Plant sulfur metabolism – the reduction of sulfate to sulfite
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Until recently the pathway by which plants reduce activated sulfate to sulfite was unresolved. Recent findings on two enzymes termed 5′-adenyllysulfate (APS) sulfotransferase and APS reductase have provided new information on this topic. On the basis of their similarities it is now proposed that these proteins are the same enzyme. These discoveries confirm that the sulfate assimilation pathway in plants differs from that in other sulfate assimilating organisms.

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Abbreviations
AK 5′-adenyl sulfate kinase
APR 5′-adenyllysulfate reductase
APS 5′-adenyllysulfate
APSSTase 5′-adenyllysulfate sulfotransferase
PAPS 3′-phosphoadenosine-5′-phosphosulfate

Introduction
This article focuses on a single aspect of a broad subject area. It is prompted by the recent findings concerning one of the primary steps in the sulfate assimilation pathway of higher plants. Readers who are interested in a comprehensive treatment of plant sulfur metabolism are referred to several recent reviews [1∗–3∗].

Higher plants are dependent on inorganic sulfate to fulfil their nutritional sulfur requirement. Sulfate is a major anionic solute and is required for the synthesis of many different metabolites and coenzymes. After uptake from the soil, sulfate is either accumulated and stored in the vacuole or it is incorporated into organic compounds in a process referred to as assimilation. Sulfur can be assimilated in one of two ways—it is either incorporated as sulfate in a reaction termed sulfation [4∗∗,5∗∗], or it is first reduced to sulﬁde, the substrate for cysteine synthesis. In plants the majority of sulfur is assimilated in the reduced form.

The plant sulfate assimilation pathways are depicted in Figure 1. The prerequisite step for all metabolic fates of sulfate is its activation by linkage to 5′-AMP, forming 5′-adenyllysulfate (APS), a reaction catalyzed by ATP sulfurylase. APS lies at a metabolic branch point—it is used either for sulfation or for the reduction pathway. It is the first reduction step that has been a matter of contention and is the focus of this review. Skipping this step for the moment, the remaining reactions in the pathway to cysteine include the reduction of sulfite to sulfide catalyzed by ferredoxin-dependent sulfite reductase [6], then assimilation of inorganic sulfide by the sulfhydration of O-acetylsereine [7∗†]. The second assimilation pathway branching from APS is used for the synthesis of a variety of sulfated compounds carried out by specific sulfotransferases (ST’s). These enzymes use the phosphorylated derivative of APS, 3-phosphoadenosine-5′-phosphosulfate (PAPS)[4∗∗,5∗∗], formed by APS kinase (AK).

This paper focuses on the latest information on the enzyme catalyzing the reduction of APS. The recent purification of APSSTase from a marine alga provides the first evidence on the catalytic properties of this enzyme. At the same time, cDNAs have been cloned from the higher plant Arabidopsis thaliana that encode an enzyme termed APS reductase that shows similarities to APSSTase. We propose that they are the same enzyme and discuss their properties in relationship to a role in sulfate assimilation.

The sulfate assimilation pathway in plants. Sulfate is activated by ATP sulfurylase (AS) forming 5′-adenyllysulfate (APS). APS is a branch point intermediate that is used for the reduction pathway leading to formation of cysteine (upper pathway) or the sulfation pathway leading to synthesis of various sulfated compounds (lower pathway). The sulfation pathway begins by phosphorylation of APS to 3′-phosphoadenosine-5′-phosphosulfate (PAPS) by APS kinase (AK). PAPS is the substrate for a variety of sulfotransferases (ST’s). In the cysteine pathway, the first reduction step is the topic of this article. We argue that the enzyme APS reductase (APR) is identical to APS sulfotransferase (APSSTase), a glutathione-dependent reductase. Its activity is driven by reduced glutathione (GSH) which is recycled from oxidised glutathione (GSSG) through the action of NADPH-dependent glutathione reductase (GR). The subsequent reactions are the reduction of sulfite to sulfide by sulfite reductase (SiR) and assimilation of sulfide into cysteine by O-acetylsereine (thiol) lyase (OASTL).
Sulfate reduction in plants

The long-held dogma concerning sulfate reduction stated that, in plants, APS is the substrate for this reaction and the enzyme APS sulfotransferase (APSSTase) [8]. For a number of reasons, however, the APS pathway in plants was not universally accepted [9]—despite years of research, definitive evidence for the existence of APSSTase was not forthcoming. One report of its purification from Euglena gracilis was confounded by the unreasonably low specific activity of the pure enzyme [10]. Subsequently, another group reported that, *in vitro*, plant APS kinase displays APS sulfotransferase activity as a side reaction [11]. This result, combined with several physical similarities, prompted the idea that perhaps APS sulfotransferase is a kinetic artefact [11]. In contrast to this uncertainty, it is widely believed that prokaryotes (including cyanobacteria) and fungi use PAPS for sulfate reduction via the enzyme PAPS reductase [12,13]. Recently, however, direct evidence was published by three independent groups, confirming the existence of an APS-dependent pathway in plants [14**–16**].

