1998

Isolation of arsenic-accumulating mutants of Arabidopsis thaliana

Phuong T. Nguyen

University of Wollongong
NOTE

This online version of the thesis may have different page formatting and pagination from the paper copy held in the University of Wollongong Library.

UNIVERSITY OF WOLLONGONG

COPYRIGHT WARNING

You may print or download ONE copy of this document for the purpose of your own research or study. The University does not authorise you to copy, communicate or otherwise make available electronically to any other person any copyright material contained on this site. You are reminded of the following:

Copyright owners are entitled to take legal action against persons who infringe their copyright. A reproduction of material that is protected by copyright may be a copyright infringement. A court may impose penalties and award damages in relation to offences and infringements relating to copyright material. Higher penalties may apply, and higher damages may be awarded, for offences and infringements involving the conversion of material into digital or electronic form.
Isolation of Arsenic-Accumulating Mutants of *Arabidopsis thaliana*

A thesis submitted in fulfilment of the requirement of the award of the degree of

HONOURS MASTER OF SCIENCE

from

THE UNIVERSITY OF WOLLONGONG

by

PHUONG T.M. NGUYEN

Bachelor of Science
(University of Hanoi, Vietnam)
Master of Science
(University of Wollongong, Australia)

DEPARTMENT OF BIOLOGICAL SCIENCES

-1998-
Chapter 1: LITERATURE REVIEW

1.1 ARSENIC IN THE ENVIRONMENT AND ITS EFFECTS ON ORGANISMS

1.1.1 The Chemistry of Arsenic Compounds

1.1.1.1 Inorganic Arsenic Compounds

1.1.1.2 Organic Arsenic Compounds

1.1.2 Environmental Transfer of Arsenic

1.1.3 Arsenic Status in soil

1.1.3.1 Arsenic Adsorption in soils

1.1.3.2 Biotransformation of Arsenic Compounds

1.1.4 Hazards to Living Organisms from Arsenic

1.2 ARSENIC CONTAMINATION SURROUNDING CATTLE TICK DIP SITES

1.3 REMEDIATION OF HEAVY METALS AND METALLOIDS

1.4 PHYTOREMEDIATION
1.4.3 Phytostabilisation

1.4.4 Cost for Phytoremediation

1.5 MECHANISMS OF ARSENIC RESISTANCE IN ORGANISMS

1.5.1 Efflux Mechanisms of Resistance in Prokaryotes and Eukaryotes

1.5.1.1 Bacteria

1.5.1.2 Yeast

1.5.1.3 Mammalian Cells

1.5.1.4 Plants

1.5.2 Metallothioeins and Phytochelatins

1.5.3 Biomethylation of Arsenic by plants and animals

1.5.4 Arsenic-Binding Proteins

1.5.5 Heavy Metal Resistance of Plants

1.6 THE BIOLOGY OF HEAVY METAL ACCUMULATION BY PLANTS

1.6.1 Root Uptake

1.6.2 Transport Within Plants

1.6.3 Arsenic Uptake and Accumulation by Plants

1.7 AIMS OF THESIS

Chapter 2: MATERIAL AND METHODS

2.1 PLANT MATERIALS

2.2 SEED GERMINATION

2.3 GROWTH OF SEEDLINGS

2.4 MEASUREMENTS OF ROOT LENGTH

2.5 INDEX OF TOLERANCE (IT)

2.6 VERTICAL MESH TRANSFER TECHNIQUE (VMT)
Chapter 3: CHARACTERISATION OF THE RESPONSE OF THREE EMS MUTANTS IN RESPONSE TO ARSENIC STRESS

3.0 INTRODUCTION.................................................................41

3.1 EFFECTS OF ARSENIC ON ROOT GROWTH OF EMS MUTANTS.........................................................41

3.1.1 Effect of As(V) on Root Growth of wt, and pho1, pho2, man1 Mutants.................................................41

3.1.2 Effect of As(III) on Root Growth of pho2 Mutants...........................................................................42

3.2 SHOOT DEVELOPMENT OF wt and pho2
IN ARSENIC SOLUTION

3.3 ARSENIC ACCUMULATION IN THE ABOVE-GROUND BIOMASS OF pho2 MUTANT

3.4 $^{73}$As(V) UPTAKE BY SEEDLINGS OF pho2

Chapter 4: SCREENING OF ARABIDOPSIS THALIANA T-DNA INSERTION MUTANTS IN RESPONSE TO ARSENIC STRESS

4.0 INTRODUCTION

4.1 SCREENING FOR ARSENIC TOLERANCE IN MUTANTS IN OF A. THALIANA GENERATED BY T-DNA INSERTION

4.2 VISIBLE PHENOTYPE OF as1 AND as2 MUTANTS

4.3 EFFECT OF As(V) ON ROOT GROWTH OF as1 AND as2 MUTANTS

4.4 ARSENIC ACCUMULATION IN as1 AND as2 MUTANTS

4.5 ARSENIC ISOTOPE UPTAKE BY GROWING SEEDLINGS

4.6 PHOSPHATE ACCUMULATION IN as1 AND as2

Chapter 5: MOLECULAR CHARACTERISATION OF as1 AND as2 MUTANTS

5.1 ISOLATION OF T-DNA TAGGED GENE(S) IN MUTANTS

5.2 SEQUENCING OF as1 T-DNA FLANKING FRAGMENT

5.3 DATABASE SEARCH OF 5LB1 SEQUENCE
Chapter 6: DISCUSSION

6.1 METHODS USED IN SCREENING FOR ARSENIC TOLERANCE MUTANTS..........................66
6.2 RESPONSE OF \textit{pho2} MUTANT TO ARSENIC STRESS.............67
6.3 ARSENIC TOLERANT MUTANTS IN T-DNA INSERTION \textit{Arabidopsis} .............................................................69
6.4 ISOLATION OF \textit{asl} DNA FLANKING FRAGMENT..................71
6.5 FUTURE DIRECTIONS.................................................................72
6.6 POSSIBLE APPLICATION OF As RESISTANCE TRAIT TO ENVIRONMENTAL SCIENCE AND AGRICULTURE........73

LITERATURE CITED.........................................................................74

APPENDIX..............................................................................................83
Soil contamination by chemicals has become a serious world-wide problem. The use of arsenic as a tickicide during the first half of this century has left hundreds of areas surrounding old cattle tick dip sites in Australia with heavily arsenic-contaminated soil. At present, no economical and effective technology is available to remEDIATE these sites. The use of specially selected and genetically engineered arsenic-accumulating plants may provide a cheap and effective way of removing arsenic from the soil. The generation of new plants with the ability to accumulate high amounts of arsenic requires fundamental knowledge about the molecular mechanism of arsenic uptake and storage. *Arabidopsis thaliana* has been used as a model plant for this study.

Evaluation of the tolerance to arsenic (As) of the *A. thaliana* mutants *pho1*, *pho2*, and *manl* (generated by ethyl methylsulfonate-EMS treatment) was carried out. The results showed that *pho1* and *manl* were sensitive to As(V) while *pho2*, a phosphate accumulator, had increased tolerance to the As(V) form of arsenic but was more sensitive to As(III) during the germination stage. During later stages of growth, shoot development of *pho2* grown hydroponically and in soil watered with 10 ppm As(V) without phosphate was significantly inhibited compared to the wild-type. Arsenic toxicity symptoms were observed in the shoots of the *pho2* mutant. On the basis of measured arsenic content in the above-ground biomass and the distribution of $^{73}\text{As(V)}$ uptake in seedlings by autoradiography, it was demonstrated that more As(V) was taken up and accumulated in the *pho2* mutant.

More than 8,000 T-DNA insertion mutant lines were screened. Two mutants, *asl* and *as2*, with enhanced root tolerance characteristics towards arsenate have been identified. Other phenotypic differences between these mutants and the wild-type were characterised. The chlorophyll content was higher than the wild-type in mutant *asl* but lower in mutant *as2*. The above-ground parts of these two mutants, when grown
hydroponically or in soils watered with 10 ppm As(V), contained more arsenic than the wild-type. Autoradiographic studies of $^{73}\text{As}(\text{V})$ uptake by these mutants confirmed the accumulation of As(V) in the above-ground parts. A 2.1 Kb DNA fragment flanking the T-DNA insertion tag in $asl$ was cloned and subjected to sequencing. Analysis of the left border fragment, 5LB1, revealed only weak homology to other known DNA and protein sequences, suggesting its isolation for the first time. The significance of isolation of these mutants with regard to the molecular genetic studies of arsenic as well as phosphate accumulation is discussed.
DECLARATION

I certify that this thesis contains no material which has been accepted for the award of any other degree or diploma in any other institution and no material previously published or written by another person, except where due reference is made in the text.

PHUONG T.M. NGUYEN
I sincerely thank my supervisors Associate Professor Ross McC. Lilley and Dr. Ren Zhang for their inspired advice, patience, continuous encouragement and support; for their generous help in all matters related to me as a student.

I am grateful to Dr. Emmanuel Delhaize, CSIRO, Canberra for providing EMS mutants and help in Pi determination assays, Dr. Csaba Koncz, Max-Planck-Institut für Züchtungsforschung, Germany for providing documents on vector map, Prof. B.K. Chopra for critically reading the thesis, Ms. Irina Mikheenko for her help to proceed with the project in the early time.

I thank the research students in the laboratory of Mark Walker for their assistance.

My thanks to all the staff and students in the Department of Biological Sciences at this University for creating a friendly environment for me.

Most of all, my deep thanks to my family and friends for their encouragement and support.

The financial support from the Australian Agency for International Development in the form of scholarship for the year 1996-1998 is gratefully acknowledged.
ABBREVIATIONS

Ap  ampicillin
As(III)  trivalent arsenic (arsenite)
As(V)  pentavalent arsenic (arsenate)
ATP  adenosine triphosphate
bp  base pairs
BSO  L-buthionin sulfoximine
DMA  dimethylarsonic acid
DMAA  Dimethylarsenic acid (cacodylic acid)
EMS  ethyl methylsulfonate
GSH  glutathione
It  index of tolerance
kb  kilobase
KDa  kilodalton
M  molar
MES  2-N-morpholino ethane sulphonic acid
manl  Arabidopsis manganese mutant
MMA  methylarsonic acid
MT  metallothionein
NAD  nicotinamide adenine dinucleotide
PC  phytochelatin
phol  Arabidopsis phosphate mutant
pho2  Arabidopsis phosphate mutant
Pi  inorganic phosphate
SAM  S-adenosyl methionine
T-DNA  transfer DNA
UV  ultraviolet
VMT  vertical mesh transfer technique
wt  wild-type
LIST OF FIGURES

Figure 1.1 Some Arsenic Compounds Illustrating the Range of Valency States..................................................................................................................2

Figure 1.2 Organic Arsenic Compounds in Marine Animals..................................................3

Figure 1.3 A Cyclic Transfer of Arsenic..................................................................................4

Figure 1.4 Biological and Chemical Transformations of Arsenic in The Soil..........................6

Figure 1.5 Efflux Systems for Arsenic in E. coli......................................................................17

Figure 2.1a Structure of The pGV3850:1003 T-DNA Used to Generate Fieldmann Arabidopsis Mutant Lines...........................................................................30

Figure 2.1b Structure of The pPCV6NF Hyg T-DNA Used to Generate Koncz Arabidopsis Mutant Lines..................................................................................30

Figure 2.1c Structure of The pD991 Enhancer Trap Vector Used to Generate Thomas Arabidopsis Mutant Lines........................................................................30

Figure 2.2 Arabidopsis thaliana Grown in Solution Culture.................................................31

Figure 2.3 Root Development of wt in Control and Arsenic Solution.................................32

Figure 2.4 The VMT System................................................................................................33

Figure 2.5 Root Phenotypes of Arabidopsis in VMT................................................................34

Figure 3.1 Index of Tolerance of Arabidopsis thaliana wt, and pho1, pho2 man1 Mutants to As(V).............................................................................................................42

Figure 3.2 Index of Tolerance of Arabidopsis thaliana wt and pho2 Mutant to As(III)......................43

Figure 3.3 Shoot Development of pho2 and wt in As(V) Solutions........................................44
Figure 3.4 Arsenic Distribution in The Root of *wt* and *pho2* Seedlings after Two Days As(V) Uptake .................................................................47

Figure 4.1 Representatives of Arsenic Tolerant Mutants Selected in *A. thaliana* T-DNA Insertion Lines *asl* and *as2* .........................................................50

Figure 4.2 Root Development of *wt* and *asl, as2* Mutants After 5 Days Growth in 5 ppm As(V) on Filter Paper...............................................................51

Figure 4.3 73As(V) Uptake by Seedlings of *wt, asl, as2* Mutants. after Two Days Uptake.............................................................................................54

Figure 5.1 Agarose Gel (0.8%) Electrophoresis of The Genomic DNA of Mutants Digested with HindIII and XbaI.................................................................55

Figure 5.2 Agarose Gel Electrophoresis of Plasmid Rescue DNA (pAS1) Digested with PvuII.................................................................56

Figure 5.3 Diagram of The Rescued Plasmid pAS1.........................................................57

Figure 5.4 Nucleotide Sequence and The Deduced Six Possible Amino Acid Sequences of The 5LB1 Fragment from *asl* Mutant............................................60

Figure 5.5 Amino Acid Sequence Comparison of 5LB1 from *asl* with Histidine-Rich, Metal Binding Protein, HPN_HEPLY from *Helicobacter pylori*, MBP ..................................................................................64

Figure 5.6 Amino Acid Sequence Comparison of 5LB1 from *asl* with Carbon Catabolite Depressing Protein Kinase, SNF1_YEAST........................................65
### LIST OF TABLES

**Table 1.1** Distribution of Arsenic in The soil Profile at A Typical Cattle Tick Dip Site ................................................................. 9

**Table 1.2** Comparison of Biological Remediation Technologies of Contaminated Soil ......................................................................................................... 10

**Table 1.3** Differential Metal Uptake by Plants .............................................................................................................................................. 12

**Table 1.4** Metal Concentration in Known Hyperaccumulators ........................................................................................................... 13

**Table 1.5** Comparative Cost for Treatment of Contaminated Soil ...................................................................................................... 15

**Table 1.6** Comparative Cost of Treatment Methods for Copper-Contaminated Water ....................................................................................... 15

**Table 3.1** Arsenic Contents in The Shoot of wt and pho2 Mutant Grown in Soil and in Hydroponic Culture Containing As(V) .............. 45

**Table 3.2** As(V) Activity in The Shoots of wt and pho2 ................................................................................................................................. 46

**Table 4.1** Chlorophyll Contents in The Leaves of wt and asl, as2 Mutants .............................................................................................................. 49

**Table 4.2** Root Lengths of wt, asl, and as2 after 5 Days Growth in As(V) Solution ................................................................................................. 51

**Table 4.3** Arsenic Contents in The Above-ground Biomass of wt and asl, as2 Mutants Grown in Soil and Hydroponic Culture Containing As(V) ........................................................................................................ 52

**Table 4.4** As(V) Activity in The Above-Ground Parts of wt and asl, as2 Mutants ........................................................................................................ 53

**Table 4.5** Phosphate Contents in The Leaves of wt and pho2, asl, as2 Mutants ........................................................................................................ 54

**Table 5.1** Nucleotide Sequence Comparison of 5LB1 Fragment from asl and Other Known Genes ........................................................................ 60

**Table 5.2** Amino Acid Sequence Comparison of 5LB1 Fragment
from asl and Other Known Proteins ................................................................. 62

Table 5.3 Comparison of 5LB1 with Some Known Proteins ............................ 64
Chapter 1

LITERATURE REVIEW
1.1 ARSENIC IN THE ENVIRONMENT AND ITS EFFECTS ON ORGANISMS

Arsenic is a common constituent of the earth's crust and a common contaminant in metallic ores and industrial materials. Although arsenic has a number of beneficial uses in manufacturing industries, wood preservation, agriculture (food additives, pesticides), electronics (solar cells) and medicine (antisyphilitic drugs) (Nriagu, 1994), contamination of soil and water from past and present use of arsenic imposes now a serious problem to the environment and living organisms.

