Arabidopsis AHL Gene Encodes a 3'(2'),5'-Bisphosphate Nucleotidase Sensitive to Toxic Heavy Metal Ions

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***Arabidopsis AHL* gene contains 4 exons encoding a putative protein highly homologous to the yeast salt-sensitive enzyme HAL2, a 3'(2'),5'-bisphosphate nucleotidase involving in reductive sulfate assimilation. *AHL* cDNA complemented yeast *met22* (*hal2*) mutant. AHL fusion protein expressed in *E. coli* exhibited Mg^{2+}-dependent, 3'-phosphoadenosine 5'-phosphate (PAP)-specific phosphatase activity. Li^+, Na^+, K^+ and Ca^{2+} ions inhibit the enzyme activity by competing with Mg^{2+} for the active site of the enzyme. The enzyme activity was also sensitive to μM concentrations of toxic heavy metal ions such as Cd^{2+}, Cu^{2+} and Zn^{2+}, but was not recovered by addition of more Mg^{2+} ions, suggesting that these ions inactivate the enzyme with a mechanism other than competition with Mg^{2+} ions. Inhibition of the AHL enzyme activity may result in accumulation of PAP, which is highly toxic to the cell. Thus, the AHL enzyme could be one of the initial targets of heavy metal toxicity in plants.**

Key words: Arabidopsis thaliana, *3'(2'),5'-bisphosphate nucleotidase, heavy metal toxicity, reductive sulfate assimilation.*

Sulfur metabolism in plants is initiated by uptake of inorganic sulfate from soil. Since the sulfate is chemically inert under biological conditions, it must be reduced to sulfide to be incorporated into sulfur-containing compounds including cysteine.[1] Accumulated evidence suggests that the reductive sulfate assimilation mechanism in plants is similar to that in *E. coli* and yeast.[2,3] In these microorganisms, sulfate is activated via coupling with ATP by ATP sulfurylase to form APS. A second ATP-coupled activation is conducted by APS kinase that converts APS to PAPS. PAPS reductase then reduces PAPS to sulfite that is further reduced to sulfide by sulfite reductase. Plants contain all of the enzymes necessary for the pathway.[3]

In general, the sulfonucleotide PAPS has been implicated as the primary sulfate donor in reduction to sulfite or in sulfate transfer reactions. In PAPS-utilizing reactions, the by-product PAP is generated. The PAP is then hydrolyzed to AMP and inorganic phosphate by a PAP-specific phosphate-releasing enzyme 3'(2'),5'-bisphosphate nucleotidase. An enzyme with such activity was purified from guinea pig liver.[4] Recently, it has been reported that the proteins encoded by yeast (*Saccharomyces cerevisiae*) *HAL2* have PAP phosphatase activity.[5,6] Three *HAL2* homologues, *SAL1*, *SAL2*, and *AHL*, have been identified from *Arabidopsis*.[7-9] The enzymes may contribute to the rapid sulfur flux through the assimilation pathway by accelerating the PAPS-utilizing reactions. More importantly, it has been suggested that the enzyme controls the level of PAP that is toxic to the cell when accumulated at high concentrations.[10,11]

PAPS is also harmful for the cell at high concentrations.[12,13] It has been suggested that a 3'(2'),5'-diphosphonucleoside 3'(2')-phosphohydrolase (DPNPase) catalyzes the conversion of PAPS back to APS to avoid accumulation of the PAPS. It was shown that proteins encoded by *E. coli* gene *cysQ* and rice *RHL* have DPNPase activity.[12,13] These proteins are similar to PAP phosphatases throughout their amino acid sequences including two motifs that are conserved in the inositol monophosphatase family. In particular, the SAL1 and RHL proteins show 68% identity with each other in their amino acid sequences. Each of the *RHL* and *SAL1* cDNAs complements the yeast *HAL2* mutant, *met22*. Moreover, the gene products of *HAL2*, *RHL*, and *SAL1* utilize both PAPS and PAP as substrates with equal preference *in vitro*. Thus, it is ambiguous whether these proteins belong to the PAP phosphatase or DPNPase enzyme class.

