

UNIVERSITY OF SOUTHAMPTON

FACULTY MEDICINE, HEALTH AND LIFE SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES

Transition metal homeostasis in *Arabidopsis thaliana*: The role of *AtHMA4* and *AtHMA1*

by

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ABSTRACT
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TRANSITION METAL TRANSPORTERS IN ARABIDOPSIS THALIANA: THE ROLE
OF ATHMA4 AND ATHMA1

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Transition metals such as Zn and Cu are essential for many physiological processes in plants, yet at elevated concentrations these and non-essential metals such as Cd can be toxic. Plants possess a number of homeostatic mechanisms that help to maintain a healthy balance of nutrient metals, whilst limiting the levels of toxic ions. Transition metal transporters such as the Heavy Metal ATPases (HMAs) may play a vital role in this process, yet our knowledge of their function and significance is limited.

This study has focussed on a number of HMA transporters present in *Arabidopsis thaliana*, with particular emphasis being placed on AtHMA4 and AtHMA1. This work was part of a study that was the first to report on a HMA of the Zn/Cd/Pb/Co sub-class in any higher plant species. This work reports on the cloning of *AtHMA4* and its functional characterisation. The cDNA has an open-reading frame of 3516 bp encoding a polypeptide of 1172 amino acids. Analysis of the 5' region of *AtHMA4* using RACE-PCR highlighted the presence of two alternative transcripts within the plant resulting from differential splicing of an intron in the 5' untranslated region. AtHMA4 contains the conserved motifs found in all P-type ATPases and also motifs characteristic of heavy-metal ATPases (also known as P_{1B}-type ATPases or CPx-ATPases). AtHMA4 clusters with the Zn/Cd/Pb/Co subclass of HMAs in phylogenetic analysis and is closely related to AtHMA2 and AtHMA3 but more distantly related to AtHMA1.

AtHMA4 was expressed in all organs of *Arabidopsis* with highest levels in the roots. *AtHMA1* was also expressed widely with highest levels in leaves, followed by stems and roots. *AtHMA4* expression was up-regulated in the roots when plants were exposed to Zn, Cu and Mn, but down-regulated when exposed to Cd and Co. *AtHMA1* expression in roots was reduced by Zn, Cu and Cd, whilst Mn, and to a lesser extent Co, tended to up-regulate expression. The expression profile in leaves was difficult to determine, but Zn was observed to cause up-regulation of both genes. A concentration range for Zn revealed that *AtHMA4* transcript abundance increased markedly in roots and leaves with increasing levels of Zn, whilst *AtHMA1* in roots displayed consistent and gradual reductions. *AtHMA2* showed no response to Zn while *AtHMA3* showed strong down-regulation and *AtHMA5* was up-regulated.

To investigate function, heterologous expression of *AtHMA4* and *AtHMA1* in *Saccharomyces cerevisiae* was carried out. *AtHMA4* conferred Cd resistance to yeast whereas *AtHMA1* conferred susceptibility. Radiolabelled uptake assays confirmed that *AtHMA4* transformed yeast accumulated lower levels of Cd and Zn and *AtHMA1* transformants higher levels of Zn, than vector control yeast. These results are consistent with AtHMA4 functioning as an efflux pump at the plasma-membrane. AtHMA1 could be functioning in metal influx at the plasma-membrane or in the influx of metals into an internal compartment such as the mitochondrion. The results are discussed in relation to the possible roles of AtHMA4 and AtHMA1 within the plant.

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Abbreviations used in text

AAP	Abridged anchor primer
ABC	ATPase binding cassette
ATP	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUAP	Abridged universal amplification primer
BAC	Clone Bacterial Artificial Chromosome
BCA	Bicinchoninic acid
bp	Base pairs
BSA	Bovine serum Albumin
CDF	Cation Diffusion Facilitator
cDNA	Complementary DNA
CNGC	Cyclic Nucleotide-Gated Channels
CSPD	Disodium 3-(4-methoxy-spiro[1,2-dioxetane-3,2'-(5'-chloro)tricyclodecan) 4-yl)phenyl phosphate
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
dH₂O	Distilled water
DIG	Digoxygenin
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotidetriphosphate
DTT	Dithiothreitol
dUTP	Deoxyuridinetriphosphate
EDTA	Ethylene diamine tetra-acetic acid
EGTA	ethylene glycol bis(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ER	Endoplasmic reticulum
EST	Expressed sequence tag
Ferrozine	3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1, 2,4-triazine, monosodium salt
GALL	Deletion variant of the galactokinase promoter GAL1 (in p426 vector)
GFP	Green fluorescent protein
GSP	Gene Specific Primer

GUS	β -glucuronidase
HM	Heavy metal
HMA	Heavy Metal ATPase
IAA	Isoamyl alcohol
IPTG	Isopropyl- β -D-thiogalactoside
kb	Kilobase pairs
kDa	Kilodaltons
LB	Luria Bertani
MBD	Metal Binding domain
MES	2-(N-Morpholino)ethanesulphonic acid
MOPS	3-(N-morpholino)propanesulphonic acid
MNK	Menkes disease protein
mRNA	Messenger RNA
NaOH	Sodium hydroxide
Nramp	Natural resistance-associated macrophage protein
OD	Optical density
PAGE	Polyacrylamide electrophoresis
PBS	Phosphate Buffered Saline
PC	Phytochelatin
PCR	Polymerase chain reaction
PM	Plasma-membrane
PMSF	Phenyl methyl sulfonyl fluoride
PVDF	Polyvinylidene fluoride
PVP	Polyvinylpyrrolidone
RACE	Rapid Amplification of cDNA Ends
rRNA	Ribosomal RNA
RNA	Ribonucleic acid
Rnase	Ribonuclease
RT-PCR	Reverse transcriptase PCR
SDS	Sodium dodecyl sulphate
SERCA	Sarcoplasmic-endoplasmic reticulum calcium ATPase
SSC	Saline-sodium citrate
SSPE	Saline-Sodium Phosphate-EDTA
TAE	Tris/acetate/EDTA
TBS	Tris Buffered Saline
TdT	Terminal deoxynucleotidyl transferase

TE	Tris/EDTA
TEG	Tris/glucose/EDTA
TMD	Transmembrane domain
Tris	Tris(hydroxymethyl)aminomethane
UV	Ultra violet
v/v	volume per volume
WND	Wilson's disease protein
w/v	weight per volume
X-Gal	5-Bromo-4-chloro-3-indolyl- β -D-galactoside
ZIP	Zrt/Irt related protein

Chapter 1 General introduction

Transition metals are ubiquitous in the environment. Life is thought to have evolved from a primeval soup that contained a background of solubilized metallic ions. Consequently living organisms have adapted to exploit the unique chemical properties of these elements. Some have become essential for the correct functioning of important biological processes, whilst others are excluded or regulated by specially developed homeostatic mechanisms (George 1991; Kieffer 1991). These mechanisms exist in all life-forms from simple bacterial cells, to fungi, plants and animals. Such is the importance of metals in biology, their behaviour forms a prominent theme in the fields of toxicology, medicine and nutrition.

Plants require a range of transition metals as essential micronutrients for normal growth and development (Hall and Williams 2003). Genes have now been discovered for transport proteins that acquire/distribute metals (Williams et al. 2000; Hall and Williams 2003), and techniques developed so that their role in plant metal homeostasis can be elucidated. Sequencing of the genome of the 'model plant' *Arabidopsis thaliana* is now complete (Ausubel 2000; The Arabidopsis Genome Initiative 2000) and this gives us the opportunity to investigate some of the transporters responsible for metal transport in higher plants. A wide range of gene families have been identified in plants that are likely to be involved in metal transport (Williams et al. 2000; Hall and Williams 2003). Despite recent progress in our understanding of their molecular mechanisms, there are many cases where clear evidence is lacking on their location, function and even substrate specificity.

With the implication of transition-metal pumps in the transport of an increasingly diverse range of ions (Silver 1996), the potential for their involvement in metal accumulation, compartmentation, and tolerance is gathering momentum. If the transporters are implicated in some way, then it is possible that genetic engineering could be used to produce plants with a 'tailored' physiology/biochemistry, desirable in certain agricultural and phytoremediation schemes (Wagner 1993; Salt et al. 1998; Meagher 2000). The latter of these two scenarios is of topical interest, with regards to the possible accumulation of metals in agro-ecosystems, resulting from sewage sludge application (Alloway 1991; McBride 1995); genetically modified plants could help reduce the burden of toxic metals in foodstuffs. For now these goals remain the ideal, to be achieved by future work. At present, interest in plant transition metal interactions is generally focussed at a more fundamental level.

Since metals are by their very nature, reactive elements (George 1991) they may undergo numerous reactions with biological molecules. A review of these reactions must be given in any discussion on putative transporters, as such systems may act competitively, to divert metallic ions from the target site in question (e.g. specific binding sites on transporters). In order to help predict metal ion behaviour, various attempts have been made to develop classification schemes, based primarily on ion chemistry. Unfortunately many of these schemes have been of limited use, with some even vague as to the description of the different classes of metal (Martin and Coughtrey 1982). Perhaps the broadest predictor of metal ion behaviour in biotic systems, at least in the first instance, is their designation as 'essential' or 'non-essential' elements.

1.1 Essential and non-essential metals

Essentiality is a term that has been defined consistently by numerous authors. Some, such as Epstein (1972) and Bowen (1979), have set out criteria in order to ascertain the essentiality of an element in question, while others have opted for a more concise statement. Mertz and Cornatzer (1971- cited in Kieffer 1991) consider essentiality to be demonstrated "when a measurable deficit in the diet reduces the growth and vitality of humans, animals or plants to a reproducible degree". However, whilst this definition remains succinct, confusion may arise from its brevity. It is plausible that even elements which are known to be toxic in the higher concentration range could be regarded as essential in minute quantities (Kieffer 1991; Mehra and Farago 1994). Such uncertainty stems from the difficulty in proving conclusively and unequivocally that an element is required for proper functioning of an organism (Pais 1993). The chemical properties of the elements are of little use in highlighting potential nutrient elements. Physical and chemical regularities are frequently modified in biological systems, such that the periodic system (table) does not provide clear answers to questions regarding the toxicity and essentiality of individual elements or their ligands (Kabata-Pendias and Pendias 1995).

In a similar way, the concentration of an element within a particular organism may yield little information on its potential significance (Markert 1998). For example, plants known as hyperaccumulators may accumulate large concentrations of elements such as Cu, Co, Pb, Ni, Mn, and Zn (Brooks 1994; Baker et al. 2000), in a mechanism that is apparently separate from normal metabolic demand. Indeed, a large portion of accumulated metals may remain bound in an inert form,

sequestered from delicate constituents such as enzymes, structural proteins and lipids (Thurman 1981; Verkleij and Schat 1990; Neumann et al. 1995). In order to avoid this problem, some authors have seen it necessary to briefly summarise the uses of elements, when discussing their requirement on a macro or micro scale (Clarkson and Hanson 1980; Kabata-Pendias and Pendias 1995). The classification presented by Markert (1998) (Table 1.1) is a simple division of the elements that highlights the importance played by certain metals, most notably those of the transition group (e.g. Mn, Fe, Co, Ni, Cu, Zn and Mo) (Borovik 1990) in biological systems. Often the term 'heavy metal' is used when describing the behaviour of metal ions in biological systems. This term has often been defined using the specific gravity or atomic number of an element (e.g. Walker et al. 1996), however there is no clearly accepted definition (<http://www.iupac.org/>). The use of the term 'transition metal' is preferred in this project, as it has a strict chemical definition. Transition metals are those elements in the transition group of the periodic table, mainly: 1st row transition metals, Sc to Zn; 2nd row transition metals, Y to Cd; 3rd row transition metals La to Hg. This project will largely focus on the transition metals which have been shown to be important micronutrients in plants and additionally Cd, which can be a substrate for some of the transport proteins discussed in this work.

There are several properties that allow transition metal ions to have such diverse roles in nature, the two most important being their ability to accept and donate electrons and to bind several donor groups. As a result of these properties, transition metals can act as regulators of biological processes (e.g. metal regulatory elements in gene expression; Rosen 1999), form important structural components (e.g. enabling specific folding of metalloproteins; Kotrba et al. 1999) and carry out functional roles as activators of enzyme catalysis (Kabata-Pendias and Pendias 1995; Borovik 1990). Indeed, roughly 30% of all enzymes are classed as metalloenzymes or require metal ions for activity (Hay 1994). Zn is a good example. This micronutrient is important for the correct functioning of superoxide dismutase (Marschner 1995), but it is also an essential component of at least another 150 enzymes, with representatives in each of the six classes of enzyme (Marschner 1995; Walker et al. 1996).

1.2 Toxic metals

In demonstrating the discrepancies that often arise between plant tissue concentration and requirement, Pb, Co, Cu, Ni and Zn have been cited as examples of elements that are found elevated (above 1000 mg/kg dry weight plant material) in

Table 1.1 Classification of the elements according to their physiological attributes

Structural elements ¹	C, H, O, N, P, S, Si, Ca
Electrolytic elements ²	K, Na, Ca, Cl, Mg
Enzymatic elements ³	V, Cr, Mo, Mn, Fe, Co, Ni, Cu, Zn, B, Sn

Source; Modified from Markert (1998)

Definitions from Markert (1998)

¹ "Elements which participate in the constitution of the functional molecular structural elements of cell metabolism"

² "Elements required for the constitution of specific physiological potentials and are important for maintaining defined osmolytic conditions in cell metabolism"

³ "Elements that exercise a catalytic function in cell metabolism as metal complex compounds"

different hyperaccumulator species (Baker et al. 2000). It is evident that a mix of essential and toxic metals may be accumulated. For example, Pb is an element of mild phytotoxicity (Davis 1995). It generally exerts deleterious effects when present in leaf tissue at 30 - 300 mg/kg (Kabata-Pendias and Pendias 1995) causing symptoms such as stunted growth and wilting (Kabata-Pendias and Pendias 1995; Wierzbicka 1999). Since it has no known essential biochemical function, Pb and other such metals, are regarded as toxic elements. This designation is somewhat incorrect, as they may in fact be less toxic (w/w basis per dry wt. of plant tissue) than some plant micronutrients (e.g. Cu). With this reasoning, Alloway and Ayres (1993) have pointed out that the term 'toxic' element should be dropped in favour of a 'non-essential' label, since there is confusion with the previous classification. Micronutrients are liable to react differently in biological systems to their non-essential counterparts, yet both may exert toxic affects.

