# Arsenic toxicity: the effects on plant metabolism

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The two forms of inorganic arsenic, arsenate (AsV) and arsenite (AsIII), are easily taken up by the cells of the plant root. Once in the cell, AsV can be readily converted to AsIII, the more toxic of the two forms. AsV and AsIII both disrupt plant metabolism, but through distinct mechanisms. AsV is a chemical analog of phosphate that can disrupt at least some phosphate-dependent aspects of metabolism. AsV can be translocated across cellular membranes by phosphate transport proteins, leading to imbalances in phosphate supply. It can compete with phosphate during phosphorylation reactions, leading to the formation of AsV adducts that are often unstable and short-lived. As an example, the formation and rapid autohydrolysis of AsV-ADP sets in place a futile cycle that uncouples photophosphorylation and oxidative phosphorylation, decreasing the ability of cells to produce ATP and carry out normal metabolism. AsIII is a dithiol reactive compound that binds to and potentially inactivates enzymes containing closely spaced cysteine residues or dithiol cofactors. Arsenic exposure generally induces the production of reactive oxygen species that can lead to the production of antioxidant metabolites and numerous enzymes involved in antioxidant defense. Oxidative carbon metabolism, amino acid and protein relationships, and nitrogen and sulfur assimilation pathways are also impacted by As exposure. Readjustment of several metabolic pathways, such as glutathione production, has been shown to lead to increased arsenic tolerance in plants. Species- and cultivar-dependent variation in arsenic sensitivity and the remodeling of metabolite pools that occurs in response to As exposure gives hope that additional metabolic pathways associated with As tolerance will be identified.

**Keywords: arsenic toxicity, arsenate, arsenite, metabolism, rice**

## INTRODUCTION

The element arsenic (As) is an environmental toxin that is found naturally in all soils (Cullen and Reimer, 1989; Smedley and Kinniburgh, 2002). The main environmental exposure to As for humans is through contaminated drinking water, for example on the Indian sub-continent (Nordstrom, 2002). Arsenic becomes part of the human solid food chain when crops and fodder become contaminated. The metalloid enters into farming systems through a variety of means that include natural geochemical processes (Smedley and Kinniburgh, 2002), the past and present use of As-based pesticides, mining operations, irrigation with As-contaminated groundwater, and fertilization with municipal solid wastes (Meharg et al., 2009).

Arsenic contamination of the human food chain is a worldwide concern that is not restricted by economic boundaries. Rice grain has been identified as a major source of human As outside of contaminated drinking water (Meharg et al., 2009). In rice samples collected from around the world, As was found to be normally distributed in samples from developing economies, but strongly skewed toward high As contents in samples from developed economies (Meharg et al., 2009). Thus, the elimination of As accumulation in grain, especially rice, is a major research goal.

Arsenic is non-essential and generally toxic to plants. Roots are usually the first tissue to be exposed to As, where the metalloid inhibits root extension and proliferation. Upon translocation to the shoot, As can severely inhibit plant growth by slowing or arresting expansion and biomass accumulation, as well as compromising plant reproductive capacity through losses in fertility, yield, and fruit production (reviewed by Garg and Singla, 2011). At sufficiently high concentrations, As interferes with critical metabolic processes, which can lead to death. Most plants possess mechanisms to retain much of their As burden in the root. However, a genotype-dependent proportion of the As is translocated to the shoot and other tissues of the plant.

Numerous physiological processes are susceptible to As toxicity. Cellular membranes become damaged in plants exposed to As, causing electrolyte leakage (Singh et al., 2006). Membrane damage is often accompanied by an increase in malondialdehyde, a product of lipid peroxidation, pointing to the role of oxidative stress in As toxicity. Arsenic exposure induces antioxidant defense mechanisms. The synthesis of ascorbate, the γ-Glu-Cys-Gly tripeptide glutathione (GSH), and the GSH oligomer ($[γ$-Glu-Cys$]_n$-Gly) phytochelatin (PC) increases throughout the plant, but particularly in the roots (Schmöger et al., 2000; Li et al., 2004; Geng et al., 2006; Singh et al., 2006; Khan et al., 2009), while anthocyanin accumulates in leaves (Catarecha et al., 2007). Plant transpiration intensity can be reduced (Stoeva and Bineva, 2003). Low As burden causes the number of nitrogen-fixing root nodules to be repressed in soybean (Vázquez et al., 2008). The molecular mechanisms

underlying these physiological responses to As exposure are not clear, but have recently attracted increased attention.

One of the many interesting paradoxes related to As toxicity is that plant growth is stimulated at low As concentrations (Woolson et al., 1971; Carbonell-Barrachina et al., 1997, 1998b; Miteva, 2002; Garg and Singla, 2011). The fact that this phenomena occurs under axenic conditions in cultured plants, such as *Arabidopsis thaliana* (Chen et al., 2010), indicates that the trait is not based on As disrupting plant-biotic interactions. Instead, it results either from a direct interaction of As with plant metabolism, or from an interaction of As with plant nutrients. While the mechanism is unknown, it has been suggested that the growth benefit arises from As stimulation of Pi uptake (Tu and Ma, 2003).

There are relatively few species of plants that are naturally As tolerant. Among these are a group of plants including *Pteris vittata* and other members of the Pteridaceae that hyperaccumulate As (Ma et al., 2001; Visoottiviseth et al., 2002; Ellis et al., 2006; Pickering et al., 2006; Zhao et al., 2009). The growth of these plants is not compromised during times when they are accumulating extremely high levels of As. In contrast to As non-hyperaccumulating plants, hyperaccumulators tend not to restrict As to the roots, instead allowing transfer of the toxicant immediately to the shoots. This is likely to be an important aspect of the hyperaccumulation phenotype. The mechanisms by which these plants are able to hyperaccumulate As are being elucidated, but it is not entirely clear how they are able to avoid As toxicity while As is actively accumulating to extremely high levels in the leaves.

A better understanding of the mechanisms responsible for As resistance and toxicity in plants is needed. A potential outcome of a better understanding would be the production of As-resistant plants for phytoremediation and safe cropping. For soil remediation and contaminated site rehabilitation, a diverse group of resistant plants suitable for growth in a wide-range of environments and able to (hyper)accumulate As in harvestable biomass are needed. In contrast, for safe cropping in areas where land and/or groundwater are contaminated, As-resistant plants that prevent As accumulation in the harvested plant product are required.

Significant advances have been achieved recently in our understanding of the physiological processes that are affected by As exposure. We are also making progress unraveling some of the biochemical mechanisms underlying the disruption of key physiological processes. Our understanding of As as an inducer of oxidative stress is a good example of such a biochemical mechanism. In recent times, insights have been gained on identifying the gene expression changes that accompany As stress. One area where much remains to be learned is in identifying the cellular metabolic processes that are most at risk from As-induced damage. In this paper, we review what is known about the metabolic consequences to the plant of As exposure, identifying key areas where further research is needed.

## ARSENIC ACQUISITION, TRANSPORT, AND METABOLISM IN PLANTS

When considering the effects of As on plant cellular metabolism, it is important to consider the As species present in soils, the ability of these chemical species to enter plant cells, the ability of the plant to transform one As species to another, and the various As transport pathways that are available within the plant. During the movement of As through the plant tissues, some cell types are likely to be exposed to and need to respond to relatively high levels of particular As species, while others will be exposed to only low levels of the metalloid. The cell types that are exposed to high levels of particular As species is a defining difference between As hyperaccumulators and non-accumulators. While non-accumulators tend to retain As in root cells, with much lower concentrations of As in shoot cells, the hyperaccumulators have incredibly high concentrations of As in the cells of aerial tissues compared to the root. Understanding the responses of different cell types to the various As species to which they are exposed and internalize will likely be important in designing ways of producing plants that are better suited to deal with high environmental As burdens. The speciation, acquisition, transport, and metabolism of As have been extensively reviewed recently (Zhao et al., 2009, 2010; Zhu and Rosen, 2009; Mendoza-Cózatl et al., 2011), so only a brief overview of these topics will be given here to provide a context for the discussion on the consequences of As exposure to cellular metabolism.

## ARSENIC ACQUISITION AND TRANSPORT

In the environment, As can exist as inorganic or organic species. Of the two inorganic forms, the more highly oxidized arsenate (AsV) predominates in aerobic environments, while the more highly reduced arsenite (AsIII) is the predominant form in anaerobic environments, such as flooded rice paddy fields. Microbes are able to biotransform inorganic As to organic forms (Zhao et al., 2010). The organic species of AsV that are found at low concentrations in most soils include monomethylarsonic acid (MMA$^V$ – superscript denotes As oxidation state), dimethylarsinic acid (DMA$^V$) and trimethylarsine oxide (TMAO$^V$). The concentrations of the methylated species are higher in anaerobic soils than in aerobic soils (Abedin et al., 2002). The corresponding mono-, di-, and trimethylated derivatives of AsIII (MMA$^{III}$, DMA$^{III}$, TMA$^{III}$) are volatile. They are produced in the soil through processes likely to be limited by the availability of MMA$^V$ (Mestrot et al., 2011). Like AsV and AsIII, the methylated forms of As are phytotoxic (Zhao et al., 2010).

AsV is an analog of inorganic phosphate (Pi) and is easily transported across the plasmalemma by Pi transporter (PHT) proteins (Ullrich-Eberius et al., 1989; Meharg and Macnair, 1990, 1991, 1992; Wu et al., 2011). AsV and Pi compete for uptake through the same transport systems in As hyperaccumulators (Wang et al., 2002; Tu and Ma, 2003), As-tolerant non-hyperaccumulators (Meharg and Macnair, 1992; Bleeker et al., 2003) and As-sensitive non-accumulators (Abedin et al., 2002; Esteban et al., 2003). Under low Pi conditions, AsV may outcompete Pi for entry into the plant, amplifying Pi deprivation symptoms. Conversely, Pi fertilization can protect plants, including the hyperaccumulator *P. vittata*, from AsV toxicity (Tu and Ma, 2003).

