Running head: Plastidial LPD mutations cause arsenate hypersensitivity

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Title: Disruption of *ptLPD1* or *ptLPD2*, genes that encode isoforms of the plastidial lipoamide dehydrogenase, confers arsenate hypersensitivity in *Arabidopsis thaliana*

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ABSTRACT

Arsenic is a ubiquitous environmental poison that inhibits root elongation and seed germination to a variable extent depending on the plant species. To understand the molecular mechanisms of arsenic resistance, a genetic screen was developed to isolate arsenate overly-sensitive (aos) mutants from an activation-tagged Arabidopsis (Arabidopsis thaliana) population. Three aos mutants were isolated and the phenotype of each was demonstrated to be due to an identical disruption of plastidial lipoamide dehydrogenase 1 (ptLPD1), a gene that encodes one of the two E3 isoforms found in the plastidial pyruvate dehydrogenase complex. In the presence of arsenate, ptld1-1 plants exhibited reduced root and shoot growth and enhanced anthocyanin accumulation compared with wild-type plants. The ptld1-1 plants accumulated the same amount of arsenic as wild-type plants, indicating that the aos phenotype was not due to increased arsenate in the tissues, but to an increase in the innate sensitivity to the poison. Interestingly, a ptld1-4 knockdown allele produced a partial aos phenotype. Two loss-of-function alleles of ptLPD2 in Arabidopsis also caused elevated arsenate sensitivity, but the sensitivity was less pronounced than for the ptld1 mutants. Moreover, both the ptld1 and ptld2 mutants were more sensitive to arsenite than wild-type plants, and the LPD activity in isolated chloroplasts from wild-type plants was sensitive to arsenite, but not arsenate. These findings show that the ptLPD isoforms are critical in vivo determinants of arsenite-mediated arsenic sensitivity in Arabidopsis and possible strategic targets for increasing arsenic tolerance.
INTRODUCTION

Arsenic (As) is a naturally occurring metalloid found in soil, water, and air, but anthropogenic activities, including smelting and fossil fuel combustion, have led to increased environmental exposure (Mandal and Suzuki, 2002). In the environment, As exists in both organic and inorganic forms. Arsenate (As(V)) is the principle inorganic form of As in aerobic soils, while arsenite (As(III)) is the main form found under anaerobic conditions (Marin et al., 1993; Onken and Hossner, 1995, 1996; Mandal and Suzuki, 2002; Masscheleyn et al., 2002).

Both As(V) and As(III) are toxic to plants, inducing symptoms ranging from poor seed germination and inhibited root growth through to death (Meharg and Hartley-Whitaker, 2002; Lee et al., 2003; Ahsan et al., 2008; Smith et al., 2009). The modes of action of As(V) and As(III) differ, owing to their distinct chemical properties. As(V), with its structural similarity to phosphate, can compete with phosphate in oxidative phosphorylation, leading to the production of adenosine-5’-diphosphate (ADP)-As(V) (Gresser, 1981). However, half-maximal stimulation of ADP-As(V) formation requires physiologically unlikely concentrations of approx. 0.8 mM As(V) (Moore et al., 1983). As(V) has been recently shown to enhance membrane fluidity, and thus membrane permeability, by binding and replacing phosphate- or choline-head groups (Tuan et al., 2008). The resulting damage to the membrane would disrupt the transport of mineral nutrients and water (Smith et al., 2009). As(V) can be promptly reduced in plants, including Arabidopsis, to As(III) by endogenous arsenate reductases, so that often more than 90 % of As in plant cells is in the form of As(III) (Zhao et al., 2009). As(III) readily forms covalent bonds with sulfhydryl (-SH) groups, especially vicinal dithiols. Binding to the free thiols of proteins is believed to be the basis of As(III) toxicity, either by inhibiting activity directly, or by disrupting protein structure. Many enzymes have been proposed to be targets leading to As(III) toxicity, and the As(III) sensitivity of some of these enzymes has been investigated in non-plant systems (Adamson and Stevenson, 1981; Cavigelli et al., 1996; Lynn et al., 1997; Hu et al., 1998; Kitchin and Wallace, 2008). Of the many potential protein targets, only the pyruvate dehydrogenase complex (PDC) has been shown to be inactivated by physiologically relevant micromolar concentrations of As(III) (Hu et al., 1998), suggesting that PDC may be the primary target for
As(III)-mediated cytotoxicity. However, little is known about the mechanism of arsenic toxicity in vivo, especially in plants.

Although As is phytotoxic, some plants species are resistant to high levels of As through avoidance mechanisms, while species of the Pteridaceae family of ferns hyperaccumulate arsenic without toxic effects (Verbruggen et al., 2009; Zhao et al., 2009). As an analogue of phosphate, As(V) is readily taken up by plants through high-affinity phosphate transporters encoded by the PHT1 gene family (Shin et al., 2004; González et al., 2005; Catarecha et al., 2007). Except for the hyperaccumulating ferns, avoidance of As toxicity by resistant species is often accomplished by a decrease in phosphate uptake activity (Meharg and Hartley-Whitaker, 2002). Unlike As(V), the transport of As(III) is facilitated by aquaporin nodulin 26-like intrinsic proteins (Bienert et al., 2008; Isayenkov and Maathuis, 2008; Ma et al., 2008; Kamiya et al., 2009). In roots and fronds of hyperaccumulating ferns, As(III) is sequestered in the vacuole (Lombi et al., 2002; Pickering et al., 2006). Much of the As(III) taken up by non-accumulating resistant species may be released back to the rhizosphere through an undefined efflux pathway (Zhao et al., 2009). As(III) that remains in tissues reacts with thiol-containing molecules, such as glutathione or phytochelatins, both of which are usually produced in greater abundance in response to As (Grill et al., 1987; Sneller et al., 1999; Schmöger et al., 2000; Schulz et al., 2008). As(III)-glutathione adducts can be sequestered in the vacuole (Dhankher et al., 2002; Bleeker et al., 2006). However, increased synthesis of glutathione or phytochelatins alone is unlikely to confer a very high level of tolerance (Zhao et al., 2009).