1.1.1 The Chemistry of Arsenic

Arsenic is classified as a metalloid and its chemistry with properties similar to those of other group V elements such as phosphorus. Arsenic is more metallic (ie, more labile in behaviour) than phosphorus. Arsenic can exist in a range of valency states ranging from -3 to +5 (Fig. 1.1). Elemental arsenic and arsine (-3) can exist in strongly reducing environments. Under moderately reduced conditions, arsenite (+3) may be the dominant form but in oxygenated environments, arsenate (+5) is the stable oxidation state (Vaughan, 1993).

Arsenic is a ubiquitous element in the environment. However, its concentrations may be higher in certain areas as a result of weathering and anthropogenic processes such as metal refining, fossil fuel combustion and pesticide use. Arsenic is a mobile element which continuously changes its form and location in the environment. The rates of change depend on the substrate in which it is found and the dynamic balance of physical, chemical, biological and geological processes acting on individual arsenic species (Nriagu, 1994).
1.1.1.1 Inorganic Arsenic Compounds

The most important commercial arsenic compound is arsenic trioxide ($\text{As}_2\text{O}_3$). It dissolves readily in alkali to form arsenite. Sodium arsenate and sodium arsenite are highly soluble in water and may change valency state in solution as well as in the presence of substances that may be reduced or oxidised. Arsenate binds more strongly to soil than arsenite (Nriagu, 1994). Under aerobic conditions there is increasing oxidation of arsenite to arsenate. The arsenate is more readily desorbed from complexes and is available for biological reduction and methylation to form volatile alkylarsines. The release of these gases from the soil is an important mechanism for the loss of arsenic from contaminated soils (Vaughan, 1993).

1.1.1.2 Organic Arsenic Compounds

There are a large number of compounds that possess carbon arsenic bonds. These arsenoarsenicals exist in both trivalent and pentavalent states (Fig. 1.2). Methylarsenic compounds, such as di- and trimethylarsines, occur naturally as a consequence of biological activity. In aqueous solution, these species may undergo oxidation to the
corresponding methylarsenic acids. Methanearsonic acid is a difunctional acid that forms soluble salts with alkali metals. Dimethylarsenic acid (cacodylic acid), also forms fairly soluble alkali metal salts. These and other higher organic arsenic compounds such as arsenobetaine and arsenocholine, which are found in marine organisms, are very resistant to chemical degradation (Cannon et al, 1981).

Figure 1.2 Some Organic Arsenic Compounds (Cannon et al, 1982).

1.1.2 Environmental Transfer of Arsenic

Numerous cycles for arsenic transformation in the environment have been proposed. A simplified, comprehensive cycle has been diagrammed by Nriagu (1994) (Fig. 1.3).

The major components of this cycle are gasses (volatiles), mining and smelting, biota (animals, men, plants and microbes), pesticides, waters, oceans, soils, rocks and sediments, and nonagricultural materials (fossil fuels, industrial and municipal wastes).
1.1.3 Arsenic Status in Soils

1.1.3.1 Arsenic Adsorption in Soils

Arsenic compounds bind strongly to soils to an extent dependent on their chemical form and the soil composition (Nriagu, 1994). Absorption of arsenic compounds is collated with the clay content of the soil, soil texture, time since exposure and soil pH (Marin et al, 1993). Heavier soils with higher clay content absorb more arsenic. Arsenic compounds also react with other elements in solution especially Fe, Al, Ca, Mg and in some soils, Mn and Pb.
Phosphate and arsenate exhibit similar physico-chemical behaviour in soils and compete directly for sorption sites on soil particles (Vaughan, 1993). In lead arsenate-contaminated orchard soils, phosphate promoted the release of arsenate. However, since arsenic is more labile than phosphate it can undergo valency state changes over a range of redox conditions and does not combine with organic matter in soils.

1.1.3.2 Biotransformation of Arsenic Compounds

Biological transformations are probably the most important environmental reactions of arsenic. Biomethylation and bioreduction are particularly important because they can produce organometallic species that are stable enough to be mobile in air and water (Fig. 1.4).

Oxidation and reduction of arsenic occur in many bacteria such as *Bacillus arsenoxydans* and *Pseudomonas fluoresces*. The reduction reactions include the conversion of arsenate or arsenite to the gaseous arsine (AsH$_3$) by bacteria such as *Pseudomonas* and *Alcaligenes* (Vaughan, 1993).

The methylation of arsenic to form the volatile alkylarsines is an important reaction in the redistribution and cycling of arsenic in the environment. The biomethylation of arsenic involves an activated methionine intermediate, S-adenosyl methionine (SAM) as a biochemical basis for the synthesis of trimethylarsine. This reaction occurs by direct transfer of a methyl group from methionine. The methylation process of arsenic in soils can be carried out by fungi such as *Candia humicola* and by bacteria such as *Methanobacterium* (Honschopp *et al*, 1996).
1.1.4 Hazards to Living Organisms from Arsenic

Biochemical mechanisms for arsenic toxicity fall into two distinct groups corresponding to As(V) and As(III) (Breed et al, 1996).

Pentavalent arsenic possesses the potential to uncouple oxidation by substitution of arsenate for phosphate. Many experiments have demonstrated that high-energy bonds of adenosine triphosphate (ATP) are not conserved in the presence of arsenate. Arsenate uncouples oxidation and phosphorylation by forming the highly labile acyl arsenate (Stryer, 1995).

Trivalent arsenic leads to enzyme inhibition. It inhibits the reduction of nicotinamide adenine dinucleotide (NAD) by deactivating critical enzymes in the tricarboxylic acid cycle (Salt et al, 1995). It complexes with two sulfhydryl groups in the same protein molecule, thereby forming a stable ring structure that is not easily ruptured by monothiols. This leads to inhibition if the protein is an enzyme. Furthermore, arsenite inhibits an unusually large, reticulocyte-specific enzyme which is important in covalent
conjugation of ubiquitin to intracellular proteins. The conjugation provides the signal for
degradation by the 26S protease (Berleth and Pickart, 1996). Therefore, arsenic may be
doubly toxic to cellular respiration by inhibiting energy-linked functions of the
mitochondria.

In mammals, many arsenicals cause toxic injury to cells of the nervous systems, blood
vessels, livers, kidney and other tissues once they are absorbed. Many enzymes can be
inactivated by arsenic (Lee and Ho, 1994). Recently, arsenic was reported to be
genotoxic to mammalian cells in vitro and is regarded as a human carcinogen (Hartwig et
al, 1997). The role of arsenicals in induction of chromosomal aberrations in cultured
human fibroblasts was demonstrated by Oyaokta et al (1996). They found that arsenic
compounds induced mainly chromatid gaps and chromatid breaks.

In plants, studies by Nriagu (1994) on the effects of different forms of arsenic
compounds on rice growth showed that the sodium and calcium salts of arsenic were the
most toxic. Arsenite is more inhibitory to crop yield than arsenate (Jiang and Singh,
1994). Arsenic exposure resulted in a drastic decrease in plant growth parameters
(Carbonellbarrachina et al, 1995; Carbonellbarrachina et al, 1998). High environmental
arsenic concentration appeared to have negative impacts on Typha latifolia growth as
indicated by decreased stand height, necrosis of leaf tips and reduced micronutrient
concentration of Cu, Mn and Zn in root tissues (Dushenko et al, 1995; Jiang and Singh,
1994). Many reports have shown inhibition of growth and phosphorus metabolism in
algae with abnormal changes in algal cell morphology by arsenic (Neff, 1997; Sanders,
1980). In canola, arsenic caused chlorosis, wilting and stunted growth symptoms (Cox
et al, 1996). Arsenic has also been found to reduce the chlorophyll content,
chlorophyllase activity and the nitrate reductase activity in greening maize leaf segments
(Jain and Gadre, 1997).
1.2 ARSENIC CONTAMINATION SURROUNDING CATTLE TICK DIP SITES

Arsenic contamination is an environmental problem in Australia because of its long-term use as a pesticide for sheep and cattle ticks (Masciantonio, 1996; William, 1995). The problem arose initially with the introduction of cattle tick late last century and arsenic was used subsequently up to mid 1950s as an effective anti-tick agent. The control of ticks in New South Wales has been achieved through a pest management program administered by the Board of Tick Control under the auspices of New South Wales Department of Agriculture (Van Zwieten, 1995; McDougall, 1996).

In the 1980's, it became clear that residues of arsenic persisted in the dip sites even though the chemicals had long gone out of use. The New South Wales Department of Agriculture undertook a survey of 100 cattle tick dip sites with results showing high levels of arsenic in many of these (Table 1.1). Contamination was found to be extensive around the cattle dip yards with very high concentrations of residues occurring on both sides of the dip bath. The areas immediately surrounding the dip were highly contaminated with arsenic and were considered to be of major concern to the environment and human health. Concentrations of arsenic in these sites exceeding 1g/kg soil are common. The potential problems faced at these former sites include ingestion of contaminated soil by children and pets and release of arsenic into surface and ground water. Under aerobic conditions, most of the arsenical pesticides have been converted to arsenate in the soils. The removal of soil by erosion may cause an increase in the concentration of arsenic in the sediments of streams, lakes and dams. The arsenic would then be available for uptake by aquatic biota, particularly sediment-dwelling organisms. This arsenic can be passed on to other organisms through the food chain. Urban growth into areas that were previously agricultural now poses an especially serious problem where there are no records of old dip sites.
So far, the environmental impacts of dip site contaminants have not yet been determined. In May, 1991, the Federal and State governments jointly announced the formation of the Cattle Tick Dip Site Management Committee (DIPMAC) to investigate this problem and advise the Government. The issues addressed by DIPMAC include the rehabilitation of dip sites and the development of strategies for the responsible disposal of dip site materials and wastes.

Table 1.1 Distribution of Arsenic in The Soil Profile at A Typical Cattle Dip Site (McDougall, 1996)

<table>
<thead>
<tr>
<th>Soil Depth (cm)</th>
<th>Average Residue (mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uncontaminated Area</td>
</tr>
<tr>
<td>0-25</td>
<td>484</td>
</tr>
<tr>
<td>50-75</td>
<td>42</td>
</tr>
<tr>
<td>100-125</td>
<td>3</td>
</tr>
</tbody>
</table>

1.3 REMEDIATION OF HEAVY METALS AND METALLOIDS

1.3.1 Remediation Technology

A variety of technologies are currently available to treat soils contaminated with hazardous materials including physical, thermal, and chemical technologies (Table 1.2). The non-biological techniques often require special equipments and operators, are expensive, can remove biological activity from the soil and can deleteriously affect the soil physical properties (Kelly, 1995).
Table 1.2 Comparison of Different Remediation Technologies of Contaminated Soil
(Mohammed et al, 1996).

<table>
<thead>
<tr>
<th>Remedial Technology</th>
<th>Relative Cost</th>
<th>Limitation</th>
</tr>
</thead>
<tbody>
<tr>
<td>In situ treatments</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Volatilisation</td>
<td>low</td>
<td>Volatile organic compounds only</td>
</tr>
<tr>
<td>Leaching</td>
<td>moderate</td>
<td>uncommon procedure</td>
</tr>
<tr>
<td>Vitrification</td>
<td>high</td>
<td>high cost</td>
</tr>
<tr>
<td>Passive remediation</td>
<td>low</td>
<td>variable performance</td>
</tr>
<tr>
<td>Isolation-containment</td>
<td>low to moderate</td>
<td>contaminants remain</td>
</tr>
<tr>
<td>Ex-situ-aboveground treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thermal treatment</td>
<td>high</td>
<td>special facilities required</td>
</tr>
<tr>
<td>Ground water treatment</td>
<td>low to moderate</td>
<td>long time frame is common</td>
</tr>
<tr>
<td>Chemical extraction</td>
<td>high</td>
<td>uncommon procedure</td>
</tr>
<tr>
<td>Excavation and landfill</td>
<td>moderate to high</td>
<td>long-term liability</td>
</tr>
</tbody>
</table>

1.3.2 Bioremediation

The term bioremediation encompasses all biological, though mainly refers to microbiological, methods for cleaning contaminated land and water. Biological methods are currently receiving much favourable publicity as promising treatment technologies for the remediation of hazardous waste sites (Anderson et al, 1993; Mohammed et al, 1996).

A major advantage of bioremediation is that it is a natural process. Another advantage is that bioremediation is theoretically useful for the complete removal of a wide variety of contaminants (Mohammed et al, 1996). Bioremediation may cost between $20,000-100,000 per contaminated site depending on whether in situ or ex situ approaches are employed, while thermal desorption or solvent washing will cost between $250,000-500,000 per site (Van Zwieten and Grieve, 1995). It is therefore, not surprising that
bioremediation is receiving widespread public attention and is the subject of much applied and basic research.

Recently, there has been some research into the use of bacteria and algae for the bioremediation and recovery of heavy metals from aqueous streams. Commercial applications of this research are still hampered by the high cost of growing pure cultures of cells and microorganisms and by the need for their immobilisation or separation from the aqueous streams. Metal-accumulating fungi and *Azolla filiculoides*, an aquatic fern, have been also proposed as metal biosorbers capable of remediating industrial effluents. However, the efficiency of metal removal by these plants is low because of their small size and small, slow-growing roots (Raskin *et al.*, 1994).

Although bioremediation has several important and distinct advantages for treating contaminated soils, it has been less successful with some compounds, especially heavy metals (Alexander, 1981; Wackett, 1994).

At present, no economical and reliable technology is available to remediate soil contaminated with heavy metals, including soil contaminated with arsenic.

1.4 PHYTOREMEDIATION

Plants that tolerate and accumulate toxic metals in contaminated soil and water have the potential to provide an efficient way of cleaning up this type of contamination. The basis of the principle of phytoremediation is that pollutants are accumulated or modified by the plant, leaving the soil or water with only minimal residual levels of the pollutants (Cunningham and Lee, 1995; Schnoor *et al.*, 1995; Raskin *et al.*, 1997). Phytoremediation is under development to overcome some of the shortcomings of bioremediation and other classical waste removal methods. This technology has emerged very recently and is not yet widely accepted by regulatory agencies and therefore is not
commonly used. There are three distinct applications of phytoremediation: phytoextraction, rhizofiltration and phytostabilization.

1.4.1 Phytoextraction

Phytoextraction is the utilisation of metal-accumulating plants, to transport metals from the soil and concentrate in the root and shoot. The ability of plants to accumulate heavy metals varies greatly between species and cultivars within a species (Table 1.3). Brassica species were found by Dushenkov et al, (1995) to be the best accumulators of lead, in which lead was concentrated in the shoot. In addition, these plants also accumulate other metals such as Zn, Cu, Mn and have a high biomass, a desirable characteristic for phytoextraction.

<table>
<thead>
<tr>
<th>Species</th>
<th>Metal</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agrostis capillaris</td>
<td>Zn</td>
<td>Shaw, 1990</td>
</tr>
<tr>
<td>A. stolonifera</td>
<td>Cu, Zn, As</td>
<td>Nriagu, 1994</td>
</tr>
<tr>
<td>A. tenuis</td>
<td>As, Cd</td>
<td>Nriagu, 1994</td>
</tr>
<tr>
<td>A. gigantea</td>
<td>Cu, Zn, Pb</td>
<td>Shaw, 1990</td>
</tr>
<tr>
<td>Festuca oviva</td>
<td>Pb</td>
<td>Shaw, 1990</td>
</tr>
<tr>
<td>F. rubra</td>
<td>Ni, Cd, Pb</td>
<td>Salt et al, 1995</td>
</tr>
<tr>
<td>Silene burchelli</td>
<td>Cu, Co</td>
<td>Shaw, 1990</td>
</tr>
<tr>
<td>Vand cerulescens</td>
<td>Zn, Ni, Cd, Cu</td>
<td>Raskin et al, 1994</td>
</tr>
<tr>
<td>Eichonia craapie</td>
<td>As</td>
<td>Nriagu, 1994</td>
</tr>
</tbody>
</table>

In Ukraine, mustard plants were found to extract radioactive caesium from the soil that was so heavily contaminated by the nuclear reactor disaster there that crops grown in it are poisonous (Bishop, 1995).
Some plants have been identified as hyperaccumulators in the above-ground parts (Table 1.4). However, there are few reports in the literature on arsenic accumulation in plants. *Agrostis tenuis*, *Agrostis stolonifera* and *Cynodon dactylon* growing on smelter wastes in Southeast England can accumulate arsenic up to 1% dry weight (Benson et al., 1981; Nriagu, 1994). *A. tenuis* accumulated a foliage concentration of between 2080 and 3470 mg arsenic/kg dry weight. These plants are promising candidates for phytoremediation of arsenic-contaminated sites.