In this paper, we present the results obtained from experiments designed to identify the biological function of the AHL protein. Complementation experiments with yeast mutant and substrate specificity assays with purified AHL protein have revealed that the *AHL* gene encodes a PAP-specific 3'(2'),5'-bisphosphate nucleotidase. In addition, the

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Abbreviations: APS, adenosine 5'-phosphosulfate; DTT, dithiothreitol; IC_{50}, concentration for inhibition of 50% activity; IPTG, isopropylthiogalactopyranoside; MBP, maltose-binding protein; PAP, 3'(2')-phosphoadenosine 5'-phosphate; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; SSC, sodium saline citrate; SD, synthetic minimal dextrose medium; SDS, sodium dodecyl sulfate.

AHL enzyme activity is extremely sensitive to toxic heavy metal ions such as Cd^{2+} and Cu^{2+}. The possibility that the enzyme is one target of metal toxicity is discussed.

Materials and Methods

Cloning and characterization of the *AHL* gene. *Arabidopsis* Lansberg genomic library was obtained from the Arabidopsis Biological Resource Center (Stock No. CD4-8). The library was screened as described[14] with a 250 bp (nucleotides 374 through 623) *AHL* cDNA probe.[9] Blots were washed twice with 2×SSC/0.2% SDS at room temperature, once with 0.2×SSC/0.1% SDS at 65°C, then twice with 0.1×SSC/0.1% SDS at 65°C.

For genomic DNA sequencing, amplified phage DNA was digested with *Pst*I and subcloned into Bluescript KS(-) vector (Stratagene, La Jolla, CA). DNA sequencing was performed using an automatic sequencer (ABI model 370). The internal 93 bp fragment between *Pst*I sites (Fig. 1) was sequenced manually from the phage DNA as described.[14]

All DNA fragments to be inserted into vectors were prepared by polymerase chain reaction (PCR) using a high fidelity *Taq* polymerase (Boeringer Mannheim, Indianapolis, IN). Orientation of the insert was examined by digestion with diagnostic restriction enzymes and partial DNA sequencing.

Yeast Functional Complementation. Yeast mutant *met22* (CD108; *MAT***a**, *his3, leu2, ura3, ade2, trp1, met22::TRP1*) was provided by Dr. Yolande Surdin-Kerjan (Centre National de la Recherche Scientifique, France). For routine growth of the yeast, minimal growth medium (SD) containing 2% dextrose (or galactose) and 0.67% yeast nitrogen base without amino acids (Difco) was prepared. It was supplemented with adenosine hemisulfate (20 μg/ml), uracil (20 μg/ml), L-histidine (20 μg/ml), L-leucine (30 μg/ml), and L-methionine (20 μg/ml). In some media, certain components were omitted as indicated.

The *AHL* cDNA was subcloned into the *Bam*HI and *Xba*I sites of the yeast expression vector pYES2 (Invitrogen, San Diego, CA). The yeast *met22* mutant cells were transformed with the construct or empty vector using lithium acetate,[15] and selected at 30°C on uracil- or methionine-free selection medium. For complementation experiments, saturated yeast cultures grown in SD dextrose media were centrifuged at 3,000 rpm for 5 min using a table-top centrifuge and resuspended in an equal volume of sterile water (OD_{600}= ~1.3; ~1×10^8/ml). Each 3 μl of the suspensions was streaked on solid (2% agar) minimal medium containing galactose.

