DOE/ER/20252

Heavy Metal Pumps in Plants

Final Report – 09/15/1996 – 09/14/2000

J. Harper

October 2000

Work Performed Under Contract No. DE-FG07-96ER20252

For
U.S. Department of Energy
Assistant Secretary for
Energy Efficiency and Renewable Energy
Washington, DC

By
The Scripps Research Institute
La Jolla, CA

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Final Report October 10, 2000
P.I. Jeff Harper
The Scripps Research Institute

The long term goal of the funded research is to understand how heavy metals are taken up from the soil and translocated throughout the plant. The potential application of this research is to create plants with better heavy metal uptake systems and thereby improve the ability of these plants to help clean up toxic metals from soils. A rate limiting step in using plants for bioremediation is the normally poor capacity of plants to concentrate toxic metals.

Our interest in metal ion transport systems includes those for essential mineral nutrients such as molybdenum, copper, iron, manganese, as well as toxic metals such as cerium, mercury, cesium, cadmium, arsenic and selenium. Our focus is on a unique heavy metal pump, AMA1p [*Arabidopsis* heavy metal) ATPase, isoform 1 protein (also known as AXA2)], identified in a model plant, *Arabidopsis.* AMA1 belongs to a subfamily of heavy metal pumps within the large superfamily of ion-translocating P-type ATPases. AMA is distinquished from other characterized metal pumps in that it 1) has numerous metal binding motifs containing cys-cys amino acid pairs, and 2) has a histidine loop positioned at the outside surface of the channel pore domain. These unique features are consistent with a working model that this pump serves as an uptake transporter for molybdenum or selenum oxyanions.

The grant funded a graduate student Yuwen Wang. She completed her Thesis in May 2000 and received a Ph.D from the Scripps Research Institute. This work is published as a thesis with the title "Identification of a Molybenum Uptake Pump in Arabidopsis Plants". The abstract is attached in the following pages. The primary significance of the research was to show that a gene disruption of the AMA1 pump resulted in plants with reduced accumulation of molybdenum.

We have post-poned publishing in a peer review journal because follow up work revealed highly variable results for molydenum accumulation when plants were grown on different media. We decided to post-pone publishing until we can determine the source of this variability. Our primary working hypotheses is that media with high sulfur is required to block secondary molydenum transport through sulfur transporters. Under conditions of low sulfur, we suspect that high levels of molybdenum can enter the cells without the AMA1 pump. We expect these studies to be completed in 6 months.

A major advance made by this work was to demonstrate that a mutant screen for plants with altered accumulation of mineral nutrients was a viable approach to identify transport systems for different metals. This resulted in an NSF functional genomics grant funded as a collaboration between Mary Lou Guerinot, Dartmouth College, David J. Eide, University of Missouri, Jeffrey F. Harper, The Scripps Research Institute, David E. Salt, Northern Arizona University, Julian I. Schroeder and Michael Gribskov, University of California at San Diego. The abstract for that collaborative program is the following:

> Title: **Gene Discovery in Aid of Plant Nutrition, Human Health and Environmental Remediation**
>
> Uptake and translocation of mineral nutrients in plants is essential for plant growth and human nutrition. In spite of recent advances in identifying genes involved in nutrient transport, the systems that control acquisition of individual nutrients remain largely unknown. The major objective of the proposed research is to identify gene networks that control uptake and

accumulation of a wide array of plant nutrients and toxic metals. The approach makes use of recent technical advances in inductively-coupled plasma atomic emission spectroscopy (ICP-AES) which now permit the measurement of up to 72 different elements in 35 seconds per sample. Identifying genes controlling solute uptake and accumulation has significance for agriculture, human health and the environment. For example, enhancing the ability of a crop plant to mobilize soil nutrients should reduce the use of fertilizers, thereby making agriculture more cost efficient and less polluting. Because plants are the primary source of food for humans, either directly or through animal feed, the nutritional value of plants is of central importance to human health. The most widespread nutritional problem in the world is iron deficiency. Increasing the ability of plants to provide higher levels of minerals, such as iron, will have a dramatic impact on human health. Furthermore, understanding the pathways by which toxic metals accumulate in plants will enable the engineering of plants to exclude toxic metals and create healthier food sources, or to extract toxic metals from the soil as a strategy to clean up polluted lands and water.