**APS sulfotransferase**

Although APSSTase has long been the focus of investigation [17] its purification to homoecity proved to be an elusive goal. Kanno et al. [14**] finally succeeded in isolating the enzyme from a marine macroalga by maintaining high levels of ammonium sulfate throughout the purification procedure. Previous publications noted the lability of APSSTase and that it could be stabilized with high concentrations of sulfate salts [18], but this finding was never incorporated into a purification scheme.

As reflected by its name, APSSTase was proposed to catalyze the transfer of sulfate from APS to a thiol acceptor molecule forming a thiosulfonate [8]. *In vitro*, many different reduced thiol compounds, including dithiothreitol, can serve as the acceptor. The physiological acceptor was envisaged to be glutathione, primarily because it is an efficient substrate and is the most abundant thiol in the chloroplast stroma in which APSSTase is localized [19]. The algal enzyme shows a kinetic constant for glutathione of 0.6 mM, a value that is well below the physiological concentration in the stroma, reported to be between 3 and 10 mM [20,21]. This is a key finding that supports much of the earlier work on this enzyme. For example, it has long been known that sulfate production from sulfate is not light dependent, requiring only ATP and glutathione (it is not influenced by NADPH or ferredoxin) [22].

Purified algal APSSTase has a high catalytic activity (10.3 µmol min⁻¹ mg⁻¹ protein) and a high affinity for APS (Kₘ = 2.1 µM). It shows a high pH optimum (9.0–9.5) and it is maximally active in the presence of high sulfate concentrations (0.5 M). The enzyme is sensitive to inactivation by heat and dithiothreitol, but not glutathione. High concentrations of sulfate salts and APS (or APS analogs) stabilize the enzyme against these inactivating treatments.

Although the reaction mechanism of APSSTase has not been addressed, this is a central issue that should be explored with the purified enzyme. As already mentioned APSSTase was proposed to be a sulfotransferase. For example, with glutathione as the acceptor S-sulfogluthathione is formed. It was acknowledged, however, that this hypothesis is formed on the basis of inconclusive data and that it is equally plausible that the enzyme is a reductase that forms free sulfite [8]. Due to its high reactivity sulfite can readily form a thiosulfonate with reduced thiols [23], possibly explaining the presence of thiosulfinate in APSSTase reactions.

The purification of APSSTase from a macroalga is the first direct and convincing evidence for an APS-dependent reduction pathway in plants. Additional and complementary evidence for such a pathway was contemporaneously obtained using molecular genetic methods as described in the next section.

**The APR cDNAs encoding APS reductase**

APS reductase (APR) was identified serendipitously by searching for cDNAs encoding plant PAPS reductase [15**–16**]. Three different cDNAs were cloned from Arabidopsis thaliana (APR1, 2 and 3) that are able to complement the cysteine auxotrophy of an *Escherichia coli* cysH (PAPS reductase) mutant strain. The APR cDNAs encode individual members of a small, highly conserved gene family.

Analysis of the APR isoenzymes revealed that, unlike PAPS reductase, they use APS preferentially over PAPS. This property was demonstrated both by *in vitro* assays and by *in vivo* functional complementation of a cysC (APS kinase) mutant of *E. coli* with the APR cDNAs. An APS reductase would be expected to bypass both the cysC and cysH genes in *E. coli*. Another key difference is that, while PAPS reductase is dependent on the redox proteins thioredoxin or glutaredoxin as a source of electrons, the activity of the APR enzymes is not stimulated *in vitro* by the addition of *E. coli* or *Spirulina sp.* thioredoxin. Also, the APR cDNAs are able to complement a thioredoxin/glutaredoxin double mutant of *E. coli*.