<table>
<thead>
<tr>
<th>Metals</th>
<th>Plant Species</th>
<th>Concentration in Shoot Material from Plants Grown in Contaminated Soil (dry weight basis)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cd</td>
<td><em>Thlaspi caerulenscens</em></td>
<td>1,800 mg kg(^{-1}) in shoots</td>
</tr>
<tr>
<td>Cu</td>
<td><em>Ipomoema alpina</em></td>
<td>12,300 mg kg(^{-1}) in shoots</td>
</tr>
<tr>
<td>Co</td>
<td><em>Haumaniastrum robertii</em></td>
<td>10,200 mg kg(^{-1}) in shoots</td>
</tr>
<tr>
<td>Pb</td>
<td><em>T. rotundifolium</em></td>
<td>8,200 mg kg(^{-1}) in shoots</td>
</tr>
<tr>
<td>Mn</td>
<td><em>Macadamia neurophylla</em></td>
<td>51,800 mg kg(^{-1}) in shoots</td>
</tr>
<tr>
<td>Ni</td>
<td><em>Psychotria douarrei</em></td>
<td>47,500 mg kg(^{-1}) in shoots</td>
</tr>
<tr>
<td>Zn</td>
<td><em>T. caerilenscens</em></td>
<td>51,600 mg kg(^{-1}) in shoots</td>
</tr>
</tbody>
</table>

1.4.2 Rhizofiltration

This technology exploits plant roots to absorb, concentrate and precipitate toxic metals from polluted water. Roots of many hydroponically grown terrestrial plants such as *Brassica juncea*, sunflower, and various grasses, effectively remove toxic metals such as Cu\(^{2+}\), Cd\(^{2+}\), Cr\(^{2+}\), Ni\(^{2+}\), Pb\(^{2+}\) and Zn\(^{2+}\) from aqueous solution (Dushenkov et al., 1995). The roots of *B. juncea* can concentrating these metals 131-563 fold above the initial
solution concentration (Dushenkov et al, 1995). Tomato and bean plants can also extract arsenic from solution (Carbonellbarrachina et al, 1996).

Rhizofiltration is particularly effective and economically compelling when low concentrations of contaminants and large volumes of water are involved. Therefore, this method may be particularly applicable to radionuclide contaminated water. Uptake of radionuclides by plants is not well studied, but Salt et al (1995) obtained results suggesting that many actinic and anion radionuclide contaminants can be substantially or completely removed from water by selected plants, cultivated in a specially developed and optimised rhizofiltration system (Entry, 1996).

1.4.3 Phytostabilization

Plants can eliminate the bioavailability of toxic metals in soils. Heavy metal polluted soils usually lack established vegetation cover due to the toxic effects of these pollutants or recent physical disturbance. Barren soils are more prone to erosion and leaching which further spreads the pollutants in the environment. Revegetation with metal-tolerant plant species is a simple solution to the stabilisation of these wastes. By extensive fertilisation and planting of the endemic metal-tolerant varieties, researchers were able to stabilise contaminated sites and establish a dense vegetative cover. For example, Agrostis gigantea has been used for acid Pb/Zn waste; Festuca rubra or Festuca merlin for acid Pb waste and Agrostis stolonifera for Cu waste (Salt et al, 1995). The potential for reducing the leaching of metals from soils into the ground water using heavy metal accumulating plants has been investigated using B. juncea. Seedlings grown in the sand-perlite mixture containing 625 mg/kg Pb were able to reduce the Pb level in the leachate from 740 mg/ml to 22 mg/ml (Nriagu, 1994). The reduction of metal leaching may be due to the formation of an insoluble oxidation state. Evidence obtained by X-ray absorbance spectroscopy suggests that roots of B. juncea are able to reduce available and
toxic Cr(VI) to unavailable and less toxic Cr(II). Thus, the phytostabilizing plants are not only tolerant to high levels of heavy metals but also immobilise these metals in the soil.

1.4.4 Cost for Phytoremediation

The costs for phytoremediation methods are much lower than other remediation methods (Table 1.5 and 1.6).

<table>
<thead>
<tr>
<th>Table 1.5</th>
<th>Comparative Cost for Treatment of Contaminated Soil (Salt et al, 1995).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Treatment</td>
<td>Cost/m^3 (US$)</td>
</tr>
<tr>
<td>Fixation</td>
<td>90-200</td>
</tr>
<tr>
<td>Landfilling</td>
<td>100-400</td>
</tr>
<tr>
<td>Soil extraction, leaching</td>
<td>250-500</td>
</tr>
<tr>
<td>Phytoextraction</td>
<td>15-40</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 1.6</th>
<th>Comparative Cost of Treatment Methods for Copper- Contaminated Water.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Treatment</td>
<td>Cost/Pound of Copper (US$)</td>
</tr>
<tr>
<td>Precipitation and flocculation</td>
<td>40</td>
</tr>
<tr>
<td>Ion exchanges</td>
<td>15</td>
</tr>
<tr>
<td>Microfiltration</td>
<td>50</td>
</tr>
<tr>
<td>Rhizofiltration</td>
<td>1-5</td>
</tr>
</tbody>
</table>

Data taken from Raskin et al, (1994)
To develop an efficient phytoremediation procedure for arsenic, it is necessary for us to have a deep understanding of the factors governing tolerance, uptake and accumulation of this element in plants which is virtually unknown. Research in these aspects is for all heavy metals and metalloids still in its infancy.

1.5 MECHANISMS OF ARSENIC RESISTANCE IN ORGANISMS

1.5.1 Efflux Mechanisms of Resistance in Prokaryotes and Eukaryotes

Prokaryotic and eukaryotic organisms have evolved an ingenious set of efflux pumps to provide resistance to arsenic. In both prokaryotes and eukaryotes there are transport systems that confer resistance to toxic metals by extrusion from the cell, reducing the intracellular concentration to subtoxic levels (Tsai et al, 1997). The mechanisms include active transport of the toxic metal, reduction to a less toxic form recognised by an efflux system and sequestration (Rosen, 1996).

1.5.1.1 Bacteria

Arsenical-resistance determinants are widely distributed in bacteria. They are found in both gram positive and gram negative bacteria, located on both plasmids and chromosomes (Diorio et al, 1995; Silver and Phung, 1996; Ji and Silver, 1992a; Kalinichenko, 1996; Wu and Rosen, 1993b). The $ars$ operon on plasmids, a single cluster of genes, was responsible for the arsenical resistance phenotype. The $ars$ operon of plasmids R773 and R46 of $E.coli$ (Fig. 1.5) each has five genes: two coding for transcriptional regulators ($arsR$ and $arsD$) and three structural genes ($arsA$, $arsB$ and $arsC$). These confer only a low level of arsenical resistance (Carlin et al, 1995; Endo et al, 1995).
arsA encodes an ATPase subunit, which is a functional dimer (Hsu et al, 1991). In the absence of substrate, arsA is mostly a monomer in the cytosol. In the presence of the inducer arsenite, arsA becomes a dimer. This has a higher affinity for ATP than the monomer, and is anchored tightly to the membrane by arsB during the catalytic cycle (Tisa and Rosen, 1990; Hsu et al, 1991).

![Diagram of E. coli efflux systems for arsenic]

**Figure 1.5** Efflux Systems for Arsenic in *E. coli*. Alignment and functions (below) of arsenic resistance genes (boxes) with lengths of gene products in amino acids (aa), (above) (Endo et al, 1995)

arsB encodes an inner membrane protein (Chen et al, 1986; Wu et al, 1992) which forms an anchor for the arsA protein and serves as an anion channel (Tisa and Rosen, 1990). When arsA is present, the arsA-arsB complex functions as an obligatory ATP-driven pump which can probably pump out far in excess of the proton equilibrium, maintaining a low arsenic concentration in the cytosol, independent of the external concentration.

arsC encodes an arsenate reductase enzyme which can reduce arsenate to arsenite before excretion from the cells (Ji and Silver, 1992b). A reactive sulfhydryl group may participate in the action of arsC (Gladysheva et al, 1994). An enzyme-substrate complex was formed by arsenylating cys12 of arsC, then the complex interacted with glutaredoxin which transferred electrons to reduce As(V) to As(III). The resulting As(III) dissociated from the complex, and the reduced glutaredoxin was regenerated by the action of glutathione reductase (Liu et al, 1995).
arsR encodes a trans-acting repressor protein and can be induced by arsenate, arsenite, antimonite and bismuth (Wu and Rosen, 1991; Endo et al, 1995; Shi et al, 1996).

The arsD protein is an inducer-independent trans-acting regulatory protein which controls the upper level of expression of the ars genes and is not needed for regulating low level ars gene expression (Wu and Rosen, 1993a).

The regulatory genes arsR and arsD share a single promoter sequence (Wu and Rosen, 1991). Studies suggest that the action of arsR and arsD forms a regulatory circuit. When an inducer is absent, constitutively expressed arsR repressor dimerizes and binds to the ars operator, preventing transcription. Upon induction by arsenic, the arsR repressor is inactivated and the ars operon is derepressed. arsD is produced along with arsA, arsB and arsC. When the amount of arsD reaches a critical level, it presumably binds to a site in the promoter region and prevents further ars expression.

1.5.1.2 Yeast

Arsenic resistance has been found in certain of the yeast Saccharomyces (Bobrowicz 1997), however, the present knowledge of arsenic resistance mechanisms in yeast is limited (Tsai et al, 1997). There are no data on any specific arsenic detoxification systems. Bobrowicz (1997) reported the isolation of three contiguous genes, acr1, acr2 and acr3 involved in resistance to arsenic compounds in the Saccharomyces cerevisiae. The acr1 gene produces an arsenite and arsenate hypersensitive phenotype. The acr2 gene is indispensable for arsenate but not for arsenite resistance. Conversely, the acr3 gene confers an arsenite but not arsenate resistance phenotype. The presence of acr3 together with acr2 on a multicopy plasmid expands the resistance phenotype into arsenate.
1.5.1.3 Mammalian Cells

Resistance has been shown to involve active extrusion of As(III). It seems that arsenite is conjugated in mammalian cells to glutathione by a transferase activity, followed by extrusion of the conjugate by a transport system known as the thiol-linked efflux pump. Efflux of glutathione S-conjugant has been observed in vesicles from rat heart and liver cells. This system is a transport ATPase. Arsenite and arsenate can react nonenzymatically with glutathione (Zaman et al, 1995). However, arsenate is reduced by glutathione to arsenite and the arsenite reacts further with glutathione to form (GS)_3As. The pump would then extrude the As(III)-thiol conjugate.

1.5.1.4 Plants

Efflux mechanisms of arsenic resistance in plants are poorly understood. No reports have been published so far on these mechanisms. Whether the efflux systems for arsenic in plants are similar to other organisms is not known.

1.5.2. Metallothioneins and Phytochelatins

The proteins of superfamily called metallothionein (MT) are cysteine-rich proteins required for heavy metal tolerance in animals, fungi and higher plants (Foley, 1997). The MTs may serve in this role by binding metal ions through closely spaced cysteine thiolate groups. Overproduction of metal-binding complexes seems to account for metal tolerance (Rauser, 1990). The hypothesis being considered centres upon the action of MT to sequester and dispense metal ions, and also to have specialised functions in normal cellular metabolism. Yeast lacking MT exhibits abnormalities in heavy metal metabolism (Hamer, 1986). This observation indicated that a major function of yeast MTs is to maintain low levels of free intracellular copper. Bacterial MT homologous at the protein and functional levels to the small, thiolate-rich metal binding proteins of animal systems, have not been widely reported. To date, the only prokaryotic cells with
well-studied MT, are two cyanobacterial strains of the genus *Synechococcus*. The order of preference of cation binding for cyanobacterial metallothionein is $\text{Zn}^{2+} > \text{Cd}^{2+} > \text{Cu}^{2+}$ (Silver and Phung, 1996). In higher eukaryotes, the main function of MT with regards to cadmium is protection against long-term toxicity (Hamer, 1986). The synthesised MT is probably sufficient to chelate most or all of the cadmium ions. The high affinity of MT for cadmium and long half-life of the cadmium-containing protein, make it well suited for this detoxifying function. The primary function of MT in regard to copper is to maintain low intracellular concentrations of the free ions while at the same time permitting the activation of copper enzymes (Hamer, 1986). Under conditions of high copper exposure, induced MT would provide a highly effective detoxifying agent because of its strong avidity to the free ion. Moreover, MT also acts as a zinc storage protein that plays a true homeostatic role in the metabolism of this ion (Hamer, 1986).

In plants, a unique family of metal-binding polypeptides has been discovered. These polypeptides are classified as class III MT (apical, nontraslationally synthesised metal thiolate polypeptides) and are called phytochelatins (PC). The molecular structure is $(\text{EC})_n\text{G}_2$ (with $n$ from 2 to 11) (Grill *et al*, 1985; Robinson *et al*, 1993; Rauser, 1995; Maitani, 1996). The biosynthesis, structure and function of these peptides have been extensively reviewed. The discovery of metal binding PCs in plants led to the proposal that PCs are the functional analog of fungal and animal MT. PCs are the simplest (since they are composed of only three different amino acids) natural compounds so far reported that may be engaged in the detoxification and homeostasis of heavy metals through metal-thiolate formation. They may serve the same purpose of binding excess heavy metals through mercaptide complexes (Grill *et al*, 1985). In wheat root cells the location of class III MT was found to be in vacuoles (Robinson, 1993).

In vivo evidence has demonstrated that PCs play an important role in heavy metal detoxification. Biosynthesis of these peptides is rapidly induced by the presence of heavy metals and appears to result from the activation by heavy metals of an enzyme,
phytochelatin synthetase, whichsynthesises the $(EC)_nG_2$ peptides from glutathione. Use of L-buthionine sulfoximine, an inhibitor of the enzyme glutamyl-cysteine synthase, and thus of glutathione biosynthesis, has provided strong evidence that this biosynthetic pathway is necessary for heavy metal detoxification.

The isolation of a Cd$^{2+}$ sensitive (cad1) mutant of *Arabidopsis thaliana* that is deficient in PC synthase demonstrated conclusively the importance of PCs for heavy metal tolerance (Howden *et al*, 1995).

Grill *et al* (1987) studied PC induction by various metals and found that As(V) induced PC synthesis but it did not bind to PC. The relationship between induced PC synthesis and arsenic tolerance in plants are not well understood.

### 1.5.3 Biomethylation of Arsenic by Plants and Animals

Aquatic plants accumulate little arsenic in the toxic form even though most of them absorb arsenate avidly in their quest for phosphate (Knowles and Benson, 1983; Takimura *et al*, 1996). Marine algae accumulate arsenate from seawater, reduce it to arsenite, and then convert the arsenite to a large number of organo-arsenic compounds. Most of the arsenic in marine organisms exists in methylated forms. Thus, the detoxification of arsenic may involve the formation of methylated derivatives which are excreted or converted to organic compounds such as arsenolipids (Andrea and Klumpp, 1979, Vahter and Marafante, 1988). The result is an arsenic cycle with reduction and alkylation in photosynthetic aquatic plants and regeneration of the oxidised forms in non-photosynthetic organisms (Knowles and Benson, 1983). The reduced and detoxified forms of arsenic may be utilised as an energy source by bacteria. Conversion of arsenous acid to phospholipid derivatives appears to be a mechanism for membrane-mediated excretion of arsenic by marine algae. Arsenosugars and dimethylated compounds were identified as the major arsenic compounds present in marine algae and
seaweed (Edmonds and Francesconi, 1987; Kaise et al; 1988; Le et al, 1994). Arsenobetaine is ubiquitously distributed and may be the end product of arsenic cycling in marine ecosystems (Nriagu, 1994; Cullen et al, 1994). In higher plants, there has been no investigation on arsenic methylation.