Expression and purification of AHL proteins. For bacterial expression, the *AHL* cDNA was cloned in translational fusion with the *E. coli malE* gene between the *Xmn*I and *Xba*I sites of the pMAL-c2 vector (NEB, Beverly, MA). The construct was then transformed into *E. coli* TB1 cells by electroporation. Expression of the fusion protein was induced by 0.3 mM IPTG at 37°C for 2 h. The fusion proteins were purified under nondenaturing condition using amylose affinity chromatography according to the manufacturer's instructions (NEB, Beverly, MA). The column buffer contained 20 mM Tris-HCl (pH 7.4), 0.2 M NaCl, 1 mM EDTA, and 1 mM DTT. Purified proteins were stored at 4°C in the presence of 0.01% (w/v) Triton X-100 and used within 2 days for enzyme assay. SDS-polyacrylamide gel electrophoresis was performed to examine the proteins,[14] which were visualized using a silver stain kit (Bio-Rad, Richmond, CA). Protein concentration was determined using Bradford assay reagent (Bio-Rad, Richmond, CA) using bovine serum albumin as a standard.

Phosphatase activity assay. Phosphatase activity was determined by mixing 20 ng protein with 0.5 mM substrate in 100 ml reaction buffer containing 50 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, 1 mM DTT, and 0.01% Triton X-100. After incubation at 37°C for 30 min, 300 μl of water and 100 μl of malachite green dye mixture[16] were added. Then, 200 μl of each reaction mixture was transferred to a 96-well flat-bottom plate. Intensity of developed green color was measured at 655 nm using an ELISA reader (Bio-Rad, Type 550). All chemical compounds used in substrate specificity tests were purchased from Sigma Chemical Co.

Results

Characterization of the AHL gene. Genomic *AHL* DNA was cloned by screening an *Arabidopsis* genomic library originally obtained from Lansberg ecotype plants. The genomic DNA (3,232 bp) consists of an upstream promoter sequence, 3 introns, 4 exons, and a polyadenylation signal (Fig. 1). In the upstream region of the coding sequence, a repeated sequence GTTGTT was found at four locations. Significance of these repeats remains to be studied, but it is possible that they are binding sites for expression regulators or transcription enhancers. The nucleotide sequence of the *AHL* genomic DNA has been deposited in the GenBank database under accession number AF016644.

Amino acid sequence deduced from the exons showed 98% nucleotide identity and 99 % amino acid identity to that of the *AHL* cDNA isolated from Columbia cDNA library.[9] The putative protein (373 aa) has a predicted molecular mass of 39 kDa and a calculated pI of 5.5. The protein contains two motifs that are highly conserved in the inositol monophosphatase family (not shown), and exhibits 38% amino acid sequence identity to yeast HAL2,[5] 45% to *Arabidopsis* SAL1,[7] 45% to rice RHL,[13] and 30% to *E. coli* cysQ[12] proteins.

Yeast mutant complementation experiment. The yeast *met22* (*hal2*) mutant[17] was transformed with pYES2 vector carrying *AHL* cDNA. The yeast cells transformed with the construct were grown on methionine-free medium, and a significant number of colonies appeared after 3-4 days (Fig. 2). In contrast, the *met22* mutants harboring no or empty vector did not grow even after 10 days.

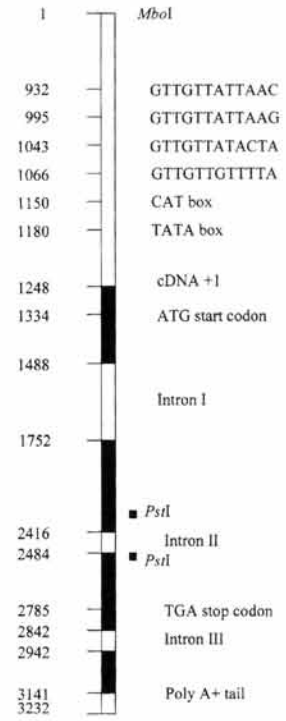

Fig. 1. Primary structure of *Arabidopsis AHL* genomic DNA. Numbers on the left side correspond to those of DNA sequence deposited in GenBank database under accession number AF016644. Exons corresponding *AHL* cDNA (accession number U55205)[9] are indicated by black boxes.

