the manufacturer's instructions (Pierce, Illinois, USA). The antiserum was produced in rabbits from purified APR2 protein overexpressed in *E. coli* by the pET His-Tag system (Novagen). The antiserum cross-reacted with recombinant proteins from APR1 and APR3 isoforms.

Feeding of [35 S]-sulphate and determination of [35 S] in thiols and proteins

Six pots of *A. thaliana* plants, precultivated in the dark for 38 h either in Hentschel nutrient solution or in N-free nutrient solution, each containing 0.75 mM sulphate, were supplemented with 0.5% glucose. After 4 h, 6 mCi [35 S]-sulphate (Hartmann, Braunschweig, Germany) was added to the nutrient solution and the plants were kept in dark for an additional 4 h. Roots of treated plants were extracted 1:5 (w/v) in glass potters in 0.1 M HCl containing 1 mM Na₂EDTA and the extracts were centrifuged for 30 min at 4 °C. The samples were analysed as described in Kopriva *et al.* (1999) and Koprivova *et al.* (2000). Thiols in the supernatant were determined according to Bernhard *et al.* (1998) and labelled with monobromobimane as described by Kranner and Grill (1996). A 100 μ l aliquot of each sample was separated by reversed-phase HPLC as previously described (Rüegsegger and Brunold, 1992) and fractions of 0.75 ml were collected in scintillation vials. [35 S] radioactivity was determined in a Betamatic V liquid scintillation counter (Kontron, Zürich, Switzerland). The radioactivity in the first five fractions of the eluate corresponded to [35 S]-sulphate. Total cysteine, γ -EC and GSH were analysed by the same HPLC system as described by Rüegsegger and Brunold (1992). Incorporation of [35 S] into proteins was measured by precipitating 200 μ l aliquots of extracts with 10% TCA, washing twice with 1% TCA and once with 96% ethanol, dissolved in 400 μ l 0.2 M NaOH determining the radioactivity in an aliquot using a liquid scintillation counter (Koprivova *et al.*, 2000)

Statistical analysis

The Student–Newmann–Keuls method (SigmaStat for Windows, Version 1.0, 1992–94, Jandel Corporation) was used to determine significant differences in the enzyme activities and the contents of labelled thiols.

Results

Effect of glucose feeding after a prolonged dark period on the activity of enzymes involved in sulphate assimilation in roots

A prolonged dark period had a significant effect on the activity of enzymes involved in sulphate assimilation in *A. thaliana* roots (Fig. 1). A decrease in activity of the first three enzymes of the pathway, ATPS, APR and SiR was detected, whereas OASTL's activity increased. APR was more affected than all other enzymes with a remaining activity of 5% of that detected in light. ATPS and SiR

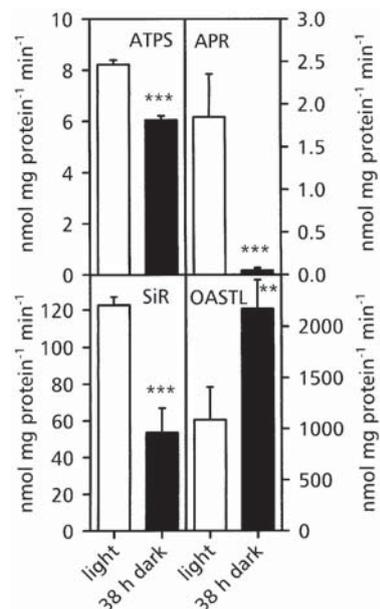


Fig. 1. Activity of the enzymes of sulphate assimilation in *A. thaliana* roots before and after a prolonged dark period. The activity of ATP sulphurylase (ATPS), APS reductase (APR), sulphite reductase (SiR), and *O*-acetyl-L-serine(thiol)lyase (OASTL) was measured before (open bars) or after (black bars) a 38 h dark period. Mean values \pm SD of four measurements are presented. Light and dark values significantly different at $P < 0.01$ and 0.05 are indicated by *** and **, respectively.

activities decreased to 75% and 40% of the control, respectively. The much greater effect of dark cultivation on APR than on the other enzymes of sulphate assimilation made it appropriate to focus the following investigations on this enzyme.