To identify genes essential for As resistance in plants, we used a genetic screen to identify mutants of Arabidopsis (Arabidopsis thaliana) that were hypersensitive to As(V). The screen was analogous to that used to isolate the salt overly-sensitive (sos) mutants of Arabidopsis (Wu et al., 1996) that led to the identification of the SOS pathway for salt tolerance (Zhu, 2000, 2003). Our hypothesis was that arsenate overly-sensitive (aos) mutants would reveal a different set of genes from those identified in mutants showing increased resistance to As(V).
RESULTS

Isolation of arsenate overly-sensitive mutants of Arabidopsis

Plants exposed to As(V) suffer a reduction in root and shoot growth. Arabidopsis is sensitive to moderate to high concentrations of As(V) (Quaghebeur and Rengel, 2004) and inhibition of root elongation is one of the most conspicuous developmental changes that occur during short-term exposure. We adapted the root-bending assay developed for isolating Arabidopsis mutants that are sensitive to toxic cations (Howden and Cobbett, 1992; Wu et al., 1996) to identify arsenate overly-sensitive (aos) mutants. The optimal concentration of As(V) for isolating aos mutants was determined by exposing 5-d-old wild-type (WT) Arabidopsis seedlings of uniform size to various As(V) concentrations in solid growth medium. Plates were placed so that seedlings were vertically orientated with the root tip pointing upward. During root elongation, gravitropism caused the roots to bend downward, allowing the increase in root length since exposure to As(V) to be readily visualized and measured. Exposure to higher As(V) concentrations generally caused a stronger inhibition of root elongation (Suplemenal Fig. S1A). The only exception was a stimulation of root elongation observed at 100 µM As(V), the lowest concentration tested. As(V) at 200 µM was the highest concentration tested that did not strongly inhibit WT root growth and was chosen for isolating aos mutants. It was expected that mutants could be recovered from the screen, because WT seedlings could be rescued after exposure to 1 mM As(V) for five days (data not shown).

Approximately 80,000 Arabidopsis seedlings representing a random selection of 40,000 activation-tagged M3 lines in the Columbia 2 (Col-2) background were screened for an aos phenotype. About 350 putative mutants were identified (Supplemental Fig. S1B), rescued and grown to seed. During re-screening of progeny from each putative mutant, only three lines, 106, 107, and 116, were confirmed to have an aos phenotype. The aos phenotype of these three mutants was transmitted into both M5 and M6 generations, indicating that the phenotype was genetically stable.
The phenotype of each aos mutant was characterized more fully by growing mutant and WT (Col-2) seedlings side-by-side on solid medium containing a range of As(V) concentrations. In the absence of As(V), the root growth of each mutant was similar to that of WT seedlings (Fig. 1, Supplemental Fig. S1C). Exposure to As(V) for four days caused a concentration-dependent inhibition of root growth in WT seedlings. At each As(V) concentration tested, root elongation for each mutant was inhibited similarly, but much more severely, than for WT seedlings (Fig. 1, Supplemental Fig. S1C). The As(V) concentration that inhibited root elongation by 50 % ($I_{50}$) compared with growth in the absence of As(V) was estimated roughly by examining the data in Figure 1 to be about 500 µM for WT seedlings and about 100 µM for each mutant.

**Genetic characterization of the aos mutants**

Each aos mutant was crossed with a WT plant to determine the pattern of aos inheritance. None of the F1 progeny had the aos phenotype (Supplemental Table S1), demonstrating that the phenotype was recessive in each mutant. The recessive nature of the mutation indicated that the aos phenotype arose from a loss-of-function mutation caused by the T-DNA insertion per se, rather than a gain-of-function mutation caused by activation of a near-by gene by the enhancer elements present in the T-DNA. The mutant phenotype segregated in a 1 : 3 (aos : WT) ratio in the progeny from selfed F1 plants from all three crosses, indicating the involvement of a single locus inherited according to Mendelian principles. Allelism among the three mutants was tested by crossing a homozygous mutant 106 plant with plants homozygous for the other two aos mutations. No phenotypic complementation was seen in the F1 progeny (Supplemental Table S1), indicating that the three aos mutations were allelic. One of the arsenate-sensitive F2 progeny from the mutant 107 x WT cross was named ptlpd1-1 based on its molecular characteristics (described below). This line was used for subsequent experimentation.

*ptlpd1* conferred enhanced As(V) sensitivity at whole plant level and at germination
Exposure of *ptlpd1-1* seedlings to As(V) for 12 days resulted in plants that were much smaller than WT plants (data not shown). When exposed to 100 µM As(V) in the growth medium, the fresh weight of *ptlpd1-1* seedlings was 63 % lower than that of WT seedlings whose growth was unaffected (Fig. 2A). In the presence of 200 µM As(V), the fresh weight of WT seedlings was 30 % lower than seedlings grown in the absence of As(V), while that of *ptlpd1-1* seedlings was 77 % lower. The *ptlpd1-1* seedlings also accumulated more anthocyanin in the shoot than WT seedlings (Fig. 2B). WT plants showed no change in anthocyanin concentration when exposed to 100 µM As(V), while the concentration in *ptlpd1-1* seedlings increased 3-fold. The *ptlpd1-1* seedlings had a higher shoot-to-root fresh-weight ratio when exposed to As(V) than WT seedlings (Fig. 2C), indicating a shift in resource allocation toward shoot growth.

Seeds from *ptlpd1-1* plants showed a significantly lower germination rate, scored as percentage cotyledon emergence (Lee et al., 2003), than seeds from WT plants when the medium contained either 100 or 200 µM As(V) (Fig. 2D). At both As(V) concentrations, the *ptlpd1-1* germinants had shorter roots and smaller shoots than WT germinants (results not shown). Germination of both *ptlpd1-1* and WT seeds was nearly completely inhibited by 400 µM As(V) in the medium.