Increasing or decreasing the rate of Pi and As uptake by increasing or decreasing PHT protein amount or activity at the plasmamembrane through genetic means can also increase or decrease, respectively, the toxicity of AsV (Shin et al., 2004; González et al., 2005; Catarecha et al., 2007; Wu et al., 2011). In the *Arabidopsis pht1–3* mutant, which has a compromised Pi uptake system, As accumulates without causing toxicity (Catarecha et al., 2007),

similar to the Pi fertilization effect in *P. vittata*. The lack of toxicity in such non-hyperaccumulating systems has been explained by postulating that a slower rate of As accumulation allows the plant to detoxify the incoming As before defense systems are overloaded and the toxicant can exert its toxic effects (Hartley-Whitaker et al., 2001a; Catarecha et al., 2007).

Once inside the plant cell, AsV can probably move easily from one cellular compartment to another, crossing internal membranes through the various Pi transporters. For example, AsV has been demonstrated to be a co-substrate for three mitochondrial dicarboxylate transporters, proteins localized to the inner mitochondrial membrane and responsible for dicarboxylate exchange with co-substrates such as Pi, between the cytosol and the organelle matrix (Palmieri et al., 2008). The outcome of this rapid movement would be the rapid equilibrium of As throughout the cell, exposing all parts of cellular metabolism to the toxicant.

AsV can be found in the xylem, having most likely been loaded into the xylem vessels by PHT proteins (Catarecha et al., 2007; Zhao et al., 2010; Mendoza-Cózatl et al., 2011; Wu et al., 2011). However, roots of As non-hyperaccumulators have the ability to strongly retain As. In *Arabidopsis*, only about 3% of the As taken up by the root was translocated to the shoot (Quaghebeur and Rengel, 2003). Similar results have been found for other plants (Lomax et al., 2012). Of the small portion of As that is translocated, no more than 40% would be expected to be in the form of AsV, based on As speciation determinations in a number of species (Zhao et al., 2009).

AsIII is able to enter root cells through nodulin26-like intrinsic proteins (NIPs, Meharg and Jardine, 2003; Bienert et al., 2008; Isayenkov and Maathuis, 2008; Ma et al., 2008). These proteins belong to the aquaporin family of major intrinsic proteins. In rice roots, the OsNIP2;1/OsLsi1 silicon transporter has been implicated as the major AsIII uptake protein, while AsIII efflux from rice root cells to the xylem is through the OsLsi2 silicon transporter (Ma et al., 2008). The localization of OsLsi2 to the proximal side of epidermal and endodermal cells (Ma et al., 2007), and OsLsi1 to the distal side of the same cells (Ma et al., 2006) is an elegant example of the heterogeneous distribution of proteins in a membrane providing directionality to solute transport across cells and tissues. Other types of proteins may facilitate the transport AsIII into cells. In yeast, the majority of AsIII uptake occurs through hexose permeases (Liu et al., 2004). While plants have proteins with strong homology to the yeast hexose permeases, it is not known if they provide a path for AsIII entry into plant cells.

In As hyperaccumulating species, such as *P. vittata*, As is not immobilized in the roots, but is instead rapidly transported as AsIII through the xylem to the fronds (Lombi et al., 2002; Pickering et al., 2006; Su et al., 2008). In the fronds, AsIII is sequestered as free AsIII in the vacuole (Lombi et al., 2002; Pickering et al., 2006), where it accumulates to extremely high levels (Zhao et al., 2009). It has been shown that PvACR3 is involved in the vacuolar sequestration of AsIII (Indriolo et al., 2010). This protein is a homolog of the yeast ScACR3p protein, a plasmamembrane protein responsible for the efflux of AsIII from the yeast cell. In *P. vittata*, the PvACR3 protein still acts to efflux AsIII from the cytosol, but instead of delivering the AsIII to the outside of the cell, PvACR3

resides on the vacuolar membrane and transports the AsIII into the vacuole. Single-copy *ACR3* genes are found in moss, lycophytes, ferns, and gymnosperms, but not in angiosperms, which may help explain the lack of As hyperaccumulators among the angiosperms (Indriolo et al., 2010).

Plants have been found to contain methylated As species (Marin et al., 1992; Quaghebeur and Rengel, 2003; Raab et al., 2005, 2007b; Xu et al., 2007). For example, when fed AsIII or AsV, roots of *Helianthus annuus* (sunflower) contained up to 14 extractable and separable forms of As, of which eight were identified (Raab et al., 2005). Among these forms were $MMA^V$ and $DMA^V$ and a $MMA^{III}$-PC complex. The finding of methylated As species inside plant tissues brought the question of their origins to recent attention. Several reports using non-axenically grown plants concluded that plants were able to methylate AsV to form $MMA^V$ and $DMA^V$ (Raab et al., 2005, 2007b; Xu et al., 2007). Moreover, it was reported that leaf extracts of *Agrostis tenuis*, again from non-axenically grown plants, had an AsV-inducible AsIII/AsV methylation activity (Wu et al., 2002). However, methylated forms of As were not found in several plant species grown under axenic conditions (Lomax et al., 2012). This study, then, indicates that the methylated derivatives of As that have been found in plants are likely to have been acquired after biotransformation by soil microbes (Lomax et al., 2012). The question of the source of methylated forms of As is important, as methylated forms of AsIII are more reactive and cytotoxic to animal cells than non-methylated forms (Styblo et al., 1997, 2000; Lin et al., 1999; Schwerdtle et al., 2003; Naranmandura et al., 2011). Moreover, methylated forms of AsV common in terrestrial environments are also a concern as they can be reduced to the more cytotoxic methylated AsIII forms. Thus, methylated forms of As have the potential to more strongly disrupt plant metabolism at lower concentrations than the inorganic species.

Like AsIII, the protonated, uncharged forms of the methylated As species $MMA^V$ and $DMA^V$ enter rice roots at least in part through the aquaporin channel OsLsi1 (Li et al., 2009). However, the rate of uptake for $MMA^V$ and $DMA^V$ is much slower than that of AsIII or AsV (Abedin et al., 2002; Raab et al., 2007a; Abbas and Meharg, 2008). In contrast, the mobility within the plant of $MMA^V$ and $DMA^V$ appears to be substantially greater than that of AsIII or AsV (Marin et al., 1992; Burló et al., 1999; Raab et al., 2007a; Li et al., 2009; Carey et al., 2010, 2011). While OsLsi1 has been implicated in the uptake of both $MMA^V$ and $DMA^V$, OsLsi2 does not seem to be involved in their efflux (Li et al., 2009). In animal systems, $MMA^{III}$ is also transported through aquaporins (Liu et al., 2006); however, it is not yet known whether OsLsi1, OsLsi2 or AsIII transporters are able to transport $MMA^{III}$.

Arsenic can also be metabolized by various organisms to form arsenocholine, arsenobetaine, and arseno-sugars. These compounds have been detected in some terrestrial plants (reviewed by Dembitsky and Levitsky, 2004). However, it has not been demonstrated that these compounds can be produced by the plant, or whether they are simply taken up in those forms from the soil. The effects of these compounds on plant metabolism are largely unknown.

## ARSENIC METABOLISM IN PLANTA

When plants were supplied AsV, typically more than 90% of the As in the roots and in the shoots was found to be in the form of AsIII (Pickering et al., 2000; Dhankher et al., 2002; Xu et al., 2007). Thus, AsV is readily reduced to AsIII by plants. This reduction is accepted as the first step in the major As detoxification pathways found in plants (Pickering et al., 2000; Schmöger et al., 2000). The reduction of AsV to AsIII occurs both enzymatically and non-enzymatically. In the non-enzymatic pathway, two molecules of GSH are able to reduce AsV to AsIII (Delnomdedieu et al., 1994). The oxidation of GSH is via the formation of a disulfide bond, producing a GSH dimer (GSSG; Delnomdedieu et al., 1994), which can be rapidly recycled to two GSH molecules by GSH reductase (Foyer and Noctor, 2011).

While AsV reduction can occur non-enzymatically, the enzymatic rate is much higher (Duan et al., 2005). AsV can be directly reduced to AsIII by arsenate reductase (ACR), an enzyme first isolated from bacteria and yeast (Mukhopadhyay et al., 2000). Based on homology between the yeast ACR gene *ScAcr2* and several homologous sequences from plants, an *HlAsr* cDNA was cloned from AsV-hypertolerant *Holcus lanatus* and shown to encode an enzyme with ACR activity (Bleeker et al., 2006). The homologous proteins from *Arabidopsis* (AtAsr/AtACR2), *P. vittata* (PvACR2), and rice (OsACR2.1 and OsACR2.2) also have ACR activity (Dhankher et al., 2006; Ellis et al., 2006; Duan et al., 2007).

The plant ACR2 protein is related to the CDC25 cell cycle dual specificity tyrosine phosphatases. Interestingly, AtACR2 has phosphatase activity, while the PvACR2 enzyme, like the yeast ScAcr2p protein, does not (Landrieu et al., 2004; Ellis et al., 2006; Duan et al., 2007). Also like ScAcr2p, the plant ACR2 enzyme uses GSH and glutaredoxin (GRX) as electron sources (Ellis et al., 2006; Duan et al., 2007), suggesting that the catalytic cycle involves the formation of a mixed disulfide between GSH and ACR2 that is resolved by GRX (Mukhopadhyay et al., 2000).

The *Arabidopsis* AsV reductase activity has an AsV-inducible component that has been attributed to *AtACR2*, as well as a constitutive component that is not diminished in *AtACR2* T-DNA insertion lines (Bleeker et al., 2006). Moreover, AsIII has been stated to remain the predominant form of As present in *AtACR2* T-DNA insertion lines supplied with AsV (reported in Zhao et al., 2009). Together, these results indicate that *Arabidopsis*, and thus in all likelihood other plants, possess enzymes in addition to ACR2 that have As(V) reductase activity.