In animals, trivalent inorganic arsenic is the main form of arsenic interacting with tissue constituents, due to its strong affinity to sulfhydryl groups. However, a substantial part of the absorbed As(III) is methylated in the body to less reactive and less toxic metabolites such as methylarsinic acid (MMA) and dimethylarsinic acid (DMA). These are rapidly excreted in the urine along with unchanged inorganic arsenate. The intermediate steps in arsenic biotransformation in mammals have not yet been elucidated, but several observations in humans indicate the identity of some of the enzymes involved in the methylation process. This process takes place primarily in the liver by transfer of methyl groups from SAM to As(III). A substantial part of absorbed As(V) is reduced to As(III) before being methylated in the liver (Vahter, 1994).

Thompson (1993) found that glutathione plays a very important role in methylation of arsenic in vivo. Glutathione (GSH) is required for reduction of arsenic(V) to arsenic(III) species in preparation for enzyme-catalyzed oxidative methylation. In addition, GSH is involved in dimethylation by reducing methylarsonic acid MMA(V) to methylarsonous acid MMA(III). GSH also has the general role of stabilising the reductive nature of the cell, which is required for the methylation of arsenic.

1.5.4 Arsenic-Binding Proteins

Protein binding has been proposed as the initial protective mechanism against arsenic (Vahter and Marafante, 1985; Bogdan et al, 1994) and may be the first step in the detoxification of arsenite prior to methylation. Studies in marmosets using [\(^{74}\text{As}\)] arsenite demonstrated that no methylated arsenic metabolites were excreted in the urine
Vahter et al, 1982; Vahter and Marafante, 1983). The binding of arsenite to proteins was shown to be an important pathway for the marmoset to detoxify administered inorganic arsenic. When concentrations of arsenite rapidly become too high for the methylation enzyme systems, the methylation pathway for arsenite and arsenate detoxification might be overwhelmed. Binding of arsenite by protein may decrease its metabolic availability, reducing the substrate concentration to a level suitable for the methylating enzymes. A reservoir of arsenite-bound proteins could slowly release small amounts of arsenite for methylation.

In vivo studies in rabbits by Marafante et al (1982) showed that most of the arsenic present in liver, kidney and lung tissues was bound to proteins in the cytosol. This binding occurred within 1 hour of either arsenite or arsenate administration and persisted for at least 16 hours. Appreciable amounts of methylated arsenic metabolites were not detected in rabbit urine until 2 hours after arsenite administration (Bertolero et al, 1981).

So far, no As-binding protein in higher plants has been isolated. Identification and characterisation of such proteins in plants could be important in understanding the mechanisms of arsenic detoxification in plants.

1.5.5 Heavy Metal Resistance of Plants

For the plants to resist the toxic effects of heavy metals, they must limit cellular uptake, detoxify the heavy metal after entry to the cells or develop heavy metal-resistant metabolisms. The vacuole is thought to be the site of heavy metal accumulation. Salt et al, (1995) reported that the vacuole volume of meristematic cells within the root tip of Festuca rubra increases during Zn exposure. Inside the vacuole, metal ions are thought to be chelated by organic acids such as oxalic acid in the case of aluminium detoxification in buckwheat (Ma et al, 1997) or by enzymatically synthesised γ-glutamylcysteinyl-isopeptides (γEC-isopeptides) to form chelatin (Maitani, et al, 1996). Moreover, the
precipitation of Zn as Zn-phytate or the existence of free histidine as a metal chelator in plants which accumulate Ni are other resistance responses to heavy metals (Kramer et al., 1996).

Generally, the specificity of metal uptake by hyperaccumulating plants and the biochemical and molecular bases of accumulation is poorly understood and requires further research.

1.6 THE BIOLOGY OF HEAVY METAL ACCUMULATION IN PLANTS

Plants employ two strategies for growing on metalliferous soils: exclusion and non-exclusion (Baker, 1981). Metal excluders effectively prevent metal from entering their aerial parts over a broad range of metal concentrations in the soil (Cumming and Taylor, 1990; Taylor, 1987; Zenk, 1996). However, they can still contain large amounts of metals in their roots. Metal non-excluders actively accumulate metals in their above-ground tissues. Some non-excluders (termed hyperaccumulators) can concentrate metals in their above-ground tissues to high levels, sometimes even exceeding those present in the soil. There are special mechanisms that enable these plants to accumulate heavy metals to these high concentrations. Investigations into this phenomenon have demonstrated the potential of using plants to remediate heavy metal contamination.

1.6.1 Root Uptake

Metal ions may be bound tightly to soil particles, thus making them unavailable to plants. Some plants mobilise soil-bound metals into the soil solution by secreting metal-chelating molecules such as proteins into the rhizosphere. These metal-chelating proteins are perhaps related to metallothionein or phytochelatins (Raskin et al., 1994). During uptake into root cells, the metal ions are reduced by specific plasma membrane-bound metal reductases. Additionally, plant roots solubilise heavy metals by acidifying the soil
environment with protons extruded from the roots. At low pH, "soil-bound" metal ions are released into the soil solution and these solubilised metal ions may enter the root either via extracellular (apoplastic) or intracellular (symplastic) pathways.

1.6.2 Transport Within Plants

When metals have entered the root they can either be stored there or transported to the shoot, probably via the xylem. However, metals chelated to either organic acids, phytochelatins or metallothioneins may redistribute in the shoot via the phloem (Raskin, 1994).

1.6.3 Arsenic Uptake and Accumulation by Plants

Not many investigations so far have focused on the mechanisms of arsenic uptake and distribution in plants. Recently, Carbonellbarrachina et al (1995) reported on the response of tomato plants to different levels of arsenic in nutrient solution. Arsenic was taken up and accumulated in the tomato roots, resulting in a significant decrease in the upward transport of arsenic. Arsenic distribution in different parts of tomato plants correlated with the arsenic levels in the nutrient solution. Some naturally-occurring plant species can accumulate very high arsenic levels. *Agrostis tenuis* and *Cynodon dactylon* have been found to be arsenic hyperaccumulators (Nriagu, 1994). Presumably, these species utilise special mechanisms to accumulate arsenic to high levels. At present, the mechanisms of arsenic uptake, accumulation, and storage in plants are still unanswered questions. Therefore, these species may be very useful in identification and characterisation of the enzymes and transporters that are central to the accumulation of arsenic.
1.7 AIM OF THESIS

At present, our knowledge of plant arsenic tolerance is only based on a few physiological studies of arsenic resistance in different growth conditions (Cox et al., 1996; Carbonellbarrachina et al., 1996). The biological basis is poorly understood. It is obvious that a molecular genetic approach in this area of research would help greatly in advancing an understanding.

*Arabidopsis thaliana* has been widely used as a model higher plant species in modern plant physiology because of its small genome size (80-120 Mb) (Meyerowitz and Summerville, 1994), rapid life cycle (8-12 weeks) and ease of transformation. An international network and database of *Arabidopsis* research has been established by hundreds of laboratories around the world and the international collaboration on sequencing the whole *Arabidopsis* genome is also progressing rapidly.

To date, *Arabidopsis* mutants that can accumulate or exclude metal and other ions have been generated using ethyl methylsulfonate (EMS) include *pho1* (Poirier, 1991), *pho2* (Delhaize and Randall, 1995), *man1* (Delhaize, 1996), *cad1* (cadmium-sensitive) (Howden et al., 1995), and *adr* (aluminum-resistant) (Larsen et al., 1998). The *man1* mutant can absorb not only high levels of Mn, but also Cu, Zn and Mg in the leaves. The mutants *pho1* and *pho2* are phosphate accumulators. *pho1* can accumulate a considerable amount of inorganic phosphate (Pi) in the root. In contrast, the *pho2* mutant can accumulate Pi in the stems, siliques (a special carpel) and seeds to levels three times higher than that of the wild type. It is interesting to examine these mutants in terms of their arsenic tolerance and accumulating ability. The *pho1* and *pho2* mutants are of particular interest because the arsenate uptake mechanism is thought to be similar to that for phosphate. Arsenate would seem to compete with phosphate for the phosphate uptake system and is taken up actively due to the chemical similarity of arsenate to phosphate (Macnair and Cumbes, 1987; Nriagu, 1994).
In addition, several laboratories have generated transfer DNA (T-DNA) mutagenized populations of *Arabidopsis* (Meyerowitz and Somerville, 1994, Koncz *et al*, 1989). Modified T-DNA from tumour inducting (Ti) plasmid in *Agrobacterium tumefaciens* was introduced into *Arabidopsis* using tissue culture or seed infection methods. From this work, more than 8,000 mutant lines have been produced and are now available in *Arabidopsis* stock centres. These have been utilised in this research project as the basis of a system to screen for arsenic tolerant mutants. The advantage of the use of these mutant populations is that the mutated gene can be isolated by using the T-DNA tag that marks the integration site. T-DNA insertion mutagenesis is one of those recently developed genetic techniques which already have a major impact on plant molecular biology. T-DNA insertions may inactivate or alter the expression of plant genes, the mutation of which usually results in a "loss of function" phenotype. The genetic and molecular analysis of these T-DNA induced mutations documents both the simplicity and the efficiency of this gene tagging approach. T-DNA tags provide dominant markers for genetic mapping, reporter genes for studies of transcriptional regulation, as well as molecular probes for gene isolation and complementation of mutations all in one (Schulz *et al*, 1995; Koncz *et al*, 1992; Koncz *et al*, 1990).

So far, no arsenic accumulating or excluding mutants in *Arabidopsis* have been reported. This project sets out to identify, by screening such mutants from available mutant populations of *Arabidopsis*. By isolation and analysis of arsenic accumulating and excluding mutants, we hope to identify and characterise genes responsible for arsenic resistance and to gain a better understanding of the genetic and physiological mechanisms resulting in such resistance. On the phytoremediation application front, the identified genes and resistance mechanisms identified in this project may prove useful for genetic engineering to generate plants suitable for field phytoremediation.
The aims of this thesis were to screen for arsenic accumulating mutants of *A. thaliana* which had been subjected to EMS treatment or T-DNA insertion and to isolate the gene(s) responsible for the phenotype change(s).
Chapter 2

MATERIALS AND METHODS
2.1 PLANT MATERIALS

All *Arabidopsis* mutant lines mutagenized by T-DNA insertion were obtained from the *Arabidopsis* Biological Resource Centre at the Ohio State University including 265 Koncz, 6500 Fieldman and 1800 Thomas lines. The vectors used to generate these mutant lines are shown in Fig. 2.1a, 2.1b and 2.1c. The seeds of mutants generated by EMS treatment including *pho1*, *pho2* and *manl* were provided by Dr. E. Delhaize, CSIRO, Division of Plant Industry, Canberra.

Both the EMS and T-DNA insertion mutants were derived from *A. thaliana* ecotype "Columbia" background and so *A. thaliana* Columbia was used as wild-type (wt) control.

2.2 SEED GERMINATION

Seeds were surface sterilised by soaking for 20 minutes in a 1:1 mixture of ethanol (96%): aqueous hydrogen peroxide (5%) and dried on filter paper at room temperature.

Surface sterilised seeds were sown in rows 5 mm apart on filter papers oriented horizontally in petri dishes soaked by a solution containing different concentrations of arsenic. After incubation for 24 hour, the dishes were placed vertically and kept in a culture room at a constant temperature of 23°C and light/dark periods of 16/8 hours (fluorescent illumination by Gro-Lux lamps, 100-150 mol m\(^{-2}\)s\(^{-1}\) in the 400-700 nm range).
**Figure 2.1a** Structure of The pGV3850:1003 T-DNA Used to Generate Feldmann *Arabidopsis* Mutant Lines. Left and right border sequences of T-DNA are flanking two copies of pBR322. The kanamycin resistance gene of Tn5 (1'NPT) was used for kanamycin selection in plants. The kanamycin-resistance gene of Tn903 was used for selection in bacteria (Schulz *et al.*, 1995).

**Figure 2.1b** Structure of The pPCV6NF Hyg T-DNA Used to Generate Koncz *Arabidopsis* Mutant Lines. aph and hph are aminoglycoside (kanamycin) phosphotransferase and hygromycin phosphotransferase genes, respectively. g5 and nos are promoters of the T-DNA gene 5 and the nopaline synthase gene, respectively. A4 and Anos are polyadenylation signals of T-DNA gene4 and nos. pBR is the pBR322 replicon within the T-DNA (Koncz *et al.*, 1990).

**Figure 2.1c** Structure of The pD991 Enhancer Trap Vector Used to Generate Thomas *Arabidopsis* Mutant Lines. The vector consists of the -60 CaMV minimal promoter fused to ildA (which encodes GUS) followed by the 3'untranslated region of the nopaline synthetase gene (3'NOS). In plants, kanamycin resistance (kanR) is encoded by the NPTII gene. These genes are flanked by right border (RB) and left border (LB) sequences (Seed and DNA stock list, *Arabidopsis* Biological Resource Center, 1996).
2.3 GROWTH OF SEEDLINGS

Seedlings were grown on soil or on plugs of rockwool that were dipped into aerated nutrient solution (Fig.2.2). The nutrient medium contained the following macronutrients: 1.25 mM KNO₃, 1.50 mM Ca(NO₃)₂, 0.75 mM MgSO₄, 0.75mM KH₂PO₄ and the following micronutrients: 50 µM KCl, 50 µM H₃BO₃, 10 µM MnSO₄, 2.0 µM ZnSO₄, 1.5 µM and CuSO₄, 0.075 µM (NH₄)₆Mo₇O₂₄ (pH 5.8). This formulation represents 1/3 of the macronutrient concentration and the full concentration of micronutrient recommended by Gibeau et al. (1997). To ensure that no nutrients unintentionally became limiting, solutions used in hydroponic culture were replaced weekly during the initial 14 days of growth and then every 4 days thereafter. The arsenic solutions were made by using Na₂HAsO₃ and NaAsO₂ (Aldrich Chemical) as As(V) and As(III), respectively. For seedlings grown on agar, agar (0.75%) and sucrose (2.9 mM) medium was autoclaved. Seedlings grown by hydroponic culture or on agar were grown under artificial lighting (fluorescent Gro-Lux lamps). Soils used for seedlings growth were mixed with vermiculite (2:1), packed in pots and irrigated with the nutrient solution. Seedlings were grown in the same culture room described above.

Figure 2.2 Arabidopsis thaliana Grown in Solution Culture.
2.4 MEASUREMENTS OF ROOT LENGTH

After 5 days of germination, those roots which were pointed downward in plates were measured (Fig. 2.3). The roots were stained with cotton blue solution containing 0.5g cotton blue in lactophenol solution (30%). The samples were destained with distilled water three times and dried in the fume hood. The roots became dark blue and were distinguished from the stems (no colour) and leaves (green). Roots on the seedlings without arsenic treatment were measured as control.

![Figure 2.3 Root Development of wt in Control and Arsenic Solution.](image)

**Figure 2.3** Root Development of wt in Control and Arsenic Solution.

2.5 INDEX OF TOLERANCE (I_t)

To determine the root growth rate of EMS mutants, the index of tolerance (I_t) was calculated (Shaw, 1990).

\[
I_t = \frac{\text{root growth in toxic solution}}{\text{root growth in control solution}}
\]

All experiments were repeated 3 times and mean differences of the independent measurement at each concentration point were subjected to a t-test.
2.6 VERTICAL MESH TRANSFER TECHNIQUE (VMT) FOR SCREENING T-DNA INSERTION MUTANTS

To screen T-DNA insertion mutants the VMT method developed by Murphey and Taiz (1995) was used. Glass plates (19x19mm) were mounted in a polypropylene rack holding 10 plates placed at the bottom of a polypropylene chromatography tank (Fig.2.4).