**Molecular identification of the genetic lesion in the aos mutants**

The T-DNA insertion site in each mutant was localized by amplifying the genomic DNA sequences adjacent to the left border of the T-DNA insertion by Thermal Asymmetrical Interlaced (TAIL)-PCR (Liu et al., 1995). Two PCR products were obtained from mutants 107 and 116, while three products were detected for mutant 106 (data not shown). Among these TAIL-PCR products was a 900 bp amplicon common to all three mutants. Moreover, the three products obtained from mutant 106 were a combination of those obtained from mutants 107 and 116. DNA sequencing showed that the various TAIL-PCR products represented three unique T-DNA integration sites (Supplemental Fig. S2). The TAIL-PCR product common to all three lines corresponded to a T-DNA insertion precisely at the same location within the At3g17250 / At3g17260 intergenic region (Supplemental Fig. S2). The
presence of these T-DNA insertions was confirmed in each mutant by PCR using gene-specific primers in combination with either gene-specific primers expected to anneal on the far side of the T-DNA or a primer specific for the left border of the T-DNA (Supplemental Fig. S2). The absence of WT amplicons from the mutant genomic DNA templates indicated further that the T-DNA insertions were in the homozygous state in the three mutants.

The common insertion in the At3g17250 / At3g17260 intergenic region in the three aos mutants indicated that this lesion was responsible for the aos phenotype. To confirm this conclusion, plants carrying T-DNA insertions only at this locus were isolated from the F2 progeny of crosses between WT Col-2 plants and mutants 107 and 116. Only seedlings homozygous for the T-DNA insertion found in the At3g17250 / At3g17260 intergenic region had the aos phenotype, establishing genetic linkage. Linkage was verified by co-segregation analysis, where all sixty aos F2 progeny obtained from the WT x mutant 107 cross were found by PCR to be homozygous for the T-DNA insertion in the At3g17250 / At3g17260 intergenic region (data not shown).

The T-DNA insertion responsible for the aos phenotype was located 1.4 kb upstream of the annotated start codon of At3g17250 (Supplemental Fig. S2), suggesting that the insertion may interrupt transcription of this gene. At3g17250 encodes a PP2C-type protein phosphatase, a class of enzyme implicated in plant stress signalling (Schweighofer et al., 2004). However, no differences in At3g17250 transcript abundance were observed by semi-quantitative reverse-transcription (RT-) PCR between WT and mutant plants (data not shown). Additionally, plants carrying At3g17250 T-DNA insertional alleles had similar sensitivity to As(V) as WT plants (data not shown), further suggesting that the aos phenotype of ptlpd1-1 was not due to disruption of At3g17250 expression.

The possibility that the T-DNA insertion caused a chromosomal rearrangement (Nacry et al., 1998; Laufs et al., 1999; Tax and Vernon, 2001) was tested by TAIL-PCR. The primers used, T1 and T2, were specific for sequences in the At3g17250 / At3g17260 intergenic region immediately adjacent to the putative right border (RB) of the T-DNA and pointing toward the insertion site (Fig. 3A). DNA sequencing of the single 1.0 kb TAIL-PCR product showed that
a DNA rearrangement had occurred that linked the upstream region of At3g17250 directly to sequences internal to At3g16950 (Fig. 3A). PCR using primers specific for At3g16950 in combination with T-DNA specific primers revealed the presence of at least two T-DNA insertions in a head-to-head orientation (Fig. 3A and B) and that a 107 kb inversion of genomic DNA had occurred during the T-DNA integration event. RT-PCR analysis showed that ptldp1-1 lacked At3g16950 transcripts (Fig. 3C). Taken together, these results suggest that the interruption of At3g16950 was responsible for the aos phenotype. At3g16950 is the plastidial lipoamide dehydrogenase 1 (ptLPD1) gene, encoding an E3 subunit of the plastidial pyruvate dehydrogenase complex (PDC).

Confirmation that the aos phenotype is due to mutation of ptLPD1

To confirm that the disruption of ptLPD1 caused the increased arsenate sensitivity of the ptldp1-1 mutant, we determined the arsenate sensitivity of three T-DNA insertional mutants obtained from the publicly available stocks (Arabidopsis Biological Resource Center). These lines were WiscDsLox388B02 (designated ptlpd1-2), WiscDsLox468E10 (designated ptlpd1-3) and SALK_092097C (designated ptlpd1-4). The ptlpd1-4 mutant contained an insertion in the 5’ untranslated region (UTR) of ptLPD1, while the other two lines contained insertions within the exons of ptLPD1 (Fig. 4A). Homozygous mutants were isolated from a segregating population of each insertion line. In the absence of As(V), the phenotype of seedlings carrying any one of these alleles was indistinguishable from that of the corresponding WT (Col-0) seedlings. When grown in the presence of 200 μM As(V), the mutants showed the aos phenotype of compromised root elongation (Fig. 4B; Supplemental Fig. S3A). Root elongation in ptlpd1-2 and ptlpd1-3 seedlings was as sensitive to As(V) as that of ptlpd1-1 seedlings, while that of ptlpd1-4 seedlings was intermediate between WT and the other mutants. To understand the intermediate nature of the ptlpd1-4 phenotype, the abundance of ptLPD1 transcripts was determined in ptlpd1-2, ptlpd1-3, ptlpd1-4, and WT seedlings. The ptLPD1 transcripts in ptlpd1-2 or ptlpd1-3 seedlings were greatly reduced (Fig. 4C) compared to WT seedlings. These results, combined with the location of the T-DNA insert in the middle of the ptLPD ORF in both mutants and their phenotypic similarities to
ptlpd1-1, indicated that ptlpd1-2 and ptlpd1-3 are loss-of-function alleles. In ptlpd1-4 seedlings, the ptLPD1 transcript abundance was intermediate between that of WT and the other mutant seedlings, indicating that the T-DNA insert in the 5'UTR of the ptLPD1 is only partially inhibitory.

Like ptlpd1-1 seedlings, ptlpd1-2, ptlpd1-3 and ptlpd1-4 seedlings exposed to As(V) were smaller (Supplemental Fig. S3B), had lower fresh weight (Supplemental Fig. S3C), increased shoot anthocyanin concentration (Supplemental Fig. S3D) and increased shoot-to-root fresh-weight ratio (Supplemental Fig. S3E) compared with WT seedlings. In addition, ptlpd1-2, ptlpd1-3 and ptlpd1-4 seeds showed decreased germination rates relative to WT at all but the highest As(V) concentration tested (Supplemental Fig. S3F). In all these experiments, the ptlpd1-4 mutant phenotype was intermediate between WT and the knockout mutants, consistent with the view that ptlpd1-4 is a knockdown allele. Taken together, these results suggest that ptLPD expression quantitatively controls Arabidopsis As(V) sensitivity.