The main aims of the proposal are to:

1) Use bioinformatics to identify genes that potentially encode transporters.
2) Use mRNA expression profiling to identify genes that change expression in response to nutrient deprivation or overfeeding.
3) Use nutrient profiling to screen for mutant plants with abnormal element compositions. ICP-AES will be used in a high-throughput strategy to determine the relative element composition of approximately 50,000 "tagged" mutagenized plants (Arabidopsis and maize).
4) Use yeast to obtain functional predictions of plant orthologs. The primary approach will be to conduct ICP-AES nutrient profiling of approximately 5,000 knockout lines of yeast.
5) Establish a Web site to provide access to data sets and enhanced annotation of genes.
6) Initiate collaborative research focused on selected mutations that control accumulation of Fe, Zn, Cu, Mn, K, Na, Ca, Ni, Se and Cd to demonstrate the power of this novel approach.
7) Establish a program to train undergraduate and graduate students in genomics, informatics and plant molecular biological techniques.
8) Provide a service to high school and college students who wish to test plants from their local environment for levels of toxic and nutritional minerals.

This project will functionally identify many important genes, including those that are involved in:

1) nutrient mobilization in the rhizosphere
2) cellular uptake and efflux systems
3) subcellular compartmentalization of solutes
4) the operation of phloem and xylem translocation systems
5) central regulation mechanisms
6) nutrient sensing
7) control of root structure

This functional genomic investigation will provide the first integrated picture of the genes involved in a fundamental feature of all living systems - the selective accumulation of essential minerals.

Paper in Preparation

Evidence from a gene disruption for a heavy metal P-type ATPase involved in molybdenum accumulation in *Arabidopsis*

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ABSTRACT

Heavy metal P-type ATPases have been identified to transport Cd^{2+}, Cu^{2+}, Co^{2+} and Pb^{2+}. They have been implicated in heavy metal resistance and regulation of essential micronutrients. Here we report the identification of a new heavy metal pump from *Arabidopsis*, called *AMA1* (*Arabidopsis* Heavy Metal ATPase-isoform 1). *AMA1*p differs from other heavy metal pumps. It contains a unique 26kDa C-terminal domain that is rich in histidine and cysteine, with the cysteines commonly found as CC pairs. *AMA1*p is localized to the plasma membrane, and is expressed at a high level in siliques and at a medium level in roots and stems. Plants containing a T-DNA disrupted allele, *AMA1*-1, grow normally under standard growth conditions. However, metal analysis indicated that they contain 20%-70% less molybdenum than wild-type plants, while other metals are present at normal levels. These results suggest *AMA1*p functions as a molybdenum uptake pump in plants. This research provides the first evidence for a P-type ATPase involved in molybdenum transport.

Ion Transporting Pumps in Plant Cells 1998, 1999 Report (copy)

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The long-term goal of this research is to learn how to engineer a plant to selectively extract metal cations such as the RCRA metals or radionuclides from the soil. The approach being pursued is based on first understanding how plants use membrane transporters (pumps) to take up metals from the soil. The specific focus is on a family of heavy metal pumps that use ATP to drive the high affinity transport of ions across membranes. Heavy metal pumps are known to transport metals such as manganese, copper, molybdenum, zinc, cadmium and silver. The objective is to understand how these pumps selectively transport various metals. With that knowledge, it is expected that new transport systems can be designed that will increase the efficiency of plants to take up various toxic elements, such as plutonium. Modified transport systems will be introduced into plants by genetic engineering.

Three metal ion pumps are being studied in a model plant system *Arabidopsis*. One pump (AMA1) is a member of a subfamily of heavy metal pumps. Genetic evidence indicates that AMA1 is involved in molybdenum uptake, as indicated by a deficiency of this micronutrient in a plant line harboring a disruption of the AMA1 gene. This nutrient deficiency is reversed in mutant plants that have been transformed with an AMA1 pump under the control of a constitutive promoter (35s). Two other pumps being studied (ECA1 and ACA2) are members of two different subfamilies of calcium pumps. Genetic evidence suggests that (i) both pumps can also transport cerium (which has chemical properties similar to plutonium), and (ii) ECA1, but not ACA2, can also transport manganese, as indicated by the ability of these pumps to provide yeast with an increased resistance to toxic levels of these metals. These molybdenum and calcium pumps may be useful in engineering plants with an increased capacity to uptake and accumulate toxic metals.