Preliminary experiments showed that after 38 h darkness the highest level of APR activity was reached 6 h after the addition of glucose to the culture medium. Therefore, the following measurements were performed 6 h after the addition of the tested compound. Addition of glucose at different concentrations to *Arabidopsis* plant roots, preincubated in darkness for 38 h and kept in the dark for an additional 6 h, did not affect APR activity in shoots and remained at approximately 0.3 nmol min⁻¹ mg⁻¹ protein (data not shown). In roots, the enzyme activity increased from a basal activity of 0.1 nmol min⁻¹ mg⁻¹ protein in control roots up to a maximum of 1.5 nmol min⁻¹ mg⁻¹ protein after treatment with 0.5% (w/v) glucose (Fig. 2A). Higher glucose concentrations also resulted in an increased APR activity, which was, however, significantly lower than that at 0.5%. Therefore, all following measurements were only performed with extracts from roots of intact *A. thaliana* plants using a glucose concentration of 0.5% (w/v).

To test the possibility that the increase of APR activity resulted from osmotic stress, plants were fed with 0.5% (w/v) sorbitol or mannitol, respectively (Fig. 2B). Both

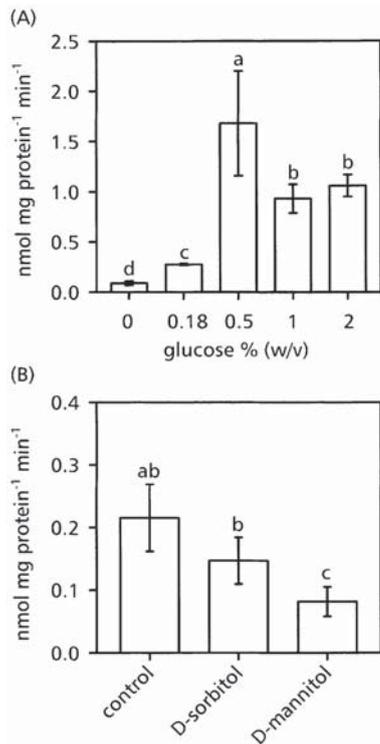


Fig. 2. Effect of various glucose concentrations (A) D-sorbitol or D-mannitol (B) on APR activity in *A. thaliana* roots. 4.5-week-old plants were cultivated in the dark for 38 h before the addition of various concentrations of D-glucose (A) or 0.5% (w/v) of D-sorbitol or D-mannitol (B) to the nutrient solution, and APR activity was measured after an additional 6 h in the dark. Mean values of at least four measurements \pm SD are presented. Bars indicated by different letters are significantly different at $P < 0.05$.

compounds decreased APR activity, demonstrating that osmotic stress alone had no inducing effect.

Regulation of APR expression and activity by glucose and OAS

Time-dependent changes in APR, protein content and activity, and APR2 mRNA after the addition of 0.5% glucose to the culture medium is presented in Fig. 3A. The enzyme activity reached a maximum 6 h after glucose addition. Afterwards, the enzymatic activity declined continuously over a period of 18 h. APR activity in control plants started at very low levels and dropped to even lower levels during this time period. Western analysis revealed that the change in APR activity correlated with APR protein accumulation, reaching a maximum 6 h after supplementation with glucose (Fig. 3A, W, +glucose). In control plants cultivated without external glucose, APR content remained low (Fig. 3A, W 8c). The APR2 mRNA level strongly increased within the first 2 h of the glucose treatment and decreased to the level of the control within 6 h (Fig. 3A, N, +glucose). The APR2 transcript level in the control plants (Fig. 3A, N, control) decreased slowly to an almost undetectable level over 8 h.