To verify that the enhanced arsenate sensitivity is due the disruption of the ptLPD1 gene, we crossed homozygous ptlpd1-1 with ptlpd1-2 and ptlpd1-3. The F1 progeny of these crosses showed decreased root growth similar to each homozygous mutant (data not shown). This result confirms that the mutations in all three lines are allelic. Moreover, the aos phenotype of ptlpd1-1 was functionally complemented by transforming the mutant with a 5464 bp genomic DNA fragment that included the WT ptLPD1 gene (Supplemental Fig. S4). These data provided further evidence that the enhanced arsenate sensitivity of ptlpd1-1 was due to disruption of ptLPD1.

The ptlpd1-1 allele does not enhance arsenic accumulation

One possible mechanism for the enhanced As(V) sensitivity of ptlpd1 seedlings is an increased accumulation of As in the tissues. The As concentrations in both shoots and roots of WT and ptlpd1-1 plants exposed to 50 μM As(V) for 3 d did not differ significantly (Fig. 5, A and B), indicating that the aos phenotype conferred by ptlpd1-1 was not due to enhanced As accumulation. Another mechanism for increased As(V) sensitivity could be alteration of the
phosphate status of the mutant (Lee et al., 2003). As(V), as an analog of phosphate, can compete with phosphate for uptake (Asher and Reay, 1979; Meharg and Macnair, 1992; Clark et al., 2000) and can replace it in some biochemical reactions (Hughes, 2002; Tseng, 2004). The total phosphate concentrations in shoot and root tissues of the *ptlpd1-1* mutant in both the presence and absence of As(V) were similar to those of WT plants (data not shown), indicating that the increased As(V) sensitivity in the *ptlpd1-1* mutants was not due to a change in tissue phosphate concentration.

**ptlpd1 mutants was also sensitive to As(III)**

To investigate whether the *aos* phenotype is As(V) specific, the sensitivity to As(III) of seedlings carrying various *ptlpd1* alleles was determined using the root-bending assay. Col-0 was used as the WT control, as there was no difference between Col-0 and Col-2 in response to As(III) (data not shown). Root elongation in WT Arabidopsis was inhibited by As(III) with the *I*₅₀ roughly estimated from the data on Figure 6 A to be 5 - 10 μM. The *ptlpd1* alleles caused a dramatic increase in the sensitivity of root elongation to As(III) (Fig. 6A), with an *I*₅₀ for As(III) of < 5 μM for the knockout alleles. As(III) caused a slight decrease in shoot-to-root fresh-weight ratio in WT seedlings (Fig. 6B). In contrast, seedlings carrying *ptlpd1* alleles had an increased shoot-to-root fresh-weight ratio when exposed to As(III) (Fig. 6B). As was the case for As(V), *ptlpd1-4* seedlings showed a response to As(III) that was intermediate between WT and the *ptlpd1* knockout alleles (Fig. 6, A and B).

The specificity of the *aos* phenotype to As anions was examined by exposing WT and *ptlpd1-1* mutant seedlings to various concentrations of Cd²⁺, Zn²⁺, Ni²⁺, and Cu²⁺. After four-days exposure, the root elongation of WT and *ptlpd1-1* mutant seedlings was similar (results not shown), indicating that the *aos* phenotype was specific for As anions and that mutation of *ptLPD1* did not affect the sensitivity of Arabidopsis to divalent heavy metal cations in the growth medium.

**ptlpd2 mutants also demonstrate an aos phenotype**
There are two ptLPD genes in the Arabidopsis genome. Having found that loss of ptLPD1 function caused increased sensitivity to As(V) and As(III), the relationship between LPD and As was further explored in two Arabidopsis lines where the second gene, ptLPD2 (At4g16155), was disrupted by T-DNA insertion. The T-DNA insertions in the alleles designated ptlpd2-1 (SALK_013426) and ptlpd2-2 (SALK_118337C) were located in the first and third exon of ptLPD2, respectively (Fig. 7A). There were no detectable ptLPD2 transcripts in seedling homozygous for either ptLPD2 allele (Fig. 7B), indicating that both ptlpd2-1 and ptlpd2-2 were knockout alleles.

Similarly to the ptlpd1 knockout mutants, the ptlpd2-1 and ptlpd2-2 mutants did not have an apparent phenotype in the absence of As(V), while root elongation was more severely inhibited by As(V) in both mutants than in WT seedlings (Fig. 7C). The presence of As(V) in the growth medium also enhanced anthocyanin accumulation in the shoots of both ptlpd2-1 and ptlpd2-2 seedlings compared with that in WT seedlings (Fig. 7D). Interestingly, root elongation in the ptlpd2 mutants was less sensitive to As(III) and As(V) compared with that in the ptlpd1 mutants, including the ptlpd1-4 knockdown mutant.

**ptLPD is sensitive to arsenite but not arsenate**

As(III) is a well known thiol group reagent that has been widely used to identify the reactive disulfide group in lipoamide dehydrogenases (Massey and Veeger, 1960; Marcinkeviciene and Blanchard, 1997). All known lipoamide dehydrogenase protein sequences, including those of ptLPD1 and ptLPD2 from Arabidopsis, contain two absolutely conserved Cys residues (data not shown) that participate in catalysis through disulfide bridge formation. To further understand the enhanced As(V) sensitivity of the ptlpd knockout mutants, the effect of As on ptLPD activity was determined. As(III) inhibited ptLPD activity in isolated chloroplasts isolated from leaves of wild-type Arabidopsis (Fig. 8A). The \( I_{50} \) for As(III) on the ptLPD activity was estimated from the data in Figure 8A to be about 100 \( \mu \)M. In contrast, As(V), even at a concentration of 1 mM, did not significantly inhibit ptLPD activity in these extracts (Fig. 8B).
DISCUSSION

Three arsenate overly-sensitive (aos) mutants were isolated from about 40,000 activation-tagged lines of Arabidopsis. The root growth of aos seedlings was about five times more sensitive to As(V) than that of the WT seedlings. Genetic and molecular analyses showed that the aos phenotype in all three mutants was due to a single mutational event that produced a recessive allele that we have designated ptlpd1-1. While T-DNA activation-tagging mutagenesis was designed to produce dominant mutations (Weigel et al., 2000), the isolation of a recessive mutation was not unexpected. T-DNA activation-tagging not only has the ability to activate genes near the insertion site, but also to disrupt a gene at the insertion site (Weigel et al., 2000).

