Effect of ATP Sulfurylase Overexpression in Bright Yellow 2 Tobacco Cells

Regulation of ATP Sulfurylase and SO$_4^{2-}$ Transport Activities

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To determine if the ATP sulfurylase reaction is a regulatory step for the SO$_4^{2-}$-assimilation pathway in plants, an Arabidopsis thaliana ATP sulfurylase cDNA, APS2, was fused to the 35S promoter of the cauliflower mosaic virus and introduced by Agrobacterium tumefaciens-mediated transformation into isolated Bright Yellow 2 tobacco (Nicotiana tabacum) cells. The ATP sulfurylase activity in transgenic cells was 8-fold that in control cells, and was correlated with the expression of a specific polypeptide revealed by western analysis using an anti-ATP sulfurylase antibody. The molecular mass of this polypeptide agreed with that for the overexpressed mature protein. ATP sulfurylase overexpression had no effect on [35S]SO$_4$ influx or ATP sulfurylase activity regulation by $S$ availability, except that ATP sulfurylase activity variations in response to $S$ starvation in transgenic cells were 8 times higher than in the wild type. There were also no differences in cell growth or sensitivity to SeO$_4^{2-}$ (a toxic SO$_4^{2-}$ analog) between transgenic and wild-type cells. We propose that in Bright Yellow 2 tobacco cells, the ATP sulfurylase derepression by $S$ deficiency may involve a posttranscriptional mechanism, and that the ATP sulfurylase abundance is not limiting for cell metabolism.

S is one of the major essential elements. It enters into the composition of the amino acids Met and Cys, and in a large variety of secondary metabolites, sulfolipids, sulfated glucides, and coenzymes (Mitchell, 1996). Plants and most of the bacteria and fungi are able to assimilate $S$ from SO$_4^{2-}$, whereas animals require organic $S$ molecules as nutrients. Because of its low redox potential, SO$_4^{2-}$ is a relatively nonreactive form, and has to be activated prior to its reduction and incorporation into organic compounds (Leyh, 1993). The first step of the SO$_4^{2-}$-activation process is catalyzed by ATP sulfurylase (ATP:sulfate adenyl transferase, EC 2.7.7.4), which associates inorganic SO$_4^{2-}$ to ATP, resulting in the formation of APS and PPi. As this reaction is the first step in an energy-expensive sequence, ATP sulfurylase is considered to be an excellent candidate for the pathway-regulating, rate-limiting enzyme (Leustek, 1996).

APS is further phosphorylated by APS kinase using ATP, releasing PAPS. PAPS is considered to be a high-energy SO$_4^{2-}$ donor for sulfation of macromolecules in higher organisms, and can be reduced to SO$_3^{2-}$ in fungi (Thomas et al., 1992) and bacteria (Kredich, 1987). An alternative pathway has been identified recently in plants, in which SO$_3^{2-}$ formation appears to come from the reduction of APS rather than from PAPS (Gutierrez-Marcos et al., 1996; Setya et al., 1996; Hell, 1997).

In plants SO$_3^{2-}$ uptake and ATP sulfurylase activity are derepressed in response to $S$ starvation, and both activities are repressed when SO$_4^{2-}$ is restored (Smith, 1975; Reuveny and Filner, 1977; Yildiz et al., 1994; Smith et al., 1995; Logan et al., 1996). To date, the regulation of SO$_4^{2-}$ uptake and ATP sulfurylase activity has been studied essentially in relation to SO$_4^{2-}$ availability as an $S$ source (Hawkesford et al., 1993; Yildiz et al., 1996; Massonneau et al., 1997). Because SO$_3^{2-}$ activation has been considered to be a limiting step in the SO$_4^{2-}$ pathway, the reaction has been postulated to be one of the main regulatory steps of this pathway (Leustek, 1996). To address this question, we constitutively overexpressed an Arabidopsis thaliana cDNA encoding a putative chloroplastic ATP sulfurylase isofrom (Logan et al., 1996) in BY2 tobacco (Nicotiana tabacum) cells. We used these cells to study the effects of three different $S$ nutritional conditions on growth, SO$_4^{2-}$ influx, and SO$_4^{2-}$ accumulation: normal SO$_4^{2-}$ availability, S deficiency, and S nutrition bypassing the ATP sulfurylase step (using $S_2O_3^{2-}$ as an $S$ source). Comparison between wild-type cells and transformed cells indicated that the level of expression of ATP sulfurylase is not a limiting factor for growth. Our results suggest that the regulation of SO$_4^{2-}$ uptake and ATP sulfurylase activity is independent of the nature of the $S$ source and of the abundance of the ATP sulfurylase protein.