To compare the effect of glucose feeding on the regulation of APR with OAS, *Arabidopsis* plants were fed with 0.5 mM OAS (Neuenschwander *et al.*, 1991; Koprivova *et al.*, 2000) under the same conditions as for glucose treatment (Fig. 3B). OAS resulted in a faster and a greater increase of APR activity than with glucose. Maximum activity was reached 2 h after OAS addition, and then the activity decreased more rapidly than after glucose feeding. Western blot analysis revealed that the time-dependent changes in APR protein accumulation, induced by OAS, did not correspond to the changes in APR activity. By 6 h after OAS addition, the roots contained maximum levels of APR protein whereas APR activity had already decreased to 50% of the maximum value (Fig. 3B, W, +OAS). Northern blot analysis showed a strong induction of the APR2 transcript level with a maximum between 4 h and 6 h after addition of OAS (Fig. 3B, N, +OAS). APR2 mRNA in the control plants decreased during the whole experimental period (Fig. 3B, N, control). The different time-courses after glucose and OAS treatment indicate that glucose did not regulate APR via OAS.

To test this hypothesis further, glucose and OAS were added simultaneously to the culture medium. The results presented in Fig. 2A show that increasing the glucose concentration in the medium above 0.5% (w/v) resulted in APR activities which were lower than those induced by 0.5%. If glucose affects APR activity via OAS, the combined treatment with the optimal glucose concentration and OAS should result in an APR activity which was lower than that induced by glucose alone. Figure 4 shows, however, that the simultaneous treatment with glucose and OAS resulted in a much higher level of APR activity and protein and APR2 mRNA than the sum of the levels obtained with either of the two compounds, indicating again that glucose did not regulate APR activity via OAS.

Table 1 shows that ATP levels in roots of *A. thaliana* cultivated in the dark for 38 h were comparable to those of roots treated with glucose or OAS for various times. These results seem to exclude a function of ATP in the regulation of APR expression before and after glucose or OAS addition.

Effect of glucose under nitrogen deficiency on the activity of nitrate reductase and APR

To test the hypothesis that glucose increased APR expression independently from OAS, feeding studies with glucose were performed under N-deficiency and the activities of nitrate reductase (NR) and APR, respectively, were determined 2, 4, and 8 h after glucose supplementation. Glucose increased NR in the presence of nitrogen (Fig. 5A, NR, +N). Under nitrogen depletion, NR activity was not affected by glucose feeding (Fig. 5A, NR, -N). By contrast, APR activity was increased by glucose feeding despite nitrogen depletion (Fig. 5B, APR, -N). Maximal activity was detected 4 h after glucose addition, and then