The ptlpd1-1 mutation was mapped to the ptLPD1 gene. The T-DNA insertion event responsible for this mutation caused the inversion of a 107 kb fragment of chromosome 3, producing a breakpoint in exon 6 of ptLPD1. Genomic DNA rearrangements associated with T-DNA insertions are common. For example, Castle et al. (1993) reported that seven out of thirty-six T-DNA-tagged embryonic mutants had indications of chromosomal translocations. Several lines of evidence demonstrated that the aos phenotype was caused by disruption of ptLPD1. First, the T-DNA causing the inversion that disrupted ptLPD1 co-segregated with the aos phenotype. Second, transcripts from ptLPD1 were absent from ptlpd1-1 mutant seedlings. Third, two independent ptLPD1 loss-of-function alleles produced phenotypes identical to that of the ptlpd1-1 allele when plants were exposed to As(V). The aos phenotype in the presence of As(V) included reduced root elongation, increased anthocyanin accumulation and decreased fresh weight compared with WT plants. Consistent with this, the ptlpd1-4 knockdown allele, where the T-DNA insertion is located up-stream of the coding region, caused a phenotype intermediate between WT and the loss-of-function alleles. Fourth, F1 seedlings derived from crosses between ptlpd1-1 and ptlpd1-2 or ptlpd1-3 mutants had a mutant phenotype, demonstrating allelism for these mutations. Finally, the As(V) sensitivity of ptlpd1-1 mutant seedlings was restored to WT levels through the introduction of a genomic DNA fragment containing the ptLPD1 gene.
The *ptLPD1* gene encodes an isoform of the plastidial lipoamide dehydrogenase, a key component of the plastidial form of the pyruvate dehydrogenase complex (ptPDC). In Arabidopsis, two genes encode ptLPD, *ptLPD1* (At3g16950) and *ptLPD2* (At4g16155) (Lutziger and Oliver, 2000; Drea et al., 2001). Another two genes, *mtLPD1* (At1g48030) and *mtLPD2* (At3g17240), encode the mitochondrial forms of LPD (mtLPD) (Lutziger and Oliver, 2001) that are found in the mitochondrial pyruvate (PDC), α-ketoglutarate (KDC), and branched-chain α-ketoacid (BCKDC) dehydrogenase complexes, and in the glycine decarboxylase complex (GDC). In non-photosynthetic eukaryotes, LPD is exclusively located in mitochondria.

The underlying cause of the increased As(V) sensitivity of the *ptlpd1* mutants was not the over-accumulation of As, because the roots and shoots of the *ptlpd1* mutant and WT plants exhibited similar concentrations of As. It was also not the result of changes in the phosphate status of the plants. Instead, we hypothesize that the higher sensitivity was due to As directly inhibiting residual ptLPD activity, thus inhibiting ptPDC activity. Consistent with this idea, we showed that As(III), but not As(V), inhibited LPD activity, in agreement with studies on LPD activity from bacteria and animals (Massey and Veeger, 1961; Searls et al., 1961; Matthews and Reed, 1963; Ide et al., 1967; Marcinkeviciene and Blanchard, 1997). The mechanism of LPD inhibition is through the binding of As(III) to a catalytic dithiol group formed by two absolutely conserved and nearly adjacent Cys residues within the LPD protein. Plants can rapidly produce As(III) through the reduction of acquired As(V) by endogenous As(V) reductase activity (Zhao et al., 2009). Therefore, the reduced amounts of ptLPD in the plastids of *ptlpd1* mutants would provide fewer targets for As(III) binding, leading to a stronger inhibition by As(III), consequently reduced ptPDC activity.

It is unclear how As enters the plastids to exert its toxic effect on ptLPD. Both As(V) and As(III) may be able to cross the plastid envelope. As(V) might enter plastids via phosphate transporters, in a manner analogous to its crossing the plasma-membrane. Biochemical studies (Fliege et al., 1978; Borchert et al., 1993) showed that As(V) suppresses phosphate uptake.
into plastids, suggesting that As(V) and phosphate compete for the same transporters. PHT2;1, a plastid-localized phosphate transporter in Arabidopsis (Versaw and Harrison, 2002), may facilitate the import of As(V) to plastids. Once As(V) is in plastids, it might be reduced to As(III) through an unknown mechanism. It is also possible that As(III) is reduced outside the plastid and then imported by an unknown mechanism.

The available evidence suggests that the aos phenotype of the ptlpd mutants is caused by lowering of ptPDC activity below a critical threshold. ptPDC provides two essential substrates, acetyl-CoA and NADH, for de novo fatty acid biosynthesis (Mooney et al., 2002). Since the plastid is the predominant site of fatty-acid biosynthesis in plants, inhibition of ptPDC would have a profound effect on fatty-acid production. Fatty acids and fatty acid-derived complex lipids are essential for plants, as for other organisms. They are the components of cellular membranes, the precursors of cellular signaling molecules, such as jasmonic acid, and major energy reserves in storage tissues, such as seeds. Fatty acids and their derivatives are so important that an Arabidopsis knockout mutant of the E2 subunit of ptPDC is early-embryo lethal in the homozygous state (Lin et al., 2003).