1.3 Toxic effects of transition metals on plants

The maintenance of membrane integrity is a crucial factor in determining the tolerance of plants to transition metals (Demidchik et al. 1997). However the plasma membrane is not always completely selective with regards to the concentrations and ionic species taken up. This means that the cytosol can experience appreciable fluctuations in the concentration of free metal ions (Rauser 1999). This may be deleterious to the plants well being, as free metal ions may disrupt a number of vital metabolic processes such as photosynthesis, respiration, transpiration (including general water balance) and nucleic acid metabolism (Van Asche and Clijsters 1990; Ross 1994; Lagriffoul et al. 1998; Ralph and Burchett 1998; Losch and Kohl 1999; Poschenrieder and Barcelo 1999). These responses may occur directly or indirectly as a result of metal stress and in such cases are termed primary or secondary effects. An example of such influences may be seen in the case of plant water relations. Barcelo and Poschenrieder (1990) have highlighted that metal ions can disrupt osmoregulation directly, such as altering ionic balance of various tissues and cellular compartments, or indirectly by affecting the number and size of leaves and stomata. Another example concerns nucleic acid metabolism. Transition metals can bind directly to DNA molecules, causing cross-linking between both DNA strands (Gebhart and Rossman 1991). Conversely, metals may also cause damage to genetic material by secondary effect; a general response of plants to metal stress is to experience a rise in the concentrations of free radicals and reactive oxygen species (Schützendübel and Polle 2002). Nucleobases and sugar moieties are the

primary site of oxidative attack, purines and pyrimidines may be destroyed and nicks may be introduced into the sugar-phosphate backbone of DNA (Gebhart and Rossman 1991; Dietz et al. 1999).

While these examples have demonstrated a definite segregation between the primary and secondary effect of metal stress, other situations may not be so clear-cut. Photosynthesis is recognised as one of the most metal-sensitive metabolic functions of higher plants (Van Assche and Clijsters 1990). Both the dark and light reactions may be affected, but it is perhaps the latter which suffers the greatest disruption. Several points along the reaction pathway (principally photosystem II) appear sensitive. Transition metal ions may affect the transport chain by scavenging electrons, inducing lipid peroxidation of chloroplastic membranes and by inhibiting key enzymes such as the water-splitting, manganese complex (Sandmann and Böger 1980; Van Assche and Clijsters 1986; Hsu and Lee 1988). Van Assche and Clijsters (1990) have reviewed the general effects of transition metals on enzymes. These authors highlight two points with particular relevance to photosynthesis (1) enzymes may suffer loss of function due to displacement of an essential metal-ion with catalytic activity and (2) that enzymes may in fact be induced in the presence of some transition metals. This latter mechanism has recently been considered to play a part in the inhibition of photosystem II. A surplus of transition metal ions have been proposed to increase the activity of galactolipase, an enzyme which erodes thylakoid membranes and eliminates the electropotential gradient needed for the formation of ATP (Ernst 1998).

Although specific transition metal-ions can usually be assigned to one or a few modes of toxic action, it must be remembered that these can vary between plant species, and even between varieties within a species (El-Jaoual and Cox 1998). As a result of this, it is useful to summarise the potential reactions that may cause toxic effects (Table 1.2). It must also be remembered that plants may employ a number of mechanisms to overcome these toxic effects.

1.4 Uptake and distribution of transition metals in plants

Essential and non-essential transition metals are obviously treated differently within plants, both in the event of acquisition, and during transport and storage in the symplasm, but it is hard to make generalised descriptions. In the rhizosphere physico-chemical factors (e.g. pH, pE cation exchange capacity (Rowell 1994)) tend to dominate, controlling the concentration and ionic species that are presented to or

Table 1.2 Classification of transition metals and metalloids according to their generalised toxic affects

Reaction	Metals involved
Changes in permeability of the cell membrane	Ag, Au, Cd, Cu, Hg, Pb,
Reactions of thiol groups with cations (highest affinity shown)	Hg, Pb, Cd
Competition for sites with essential metabolites	As, Sb, Te, W
Affinity for reacting with phosphate groups and active groups of ADP or ATP	Be, Sc, Y, Zr, potentially all transition metals
Occupation of sites for essential groups such as phosphate and nitrate	As, B, W

Source; Modified from Kabata-Pendias and Pendias (1995)

'available' at the root plasma membrane. Unfortunately the properties of availability and essentiality do not necessarily coincide, and a plant may be swamped with non-essential metal ions. In some instances, the soil environment may be conducive to the movement or fixation of a particular metal, acting in an almost specific manner and independent of plant requirements (Alloway 1995). Plants themselves may attempt to ameliorate the situation by manipulating soil chemistry. The extrusion of protons and bicarbonate ions, electrons (Fergusson 1990; Ross 1994) and phytosiderophores (nonprotein amino acid chelators) (Marschner 1995) from the roots are perhaps more common responses, but there are reports that some species effect changes on soil chemistry (largely the pH) by switching to a different nitrogen source (Klobus et al. 2002). Despite the variations, these responses are still largely unspecific (in terms of plant requirement) and as a consequence, increase the availability of a number of metal ions, including those of non-essential elements. In addition, the mechanisms tend to function by enhancing the effect of general uptake phenomenon such as mass flow and ion diffusion, already established in the rhizosphere (Ross 1994).

1.4.1 Uptake into the apoplasm

The previous section has highlighted how a number of processes operating in the bulk soil/ rhizosphere can lead to a melee of ions at the soil-root interface. The plant has a limited capacity to select for its specific requirements at this level, and the same appears to be true within the root compartment known as the apoplasm (Marschner 1995; Greger 1999). The flow of ions in this porous network of cellulose, hemicellulose, pectins, carboxylic acids and proteins (components of the cell wall continuum) is a non-metabolic, passive process. Binding of metals to negatively charged or nitrogen containing groups restricts their movement and increases their concentration in the vicinity of the plasma membrane. Cation exchange sites are extremely important although not essential, to the uptake process. Such is the effect of cation exchange sites that they contribute significantly to the overall metal 'content' of the root. In fact, whilst it is generally agreed that the plant has little or no influence over the root-cation exchange capacity, an early study has suggested that an increased binding potential may be employed by metal tolerant plants to reduce their uptake of Zn^{2+} (Turner and Marshal 1972). Certain aspects of this mechanism remain unexplained and a volume of evidence is still lacking (Hall 2002). Metal exposure has been observed to alter the composition of plant cell walls, namely by increasing wall thickness and rigidity (Csech et al. 2002),

however this is thought to be a manifestation of a toxic effect on cell wall synthesis, rather than a 'protective' response (Csech et al. 2002).

1.4.2 Uptake at the plasma membrane

The plasma membrane is the first 'living' structure of the plant to be exposed to potentially toxic transition metal ions. It is also the first compartment where the plant may exert some degree of regulation on the ionic contents of the cell interior (Ernst 1998). The presence of numerous transport mechanisms, (pumps, carriers and channels), each with a limited substrate range allows plants to customise their uptake in line with requirements (Taiz and Zeiger 1998; for a discussion see later sections). However, metal selection by these various mechanisms is not entirely efficient and excess nutrient, or toxic metal ions may gain entry to the plant symplasm. This appears to occur both passively and actively (Costa and Morel 1994). Passive uptake, whereby ion movement into plant tissue is driven by a (chemical) concentration gradient is rather limited, by far the greatest contribution derives from transport processes that are linked with metabolism and the expenditure of energy. This poses the question: why would a plant expend energy to poison itself? Initially, it would seem that the uptake of 'unwanted' metal ions occurs via less specific mechanisms (Verkleij and Schat 1990; Clemens 2001). Breach of the membrane through channel and carrier proteins, in a facilitated diffusion process, is likely to be responsible. A link with metabolism would be present in secondary active transport processes via the proton motive force (Δp) and its generation by hydrogen ion extrusion by plasma membrane H^+ ATPases. Indeed several (secondary active) transporters from different families have been shown to transport a number of ions (Clemens 2001; Hall and Williams 2003). Lack of substrate specificity by these proteins could explain the carrier-mediated uptake kinetics observed for some toxic ions (Clemens 2001). Other mechanisms may contribute however. Most intriguing is the possible role played by heavy metal ATPases (HMAs; also referred to as CPx-type ATPases or P_{1B} ATPases). These primary active pumps can transport their substrates against chemical and electropotential gradients, allowing the accumulation of ions at the expense of ATP. Until recently, transporters of this group have been poorly characterised in plants but work with other organisms such as bacteria and yeast suggest that they may allow metals to traverse both intracellular and extracellular membranes, with individual transporters being dedicated to either import or export of a particular ion. Up until recently the limited evidence available on plant HMAs has suggested that

these proteins may be more involved with the intracellular trafficking of metal ions. The loading of copper into post Golgi vesicles by RAN 1 and the chloroplast by PAA1 are clear examples (Hirayama et al. 1999; Shikani et al. 2003). However, work by van Hoof et al. (2001) has shown that an ATP-dependant Cu-efflux protein exists in the root plasma membrane of copper-sensitive and copper-tolerant ecotypes of *Silene vulgaris*. Studies of enzyme activity suggest that this could well be a member of the HMA family of transporters (van Hoof et al. 2001).

1.5 Mechanisms used by plants to deal with transition metals

Several mechanisms have been implicated which may help to make plant cells resistant to elevated levels of transition metals and these will be discussed below.

1.5.1 Metal chelators

Despite the absence of genetic evidence conclusively linking the characteristics of heavy metal tolerance and hyperaccumulation, it is obvious to assume that hyperaccumulators possess some degree of tolerance (Macnair and Baker 1994; Baker et al. 2000). Since there appears to be no difference in the metal sensitivity of enzymes from metal tolerant or intolerant plants it appears likely that, for hyperaccumulators at least, internal sequestration of metals is responsible for an important part of tolerance (Baker et al. 2000). Following the terminology of Nieboer and Richardson (1980), Baker et al. (2000) have classified intracellular metal-binding ligands with regards to their electron donating centers, namely sulphur, nitrogen and oxygen donor ligands. Generally these equate to proteins and phytochelatins, amino acids, and organic acids, and follow the same order for reducing metal-ion affinity and complex stability. Various studies have shown that metal tolerant plants may possess greater quantities of these complexing substances in comparison to metal-sensitive lines (Krämer *et al.* 1996). Some have even demonstrated strong correlations between the amount of chelate production and the rate of metal supply (Thurman and Rankin 1982). However, despite these observations, there are no *a priori* reasons to suggest that these responses form part of a tolerance mechanism (Thurman 1981). Even if they are, their involvement may still only be of a secondary nature. Such secondary tolerance mechanisms are present in both tolerant and non-tolerant plants, and do not contribute to resistance of metal stress *per se* (Macnair and Baker 1994). They tend not to be displayed by

sensitive plants upon metal exposure, simply because injury prevents them from doing so (Macnair and Baker 1994).

Caution has been heeded in the interpretation of a number of studies looking at intracellular chelates. For example, zinc tolerant clones of *Deschampsia caespitosa* have been shown to accumulate higher concentrations of malate and citrate than non-tolerant clones, leading to the view that these may be involved in a tolerance mechanism (Mathys 1977). This does not appear to be the case for two main reasons (1) the plants are still sensitive to copper, despite the complexes having similar stability constants as those for zinc (Thurman and Rankin 1982) and (2) inhibition and stimulation of citrate production fails to have any effect on the metal sensitivity of both the metal tolerant and intolerant clones (Thurman 1980; Thurman and Rankin 1982). In another case, an alternative explanation has also been produced to account for the elevated levels of organic acids in the nickel hyperaccumulators *Alyssum bertolonii* and *Pearsonia metalliferasin* (Peterson 1993). Initially it would seem that organic acid levels may be stimulated in order to improve metal tolerance, but it has been pointed out that this effect may be a result of iron deficiency (caused by high concentrations of Ni) (Peterson 1993). Indeed iron chlorosis is known to have such an effect (Marschner 1995). Misinterpretation of data has not remained exclusive to the organic acids. Phytochelatins and metallothioneins (class I and II) were once thought to be the sole factors contributing to plant heavy-metal tolerance, although evidence now suggests otherwise. This is especially true for phytochelatins which may be produced to a greater extent in metal-sensitive plants (de Knecht et al. 1994; 1995), and have no effect on tolerance when their production is inhibited (Reese and Wagner 1987; Shultz and Hutchinson 1988). Data on metallothioneins (MTs) may be particularly confusing, since they can display a complex induction pattern not only with regard to different tissues but also to different metal ions. For example increased Cu has been shown to cause elevated levels of MT-like mRNA in *Brassica juncea*, whilst raised Zn causes an opposite effect (Schaffer et al. 1997).

Despite the lack of evidence to substantiate claims that intracellular chelating compounds play any significant role in heavy metal tolerance of plants, it is clear that they do bind to metals (e.g. Maitani et al. 1996), and so may play important roles in metal-ion homeostasis (Kotorba et al. 1999; Rauser 1999; Baker et al. 2000). Phytochelatins, which have been the subject of extensive study particularly in relation to Cd, are a good example. It has been demonstrated that these compounds may protect metal-sensitive enzymes by sequestering free ions

(Kneer and Zenk 1992), but importantly, they have the ability to re-supply these ions to metal-deficient metalloenzymes (Thumann et al. 1991).

1.5.2 Possible importance of transport processes

The previous section has cited work demonstrating the importance of various organic ligands in plant-metal ion homeostasis. While sequestration is of undeniable importance in regulating metal ion activity, recent work has hinted at the, overriding importance of metal transport processes in both stressed and healthy plants (Vogeli-Lange and Wagner 1990; Clemens 2001; Hall and Williams 2003). Indeed in some cases, there seems to be a crossover between these two phenomena. It has been known for many years that plants transport a range of elements in the xylem as relatively inert organic complexes with compounds such as malate and citrate (Tiffin 1965a,b, 1967) and that competition may occur between elements for uptake by these 'xylem carriers'. Additionally, the importance of these mobile complexes has also been demonstrated at a cellular level, where work has shown that they may act as shuttles to facilitate the transport of certain elements (e.g. Zn) across the tonoplast to the vacuole (Harmens et al. 1994). Similar behaviour has also been observed for phytochelatins in yeast with an ABC-type vacuolar membrane transporter (Ortiz et al. 1995). This protein has been postulated to exist in plants, where other transporters may complement in the metal sequestration process. A $\text{Cd}^{2+}/\text{H}^{+}$ antiporter characterised in oat (*Avena sativa*) vacuolar membranes and sulphide (S^{2-}) carriers (as yet undiscovered), could act to transform the low molecular weight (LMW) PC into a medium or high molecular weight (M/HMW) complex (Rauser 1999). These complexes, with their crystal-like structure (due to their higher S:PC ratio) possess greater metal binding stoichiometries, and acid pH stability (more suitable for the vacuolar environment) and could thus lead to greater metal resistance (Rauser 1999). Whilst the existence of the ABC-transporter mediated, LMW-HMW model has been questioned (Kneer and Zenk 1997), the importance of plant metal transporters in intracellular partitioning is now starting to be elucidated and the next section will describe some of the main transition metal transporters identified to date.