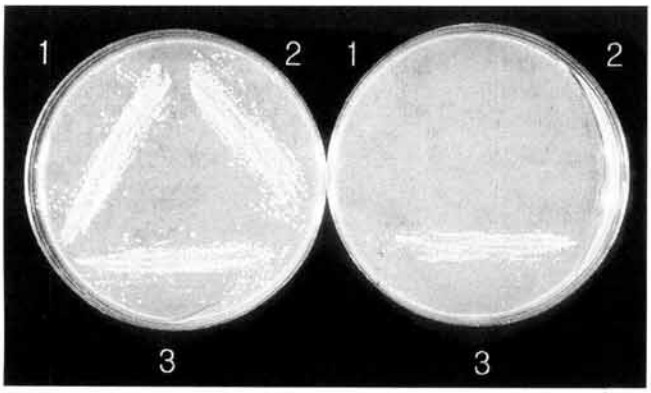

Fig. 2. Complementation of yeast *met22* mutant. 1, the *met22* mutant;[17] 2, the *met22* mutant harboring vector pYES2 only; 3, the *met22* mutant harboring vector pYES2 containing *AHL* cDNA. Saturated yeast cultures (each 3 μl) grown in SD media were washed with sterile water and streaked on solid minimal galactose medium with (left) or without (right) 20 μg/ml methionine. Growth was for 4 days at 30°C.

Enzymatic characteristics of AHL proteins. *E. coli* TB1 cells transformed with the pMAL-c2 vector containing *AHL* cDNA expressed 80 kDa MBP-AHL fusion proteins (Fig. 3). The AHL protein exhibited its highest PAP phosphatase activity at 37°C and pH 7.6-8.0 (data not shown). The AHL enzyme activity required Mg^{2+}, with an optimal concentration of 2 mM in the presence of 0.5 mM PAP (Fig. 4). Mg^{2+} concentrations higher than 2 mM were inhibitory for

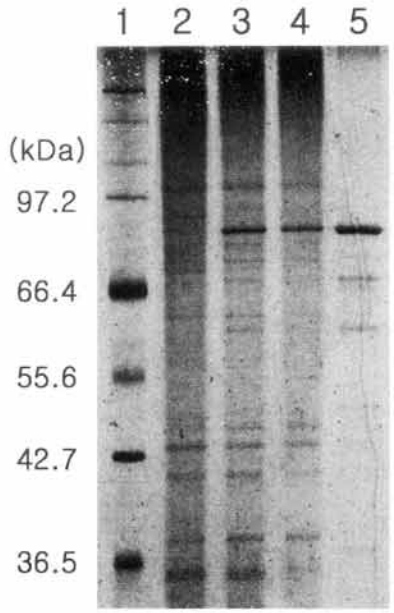

Fig. 3. Expression and purification of MBP-AHL fusion protein. Proteins were separated on a 10% SDS-polyacrylamide gel and stained with a silver staining kit (Bio-Rad). Lane 1, molecular weight markers; lane 2, total homogenate from uninduced *E. coli* TB1 cell harboring pMAL-c2 vector containing *AHL* cDNA (~1×10^7 cells); lane 3, total homogenate from the *E. coli* TB1 cells treated with 0.3 mM of IPTG for 2 h (~1×10^7 cells); lane 4, soluble fraction of the lysate of the *E. coli* TB1 cells overexpressing the MBP-AHL fusion proteins (2 μg proteins); lane 5, MBP-AHL proteins purified by amylose affinity chromatography (2 μg proteins).