The two Arabidopsis genes encoding ptLPDs are likely to be paralogs resulting from a relatively recent gene duplication (Mooney et al., 2002). ptLPD1 and ptLPD2 have similar tissue-dependent transcript profiles, although the relative abundance of transcripts from the two genes differs somewhat among some tissues (Lutziger and Oliver, 2000). The lack of an apparent phenotype for ptLPD1 and ptLPD2 loss-of-function mutants grown in soil or on plates in the absence of As(V) indicates that, under these growth conditions, each ptLPD gene can compensate for the loss of the other. However, the As(V) hypersensitive phenotype of the ptlpd1 knockout mutants indicates that ptLPD2 cannot fully compensate for the loss of ptLPD1 in the presence of As(V). A similar situation exists in the ptlpd2 knockout mutant, although the hypersensitivity is much less severe. Microarray data (Geneinvestigator; Zimmermann et al., 2004) indicate that ptLPD1 transcripts are more abundant in the root tip than are ptLPD2 transcripts. Thus, the ptlpd1 mutants may be more sensitive to As(V) than the ptlpd2 mutants simply because ptLPD1 is more highly expressed than ptLPD2 in root tips.
The presence of both ptLPD genes is vital for maximizing As resistance, despite their appearance of being largely redundant under normal growth conditions. The absence of ptLPD transcriptional induction in an analysis of Arabidopsis responses to As(V) using microarrays (Abercrombie et al., 2008) suggests that the compensatory effect of one ptLPD gene in the absence of the other is due to the additive nature of their expression, rather than an ability to substitute for one another. The ptlpd1-4 knockdown mutant, which produces ptLPD transcripts, but in lower abundance than WT seedlings, displayed an intermediate sensitivity to As(V), further suggesting that loss of ptLPD1 expression cannot be compensated for by an endogenous change in ptLPD2 expression. Therefore, knockout of ptLPD1, with its higher contribution to the ptLPD transcript pool in the root, resulted in greater sensitivity to As(V) than the loss of ptLPD2, which contributes proportionally less to the ptLPD transcript pool.

This study has shown that the amount of ptLPD probably plays an essential role in determining the As resistance of Arabidopsis. Thus, the possibility that an increase in the amount of ptLPD through transgenic over-expression would result in greater As tolerance warrants further investigation.

In conclusion, the ptlpd1 mutant is hypersensitive to both As(V) and As(III), due to a loss-of-function mutation in ptLPD1. ptLPD1 and ptLPD2 play largely redundant but additive roles, as loss-of-function of either ptLPD gene only results in a phenotype when exposed to As(V) or As(III). It is likely that a lower amount of ptLPD in the ptlpd1 mutants resulted in their greater sensitivity to As, and that As sensitivity is caused by As(III) binding directly to the catalytic dithiol group of ptLPD. To our knowledge, this is the first study indentifying a molecular target for As toxicity in plants. Future over-expression of ptLPD should shed light on its possible ability to confer As tolerance.

MATERIALS AND METHODS

Plant Materials
Seeds of WT Arabidopsis (*Arabidopsis thaliana*) Col-0 and Col-2 accessions, as well as T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center (ABRC; http://www.arabidopsis.org/abrc/). The mutant population used in this study was activation-tagged (Weigel et al., 2000) M3 seeds in Col-2 background. Individual T-DNA insertion lines used in this study were *WiscDsLox388B02 (ptlpd1-2)* and *WiscDsLox468E10 (ptlpd1-3)* from the Wisconsin collection (Krysan et al., 1999; Woody et al., 2007) and SALK_092097C (*ptlpd1-4*), SALK_013426 (*ptlpd2-1*) and SALK_118337C (*ptlpd2-2*) from the Salk collection (Alonso et al., 2003).

**Plant Growth Conditions and Mutant Isolation**

Seeds were surface sterilized in 70 % (v/v) ethanol for 2 min and in 5 % (v/v) hypochlorite with 0.1 % (v/v) Tween 20 for 10 min followed by rinsing five times with sterile, distilled water. All plants were grown at 22°C with a 16-h-light (70-100 μmol photons m$^{-2}$ s$^{-1}$) / 8-h-dark cycle.

A modified root-bending assay (Howden and Cobbett, 1992; Wu et al., 1996) was used to screen for arsenate overly-sensitive mutants. Surface-sterilized seeds were suspended in 0.1 % (w/v) agarose and stratified at 4°C for 2 to 3 d. Stratified seeds were sown in rows on plates containing 2/3-strength Gamborg B-5 basal salts medium (Phytotechnology Laboratories, Shawnee Mission, KS, USA) supplemented with 1 % (w/v) agar and 3 % (w/v) sucrose (Amresco, Solon, OH, USA). Plates were placed vertically to allow roots to grow along the surface of the agar medium to facilitate transfer to fresh plates. Four- to five-day-old seedlings were transferred, one by one, to germination medium supplemented with As(V) (NaH$_2$AsO$_4$) or As(III) (NaAsO$_2$) and placed vertically with root tips pointing upward. After 3- to 4-d exposure to As(V), seedlings with apparently inhibited root growth were recovered by transferring to fresh germination medium lacking As for about 2 wk before transfer to soil.

For hydroponic growth, 7-d-old seedlings grown vertically on plates of germination medium were transferred to fresh medium and grown for another 3 wk with the plate in a horizontal
position. Plants were transferred to hydroponics and grown in nutrient solution containing 1.25 mM KNO₃, 1.5 mM Ca(NO₃)₂, 0.75 mM MgSO₄, 1 mM KH₂PO₄, 50 µM KCl, 50 µM H₂BO₃, 10 µM MnSO₄, 2.0 µM ZnSO₄, 1.5 µM CuSO₄, 0.075 µM (NH₄)₆Mo₇O₂₄, 0.1 mM Na₂SiO₃ and 25 µM FeNaEDTA. Solutions were changed every 3 d. After 10 d in hydroponics, plants were transferred for 3 d to fresh nutrient solution with or without supplementation with 50 µM As(V).

**Growth Measurements**

In the root-bending assay, the length of newly elongated root was measured with a ruler from the top of the curl to the root tip after exposure of plants to As-containing medium. For measuring shoot and root fresh weights, shoots were excised in the middle of the hypocotyls. Shoots and roots from a number of plants were pooled and treated as one biological replicate. The seed survival rate was determined according to Lee *et al.* (2003) except the plates containing seeds on growth medium were placed vertically.