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Abbreviations: APS, adenosine 5'phosphosulfate; BY2, Bright Yellow 2; PAPS, 3'phosphoadenosine 5'phosphosulfate.
MATERIALS AND METHODS

Culture Conditions of Isolated Tobacco Cells

BY2 tobacco (Nicotiana tabacum) cells were cultivated at 28°C in the dark on an orbital shaker at 120 rpm. The suspension was diluted to 1/50 every 7 d with a modified Murashige and Skoog medium (Nagata et al., 1992) devoid of \( \text{SO}_4^{2-} \) salts. To achieve \( \text{S} \)-starvation conditions, \( \text{S} \)-free medium was maintained in medium without antibiotics. All of the transformed cell line was considered to be established after five additional culture cycles in selective medium, and was maintained in medium without antibiotics. The bacteria were pelleted and cultured on solid Luria-Bertani medium supplemented with 200 mg L\(^{-1}\) rifampicin. The cells were diluted into 1 mL of Luria-Bertani medium and grown in 1 volume of the same medium at 120 rpm for 24 h at 28°C in the dark. The composition of the solid medium for callus cultures was identical to the composition of liquid medium, except that 0.8% (w/v) agar-agar (Merck, Darmstadt, Germany) was added before sterilization. Drops (100 \( \mu \text{L} \)) of 7-d-old cell suspensions containing the same amount of fresh weight (40 mg) were deposited on solid medium in a Petri dish, wrapped with polyethylene film, and grown at 28°C in the dark.

Transformation of BY2 Tobacco Cells

The complete cDNA encoding \( \text{APS2} \) (previously named \( \text{ASA1} \), an Arabidopsis thaliana putative chloroplastic ATP sulfurylase isoform (Logan et al., 1996), was introduced between the cauliflower mosaic virus 35S promoter and the terminator of the nopaline synthase gene in place of the GUS gene of the binary vector PBI121 (Clontech, Palo Alto, CA). Agrobacterium tumefaciens LBA4404 was cultivated at 28°C in Luria-Bertani medium supplemented with 200 mg L\(^{-1}\) streptomycin and 150 mg L\(^{-1}\) rifampicin (Sigma). For transformation, an overnight 2-mL culture of \( A. \) tumefaciens was cooled in melting ice for 10 min, and then pelleted at 1500g for 5 min at 4°C. The pellet was resuspended into 100 \( \mu \text{L} \) of cold, sterile 20 mM CaCl\(_2\) and 2 \( \mu \text{L} \) of purified plasmid suspension was added. To achieve S-starvation conditions, 5-d-old cells were washed under sterile conditions with 3 volumes of S-less medium on a 150-μm polyamide filter (Tissages Tissus Techniques, Saillly-Saillissel, France) and grown in 1 volume of the same medium at 120 rpm for 24 h at 28°C. The bacteria were pelleted and cultured on solid medium in a Petri dish, wrapped with polyethylene film, and grown at 28°C in the dark.

ATP Sulfurylase Activity and Protein-Content Assay

Isolated tobacco cells corresponding to about 0.8 g fresh weight were washed with 0.2 mM CaCl\(_2\) by vacuum filtration through a 48-μm polyamide filter. The cells were frozen with liquid N\(_2\) and homogenized into a 100 mM Tris-HCl, pH 8.0, 10 mM EDTA, and 20 mM DTT buffer at 4°C. Extracts were centrifuged at 12,000g for 15 min at 4°C, and the supernatant containing the soluble proteins was collected and stored on ice until use. The ATP sulfurylase activity of the protein extract was determined using the molybdatopolyphosphate procedure described by Osslund et al. (1982). Protein content of the extract was quantified by the method of Schaffner and Weissmann (1973) using BSA as a standard.

\( \text{SO}_4^{2-} \)-Content Assays

Isolated tobacco cells were washed as previously described, disrupted by incubation in 0.1 n HCl at 80°C for 30 min, and then kept at 4°C for 24 h. Total cellular \( \text{SO}_4^{2-} \) was quantified by the BaCl\(_2\) turbidimetric procedure described by Tabatabai and Bremner (1970).