1.6 Plant transition metal transporters

Three main classes of transport protein can be identified in plant membranes; channels, carriers and pumps. All potentially play a vital role in plant

metabolism. For example only around 15% of *Arabidopsis* transport proteins fall within the first category, whilst the latter two, often classed collectively as [true] 'transporters', comprise over 80 % (The Arabidopsis Genome Initiative 2000). Channels can be distinguished from transporters by the rate at which they mediate ion flux. The mechanism is several orders of magnitude faster in channels (Fox and Gueriot 1998), and does not appear to involve a fixed stoichiometry of solute movement (Hediger 1997). This behaviour is due to the underlying fact that channel-mediated translocation does not involve the binding of solutes to a specific substrate binding site(s) (Hediger 1997). It is always a passive process, with net transport occurring in the direction of the electrochemical gradient. This is in contrast to pumps, which require to be coupled directly to a metabolic source of energy, such as ATP hydrolysis, redox reactions, or (as in certain bacteria) the absorption of light by the carrier protein (Taiz and Zeiger 1998; Palmgren and Harper 1999). The three major types of pumps present in plants are the ATP-binding cassette (ABC) transporters (mentioned above), the P-type ATPases and the vacuolar or V-type ATPases. A distinction is often made between these proteins since they appear to be associated with different functions. V-type ATPases may participate in the co-transport of a number of substrates (e.g. amines and amino acids etc.) however they all have the primary role of proton transport (Pederson and Carafoli 1987; Ratajczak 2000). The assignment of ABC transporters to one particular (generalised) function is not so simple. In addition to the diverse array of potential substrates, some members of the group may also behave as ion channels and channel regulators (Theodoulou 2000). Despite this, current evidence suggests that organic compounds tend to be the most commonly transported substrate in plants (Theodoulou 2000). In addition they play an important role in the movement of phytochelatin and glutathione complexes (Ortiz et al. 1995; Taiz and Zeiger 1998) as well as having possible roles in the compartmentalisation of organic nutrients, unusual metabolites and drugs (The Arabidopsis Genome Initiative 2000). P-type ATPases, on the other hand are a major family of ion transporting ATPase, involved primarily in the transmembrane movement of different inorganic cations including for example H^+ , Ca^{2+} , K^+ , Na^+ and also transition metal ions such as Cd^{2+} , Zn^{2+} and Mn^{2+} (Kuhlbrandt et al. 1998; Scarborough 1999).

Whilst the importance of P-type ATPases in the movement metal cations in plant cells is not in any doubt, it is important to stress that they are by no means the only transport proteins to participate in this role. *Arabidopsis* in particular, possesses around 600 genes dedicated to transport, occupying quite a large portion of its (protein-coding) genome (Van Belle and Andre 2001; Martinioa et al. 2002). It is

proposed that functionally overlapping but structurally distinct transporters can provide plants with the ability to transport nutrients under various conditions, including differing energetic conditions, genetic defects and in the presence of toxic blocking cations (Mäser et al. 2001). What is more, they may also be co-regulated by the same metallo-regulatory protein (e.g. Nramps and ABC transporters in the bacteria *B. subtilis*; Jakubovics and Jenkinson 2001). Yeast, a relatively simple eukaryotic organism possesses no less than 5 different transporters belonging to two different families, that are involved in the movement of Zn (MacDiarmid et al. 2000) and more are still suspected to exist (Guerinot and Eide 1999). Besides the P-type ATPases, there are around nine different families of transporter protein may be involved in the movement of transition metal ions in plant tissues (Clemens 2001). These include the Nramps, CDFs, ZIPs and ABC transporters (Williams et al. 2000; Clemens 2001; Hall 2002; Hall and Williams 2003).

1.6.1 ABC transporters

Energetically, ABC transporters are not dissimilar from their P-type ATPase counterparts. These membrane-associated proteins have a direct dependence on MgATP, which binds to a nucleotide binding fold (NBF), a large cytosolic 'loop', in order to couple ATP hydrolysis with membrane transport (Theodoulou 2000; Martinoia et al. 2002). The catalytic cycle also shares some similarity, both form a phosphorylated intermediate and thus both are inhibited by micromolar concentrations of vanadate (Martinoia et al. 2002; <http://www.plantphys.net/>). In terms of structure ABC transporters are an extremely diverse group. All members consist of one or two pairs of basic structural elements – integral membrane spanning domains (MSDs) which contain multiple (usually four to six in plants) transmembrane spans and the cytoplasmic nucleotide binding folds, mentioned above (Rea 1999). These different domains may be encoded as separate subunits and expressed as separate polypeptides or they may be fused together to form multi-domain proteins. (Rea 1999). In eukaryotic organisms such as plants, ABC transporters are encoded by conserved gene families, producing proteins in which the core domains are contiguous on a single multi-domain polypeptide (Rea 1999; Martinoia et al. 2002). Fifty four genes encoding these so called 'full-size' ABC proteins have been found in the *Arabidopsis* genome (Jasinski et al. 2003). Each belongs to one of four different gene families, depending upon their structure. These include multidrug-resistance (MDR) and multidrug-resistance related proteins (MRPs), and the pleiotropic drug resistance (PDR) and ABC1-like proteins (the

latter two show mirrored topology). The substrates of these various sub-families are as diverse as their structure. Transport of compounds such as peptides, sugars, lipids, polysaccharides, alkaloids, steroids, inorganic acids, glutathione conjugates and most importantly heavy metal chelates, may all be mediated by ABC transporters (Hall and Williams 2003). Moreover whilst some show relative specificity, others are able to mediate the transfer a variety of chemically dissimilar compounds. Early reports on plant ABC transporters highlighted their importance in detoxification processes, in particular via their ability to concentrate compounds within the vacuole. Despite the involvement of HMT1p in the movement of Cd-phytochelatin (Cd-PC) complexes in yeast (Ortiz et al. 1992, 1995), it still remains the case that no HMT1 homologues have yet been found in plants (Rea 1999). One reason for this could be due to the presence of another ABC transporter AtMRP3, which is thought to load Cd-glutathione (Cd-GS) complexes into the vacuole (Tommasini et al. 1998). Since phytochelatins are composed of multiple glutathione units, it has been suggested that AtMRP3 could have the ability to recognise Cd in both PC and GS complexes (Rea 1999). AtMRP3 has been shown to suppress the Cd²⁺ hypersensitivity of *ycf 1Δ* yeast strains, but the biochemical mechanism of this is not known (Tommasini et al. 1998). Doubt has been cast over its role in Cd-GS transport. The closely related AtMRP1 and AtMRP2 proteins neither confer resistance to nor mediate GSH dependent transport of Cd²⁺ (Lu et al. 1997, 1998). Perhaps the protective action of AtMRP3 is achieved in some other way. For instance it may ameliorate Cd toxicity by vacuolarly sequestering the products of toxic metal action, for example reactive oxygen species (ROS), rather than Cd ions itself (Rea 1999). Bovet et al. (2003) have recently demonstrated that the up-regulation of four *Arabidopsis* MRP genes was in response to Cd and not to oxidative stress. Despite this however, there is still no firm evidence that AtMRP3 functions directly in Cd transport (Bovet et al. 2003).

The fact that some metal ions function as essential plant nutrients, highlights another important aspect of ABC transporters, one that has been alluded to only recently. Studies have shown that they may play an important role in plant growth and development processes. This may occur indirectly via modulation of ion channel activity (Martinoia et al. 2002), or directly by normal ATP-fuelled transport. In the latter case, transport of metals such as Fe, Mn and Zn have been observed. In yeast the mitochondrial transporter ATM1 has been shown to be essential for the synthesis of Fe/S clusters (Leighton and Schatz 1995). Mutation of the *Arabidopsis* orthologue *Sta1* produced cells with 1.5 – 1.8 fold higher levels of free iron in their mitochondria, and consequently less was associated with protein. Mutant plants

exhibited both dwarfism and chlorosis, suggesting that Sta1 plays a vital part in an Fe shuttling pathway. In the bacteria *Streptococcus* several putative ABC transporters have been linked to the transport of Zn and Mn. Mutations in *sca* genes of *S. gordonii* and *psa* genes of *S. pneumoniae* have been shown to reduce Mn^{2+} uptake and reduce the ability of cells to undergo DNA mediated transformation. ABC transporter-mediated Mn^{2+} movement has also been observed in the cyanobacterium *Synechocystis*.

1.6.2 Nramp transporters

The Nramp proteins are a class of pH dependent divalent transition metal cation transporters that are found in a wide range of different organisms, such as bacteria, fungi, plants and animals (Jakubovics and Jenkinson 2001; Mäser et al. 2001). Each member of the group is predicted to have 12 membrane-spanning domains and possess a fairly large (around 20 amino acids) conserved region, known as a consensus transport signature. This is located in the fourth intracellular loop between the 8th and 9th transmembrane domains (Williams et al. 2000; Hall and Williams 2003) and its function is not known. Whilst in the majority, not all Nramps have been linked to the transport of metal cations. *EIN2* (ethylene insensitivity gene 2), one of the only plant Nramps to be characterised, functions in the transduction of multiple stress signals and has no metal-transport component (Alonso et al. 1999; <http://plantst.sdsc.edu/plantst/html/cation.shtml>). This protein, whilst displaying the characteristic conserved motifs, is of much lower sequence similarity to a further 6 Nramps known to exist in the *Arabidopsis* genome (Mäser et al. 2001; Grotz and Gueriot 2002). AtNramps 1-6, themselves display subtle differences in structure, with the closely related AtNramp 1 and 6 being distinct from a second phylogenetic grouping containing AtNramp 2,3,4 and 5 (Mäser et al. 2001; <http://plantst.sdsc.edu/plantst/html/cation.shtml>; Curie et al. 2000). *AtNramp 1,3* and *4* have all been shown to complement an Fe-uptake mutant of yeast (*fet3fet4* and *smf1* mutants), and are induced in Fe-deficient plants (Curie et al. 2000; Thomine et al. 2000). This suggests a possible function in Fe uptake. However, further evidence may point to their involvement in different roles. Overexpression of *AtNramp1* leads to an increase in plant resistance to Fe toxicity, suggesting that AtNramp1 may be targeted to an intracellular membrane where it plays a key role in Fe distribution/homeostasis within the cell (Curie et al. 2000; Grotz and Gueriot 2002). In addition, disruption of *AtNramp3* produced plants with no obvious defects in Fe metabolism (Grotz and Gueriot 2002; Thomine et al. 2000). It did however promote

slightly greater resistance of root growth to Cd^{2+} treatment and increased sensitivity to this metal ion when overexpressed (Thomine et al. 2000). This, along with other work on mammalian DMT1 (Nramp2) and the yeast homologues (SMF 1 and 2) suggests that Nramps are rather 'promiscuous' (unspecific) in their transport of transition metal ions (Lui et al. 1997; Chen et al. 1999; Andrews 2001; Mäser et al. 2001). Each has displayed the *uptake* of a range of metal ions. DMT1, which was isolated in a functional screen for Fe transport systems has subsequently been shown to transport Zn^{2+} , Mn^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Ni^{2+} and Pb^{2+} . Similarly in yeast, SMF1 and 2 which were initially characterised in the transport of Mn^{2+} have since been shown to transport Cu^{2+} , Co^{2+} , Cd^{2+} and Fe^{2+} (Supek et al. 1996; Lui et al. 1997; Chen et al. 1999). What this means with regards to their physiological role in plants is uncertain. Expression profiles suggest that they contribute to constitutive metal transport and up-regulation in response to Fe starvation suggests that (for some members at least) this ion is of primary importance. It is conceivable that the AtNramps, primarily associated with the movement of Fe, can be used to transport other ions during periods of stress or altered metabolism which may result from an unbalanced ionic environment. For example, AtNramp1 distributes Fe^{2+} under normal physiological conditions, but when concentrations of various other free ions rise, its lack of specificity means that these will also be dealt with, perhaps by sequestration and/or compartmentalisation. For Nramps thought to be involved in uptake at the root plasma membrane a similar situation may arise. When transport pathways for different ions become blocked or reduced (e.g. ion competition etc.), certain Nramps may act to provide an alternative, but limited, route of entry (Curie et al. 2000). This mechanism has been suggested to work in support of some members from the ZIP transporter family (Curie et al. 2000).

1.6.3 ZIP transporters

The ZIP (ZRT-IRT-like) transporter family of proteins derives its name from the founding members of the group IRT 1, the Iron Regulated Transporter, first isolated from *Arabidopsis* and ZRT 1 and ZRT 2 the Zinc Regulated Transporters subsequently identified in yeast (Eide et al. 1996; Guerinot and Eide 1999; Maathuis and Sanders 1999; Guerinot 2000). As with the Nramps, the ZIP family of proteins require a pH (H^+ ion) gradient in order to mediate transport across the membrane (Thomine et al. 2000). In addition, they also display some diversity in the type of transition metal that may be accepted as the substrate ion. The group is thought to be much larger however, with over 85 members identified in bacteria, archaea,

fungi, protozoa, insects, plants and animals (Mäser et al. 2001). These divide into four main subfamilies, with all plant ZIP transporters identified to date (less than 20) being found within the same group (group I) (Guerinot and Eide 1999; Mäser et al. 2001; Guerinot 2000). Their predicted structures are very similar. Each is proposed to consist of 8 transmembrane domains, orientated so that both the amino and carboxyl termini are located on the outside surface of the plasma membrane (Guerinot and Eide 1999; Guerinot 2000; Mäser et al. 2001). The transmembrane domains are grouped in a (N → C terminus) 3 + 5 arrangement with the clusters separated by a variable length region. This is largely responsible for the diverse range of protein sizes seen in the ZIP family of transporters. In most cases the 'variable region' contains a potential metal binding domain rich in histidine residues that is predicted to be cytoplasmic. In IRT1 the sequence is HGHGHHG whilst in ZRT1 it is HDHTHDE. Although its function is not known, this motif is thought to be important in transition metal-ion uptake or its regulation (Guerinot and Eide 1999; Mäser et al. 2001). Further conserved regions also occur in putative transmembrane spans 4, 5 and 6 (Maathuis and Sanders 1999). Of these, that present in transmembrane domain 4 is conserved most strictly. Consisting of histidine and an adjacent (semi) polar residue (often serine; Rogers et al. 2000) this site is predicted to form an amphiphatic helix and may comprise part of an intramembranous heavy metal binding site that is part of the transport pathway (Mäser et al. 2001; Guerinot and Eide 1999). In agreement with this model, Rogers et al. (2000) have shown that mutations in the conserved histidine or adjacent polar/charged residues of IRT1 eliminates transport function. Interestingly the same authors were also able to modify the substrate specificity of this transporter by altering residues found in the loop region outside of the cell (plasma membrane) between transmembrane domains 2 and 3 (Rogers et al. 2000).