**Chloplast Isolation**

Chloroplasts were isolated according to Kunst (1998) with modifications. Four-week-old Arabidopsis leaves were soaked in ice-cold water for 20 min, blotted dry and homogenized in 0.45 M sorbitol, 20 mM Tricine-KOH, pH 8.4, 10 mM EDTA, 10 mM NaHCO₃, 0.1% (w/v) BSA, 5 mM Cys, and 5 mM glutathione. The homogenate was filtered through four layers Miracloth™ (CalBiochem, La Jolla, CA) and chloroplasts collected by centrifugation at 2000 xg for 5 min. The chloroplast pellet was gently resuspended in ice-cold resuspension buffer (0.3M sorbitol, 20 mM Tricine-KOH, pH 7.6, 5 mM MgCl₂ and 2.5 mM EDTA), followed by layering of the suspension onto a pre-formed linear density gradient made by centrifugation of 50 % (v/v) Percoll™, 0.3 M sorbitol, 20 mM Tricine-KOH, pH 7.6, 5 mM MgCl₂, 2.5 mM EDTA and 0.6 mM glutathione at 43,000 xg for 30 min followed by deceleration without a brake. Chloroplasts were separated by centrifugation at 12100 xg for 20 min, withdrawn in the lower band, collected by centrifugation 2000 xg for 5 min and washed twice with resuspension buffer.
LPD Activity Determination

Isolated chloroplasts were incubated 1 h at 65 °C in the presence of 1 % (v/v) Triton X-100 and the lysate clarified by centrifugation at 20,000 xg for 20 min to remove pigment and protein aggregates (Conner et al., 1996). The protein concentration in the supernatant was determined using a commercial kit (Coomassie Plus Reagent, Pierce Chemical, Rockford, IL) according to the supplier's instructions. LPD activity was determined as NADH consumption at 25 °C by measuring the change in absorbance at 340 nm (Cary 300 Biospectrophotometer, Varian, Palo Alto, CA). The reaction mixture contained 50 mM TES-KOH, pH 7.5, 2 mM MgCl₂, 0.05 mM NADH, 1 mM DL-α-lipoamide (Sigma Aldrich) and 12 µg ml⁻¹ clarified chloroplast protein. The reaction was initiated by adding the clarified chloroplast protein.

Anthocyanin Extraction and Estimation

Total anthocyanins were extracted according to Lange et al. (1971). Shoots of seedlings were weighed fresh and placed in 1.5 ml of 1-propanol : HCl : water (18 : 1 : 81). Samples were submerged in boiling water for 3 min, followed by incubation in the dark at 22°C for 24 h. Insoluble material was removed by centrifugation at 14,000 xg for 15 min at ambient temperature, before the optical density of the supernatant was measured at 535 and 650 nm. The absorbance due to anthocyanins was calculated as \( A = A_{535} - 0.24 A_{630} \) (Lange et al., 1971). The quantity of anthocyanins was determined from the corrected absorbance using a molar extinction coefficient (\( \varepsilon \)) of 38,000 L mol⁻¹ cm⁻¹ and normalized to the fresh weight of each sample.

Measurement of Arsenic

Shoot and root tissues were harvested separately, rinsed three times with distilled water and dried at 70°C for 3 d. Dried tissues were weighed and digested with nitric acid at 250°C for 5 min and 500°C for 5 min in a microwave extraction system (Microwave Laboratory Station
Milestone ETHOS 900, Milestone s.r.l., Sorisole, Italy). The arsenic content of the digested samples was analyzed by inductively coupled plasma optical emission spectrometry (ICP-OES), and normalized to the dry weight of each sample.

**PCR and DNA Sequencing**

DNA samples were amplified in a thermal cycler (Eppendorf, Hamburg, Germany) using a thermostable DNA polymerase (GoTaq Flexi DNA polymerase, Promega, Madison, WI, USA) according to the enzyme manufacturer’s instructions. Unless specified otherwise, the thermal cycling parameters used were: 1 cycle of 95°C for 2 min; 23 to 40 cycles of 30 s at 95°C, 30 s at 55°C and 1 to 3 min at 72°C; 1 cycle of 10 min at 72°C. PCR products were separated by electrophoresis on agarose gels and purified from excised gel slices using a commercial kit (AxyPrep™ DNA Gel Extraction Kit, Axygen, Union City, CA). The purified PCR products were used in dye-terminator sequencing reactions using a commercial kit (BigDye terminator version 3.1 Sequencing Kit, Applied Biosystems, Foster City, CA) according to the manufacture’s instructions followed by separation and analysis (Lottery West Biomedical Facility: Genomics, Perth, Australia).

**TAIL-PCR**

TAIL-PCR was done according to Liu et al. (1995). Genomic DNA was prepared using a commercial kit (PhytoPure™ Plant Genomic DNA Extraction Kit, Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer’s instructions. The primers (Supplemental Table S2) used to recover genomic DNA sequences flanking the T-DNA left border of pSKI015 were LB1, LB2, and LB3, in combination with DEG1. In other TAIL-PCR experiments, T1 and T2 were used in combination with DEG1. PCR conditions were those of Liu et al. (1995).

**Verification of T-DNA Insertion Sites**

The annotated positions of T-DNA insertions were confirmed by PCR using the T-DNA insert-specific primers LBa1 for the SALK lines, p745 for the Wisconsin (Wisc) lines and
LB3 for the activation-tagged mutants, in combination with the sequence-specific primers 66F and 66R for genotyping WiscDsLox388B02 and WiscDsLox468E10; 67F and 67R for genotyping SALK_118337C; 8F and 8R for genotyping ptlpd1-1; 69F and 69R for genotyping SALK_118337C and SALK_013426 (Supplemental Table S2).

Reverse Transcription (RT)-PCR

Total RNA was extracted from 7-d-old seedlings using a commercial kit (RNeasy Plant Mini Prep Kit, Qiagen, Clifton Hills, VIC, Australia) according to the manufacturer’s instructions and treated with DNase I (DNA-free; Ambion, Austin, TX, USA) according to the manufacturer’s instructions. First-strand cDNA was synthesized from 1 µg of total RNA with an oligo(dT) primer using a commercial kit (MonsterScript™ 1st-Strand cDNA Synthesis Kit, Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer’s instructions, and the primers ptLPDF and ptLPD1R for ptLPD1, ptLPDF and ptLPD2R for ptLPD2, and actin2F and actin2R for ACTIN2 (Supplemental Table S2).