1997 Progress Report (copy)

The long term goal of the proposed research is to understand how heavy metals, such as copper and cadmium, are taken up from the soil and translocated throughout the plant. The focus is on a putative heavy metal pump, AXA2p [*Arabidopsis* X (unknown heavy metal) ATPase, isoform 2 protein], identified in a model plant, *Arabidopsis.* AXA2 belongs to a large family of ion-translocating P-type ATPases. AXA2p is the first heavy metal pump cloned from plants and is most similar to a subfamily of heavy metal pumps recently identified in bacteria, yeast and humans.

Specific Aims

1) Determine which ions are translocated by the AXA2 pump. The approach is to over-express AXA2 in yeast, and use purified membrane vesicles to evaluate ion stimulated ATPase activity. The metals to be tested include copper, cadmium, cesium, cobalt, chromium, lead, manganese, molybdenum, nickel and zinc.

Progress:
We have successfully expressed AXA2p in yeast and shown that it accumulates in a yeast endomembrane system. However, at present we have be unable to detect any metal stimulated ATPase activity, using Cu(I), Cu (II), Zn, Co, Cd, Fe, Mn, or Mo. In parallel studies (partially supported by this grant), we have successfully demonstrated Mn^{2+} and Ca^{2+}-pumping activities for two other P-type ATPases identified in plants.

Continuation Plan:
This biochemical approach will be continued in two directions: 1) We will test whether any of the above metals stimulates a phospho-intermediate in AXA2p. This would indicate that a specific metal was recognized by the pump. Although this assay is similar to the ATPase assays already employed, it may avoid the complication that even the correct heavy metal will inhibit the ATPase activity of the pump when used at an "inappropriately high" concentration. 2) We will exhaustively analyze all possible assays for the specific metals showing changes in uptake properties in an AXA2 "knock-out" plant (see below).

2) Determine whether a deletion of the C-terminal or N-terminal domain will generate a hyper-active pump. The approach is to express deletion mutants in yeast, and to evaluate their metal stimulated ATPase activity in purified membrane vesicles. The hypothesis being tested is that an autoinhibitor is located in the N- or C-terminal domain.

Progress:
We have engineered and successfully expressed C- and N-terminal truncations in yeast. However, as with the full-length enzyme, we have been unable to detect an ATPase activity. Nevertheless, our preliminary results indicate that a C-terminal truncation mutant does appear to provide a growth phenotype in yeast.

Continuation Plan:
The exciting result that yeast growth properties can be altered by a mutant AXA2 will be pursued to obtain functional evidence to test the hypothesis that the pump translocates copper, and is regulated by a C-terminal autoinhibitor.

3) **Determine if heavy metal uptake can be increased in a transgenic plant by expressing a hyper-active heavy metal pump**. The approach is to transform plants with an AXA2 mutant in which the putative autoinhibitory domain has been disrupted.

Progress:
The most significant progress has been the identification of the first "knock-out" of a heavy metal pump in plants. We are now characterizing a homozygous plant line harboring a T-DNA insertion in the N-terminal end of the AXA2 coding sequence (i.e. "knock-out"). In addition, we have obtained 4 plant lines which are over-expressing AXA2p, as determined by immunodetection on Western blots.

Continuation Plan:
The knock out and over-expression plants should allow us to evaluate the role of this pump in heavy metal ion accumulation. Plants are currently being grown for ion measurements using an ICP-coupled Mass Spectrometry. If any of the plants show changes in the accumulation of a specific heavy metal, we will refocus our biochemical investigations to exhaustively study that metal. At present, our working hypothesis is still that AXA2 is an uptake pump for copper or molybdenum.

Summary.
We are making significant progress towards our original three specific aims. In addition, the identification of an AXA2 knock-out plant line provides an unprecedented opportunity to study the role of heavy metal pumps in the uptake and accumulation of metals in plants.

We therefore request continued funding.

Thank you for your support.

Sincerely

Jeff Harper