The relatively unspecific nature of the ZIP transporter proteins has been mentioned briefly in the preceding section, however the significance of this is best considered alongside information regarding their proposed function *in planta*. IRT1, the first ZIP gene to be identified was discovered by functional complementation of the *fet3 fet4* iron uptake mutant of *Saccharomyces cerevisiae* (Eide et al. 1996). Yeast cells expressing IRT1 exhibited novel Fe(II) uptake activity and so provided good evidence that this gene encodes an iron transporter, perhaps targeted to the plasma membrane in plants. Further work has shown that IRT1 is expressed in the epidermal cells of Fe-deficient roots and that *irt1-1* mutants are severely impaired in Fe uptake and die before setting seed (Grotz and Guerinot 2002). Whilst this additional information appears to provide conclusive information as to the function of

IRT, conflicting studies show that this protein may also mediate the transport of Mn and Zn, since *IRT* could also rescue *smf1* and *zrt1 zrt2* mutants of *S. cerevisiae* (Rogers et al. 2000). Cadmium is also another potential substrate. Cadmium has been shown to inhibit the uptake of both of these metals by IRT1, and expression of the *IRT1* gene in yeast results in increased sensitivity to Cd (Mäser et al. 2001). Radiotracer techniques used on the IRT1 isolog in pea plants (RIT1) has demonstrated directly an ability to transport Cd (Guerinot 2000). Rather interesting, IRT2, another member of the ZIP family that is also expressed in root epidermal cells upon Fe deficiency, seems to have greater specificity regarding its substrate ions (Grotz and Guerinot 2002). Whilst it may complement both Fe and Zn uptake mutants in yeast, it appears not to transport either Mn or Cd (Mäser et al. 2001). This poses the question: why do plants possess two similar Fe transporters that have slightly different substrate specificities? The answer appears to be because they have different functions. IRT2 cannot substitute for the loss of IRT1 (Grotz and Guerinot 2002). This suggests that the former protein may be localised elsewhere in the cell (i.e. not at the plasma membrane) or, perhaps it may form part of variable *affinity* uptake system, such has been found to be the case with the Zn-transporting ZIP proteins ZRT1 and ZRT2 in yeast (Eide 1997; Guerinot and Eide 1999), and may well apply to ZIP1 and 3 in *Arabidopsis*. Using a method similar to the one used to isolate *IRT1*, the *ZIP1*, *ZIP2* and *ZIP3* genes of *Arabidopsis* were isolated by functional expression cloning in the Zn uptake mutant *zrt1 zrt2* of yeast (Grotz et al. 1998; Guerinot and Eide 1999; Maathuis and Sanders 1999). A further transporter *ZIP4*, identified in the sequence database, could not restore Zn uptake activity (Guerinot and Eide 1999; Mäser et al. 2001). Despite this, the group is predicted to share the primary role of Zn transport, although the evidence suggests they have slightly different roles. ZIP1, ZIP2 and ZIP3 display different time, temperature and concentration-dependent Zn uptake activities with apparent K_m values similar to the levels of free Zn^{2+} thought to exist in the rhizosphere (Grotz et al. 1998; Guerinot and Eide 1999). *ZIP1* and *ZIP3* are known to be expressed in root tissue upon Zn deficiency, whilst *ZIP4* is present in both roots and leaves and is enhanced under the same conditions (Grotz et al. 1998; Maathuis and Sanders 1999; Mäser et al. 2001). Based on this information, ZIP1 and ZIP3 are prime candidates for transporting Zn from soil to plant via the root plasma membrane. ZIP4 on the other hand can be expected to play a different role, perhaps in transporting Zn intracellularly or between plant tissues. The proposed role of ZIP4 in *Arabidopsis* has been supported somewhat with the discovery of a further ZIP gene, *ZNT1* in *Thlaspi caerulescens* and *Thlaspi arvense*. Studies of these plants

revealed that constitutively high expression of *ZNT1*, a *ZIP4* homolog, in roots and shoots contributed to the hyperaccumulator trait of the former species (Lasat et al. 2000; Pence et al. 2000). The protein's primary substrate was Zn, although Cd was also transported to a small extent.

1.6.4 CDF transporters

The CDF family of proteins is a small but ubiquitous family of transporters dedicated exclusively to the transport of transition metal ions (Paulsen and Saier 1997; http://www-biology.ucsd.edu~msaier/transport/2_A_4.html). Members of this diverse group are found in bacteria, fungi, plants and animals (Mäser et al. 2001). Whilst the first examples from this protein family were identified some time ago (Nies 1992), relatively little is still known about these transporters. In particular, there seems to be a lack of information regarding the energetics of transport (Mäser et al. 2001; http://www-biology.ucsd.edu~msaier/transport/2_A_4.html). A lack of an obvious nucleotide binding domain indicates that they are 'powered' by some form of secondary active transport (Palmiter and Findley 1995; van der Zaal et al. 1999), however the mechanism is not clear. Palmiter and Findley (1995) suggest that the substrate cation is transported in symport with an anion, whilst others consider cation-proton antiport to be more likely (van der Zaal et al. 1999). Recent work on the reconstituted protein of an *Arabidopsis* CDF transporter has shown that a proton gradient was not required for substrate movement into proteoliposomes (Bloß et al. 2002). Whatever the nature of ion movement, their general description as cation diffusion facilitators still appears correct. The term cation efflux (CE) transporters has been suggested as an alternative name for this group, since all characterised members appear to be involved in the removal of ions from the cell (cytosol) (Mäser et al. 2001). This name, however should be used with caution. The CDF family of proteins have a rather diverse structure with variable polarity, and as a result could be involved in both influx and efflux (Paulsen and Saier 1997; http://www-biology.ucsd.edu~msaier/transport/2_A_4.html).

A good deal of information may be gleaned from studying protein structure. Hydropathy analysis shows that the CDF proteins have six putative transmembrane domains, with N and C termini that are likely to be intracellular (Palmiter and Findley 1995). Membrane-spanning regions are of variable polarity, transmembrane domains 3 and 4 are most hydrophobic, with 5 and 6 being most hydrophilic. Detailed analysis of these helical regions has revealed that transmembrane domains 1,2,5 and 6 are remarkably amphiphatic and are proposed to form part of

an inner core of the protein that presumably acts as a channel to substrate ions (Paulsen and Saier 1997; Williams et al. 2000). The presence of conserved aspartate residues in helices 2,5, and 6 ties in with this theory, as they may well function as cation binding sites within the proposed channel. Between each of the transmembrane domains exists five interconnecting loops. Loops 1-3, nearest the N-terminus tend to be shorter and less variable in length, whilst loops 4 and 5 along with the adjacent C terminus are longer and may show substantial variability. Indeed size differences in these regions may be so large that they have been pin-pointed as the major cause of size variation in the whole CDF family (Paulsen and Saier 1997). This is not their only remarkable trait however; many of these hydrophilic loops accommodate conserved motifs that are thought to have a role in a variety of functions. Perhaps most significant of these is the CDF signature sequence that begins in loop 1 and terminates near to the centre of transmembrane domain 2. For a signature sequence this motif does deviate somewhat between different members of the group, however an area of high similarity always appears to occur between fully conserved serine and aspartate residues (Paulsen and Saier 1997). The function of this domain remains unknown, and no work is currently available that tests its function by mutation or deletion of the appropriate residues. One possible function of this domain could be in the coupling of ion movement to an energy source. In this way, it may also interact with other conserved parts of the protein. For example the C-terminal cation efflux domain (<http://plantst.sdsc.edu/plantst/html/cation.shtml>; Mäser et al. 2001), whose function is also unknown. The role of a histidine-rich region ($[HX]_n$ where X = G or C) present in the long loop between transmembrane domains 4 and 5, has long been the subject of speculation. ZIP proteins have a similar region which is suspected to have either a functional or regulatory role. Experience with other transporters has created the consensus view that these sites are the location of metal binding. Recent work on reconstituted ZAT1 (from *Arabidopsis*) has confirmed this to be the case, as Zn was shown to bind to this region (Bloß et al. 2002). No comment however, was made on the presence of other putative metal binding sites, for example the small cysteine-rich loop between transmembrane domains 5 and 6 that has been found in the related mammalian transporter Znt-1 (Palmiter and Findley 1995). The fact that the HX motif has been observed to bind Zn is interesting. The CDF family, in eukaryotes at least, is thought to be involved primarily in the movement of Zn^{2+} (van der Zaal et al. 1999; Miyabe et al. 2000). The transport of other cations such as Cd^{2+} and Ni^{2+} has been observed, although it appears that this is due to their affinity with Zn-transporting CDF proteins (Mäser et al. 2001). At

present, cobalt (Co^{2+}) is the only other 'true' substrate of eukaryotic CDFs with transport being carried out (exclusively) by the yeast protein COT1. It is not yet clear if this metal binds to the HX region of the protein during transport or if other binding sites are involved. If the HX region is involved, this would ask further questions about the functioning of prokaryotic CDFs which do not possess this motif.

1.6.5 P-type ATPases

The term 'P-type', used to describe these proteins, refers to the existence of a covalent high-energy phosphoryl-enzyme intermediate in the chemical reaction pathway of ion transfer (Kuhlbrandt et al. 1998). This is formed when the phosphate of ATP spontaneously reacts with an aspartic acid residue in the central region of the polypeptide chain (Pedersen and Carafoli 1987). Several changes in the protein's conformation are induced by this process, leading initially to the channelling of substrate ions to specific binding sites, then disruption of these sites to allow access to a translocation domain (MacLennan and Green 2000; Xu et al. 2002). This general reaction is often described by an E1-E2 – cycle model (Jencks 1989; Tsai and Linet 1993; Toyoshima and Mizutani 2004).

In a survey of protein sequences, Axelsen and Palmgren (1998) identified 159 different P-type ATPases from a number of different organisms. These grouped together into five major families, segregating not because of any evolutionary division, but as a result of their transported ion. The database search was conducted on partial-length sequences, and focused on the identification of a string of seven amino acids D-K-T-G-T-X-X (where X-X = I-T, V-T, L-S, M-T, I-I), a characteristic which is often used as a prerequisite in the classification of P-type ATPases (Kaplan 2002). Although perhaps the definitive motif, there are a total of eight conserved regions, interspersed with variable segments, present in all P-type ATPases (Palmgren 1998). Some are shown in Table 1.3.

In all, around 265 residues are conserved across the group (Axelsen and Palmgren 1998). Despite this, the P-type ATPases do show appreciable variation in their sequence identity and predicted structures. For example Ca^{2+} ATPases, considered by many to be the model transport protein of this group (Solioz and Odermatt 1995), possess a greater number of hydrophobic regions (membrane spanning domains), than the HMAs that transport transition metal ions; calcium ATPases typically have 10 transmembrane domains, whilst the HMAs are unusual in possessing only 8 (Serrano 1988; Williams et al. 2000; Cobbett et al. 2003). Fortunately, however, there appears to be some consistency in transporter

attributes when grouped according to 'substrate families' described by Axelsen and Palmgren (1998).

1.6.5.1 HMAs and sequence motifs

Whilst there is some evidence for the involvement of the putative phospholipid transporter (P-type ATPase group P IV) in the translocation of heavy metal ions, it is clear that those of class IB (*sensu* Axelsen and Palmgren 1998), are the principal group with such function. A striking feature of these proteins is the presence of a conserved intramembranous cysteine-proline-cysteine, cysteine-proline-serine or cysteine-proline-histidine (CPx) motif, centred 43 amino acid residues N-terminal to the phosphorylatable D-K-T-G-T residue (Moller et al. 1996; Williams et al. 2000,). Initially, this motif was speculated to have a role in energy transduction, a hypothesis that focused on the internal proline of the sequence. However it now appears that it is important for the translocation of metal ions. This is supported by evidence that highlights the importance of the 6th transmembrane domain (4th in the smaller bacterial HMA, CadA; Silver 1996) in membrane permeation, and the possibility of other motifs in initial ion capture (Gitschier et al. 1998). These additional metal-binding motifs are also novel to the HMA - transporter proteins. They include a cysteine-rich CxxC motif, repeated up to 5 times (e.g. in human Menkes disease protein, ATP7A), in the amino terminus, and a histidine-proline dipeptide, 34 to 43 amino acids from the CPx sequence (Solioz and Vulpe 1996). Although a functional role for these sites has been suggested, this is one of many areas where conclusive evidence is unavailable. Due to their generally higher frequency in copper transporters, the CxxC motif has been suggested to be involved more specifically in copper binding (Lutsenko et al. 1997; DiDonato and Sarkar 1999). Others have pointed out that this is probably not the case, as these motifs are also present, as metal binding (metal capture) domains, in HMAs known to transport other metals (Solioz and Odermatt 1995). Work with yeast (*S. cerevisiae*) has pointed to other motifs with specificity for Cu binding. The discovery of the MxM and MxxM motifs (Camakaris et al. 1999) could help explain past discrepancies in relating CxxC to Cu transport. It is possible that these groups, which can be present in addition to CxxC repeats, may have some interactive effect or overriding influence determining the specific metal that acts as the substrate ion. This may have gone unnoticed in previous work, and led to erroneous conclusions regarding the CxxC repeats in some cases.

The suggestion that certain metal-binding motifs contribute to the specificity and/or affinity of the protein to a specified ion could prove useful in explaining the different functions of the various HMAs. Indeed, whilst several unique features for these transporters have been highlighted, it must be remembered that, apart from CPx, not all motifs are conserved in all members. For example AtHMA4, a transporter on which this work will focus, possesses a histidine chain and multiple CC repeats near its carboxyl terminus. This is in stark contrast to the related transporters, AtHMA1 and AtHMA5, which present their (respective) putative metal-binding domains, a histidine stretch and CxxC repeats, close to the amino tail. What implications these motifs may have on protein structure or function are unknown.

1.6.5.2 Protein structure

Much information on the structure of HMAs has been taken from data obtained with Ca²⁺ ATPases. Acknowledging that there are differences between the two types, it is assumed that those motifs conserved in all P-type ATPases function in the same manner. Hydropathy analysis, a method for displaying the hydrophobic character of a protein (Serrano 1988), has predicted that HMA transporters have fewer transmembrane domains than other P-type ATPases. It appears that eight regions exist, with a small cytoplasmic domain (β – loop) present between domains 4 and 5 and a larger one linked to domains 6 and 7 (Solioz and Vulpe 1996). These cytoplasmic regions generally possess a surplus of positive charge, whilst the transmembrane domains are largely neutral in order to obtain a hydrophobic character, compatible with the lipid bilayer (Zhang et al. 1998). However, unstable ionisable groups are apparent in the transmembrane segments, and these are supposedly tolerated by ion pair formation (i.e. +/-, to cancel out net charge) and, most importantly, an alpha helical arrangement (Serrano 1988; Moller et al. 1996). The larger of the two loops may be smaller in the HMAs (Williams et al. 2000), but the cytoplasm still remains the location for over 70% of the membrane bound protein (Kuhlbrandt et al. 1998; Palmgren 1998). This is in contrast to the portion traversing the membrane, which despite tight packing due to the tilting configuration (Kuhlbrandt et al. 1998; Zhang et al. 1998; Scarborough 1999) of the alpha helices, only represents around 10% of the protein. Stalk regions adjacent to the cytoplasmic surface of the membrane, are a factor contributing to the size of the cytosolic portion. These structures are likely to mediate some form of communication between the cytosolic and membrane regions, probably via shifts in protein conformation (Toyoshima et al. 2000).

The loops are extremely important in the functioning not just of the HMAs, but for the P- type ATPases in general, since they contain a number of conserved motifs. Techniques such as site-directed mutagenesis have linked certain domains with specific roles within the translocation reaction pathway (Table 1.3), but often evidence is only circumstantial. A lack of information with regards to the structural arrangement of the protein, has hampered progress in the field, since such data is invaluable in clarifying those processes/domains that act together in a concerted fashion. Toyoshima et al. (2000) solved the crystal structure of a Ca^{2+} ATPase at 2.6 Å resolution. Their study has confirmed various aspects of previous work, and has shown that at least three conformational changes are required to transport two atoms of calcium. The phosphorylation and nucleotide binding domains of the large cytosolic loop, come together to allow phosphorylation. The TGES motif on the β loop subsequently closes to the DKTGT phosphorylation site, and this triggers the next movement of the 4th and 5th transmembrane domains (unwinding of the alpha helix in the case of M4) which in turn cause alterations in the cation binding sites (Toyoshima et al. 2000). This latter mechanism has yet to be fully explained, but it is thought to alter the affinity of the binding site, so it first occludes, and then disrupts Ca^{2+} binding (Maclennan and Green 2000). This allows release of the ion to the opposite side of the membrane. It is conceivable that a similar process occurs during CPx - mediated translocation, although it is obvious that the 6th and 7th transmembrane domains would be of importance in instigating the changes in conformation required at the CPx domain. A two-dimensional diagram showing the salient features of a SERCA-type Ca^{2+} -ATPase and a HM-ATPase is shown in Figure 1.1.