Following the initial incubation period (72 hours), the VMT plates were removed from the tank and replaced onto the plastic support. The mesh was lifted off the paper and transferred to the second paper glass plate assembly saturated with the same nutrient solution. During the transfer, the mesh was rotated 90° from its original orientation so that the roots were horizontal on the plate. The assembled VMT plate was then replaced in the same position in a tank containing fresh nutrient solution. When all of the seedlings had been transferred, the apparatus was returned to the growth room for 36 hours. At the end of this period, the roots of normal seedlings had grown sufficiently downward (5-7 mm) to form a distinct right angle. Seedlings that did not form this characteristic right angle were ignored for the remainder of the assay.

Figure 2.4. The VMT System. Seedlings were grown vertically down the surface of a nylon mesh placed on medium-saturated 3-mm chromatography paper. The mesh and paper were supported on a glass plate and mounted in a rack at the base of a covered chromatography tank (Reproduced from Murphey and Taiz, 1995).
This 90° rotation procedure was repeated again and seedlings were grown for further 36 hours. At the end of this incubation, inhibited seedlings that failed to form right angle turns were termed "no counts" and were discarded. Seedlings whose roots formed the first right angle turn but failed to form the second turn were selected or counted as "sensitive". In the "low-stringency" version of the assay, the root was considered to have formed a second turn if it extended 1 mm beyond the right angle. In the "high-stringency" assay, the roots must extend at least 2 mm beyond the second right angle to be scored or selected as "tolerant" (Fig. 2.5)

![Diagram of root phenotypes](image)

**Figure 2.5** Root Phenotypes of *Arabidopsis* in VMT

Tolerant seedlings were removed from the mesh with forceps and floated in a beaker containing nutrient solution. The seedlings were then transferred into pots containing soil and vermiculite and placed in a culture room to grow to maturity.

### 2.7 ARSENIC UPTAKE ASSAY

*Wild-type* and mutant plants were grown in 7x7 cm plastic jars containing sterilised agar medium (0.7%). Plants were grown for 12-14 days at 23°C in light /dark periods of 16/8 hours (fluorescent illumination 100-150 μmol m⁻² s⁻¹). The day before the labelling experiments, the jar lids were opened to permit the plants to adjust to the ambient RH. At the start of an experiment, the plants were gently pulled out of the agar. The roots were washed and placed in MES (2-N-morpholino ethane sulphonic acid, Sigma Chemical
Company) buffer solution used for labelling experiments containing 10 ppm of As(V) but without the radioisotopes, for 1 hour. The plants were transferred to 20 ml of an MES solution containing 0.5 μCi/ml of 73As (Los Alamos National Laboratory) and 10 ppm of As(V), incubated under 16-h light/8-h dark cycle. Care was taken to avoid contact between the shoots and the labelled solution. After 2 days, plants were rinsed with 20 ml of unlabelled experimental medium and were dried on paper. The samples were covered with a Molecular Dynamics cassette (22 x 27 cm) and exposed to Molecular Dynamics Phosphor Screen at room temperature for 16 hours. Analysis was performed using STORM 860 Phosphor Screen, ImageQuant™ software version 1.1 (Molecular Dynamics, 1995). 73As(V) levels in the shoots of the dry samples were measured using an automatic γ-counter (Beckman 1480 wizard™).

2.8 ARSENIC DETERMINATION

Plant materials were dried at 60°C and ground to powder. Acid digestion was performed in Kieldahl flasks with sulphuric and nitric acids (1:5) at 280-300°C. The digested solution was transferred to a 50 ml calibrated flask and make up to volume with water. The arsenic content was determined by hydride atomic absorption spectrophotometry (HAAS) using sodium borohydride as a reductant (Allen, 1989). Most samples of this project were analysed at the Environmental Analytical and Service Testing laboratory (EAST lab). The significant cost of analysis ($20/sample), limited the number of replicates that could be performed.

2.9 INORGANIC PHOSPHATE DETERMINATION

Inorganic phosphate was determined using acid extraction of fresh material by a method described by Delhaize and Randall (1995). One to five leaves were collected and weighted, and approximately 40 μl of 5M H₂SO₄ was added per 20 mg of fresh sample. The samples were ground in a hand-held device and then 3 ml of water were added. The
mixture was filtered (Whatman No. 4 filter paper) and a subsample, ranging from 20 µl to 1.5 ml, depending on Pi concentration, was analyzed for Pi. The subsample was made up to 1.5 ml with water, added to 0.5 ml of Malachite green reagent, and then mixed vigorously. After at least 30 minutes, the $A_{550}$ of the solution was measured. Standards in the range of 125 to 500 ng of Pi as $K_2HPO_4$ were used.

2.10 CHLOROPHYLL DETERMINATION

100 mg of fresh leaves was ground thoroughly using an ice cold mortar and pestle. Three ml 80% acetone in 25 mM HEPES (N-[2-hydroxyethyl]piperazine-N-[2-ethanesulfonic acid] (Sigma Chemicals), pH 7.5 was added. The material was transferred to Eppendorf tubes and centrifuged at 13,000 rpm for 10 minutes. Chlorophyll supernatant was collected and the absorbance was measured at 647, 664, and 750 nm using an Ultrospec III (Pharmacia LKB) spectrophotometer. Total chlorophyll ($\mu$g/g) was calculated as following:

$$[\text{Chlorophyll}] = D \{ 7.34(A_{664}-A_{750}) + 17.76(A_{647}-A_{710}) \}$$

(where $D$= total volume (ml)/fresh weight (g) of fresh leaves) (Porra et al, 1989)

2.11 IDENTIFICATION OF THE TAGGED DNA REGIONS OF MUTANTS

2.11.1 Isolation of Genomic DNA

The method described by Schulz et al. (1995) was used. Plant tissue samples from homozygous mutants were frozen in liquid nitrogen and ground to a powder using a mortar and pestle. The material was transferred to a centrifuge tube and incubated at 65°C for 3 minutes after adding buffer B (1 ml/g). The mixture was briefly cooled, one volume of chloroform : isoamylalcohol (24:1) added and the resulting solution mixed
well by inversion. Centrifugation was done at 5,000 g for 15 minutes at 4°C. The supernatant was transferred to another tube and a 10% solution of preheated (65°C) cetyltrimethyl ammonium bromide (CTAB) (1/10V) added. The tube was placed at 65°C for 3 minutes, briefly cooled and one volume of chloroform: isoamylalcohol (24:1) added. The mixed solution was centrifuged at 5,000 g for 15 minutes at 4°C. The aqueous phase was transferred to new tubes, one volume of buffer C added, and then mixed by inversion. The pellet was collected and dissolved in 1.5-4 ml (depending on pellet size) of high salt TE. The tube was incubated at 65°C for 5-10 minutes. Two volumes of 100% ethanol were added to the tube after incubation and then left for 1 hour at room temperature. DNA was spooled out by sealed glass pipette and was then dissolved in sterile water and quantified by UV spectrometer or fluorometer. The bulk samples were stored at -20°C.

2.11.2 Digestion of Genomic DNA with Restriction Endonuclease

DNA restriction digestion was carried out typically in a 50 μl reaction solution containing 5 units of an appropriate enzyme, 2 μl of DNA and 5 μl of spermidine buffer in an Eppendorf tube. Digestion was carried out for 3 hours at 37°C. The enzyme was inactivated by incubating at 70°C for 10 minutes followed by placing the sample on ice. Five μl of this digested sample was loaded onto a 0.7% TAE agarose gel for electrophoresis to verify completion of digestion. The rest of digested sample was purified with an equal volume of chloroform, followed by ethanol precipitation. The pellet was washed with 75% ethanol and dissolved in 50 μl water.

2.11.3 Ligation

Two ligations with 5 μl and 40 μl of the above DNA solution were carried out each containing 1X ligation buffer (Promega) and 10 units of T4 ligase (Promega). The solution was incubated overnight at 10°C to allow ligation to proceed. Ligated DNA
samples were repurified by ethanol precipitation and the pellets were each resuspended in 5 µl water.

2.11.4 Transformation of *E. coli*

Transformation into *E. coli* was done by electroporation. *Escherichia coli* strain DH5α (Clontech) was prepared according to the protocol by Zabarovsky *et al* (1990), and stored at -70°C. The frozen cells were thawed at room temperature and placed on ice. The cells (400 µl) were transferred into a cold 1.5 ml Eppendorf tube and 1 µl of the DNA sample was added. The cell/DNA mixture was transferred to an ice-cold microcuvette (Biorad), and placed in position in the safety chamber of the BioRad Gene Pulser™ (Biorad). The instrument was set to 25 F capacitance, 2.5 KV, 200 Ω and the sample was subjected to a pulse of 12.5 KV/cm with a time constant of 4.5 msec. The cells were removed from the cuvette, mixed with 960 µl SOC (see appendix) medium, and incubated with shaking for 1 hour at 37°C. Samples of the cells were plated on 9 cm LB agar plates containing 200 µg/ml of ampicillin and incubated at 37°C overnight. Only bacteria containing the rescued plasmids can survive and are selected on the medium containing ampicillin because the plasmids carry the pBR322 sequence which flanks the T-DNA tag. The recombinants were identified through plasmid miniprep digestion.

2.11.5 Plasmid Miniprep Restriction Digestion to Verify the Putative Clones

Using a sterile toothpick, a white bacterial colony was randomly touched and the toothpick dropped into a tube containing 2.5 ml of 2YT (see appendix) containing appropriate antibiotics. The cells were incubated for 24 hours at 37°C on a mechanical shaker. The culture was poured into the 1.5 ml Eppendorf tubes and spun in a microfuge.
for 1 minute at 14,000 rpm. The supernatant was discarded and the cell pellet resuspended by vortexing.

200 μl of 0.1 M NaOH/0.5% SDS was added to the cell suspension, gently mixed, and left to stand at room temperature for 5 minutes. 200 μl of 3M sodium acetate, pH 5.4, was added and the cell suspension was gently mixed and placed on ice for 10 minutes followed by centrifugation at 14,000 g for 10 minutes. The resulting DNA pellet was washed with 500 μl of 75% ethanol, air-dried, and dissolved in 40 μl of sterile deionised water.

For restriction digestion, each reaction mix consisted of 1 μl of 10X multi-core buffer (Promega), 0.1 μl of RNase A (Sigma) from a stock concentration of 6 mg/ml (see appendix), and 2 units of restriction enzyme (Promega), with the volume made up to 7.5 μl with water. DNA sample (2.5 μl) was added and the digestion carried out for 3 hours at 37°C. Samples of 5 μl were analysed by agarose gel electrophoresis.

2.11.6. DNA Sequencing

2.11.6.1 Preparation of Double-Stranded DNA Template

A polyethylene glycol (PEG) precipitation method was used to purify the DNA templates. The mini prepared plasmid DNA was suspended in 90 μl of water was mixed with 1 μl of RNase A (10 mg/ml) and incubated at 37°C for 20 minutes. This was followed by extraction with chloroform. The upper aqueous phase was transferred to a new tube and an equal volume of 26.6% PEG (8,000) in 20 mM MgCl₂ was added, mixed, and left at room temperature for 10 minutes. The tube was centrifuged at 14,000g for 10 minutes. The supernatant was aspirated and the pellet washed in 70% ethanol, air-dried and dissolved in 10 μl of water. One μl of sample DNA was analysed for quantity and quality by agarose gel electrophoresis.
2.11.6.2 Sequencing Primers

The sequencing primer design were based on the sequence of pPCV6NFHyg T-DNA. The forward primer LB1(5'-TCTCCATATTGACCATCATA-3') faced the left T-DNA border and the reverse primer RB1(5'-GCCGAATAGCCTCTCCAC-3') faced the right T-DNA border. These primers were synthesised by Life Technologies.

2.11.6.3 Sequencing Reaction and Analysis

Sequencing analysis was performed using the ABI PRISM™ 337 DNA Sequencer. All reagents were supplied by Applied Biosystems.

2.11.6.4 Sequence Data Analysis

Data was first assembled and "cleaned" using a "Gel Assembler" (ABI). Database homology searching was conducted using ANGIS (Australian National Genomic Information Service) programs.
Chapter 3

CHARACTERISATION OF THE RESPONSE OF THREE EMS MUTANTS TO ARSENIC STRESS
3.0 INTRODUCTION

Ethyl methansulphate (EMS) is one of the most efficient mutagen. Using this chemical, a large number of mutants of *A. thaliana* have been generated. They have been used in many studies to screen for phenotypes of interest. Three identified mutants, *pho1, pho2*, and *man1* are phosphate and manganese accumulators, respectively. The *pho1* and *pho2* mutants are of particular interest because they can accumulate phosphate. It is possible that they can also accumulate arsenic if phosphate and arsenate share the same transport systems. This provides one approach to elucidating the mechanisms of arsenic uptake in higher plants. Therefore, these mutants were studied in this project.

3.1 EFFECTS OF ARSENIC ON ROOT GROWTH OF THE EMS MUTANTS

3.1.1 Effect of As(V) on Root Growth of *wt, and pho1, pho2, man1* Mutants

To examine the degree of response to arsenic of the *pho1, pho2*, and *man1* mutants, seeds were first exposed to different concentrations of As(V), the common form of inorganic arsenic in contaminated soils (Smith *et al.*, 1995). The root growth rate shortly after germination was then measured, calculated as an index of tolerance (Iₜ) and plotted versus the As(V) concentration (Fig.3.1). *wild-type* seedlings were used as controls.

At 10 ppm As(V), the root growth of all lines except *pho2* were completely inhibited. The Iₜ of *wt* and the *pho1*, and *man1* mutants were nearly similar. *pho2* had a significantly higher Iₜ (t test α = 0.05) as compared to *wt, pho1*, and *man1*, indicating that this mutant was the most tolerant to As(V).
Figure 3.1. Index of tolerance of Arabidopsis thaliana wt compared with the pho1, pho2, and man1 mutants to As(V). The root growth rates (length) were measured (n=20) 5 days after the mutant seeds had been sown.

3.1.2 Effect of As(III) on Root Growth of pho2 Mutant

After finding the higher tolerance of pho2 mutant to As(V), an experiment was conducted to examine the behaviour of this mutant when exposed to As(III), more toxic form of inorganic arsenic, which also occurs in soils.

Root length plots for the wt and pho2 mutant are shown in Fig. 3.2 The results showed, surprisingly, that pho2 was more sensitive to As(III) than the wt (different significance at α=0.05). The root development of both wt and pho2 was inhibited completely at 8 ppm concentration. At 5 ppm As(III), the wt plant roots grew markedly better than the pho2.
Figure 3.2. Index of tolerance of Arabidopsis thaliana, \( wt \) and \( pho2 \) mutant to As(III). The root growth rates (length) were measured (n=20) 5 days after the \( wt \) and mutant seeds had been sown.

3.2 SHOOT DEVELOPMENT OF \( wt \) AND \( pho2 \) IN As(V) SOLUTION

The effects of As(V) on shoot development were determined to see whether As(V) has the similar effects on this organ of \( pho2 \). Four week old plants which had been grown on normal medium were transferred to solutions containing 2.5 and 5.0 ppm As(V) without phosphate to avoid the competitive uptake of phosphate in place of arsenate. After two weeks, shoot development of \( pho2 \) was clearly inhibited as compared to \( wt \) (Fig. 3.3). Observations of changes in shoot colour showed the symptoms of arsenic toxicity, chlorosis, wilting and stunted growth. This finding suggests that more As(V) was absorbed and accumulated in the shoot of \( pho2 \) seedlings resulting in greater inhibition of shoot development in this mutant.
Figure 3.3 Shoot Development of wt and pho2 in As(V) solutions
3.3 ARSENIC ACCUMULATION IN THE ABOVE-GROUND BIOMASS OF *pho2* MUTANT

To examine whether the *pho2* mutant accumulates more As(V) in its shoots than the *wt* as it does for phosphate, the arsenic contents in the shoots of *wt* and *pho2* were compared. Four week old seedlings grown in soil were watered with 10 ppm of As(V) every third day for two weeks. Four week old plants grown in hydroponic culture were transferred to fresh culture solution containing 10 ppm of As(V) without phosphate for two weeks. The data (Table 3.1) show that the arsenic contents in the shoots of *pho2* were higher than that of *wt* in two out of three soil-grown experiments and greatly increased in one hydroponic growth experiment.