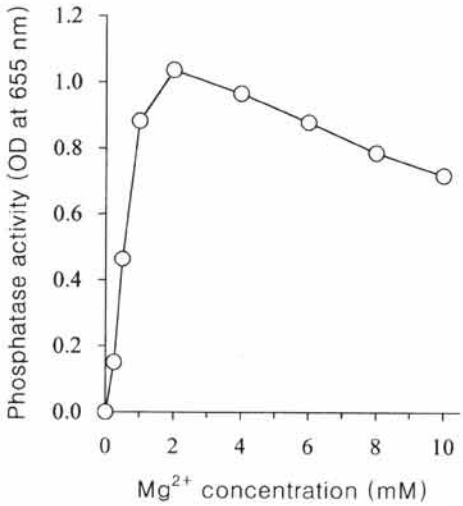

Fig. 4. Effect of Mg^{2+} concentration on PAP phosphatase activity of AHL protein. Reactions were performed at 37°C with 50 mM Tris-HCl buffer, pH 7.8. Amount of phosphate released from 0.5 mM PAP by 20 ng of AHL enzyme was determined by reading absorbance of the reaction mixture at 655 nm after treating with malachite green dye solution.[16] Each point in the data represents the average of three replicates.

the enzyme activity, with 50% of the enzyme activity abolished at 15 mM Mg^{2+} (not shown). When stored at 4°C, PAP phosphatase activity of the proteins was reduced by

Table 1. Substrate specificity of AHL enzyme.

Substrate	Activity (%)*
3'-PAP	100
2'-PAP	10
3'-PAPS*	31
2'-PAPS**	67
Inositol-1,4-bisphosphate	9
Inositol-1,3,4-trisphosphate	3
Inositol-1-monophosphate	0
ATP	0
APS	0

*Each substrate (0.5 mM) was tested with 20 ng of AHL enzyme under standard conditions as described under Materials and Methods. The relative phosphatase activity was calculated with respect to the activity for 3'-PAP (6.7 μmole/min/mg protein). The results are the means of three independent experiments with values differing by less than 5%.
**Since 3'-PAPS and 2'-PAPS (from Sigma) contain 4 moles of lithium per mole, the activity for these compounds was compared to the activity for 3'-PAP in the presence of 2 mM lithium.

approximately 20% per day in the presence of 1 mM DTT and 0.01% Triton X-100. Storage of the protein in the presence of 10-20% glycerol either at 4°C or -20°C for 1 day abolished more than 90% of the enzyme activity.

Under the optimal assay conditions (see Materials and Methods), PAP (3'-PAP) was the best substrate for the enzyme. When compared to the amount of phosphate released from PAP by the AHL proteins, 10% of phosphate was released from 2'-PAP, 9% from inositol 1,4-diphosphate, and 3% from inositol 1,3,4-triphosphate (Table 1). APS, inositol 1-monophosphate (IMP), and ATP did not release detectable amounts of phosphate. In the experiments testing PAPS (3'-PAPS) or 2'-PAPS as substrates, 2 mM Li^+ was added in the control reaction with PAP, since commercial PAPS and 2'-PAPS contain 4 moles of Li^+ per mole. In the presence of 2 mM Li^+, PAP phosphatase activity was reduced by 12%. Compared to the amount of phosphate released from PAP by the AHL protein, 31% of phosphate was released from PAPS and 67% from 2'-PAPS. Two stocks of PAPS were tested separately, giving identical results. The pH profile of the AHL phosphatase activity for PAPS was identical to that for PAP (data not shown).

Sensitivity of the AHL enzyme activity to cations. The enzyme activity was inhibited by monovalent ions such as Li^+, Na^+, and K^+ (Table 2). Concentrations for inhibition of 50% activity (IC_{50}) for Li^+, Na^+, and K^+ were 25, 120, and 150 mM, respectively. The enzyme activity was also sensitive to divalent ions such as Cd^{2+} (IC_{50} = 0.01 mM), Cu^{2+} (0.1 mM), Hg^{2+} (0.25 mM), Zn^{2+} (0.12 mM), Ca^{2+} (0.04 mM), and Mn^{2+} (0.16 mM). Each ion at concentrations tested did not interfere with the phosphate assay without enzyme.