1.6.5.3 Function

With increasing evidence pointing to a diverse distribution in various membranes (e.g. Havelaar et al. 1998; Hirayama et al. 1999; Shikanai et al. 2003; Gravot et al. 2004), it has been hard to propose a definitive function for the HMAs. Indeed, Palmgren and Harper (1999) suggest three functions (1) uptake of essential metals from the apoplast, (2) efflux of toxic metals from the cytoplasm, and (3), compartmentalisation of essential metals for specific biochemical pathways. RAN1/AtHMA7 (responsive to antagonist) was the first HMA to be characterised. Its function was highlighted in a study of *Arabidopsis* mutants deficient in certain aspects of their ethylene signalling pathway. Several experiments demonstrated the involvement of the RAN 1 protein, which has significant similarity to Menkes and

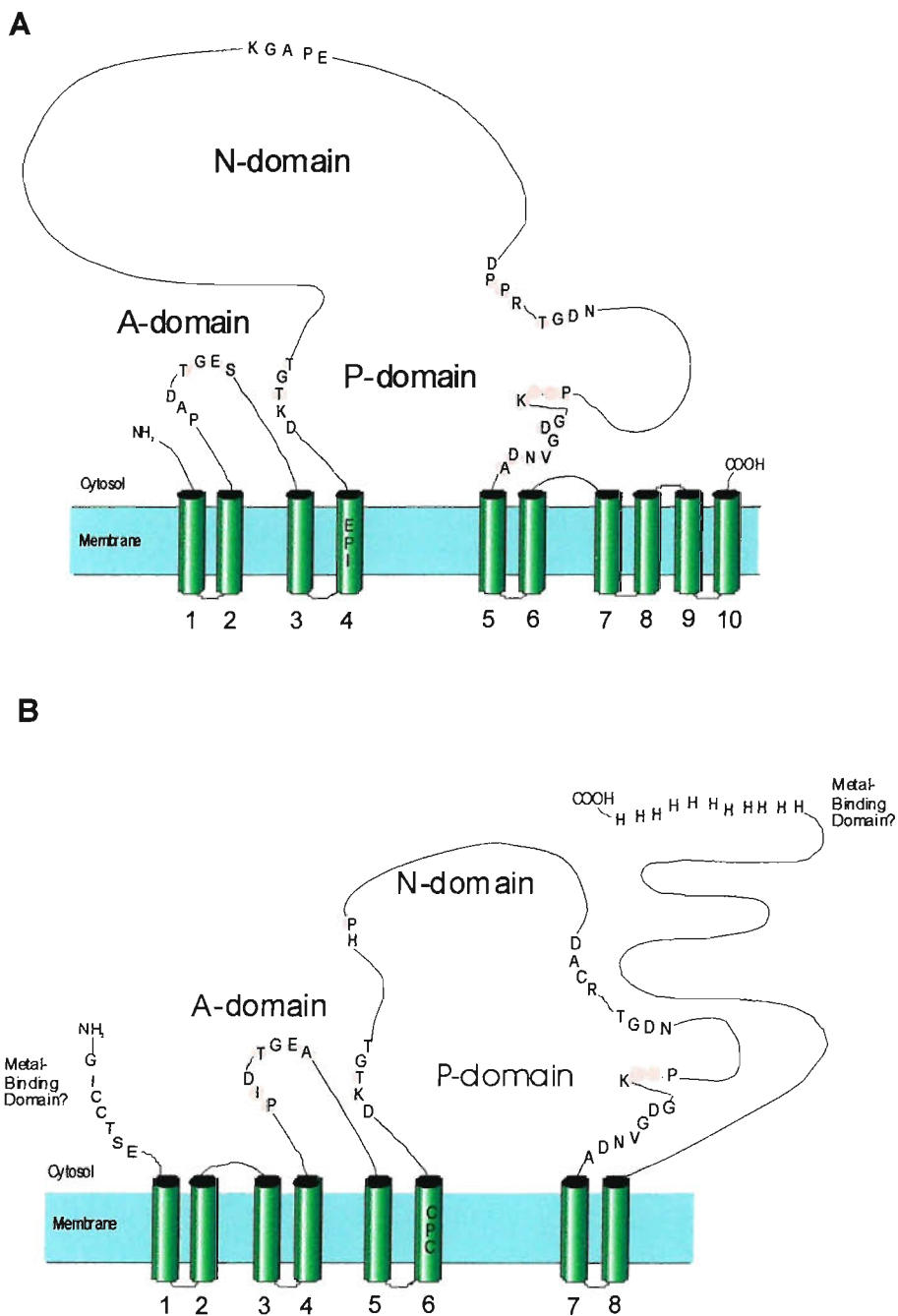


Figure 1.1 Two-dimensional diagram showing the typical features and amino acid motifs of a SERCA-type Ca^{2+} -ATPase (A) and a HM-ATPase (AtHMA4) (B). Transmembrane domains are shown as cylinders (numbered 1-8 and 1-10). Regions known as the A (actuator or energy transduction)-domain, the P (phosphorylation)-domain and the N (nucleotide-binding)-domain are also shown (Source: L.E. Williams unpublished).

Table 1.3 Putative functions of conserved motifs found in P-type ATPases

Motif	possible function*
DKTGT	Aspartate residue is phosphorylated during reaction cycle.
GDGxNDx	Forms part of the ATP binding site
GD	Phosphorylation/ATP binding?
PxxK	Charge stabilisation (protein-protein interaction?)
<u>TGE(S/P)</u>	<u>??</u>

*Note; Specific functions have been assigned to the motifs presented in the table. Some (e.g. Moller et al. 1996) consider that many (smaller) motifs are conserved because they play a role in protein folding, and thus help form a general 'scaffolding'.

Wilson's disease transporters, in delivering copper ions to create functional ethylene receptors. Expression in yeast has shown that RAN1 can complement the CCC2 deletion mutant of yeast and so is likely to be located at the post-Golgi body and it suggests that the protein may interact with Cu chaperones as has been shown for CCC2 (Himmelblau and Amasino 2000). Another HMA from *Arabidopsis* that has been characterised is PAA1 (AtHMA6). This has also been suggested to have a role in Cu transport. Studies of *paa1* mutants revealed plants that exhibited a high-chlorophyll-fluorescence phenotype, impaired photosynthetic electron transport and a lower chloroplast Cu content (Shikanai et al. 2003). This suggests that PAA1 functions in the delivery of Cu across the plastid envelope under normal conditions (Shikanai et al. 2003). The functions of the other HMAs of *Arabidopsis* are less clear. The question still remains if any members may be involved in metal resistance mechanisms, rather than in the supply of ions to enzymes/compartments as part of normal metabolism. There are members of the HMA group that are predicted to transport ions such as Zn/Cd/Pb/Co rather than Cu/Ag and this thesis will examine properties of some of the members of this sub-group.

1.7 Project aims

Transporters may play an important role in plant transition metal homeostasis and there are potentially a number of families of carriers, channels or pumps that are dedicated to such functions. The HMAs from *Arabidopsis thaliana* are a prominent transporter group and may be of particular importance to metal homeostasis and/or tolerance since they have the potential to transport substrates against concentration gradients. The aim of this thesis is to characterise a number of HMAs, particularly AtHMA4 and AtHMA1, in order to provide information about their physiological role. In order to achieve this, full-length *AtHMA4* cDNA will be cloned and its properties studied following heterologous expression in yeast. To further investigate the roles of AtHMA4 and AtHMA1 in *Arabidopsis thaliana*, the expression pattern of the genes will be studied in detail. Transcript levels are to be examined in different tissues and in response to a number of metal and metal-chelator treatments.

Chapter 2 Materials and methods

Unless otherwise stated all chemicals used were supplied by the following: Sigma-Aldrich Co. Ltd. (Poole, Dorset, UK), Fisher Scientific Ltd. (Loughborough, Leicestershire, UK), Difco (Becton Dickinson, Oxford, UK) and GibcoBRL Life Technologies Ltd. (Paisley, Scotland, UK). Molecular biology enzymes were from Promega UK Ltd. (Southampton, Hampshire, UK), Hybaid Ltd. (Ashford, Middlesex, UK), Stratagene Ltd. (Cambridge, UK), Invitrogen (Paisley, Scotland, UK), Biotin (London, UK), Takara (Cambrex Bio Science Ltd., Wokingham, UK), GRI (Braintree, UK), Eurogentec (Romsey, UK). Radiolabelled chemicals were from Amersham Life Science Ltd. (Chalfont St. Giles, UK).

2.1 Plant material

Arabidopsis thaliana L., cultivar Columbia (ecotype Col-0) were grown in hydroponic culture to allow greater control of metal exposure and the harvesting of undamaged root tissues. Seed were surface sterilised in 10% (v/v) Domestos™ bleach for 20 min and then washed in two volumes of deionised water (dH₂O) three times. Subsequent handling and sowing of seeds was carried out under sterile conditions in a laminar flow cabinet. Seed were transferred individually to 0.5 mL black Eppendorf tubes containing 300 µL of 0.5% (w/v) agar. The caps and bottom of the Eppendorf tubes had been removed. These were placed into black foam rafts (approximately 70 tubes per raft) and floated on approximately 1.5 L of nutrient solution (see below and Table 2.1) contained in a plastic vessel (black masking tape was used on this to prevent light entry). This was placed in a cold room at 4-8°C for two days and then transferred to a controlled-environment growth room and grown under the conditions shown in Table 2.2. The nutrient solution used in this work is a modified version of the ¼ strength solution used by Arteca and Arteca (2000) the composition of which is outlined in Table 2.1. Iron-ethylenediamine tetraacetic acid (Fe[EDTA]) was used as a substitute for 'sequestrine', a compound not defined chemically by Arteca and Arteca (2000), whilst the pH was adjusted to 5.2, instead of 5.7. This latter modification was necessary as it was found in earlier trials that pH values greater than ca. 5.2 caused precipitation of metallic ions. This was most noticeable with Zn and Cd, which reacted to form hydroxides when NaOH was used in pH adjustment. Nutrient solutions of pH 5.2 have been used previously to cultivate *Arabidopsis thaliana* seedlings (Toda *et al.* 1999).

Table 2.1 Concentrations of elements present in the hydroponic nutrient solution used to grow *Arabidopsis thaliana*, based on Arteca and Arteca (2000).

Nutrient salts	Concentration
Macronutrients	
KNO ₃	1.25 mM
KH ₂ PO ₄	0.625 mM
MgSO ₄	0.5 mM
Ca(NO ₃) ₂	0.5 mM
Micronutrients	
Fe(EDTA)	42.5 μM
H ₃ BO ₃	17.5 μM
CuSO ₄	0.125 μM
MnCl ₂	3.5 μM
ZnSO ₄	0.25 μM
Na ₂ MoO ₄	50 nM
NaCl	2.5 μM
CoCl ₂	2.5 nM

Table 2.2 Ambient growth room conditions used to grow *Arabidopsis* plants. The same conditions were also used during metal exposure experiments.

Environmental Variable	Value/units
Light intensity	120 μmol m ⁻² s ⁻¹
Temperature (Photoperiod)	21 °C ± 1 °C
Temperature (Dark period)	18 °C ± 1 °C
Relative Humidity	75 %
Photoperiod	16 hr

The nutrient solution was replaced every 3–5 days, depending upon the size and growth stage of the plants. After approximately 3 weeks, the tubes containing healthy plants were transferred to smaller floats holding five plants in Magenta culture vessels (Sigma-Aldrich Co. Ltd.) containing 250 mL of nutrient solution. The plants were grown for a further week before treatment with different metal ions or metal ion-chelators (see Table 2.3). All metals were supplied as their sulphate salts. For the general metal screen, plants were exposed to nutrient solutions for a period of 30 hr. A duration of 30 hr in metal-treated nutrient solution, has been shown to be sufficient for the induction of the metallothionein II (MTII) gene in *Arabidopsis thaliana* (Col-0) seedlings (Zhou and Goldsbrough 1994). Later experiments examined the effects of varying the exposure period to 15, 30 and 60 hr.

2.2. Isolation of RNA from *Arabidopsis thaliana* organs

2.2.1 Isolation of total RNA

The preparation of pure, intact RNA is complicated by the presence of contaminating RNA degrading enzymes (RNases) that are present not only within the cells from which the RNA is being extracted but also possibly present on all equipment that comes into contact with the RNA preparation. It is important therefore that precautions are taken to prevent the likelihood of contamination by RNases. Therefore, all glassware, Eppendorf tubes and pipette tips used in the isolation and handling of RNA were baked at 140 °C for at least 8 hr, and sterile disposable centrifuge tubes were used. Spatulas and magnetic stirrers were also baked at 140 °C for at least 8 hr. Additional precautions included the autoclaving of all buffers and solutions (except those containing Tris or SDS), which inhibits several types of RNases. Tris-containing buffers and SDS solutions were prepared using autoclaved diethyl pyrocarbonate (DEPC)-treated water. Plastic-ware was soaked in 0.1 M sodium hydroxide, again to prevent RNase contamination. Finally gloves were worn at all times and changed at regular intervals.

Total RNA was isolated using a phenol/SDS extraction and LiCl precipitation based on the method by Verwoerd et al. (1989). Approximately 1g of plant material was ground in liquid nitrogen until a fine, light green powder was produced. This was transferred to a chilled 50 mL Falcon tube and 8 mL LiCl extraction buffer:phenol (1:1) added. The LiCl extraction buffer contained 0.1 M LiCl, 0.1 M Tris/HCl (pH 8.0), 10 mM EDTA and 1% (w/v) SDS. The material was mixed by

Table 2.3 Concentrations of metals and chelators added to the nutrient solution. All metals were supplied as sulphate salts, in addition to the nutrient ions present in the hydroponic solution (see Table 2.1).

Treatment type	Treatment	Concentration added
Metal ions	Cd	0.5 mM→0.1 mM
	Co	0.5 mM
	Cu	0.5 mM→0.1 mM
	Ni	0.5 mM
	Fe	20 mM
	Mn	0.8 mM
	Zn	1.0 mM
	K	1.0 mM
Chelators	Ferrozine	1.0 mM
	Na ₂ EDTA	5.0 mM

vortexing for approximately 2 min before an equal volume of chloroform was added. The material was again mixed by vortexing for approximately 30 s before standing on ice for 15 min at 4 °C. The solution was then centrifuged at 5,000 $\times g$ for 10 min at 4 °C, and the aqueous phase collected and transferred to Eppendorf tubes. An equal volume of 4 M LiCl was added and the solution incubated at -20 °C overnight. The preparation was centrifuged at 12,000 $\times g$ for 10 min at 4 °C. The pellet formed was then resuspended in 250 μ L of DEPC-treated water, and 2 volumes 100% (v/v) ethanol and 0.1 volume 3 M sodium acetate (pH 5.2) were added to precipitate the RNA. This was stored at -20 °C overnight. The RNA was retrieved by centrifugation at 13,000 $\times g$ for 10 min, 4 °C in an Eppendorf 5415R (Eppendorf Ltd. Cambridge UK) chilled centrifuge. The pellet was rinsed in 70% (v/v) ethanol before being resuspended in DEPC-treated water, the volume depending on the size of the pellet but typically 25 μ L. This was stored at -70 °C, to be thawed on ice when required.