Multiple enzymes from other systems have been shown to exhibit AsV reductase activity. These include glyceraldehyde-3-phosphate dehydrogenase (GAPDH), polynucleotide phosphorylase, purine nucleoside phosphorylase (PNP), glycogen phosphorylase, and the mitochondrial $F_1F_o$ ATP synthase (Németi and Gregus, 2002; Radabaugh et al., 2002; Gregus and Németi, 2005; Németi et al., 2010). Each of these enzymes can incorporate AsV instead of Pi into biological molecules, forming an arsenoester that would readily undergo hydrolysis. In the presence of a suitable thiol group, for example GSH, the hydrolysis can result in the reduction of AsV to AsIII. It is not known if the analogous plant enzymes can also reduce AsV in the presence of thiols. However, one form of the plant GAPDH is known to interact with GSH

(Zaffagnini et al., 2007), suggesting it as a candidate ACR. Moreover, a cytosolic triose-phosphate isomerase (cTPI) from *P. vittata* has also been shown to have ACR activity (Rathinasabapathi et al., 2006). Since the TPI reaction does not involve the transfer of a Pi group, the mechanism by which PvcTPI promotes the production of AsIII is unclear. However, like the enzymes mentioned above, the plant TPI interacts with GSH (Ito et al., 2003; Rathinasabapathi et al., 2006). The number of enzymes that could misincorporate AsV for Pi, and therefore have the capacity to form arsenoesters, is large, providing many opportunities for the enzymatic reduction of AsV to AsIII. However, it is not known whether these enzymes affect the redox status of As *in vivo*. It is likely that any contribution that they make to the reduction of AsV to AsIII will depend on the concentrations of substrates and effectors in the cell (Gregus and Németi, 2007; Németi et al., 2011).

In addition to being able to reduce AsV to AsIII, plants also appear to be able to reduce MMA$^V$ taken into the roots to MMA$^{III}$. In two experiments, about 15% of the aqueous As extracted from rice roots fed MMA$^V$ and washed prior to extraction was in the form of MMA$^{III}$ (Li et al., 2009; Ye et al., 2010). The lack of MMA$^{III}$ in the medium led to the conclusion that MMA$^V$ was reduced to MMA$^{III}$ within the roots (Li et al., 2009). Another experiment using rice grown in axenic culture found that 65% of the As associated with the roots in MMA$^V$-treated plants was in the form of MMA$^{III}$ (Lomax et al., 2012). Moreover, 9% of the As in the shoots of these plants was in the form of MMA$^{III}$, demonstrating that MMA$^{III}$ can be found throughout the plant. While it has not been formally demonstrated that the observed MMA$^{III}$ was produced inside the cells of the root, the finding of MMA$^{III}$ in the shoots suggests that MMA$^{III}$ is able to penetrate cell membranes at least at some point along the transpiration stream. The presence of MMA$^{III}$ inside plant cells could have important implications for metabolism because of its higher reactivity and cytotoxicity (Styblo et al., 1997, 2000; Lin et al., 1999; Schwerdtle et al., 2003; Naranmandura et al., 2011).

Inorganic As has only limited mobility in most plants, demonstrated by the steep decline in As concentration from roots to stems to leaves to grain (Liu et al., 2006; Zheng et al., 2011; Zhao et al., 2012). However, As is clearly mobile throughout the plant, as it readily appears in leaves and grain. Since no As-sulfhydryl complexes, indicative of As-GSH and As-PC complexes, were found in sap exuded from sunflower, it appears that AsIII and AsV are the sap-mobile forms of As (Raab et al., 2005). The movement of AsIII across membranes seems to be mediated by silicon transport proteins such as OsLsi1 and OsLsi2, while the transport of AsV would likely be mediated by Pi transporters.

## THE TOXICITY OF ARSENIC

The results from a number of hydroponic experiments agree that As phytotoxicity depends on the chemical species supplied to the plant, but disagree on the identity of the most phytotoxic form of As (Marin et al., 1992; Carbonell-Barrachina et al., 1998a; Abbas and Meharg, 2008). These hydroponic experiments provide the clearest insights into the potency of externally supplied As on whole plant growth because they eliminate the complex and confounding phytoavailability issues that arise from differences in the

mobility of various As species through the diverse growth substrates. The studies generally agree with the hydroponic survey of 46 different plant species (Raab et al., 2007a) that the uptake of As by plants has the order AsIII > AsV > MMA$^V$ > DMA$^V$, while translocation from the roots to the rest of the plant has the order DMA$^V$ > MMA$^V$ > AsV ≥ AsIII. However, no one As form appears to be consistently most phytotoxic. In two *Spartina* species, where the order of uptake was AsIII > AsV ≈ MMA$^V$ > DMA$^V$, the order of phytotoxicity was DMA$^V$ ≈ MMA$^V$ > AsIII ≈ AsV (Carbonell-Barrachina et al., 1998a). This would suggest that DMA$^V$, with lowest uptake and high phytotoxicity, exerted the most highly toxic effects within the plant. In contrast, the uptake order in rice was AsIII > MMA$^V$ > AsV > DMA$^V$, an order that is similar to the order of phytotoxicity, which was MMA$^V$ > AsIII > AsV = DMA$^V$ (Marin et al., 1992). Finally, the order for phytotoxicity in maize, a species with the typical order for uptake (Raab et al., 2007a), was AsV > AsIII > DMA$^V$ (Abbas and Meharg, 2008).

The inconsistent order of phytotoxicity of the various As species could be an indication that As has interacted differently with the available nutrients, or that the phytotoxic form of As is plant species dependent. Alternatively, the apparent inconsistency of the above results may be due to our incomplete understanding of the relative importance of the various As species to the mechanism of As toxicity. After all, the mechanism through which As causes phytotoxicity has not been definitively identified and the exact species of As that is the primary cause of toxicity is unknown. In this regard, the finding of MMA$^{III}$ in plants (Li et al., 2009; Ye et al., 2010; Lomax et al., 2012) is particularly intriguing. This As species is up to 18 times more cytotoxic to animal cells than AsIII (Styblo et al., 2000; Naranmandura et al., 2011). The phytotoxicity of MMA$^{III}$ has not been tested.

Another issue that needs to be included when considering the toxicology of As is that some forms of As may be under-represented or even missing from the evaluations of As speciation in plants. In a study on sunflower, 15–20% of the total tissue As was not recovered from the plant tissue, depending on the tissue and post-harvest storage time (Raab et al., 2005). In beetroot, As was nearly quantitatively extractable from tissues with low As burden, but in tissues with higher As burdens, up to 75% of the As was not extracted (Száková et al., 2010). Perhaps the speciation of this missing As, or indeed the molecules to which it may be bound, will give us important insights into the underlying mechanism of As toxicity.

The above arguments indicate that we need a fuller understanding of the action of As at the cellular level. The structural similarities of AsV and Pi allow this form of the metalloid to substitute for Pi in biochemical reactions, potentially disrupting vital cellular processes. On the other hand, AsIII is highly reactive toward thiol groups, as are MMA$^{III}$ and DMA$^{III}$. The binding of AsIII to the thiol groups of proteins or enzyme co-factors may alter or inhibit their activity, also exposing cellular processes to risk. Oxidative stress brought about by the inevitable production of reactive oxygen species (ROS) that occurs during As exposure has most recently gained favor as the main driver of As toxicity in plants. However, which cellular processes are most sensitive to As toxicity and which As species pose the greatest threats to plant cell health remain unclear.

## REPLACING PHOSPHATE IN REACTIONS

An important mode of action of AsV toxicity may be the replacement of Pi in critical biochemical reactions. Substitution of Pi by AsV has been demonstrated to occur in numerous biochemical reactions, and any reaction with Pi or a Pi-ester as a substrate is a potential target for AsV disruption (Orsit and Cleland, 1972; Park and Agrawal, 1972; Long and Ray, 1973; Gresser, 1981). Potential AsV-sensitive reactions would include those central to cellular metabolism (i.e., glycolysis, oxidative phosphorylation) and biosynthesis (i.e., phospholipid metabolism), information storage and retrieval (DNA, RNA metabolism), and cellular signaling (i.e., protein phosphorylation/dephosphorylation).

When AsV comes into contact with the surface of a cell within the plant root, it is probable that a Pi transporter will be the first enzyme where AsV will compete with Pi. Plants have both low- and high-affinity Pi transport systems. High-affinity transport is mediated by PHT1 proteins. The protein responsible for the low-affinity transport is unknown, although some PHT1 proteins also have a low-affinity activity. Competition by AsV with Pi for entry into the cell through both of these transport systems has been demonstrated in numerous plants, both monocots and dicots, and both As-hyperaccumulators and non-hyperaccumulators (Ullrich-Eberius et al., 1989; Meharg and Macnair, 1992; Clark et al., 2000; Abedin et al., 2002; Wang et al., 2002; Bleeker et al., 2003; Esteban et al., 2003; Tu and Ma, 2003). Since Pi competes with AsV for uptake, AsV toxicity is lower under high Pi conditions. On the other hand, AsV may outcompete Pi for uptake under low Pi conditions, exacerbating Pi deprivation (Tu and Ma, 2003). Other transporters besides PHT1 can also be fooled into utilizing AsV instead of Pi. For example, AsV is able to move across the plant inner mitochondrial membrane through the Pi translocator (De Santis et al., 1975) and the dicarboxylate carrier (Palmieri et al., 2008). The toxicant can also pass through the *Arabidopsis* AtPHT4 family of Pi transporters localized to the plastid and golgi (Guo et al., 2008a).

Relatively few enzymes use Pi as a substrate due to the irreversible nature of most Pi-liberating reactions. Therefore, few enzymes are expected to use AsV directly as a substrate (Tawfik and Viola, 2011). Perhaps the predominant Pi-requiring reaction is the phosphorylation of ADP to ATP by the $F_1F_o$-type ATP synthases found in the mitochondrial inner membrane and the plastid thylakoid membrane. The mitochondrial enzyme uses AsV in a reaction that produces ADP-AsV (Gresser, 1981). The $K_M$ and $V_{max}$ of this reaction are remarkably similar for both Pi and AsV (Moore et al., 1983), demonstrating that at least some enzymes are capable of recognizing and reacting equally well with AsV and Pi. These characteristics are most probably shared with the plastid $F_1F_o$-type ATP synthase (Avron and Jagendorf, 1959; Watling-Payne and Selwyn, 1974), although this has not been demonstrated directly.