Under standard reaction condition with 2 mM Mg^{2+}, addition of 0.2 mM Ca^{2+}, 100 mM Li^+, 400 mM Na^+ or 400 mM K^+ to the reactions inhibited the AHL enzyme to show 6 to 16% of its maximal activity (Fig. 5). The enzyme activity

Table 2. Sensitivity of AHL enzyme to various cations.

Cations*	IC_{50} (mM)**
Cd^{2+}	0.01
Cu^{2+}	0.10
Zn^{2+}	0.12
Hg^{2+}	0.25
Mn^{2+}	0.16
Ca^{2+}	0.04
Li^+	25
Na^+	120
K^+	150

*Chloride salt of each cation was added to the phosphatase assay reaction mixture described under Materials and Methods.
**Concentration of each cation required to inhibit 50% of the enzyme activity in the presence of 2 mM Mg^{2+}. The results are the means of three independent experiments with values differing by less than 10%.

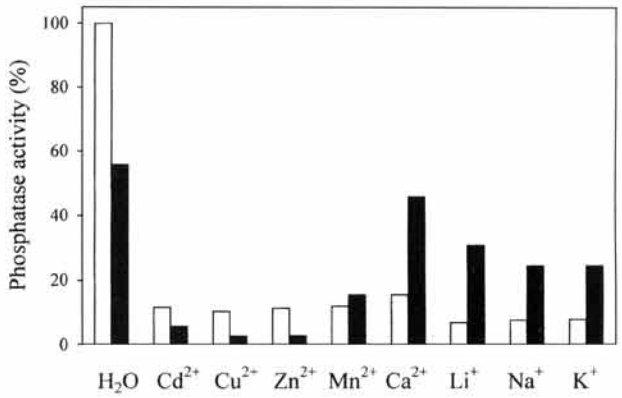

Fig. 5. Effect of Mg^{2+} concentration on cation inhibition of PAP phosphatase activity of AHL protein. Enzyme activity is expressed as a percentage of that without any ions tested. Phosphatase activities in the presence of 2 and 15 mM Mg^{2+} are expressed by gray and black bars, respectively. Cation concentrations in the reactions were 0.1 mM Cd^{2+}, 0.5 mM Cu^{2+}, 0.5 mM Zn^{2+}, 2.5 mM Mn^{2+}, 0.2 mM Ca^{2+}, 100 mM Li^+, 400 mM Na^+, and 400 mM K^+. Each point in the data represents the average of two replicates.

inhibited by these ions was recovered to show 24 to 45% activities in the reaction with 15 mM Mg^{2+}. In addition, 0.1 mM Cd^{2+}, 0.5 mM Cu^{2+}, and 0.5 mM Zn^{2+} also reduced the enzyme activity by 90%. However, the inhibition effects of these toxic heavy metal ions were not overcome by addition of 15 mM Mg^{2+} in the reaction mixture, but rather enhanced by the inhibition effect of high Mg^{2+} concentration. Inhibition by 2.5 mM Mn^{2+} was slightly, but not significantly, recovered by the additional Mg^{2+}.

Discussion

The *AHL* cDNA encoded by exons of the AHL gene (Fig. 1) complements yeast *met22* (Fig. 2) and another *hal2* mutant strain JRM4,[8] suggesting that the gene product has biological functions similar to those of yeast HAL2. The HAL2, SAL1, and RHL proteins showed the same or higher

activity with PAPS compared to PAP, but the AHL enzyme strongly prefers PAP as a substrate (Table 1). When compared to the amount of phosphate released from PAP by the AHL enzyme, only 31% of the phosphates were released from PAPS. According to the manufacturer's (Sigma) specifications, PAPS contains 20% impurities consisting mainly of PAP, thus utilization of PAPS compared to PAP may be as low as 11%. Relative to PAP, 2'-PAPS is used 67% as well; however, 2'-PAPS is not a biological substrate for phosphatases. The AHL enzyme had some activity (10%) with 2'-PAP. Therefore, the AHL gene encodes a PAP-specific 3'(2'),5'-bisphosphate nucleotidase (E.C. 3.1.3.7).