2.2.2 DNase treatment of total RNA

Contaminating genomic DNA was removed from RNA preparations with the use of the DNA-free, DNase treatment kit (Ambion Ltd. Huntingdon UK). The contaminated sample ($\leq 100 \mu$ L) was pipetted into a 0.5 mL Eppendorf tube, followed by 0.1 volumes of 10 \times DNase I buffer and 2 μ L of DNase I enzyme (1 unit/ μ L). The reactants were gently mixed and then incubated in a water bath at 37 °C for 30 min. After gently mixing and brief centrifugation the sample was returned to the water bath for another 30 min. The reaction was terminated with the addition of 0.1 volumes of DNase inactivation reagent (Ambion Ltd.) which was mixed with the sample for 1 min. The tube was incubated at room temperature for 2 min with occasional mixing. The sample was then centrifuged at 10,000 $\times g$ for 1 min in order to remove the spent reagent. Clean RNA was pipetted from the top of the waste pellet and transferred to a new tube prior to storage/use.

2.3 Isolation of genomic DNA from *Arabidopsis*

Genomic DNA was extracted from tissue frozen in liquid N₂ and ground to a powder as described by Carroll *et al.* (1995) in 200 mM Tris (pH 7.5), 50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB, 0.38% (w/v) sodium bisulphite, 15 μ g RNase A and 1% (w/v) lauroylsarcosine at 65 °C. DNA was further purified by extraction in phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated in an equal volume of isopropanol at room temperature. DNA pellets were resuspended using ~50 μ L

prewarmed (50 °C) dH₂O. All centrifugation steps were at 13,000 x *g* in a Sorvall MC12V microfuge (Kendro Laboratory Products Plc., Bishop's Stortford, UK).

2.4 DNA purification

Certain experiments required DNA to be further purified and/or precipitated. This was carried out using the QIAquick purification kit (Qiagen Ltd. Crawley, UK) according to the manufacturer's instructions. If DNA from an agarose gel was to be purified it was cut from the gel using a scalpel. Five volumes of binding solution were added to the sample, mixed thoroughly and then pipetted onto the surface of a QIAquick spin column. The sample and column were then centrifuged at 10,000 x *g* for 1 min and the eluent discarded. Wash buffer (750 µL) was added on to the top of the spin column and the sample centrifuged again at 10,000 x *g* for 1 min. The eluent was discarded before an additional spin in the centrifuge was carried out. This was done in order to remove any residual traces of ethanol (a constituent of the wash buffer) which may interfere with the subsequent step in the procedure. Finally, the spin column was placed inside a 1.5 mL Eppendorf tube and 30- 50 µL of water pipetted onto the surface of the column. After 2 min incubation at room temperature, the column was spun at 10,000 x *g* for 1 min and the eluted DNA collected in the bottom of the Eppendorf tube.

2.5 Spectrophotometric determination of nucleic acid concentration

The purity and concentration of DNA or RNA samples was determined by spectrophotometry as described by Sambrook et al. (1989). One microlitre of a nucleic acid sample was diluted to 250 µL in sterile deionised water (DEPC-treated in the case of RNA samples). Optical density (OD) readings were taken at 260 nm and 280 nm against a water control in a quartz cuvette with a 0.5 cm path-length. The OD_{260nm} gives the concentration of nucleic acid and concentrations were calculated based on the fact that the absorbance of 1 mg/mL of double-stranded DNA at 260nm is known to be 20. The concentration of the sample was therefore given by :-

$$\frac{(\text{OD}_{260\text{nm}}) \times (\text{dilution factor})}{20} \times 2 = \text{concentration of DNA in } \mu\text{g}/\mu\text{l}$$

20

Similarly the absorbance of 1 mg/mL at 260nm of single-stranded DNA and RNA is known to be 25. The concentration was therefore: -

$$\frac{(\text{OD}_{260\text{nm}}) \times (\text{dilution factor})}{25} \times 2 = \text{concentration in } \mu\text{g}/\mu\text{l}$$

The purity of nucleic acid samples was calculated using the ratio of the absorbance measurements at 260nm and 280nm. Where $\text{OD}_{260}/\text{OD}_{280} = 1.8 - 2.0$ the sample was considered pure. A lower ratio may indicate protein, phenol or ethanol contamination whereas a higher ratio could be indicative of polysaccharide contamination or sample degradation, the latter being particularly relevant to RNA (Sambrook et al. 1989).

2.6 Gel electrophoresis of nucleic acid samples

2.6.1 RNA

Gel electrophoresis of RNA was carried out as described by Sambrook et al. (1989). RNA was separated on a 1% (w/v) agarose gel containing 0.9 M formaldehyde, 20 mM MOPS and 100 μM EDTA (pH 8.0). Prior to loading, RNA was denatured at 65 °C for 15 min in 0.4 volumes of 10 × MOPS buffer (containing 200 mM MOPS, 50 mM sodium acetate and 1 mM EDTA, pH 8.0), 0.8 volumes of formaldehyde and 2 volumes of formamide, followed by cooling immediately on ice. RNA was loaded in sample buffer containing 50 % (v/v) glycerol and 0.025% (w/v) bromophenol blue, and electrophoresis was carried out at 80 V for 1 to 2 hr. The RNA was visualised under UV light after incubating the gel in staining solution containing 1 μM ethidium bromide and 100 mM ammonium acetate for 30 min, then incubating in destaining solution containing 100 mM ammonium acetate for 2 hr.

2.6.2 DNA

Gel electrophoresis of DNA was carried out as described by Sambrook et al. (1989). For DNA samples over approximately 500bp a 1% (w/v) agarose gel was prepared in TAE (40 mM Tris/acetate pH 8.0, 1 mM EDTA). Ethidium bromide (5 mM) was included in both gel and TAE-running buffer. The sample was mixed in a 5:1 ratio with loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF and 15% (w/v) FICOLL). Electrophoresis was carried out at 90 V for 45-

90 min, depending on the size of the DNA fragments and the gel concentration. DNA was visualised under UV.

2.6.3 Agarose gel imaging and integrated densitometry values

In order to study gene expression, electrophoretically separated RT-PCR products were analysed by measuring integrated densitometry (IDV) values. Ethidium-bromide-stained agarose gels were visualized in a Multi-Image UV Light Cabinet (Alpha Innotech Corporation, San Leonardo, USA) and the image analysed using the Alphamager software programme 1220, version 5.04. Product bands generated by the same PCR primers were analysed together on the largest possible image size. Care was taken to ensure that no pixels were 'saturated' on any of the product bands of the captured image. IDV values were taken from highlighted boxes placed around the product bands. These were kept at a uniform size for each sample type on a particular gel.

2.7 Reverse transcriptase (RT) reaction

First strand complementary DNA (cDNA) was prepared from total RNA extracted from root and leaf tissues of *Arabidopsis thaliana* exposed to various metal ion and metal ion-chelator treatments. Five micrograms of total RNA were used in each RT reaction, usually as a pre-prepared solution of 2.5 µg/µL total RNA. The RNA was added to a small PCR thermo-tube (ABgene Ltd., Epsom, UK) containing 8 µL of sterile deionised water. This was followed by 1 µL (0.5 µg) of oligo dT¹²⁻¹⁸ primer and 1 µL of (10 mM) dNTP mix. After mixing, and a short pulse at 13,000 x g in a microfuge, the sample was denatured on a heated-block at 70°C for 10 min and then cooled immediately on ice. Four µL of 5 x First-strand Buffer (consisting of 250 mM Tris-HCl (pH 8.3), 375 mM KCl and 15 mM MgCl₂), 2 µL of 0.1 M DTT and 1 µL (40 units) of 'RNAGuard' (all from Gibco BRL Life Technologies Ltd.) were added, and the mixture incubated at 37 °C for two min. One microlitre (200 units) of 'Superscript' reverse transcriptase (Gibco BRL Life Technologies Ltd.) was subsequently added, mixed thoroughly and the tube returned to the heat-block at 37 °C for 50 min. Finally, the reaction was terminated by increasing the block temperature to 70 °C for 15 min.

Where desired, the cDNA product was cleaned of any residual RNA by adding 1 µL (2 units) of *E. coli* RNAase H and incubating at 37 °C for ≤ 20 min. The reaction was terminated by raising the temperature to 70 °C and holding this for a further 15 min

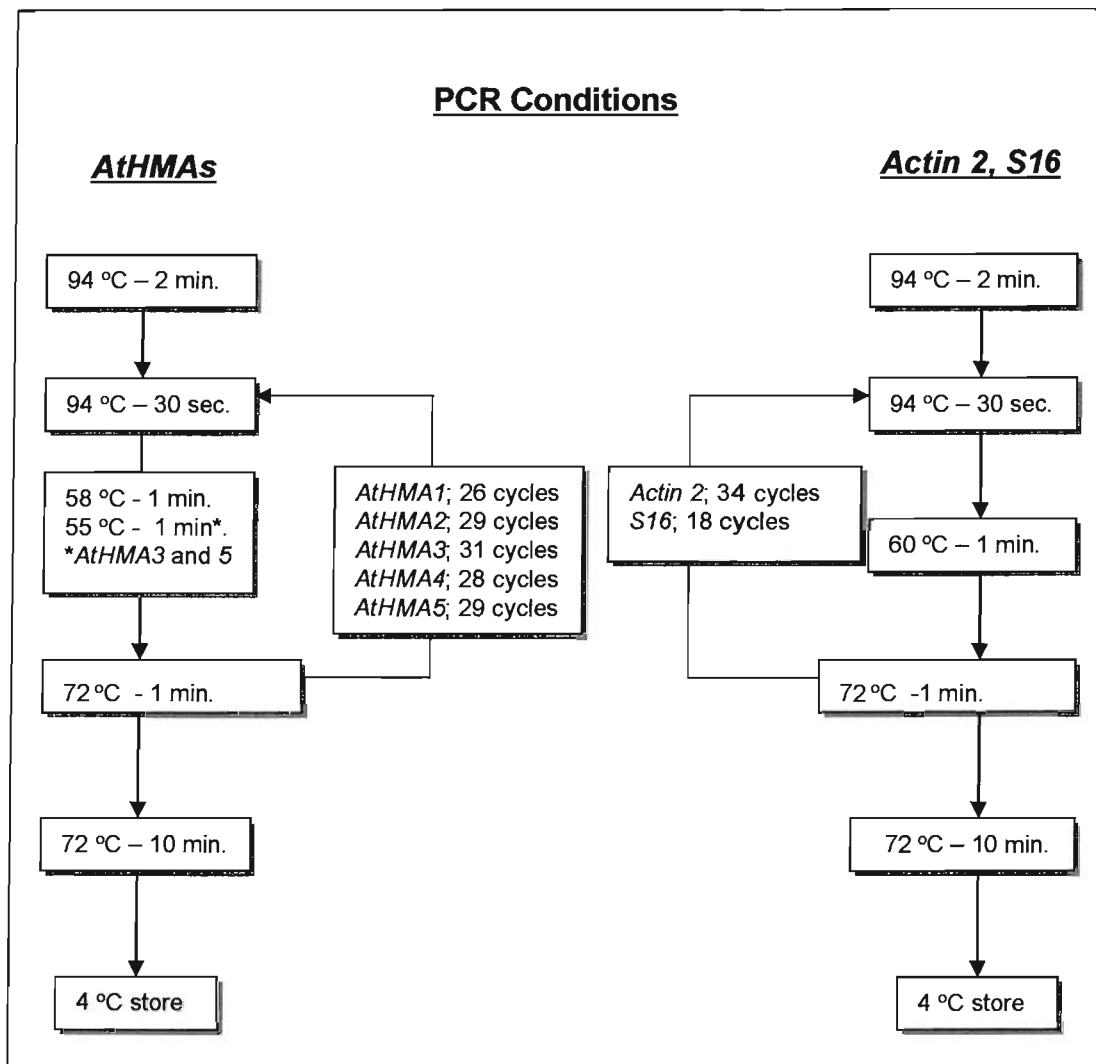


Figure 2.1 PCR reaction conditions used for *AtHMAs*, *Actin 2* and *S16* PCR conditions shown above apply to those reactions carried out using AGS Gold Taq polymerase (Hybaid, Germany). For other work Takara r-Taq (Takara Shuzo co. Ltd. Shiga, Japan) was used. Optimised conditions for this enzyme were exactly the same, however the optimal number of cycles were: 25, 35 and 21 for *AtHMA1*, *AtHMA4* and *Actin 2* respectively. All PCR reactions were carried out using a Hybaid UNO II or Perkin Elmer 9700 thermocycler set at standard ramp times.

2.8 Polymerase chain reaction (PCR)

PCR was used to amplify target sequences from cDNA constructed from RT reactions, cDNA libraries or genomic DNA. PCR reactions were carried out using various *AtHMA1*- and *AtHMA4* -specific primers (Table 2.4) and a variety of cycling programmes in order to optimise the amplification conditions. PCRs were also carried out for *Actin 2*, *S16* and *AtHMA 2*, *3* and *5* (see Chapter 5). For semi-quantitative RT-PCR non-saturated products were required, so that differences in the relative amounts of each product could be assessed. The conditions used for each gene are shown in Figure 2.1. Reactions were carried out in PCR tubes (ABgene Ltd.) using a high yielding PCR kit (AGS Gold; Hybaid Ltd.) in volumes of 10 μ L. A 'bulk-mix' was prepared for processing multiple samples in order to minimise variation in reaction conditions and to obtain representative results. For each reaction the following reagents were used: 1 μ L buffer complete (containing $MgCl_2$ at a final concentration of 1.5 mM), 0.7 μ L enhancer, 0.15 μ L dNTPs (A,C,G,T at a final concentration of 200 μ M), 0.1 μ L Taq (AGS Gold) polymerase (10 units/ μ L), 6.05 μ L sterile deionised water and 0.5 μ L of both forward and reverse primers (at 10 pmol/ μ L). Nine microlitres of the 'primed' bulk mix were pipetted into PCR tubes and cooled on ice before the sample cDNA was added. After mixing, and a short pulse in a microfuge at 13,000 \times g, the tubes were loaded into the thermal cycler block pre-heated to 94 $^{\circ}$ C, and the programme started immediately. Once the cycling reactions had been completed the PCR products were examined by agarose gel electrophoresis.