Other Pi-dependent enzymes that are able to use AsV include the glycolytic enzyme GAPDH. Like the ATP synthase reaction, the GAPDH reaction where AsV replaces Pi has remarkably similar kinetic constants to the Pi-dependent reaction (Orsit and Cleland, 1972). Aspartate-β-semialdehyde dehydrogenase has a critical role in the biosynthesis of essential amino acids in plants, catalyzing the reversible reductive dephosphorylation of β-aspartyl phosphate

to L-aspartate-β-semialdehyde. This enzyme, too, is able to use AsV nearly as efficiently as Pi, judged from the $K_M$ and $k_{cat}$ values (Kish and Viola, 1999). PNP catalyzes the phosphorolysis of various nucleosides, producing the free nucleotide base and ribose-1-phosphate. The substitution of AsV for Pi in the PNP reaction releases ribose-1-AsV in an arsenolysis reaction with a $K_M$ that is again quite similar to that of the Pi-dependent reaction (Park and Agrawal, 1972). While much of the above information comes from non-plant systems, there is little reason to believe that the behavior of homologous plant enzymes would be substantially different.

The AsV-esters produced by these and other reactions are highly unstable in water and undergo spontaneous and rapid hydrolysis (Rosen et al., 2011; Tawfik and Viola, 2011). The hydrolytic products are generally free AsV and the corresponding carbon compound. The instability of AsV-esters is highlighted by the rates of hydrolysis for glucose-6-AsV and ADP-AsV, which are at least $10^5$ greater than for the corresponding Pi-ester (Moore et al., 1983). As pointed out above, some of the spontaneous ADP-AsV hydrolysis reactions, for example, those catalyzed by GAPDH and PNP in the presence of a suitable thiol group, are reductive, releasing free AsIII instead of AsV (Radabaugh et al., 2002; Gregus and Németi, 2005). The instability of AsV-containing compounds is probably increased by the likelihood of such reductions (Rosen et al., 2011).

As a consequence of the instability of As-esters, the enzymatic reactions that produce them are essentially irreversible. The products simply do not stay around long enough to allow the reverse reaction to proceed at an appreciable rate. The unstable nature of the AsV-esters also creates futile reaction cycles around enzymes that use free AsV to produce AsV-esters. The AsV and ADP released by the auto hydrolysis of ADP-AsV produced by mitochondrial and plastid ATP synthases, for example, can be used in subsequent reaction cycles. Such futile cycling uncouples respiratory electron transport in the mitochondrial inner membrane and photosynthetic electron transport in chloroplast thylakoid membranes from ATP synthesis (Avron and Jagendorf, 1959; Ter Welle and Slater, 1967; Watling-Payne and Selwyn, 1974; Wickes and Wiskich, 1975; Gresser, 1981; Berry and Rumberg, 1999). This collapse of ATP production has potentially grave consequences for the energy status of the cell.

While AsV-esters are unstable, enzymes can utilize them when they are available. Hexokinase can use ADP-AsV to arsenolate glucose to glucose-6-AsV. This product, in turn, is a substrate for glucose-6-phosphate dehydrogenase, an enzyme in the reductive pentose phosphate pathway (Gresser, 1981). Glucose-6-AsV is also a substrate for phosphoglucomutase in the formation of glucose-1-AsV (Long and Ray, 1973). However, the lability of As-esters calls into question the physiological relevance of these metabolic reactions during AsV exposure.

## BINDING THIOLS
The mode of action of AsIII differs substantially from that of AsV. AsIII is a thiol reactive compound that can bind up to three sulfhydryl groups (Kitchin and Wallace, 2006b). This allows AsIII to act as a cross-linking agent by binding up to three monothiol molecules, such as the antioxidant GSH. Alternatively, it could bind to a single molecule of a poly-thiol compound, such as PC,

the Cys-rich polymerization product of GSH. AsIII can also bind to thiol-containing proteins and co-factors. Dihydrolipoamide, which in plants is a co-factor associated the mitochondrial and plastid pyruvate dehydrogenase complexes (mtPDC, ptPDC), the 2-oxoglutarate dehydrogenase complex (OGDC), the Gly decarboxylase complex (GDC) and the branched-chain 2-oxoacid decarboxylase complex (BCOADC), has been long-thought to be an important cellular target for AsIII binding (Peters et al., 1946; Bergquist et al., 2009).

The stability of AsIII complexes increases with the number of bonds formed. The half-life of an AsIII-monothiol peptide complex is only about 1–2 s. The half-life increases to about 1.3 and 155 min when two or three intramolecular thiols are bound (Kitchin and Wallace, 2006b). The high stability of AsIII-trithiol complexes is supported by the finding that AsIII preferentially binds zinc-finger proteins containing three or more Cys residues in the zinc-finger motifs (Zhou et al., 2011). This study did not find AsIII binding to zinc-finger motifs with only two Cys residues, possibly due to the time needed to process the samples (Zhou et al., 2011). Complexes where AsIII forms intramolecular links between peptides are more stable than those with intermolecular bonds (Kitchin and Wallace, 2006b). The binding of AsIII to dithiols is enhanced when the sulfhydryl groups are in close proximity to one another (CX$_{0-14}$C; Kitchin and Wallace, 2006a), but the optimal spacing for trithiols is unknown.

The binding of AsIII to proteins can have profound effects on their folding (Cline et al., 2003; Ramadan et al., 2007). More than 100 enzyme activities that were sensitive to As compounds were identified by 1966 (Webb, 1966), and that number will have grown considerably. In various systems, proteins that are known to bind AsIII include transcription factors, signal transduction proteins, proteolytic proteins, metabolic enzymes, redox regulatory enzymes, and structural proteins. Among the 35,386 predicted translation products from the *Arabidopsis* genome sequence (TAIR10 release), there are 64,335 dithiols with optimal spacing for AsIII binding (CX$_{0-14}$C, Kitchin and Wallace, 2006a) on 23,578 proteins. About one-third of these dithiols, residing on 11,559 proteins, form part of a trithiol that may be optimally spaced for AsIII binding, assuming that the optimal sulfhydryl spacing for AsIII binding to trithiol groups is symmetrical with the dithiol spacing (CX$_{0-14}$CX$_{0-14}$C). This analysis ignores the potential for intramolecular cross-links, but raises two intriguing questions: What types of proteins in *Arabidopsis* are among the 2123 proteins lacking a Cys residue? Are there evolutionary pressures for these proteins in particular to lack the ability to interact with AsIII? One conclusion to be drawn from these values is that AsIII has the capacity to interact with a large proportion of any cellular proteome and it will be a large task to identify which proteins among the AsIII targets are most critical to cell survival.

While AsIII is an inhibitor of many enzymes, the recent finding of methylated forms of AsIII in plant cells (Raab et al., 2005; Li et al., 2009; Ye et al., 2010; Lomax et al., 2012) has important implications in this respect. Half maximal inhibition of pyruvate dehydrogenase was found to occur at about 115 μM AsIII, while two- to six-fold less of several methylated AsIII derivatives was need for similar inhibition (Petrick et al., 2001). Compared to AsIII, MMA$^{III}$ is a more potent inhibitor of other enzymes,

including GSH reductase (Styblo et al., 1997) and thioredoxin reductase (Lin et al., 1999). MMA$^{III}$ and DMA$^{III}$ in the low to mid micromolar range were able to displace $Zn^{2+}$ from a zinc-finger protein (Schwerdtle et al., 2003), an important class of proteins involved in gene expression and DNA repair. Both methylated forms were also more potent inhibitors of zinc-finger protein activity than AsIII, again highlighting the necessity to critically evaluate the ability of plants to methylate inorganic As into more toxic forms or reduce methylated-As$^V$ compounds to their As$^{III}$ counterparts.

The binding of AsIII to thiols is the basis for what is considered to be the main detoxification pathway for both AsV and AsIII and explains the retention by roots of up to 90% of the As taken into a plant (Quaghebeur and Rengel, 2003; Raab et al., 2007a; Zhao et al., 2012). AsIII is either taken directly into the roots from the soil solution or rapidly produced within the root by the efficient reduction of AsV. AsIII then combines rapidly with sulfhydryl-rich protective molecules like GSH and PC (Pickering et al., 2000; Schmöger et al., 2000; Liu et al., 2010). The preference of AsIII to bind polythiols favors the formation of AsIII-PC$_3$ complexes over complexes with PC$_2$ or oxidized GSH (Raab et al., 2005). However, a variety of AsIII conjugates can exist in a plant, with 14 different complexes being isolated from sunflower (Raab et al., 2005). The AsIII-PC conjugates can then be transported from the cytosol into the vacuole. In *Arabidopsis*, this transport is via the ABC transporters MRP1/ABCC1 and MRP2/ABCC2 (Song et al., 2010). Homologs of *Arabidopsis* ABCC1 and ABCC2 are found throughout the plant kingdom (Mendoza-Cózatl et al., 2011). A homolog of ABCC2 was among several ABC transporters to be up-regulated at the transcript level in response to As in rice (Norton et al., 2008; Chakrabarty et al., 2009). Thus, in many plants, AsIII present in root cells is rapidly complexed to PC and sequestered in the vacuole, severely restricting the transport of As from the root (Liu et al., 2010; Mendoza-Cózatl et al., 2011) and preventing its interaction with cellular metabolism. The impact of As on cellular metabolism in many plants, then, depends on how efficiently AsIII can be neutralized by thiol binding and sequestration in the vacuole. Interestingly, this is not the case in hyperaccumulating species such as *P. vittata*, where there is little PC binding of AsIII in the roots (Zhao et al., 2003). Instead, AsIII that is taken into the plant cell from the soil solution or is produced by the reduction of AsV is rapidly translocated to the xylem and fronds for sequestration as free AsIII in the vacuole (Lombi et al., 2002; Pickering et al., 2006; Su et al., 2008). This transport of AsIII through the plant body raises the intriguing question of how *P. vittata* is able to avoid the toxic effects of AsIII binding to enzymatic thiols.