**Table 3.1** Arsenic Contents in The Shoot of *wt* and *pho2* Mutant Grown in Soil and Hydroponic Culture Containing As(V) for 14 Days.

<table>
<thead>
<tr>
<th>Experimental Number</th>
<th>Growth Medium</th>
<th>Plant Type</th>
<th>Arsenic Content (μg/g dry weight)</th>
<th>% of <em>wt</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>soil</td>
<td><em>wt</em></td>
<td>59.2</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>pho2</em></td>
<td>77.7</td>
<td>131</td>
</tr>
<tr>
<td>2</td>
<td>soil</td>
<td><em>wt</em></td>
<td>31.5</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>pho2</em></td>
<td>45.4</td>
<td>144</td>
</tr>
<tr>
<td>3</td>
<td>soil</td>
<td><em>wt</em></td>
<td>16.3</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>pho2</em></td>
<td>16.5</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>hydroponic</td>
<td><em>wt</em></td>
<td>82.9</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>pho2</em></td>
<td>315.2</td>
<td>380</td>
</tr>
</tbody>
</table>

Arsenic analyses were performed in the Department of Chemistry, University of Wollongong (Expts. 1 and 2) or the EAST laboratory, Port Kembla (Expt 3). These three experiments were carried out independently at different times.
3.4 $^{73}$As(V) UPTAKE BY *pho2* SEEDLINGS

$^{73}$As(V) was used to determine the uptake and distribution of As(V) in the shoots of *pho2* seedlings. Fourteen day old seedlings grown in rockwool hydroponic or in agar medium were transferred to MES buffer containing 500 nCi of $^{73}$As(V) supplemented with 10 ppm As(V) without phosphate. Radioactive distributions were determined after two day exposure using the phosphorimager. These autoradiograph show that $^{73}$As(V) was taken up by *pho2* mutant in the leaves to a markedly higher extent than *wt* (Fig. 3.4). This is very strong evidence indicating that *pho2* accumulated arsenic in the leaves.

The $^{73}$As(V) activity was determined in leaves of the seedlings (Table 3.2). The amount of radioisotope taken up by *pho2* leaves was about 2.5 and 5 times that of the wild-type in the two experiments.

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>Agar Medium</th>
<th>Rockwool Hydroponic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts/mg</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>dry weight</td>
<td><em>wt</em></td>
</tr>
<tr>
<td></td>
<td>counts/mg</td>
<td>%</td>
</tr>
<tr>
<td></td>
<td>dry weight</td>
<td><em>wt</em></td>
</tr>
<tr>
<td><em>wt</em></td>
<td>2,577</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>808</td>
<td>100</td>
</tr>
<tr>
<td><em>pho2</em></td>
<td>6,681</td>
<td>259</td>
</tr>
<tr>
<td></td>
<td>4,102</td>
<td>508</td>
</tr>
</tbody>
</table>
Seedlings Grown in Hydroponic Culture

Seedlings Grown in Agar

Figure 3.4 Arsenic Distribution in wt and pho2 Seedlings after Two Days $^{73}$As(V) Uptake. The root is the heavily labelled structure in the lower part of each autoradiograph.
Chapter 4

SCREENING OF ARABIDOPSIS THALIANA T-DNA INSERTION MUTANTS IN RESPONSE TO ARSENIC STRESS
4.0 INTRODUCTION

T-DNA insertion mutant lines can be produced by insertion of T-DNA fragment (25 Kb) into the genome of *A. thaliana*. Using this technique, more than 8,000 mutant lines have been generated and are now available in *Arabidopsis* stock centres. Once mutants of interest are detected by screening in these mutant lines, the mutated gene(s) can readily be isolated by using the T-DNA tag. We screened a large number of seedlings from these mutant lines obtained from stock centres for arsenic tolerance.

4.1 SCREENING FOR ARSENIC TOLERANCE IN MUTANTS OF *A. thaliana* GENERATED BY T-DNA INSERTION

The VMT technique (see chapter 2) was adapted to screen more than 8,000 T-DNA insertion lines of *A. thaliana* for arsenic tolerant mutants.

Approximately 100,000 M2 seeds from 265 Koncz lines, 6500 Feldmann lines and 1300 Thomas lines have been screened for stress response at 10 ppm of As(V). Seed development in a majority of these lines were either completely inhibited at the start of the germination or severely inhibited later during the root development. Of these, 33 individuals from 16 Koncz mutant lines, 32 individuals from 24 Feldmann lines, and 7 individuals from 6 Thomas lines tolerant to As(V) were selected and potted in soil to produce seeds for the next round of screening. Due to the death or infertility of some plants, 65 were rescreened. From the rescreening of M3 seedlings two individuals from Koncz lines and nine individuals from Feldmann lines were isolated which again showed tolerance to 10 ppm of As(V) as compared to *wt*. The Koncz line mutants, designated *asl* and *as2*, were subjected to further study because they are separate mutant lines and are listed by *Arabidopsis* stock centres to be homozygous. The other mutants segregated and the progeny therefore need to be rescreened and confirmed for tolerance to As(V) by genetic analysis.
4.2 VISIBLE PHENOTYPE OF \textit{asl} AND \textit{as2} MUTANTS

Apart from the observed responses to arsenic, the visible phenotypic characters of \textit{asl} and \textit{as2} mutants were compared to the \textit{wt} (Fig. 4.1). \textit{asl} mutant had bigger and thicker leaves and stems, darker green colour, larger seeds and later flowering. In contrast, \textit{as2} had less green colour of in the leaves and early flowering. Chlorophyll contents of the two mutant in the leaves were determined to obtain a quantitative comparison of the visible colour difference between the mutants and \textit{wt} (Table 4.1).

\begin{table}[h]
\centering
\caption{Chlorophyll Content in Leaves of \textit{wt} and \textit{asl}, \textit{as2} Mutants.}
\begin{tabular}{ll}
\hline
Plant Type & Chlorophyll Contents (\textmu g/g) \\
\hline
\textit{wt} & 602.155 \pm 4.800 \\
\textit{asl} & 675.130 \pm 18.038 \\
\textit{as2} & 519.280 \pm 14.786 \\
\hline
\end{tabular}
\end{table}

\textit{Mean chlorophyll content in leaves taken from three replicates (different plants) \pm SE.}

4.3 EFFECT OF As(V) ON ROOT GROWTH OF \textit{asl} AND \textit{as2} MUTANTS

Seeds of these mutants and \textit{wild-type} were sown on filter paper wetted with nutrient solution supplemented with 5 ppm As(V). Root lengths of these mutants after 5 days growth were observed and found to be longer and than \textit{wt} demonstrating they were tolerant to As(V) (Fig. 4.2 and Table 4.2).
Figure 4.1 Represents of Arsenic Tolerant Mutants Selected in *A. thaliana* T-DNA Insertion Lines *as1, as2*. A wt plant grown under identical condition is shown for comparison.
### Table 4.2 Root lengths of *wt*, *asl*, and *as2* after 5 days Growth in As(V) Solution.

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>Root Length (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>wt</em></td>
<td>0.646 ± 0.008</td>
</tr>
<tr>
<td><em>asl</em></td>
<td>0.892 ± 0.009</td>
</tr>
<tr>
<td><em>as2</em></td>
<td>0.730 ± 0.008</td>
</tr>
</tbody>
</table>

Mean root lengths (± SE) from two experiments. There were 20 plants per line in each experiment.

#### Figure 4.2 Root Development of *wt* and *asl*, *as2* Mutants after 5 Days Growth in 5 ppm As(V) on Filter Paper

5 ppm As(V)
4.4 ARSENIC ACCUMULATION IN \textit{asl} AND \textit{as2} MUTANTS

An experiment similar to that described in section 3.3 was carried out to determine whether \textit{asl} and \textit{as2} are arsenic accumulator or excluder mutants. Arsenic contents in the shoots of the seedlings grown for 2 weeks in either soil or in hydroponic culture containing 10 ppm As(V) were examined. Seedlings which had been grown in normal soils for 4 weeks were watered with As(V) every third day. Four week old seedlings which had been grown hydroponically in normal medium were transferred to nutrient medium containing As(V) without phosphate. After growing for two weeks, seedlings showed arsenic toxicity symptoms in the shoots. The data (Table 4.3) showed that the arsenic content in the shoot of the mutants was considerably higher than that of \textit{wt}. These results suggest that \textit{asl} and \textit{as2} are arsenic-accumulating plants.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Experimental Number & Growth Medium & Plant Type & Arsenic Content (\mu g/g dry weight) & \% of wt \\
\hline
1 & soil & \textit{wt} & 31.5 & 100 \\
 & & \textit{asl} & 42.3 & 134 \\
 & & \textit{as2} & 71.4 & 227 \\
\hline
2 & soil & \textit{wt} & 16.5 & 100 \\
 & & \textit{asl} & 17.1 & 104 \\
 & & \textit{as2} & 63.6 & 386 \\
 & hydroponic & \textit{wt} & 82.9 & 100 \\
 & & \textit{asl} & 141.3 & 171 \\
\hline
\end{tabular}
\caption{As Contents in The Above-Ground Biomass of \textit{wt} and Mutants Grown in Soil or in Hydroponic Culture Containing As(V) for 14 Days.}
\end{table}

Arsenic analyses were performed in the Department of Chemistry, University of Wollongong (Expts. 1) or the EAST laboratory, Port Kembla (Expt 2). Arsenic content in hydroponically cultured \textit{as2} plants was not determined due to some experimental accident.
4.5 ARSENIC ISOTOPE UPTAKE BY GROWING SEEDLINGS

Wild-type, asl and as2 mutants were further examined for their capability to take up and accumulate As(V) by using the $^{73}$As(V) isotope. Autoradiographs were prepared of roots and leaves of plants grown in either hydroponic culture or agar medium for 2 days in the presence of 10 ppm of As(V) supplemented with 500 nCi of $^{73}$As(V) without phosphate (Fig. 4.3). Wild-type leaves exhibited a low radioactive levels. In contrast, high radioactive levels were observed in the leaves of the mutants. The data showed that $^{73}$As(V) activity in the above-ground parts asl and as2 mutants were markedly higher compared to wt (Table 4.4). This result confirms that As(V) was taken up and accumulated in the shoot of asl and as2 mutants.

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>Agar Medium</th>
<th>Hydroponic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>counts/mg</td>
<td>% of</td>
</tr>
<tr>
<td></td>
<td>dry weight</td>
<td>wt</td>
</tr>
<tr>
<td>wt</td>
<td>2,577</td>
<td>100</td>
</tr>
<tr>
<td>asl</td>
<td>3,141</td>
<td>122</td>
</tr>
<tr>
<td>as2</td>
<td>8,492</td>
<td>330</td>
</tr>
</tbody>
</table>

**Table 4.4 $^{73}$As (V) Accumulated in The Above-Ground Parts of wt and asl, as2 Mutants**

Seedlings Grown in Agar

![Autoradiographs of roots and leaves of plants grown in agar medium](image)
Figure 4.3 $^{73}$As(V) Uptake by Seedlings of wt, as1 and as2 after 2 Days Uptake. The root is the heavily labelled structure in the lower part of each autoradiograph.

4.6 PHOSPHATE ACCUMULATION IN as1 AND as2

Phosphate contents in the leaves of as1 and as2 mutants were compared to wt and pho2 mutants as an indication of any relationship between the As(V) and Pi accumulation in these mutants. The data show that Pi concentrations in wt and asl leaves were similar but Pi in as2 and pho2 were significantly higher than wt (Table 4.5).

<table>
<thead>
<tr>
<th>Plant Type</th>
<th>Pi Contents (mg/g fresh weight)</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt</td>
<td>0.37 ± 0.03</td>
<td>100</td>
</tr>
<tr>
<td>pho2</td>
<td>0.90 ± 0.05</td>
<td>243</td>
</tr>
<tr>
<td>asl</td>
<td>0.43 ± 0.05</td>
<td>116</td>
</tr>
<tr>
<td>as2</td>
<td>1.02 ± 0.04</td>
<td>276</td>
</tr>
</tbody>
</table>
Chapter 5

MOLECULAR CHARACTERISATION OF *asl, as2* MUTANTS
5.1 ISOLATION OF T-DNA TAGGED GENE IN MUTANTS

Genomic DNA from young leaves of *asl* and *as2* mutants was isolated and digested with HindIII or XbaI (Fig. 5.1). After self-ligation, these circularised fragments were introduced into *E.coli* cells and the rescued plasmids were selected on media containing ampicillin. Only HindIII fragments from the *asl* mutant were recovered. Five transformants were examined and after digestion with PvuII all exhibited three fragments with sizes of 6.0, 3.5 and 1.4 Kb, indicating that the five transformants were identical (Fig. 5.2). This also confirmed that a fragment of about 2.1 Kb flanking DNA from the tagged gene was cloned. This plasmid, designated pAS1, was purified and subjected to sequencing.

![Figure 5.1 Agarose Gel (0.8%) Electrophoresis of The Genomic DNA of *asl* Digested with HindIII and XbaI. Lane 1: Marker Lambda DNA cut with HindIII and EcoRI. Lane 2: *asl* DNA digested with HindIII. Lane 3: *asl* DNA digested with XbaI](image)

**Figure 5.1** Agarose Gel (0.8%) Electrophoresis of The Genomic DNA of *asl* Digested with HindIII and XbaI. **Lane 1**: Marker Lambda DNA cut with HindIII and EcoRI. **Lane 2**: *asl* DNA digested with HindIII. **Lane 3**: *asl* DNA digested with XbaI
**Figure 5.2** Agarose Gel Electrophoresis of The Rescued Plasmid DNA (pAS1) Digested with Pvull Showing 3 Fragments of 6.0; 3.5 and 1.4 Kb. **Lane 1:** The rescued plasmid DNA without restriction digestion; **Lane 2:** Rescued DNA digested with Pvull.

### 5.2 SEQUENCING OF *asl* T-DNA FLANKING FRAGMENT

The rescued plasmid DNA was sequenced with primers (LB1 and RB1) designated according to the sequences near the left and right T-DNA borders of pPCV6NFHyg vector (Fig. 5.3).
Only a DNA flanking fragment (5LB1) near the left border has been successfully sequenced using the LB1 primer. The sequencing reaction using the RB1 primer failed for unknown reasons. The DNA sequence of 699 bp of 5LB1 with the six open reading frames are shown in Fig. 5.4.

CACGGGCTCACAAGCTGATATACAGCACAAGTCAGATTTAGACACCTAGAAAAGGGGAA
1    — ------1 —-----— --------1  —---------— 4-------------------I — ---------1  — — — + 60

GTGCCGAGTGTCGACATATGGTCGTGTTCAGTTGTCTAAGTACTGTGGATCTTTTCCCCTT
a
b
c
d
e
f

GAATAAAGCAGATTCATGTGTTTAAGTAAATATATGAGAATAATATCATCTTCAAAATGA
6 1 -----------+-----------+-----------4------------+-----------+-----------+ 120

HGSQLYTAQVNRFMTPRKGE -
TAHSCIQHKSTDS*HELKGRK -
RLTAVYSTSQOIHDT*KRGBR -
1 ___________4------------4------------4------------+-----------+-----------+ 60
RSVATYLVL*CI*SV*FLP -
PENCYVACATTLLNMMVGFLPS -
VA*LQICCLDLVDSEHCRSFPF -

GAATAAAGCAGATTTGATGTTTAAGTAAATATGAGAATAATATCATCTTTCCAAATGA
61 120
Figure 5.4 Nucleotide Sequence and The Deduced Six Possible Amino Acid Sequences of The 5LB1 Fragment from \textit{as1} Mutant. The deduced amino acid sequences are shown in single letter code. A region rich in histidine is underlined. Nucleotide numbers are indicated on the left and right.