2.9 Rapid Amplification of cDNA Ends (RACE)

Five-prime RACE was carried out using the GIBCO BRL Life Technologies Ltd. kit, version 2.0 according to the manufacturer's instructions. DNase treated total RNA (0.8 – 1.5 μ g) was loaded into a PCR tube (ABgene Ltd.) along with 2.5 p moles of the gene-specific primer (RACE-rev A, Table 2.4). Sterile water was added to give a final reaction volume of 15.5 μ L; the contents were gently mixed and incubated for 10 min at 70 $^{\circ}$ C. After denaturation the samples were immediately cooled on ice for 1 min before brief centrifugation (> 5 s at 13,000 \times g). Once back on ice, 2.5 μ L of 10 \times PCR buffer, 2.5 μ L of 25 mM $MgCl_2$, 1.0 μ L of 10 mM dNTPs (mixed solution), 2.5 μ L of 0.1 M DTT and 1 μ L of RNAGuard RNase inhibitor (Roche Products Ltd. Welwyn Garden City, UK) were added before gentle mixing

Table 2.4 Primers used in various experimental procedures during the course of this work. Primers are grouped according to their corresponding genes, and are complimentary to *AtHMA4* unless otherwise stated. Subgroups have been devised in accordance with their general usage which is indicated in brackets in the top right corner of the primer sequence boxes; (5') = 5'-PCR, (R) = RACE, (S) = sequencing, (E) = Expression analysis, (C) = cloning

Primer name	Primer sequence (5' - 3')	In frame Protein target
Forward A	AAGCCGCATACGTCTTTACTC (5')	Non-coding
Forward B	TTGGTGCGTTGCGTTAAAAG	Non-coding
Forward C	CTAATGTGCCTAAACTCGTGAC	Non-coding
Forward D	CTTGAAGATAGGAACCGGAAAC	Non-coding
Forward E	TAAAACCCCATAAAACC	Non-coding
Forward F	TTATTAACTTTCAACAACC	Non-coding
Forward G	CGGCTTCTTCCTTTTGCTAC	Non-coding
Reverse A	TGAGAGTGTGTCAAGATAATCAGC (S)	D Y L D T L S
Reverse B	AATCCAGCTAGTCTTTCACCTAC	G E R L A G F
Reverse C	TCAACAACCTTTGCGTCGTGCTC	A R R K V V E
Reverse D	ATCTTTCACAAGCGGCTTCAC	K P L V K D
Reverse E	TTTCTCCAGGTAAGGTGTGTTCTC	N T P Y L E K
Reverse F	TTGTATTTCTCGCCGAGCAACC	C C G E E I Q
Forward(nest)	(EcoRI)GCAGCAGTTGTGTTCTATTACC (E)	A V V F L F T
Reverse(nest)	(EcoRI)GAGATTTGGTTTTACTGCTCTGAGC	Q S S K T K S
Forward(out)	(EcoRI)TTTTTCCTCCGCAAACAGTC (C)	Non-coding
Forward-start	(EcoRI)ATGGCGTTACAAAACAAAG	M A L Q N K
Reverse(out)	CATTCACGGAATGAGACTAAAC(EcoRI)	Non-coding
RACE-rev A	CTCCTCATCTCTCCCTTCCA (R/5')	
RACE-rev B	CTTCGATGTTCTCGGAATCTG	F D V L G I
RACE-rev C	TCTTTTGCAACTTCTTCACTTTCTTTTCT (R)	K K K V K K L Q K
RACE AAP(for)	GGCCACGCGTCGACTAGTACGGGIIIGGGIIG	N/A
RACE AUAP(for)	GGCCACGCGTCGACTAGTAC	N/A
HMA1-for(out)	TGCAACTCTTACTCGTTCCTCCTCTTA (E)	Non-coding
HMA1-rev(nest)	CTACAAAGGCATGGAATGATTCGACGCTTA	T K A W N D S T L
HMA2-for(nest)	AGACTGGAGAAGAAGTTGAAGTGGAT (E)	T G E E V E V D
HMA2-rev(nest)	TGGCTACTGGTGTGGAGAGGATA	L S T P V A
HMA3-for(nest)	AGCATGTTTTGCGGTAATCC (E)	A C F A V I P
HMA3-rev(nest)	GTGCATCGTTAAGCCCATCT	D G L N D A P
HMA5-for(nest)	CAGTGGCAAAGAGAAAGGG (E)	P V A K R K
HMA5-rev(nest)	TGATGGACACAAAATCACAGG	A C D F V S I
Actin 2(for)	GGTAACATTGTGCTCAGTGGT (E)	G N I V L S G
Actin 2(rev)	CTCGGCCTTGGAGATCCACAT	M W I S K A E

and brief centrifugation. The reactants were incubated at 42 °C for 1 min before 1 µL of Superscript reverse transcriptase enzyme was added. After gently mixing the sample once more, the reactants were collected by brief centrifugation and incubated in a thermal cycler heated block (UNO II thermocycler). The reaction was held at 42 °C for 50 min prior to a termination step of 15 min at 70 °C. The sample was centrifuged for 20 s at 13,000 $\times g$ and placed at 37 °C. One µL of RNase mix (GIBCO BRL Life Technologies Ltd. RACE kit, version 2.0) was added to the tube, mixed thoroughly and incubated for 30 min. The sample was centrifuged briefly before being stored at – 20 °C prior to the next step.

Once thawed, the sample was cleaned using a QIAQuick PCR clean-up column (Qiagen Ltd.), following the manufacturer's instructions and eluted into 30 µL of sterile deionised water. 10 µL of the purified sample was added to a new PCR tube containing 6.5 µL of sterile deionised water, 5.0 µL of 5 X tailing buffer and 2.5 µL of 2 mM dCTP solution (GIBCO BRL Life Technologies Ltd. RACE kit, version 2.0). The mixture was incubated for 3 min at 94 °C, snap cooled on ice for 1 min and then collected by brief centrifugation. 1 µL of TdT (terminal deoxynucleotidyl transferase) enzyme was added to the tube and the solution gently mixed. The reaction was incubated for 10 min at 37 °C before heat inactivation at 65 °C for 10 min. After brief centrifugation the sample was stored at – 20 °C. Aliquots were then used in the subsequent PCR reaction.

In the initial step of the 5' RACE procedure, cDNA is synthesised from an RNA template, using a reverse-acting gene specific primer (GSP 1 = RACE-rev A; Figure 2.2, Table 2.4). In the second step, the gene specific cDNA is capped with a poly-C tail at its most 5 prime region. Subsequent PCR steps are then carried out: first with a primer complementary to the cap region (abridged anchor primer- AAP) and a gene specific primer (GSP 2 = RACE-rev B; Figure 2.2, Table 2.4) nested in respect to GSP1 and second, using a primer nested within the cap region (abridged universal amplification primer - AUAP) and a further nested gene specific primer (GSP 3 = RACE-rev C, Figure 2.2, Table 2.4).

For PCR reactions the Takara r-Taq polymerase (Cambrex Bio Science Ltd.) was used. Care was taken to keep all reaction constituents cool whilst the PCR mixture was prepared. 22.5 µL of water, 10 µL of 10 x Taq buffer, 8 µL of 2.5 mM dNTP solution (both supplied with Takara) were added to a PCR thermo-tube followed by 2 µL of AAP and RACE-rev B (10 µM solutions) and 5 µL of capped cDNA. Just prior to the PCR, 0.5 µL of r-Taq was added and gently mixed in with the solution. PCR conditions were used in accordance with the manufacturers

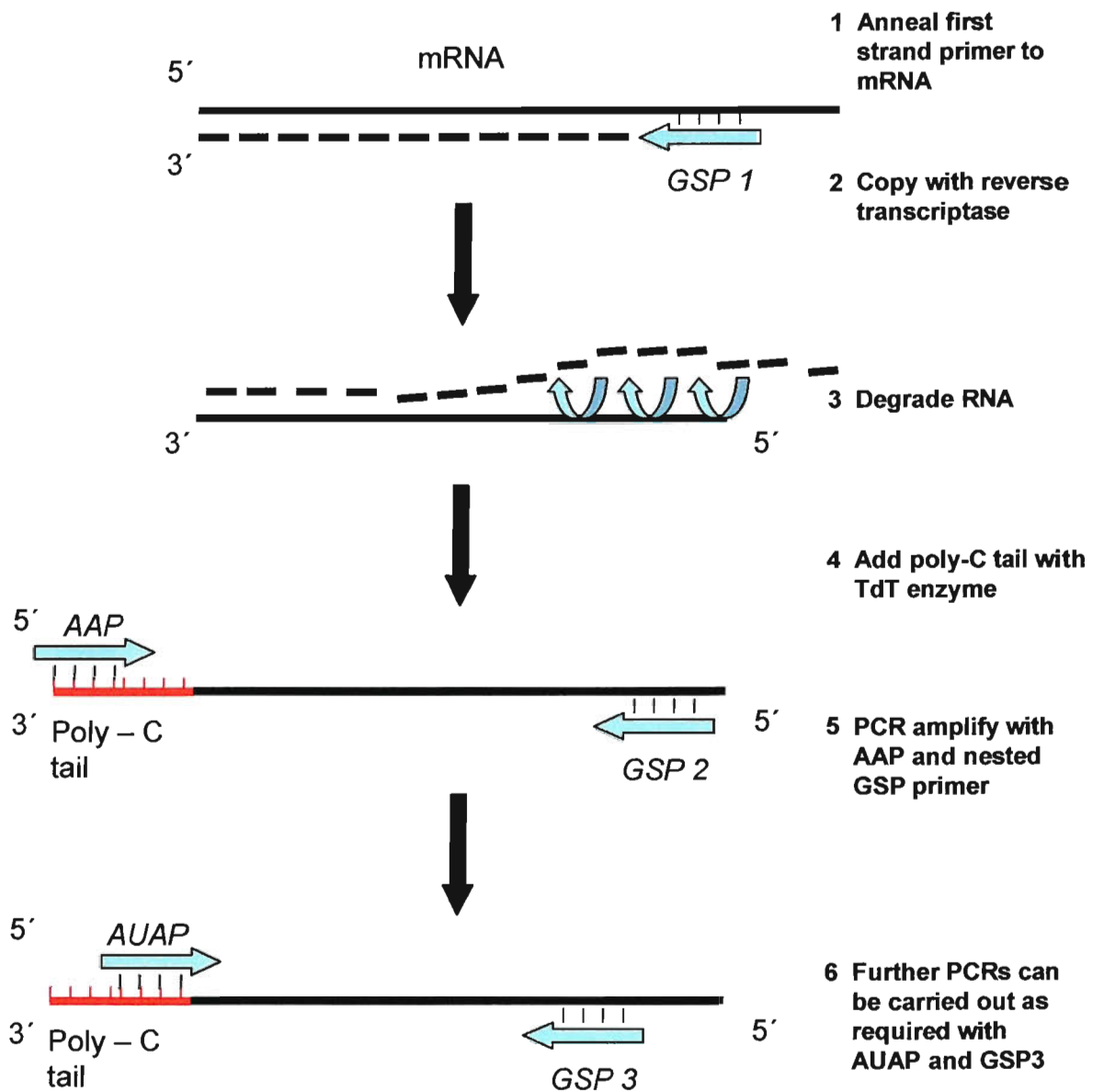


Figure 2.2 Summary of the reaction steps in the 5' RACE procedure. See text for details. GSP = Gene specific primer, AAP = Abridged anchor primer, AUAP = Abridged universal amplification primer

instructions and performed using Perkin Elmer 9700 thermocycler with the heating block set at standard ramp times for 50 μ L reaction volumes.

After the reaction was completed a 1:1 dilution of the products was made using sterile water. Another PCR was then carried out on 5 μ L of the diluted products using exactly the conditions as before. The reaction constituents were also the same except that the primer pair was substituted by AUAP and RACE-rev C, as mentioned in the above section. The products of this PCR were then analysed by agarose gel electrophoresis and ligated into the pGEM Easy-T vector before cloning into *E. coli* DH5 α (see below).

2.10 Cloning of DNA products

2.10.1 Ligation into plasmid vector

Cloning of the RACE-PCR products was carried out using a pGEM[®]-T Easy Vector System (Promega UK Ltd.) following the manufacturer's instructions. The vector uses the pGEM[®]-T Easy Vector, and JM109 competent *E. coli* cells. This kit greatly facilitates cloning of DNA products, since it eliminates the need to digest, dephosphorylate and purify the vector DNA, as well as the need to digest the insert DNA. It also allows blue/white colony screening, and the release of the insert by a choice of three different single-enzyme digestions.

2.10.2 Preparation of competent *E. coli* cells

Cultures of DH5 α cells were grown overnight at 37 °C in Luria Bertani (LB) broth. Fresh LB broth was isolated with 1% (v/v) of overnight culture and incubated until the OD₅₅₀ reached 0.4-0.5. The cells were pelleted by centrifugation at 3,000 \times *g* for 5 min at 4 °C in a Chillspin centrifuge and resuspended in 0.5 volume of pre-cooled 100 mM CaCl₂. The cells were incubated on ice for 1 h before repelleting as before. The cells were resuspended in 0.1 original volume of pre-cooled 100 mM CaCl₂. Cells could be stored at -70 °C in 15% (v/v) glycerol (final concentration) for later use but as far as possible fresh competent cells were made up for each transformation.

2.10.3 Transformation of *E. coli* with cDNA clones

Five μL of ligation reaction (see above) was added to 300 μL of competent cells and incubated on ice for 1 h before being given a heat shock at 42 °C for 90 s then returned immediately on ice for 5 min. Three hundred μL of LB broth was added and the cells were incubated at 37 °C for 1 h. Two hundred μL of cells were grown overnight at 37 °C on separate LB agar plates containing 50 $\mu\text{g}/\text{mL}$ ampicillin, 0.5 mM Isopropyl- β -D-thiogalactoside (IPTG) and 40 $\mu\text{g}/\text{mL}$ 5-bromo-4-chloro-indoyl- β -D-galactoside (X-Gal) so that recombinant colonies could be selected by the blue/white colour screening method. White colonies represented successfully transformed cells containing a plasmid with the ligated fragment. Such colonies were removed and grown overnight at 37 °C in 5 mL of LB broth. Plasmid DNA was isolated from each set of colonies and an *EcoRI* digest (see later) was performed to check whether they contained the vector with the insert DNA.

2.10.4 Small-scale isolation of plasmid DNA from *E. coli*

Plasmid DNA was isolated using the Qiagen QIAprep Spin Miniprep (Qiagen Ltd.), following the manufacturers instructions. Cultured cells (1.5 mL) were centrifuged at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 1 min. The resulting pellet of cells was resuspended in 250 μL of cell suspension solution (50 mM Tris/HCl (pH 8.0), 10 mM EDTA, 100 mg/mL RNaseA). 250 μL of cell lysis solution (200 mM NaOH, 1% (w/v) SDS) was then added to the Eppendorf tube. The contents were mixed by gentle inversion and left at room temperature for 5 min. Three hundred and fifty μL of neutralisation solution (containing acetate and guanidine hydrochloride) was then added and the Eppendorf was immediately inverted and a white precipitate formed. The contents were centrifuged for 10 min at 13,000 $\times g$ in a Sorvall MC 12V microfuge. The resulting supernatant was transferred to a filter column in a receiver tube. After centrifugation at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 1 min the filtrate was discarded. Five hundred microlitres of wash solution (containing ethanol, NaCl, EDTA and Tris/HCl) were added to the filter column and centrifuged through to the receiver tube at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 1 min. This step was designed to remove trace nuclease activity. After discarding the spent solution, the column was rinsed of any residual wash solution by adding 75 μL of secondary wash solution (contained chaotropic salt) to the column, and centrifuging at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 1 min. Finally, the remaining contents of the column (i.e. the plasmid

DNA) were recovered in a new receiver tube by eluting with 50 μL of sterile deionised water. This was done by carefully pipetting the water onto the surface of the column, allowing a 1 min equilibration time, followed by a further 1 min in the centrifuged at 13,000 $\times g$.