## OXIDATIVE STRESS

It is well documented that exposure of plants to AsIII and AsV induces the production of ROS, including superoxide ($O_2\bullet^-$), the hydroxyl radical ($\bullet OH$), and $H_2O_2$ (Hartley-Whitaker et al., 2001a; Requejo and Tena, 2005; Singh et al., 2006; Ahsan et al., 2008; Mallick et al., 2011). ROS can damage proteins, amino acids, purine nucleotides and nucleic acids and cause peroxidation of membrane lipids (Møller et al., 2007). Lipid peroxidation not only compromises cellular function, but leads to the production of lipid-derived radicals (Van Breusegem and Dat, 2006; Møller et al., 2007). Induction of lipid peroxidation by AsV was also observed in the As hyperaccumulator *P. vittata* (Srivastava et al., 2005; Singh et al., 2006), indicating that ROS production is a feature of the general plant As response and that the magnitude of the redox imbalance in the cell may be an important determinant of ROS-induced toxicity. Although the mechanism of the As-induced production of ROS is not well understood, it has been proposed that As detoxification processes, including the reduction of AsV to AsIII and the induction of PC synthesis (Meharg and Hartley-Whitaker, 2002), have roles to play in ROS production. The molecular targets that are most sensitive to the ROS produced by As exposure are not yet clear, although there are many candidates (Møller et al., 2007).

Under normal cellular conditions, ROS homeostasis is delicately balanced. Relatively small changes in nutrient availability or environmental conditions such as temperature and light can cause small ROS imbalances that act as signals of cellular status and are easily managed by pre-existing antioxidant defense mechanisms (Van Breusegem and Dat, 2006; Møller et al., 2007; Foyer et al., 2011). However, under stronger stresses, such as As exposure, where ROS generation increases, these defense mechanisms may be overwhelmed, leading to cellular damage. This damage can lead to cell death (Van Breusegem and Dat, 2006). Unless the cell death is part of a developmental program, cellular responses must seek to restrict ROS-mediated damage or the survival of the organ or individual will be jeopardized.

Several enzymes are involved in ROS defense strategies. Highly reactive superoxide can be converted to less active but longer-lasting $H_2O_2$ through the action of superoxide dismutase (SOD). SOD activity in plants varies quite widely with As treatment. In some plants, like *Zea mays*, As-sensitive clones of *H. lanatus*, and the As-hyperaccumulator *P. vittata*, the enzyme is induced by low As exposure, and either stays at the same level or decreases in activity at higher As levels (Mylona et al., 1998; Hartley-Whitaker et al., 2001a, Cao et al., 2004). One explanation put forward for this variation in activity is that SOD is a metallo-enzyme (Meharg and Hartley-Whitaker, 2002). However, part of the explanation may also be at the level of gene expression. In *Arabidopsis*, genes encoding the three classes of SOD (FeSOD, MnSOD, Cu/ZnSOD) responded to AsV differentially at the transcript level (Abercrombie et al., 2008). Transcripts for genes encoding a chloroplastic and a cytosolic Cu/ZnSOD were induced more than two-fold by AsV exposure, while transcripts for an FeSOD were down-regulated about five-fold (Abercrombie et al., 2008). These observations raise the question of what effects these changes in the SOD transcript pool have on the characteristics of the SOD activity, and, if the characteristics of the SOD activity changes, what are the adaptive advantages, if any, and the underlying mechanisms, of those changes.

$H_2O_2$ produced in a plant cell either directly or enzymatically through enzymes such as SOD can be neutralized by catalase, an enzyme that is often induced by As exposure (Mylona et al., 1998; Cao et al., 2004; Srivastava et al., 2005; Geng et al., 2006; Duman et al., 2010). In addition to catalase, plants have a two-component system for regulating the balance of $H_2O_2$, and therefore of ROS, within cells. One component encompasses a group

of non-enzymatic antioxidants that includes GSH, PC, ascorbate, carotenoids, and anthocyanin. These antioxidants generally accumulate during As exposure (Schmöger et al., 2000; Hartley-Whitaker et al., 2001a; Bleeker et al., 2003, 2006; Khan et al., 2009; Song et al., 2010). The production of these molecules requires metabolic acclimations, including the diversion of carbon, nitrogen, sulfur, and metabolic energy from normal growth and development.

GSH and ascorbate are fairly unique among the non-enzymatic antioxidants in that they can form a redox cycle. The ROS produced during As treatment typically induces an increase in the oxidation state of the redox active pools of GSH and ascorbate in favor of GSSG dimers and dehydroascorbate over the more reduced GSH and hydroascorbate (Singh et al., 2006). This shift in redox state arises on at least two levels (Foyer et al., 2011). Superoxide and the hydroxyl radical can directly oxidize both GSH and ascorbate. In this way, GSH and ascorbate act as nucleophilic scavengers. Alternatively, $H_2O_2$ can oxidize GSH and ascorbate through the action of specific peroxidases, or in the case of GSH, also through the action GRXs and GSH-S-transferases (GST). Like SOD and catalase, GST, GRX and/or peroxidase transcript or protein abundance, or enzymatic activity often increase in response to As exposure (Mylona et al., 1998; Stoeva et al., 2003; Srivastava et al., 2005; Geng et al., 2006; Abercrombie et al., 2008; Ahsan et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009). As an example, in rice, at least 10 GST genes are up-regulated in response to AsV exposure, while no more than two GST genes are down-regulated (Norton et al., 2008; Chakrabarty et al., 2009). Changes in GST gene expression do not seem to have as pronounced a role in the AsIII response, as fewer transcripts changed in abundance (Chakrabarty et al., 2009), potentially highlighting the differential effects of the two inorganic As forms on cellular metabolism. Important questions to be addressed are: Why are there changes in the isoforms of the various enzymes that are expressed? What are the energetic and metabolic costs and benefits of such shifts?

The second component of the two-component $H_2O_2$ neutralizing system is made up of monodehydroascorbate reductase, dehydroascorbate reductase, and GSH reductase. Together, these enzymes efficiently recycle oxidized GSH and ascorbate to allow further cycles of $H_2O_2$ reduction. The reduction of $H_2O_2$ through the interdependent ascorbate-GSH cycle requires reducing power in the form of NAD(P)H, diverting this energy from other metabolic processes. The enzymes involved in the recycling of oxidized GSH and ascorbate are also often induced upon exposure of plants to As (Ahsan et al., 2008; Khan et al., 2009). Thus, the interdependent ascorbate-GSH cycle, when it can be established, has an important role in maintaining ROS balance in plants (Foyer et al., 2011), probably even during As exposure. The reliance of the ascorbate-GSH cycle on the diversion of carbon to ascorbate biosynthesis, plus the diversion of carbon, nitrogen, and sulfur in the form of Glu, Cys, and Gly to support the biosynthesis of GSH and PC, requires a remodeling of metabolism to focus on the production of the precursors for these compounds.

The oligomerization of GSH to produce PC is also induced during As exposure (Schmöger et al., 2000; Geng et al., 2006; Singh et al., 2006; Khan et al., 2009). PC was found to have an important role in controlling As toxicity when the increased sensitivity of the cad1–3 Arabidopsis mutant to AsV was mapped to the disruption of the PC synthase gene (Ha et al., 1999). Conversely, increased PC synthase in Arabidopsis, Brassica juncea, and tobacco conferred increased As tolerance to these plants (Li et al., 2004; Gasic and Korban, 2007; Wojas et al., 2010). However, at higher levels of As, thiol metabolism was disrupted in some over-expressing lines (Wojas et al., 2010). Moreover, the synthesis of PC can deplete cellular GSH pools, decreasing the antioxidant capacity of the cell (De Vos et al., 1992; Sneller et al., 1999; Hartley-Whitaker et al., 2001b). These depleted GSH pools can only be rejuvenated by an influx of Glu, Cys, and Gly.

## DIRECT METABOLIC CONSEQUENCES OF ARSENIC

The ability of AsV to substitute for Pi, the propensity of AsIII-based compounds to bind to and change the activity of enzymes and the damaging effects of ROS all have direct and important consequences for plant metabolism. The need by the plant for sufficient metabolite-based compounds to combat the ROS produced by As exposure has more indirect, but no less important, consequences on metabolism. The plant response to these factors predominantly impacts on carbon, nitrogen, and sulfur metabolism. Much more information is needed at the biochemical and molecular levels to understand the full influence of As on these and other aspects of metabolism. For example, which effects are driven specifically by AsV and which are driven by AsIII, or perhaps even methylated-AsIII species? Which steps in metabolism are the most sensitive to the effects of As and why?

Caution is needed in assessing the literature on the effects of As on plant metabolism. The number of studies that examine the biochemical and molecular aspects of As toxicity in plants remains limited. It is also challenging to identify trends in the existing literature, and exceptions to those trends, due to the variability in experimental approaches. Experiments have been done using plants grown in soil, washed sands, artificial matrices and in hydroponics, with each approach uniquely influencing As phytoavailability. Additional experimental variables, such as plant size and nutritional status at the time of As supply, and the growth conditions and timing of sampling after supply would have also influenced the observed effects of As exposure on plant metabolism. The plant-dependent variation would then have been superimposed onto this landscape. Highlighting some of these issues, Wang et al. (2012) carried out a study in Pteris over a longer than average time span, using two As hyperaccumulators and two non-accumulators. They found that some As-dependent differences between plants in parameters such as quantum yield, chloroplast ATPase activity, and Rubisco activity were transitory, species specific and did not correlate with the plant classification as a hyperaccumulator or a non-hyperaccumulator. These transient effects may well be short-term stress responses initiated upon moving a plant from an As-free environment to an As-contaminated environment. Thus, this study illustrates the need to understand the kinetics of observed differences, and to avoid a reliance on single time points which may or may not reveal physiologically important differences in responses. Keeping these difficulties in mind, some clear trends do appear to emerge when examining the effects of As on plant metabolism.

## CARBON METABOLISM

A main effect of AsV on plant carbon metabolism is in stimulating the accumulation of ascorbate (Srivastava et al., 2005; Singh et al., 2006; Khan et al., 2009), presumably to bolster protection against ROS. The effects of As on primary carbon metabolism in plants is largely unknown. However, the transcriptional profiles of genes encoding proteins involved in carbon metabolism are largely unaffected both in *Arabidopsis* and rice (Abercrombie et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009). Proteomic studies in the non-hyperaccumulating plants rice and maize (Requejo and Tena, 2005, 2006; Ahsan et al., 2008, 2010) have shown that there are some changes in the abundance of proteins that participate in glycolysis and the citric acid cycle, but the changes are not consistent or systemic throughout either pathway. There are too few studies to fully understand the relevance of the changes that are observed. However, it appears that As does not have strong effects on gene expression related to carbon metabolism. This suggests that plant metabolism has sufficient plasticity to maintain adequate carbon flow without the need to adjust enzyme amounts in these central pathways. Robust assessment of the metabolite pools associated with primary metabolism is needed in plant tissues exposed to As to address this point directly.