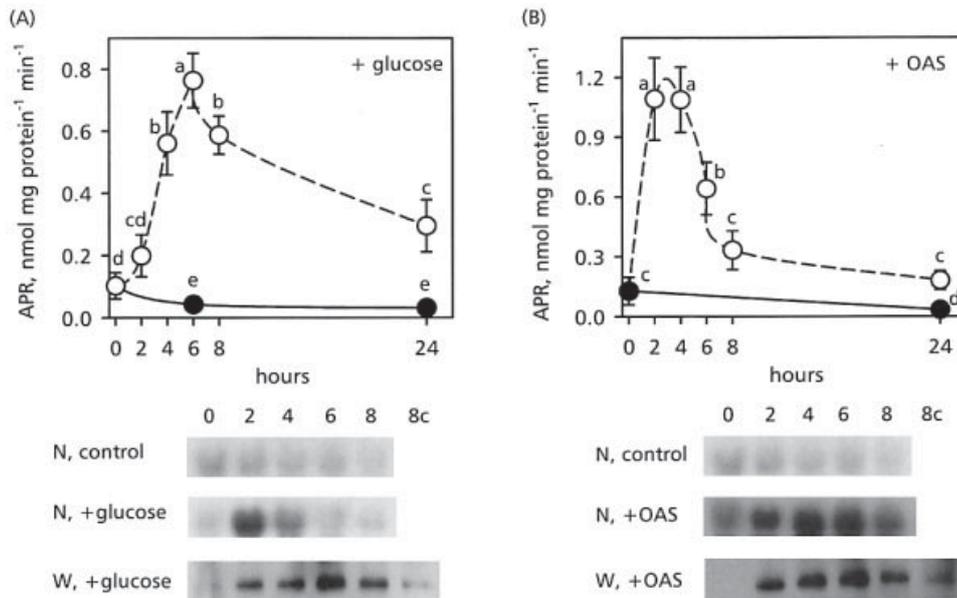


Fig. 3. Effect of glucose and OAS feeding on APR activity. (A) Determination of APR activity (open circles), APR2 mRNA (N, +glucose) and APR protein (W, +glucose) in *A. thaliana* roots. Before the addition of 0.5% (w/v) glucose, the plants were cultivated in the dark for 38 h and the various measurements were made after additional periods in the dark as indicated. Controls (closed circles; N, control; W, 8c) were cultivated without any additions to the nutrient solutions. Mean values of at least four APR activity measurements \pm SD are presented. Values significantly different at $P < 0.05$ are indicated with different letters. (B) Effect of OAS on APR activity (open circles), APR2 mRNA (N, +OAS), and APR protein (W, +OAS) levels in *A. thaliana* roots. Before the addition of 0.5 mM OAS, the plants were cultivated in the dark for 38 h and the various measurements were made after additional periods in the dark as indicated. Controls (closed circles; N, control; W, 8c) were cultivated without any additions to the nutrient solutions. Mean values of at least four APR activity measurements \pm SD are presented. Values significantly different at $P < 0.05$ are indicated by different letters.

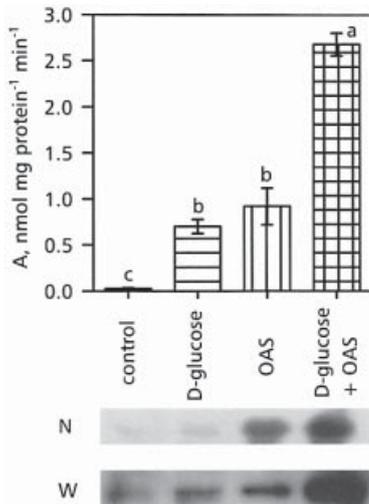


Fig. 4. Effect of D-glucose and O-acetyl-L-serine (OAS) on APR activity (A), APR2 mRNA accumulation (N), and APR protein (W) levels in *A. thaliana* roots. Before the addition of glucose (0.5%, w/v), OAS (0.5 mM), or a combination of both, the plants were cultivated in the dark for 38 h and the various measurements were made after an additional 6 h in the dark. Mean values of at least four APR activity measurements \pm SD are presented. Different letters above the bars indicate a statistically significant difference at $P < 0.01$.

APR activity decreased. These changes in APR activity were similar to the ones obtained in the presence of nitrogen (Fig. 3A) and are consistent with the idea that

Table 1. Effect of prolonged darkness, glucose, and OAS on the ATP content of *A. thaliana* roots

Mean values \pm SD of four measurements are presented. Glucose or OAS was added to the culture medium at the end of the 38 h dark period.

Treatment	ATP (nmol g ⁻¹ FW)
Control, light	30.58 \pm 5.56
38 h dark	30.68 \pm 8.98
39 h dark, 1 h 0.5% glucose	35.27 \pm 12.02
40 h dark, 2 h 0.5% glucose	30.61 \pm 13.63
41.75 h dark, 3.75 h 0.5% glucose	35.18 \pm 5.26
39 h dark, 1 h 0.5 mM OAS	32.53 \pm 8.41
40 h dark, 2 h 0.5 mM OAS	28.93 \pm 9.34

glucose regulated APR expression independently from nitrate assimilation.