5.3 DATABASE SEARCH OF 5LB1 SEQUENCE

The sequence analysis of 5LB1 was performed using the BLAST computer programs (ANGIS, Australian National Genomic Information Service).

A BLASTN search (Table 5.1) against all known nucleotide sequences (Genebank and EMBL including Arabidopsis ESTs-Expressed Sequence Tags) did not reveal any identical sequence with significant homology to 5LB1. Thus 5LB1 appears to be a DNA fragment which has been sequenced for the first time.

<table>
<thead>
<tr>
<th>Sequences Producing High-Scoring Segment Pairs</th>
<th>High Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score  P(N)  N</td>
<td></td>
</tr>
<tr>
<td>__________________________________________________________________</td>
<td></td>
</tr>
<tr>
<td>emb][AL008980</td>
<td>PFSC03050 Plasmodium falciparum DNA *** SEQU...</td>
</tr>
<tr>
<td>gb][L09637</td>
<td>DDICAR Dictyostelium discoideum cAMP rece...</td>
</tr>
<tr>
<td>gb][U18796</td>
<td>SC9379 Saccharomyces cerevisiae cromosom...</td>
</tr>
<tr>
<td>emb][X16876</td>
<td>GMENOD2B Soybean ENOD2B gene for Ngm-75</td>
</tr>
<tr>
<td>emb][X16875</td>
<td>GMENOD2A Soybean ENOD2A gene for Ngm-75</td>
</tr>
<tr>
<td>emb][AL010167</td>
<td>PFSC03094 Plasmodium falciparum DNA *** SEQU...</td>
</tr>
<tr>
<td>gb][U95738</td>
<td>HSU95738 human chromosome 16p13.11 BAC clon...</td>
</tr>
<tr>
<td>emb][AL021921</td>
<td>HS357T16 Homo sapiens DNA sequence from PAC...</td>
</tr>
<tr>
<td>emb][Y17045</td>
<td>PFY17045 Plasmodium falciparum gltS gene</td>
</tr>
<tr>
<td>gb][U83568</td>
<td>SPU83568 Schizosaccharomyces pombe delta-am...</td>
</tr>
<tr>
<td>gb][AC003063</td>
<td>AC003063 Mus musculus Chromosome 16 BAC Clo...</td>
</tr>
<tr>
<td>db][AB005236</td>
<td>AB005236 Arabidopsis thaliana genomic DNA, ...</td>
</tr>
<tr>
<td>gb][AE000784</td>
<td>AE000784 Borrelia burgdorferi plasmid lp28-...</td>
</tr>
<tr>
<td>emb][AL010243</td>
<td>PFSC04060 Plasmodium falciparum DNA *** SEQU...</td>
</tr>
<tr>
<td>gb][AC002343</td>
<td>ATAC002343 Arabidopsis thaliana BAC T19F06 ge...</td>
</tr>
<tr>
<td>emb][Z47357</td>
<td>CZ47357 Caenorhabditis elegans cosmid ZG11...</td>
</tr>
<tr>
<td>emb][AL008982</td>
<td>PFSC03052 Plasmodium falciparum DNA *** SEQU...</td>
</tr>
<tr>
<td>emb][275741</td>
<td>HS107N3 Human DNA sequence from PAC 107N3,...</td>
</tr>
<tr>
<td>emb][AJ004917</td>
<td>CAAJ4917 Cicer arietinum mRNA for endoxylog...</td>
</tr>
<tr>
<td>gb][AP065616</td>
<td>AF065616 Capsicum annum nitrite reductase ...</td>
</tr>
</tbody>
</table>
emb|Z82200|HS333E23 Homo sapiens 12q24 PAC RPCI1-46F2 ... 130 0.48 3
emb|AL010138|PFSC03066 Plasmodium falciparum DNA *** SEQU... 119 0.52 2
emb|AL010165|PFSC03093 Plasmodium falciparum DNA *** SEQU... 119 0.52 2
emb|Z98552|PFSC03022 Plasmodium falciparum DNA *** SEQU... 124 0.53 2
gb|X94355|CV41KBPL Cowpox virus 41kb fragment from l... 135 0.53 2
gb|U15669|MBU15669 Myrmecia banksi mitochondrion cyto... 136 0.54 1
emb|AL010225|PFSC04007 Plasmodium falciparum DNA *** SEQU... 121 0.57 2
gb|AC002368|AC002368 Homo sapiens Xq28 BAC PAC and cosm... 131 0.59 3
emb|Y11842|CVGRI90 Cowpox virus strain GRI-90 DNA (52... 135 0.60 2
gb|M19032|XELCAM X.laevis alpha-amidating enzyme (A... 135 0.61 1
dbj|AB000901|AB000901 Oryzias latipes mRNA for membrane ... 136 0.61 1
gb|AC003666|AC003666 Homo sapiens Xp22 BAC GS-551019 (G... 134 0.68 1
gb|AC002351|AC002351 Homo sapiens 12q24 PAC RPC11-46F2 ... 145 0.73 2
gb|Z68134|CET27A8 Caenorhabditis elegans cosmid T27A... 133 0.75 1
gb|X57558|GSNAT1 O.sativa taichung native 1 DNA ... 133 0.75 1
gb|AF013242|AF013242 Xenopus laevis homeobox protein Li... 132 0.81 1
gb|AC004521|ATAC004521 Arabidopsis thaliana chromosome II... 128 0.84 2
gb|AC004613|AC004613 Homo sapiens PAC clone DJ0651K02 f... 122 0.85 2
gb|X04465|CHMPPX Liverwort Marchantia polymorpha ch... 121 0.85 4
gb|AL010215|PFSC04006 Plasmodium falciparum DNA *** SEQU... 118 0.85 2
gb|AF003385|CEL088F11 Caenorhabditis elegans cosmids R08F11... 131 0.87 1
gb|L8901|ATACAH Arabidopsis thaliana carbonic anhy... 131 0.87 1
ebh|Z70042|HSU96H11 Human DNA sequence from cosm... 136 0.87 2
ebh|AL010213|PFSC03109 Plasmodium falciparum DNA *** SEQU... 124 0.88 2
gb|AC000437|AC000437 Homo sapiens chromosome 5, BAC clo... 113 0.88 2
gb|AC000129|F5114 Sequence of BAC F5114 from Arabid... 123 0.89 3
gb|AC000259|AC000259 Homo BAC clone RG161N06 from 7q31 ... 138 0.90 5
gb|AF007261|AF007261 Reclinomonas americana mitochondri... 139 0.91 2
ebh|AL010247|PFSC04062 Plasmodium falciparum DNA *** SEQU... 101 0.91 3
ebh|AF0024497|CELZC308 Caenorhabditis elegans cosmids ZC308... 130 0.91 1
ebh|AL010280|PFSC04106 Plasmodium falciparum DNA *** SEQU... 130 0.91 1
gb|U95740|HSU95740 Human chromosome 16p13.1 BAC clone... 130 0.91 1
gb|U87145|TG87145 Toxoplasma gondii chloroplast, com... 111 0.93 2
ebh|AF015567|AF015567 Dicyostelium discoideum PotA (pot... 129 0.95 1
dbj|AP000034|AP000034 Homo sapiens genomic DNA, chromoso... 129 0.95 1
gb|AC0002307|HUAC002307 Homo sapiens Chromosome 16 BAC clo... 129 0.95 1
ebh|AF035397|AF035397 Homo sapiens cosmids Qc15C1 and 94... 112 0.96 4
gb|AC0003089|AC0003089 Homo BAC clone RG180F08A, complet... 118 0.96 2
ebh|AL0008972|PFSC03020 Plasmodium falciparum DNA *** SEQU... 107 0.96 3
ebh|AC002990|HUAC002990 Human Chromosome 16 BAC clone CTT9... 130 0.97 2
ebh|Z99169|CEC2659A Caenorhabditis elegans cosmids C26H... 133 0.97 2
ebh|AC002089|HASAC002089 Human BAC clone RG308B22 from 7q22 ... 113 0.97 3
ebh|X64962|CEFEM3R C.elegans fem-3 mRNA for sex deter... 128 0.97 1
ebh|X64963|CEFEM3G C.elegans gene fem-3 ... 128 0.97 1
ebh|U97194|CELC37A2 Caenorhabditis elegans cosmids C37A2... 128 0.97 1
ebh|Z68213|CEC01F6 Caenorhabditis elegans cosmids C01F... 128 0.97 1
ebh|M11104|HUMRSSPC HeLa cell small polydisperse circu... 128 0.97 1
ebh|AC002539|AC002539 Homo sapiens chromosome 17, clone ... 128 0.97 1
ebh|M57944|DDODI5KA D.discoideum protein kinase 2 mRNA... 128 0.97 1
ebh|AC0004562|AC0004562 Homo sapiens chromosome 17, clone ... 128 0.97 1
ebh|AC0002427|AC0002427 Homo sapiens chromosome GS011E15 from 5g31 ... 121 0.98 3
ebh|AC0003958|AC0003958 Homo sapiens chromosome 17, clone ... 133 0.98 2
ebh|M16345|DDHPHERA D.discoideum protein 253 gene, 5' ... 100 0.98 2
ebh|U97017|CELF47B3 Caenorhabditis elegans cosmids F47B3... 127 0.98 2
ebh|AL010228|PFSC04016 Plasmodium falciparum DNA *** SEQU... 113 0.99 2
ebh|U67500|U67500 Methanococcus jannaschii section 4... 127 0.99 1
ebh|Z297192|HS29C18 Homo sapiens DNA sequence from PAC... 127 0.99 1
dbj|AB001684|AB001684 Chlorella vulgaris C-27 chloroplasts ... 133 0.99 2
ebh|AF001549|HSAP001549 Homo sapiens chromosome 16 BAC clo... 109 0.992 3
ebh|Z35601|CER10E9 Caenorhabditis elegans cosmids R10E... 114 0.994 2
ebh|AL021749|ATT2109 Arabidopsis thaliana DNA chromosom... 114 0.994 2
ebh|U39649|CEL232F2 Caenorhabditis elegans cosmids T23F2... 112 0.994 2
ebh|U41625|CELIK03A1 Caenorhabditis elegans cosmids K03A1... 105 0.995 3
A BLASTX search was then conducted against all known protein sequences (Table 5.2) and again no significant homologies appear to be present. However, worth noting are some histidine-rich proteins and carbon catabolite derepressing protein appeared by this search (underlined in Table 5.2 and Table 5.3). Of particular interest is the metal-binding protein from *Helicobacter pylori* and carbon catabolite derepressing protein from yeast.

### Table 5.2 Amino Acid Sequence Comparison of 5LB1 from asf1 and Other Known Proteins.

<table>
<thead>
<tr>
<th>Sequences Producing High-Scoring Segment Pairs</th>
<th>Reading High Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frame</td>
<td>Score</td>
</tr>
<tr>
<td>-------</td>
<td>-------</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>pir</td>
<td></td>
</tr>
<tr>
<td>sp</td>
<td>SNF1 YEAST CARBON CATABOLITE DEREPRESSING PROTEI...</td>
</tr>
<tr>
<td>sp</td>
<td>HHP HELPY HISTIDINE-RICH, METAL BINDING POLYPEP...</td>
</tr>
<tr>
<td>sp</td>
<td>YM P4 CA EEL HYPOTHETICAL 42.9 KD PROTEIN B0361.4 ...</td>
</tr>
<tr>
<td>sp</td>
<td>HHP HELPY METAL BINDING POLYPEP...</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>gnl</td>
<td>tre</td>
</tr>
<tr>
<td>sp</td>
<td>MNB HUMAN SERINE/THREONINE-SPECIFIC PROTEIN KIN...</td>
</tr>
<tr>
<td>sp</td>
<td>Q14979 NUCLEAR RECEPTOR</td>
</tr>
<tr>
<td>sp</td>
<td>Q90888 TRANSCRIPTION ACTIVATOR MAFB</td>
</tr>
<tr>
<td>sp</td>
<td>Q23038 SIMILAR TO PRUNUS PECTINESTERASE</td>
</tr>
<tr>
<td>sp</td>
<td>Q24162 STRIPE B PROTEIN</td>
</tr>
<tr>
<td>sp</td>
<td>Q24163 STRIPE A PROTEIN</td>
</tr>
<tr>
<td>sp</td>
<td>Q1066989 Caenorhabditis elegans cosmid F59B2, ...</td>
</tr>
<tr>
<td>sp</td>
<td>HRP3 PLAFS HISTIDINE-RICH PROTEIN</td>
</tr>
<tr>
<td>sp</td>
<td>Q14979 NUCLEAR RECEPTOR</td>
</tr>
<tr>
<td>gp</td>
<td>Q24282 Human mitogen induced nuclear orphan ...</td>
</tr>
<tr>
<td>pir</td>
<td>S71930 neuron-derived receptor NOR-1 - human</td>
</tr>
<tr>
<td>sp</td>
<td>NOR1 HUMAN NUCLEAR HORMONE RECEPTOR NOR-1 (NEURO...</td>
</tr>
<tr>
<td>GeneBank Accession</td>
<td>Description</td>
</tr>
<tr>
<td>-------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>GP</td>
<td>-</td>
</tr>
<tr>
<td>GP</td>
<td>-</td>
</tr>
<tr>
<td>PIR</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>SP</td>
<td>-</td>
</tr>
<tr>
<td>GN</td>
<td>-</td>
</tr>
</tbody>
</table>

Searched (Genebank and EMBL including Arabidopsis ESTs-Expressed Sequence Tags) using BLASTX program. Sequences (90) with high-scoring segment pairs are listed.
<table>
<thead>
<tr>
<th>Percent Identity</th>
<th>Source</th>
<th>Description</th>
<th>Accession</th>
<th>Protein Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>45% (10/22 aa)</td>
<td><em>Helicobacter pylori</em></td>
<td>Histidine-rich metal binding protein</td>
<td>Q48251</td>
<td>HPN_HELHY</td>
</tr>
<tr>
<td>69% (9/13 aa)</td>
<td><em>Plasmodium lophurae</em></td>
<td>Histidine-rich glycoprotein precursor</td>
<td>P04929</td>
<td>HRPX_PLALO</td>
</tr>
<tr>
<td>69% (9/13 aa)</td>
<td><em>Plasmodium falciparum</em></td>
<td>Histidine-rich protein</td>
<td>P14586</td>
<td>HRP3_PLAFS</td>
</tr>
</tbody>
</table>

The comparisons of the 5LB1 ORFd amino acid sequence with the *H. pylori* deduced histidine-rich, metal binding protein (MBP) and yeast carbon catabolite depressing protein kinase (SNF1_YEAST) using FASTA program are shown in Fig. 5.5 and Fig. 5.6.

**Figure 5.5** Amino Acid Sequence Comparison of 5LB1 from *as1* with Histidine-Rich, Metal Binding Protein, HPN_HELHY from *Helicobacter pylori*, MBP (AC. Q48251. Gilbert *et al.*, 1995). Identical residues are shown as dash (-), similar residues as (:). Comparison was made using the program FAST program from the GCG sequence analysis package. Amino acid numbers corresponding to each protein are indicated.
Figure 5.6 Amino Acid Sequence Comparison of 5LB1 from *as1* with Yeast Carbon Catabolite Depressing Protein Kinase, SNF1_YEAST (AC. P06782. Celenza and Carlson, 1986). Identical residues are shown as dash (-), similar residues as (.). Comparison was made using the program FAST program from the GCG sequence analysis package. Amino acid numbers corresponding to each protein are indicated.
Chapter 6

DISCUSSION
6.1 METHODS USED IN SCREENING FOR ARSENIC TOLERANCE MUTANTS

To study the three mutant lines by EMS treatment, a simple petri dish method was developed. In these experiments, roots were stained with cotton blue solution to clearly distinguish the root from the stems and leaves, which would otherwise require microscopic observation. Determination of \( I_t \) allowed us to not only select mutants tolerant to arsenic but also identify the concentrations which caused inhibition of root development. However, this method applies only to the very young stage of root development and is effective only for selection of a small number of mutants which are already homozygous.