At the level of carbon input into metabolism, AsV, AsIII, and MMA$^{III}$ application are all able to inhibit net photosynthesis (Porter and Sheridan, 1981; Marin et al., 1993; Stoeva and Bineva, 2003). The light harvesting apparatus can be affected, with decreases seen in chlorophyll content (Porter and Sheridan, 1981; Stoeva and Bineva, 2003; Singh et al., 2006; Rahman et al., 2007; Duman et al., 2010) and photosystem II activity (Stoeva and Bineva, 2003). These results are largely consistent across studies and suggest that As may reduce photosynthetic electron flow through the thylakoid membranes, decreasing the potential to produce ATP and NADPH, both of which are needed to fuel the carbon fixation reactions. Adding to this potential energy deficiency would be the likelihood of uncoupling thylakoid electron transport from ATP synthesis by the replacement of Pi by AsV in photophosphorylation (Avron and Jagendorf, 1959; Watling-Payne and Selwyn, 1974). On the other hand, chlorophyll can be used as a source of metabolic carbon when carbohydrate availability is low (Araújo et al., 2011). This then raises the question of whether chlorophyll degradation limits photosynthesis or whether limitations on the supply of photosynthetic carbon trigger chlorophyll degradation.

On the carbon fixation side of photosynthesis, the Rubisco large subunit content of rice leaves decreased with AsV treatment (Ahsan et al., 2010). The Rubisco large subunit is encoded by the plastid DNA (Bock, 2007). Therefore, the decreased abundance of this protein not only indicates that As interferes with carbon fixation capacity, but raises the question of whether As also interferes with chloroplast DNA gene expression. However, in contrast to the decrease in Rubisco large subunit amount in rice, Rubisco small subunit transcripts increased in AsV-treated *Arabidopsis* (Abercrombie et al., 2008). Whether the increased transcript abundance results in more Rubisco small subunit, or is a response to a decrease in active Rubisco is not yet known. AsIII inhibits the light activation of photosynthetic $CO_2$ fixation in isolated pea chloroplasts

(Marques and Anderson, 1986). The inhibition is at the level of the light activation of enzyme activities associated with the reductive pentose phosphate pathway. While the extent of the effects of As on photosynthetic carbon metabolism are not fully understood, it appears likely that the toxicant decreases the amount of carbon available to the plant through decreased $CO_2$ fixation.

Photorespiration is a prominent path of carbon flow in most plants and includes the activity of the lipoamide-containing GDC. The dithiol group of dihydrolipoamide is a well known target for AsIII binding in animals (Dixon, 1996). Addition of this co-factor to animal cells is able to ameliorate AsIII toxicity (Wang et al., 2011). In plants, lipoamide is found not only in GDC, but also in the four enzyme complexes mtPDC, ptPDC, OGDC, and BCOADC. All five lipoamide-containing complexes contain lipoamide dehydrogenase (LPD), an enzyme that catalyzes the transfer of electrons from the reduced dihydrolipoamide co-factor to NAD$^+$ as part of the enzymatic reaction cycle of the complex. Like the lipoamide co-factor, the plant LPD enzymes are inactivated by AsIII, but not AsV (Chen et al., 2010), presumably because of the binding of AsIII to the absolutely conserved dithiol present in LPD that takes part in the reaction cycle. Knock-out lines of *Arabidopsis* with decreased levels of mtLPD were more sensitive to AsV treatment and produced much higher levels of Gly when exposed to AsV than wild-type lines (Chen and Finnegan, unpublished), indicating that LPD is an important target for As toxicity in plants. LPD increased in amount in leaves of AsV-treated rice (Ahsan et al., 2010), perhaps in response to the inhibition of one or more of the LPD containing complexes.

Higher plants are likely to have photorespiratory pathways that are independent of the main GDC-dependent pathway (Peterhansel and Maurino, 2011). These alternative pathways are likely to come into play under various stress conditions. One of these pathways involves the non-enzymatic oxidative decarboxylation of glyoxylate to formate in the presence of $H_2O_2$ (Wingler et al., 1999). The formate can then be oxidized to $CO_2$ by NAD$^+$ formate dehydrogenase, which has been found among both plastid and mitochondrial proteomes (Heazlewood et al., 2007). The NADH that is produced is then available to donate electrons to the mitochondrial electron transport chain, linking formate oxidation to ATP production. While this pathway is not prominent under most conditions, it may become more important when GDC is inhibited (Wingler et al., 1999), such as during As exposure. Not only is it possible that this pathway may be driven by ROS produced by As exposure, but the NAD$^+$ formate dehydrogenase protein accumulates in leaves of rice exposed to AsV (Ahsan et al., 2010).

During daylight hours, a large proportion of newly fixed carbon is stored as starch. Starch hydrolysis to glucose, maltose, and malto-oligosaccharides, followed by phosphorylation of glucose by hexokinase is most likely the main path for the entry of glucose into glycolysis (Zeeman et al., 2004). In wheat, it has been found that AsIII, and to a lesser extent, AsV, are able to decrease the liberation of maltose from starch by inhibiting amylolytic activity (Liu et al., 2005). Phosphorolysis reactions where Pi is the attacking group to cleave a covalent bond may also contribute to the breakdown of starch, maltose, or malto-oligosaccharides (Zeeman et al., 2004), liberating glucose-1-phosphate. AsV can substitute for Pi in

this phosphorolysis reaction, yielding glucose-1-AsV that quickly hydrolyzes to glucose (Levi and Preiss, 1978). Before this free glucose can enter glycolysis, it would need to be phosphorylated by hexose kinase, at the expense of ATP, decreasing the energetic yield of glycolysis.

Glyceraldehyde-3-phosphate dehydrogenase has key roles in both photosynthesis and glycolysis. In AsV-treated rice, the amount of GAPDH protein decreased in roots and increased in shoots (Ahsan et al., 2008, 2010). The activity of the enzyme decreased with AsV treatment in non-As hyperaccumulator *Pteris* spp., remaining constant in two As hyperaccumulators (Wang et al., 2012). As discussed above, GAPDH may be an AsV reductase (Gregus and Németi, 2005). The activity of GAPDH in *Arabidopsis* was also inhibited in the presence of $H_2O_2$, suggesting that it is a direct target of $H_2O_2$ (Hancock et al., 2005). GAPDH is another example where AsV and Pi are alternative substrates. The 3-arsenoglycerate product rapidly hydrolyzes, uncoupling the GAPDH reaction (Dixon, 1996). In this way, AsV may be able to uncouple ATP synthesis from glycolysis, decreasing the energy yield of the pathway. The impact of AsV-mediated uncoupling of glycolysis through the potential production of unstable reaction products throughout the pathway has not been investigated *in vivo*. In this regard, the suppression by AsV of genes involved in the Pi starvation response in *Arabidopsis* and rice (Abercrombie et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009) may mean that the replacement of Pi by AsV may be enhanced by a repression in the ability of the plant to acquire Pi.

The increase in alcohol dehydrogenase transcripts in AsV-treated *Arabidopsis* (Abercrombie et al., 2008), indicating an increase in fermentative capacity, suggests that AsV blocks the flow of carbon from pyruvate into the citric acid cycle. The likely site of the block in plants, as in animal systems, is at mtPDC, another target of AsIII binding, as the enzyme complex contains both lipoamide and LPD. The citric acid cycle enzyme OGDC, like GDC and PDC, also contains lipoamide and LPD, making it susceptible to AsIII inhibition. This suggests that reduction of AsV to AsIII would also alter respiratory carbon flow at OGDC, an enzyme that may be rate limiting for respiration (Araújo et al., 2008), leading to changes in the 2-oxoglutarate pool and the pools of other metabolites linked to it. In roots of maize seedlings, AsV and AsIII exposure caused an increase in abundance of the succinyl-CoA synthetase α subunit (Requejo and Tena, 2005), the enzyme directly downstream of the OGDC complex in the citric acid cycle, as well as a mitochondrial ATP synthase subunit that is likely to be equivalent to the $F_A$d subunit described by Heazlewood et al. (2003). The genetic, physiological, or pharmacological disruption of the citric acid cycle or respiratory function generally caused moderate changes in several steady-state glycolytic and citric acid cycle metabolite pools (Araújo et al., 2008; Garmier et al., 2008; Meyer et al., 2009; Rocha et al., 2010; Sienkiewicz-Porzucek et al., 2010). The same might be expected of AsV-treated plants. However, metabolomic and detailed metabolic flux analyses are needed to determine directly if AsV treatment inhibits carbon flow through the citric acid cycle, and whether any restriction in carbon flow is due to decreased activity of enzymes in the cycle or to decreased entry of carbon into the cycle due to the inhibition of mtPDC.

The same trends for pyruvate and 2-oxoglutarate metabolism would be expected to operate in both roots and shoots of plants exposed to AsV. However, the effect in shoots is likely to be less intense due to the ability of roots to generally sequester As, lowering the As burden in the shoots (Chen et al., 2010). However, maize shoots exposed to inorganic As, in contrast to roots, lost malate dehydrogenase and the ATP synthase $F_A$d subunit (Requejo and Tena, 2006). These results suggest that the mitochondrial enzyme complement involved in carbon and energy metabolism in shoots may decline in response to As exposure. Such a loss may be a consequence of the oxidative stress caused by ROS generated during As exposure. The exposure of cultured heterotropic *Arabidopsis* cells to $H_2O_2$, a ROS produced in plants exposed to As, resulted in the breakdown of mtPDC E2 subunit, OGDC E2 subunit, succinyl-CoA synthetase β subunit, aconitase, malate dehydrogenase, and fumarase, as well as mitochondrial $F_1F_o$ ATP synthase α and β subunits and GABA aminotransferase (Sweetlove et al., 2002).