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Figure 1. Isolation and phenotypic characterization of arsenate overly-sensitive mutants. Arabidopsis WT and aos mutant 106, 107 and 116 seed were germinated on plates containing solid As-free medium and placed in a vertical orientation. Five-day-old seedlings were transferred in rows to growth medium containing As(V). Plates of seedlings were placed vertically, with the seedling roots pointing upward. Growth was allowed to continue for four days before root elongation was measured from the top of the ‘hook’ to the root tip. Means ± SE (n = 20 to 30 seedlings) are shown.

Figure 2. Increased sensitivity of the ptlpd1-1 mutant to As(V). A to C, Five-day-old wild-type (WT) and ptlpd1-1 seedlings germinated on solid medium (see legend to Fig. 1) were transferred to medium containing the indicated As(V) concentrations and allowed to grow for 12 days in a vertical orientation. Mean values ± SE (n = 4 plates; two to four seedlings from each plate were pooled for each replicate) are presented for fresh weight (A), shoot anthocyanin concentration (B), and shoot-to-root fresh-weight (FW) ratio (C). D, ptlpd1-1 and WT seed was germinated on solid medium containing the indicated As(V) concentrations and scored for the emergence of green cotyledons after 5 days. Values are means ± SE (n = 4 plates of 50 to 100 seeds).

Figure 3. A paracentric inversion underlies the ptlpd1-1 phenotype. A, Schematic representation of the paracentric chromosomal inversion in ptlpd1-1. The upper diagram represents the gene organization in the region of wild-type (WT) genomic DNA involved in the inversion event. Genes are represented by thick arrows indicating the direction of transcription. BP1 and BP2 are the break points of the chromosomal inversion. For clarity, the fragment involved in the inversion is shown in gray. The lower diagram represents the gene arrangement in the ptlpd1-1 mutant. LB represents the left border of the T-DNA in pSKI015. The T-DNA insert is not drawn to scale. Small arrows indicate the annealing sites of primers used to confirm the genome organization. B, PCR-based confirmation of the genomic inversion in ptlpd1-1. Fragments were amplified using the primer pairs indicated on the left and WT or ptlpd1-1 mutant genomic DNA as the template. The annealing position of each primer used is shown in A. C, Abundance of At3g16950 (ptLPD1) transcripts in ptlpd1-1 mutant and WT seedlings. At3g18780 (ACTIN2) was used as amplification control. Numbers in brackets are the numbers of cycles used in the PCR.

Figure 4. As(V) sensitivity of root elongation in seedlings with ptlpd1 alleles. A, Schematic diagram showing the T-DNA insertion sites in the ptlpd1-2, ptlpd1-3 and ptlpd1-4 alleles of At3g16950. Black boxes and lines represent exons and introns/intergenic regions, respectively, with the direction of transcription being left to right. B, Five-day-old seedlings (see legend to Fig. 1) of wild-type (WT), ptlpd1-2, ptlpd1-3, and ptlpd1-4 were transferred to solid medium containing As(V). Increases in root length were measured after growth in the presence of As(V) for four days in a vertical orientation. Values are means ± SE (n = 15 to 20 seedlings). C, RT-PCR determination of ptLPD1 transcript abundance in plants with WT, ptlpd1-2, ptlpd1-3 or ptlpd1-4 alleles. Total RNA was extracted from 5-day-old seedlings and reverse transcribed. ACTIN2 (At3g18780) was used as an amplification control.
Figure 5. Arsenic accumulation in wild-type and ptlpd1-1 plants. Arabidopsis plants (38-day-old) grown in the absence of As (see Materials and Methods) were transferred to medium containing 0 or 50 µM As(V) and grown for a further three days. The As concentration in the shoots (A) and roots (B) were determined. Means ± SE (n = 4 plants) are shown.

Figure 6. ptlpd1 alleles confer increased sensitivity to As(III). Five-day-old seedlings (see legend to Fig. 1) of wild-type Col-0 (WT), ptlpd1-1, ptlpd1-2, ptlpd1-3 or ptlpd1-4 were transferred to solid medium containing As(III) and grown in a vertical orientation. A, Root-length increase was measured after four-days growth in the presence of As(III). Means ± SE (n = 10 – 15 seedlings) are shown. B, Shoot-to-root fresh-weight (FW) ratios were determined after ten-days growth on As(III)-containing medium. Means ± SE (n = 4 plates. Two to four seedlings from a plate were pooled for each replicate) are shown.

Figure 7. Response of ptlpd2 mutants to As(V) and As(III). A, Schematic diagram showing the T-DNA insertions in the ptlpd2-1 and ptlpd2-2 alleles of At4g16155. Black boxes and lines represent exons and introns/intergenic regions, respectively, with the direction of transcription being left to right. The size of T-DNA is not drawn to scale. B, Semi-quantitative RT-PCR determination of ptLPD2 transcript abundance in plants with WT, ptlpd2-1 or ptlpd2-2 alleles. Total RNA was extracted from 5-day-old seedlings and reverse transcribed. ACTIN2 (At3g18780) was used as an amplification control. C and D, Five-day-old seedlings (see legend to Fig. 1) homozygous for WT, ptlpd2-1 and ptlpd2-2 alleles were transferred to media supplemented with As(V). Plants were grown for four days with plates in a vertical position, then for ten days with plates in a horizontal position. Root growth (C) was measured four days after transfer. Means ± SE (n = 15 to 20 seedlings) are shown. Anthocyanin accumulation in the shoots (D) of wild-type and ptlpd2 mutants was determined 14 days after transfer. Means ± SE (n = 4 plates; two to four seedlings from a plate were pooled for each replicate) are shown. E, Root elongation response of ptlpd2-1, ptlpd2-2 and WT to As(III). Five-day-old seedlings were transferred to solid medium containing As(III). After four-days exposure, the increase in root length was measured. Means ± SE (n = 15 to 20 seedlings) are shown.

Figure 8. Inhibition of ptLPD by arsenite (A) and arsenate (B). Chloroplasts were isolated from four-week-old rosette leaves of wild-type Arabidopsis grown in soil. 12 µg of heat-treated chloroplast protein were used to determine the effects of arsenite (A) and arsenate (B) on LPD activity. Mean ± SE (n ≥ 3) are shown.
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