\( ^{35}\text{S}\text{SO}_4^{2-} \)-Uptake Assay

The \( \text{SO}_4^{2-} \)-uptake assay was performed on 5-mL aliquots of 6-d-old cultures corresponding to about 2 g fresh weight. Cell samples were deposited on a 150-μm polyamide filter, washed three times by gravity flow of 5 mL of a solution containing only the Murashige and Skoog major elements 2% (w/v) Suc and 0.2 mM K\(_2\)SO\(_4\), and resuspended into the same medium. After preincubation at 120 rpm for 17 min at 28°C, 0.33 μCi of \( ^{35}\text{S}\text{SO}_4^{2-} \) (ICN Biochemicals) was added, and cells were incubated at 120 rpm for 5 min at 28°C. The radioactive medium was then removed by vacuum filtration through a 48-μm polyamide filter, and the cells were washed three times with 10 mL of ice-cold 0.2 mM CaSO\(_4\). The cell solutes were extracted by incubation into 5 mL of 0.1 n HCl for 1 h at room temperature. The radioactivity in 1 mL of the extract was quantified using a liquid-scintillation counter (Tri-Carb 2101 TR, Packard, Meriden, CT) in the presence of 3 mL of scintillation liquid (Ultima Gold, Packard).

Protein Electrophoresis and Immunoblotting

Soluble proteins were extracted as for ATP sulfurylase activity and were separated by SDS-PAGE, as described by Laemmli (1970). Electrophoresis on nitrocellulose membrane (Sartorius) was performed in a 0.22% (w/v) 3-[cyclohexylamino]-1-propanesulfonic acid and 10% (v/v) methanol buffer, and run at 35 V during 16 h. The membrane was washed into a PBS buffer containing 10 mM K\(_2\)PO\(_4\), pH 7.5, 150 mM NaCl, and 0.5% (v/v) Tween for 1 h at 4°C. The membrane was then incubated in PBS buffer containing 5% (w/v) nonfat dry milk for 1 h at 22°C, and the milk excess was removed by several washes with PBS. Proteins were hybridized overnight at 22°C with a rabbit polyclonal antibody directed against the ATP sulfurylase isoform \( \text{APS3} \) of \( A. \) thaliana. Identification of hybridized proteins was achieved using a liquid-scintillation counter (Tri-Carb 2101 TR, Packard, Meriden, CT) in the presence of 3 mL of scintillation liquid (Ultima Gold, Packard).
Expression of the Results

Experiments were repeated two to three times on independent cultures of transformed and wild-type tobacco cell lines. Typical results obtained for each experiment are presented. Unless specified, ATP sulfurylase activity and protein and $SO_4^{2-}$ content data are the means of three extractions from the same culture. [$^{35}$S]$SO_4^{2-}$-uptake results are means of five extractions from the same culture. $s$es are calculated at the level of 0.05.

RESULTS

Effect of APS2 Overexpression on Cell Growth and ATP Sulfurylase Protein Abundance

The growth kinetics and the fresh yield of the transformed APS2 cell line were identical to that of the untransformed BY2 cell line (Fig. 1). ATP sulfurylase-specific activity of 6-d-old cultures (late growing stage) of the transformed line was approximatively 8-fold that in nontransformed BY2 cells (Fig. 2A). Western analysis of protein extracts from the control BY2 line using the anti-APS3 antibody revealed a major band with a molecular mass of approximately 47 kD (Fig. 2B). In the APS2 line, this band was associated with a darker band with a molecular mass of between 47 and 50 kD. The latter band was presumably associated with the increased ATP sulfurylase activity. The same antibody recognized a 54-kD protein in the soluble protein extract of an Escherichia coli strain overexpressing APS2.

Effect of S Starvation on $SO_4^{2-}$ Uptake, $SO_4^{2-}$ Content, and ATP Sulfurylase Activity

Five-day-old, nontransformed (control BY2 line) or transgenic (APS2 line) isolated tobacco cells cultivated in standard medium were washed and transferred into S-less but otherwise complete medium. $SO_4^{2-}$ uptake was derepressed progressively, correlating with a decrease of the cell $SO_4^{2-}$ content (Fig. 3). Derepression of the ATP sulfurylase activity began after 4 h of S starvation. $SO_4^{2-}$ uptake and ATP sulfurylase activity increased as long as the S deficiency was maintained. When $SO_4^{2-}$ was added to the medium, cell $SO_4^{2-}$ content increased immediately. $SO_4^{2-}$ uptake was fully repressed within 2 h, and ATP sulfurylase activity returned to its basal level within 4 h. Both BY2 control and transgenic APS2 lines reacted in similar ways.