In vivo flux through the sulphate assimilation pathway

The effect of external glucose on the flux through assimilatory sulphate reduction was analysed by feeding *A. thaliana* plants radioactive [³⁵S]-sulphate 4 h after glucose addition and measuring the steady-state radioactivity of sulphate, cysteine, γ -EC, GSH, and protein in the roots after an additional 4 h. Consistent with the results presented above, glucose feeding resulted in an increased APR activity independently from N-availability (Fig. 6).

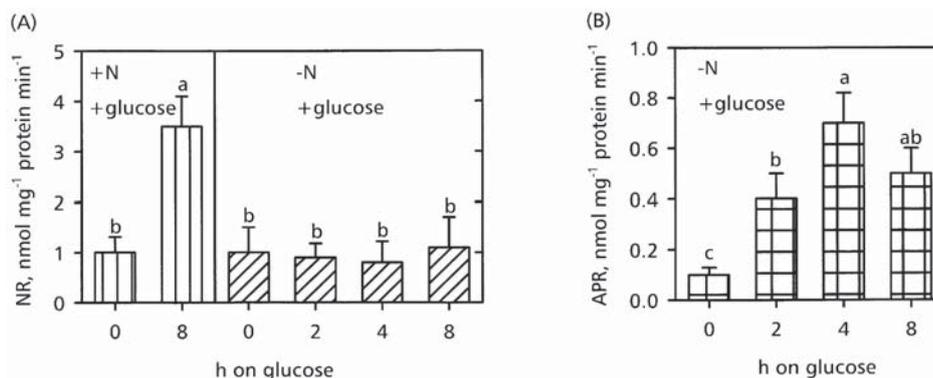


Fig. 5. Effect of glucose on NR (A) and APR (B) activity in roots of *A. thaliana* under nitrogen deficiency. *Arabidopsis* plants were cultivated with nutrient solution without a nitrogen source for 3 d. NR and APR activities were measured in roots of plants kept in the dark afterwards for 38 h (-N, 0 h) and 2, 4 and 8 h after supplying 0.5% (w/v) glucose. Controls cultivated in the presence of nitrate as a nitrogen source were kept in the dark for 38 h, and NR activity was measured 0 h (+N, 0 h) and 8 h (+N, 8 h) after the addition of glucose. Mean values of at least four independent measurements \pm SD are presented. Values significantly different at $P < 0.05$ are indicated by different letters.

Compared with control plants, the uptake of [³⁵S]-sulphate from the nutrient solution was significantly enhanced in glucose-treated roots when nitrogen was present, but no significant increase was detected in the N-deficient plants (Fig. 6). Labelling of cysteine, γ -EC and GSH was significantly increased by glucose compared to control plants, nitrate availability had no effect. The incorporation of [³⁵S] into proteins was three times higher in roots cultivated with a nitrogen source than in N-deficient roots (Fig. 6).

Discussion

Previous physiological analysis of plants has indicated that APR catalyses a highly regulated step in sulphate reduction (Brunold, 1993; Brunner *et al.*, 1995; Brunold and Rennenberg, 1997; Koprivova *et al.*, 2000; Leustek *et al.*, 2000; Bick *et al.*, 2001; Vauclare *et al.*, 2002). In the context of the present discussion, it is especially important to note that APR is regulated by nitrogen (Brunold and Suter, 1984; Haller *et al.*, 1986; Koprivova *et al.*, 2000) and sugar availability (Kopriva *et al.*, 1999). Therefore, APR seems to have an important function in co-ordinating sulphate assimilation with nitrate and carbon assimilation. In this study, the effect of glucose and nitrogen nutrition on APR was analysed in order to elucidate this co-ordinating function.