The VMT method was employed to screen the *Arabidopsis* mutant lines generated by T-DNA insertion because it allows the screening of a great number of individuals as well as rapidly and directly isolation of arsenic tolerance mutants. In our experiment, more than 100,000 seeds were screened in a relatively short period of time. The tolerant mutants isolated in our experiments, based on the root phenotype in the early stage of development were subsequently to be arsenic accumulators. Therefore, the VMT technique appears to be a good method for screening for mutants of arsenic accumulation in the shoot.

This method should also allow the isolation of sensitive mutants responding to arsenic stress. To isolate the sensitive ones, the screening would need to be done in a lower concentration of arsenic which allows the *wild-type* to grow better and the sensitive mutants to also grow to a certain extent. Due to time constraints, this procedure was not performed.
6.2 RESPONSE OF *pho2* MUTANT TO ARSENIC STRESS

Phosphate and arsename were thought to share the same transport system because they are chemically similar (Nriagu, 1994; Gonzalez et al, 1995). However, the knowledge about this relationship is very limited. In plants, the uptake of phosphate is well-documented at the physiological level. Both the uptake of Pi from the soil to the root and internal Pi transport processes within the plant occur via a symport mechanism (Leggewie et al, 1997; Schachtman et al, 1998). A dual-mechanism model for uptake of ions, including phosphate, has been proposed. This is characterised by a high-affinity transport system operating at low (μM) concentrations and a low-affinity system functioning at high concentration (mM) of ions. Phosphate stress in yeast results in activation/inactivation of several associated genes called the Pho-regulon, leading to enhanced synthesis of the high-affinity phosphate transporters and acid phosphatase to increase the availability of Pi by cleaving off phosphate groups esterified to organic compounds. Recently, several phosphate transporters have been isolated and molecularly characterised in higher plants. Muchhal et al (1996) isolated two cDNAs encoding phosphate transporters in *Arabidopsis* (*APT1* and *APT2*) which were induced in response to phosphate starvation. The DNA sequences of these transporters exhibited significant sequence identity to a yeast and mycorrhizal counterparts (Bun-ya et al, 1991; Mann et al, 1989). More recently, two cDNAs, *StPT1* and *StPT2*, that encodes a proton/phosphate cotransporter were isolated from potato (Leggwei et al, 1997).

Arsenical-resistance genes are widely found in bacteria (Diorio et al, 1995; Rosentein et al, 1992; Rosen, 1996). Bobrowicz et al, (1997) isolated three contiguous genes (*acr1, acr2, and acr3*) involved in resistance to arsenic compounds in *Saccharomyces cerevisiae*. Sequences of these genes were more or less similar to the genes of the *ars* operon in bacteria, demonstrating the conservation of these genes in organisms. Arsenical-resistance genes are rarely found in eukaryota. Generally, arsenical resistance mechanisms in eukaryota are not yet well understood.
Bun-ya et al (1996) reported mutations of Saccharomyces cerevisiae PHO84 gene encoding Pi-transporter to confer an arsenate resistance phenotype. But so far, no report has been published on arsenic transporters in plants or the relationships of phosphate-arsenic transporters in transport systems. Therefore, the pho2 mutant is a good candidate to study the arsenic uptake/accumulation/store mechanisms in plants based on knowledge about phosphate transporters in this mutant.

In the work reported here, roots of the pho2 mutant were more resistant to As(V) but shoot development of this mutant was inhibited when it was grown hydroponically in medium containing this form of arsenic. This mutant can transport larger amount of As(V) to its shoots as it does for Pi. Consequently, shoot development was inhibited while root growth was not affected at the young early stage. Observations on the changes in the colour of shoots were consistent with the physiologically toxic effects of this chemical on plants. A minimal number of replicates were used in this experiment because arsenic determination is expensive and time consuming. However, the multiple experiments conducted support the conclusion that more As(V) was taken up and accumulated in shoot of the pho2 mutant even though it seemed that the mutant did not take up as much As(V) as it did for Pi. This result might be due to the use of nutrient medium containing phosphate which is necessary for the development of seedlings in the young stage, before setting up the arsenic determination experiments. The arsenic content of the root is a very important parameter to confirm that As(V) was not accumulated in the root of pho2 mutant as it does for phol (Poirier et al, 1991), and that it was transported to the upper biomass. Unfortunately, the roots of this plant were so tiny and few that we could not get enough samples for arsenic analysis. Results on $^{73}$As(V) uptake over a shorter time period by young seedlings also strongly supported the conclusion that pho2 is an As(V) accumulator in the upper parts. Another possibility of young roots of pho2 being resistant to As(V) is that, the seeds germinated contained
more Pi accumulated during its formation. Consequently, less arsenic was taken up by the germinating seeds and seedlings.

The effects of As(III) were also investigated in this study because this form is also available in contaminated soils and is even more toxic (Smith et al, 1995). The As(V)-resistant mutant \textit{pho2} exhibited quite different behaviour with As(III), being more sensitive than the \textit{wild-type}. The basis for the marked difference is likely to be in the very different chemical behaviour of As(III) and As(V) (Breed \textit{et al}, 1996). Therefore, this form of arsenic might be accumulated in the root and not transported to the shoots. As a result, the root development would be inhibited. The arsenic concentration in roots and shoots of \textit{pho2} seedlings in As(III) solution needs to be measured to confirm this hypothesis.

Recently, Carswell \textit{et al} (1997) used phosphonate to screen for phosphate mutants. The idea is that this chemical signals to the plant that phosphate is abundant but phosphonate is actually toxic because it competes with phosphate for binding sites. Mutants which are able to grow in the presence of phosphonate would have constitutive expression of high affinity phosphate transporters due to either a mutation occurring in some regulatory domain or in a regulatory protein making it insensitive to phosphonate. Thus, our data presented here on As(V) uptake in the \textit{pho2} mutant will be valuable in studying the basis of competitive uptake between arsenate and phosphate as well as the mechanism of arsenate uptake.

\section*{6.3 ARSENIC TOLERANT MUTANTS IN T-DNA INSERTION \textit{Arabidopsis} LINES}

Although research on the molecular and physiological basis of resistance to heavy metals in higher plants is rapidly expanding, little is known of arsenic in this regard. We have chosen a molecular genetic approach to studying arsenic tolerance mechanism by the
isolation of arsenic mutants of *Arabidopsis thaliana*. The purpose of our study is to isolate arsenic accumulators/excluders and through analysis of these mutants we are attempting to identify genes that function in arsenic resistance.

We chose the T-DNA insertion population to isolate arsenic mutants because of its advantage in gene isolation as compared to EMS mutants. No metal tolerance/sensitive *Arabidopsis* so far has been isolated in this population. The screening described here has led to the isolation of a number of As-tolerant mutants of *Arabidopsis*, out of the more than 8,000 mutant lines available to us. Two mutants, *asl* and *as2* from Koncz lines have been characterised further for arsenic uptake/accumulation and molecular studies.

The data on arsenic content in the shoots, especially autoradiograph images and data on isotope levels in the leaves, indicated that *asl* and *as2* mutants are arsenate accumulators. The tolerant characteristics of *asl* and *as2* mutants were determined based on the phenotype of the root at an early stage of development. During later stages, the mutants especially *as2* showed arsenic toxicity symptoms similar to the *pho2* because arsenate was transported and accumulated in the shoots. It is likely that the transport and resulted accumulation of arsenate within *as2* is similar to these within the *pho2* mutant. This even raises the possibilities that *as2* and *pho2* mutants were defected at the same locus.

At present, the mechanism for Pi accumulation in the *pho2* mutant is not understood. Two possibilities proposed by Delhaize *et al.* (1995) are i) a specific impairment in phloem transport of Pi between shoots and roots, which may be the result of a mutation in a gene that encodes a Pi transporter in these cells, and ii) a defect in either a gene encoding a shoot-specific Pi transporter or a gene encoding a protein that senses Pi concentration, which inturns to regulate the activity and expression of Pi transporter, resulting Pi transporter remains active even under P-sufficient conditions. Isolation of the tagged gene in *as2* will facilitate the elucidation of the mechanism.
The data on Pi content in the leaves of the \textit{pho2}, \textit{as1}, and \textit{as2} mutants indicated that \textit{as2} excessively accumulated phosphate as well as arsenate while \textit{as1} did not. Thus, \textit{as1} probably exploited another mechanism to uptake arsenate only. The evidence that \textit{pho2} and \textit{as2} accumulated both Pi and arsenate supports the hypothesis that phosphate and arsenate share common uptake and accumulation mechanisms. Further analysis of the \textit{pho2}, \textit{as1}, and \textit{as2} mutants is required to clarify the roles of the genes involved in these processes.

Vanvliet \textit{et al}, (1995) suggested that the copper-sensitivity of a mutant of \textit{A. thaliana} is associated with and probably due to, increased accumulation of copper as compared to the \textit{wild-type}. However, cadmium-sensitivity in mutants of \textit{A. thaliana} (Howden and Cobbett, 1992) seemed to have resulted from reduced capability for sequestering cadmium. Both leaves and roots of the mutant showed reduced accumulation of cadmium. More recently, Larsen \textit{et al} (1998) reported the isolation of aluminium-resistant \textit{Arabidopsis} mutants based on the enhanced root growth in the presence of an elevated level of aluminium that strongly inhibited root growth of \textit{wild-type} seedlings. These mutants excluded aluminium from the roots by enhancing organic acid exudation or avoided aluminium uptake by enhancing rhizosphere pH. Thus the detoxification of metals in plants is a complex process requiring the interaction of a number of different mechanisms. Unfortunately little is known about the molecular biochemical and physiological processes that result in the arsenic accumulator phenotype. Our mutants \textit{as1} and \textit{as2} along with the \textit{pho2} mutant promise to be valuable tools in this research area.

\textbf{6.4 ISOLATION OF \textit{as1} DNA FLANKING FRAGMENT}

We have isolated and partially characterised a DNA clone from the \textit{as1} mutant, a flanking fragment near the left border of T-DNA tag. Sequencing analysis revealed a number of
stop codons inside the 699 bp sequence, which are characteristic of genomic DNA. Furthermore, the 5LB1 sequence did not show good homology to other known DNA and protein sequences from all organisms, suggesting that its new identity. Due to time constraints, further DNA sequence analysis was not carried out. Since 5LB1 sequence data was obtained from only one direction and the similarity between 5LB1 ORFd and MBP as well as SNF1 was only in the poly-His regions, the nature of the tagged DNA region in asl remains to be elucidated.

6.5 FUTURE DIRECTIONS

The segregation ratios of progeny resulting from genetic crosses between mutant and wild-type lines to confirm the gene tagging in the mutants is an experimental priority. This work has not been done yet due to time constraints. Even though our mutants were described as homozygous, further work is required to confirm the genetic characterisations of these mutants. The mutants should be back-crossed with the wild type. F1 progeny would be grown under nonselective conditions, allowed to self-pollinate, and harvested individually. F2 progeny would then be examined for genetic segregation of As-tolerant phenotypes. The segregation ratio of 3:1 is expected in this generation.

Following the isolation and characterisation of the tagged gene in the mutants, genetic complementation should be carried out. The homozygous mutant plants should be transformed with the isolated genomic or cDNA clones to observe the restoration of a wild-type phenotype. This would be the final proof to demonstrate that the cloned gene caused the mutant phenotype.

Finally, the mechanisms of arsenate and Pi uptake and accumulation in pho2 and asl, as2 mutants will be elucidated, which should advance our general understanding of plant biology in relation to these two elements.
6.6 POSSIBLE APPLICATION OF AS RESISTANCE TRAIT TO ENVIRONMENTAL SCIENCE AND AGRICULTURE

Arsenic contaminated soil in Australia has become an environmental concern. At present, no economical and effective technology is available to remediate these arsenic-contaminated sites. The use of specially selected and engineered plants that can tolerate and accumulate arsenic may provide an economic and effective way of remediating arsenic from the soil. To this end, our study could provide valuable target genes.

In our experiment, no arsenic excluding mutant has been isolated yet. Such mutants would be attractive candidates for additional study of the mechanisms of arsenic resistance. Considering that arsenic is present naturally anywhere in soil although mostly concentrations today's safety standard. Arsenic exclusion would permit the creation of plants capable of growing and producing biomass in arsenic-contaminated soils with safe level of arsenic in their grains, fruits and vegetative parts which are human and animal consumable. This may also present an alternative strategy of utilisation, rather than remediation, for arsenic contaminated sites.
LITERATURE CITED
Bishop EJ (1995) Pollution Fighters hope a humble weed will help reclaim contaminated soil. The wall street journal.


Leggewei G, Willmitzer L, Riesmeier WJ (1997) Two cDNAs from potato are able to complement a phosphate uptake-deficient yeast mutant: identification of phosphate transporters from higher plants. The Plant Cell. 9: 382-392


export of compounds from cells by the multidrug-resistance-associated protein.


APPENDIX
2.10.1

**Buffer B:**
2%(W/V) CTAB
100 mM Tris-HCl (pH 8.0)
20 mM EDTA (pH 8.0)
1.4 M NaCl
1% (w/v) PVP (MW 40,000)

**10% CTAB solution:**
10% (w/v) CTAB
0.7 M NaCl

**Buffer C:**
1% (w/v) CTAB
50 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

**High-salt TE:**
10 mM Tris-HCl (pH 8.0)
1 mM EDTA (pH 8.0)
1M NaCl

2.10.2

**Agarose Gel Electrophoresis**

A Biorad Gel Electrophoresis apparatus was used to perform agarose gel electrophoresis. 1% agarose gel was made in 1 x TAE buffer. After restriction digestion DNA samples were mixed with 1 μl of 10 x Flcoll dye and loaded into the wells. The gel was run in the 1 x TAE at 80-90 V and then stained in a solution containing ~3 μl ethidium bromide in ~100 ml 1 x TAE for 5 minutes. The gel was photographed over UV light, using 667 polaroid film in the polaroid camera. SPP1 bacteriophage DNA restricted with ECoR1 was used as molecular weight marker.
50 x TAE (1 litre):
Tris base 242 g
Glacial acetic acid 57.1 ml
EDTA (pH 8.0) 0.5 M
pH 7.0-7.5

Agarose Loading Dye (10 ml):
Bromophenol blue 0.025 g
Xylene cyanol FF 0.025 g
Ficoll 1.5 g

2.10.4
SOC Medium (100 ml):
SOB medium 100 ml
Glucose 20 mM

SOB Medium (1 litre):
Triptone 20 g
Yeast extract 5 g
NaCl (1 M) 10 ml
KCl (1 M) 2.5 ml
Autoclave and add MgSO4 10 mM

LB agar (1 litre):
Bacto-triptone 10 g
Yeast extract 5 g
NaCl 10 g
pH 7.5
Agar 15 g

2.10.5

2YT media (for two litres):
Bacto-triptone 32 g
Yeast extract 20 g
NaCl 10 g
pH 7.0
2.10.6

6% Polyacrylamide Sequencing Gel:
30% Acrylamide 6.5 ml
Urea 18 g
1X TBE 50 ml
MQ dH₂O 25 ml
10% Amonium persulfate 300 µl
TEMED 30 µl

30% Acrylamide (300 ml):
Acrylamide 87 g
N,N' methylene bisacrylamide 3 g
pH 7.0 or less

30X TBE (1 litre):
Tris base 324 g
Boric acid 165 g
0.5 M EDTA (pH 8.0) 120 ml