Effect of S Source on $SO_4^{2-}$ Uptake, $SO_4^{2-}$ Content, and ATP Sulfurylase Activity

Nontransformed (control BY2 line) or transgenic (APS2 line) isolated tobacco cells were cultivated for 24 h in S-less medium or for 6 d with $SO_4^{2-}$ or $S_2O_3^{2-}$ as the sole source of S. In $SO_4^{2-}$-containing medium, $SO_4^{2-}$ uptake and ATP sulfurylase activity were repressed, and cells contained large amounts of $SO_4^{2-}$ (Fig. 4). After 24 h of S starvation, $SO_4^{2-}$ uptake and ATP sulfurylase activity were fully derepressed, in correlation with the disappearance of intracellular $SO_4^{2-}$. In $S_2O_3^{2-}$-containing medium, BY2 cells had a relatively high content of $SO_4^{2-}$; $SO_4^{2-}$ uptake was partly derepressed, and ATP sulfurylase activity was fully dere-
pressed. The ATP sulfurylase activity correlated with the relative abundance of the protein (Fig. 5). The APS2 line showed the same reactions as the BY2 cells to both S and $\text{SO}_4^{2-}$ deficiencies, except that the ATP sulfurylase activities were approximately 8-fold higher.

**Effect of $\text{SeO}_4^{2-}$ on Callus Culture**

Control BY2 and transgenic APS2 tobacco cells were grown as calli for 50 d on normal solid medium containing 0, 100, and 500 $\mu$M Na$_2$SeO$_4$ at three different initial dilutions for each of the cell cultures (Fig. 6). No growth differences could be noticed between the two cell lines.

**DISCUSSION**

The expression of the APS2 cDNA, an *A. thaliana* ATP sulfurylase, in transgenic tobacco cells was associated with an 8-fold increase in ATP sulfurylase activity (Fig. 2A) and with the appearance of a peptide that reacted with an antibody directed against the APS3 ATP sulfurylase (Fig. 2B). The relative molecular mass of this peptide (approximately 47 kD) was close to that predicted for the mature APS2 protein (Logan et al., 1996). Thus, we conclude that the transgenic cells contain increased amounts of ATP sulfurylase compared with the control cells.

After 18 h of S deprivation (Fig. 3), the relative increase of ATP sulfurylase activity was approximately the same (35–40%) in both cell lines, in spite of the 8-fold difference in the absolute value of enzymatic activities (Fig. 2A). Thus, the absolute increase in activity upon S starvation in APS2...
cells was approximately 8-fold that in control cells, exceeding the activity in the latter cells in $SO_4^{2-}$-less medium. This result implies that the transgenic ATP sulfurylase is involved in the increase in enzymatic activity upon S starvation in APS2 cells. The augmentation of the enzymatic activity upon S deficiency could be partly due to an increase in protein concentration, as suggested by the correlation between the relative enzymatic activity and immunostaining (Fig. 5). The response of the cauliflower mosaic virus 35S promoter used on the APS2 line to S-nutrition conditions is not known, but it seems unlikely that this promoter would respond to the S nutritional status in a way similar to the ATP sulfurylase promoter (Fig. 3). Therefore, the observed increase in ATP sulfurylase protein abundance in response to S starvation probably resulted from a posttranscriptional effect. An increase in the stability of the ATP sulfurylase in S-limiting conditions has been demonstrated in tobacco cells (Reuveny and Filner, 1977). Such a mechanism could be involved in the observed derepression in transgenic cells.

The kinetics of growth and $SO_4^{2-}$ uptake of the transgenic cells overexpressing the APS2 cDNA (APS2) are similar to those of wild-type cells in $SO_4^{2-}$-containing medium, during S-starvation periods, or during return to normal nutrition (Figs. 1, 3, and 4). Since the biomass production rate is insensitive to the concentration of the ATP sulfurylase in the cells, we may infer that the cell multiplication in normal $SO_4^{2-}$ conditions is not limited by the ATP sulfurylase abundance. Several explanations for this situation can be imagined. Cell growth should not be limited by S metabolism by itself in our culture conditions. The enzyme might already be present in excess in wild-type cells, or its activity might be limited by $SO_4^{2-}$ or ATP availability. In plants the cytoplasmic concentrations of $SO_4^{2-}$ and ATP are estimated at approximately 10 and 2 mm, respectively (Cram, 1983; Roby et al., 1987). Since the $K_m$ for $SO_4^{2-}$ and ATP of the ATP sulfurylase are about 0.87 to 0.25 mm and 0.31 to 0.046 mm, respectively (Osslund et al., 1982; Renosto et al., 1993), it is unlikely that ATP sulfurylase would be limited by the availability of its substrates.