Exposure of plants to light resulted in an up-regulation of gene expression (Kocsy *et al.*, 1997; Kopriva *et al.*, 1999). Diurnal rhythms of APR disappeared, however, when *Arabidopsis* plants were subjected to continuous light or darkness, respectively, indicating that APR is not under the control of an endogenous circadian rhythm (Kopriva *et al.*, 1999). Light alone cannot be the signal for the induction of changes in APR expression, however, since the levels of APR mRNA were already increased

before the light period (Kopriva *et al.*, 1999). Clearly, an endogenous signal is required to trigger APR regulation. Apart from light, the cell's metabolic state, particularly the availability of photosynthetic products, might be involved in this transcriptional modulation as has been shown for plant genes encoding enzymes of nitrogen and sulphur assimilation (Small and Gray, 1984; Edwards and Coruzzi, 1989; Melzer *et al.*, 1989; Hesse *et al.*, 1999; Kaiser *et al.*, 1999; Riedel *et al.*, 1999).

The results of the present paper show that glucose but not sorbitol or mannitol induced APR. This clearly demonstrates that the carbohydrate effect is not mediated via an increase in the osmotic potential of the nutrient solution. The possibility that glucose regulates sulphate assimilation through another signalling cascade, for example, SNF1-related protein kinases, cannot be excluded. SNF1-related protein kinases, known as key components of environmental stress-response pathways in yeast and mammalian systems, were also identified in plants (Ikeda *et al.*, 1999). Several enzymes playing important roles in isoprenoid biosynthesis, nitrogen metabolism, and sucrose synthesis, such as 3-hydroxy-3-methylglutaryl coenzyme A reductase, nitrate reductase and sucrose phosphate synthase, respectively, were shown to be regulated by SNF1-related protein kinases (Sugden *et al.*, 1999; Ohba *et al.*, 2000). The possibility that SNF1-like protein kinases are involved in the regulation of sulphate assimilation in plants is attractive since in *Chlamydomonas* Sac3, a SNF1-like protein kinase, is involved in activating the sulphate transport system during sulphur limitation (Davies *et al.*, 1999).

Since sucrose (Cheng *et al.*, 1992) and glucose (Fig. 5A) also induced nitrate reductase transcription, the effect of carbohydrates on APR activity might be triggered by the accumulation of products of nitrate assimilation. The assimilation of sulphate and nitrate is interconnected.

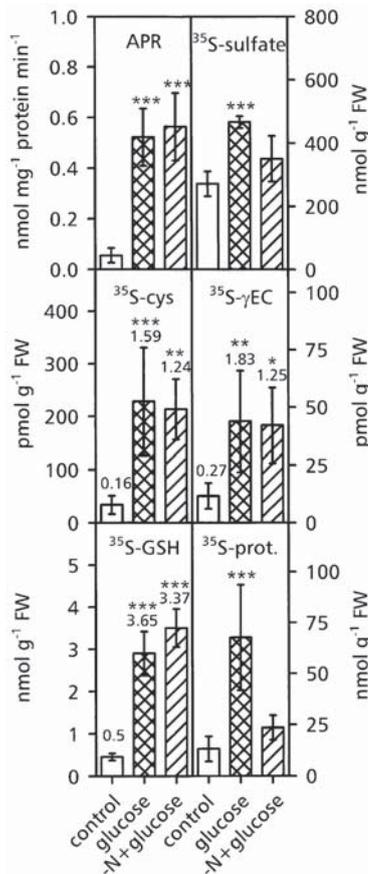


Fig. 6. APR activity and radioactive sulphur in various fractions from roots of *A. thaliana* cultivated with [^{35}S]-sulphate. The plants were kept in the dark for 38 h before the addition of glucose (0.5%, w/v). N-deficient plants (-N, +glucose) were cultivated on nitrogen-free nutrient solution for 3 d before the dark period. For the radioactive labelling, [^{35}S]-sulphate was added to the nutrient solution 4 h after glucose and the radioactivity in the various fractions was determined 4 h later. The mean APR activity during the labelling period was measured in parallel experiments. Mean values of at least four measurements \pm SD are presented. The figures above the bars representing thiols give the percentage of labelling. Values significantly different from the controls are indicated by *** ($P < 0.01$), ** ($P < 0.05$) and * ($P < 0.1$).