The properties of the APR enzymes are remarkably similar to those of APSSTase with respect to optimum assay conditions, kinetic constants and inhibitors. A key feature is that like APSSTase the APR enzymes are able to use a variety of reduced thiol compounds such as dithiothreitol and glutathione as a sole source of electrons. The enzyme shows a Kₘ for glutathione of 0.6 mM and can only complement the cysH mutation of *E. coli* if the strain produces glutathione (Bick and Leustek, unpublished data). Although the APR enzymes have not yet been directly linked with APSSTase the similarity of their catalytic features is a strong argument that they are the
same enzyme. The derived amino acid sequences of the APR cDNAs may reveal how APS reductase (and probably APSSTase) might function with only glutathione as a reductant. The APR enzymes are composed of three domains (Figure 2). At the amino terminus is a region that resembles a transit peptide; this would be cleaved from the protein following import into the chloroplast. Adjacent to this is the amino-terminal domain of the mature protein that is homologous with PAPS reductases from a variety of sulfate assimilating organisms. At the carboxyl end is a domain that resembles thioredoxin. The fusion of reductase and cofactor into a single protein suggests that the thioredoxin-like domain may serve as an exclusive electron donor for the reductase domain. But what about the interaction with glutathione?

**Figure 2**

![Diagram](https://via.placeholder.com/150)

**Figure 2**

Domain structure of the APR enzymes. The APR enzymes are composed of three domains. The amino terminal domain is a transit peptide (TP) required for localization to chloroplasts and is proteolytically removed to form the mature enzyme. The mature enzyme is composed of a reductase domain (shaded dark) homologous with bacterial PAPS reductase, and a carboxyl terminal domain (shaded light) homologous with thioredoxin (trx).

Thioredoxin and related proteins are redox-active proteins that function with a number of different reductases [24,25]. A hallmark of these proteins is an active site consisting of two cysteine residues, spaced two amino acids apart, that switch between the dithiol and disulfide. Plants contain thioredoxin proteins in both the cytoplasm and chloroplast and these are reduced by NADPH-dependent or ferredoxin-dependent thioredoxin reductases. The closely related protein, glutaredoxin, is reduced by glutathione, which is itself reduced by NADPH-dependent glutathione reductase. The natural redox systems used by these proteins are highly specific. For example, in contrast to glutaredoxin, thioredoxin is unable to accept electrons from glutathione. Interestingly, although the carboxyl domain sequence shows greater overall homology with thioredoxin, the amino acid residues between the active site cysteines are more typical of glutaredoxin. It has been demonstrated that these amino acids influence the redox potential of the cysteines and, therefore, the redox system that the proteins can react with [26]. Indeed, we have shown that the carboxyl domain functions more like glutaredoxin than thioredoxin. The implication of this result is that sulfate reduction in plants may ultimately be driven by NADPH through the activity of a glutathione reductase as depicted in Figure 1.

**Evolution of APR enzymes**

Despite the difference in substrate specificity the APR enzymes are clearly related in sequence to PAPS reductase from prokaryotes and fungi, but they are not related to APS reductase from dissimilatory sulfate reducing bacteria; this enzyme is a heteromeric iron-sulfur flavoprotein [27]. To avoid confusion, we propose that the plant enzyme be referred to as the plant-type APS reductase. A recent search of the DNA sequence databases revealed that PAPS reductase from *Pseudomonas aeruginosa* (GenBank accession #U95379) and *Rhizobium tropici* (AJ001223) has the highest homology with the reductase domain of the APR enzymes (~70% amino acid similarity), far greater than that with PAPS reductase from enteric bacteria, cyanobacteria or fungi (with which the reductase domain shares only ~50% amino acid similarity).

The evolution of the APR gene family in *A. thaliana* was explored by analysis of its genomic sequences [28]. Figure 3 shows that APR1 is encoded by three exons that are separated by two introns. In contrast, APR2 and APR3 are composed of four exons that are separated by three introns: the salient difference being that APR1 lacks the intron that separates exons 2 and 3 in APR2 and APR3. At the level of nucleotide homology, the coding sequence of APR1 is more closely related to APR3 (78% identity) than to APR2 (68% identity). This suggests that APR1 emerged by duplication of an ancestral APR1/APR3 gene followed by the loss of exon 2. The only PAPS reductase gene that is known to be interrupted is from the filamentous fungus *Emericella nidulans* (GenBank accession #X82555). This gene contains a single intron that interrupts the coding sequence at a position very close to the position of intron 2 in APR1 and intron 3 in APR2 and APR3.