As do the HAL2, SAL1, and RHL proteins, the AHL protein requires Mg^{2+} for activity (Fig. 4). These proteins contain two motifs that are highly conserved in the inositol monophosphatase family, although inositol monophosphate is not a substrate for the proteins. Crystal structure studies with human inositol monophosphatase indicated that the two motifs form the active site at which two Mg^{2+} ions bind for the enzyme activity.[18] The PAP phosphatases are sensitive to Li^+, Na^+, and Ca^{2+}, as also observed in the present study (Table 2). It has been demonstrated that Li^+ ions bind at one of the Mg^{2+} binding sites in inositol monophosphatase, resulting in inhibition of the enzyme activity.[18] Kinetics with RHL protein has also suggested that Ca^{2+} ions compete with Mg^{2+} ions for the active site.[13] Similarly, inhibition of the AHL enzyme activity by the monovalent ions and Ca^{2+} was recovered by addition of more Mg^{2+} to the reactions (Fig. 5). Thus, these cations compete with Mg^{2+} for its binding site(s) in the AHL enzyme.

The AHL enzyme is also sensitive to toxic heavy metal ions (Table 2). In particular, Cd^{2+} completely inhibits the AHL enzyme activity at 100-200 μM (IC_{50}=10 μM). It was observed that growth of *Arabidopsis* root was partially inhibited on medium containing 90 μM Cd^{2+}, and completely inhibited when the concentration was increased to 210 μM.[19] Inhibition effect exerted by the heavy metal ions were not recovered in the presence of high concentration of Mg^{2+} (Fig. 5). This result suggests that inhibitory mechanism of the heavy metal ions are different from that exerted by monovalent ions or Ca^{2+}. Biological significance of the heavy metal sensitivity of the AHL enzyme remains to be further investigated.

The PAP-specific phosphatase encoded by the *AHL* gene may contribute to the rapid sulfur flux in reductive sulfate assimilation in plant by converting PAP into AMP and inorganic phosphate, as suggested by studies of similar enzymes in microbial systems. Thus, inhibition of the AHL enzyme activity may limit the supply of cysteine, a major sulfide acceptor. Cysteine is a main component of cysteine-rich peptides such as glutathione and phytochelatins that are involved in heavy metal tolerance.[20] The Cd^{2+}-sensitive mutant of *Arabidopsis*, *cad1*, was deficient in phytochelatin synthase activity,[21] demonstrating the importance of phytochelatins for cadmium tolerance in plants.

A more likely role for AHL in heavy metal toxicity is to avoid accumulation of its substrate PAP that is highly toxic to the cell. In yeast, inhibition of the HAL2 enzyme by Li^+ or Na^+ treatment raised the intracellular concentrations of PAP and PAPS.[10] Recently, it was reported that PAP accumulation in yeast *hal2* mutants or in Li^+-treated wild type yeast strains inhibited exoribonucleases involved in RNA processing.[11] One of the plant metabolic reactions that is known to be sensitive to PAP accumulation is the import of acyl carrier protein precursor into chloroplasts for the fatty acid synthesis.[22]

In summary, inhibition of the AHL enzyme by heavy metal ions may result in either insufficient levels of metal-detoxifying thiol peptides or accumulation of the toxic compound PAP in plant cells. Thus, it is possible in either case that the AHL enzyme may be one of the initial targets for metal toxicity in plants. Metal sensitivity of the HAL2 nucleotidase is an important determinant of yeast growth in high concentrations of Li^+ and Na^+.[6,10] Thus, overexpression of this enzyme in yeast significantly improved yeast growth at high salt concentrations.[5] Assuming similar molecular bases of metal tolerance between yeast and plant cells, it may be possible to generate plants tolerant to high concentrations of salts or heavy metal ions by overexpression of the AHL protein.

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