When nitrogen is not available, APR activity is decreased, the addition of a nitrogen source leads to the induction of APR and to an increased flux through assimilatory sulphate reduction (Brunold and Suter, 1984; Brunold, 1993; Koprivova *et al.*, 2000). OAS is a good candidate for regulating APR and thus co-ordinating nitrate and sulphate assimilation (Neuenschwander *et al.*, 1991; Brunold, 1993; Hawkesford and Wray, 2000; Leustek *et al.*, 2000). Addition of OAS to the nutrient solution not only increased APR activity in various systems, but also cysteine and GSH (Neuenschwander *et al.*, 1991; Koprivova *et al.*, 2000). GSH has been shown to be a negative signal for APR expression in *A. thaliana* root cultures (Vauclare *et al.*, 2002). This finding may be used for explaining the rapid decrease in APR activity starting 4 h after the

addition of OAS (Fig. 3B). As shown in Fig. 3, feeding of OAS to *Arabidopsis* roots in the dark led to a similar increase in APR activity as glucose. These findings lead to the question, whether glucose acted on APR expression via OAS. Three independent lines of evidence make it very likely that glucose induced APR independently from OAS. (1) The time-courses of changes in APR mRNA, protein and activity induced by glucose or OAS were different (Fig. 3). APR activity was induced by OAS more rapidly than by the glucose-feeding experiments. APR2 mRNA and APR protein and activity decreased at comparable rates in the glucose-treated roots, whereas after OAS treatment, APR activity decreased more rapidly than APR2 mRNA and APR protein. The different decrease in APR protein and activity may be explained by assuming that part of the newly formed APR apo-protein did not contain the iron-sulphur cluster necessary for enzymatic activity (Kopriva *et al.*, 2001). (2) When OAS was fed together with an optimal glucose concentration, the increase of APR activity was greater than the sum of the levels induced by the individual treatments, indicating a synergy. If glucose acted on APR expression via OAS, the addition of OAS together with an optimal glucose concentration should result in lowered APR expression. The synergy of glucose and OAS in inducing APR expression, however, is consistent with the hypothesis that glucose was not acting via OAS. (3) APR activity was also induced by glucose in roots of N-deficient plants, whereas NR remained at a low level (Fig. 5). As demonstrated by the low rate of protein synthesis in the labelling experiments (Fig. 6) the level of amino acids was low in these roots due to lack of a nitrogen source and low NR activity. In roots cultivated in the presence of nitrate, protein labelling was at comparable rates as in previous experiments (Koprivova *et al.*, 2000). In these roots, glucose had three functions: it induced NR and APR activity and possibly other enzymes, then it was the source of carbon skeletons which functioned as acceptors for ammonium and sulphide, the products of assimilatory nitrate and sulphate assimilation and, finally, it was used for the production of reduction equivalents and ATP needed in both pathways.

In conclusion, the data presented here demonstrate that regulation of sulphate assimilation is not only aimed at the co-ordination with nitrate assimilation, but also with carbon assimilation. Taken together with previous results (Farago and Brunold, 1990; Rügsegger *et al.*, 1990; Neuenschwander *et al.*, 1991; Brunold, 1993; Brunner *et al.*, 1995; Kocsy *et al.*, 1997; Leustek *et al.*, 2000; Westerman *et al.*, 2001) the present findings indicate that APR plays an important role in this co-ordination. In addition, the present results show that signals from nitrogen and carbon metabolism act synergistically and that positive signalling from sugars can override negative signalling from nitrate assimilation.

Acknowledgements

We thank M Stalder for making preliminary experiments and A Rawlyer, S Täschler, I Anders, and M Eiblmeier for technical assistance. This work was supported by grants from the EU commission, the Swiss National Science Foundation and the Körper Foundation, Hamburg, Germany.

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