A question of key significance in the evolution of the APR enzymes is how three distinct domains—the plastid transit peptide, the reductase domain, and the thioredoxin/glutaredoxin domain—became fused into a single polypeptide. One popular idea on gene evolution is that the domains of proteins arise through the accretion of separate exons [29]. If this were the case in APR evolution one might expect to find an intron separating the exons encoding individual domains. This appears to be the case with respect to the transit peptide. In all three genes it is encoded by exon 1 although the protease cleavage site is encoded at the beginning of exon 2. In contrast, the other domains are not separated by an intron, rather a portion of the reductase domain and the thioredoxin/glutaredoxin domain—became fused into a single polypeptide. This finding does not help to clarify the evolutionary origin of the gene. An interesting point, however, is that in APR1 there is a nearly perfect 12basepair sequence duplication located at the 5’ end of intron 2 (gtatattcgat) and another within exon 3 (gtatatatcgat) at a position that is approximately at the border between the reductase and thioredoxin/glutaredoxin-encoding sequences. Similar, but less well conserved sequences, are present at the...
same position in APR2 and APR3. It is an intriguing possibility that this repeat is the remnant of an intron that divided the last exon of these genes, isolating the thioredoxin/glutaredoxin-like domain in a separate exon.

### Contribution of APR gene expression to the regulation of sulfate assimilation

Although the sulfate assimilation pathway in plants appears to be regulated, the published evidence is not very clear on which steps are most important. This is in contrast to the situation in *E. coli* where the genes encoding all the pathway enzymes are co-ordinately regulated by gene expression in response to the availability of cysteine [13]. Thus, we must consider whether regulation in plants might be highly co-ordinated at the level of tissues and cells rather than at the whole plant level. Recently, the first attempt at defining the specific cells and tissues in which sulfate assimilation genes are expressed was published [30**]. In the future, more work of this kind is needed.

APSSTase has long been the focus of investigation because it is believed to be a key point for regulation of the sulfate assimilation pathway. Most importantly, the published literature indicates that changes in enzyme level are the primary means by which APSSTase activity is regulated [31]. Recent investigations on the affect of sulfate starvation on *Arabidopsis thaliana* showed that the steady state level of mRNA for sulfate permease and the APR enzymes increases markedly in roots and to a lesser extent in leaves [32*]. The mRNA level for all of the other sulfate assimilation genes was not increased or, in some cases, it decreased. This result is consistent with the idea that APSSTase and APS reductase are the same enzyme and may provide a basis for regulation of enzyme level, namely that changes in gene expression alter the protein level.

### Conclusions

The next few years promise to be an exciting time for the community of plant sulfur researchers. Gene cloning has emerged as a powerful tool to define the genes and enzymes of the sulfate assimilation pathways in plants and the effort of cataloging genes continued in 1997 [33*,34**,35**,36*]. The full potential of molecular methods, however, has yet to be realized. We expect that soon the biochemistry, enzymology and cell biology of the sulfur assimilation pathway will be known at a level of detail that will rival what is known about nitrogen assimilation.

### Note added in proof

The paper referred to in the text as (Bick and Leustek, unpublished data) has now been accepted for publication [37**].

### References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest


See annotation [1*].


See annotation [1*].


An important contribution to the field of sulfation in plants, a poorly understood area of sulfate metabolism.


See annotation [4*].


Not discussed in our review is the topic of complex formation between thiol and thioredoxin/glutaredoxin domains.


This reference is the first convincing purification of APS sulfotransferase.
References [15*] and [16*] simultaneously described the isolation of APS reductase cDNAs. The information in these papers confirms the existence of an APS dependent sulfate reduction pathway in plants.
See annotation for [15*].
This paper provides information on the interesting topic of evolution of the APR enzymes.
This is the first attempt to define the tissue and cellular specificity of the expression of a sulfate assimilation gene.
This is the first attempt to present a systematic analysis of how the expression of sulfate assimilation genes in plants is co-ordinated. In addition, information on the sulfate permease gene family in Arabidopsis thaliana is presented.
These four papers [33*,34*,35*,36*] report the most recent sulfate assimilation genes to be cloned.
Papers [34**,35**] and [36*] report for the first time on the gene and enzyme involved in the synthesis of siroheme, the prosthetic group of sulfite and nitrite reductase.
See annotation [34**].
See annotation [33**].
This report shows that the two domains of APS reductase play interactive but independent roles in catalysis and confirms that the carboxy-domain functions as a glutaredoxin.