Dark respiration is inhibited in alfalfa by AsV exposure, but this process is more resistant to the toxicant than photosynthesis (Porter and Sheridan, 1981). Marin et al. (1993) also found that respiratory $O_2$ consumption was more resistant to AsV supply, in the form of $DMA^V$, than photosynthetic $O_2$ evolution in a study that did not differentiate photorespiration from mitochondrial respiration. The main effect of As on plant respiration may be the AsV-dependent uncoupling of ATP synthesis from electron transport (Wickes and Wiskich, 1975) that results from the synthesis of highly unstable ADP-AsV (Moore et al., 1983). The decrease in ATP synthesis brought about directly by the futile cycling of ADP through ADP-AsV and indirectly by the decrease in the proton motive force by the uncoupling reaction would be expected to decrease the energy status of the cell. The finding that the $K_M$ and $V_{max}$ of the mitochondrial $F_1F_o$ ATP synthase are remarkably similar for both Pi and AsV (Moore et al., 1983) indicates that uncoupling may well occur *in vivo*. Moreover, it is likely that glycolysis and the citric acid cycle would process substrates more rapidly in an attempt to maintain the proton gradient at a sufficient level. The overall expected outcome would be a cellular energy crisis.

Surprisingly, the alternative pathways of respiratory electron transport do not seem to be involved in the response of plant metabolism to As. The alternative pathways are non-phosphorylating alternative avenues for electron entry into and exit from the ubiquinone pool within the inner mitochondrial membrane (Finnegan et al., 2004; Millar et al., 2011). The alternative electron entry points are provided by a number of NAD(P)H dehydrogenases embedded on both sides of the mitochondrial inner membrane. These enzymes transfer electrons directly from NAD(P)H to ubiquinone. The alternative exit point is provided by the alternative oxidase (AOX), an enzyme that transfers electrons directly from ubiquinol to molecular $O_2$. Electron transfer through the alternative NAD(P)H dehydrogenases and the AOX is not coupled to proton translocation across the inner membrane, so these pathways do not contribute to the proton motive force and do not contribute to the synthesis of ATP (Finnegan et al., 2004; Millar et al., 2011).

Alternative oxidase gene expression is dramatically increased at the transcript and protein levels by various treatments that are generally considered to cause stress, especially oxidative stress

(Finnegan et al., 2004; Millar et al., 2011). Its role during stress is not fully understood, but it is believed to relate to an ability to decrease the production of ROS by mitochondria (Maxwell et al., 1999). Treatments that induce AOX transcript and protein amounts include a number that either involve the application of ROS or induce the production of ROS. Thus, it is surprising that As treatment, which dramatically induces ROS production, failed to induce AOX transcripts during microarray studies in *Arabidopsis* and rice (Abercrombie et al., 2008; Norton et al., 2008; Chakrabarty et al., 2009). It is also surprising that there do not seem to be any independent reports of AOX induction by As. The lack of AOX induction could either mean that the increase in ROS in these experiments was not sufficiently intense, or that the ROS generated by As treatment is sensed differently to the ROS induced by other treatments.

In *Arabidopsis*, there is strong co-expression of AOX with one particular alternative NAD(P)H dehydrogenase, NDB2 (Clifton et al., 2005), an enzyme that resides on the external face of the inner mitochondrial membrane with access to external NAD(P)H (Millar et al., 2011). The gene for this enzyme, like that for AOX, also seems to be non-responsive to As treatment. However, a homolog to the *Arabidopsis* NDA1 alternative NAD(P)H dehydrogenase was induced at the transcript level by both AsV and AsIII in rice seedlings (Chakrabarty et al., 2009). The *Arabidopsis* NDA1 protein sits on the inner face of the mitochondrial inner membrane, with access to matrix NAD(P)H (Millar et al., 2011) and is not strongly co-expressed with AOX. It therefore appears that the response to the ROS produced by As treatment may be perceived differently from the ROS produced by other treatments.

Plant carbon metabolism relies on efficient shuttling of molecules across cellular membranes. In AsV-treated rice seedlings, a triose-phosphate/Pi translocator gene was transcriptionally up-regulated (Chakrabarty et al., 2009). This protein would be expected to transport Pi and AsV across the plastid inner membrane in exchange for triose-phosphate. In AsV-treated *Arabidopsis*, transcript abundance for a mitochondrial substrate carrier protein identified as dicarboxylate carrier 2 (DIC2) by Palmieri et al. (2008) was repressed (Abercrombie et al., 2008). DIC2 is located in the inner mitochondrial membrane and is likely to exchange Pi, sulfate, or AsV for a number of dicarboxylates (Palmieri et al., 2008). Malate/oxaloacetate exchange catalyzed by DIC2, coupled with cytosolic and mitochondrial $NAD^+$-dependent malate dehydrogenase activities, allow the flow of redox equivalents from one compartment to the other (Palmieri et al., 2008). Thus, AsV interacting with DIC2 may have a negative impact on the redox balance between the mitochondrial matrix and the cytosol by inhibiting efficient malate/oxaloacetate exchange.

While decreases in carbon metabolism would have negative impacts on cellular energy flow and the production of biosynthetic intermediates, a decrease in leaf carbon metabolism may be an adaptive response to minimize As toxicity. The As hyperaccumulator *P. vittata* has a pronounced decrease in proteins associated with carbon metabolism in its ariel tissues when challenged with As. These proteins include enzymes involved in energy conversions (an organellar DNA-encoded subunit from each the chloroplast and mitochondrial $F_1F_o$ ATP synthase), carbon fixation (Rubisco large

and small subunits, seduheptulose-1,7-bisphosphatase) and carbohydrate metabolism (malate dehydrogenase, triose-phosphate isomerase, a subunit of pyruvate dehydrogenase; Bona et al., 2010). The As-tolerant monocotyledon *A. tenuis* also had decreased amounts of Rubisco large and small subunits and a subunit on the mitochondrial ATP synthase when exposed to AsV or AsIII (Duquesnoy et al., 2009). The possible value of such an adaptive response is not clear.

## NITROGEN METABOLISM

Biological nitrogen fixation, including the contribution made by symbioses in the root nodules of legumes, supplies a large proportion of the nitrogen in biological systems. The exposure of alfalfa root systems supporting well-established $N_2$-fixing symbioses with rhizobia to AsV demonstrated that symbiotic $N_2$ fixation is sensitive to As toxicity (Porter and Sheridan, 1981). Moreover, alfalfa either grown in As-contaminated soil or exposed to AsIII had less than half of the total number of root nodules formed in the absence of added As (Carrasco et al., 2005; Pajuelo et al., 2008). Under controlled conditions, this reduction was due to a 90% decrease in the number of rhizobial infections (Pajuelo et al., 2008). The use of an As-resistant strain of rhizobia in these experiments demonstrated that the decreased establishment of the symbiosis was not due to bacterial death. Rather, plant traits including root necrosis, root hair damage, and a shorter length of the root zone that was subject to infection were implicated (Pajuelo et al., 2008). Transcript analysis indicated that AsIII exposure interferes with the expression of genes involved in early nodule development (Lafuente et al., 2010). Together, these results suggest that As contamination of soil has the potential to strongly decrease $N_2$ fixation in ecosystems involving legume-rhizobium symbioses.

AsV also seems to disrupt N assimilation. Non-legumes obtain N from the soil predominantly as nitrate ($NO_3^-$) or ammonium ($NH_4^+$). Inorganic N in the form of $NH_4^+$, is assimilated by the combined action of glutamine synthase (GS) and glutamate synthase (GOGAT) for entry into the organic molecule pool as glutamate (Foyer et al., 2011). Roots of AsV-treated rice had decreased amounts of transcripts for a $NO_3^-$ transporter and for an $NH_4^+$ transporter (Norton et al., 2008). Another experiment using whole rice seedlings also found a decrease in transcripts for a different $NH_4^+$ transporter (Chakrabarty et al., 2009). The amount of nitrate reductase was repressed in whole seedlings of rice (Chakrabarty et al., 2009), but induced in whole seedlings of *Arabidopsis* (Abercrombie et al., 2008). Nitrate reductase, in combination with nitrite reductase, supplies $NH_4^+$ to GS for N assimilation. GS protein amount was lower in rice roots treated with AsV (Ahsan et al., 2008). Although it is not clear if it was the cytosolic or plastid isoform that was reduced, the chloroplastic form was sensitive to oxidative fragmentation by hydroxyl radical ($\bullet OH$; Ishida et al., 2002), a ROS species produced during AsV exposure. While the details are far from clear, it appears that AsV interferes with both the supply of inorganic N to the assimilation pathway and the activity of the pathway itself.

Arsenic exposure has been reported to cause dramatic changes in amino acid pools (Dwivedi et al., 2010; Pavlík M. et al., 2010). A key question is the extent to which the changes in the amounts of these amino acids is due to changes in amino acid biosynthesis or

changes to protein metabolism. Exposure to As caused decreases in total plant protein abundance in *P. ensiformis* and *P. vittata* (Singh et al., 2006), in total shoot protein abundance in red clover (Mascher et al., 2002) and in soluble protein in maize (Stoeva et al., 2003). Proteomic studies demonstrated that Rubisco, with its high capacity due to its great abundance to store N in the form of amino acids, can be targeted for destruction in AsV treated plants (Duquesnoy et al., 2009; Ahsan et al., 2010; Bona et al., 2010). In *Lemna minor*, total protein increased at low AsV supply, but decreased at high As supply (Duman et al., 2010), a relationship that may be linked to the stimulation of growth at low As supply that has been observed for numerous plants (Woolson et al., 1971; Carbonell-Barrachina et al., 1997, 1998b; Miteva, 2002; Chen et al., 2010). Protein degradation has been recognized as an important source of respiratory carbon when carbohydrate levels are low (Araújo et al., 2011). Thus, the lower protein abundance that generally accompanies As exposure, coupled with a likely As-induced decrease in carbohydrate metabolism that would hinder the biosynthesis of amino acids, suggests that any changes in the size of amino acid pools would be due to amino acids flowing from protein degradation.