Another explanation of the insensitivity of growth to ATP sulfurylase level would be that the activity of this enzyme might already be present in excess in wild-type cells, or its activity might be limited by $SO_4^{2-}$ or ATP availability. In plants the cytoplasmic concentrations of $SO_4^{2-}$ and ATP are estimated at approximately 10 and 2 mm, respectively (Cram, 1983; Roby et al., 1987). Since the $K_m$ for $SO_4^{2-}$ and ATP of the ATP sulfurylase are about 0.87 to 0.25 mm and 0.31 to 0.046 mm, respectively (Osslund et al., 1982; Renosto et al., 1993), it is unlikely that ATP sulfurylase would be limited by the availability of its substrates.

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enzyme is strictly regulated by its product, or by those of the downstream steps of the pathway. Potent retro-inhibition of ATP sulfurylase by micromolar-range concentrations of APS is well known in fungi and higher plants (Osslund et al., 1982; Renosto et al., 1993; Foster et al., 1994). Furthermore, the ATP sulfurylase reaction toward the formation of APS is thermodynamically not favored, and is thought to depend upon the removal of the reaction products Pi and APS by pyrophosphatases and APS reductase/APS kinase, respectively (Leyh, 1993).

SeO$_2$$^-$ can be used as a substrate by ATP sulfurylase, which results in toxic overproduction and misincorporation of selenocysteine into the proteins in place of Cys (Wilson and Bandurski, 1958; Reuveny, 1977; Cherest et al., 1997). Sensitivity to SeO$_2$$^-$ was identical in transgenic and control tobacco cells (Fig. 6). We conclude that Se assimilation was not enhanced by the overexpression of a functional ATP sulfurylase. To investigate whether a difference between the two lines would be detected when increasing the demand for reduced S compounds such as the Cys-rich phytochelatins, which are involved in heavy-metal detoxication (Rauser, 1990; Steffens, 1990), or Met, which has been shown to have a protective effect against NaCl stress (Gläser et al., 1993; Kwon et al., 1995), calli were grown, respectively, on Cd (0, 50, and 100 µM CdCl$_2$) and Na (0, 100, and 500 mM NaCl)-containing solid medium. Results were very similar to those presented in Figure 6, and no difference in the sensitivity to these two compounds was noticed between the nontransformed and the transgenic cell lines (data not shown). These results would not have been expected if the ATP sulfurylase abundance was limiting to the SO$_4$$^2-$-assimilation pathway in the nontransformed cell line.

SO$_4$$^2-$ uptake is thought to be repressed by SO$_4$$^2-$ accumulated in the cytoplasm (Smith, 1975, 1980). In both transgenic and wild-type cells, SO$_4$$^2-$ influx changed in a manner opposite to that of the whole-cell SO$_4$$^2-$ content because the latter was manipulated by various treatments (Figs. 3 and 4). We used S$_2$O$_3$$^2-$, a good S source for BY2 tobacco cell growth, to bypass the SO$_4$$^2-$-activation step without causing S starvation. In yeast, S$_2$O$_3$$^2-$ uptake is mediated presumably by the SO$_4$$^2-$-transport system (Alonso et al., 1984). The molecule is then split into SO$_4$$^2-$ and S$^2-$, which enter the S-assimilation pathway at the SO$_4$$^2-$ reduction and S$^2-$ incorporation steps, respectively (Thomas et al., 1992). The similar behavior of the two cell lines suggests that the level of ATP sulfurylase did not affect the level of its substrate, cytosolic SO$_4$$^2-$. Furthermore, the SO$_4$$^2-$ level in S$_2$O$_3$$^2-$-grown cells was the same in both cell lines, indicating that the abundance of ATP sulfurylase did not control the equilibrium between production of SO$_4$$^2-$ (via S$_2$O$_3$$^2-$ oxidation) and SO$_4$$^2-$ assimilation. These results indicate that in BY2 tobacco cells the amount of SO$_4$$^2-$ in the whole cell (and probably the amount of SO$_4$$^2-$ in the cytosol) is not dependent on the level of ATP sulfurylase activity.

In conclusion, the ATP sulfurylase abundance does not seem to regulate SO$_4$$^2-$ acquisition or SO$_4$$^2-$ use for growth in tobacco cells. These results suggest that this enzyme is under strict control by some products of its activity or of downstream steps of the SO$_4$$^2-$-assimilation pathway, either by retro-inhibition or by mass-action law effects.

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