A number of genes involved in N transport appear to change expression in response to As. Amino acid transporters were down-regulated in response to AsV in roots and seedlings of rice (Norton et al., 2008; Chakrabarty et al., 2009). Interestingly, amino acid transporter gene transcript levels were not influenced by AsIII (Chakrabarty et al., 2009), suggesting an area where the different modes of action of these compounds are manifested. Peptide and oligopeptide transporters have also been reported to be AsV responsive in rice, but reports disagree on the direction (Norton et al., 2008; Chakrabarty et al., 2009). Clearly, more work is needed in understanding the movements of N-containing compounds through the plant tissues during As exposure.

## SULFUR METABOLISM

The central role played by the binding of AsIII to sulfhydryl groups in GSH and PC in the detoxification of the metalloid indicates a critical importance for sulfur metabolism in determining plant survival in As-contaminated soils. The biosynthesis of GSH and PC that is typically induced by As exposure requires adequate supplies of the GSH-building blocks Glu, Cys, and Gly. In both shoots and roots of wild-type *Arabidopsis*, the mass ratios of free Glu:Gly:Cys were about 20:3:1 (Muñoz-Bertomeu et al., 2009). Thus, at least in *Arabidopsis*, Cys is by far the limiting substrate for GSH biosynthesis. Plants that over-express enzymes involved in GSH and PC biosynthesis have higher levels of non-protein thiols than wild-type lines (Guo et al., 2008b). However, other studies indicate that AsV exposure can decrease cellular Cys pools (Sung et al., 2009) and that under some growth conditions it is possible that the synthesis of PC can deplete GSH pools, decreasing the antioxidant capacity of the cell (De Vos et al., 1992; Sneller et al., 1999; Hartley-Whitaker et al., 2001b). These observations, combined with the possible limiting availability of Cys, suggests that increased Cys biosynthesis to support GSH and PC production would add to the effectiveness of approaches designed to increase non-protein thiols within plants, a process that would also require inputs from sulfur metabolism.

The first step that would be necessary to support increased biosynthesis of GSH and PC is the acquisition of sulfur from the soil. The main form of sulfur available to plants is sulfate. In AsV-treated rice, up to five sulfate transporter genes are up-regulated in roots (Norton et al., 2008), and at least one sulfate transporter is up-regulated in *Arabidopsis* (Sung et al., 2009). AsIII also induces a sulfate transporter gene in rice and *B. juncea* seedlings (Chakrabarty et al., 2009; Srivastava et al., 2009). It is not yet clear whether AsV and AsIII affect the expression of these transporters equally, although at least one of the transporter genes is induced by both forms of As (Chakrabarty et al., 2009). The up-regulation of this small number of transporters may be enough to move sulfate from the soil solution throughout the plant (Takahashi et al., 2011). The efflux of sulfate from cells that is required for transport to the tissues is likely to be down a concentration gradient and powered by the positive-outside membrane potential of the plasma membrane (Takahashi et al., 2011).

Before sulfate acquired from the soil can be used for the biosynthesis of Cys, and thus the biosynthesis of GSH and PC, it must be reduced via sulfite to sulfide (Takahashi et al., 2011). The reduction of sulfate to sulfite is a two step pathway. The second step is catalyzed by 5′-adenylylsulfate reductase. Transcripts of a 5′-adenylylsulfate reductase gene were elevated in *Arabidopsis* in response to AsV supply (Abercrombie et al., 2008), suggesting that the sulfate assimilation pathway is induced by AsV in plants as it is by AsIII in yeast (Thorsen et al., 2007). The 5′-adenylylsulfate reductase reaction uses GSH as a reductant. It would be expected that AsV exposure would lead to a lowering of GSH availability as it is diverted to As detoxification, with unknown consequences to the sulfate reduction pathway.

Once sulfate is reduced to sulfide, the sulfide is combined with *O*-acetylserine to form Cys in a reaction catalyzed by *O*-acetylserine (thiol)-lyase (OAS-TL), also known as Cys synthase. The *O*-acetylserine is formed from Ser in a reaction catalyzed by Ser acetyltransferase. OAS-TL exists either as a free active homodimer or in association with Ser acetyltransferase as an inactive subunit of the Cys synthase complex (Takahashi et al., 2011). It appears that AsV and AsIII exposure may cause a down-regulation of OAS-TL in As-sensitive plants. OAS-TL protein disappeared from maize shoots exposed to As (Requejo and Tena, 2006), while OAS-TL activity was repressed in an As-sensitive line of *B. juncea* (Srivastava et al., 2009). Thus, it seems likely that Cys biosynthesis, and therefore, As detoxification through GSH and PC, would be compromised. Indeed, the Cys and GSH levels in an As-sensitive variety of *B. juncea* were lower upon As exposure than in the untreated control (Srivastava et al., 2009). In contrast, a *B. juncea* variety with increased tolerance to As showed a more general induction of the sulfate assimilation and GSH biosynthetic pathways (Srivastava et al., 2009), similar to that seen in yeast (Thorsen et al., 2007). In the As-tolerant variety, there was an induction in Cys synthase activity, as well as in the activities of Ser acetyltransferase and γ-glutamylcysteine synthetase, the penultimate enzyme in the biosynthesis of GSH. These increases in enzyme activity were accompanied by increased levels of both Cys and GSH, indicating that increased sulfur metabolism may be a viable mechanism for increasing As tolerance in plants. *Arabidopsis* may behave similarly, with transcripts for a γ-glutamylcysteine synthetase, GSH

synthetase, and PC synthase being induced during As exposure (Sung et al., 2009). While GHS levels in *Arabidopsis* increased as they did in the As-tolerant variety of *B. juncea*, the PC levels declined (Sung et al., 2009).

In rice, several methyltransferase genes are induced by AsV-treatment (Norton et al., 2008). Two of these are homocysteine S-methyltransferases, which catalyze the formation of *S*-adenosyl-ʟ-homocysteine and Met from *S*-adenosylmethionine and ʟ-homocysteine. The enzyme is involved in the synthesis of *S*-methylmethionine (Ranocha et al., 2001), and may play a role in maintaining a pool of soluble Met, in the cycling of methyl groups within cells, or as a phloem-mobile form of Met that can be used to translocate sulfur derived from protein degradation (Bürstenbinder and Sauter, 2012). In the context of the As response, it is tempting to speculate that the translocation of Met as *S*-methylmethionine from remote sites of protein degradation (i.e., the leaves), aided by the action of homocysteine S-methyltransferases, can be used to increase the availability of Cys at sites where GSH biosynthesis is required for the binding of AsIII (i.e., the roots).

## CONCLUSIONS AND PERSPECTIVES

Much excellent work has been done on the metabolism of As in plants: how it is acquired and moved through the plant; how it is reduced, detoxified, and sequestered; how it mimics Pi, binds sulfhydryl groups, and causes oxidative stress. We have a fairly firm grasp of the mechanism used by As hyperaccumulators to accumulate large amounts of the toxicant without poisoning. These plants take up the metalloid more quickly than non-hyperaccumulators, do not sequester it in the root, but rather transport it quickly to the aerial tissues where it is sequestered in the vacuole as AsIII. The rapid rate of uptake and translocation to the frond and a higher antioxidant capacity to maintain lower ROS levels (Cao et al., 2004; Zhao et al., 2009), perhaps coupled with relatively rapid dilution in the bulk of the aerial tissues, together seem to provide the hyperaccumulators with adequate time and resources to neutralize the toxic effects of As. An interesting question that remains is how the cells in As-hyperaccumulators are physically capable of keeping AsIII away from vital metabolic targets during the translocation and sequestration process? Or is the As translocation rate so rapid that intracellular concentrations of biologically active AsIII are never high enough to exert a negative effect?

Despite a firm knowledge of the interactions between plant cells and As, we still do not have a good understanding of the exact nature of why As is toxic: Which combination of mechanisms for toxicity, Pi replacement, sulfhydryl binding, or ROS production, is the most damaging in the short and long terms to plant growth and productivity? Which parts of plant metabolism are most vulnerable to As toxicity and why? What are the most critical molecular targets for As and can we do anything to protect these targets through breeding or direct engineering?

An interesting observation that has not been explored in any detail is why does As at low concentrations stimulate growth? This trait is not due to As interacting with biotic factors other than the plant itself, as it is seen in both axenic and non-axenic plant cultures. Instead, the trait must be due to the interaction of As with plant nutrition, but the mechanism is unknown. Is this growth stimulation sustainable throughout the life of the plant? Can it give rise to higher seed production as well as plant biomass? Are there ways of harnessing this stimulation? Can we develop crops, i.e., grains, where the increase in biomass can be converted into increased yield where the product (i.e., seed) is free of As contamination? The mechanistic answers to these questions no doubt lie at the interface between As and plant metabolism. It is interesting to speculate on possible mechanisms: stimulation of metabolic flux by uncoupling metabolism from energy production, uncoupling Pi status from growth by disrupting Pi allocation patterns, repression/inactivation of a growth-regulating protein, and the promotion of ROS-dependent signaling are just four among many possibilities.

Central to many of these open questions is the form of As that is most detrimental to plant health. It is agreed that AsIII is more toxic than AsV. However, is it AsIII that actually targets plant growth, or is it perhaps a metabolic product of AsIII? Two issues are currently central to addressing this question. Firstly, can plants transform inorganic species into organic species? Recent studies cited above indicate not, but do indicate that plants can reduce methylated-As[V] species to more reactive methylated-As[III] species. To date, it has been fairly difficult to detect methylated-As species in plants, raising the question of whether these species are really very important determinants of As toxicity. On the other hand, a critical question relevant to this judgment is the nature of the As species that may have avoided detection because of technical limitations or biological reactivity. In this regard, the efficiency of As recovery during speciation studies needs to be considered. In all studies where a comment has been made, a significant part of the total As burden of the plant has not been recovered. The question of the form of the unrecovered As, then, becomes important, because some relatively rare As species may be highly reactive toward equally rare, but critical, cellular components. An extreme scenario would be a critical zinc-finger transcription factor active in the relatively few cells of root meristems.

The combination of detailed physiological and biochemical studies will continue to give us great insights into the mode of action of As in plants. The recent addition of global transcript analyses and proteomics approaches has added important new dimensions to our understanding of plant responses to As exposure. The combination of these methods with ever more sensitive and informative physical and biochemical assays and transcriptome and proteome analyses are likely to provide answers to some of the critical questions raised here.

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