Alternatively a 1.5 mL overnight culture was centrifuged at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 1 min. The pelleted cells were resuspended in 100 μL TEG (25 mM Tris, 50mM EDTA, 1% (w/v) glucose at pH 8) and left at room temperature for 5 min. Two hundred μL of freshly prepared NaOH/SDS (200 mM NaOH and 1% (w/v) SDS) were then added by carefully pipetting the solution on to the sidewall of the Eppendorf vial. The contents were mixed gently by inversion and left at room temperature for 5 min, until the mixture obtained a cloudy appearance. Next, 150 μL of 3 M potassium acetate (pH 5.5) were added and the contents mixed again by gentle inversion. This was left on ice for 10 min before centrifuging at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 10 min. The supernatant was transferred to a new container, avoiding transfer of any white precipitate. The centrifugation step was then repeated, and the 'double' supernatant transferred to another clean Eppendorf vial. Nine hundred microlitres of 100% (w/v) ethanol and 45 μL of 3 M sodium acetate were added and the mixture left to precipitate at $-20\text{ }^{\circ}\text{C}$ for approximately 30 min. The contents were centrifuged at 13,000 $\times g$ in a Sorvall RCB4 (Kendro Laboratory Products Plc.) at $4\text{ }^{\circ}\text{C}$ for 20 min. The supernatant was discarded and the pellet was washed in 500 μL of 70% (v/v) ethanol and pelleted again by brief centrifugation. The ethanol was pipetted off and the pellet resuspended in 200 μL of sterile deionised water. Two microlitres of RNAase-A (2 mg/mL) were added to the solution, vortexed-mixed, and then incubated in a water bath at $37\text{ }^{\circ}\text{C}$ for 30 min. The solutions were returned to the ice once more and an equal volume (200 μL) of phenol: chloroform: isoamyl alcohol (25:24:1) added. They were then vortex-mixed and centrifuged at 13,000 $\times g$ in a Sorvall RCB4 at $4\text{ }^{\circ}\text{C}$ for 10 min. The upper phase was carefully removed and transferred to a new Eppendorf vial, before fresh phenol chloroform IAA was added and the mixing and centrifugation step repeated. Two volumes (400 μL) of 100% (v/v) ethanol and 0.1 volume (20 μL) 3M sodium acetate were added, mixed thoroughly and precipitated at $-20\text{ }^{\circ}\text{C}$ for approximately 1 hr. The DNA was pelleted by centrifuging at 13,000 $\times g$ in a Sorvall MC 12V microfuge, $4\text{ }^{\circ}\text{C}$ for 20 min, before the supernatant removed and an extra cleaning step carried out. Two hundred μL of 70% (v/v) ethanol were added, mixed thoroughly and centrifuged at 13,000 $\times g$ at room temperature for 5 min. After pipetting off the liquid phase, the pellet was allowed to air-dry in order to

remove further residue. Finally, the DNA pellet was resuspended in 25 μL of sterile deionised water and used immediately, or stored at $-70\text{ }^{\circ}\text{C}$ prior to use.

2.10.5 Medium-scale isolation of plasmid DNA from *E.coli*

Twenty-five mL of overnight culture were centrifuged at $2,800 \times g$ in a Sorvall MC 12V microfuge for 5 min. The pelleted cells were resuspended in 2 mL of TEG (25 mM Tris, 50 mM EDTA, 1% (w/v) glucose (pH 8)) and left at room temperature for 5 min. Sterile water (3280 μL), 600 μL of 10% w/v SDS and 120 μL of 10 M NaOH were then added to give a final mixture of 200 mM NaOH and 1% (w/v) SDS. The contents were mixed by inversion and left on ice for 5 min. Three mL of 3M potassium acetate (pH5.5) were added and again the contents mixed by inversion. This was left on ice for 10 min before centrifuging at $2,800 \times g$ in a Sorvall MC 12V microfuge for 1 hr. The supernatant was transferred to a new container, avoiding transfer of any white precipitate and 10 mL of 100% (v/v) ethanol were added and the mixture left to precipitate at $-20\text{ }^{\circ}\text{C}$ for at least 15 min. The contents were centrifuged at $2,800 \times g$ in a Sorvall MC 12V microfuge for 1 h and then the pellet was washed in 5 mL of 70% (v/v) ethanol and pelleted again by brief centrifugation. The pellet was resuspended in 200 μL of Tris EDTA (TE). Two μL of RNaseA (2 mg/mL) was added and incubated at $37\text{ }^{\circ}\text{C}$ for 30 min. This removed any RNA contamination.

2.11 Restriction digest of DNA

Between 1 and 5 μg of DNA (in distilled H_2O) to be cut was added to 2 μL of the appropriate $10 \times$ enzyme buffer (Promega UK Ltd.) and pulsed in a Sorvall MC 12V microfuge. Ten $\mu\text{g}/\text{mL}$ acetylated bovine serum albumin (Promega UK Ltd.) and 5 units of the desired restriction endonuclease (Promega UK Ltd.) were then added. The reaction volume was then made up to 20 μL with sterile distilled H_2O and incubated at $37\text{ }^{\circ}\text{C}$ for between 3 and 18 hr, depending on the enzyme used (refer to manufacturer's manual).

2.12 DNA sequencing

The chain termination method based on Sanger *et al.* (1977) was used for sequencing. This method, which relies on the use of chain-terminating

dideoxynucleotides was conducted using the ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Warrington, UK). This uses fluorescently labelled dideoxynucleotides to visualise the chain-terminating base. The specific kit formulation contains the sequencing enzyme Amplitaq DNA polymerase, which contains a point mutation at its active site. This results in less discrimination against dideoxynucleotides, and helps to achieve a more uniform peak intensity pattern from the fluorescent labels. The BigDye terminator reaction was made up containing 200-250 ng double-stranded template DNA, 4 μL Terminator ready reaction mix, 1 μL of primer (2 p mol) and then made up to 10 μL with deionised sterile H_2O . The reaction was vortexed then centrifuged briefly to collect the contents at the bottom of the tube. PCR was used to produce material for sequencing. The PCR follows the same basic principles as a normal PCR but incorporates chain-terminating fluorescent dNTPs. This produces fragments with a colour representing the chain-terminating base. This is translated by the gel reading equipment as red for T, green for A, blue for C and black for G. Once the thermal cycling was complete the reactions were precipitated. The sample was made up to 20 μL with sterile deionized H_2O , then 2 μL 3M sodium acetate (pH 4.6) and 50 μL 95% (v/v) ethanol were added before incubation at room temperature for 1 h. The sample was centrifuged at 13,000 $\times g$ in a Sorvall MC 12V microfuge at 4 $^\circ\text{C}$ for 20 min in order to pellet the precipitate. The pellet was washed in 250 μL 70% (v/v) ethanol then re-pelleted by centrifugation at 13,000 $\times g$ in a Sorvall MC 12V microfuge for 1 min. The 70% (v/v) ethanol was removed with a pipette and the pellet dried in a heated block at 94 $^\circ\text{C}$ for 2 min. The dried pellet was stored at -20 $^\circ\text{C}$ before being loaded onto the sequencing gel. The pellet was resuspended in 80 μL of a formamide: EDTA mix (5:1, de-ionised formamide and 25 mM EDTA pH 8 containing 50 mg/ml blue dextran). The sample was vortexed and briefly centrifuged. The samples were incubated at 95 $^\circ\text{C}$ for 2 min to denature the sample. The sample was stored on ice until it was loaded onto the gel (5 % GENE PAGE plusTM, 6 M urea (J437); Amresco Inc., Ohio, USA). The gel was run at 3kV for 2 hs. The position the fragment takes up on the gel is relative to its length. This property, correlated with the colour, describes the sequence. This was visualised graphically using the program Chromas (version 1.45) to give an initial sequence.

2.13 Sequence analysis

Primers were designed (Table 2.4) using software from the GCG computer programme (Program Manual for the Wisconsin Package, Version 10, 1999,

Genetics Computer Group, Madison, Wisconsin, USA), in accordance with criteria given by Taylor (1991). Sequence analysis was performed using software from the GCG package (Program Manual for the Wisconsin Package, Version 9, 1999, Genetics Computer Group, Madison, Wisconsin, USA) on the Seqnet facility, Daresbury Laboratory, Daresbury, UK. Sequence identities were performed using the GAP program (GCG package). Sequence alignments were performed using the PILEUP (GCG package) or CLUSTALW (1.82) programs and visualised in Ghostscript. EMBL/GenBank and EST sequence databases were searched using the BLAST (GCG package and NCBI on the World Wide Web at <http://www.ncbi.nlm.nih.gov/BLAST>) program. Locations of predicted introns and exons in *AtHMA4* were obtained from the MIPS *Arabidopsis thaliana* database (<http://mips.gsf.de>) and checked with the cDNA sequence (accession no. AJ297264; <http://srs.ebi.ac.uk/>) obtained from this work. Amino acid sequence alignments were performed using Clustal W 1.82 (Thompson et al. 1994, <http://www.ebi.ac.uk/clustalw>). The presence and arrangement of transmembrane domains in proteins were predicted using the most recent versions of commonly used, internet accessible transmembrane helix prediction programmes: PHDhtm, DAS, HMMTOP, EMBOSS (Kyte-Doolittle), Pred-tmr2, Psi (MEMSAT), SOSUI, THMHMM, TMAP, TMPred and TopPred. The percentage identities and percentage similarities at the amino acid level were calculated using the EMBOSS (European Molecular Biology Open Software Suite) programme 'Matcher' accessed through the Human Genome Mapping Project resource centre (<http://www.hgmp.mrc.ac.uk/Software/EMBOSS>).

For phylogenetic analysis of HMAs, sequences from various organisms were obtained from a protein families database (<http://www-biology.ucsd.edu/~ipaulsen/transport/index.html>), a P-type ATPase database (<http://biobase.dk/~axe/Patbase.html>) and by searching general databases with P-type ATPase and HMA-specific domains. Sequences were aligned using Clustal W 1.82 and a phylogenetic tree was created using the Phylip 3.6a3 neighbour-joining method with the data set bootstrapped 1000 times (Felsenstein, 2002). The tree was rooted at the midpoint and displayed using Treeview 1.6.6 (Page, 2001). The PROSITE (Falquet et al. 2002; <http://ca.expasy.org/prosite>) and Pfam databases (www.sanger.ac.uk/software/pfam) were used to search for conserved motifs/domains

2.14 Northern analysis

2.14.1 RNA transfer

Northern analysis was carried based on Sambrook *et al.* (1989). Total RNA (25-30 µg) was separated on a 1.5% (w/v) formaldehyde agarose gel as described previously, then transferred to Hybond-N nylon membrane (Amersham Pharmacia Biotech Ltd., Little Chalfont, UK) by capillary blotting (using 20 x SSPE: 0.5 M Na₂HPO₄, 1 M NaH₂PO₄, 2 mM Na₂EDTA and 3.6 M NaCl, pH 7-8). However, before transfer, the agarose gel was rinsed in multiple washes of DEPC-treated dH₂O for 1 to 2h to remove formaldehyde which can interfere with the transfer of RNA. RNA was visualized on the membrane with methylene blue (Herrin and Schmidt, 1988). The membrane was pretreated in 5% (v/v) acetic acid for 15 min, then stained in 0.04% (w/v) methylene blue in 0.5 M sodium acetate (pH 5.2) for 1 min. Before hybridization, the membrane was destained by washing with DEPC-treated dH₂O.

2.14.2 Probe labelling

2.14.2.1 Digoxigenin-labelled probes

Two cDNA clones encoding full-length *AtHMA1* and partial-length *AtHMA4* were used as templates for the preparation of Digoxigenin-labelled probes. In addition EST 5, the expression sequence tag of *AtHMA4* was also used. All material was purified of extraneous sequence prior to use. The synthesis reaction used was essentially the same as the gene-specific PCR reaction conditions outlined previously. The reaction or 'bulk' mix was prepared in exactly the same manner, aliquoted into 0.2 µL PCR tubes (ABgene Ltd.) and the template cDNA added. Subsequent to this 0.4 µL of Digoxigenin-dUTPs were also added and the solution mixed before being placed in a pre-warmed thermal cycler heated block at 94 °C for thermal cycling. For probe synthesis, maximal product yield is desired, and so each reaction mix was subjected to 40 cycles of PCR. A PCR control was included for each different probe product synthesized. This consisted of exactly the same constituents as the sample except that 0.4 µL of sterile deionised water was substituted in place of the Digoxigenin-dUTPs. Examination of the controls by agarose gel electrophoresis allowed the PCR product(s) to be assessed, indicating the 'target' of Digoxigenin-dUTP incorporation.

2.14.2.2 ³²P – labelled probes

cDNA fragments of *AtHMA4* were obtained from a mixture of root and leaf tissue using the nested(for) and nested(rev) primers (Table 2.4) in RT-PCR. Products were extracted from agarose gels, purified with a QIAQuick clean-up column and quantified before use. *Actin 2* fragments were generated in exactly the same way using the primer pair given in Table 2.4. For *AtHMA1*, DNA was derived from a full-length clone using primers designed to the 5 and 3 prime untranslated regions. To make the probe 25 ng of the purified DNA was placed in a 1.5 mL Eppendorf tube, suspended in 45 µL of TE buffer (10 mM Tris HCL and 1 mM EDTA). The mixture was heated to 95 – 100 °C in a boiling water bath for 5 min before cooling on ice for a further 5 min. After brief centrifugation the denatured DNA was carefully pipetted into a *rediprime* II reaction tube containing a buffered solution of dATP, dGTP, dTTP and Klenow enzyme (Amersham Pharmacia Biotech Ltd.). Next, 5 µL of Redivue [³²P] dCTP was added and the reactants carefully mixed with a pipette. The tube was incubated at 37 °C for 1 min in a water bath before the reaction was terminated by the addition of 5 µL of 0.2 M EDTA. Prior to its use in hybridisation the labelled DNA was denatured once more. The tube was incubated at 95-100 °C for 5 min followed by cooling on ice, as before.

2.14.3 Dot-blot to test probes

Probes were tested for sensitivity, specificity and compatibility with hybridisation and washing conditions by probing a nylon membrane (Hybond N; Amersham Pharmacia Biotech Ltd.) impregnated with known DNA fragments. Probes for *AtHMA1* and *4* were tested for hybridisation against varying amounts of the corresponding cDNA taken from the full- length and partial-length clones respectively (Digoxigenin-probe test), and against *ZAT2* cDNA, pAVA, and p426 plasmid DNA and *Arabidopsis thaliana* genomic DNA (³²P-probe test). Cross-hybridisation between *AtHMA1* and *AtHMA4* was also tested (Digoxigenin probe test). The cDNA in 1 µL of sterile deionised water was carefully pipetted on to the nylon membrane and allowed to air dry. The membranes were baked in an oven at 80 °C for 30 min before use.

2.14.4 Hybridisation and washing conditions

Before starting the procedure 2 x 25 mL aliquots of hybridisation solution (12.5 mL Formamide, 7.5 mL 20 x SSPE, 2.5 mL 50 x Denhardt's solution and 1.25 mL sterile water) were equilibrated in a hybridisation oven set at 42 °C. Five hundred µL of salmon sperm DNA (5 mg/mL solution, Sigma-Aldrich Co. Ltd.) was pipetted into a 1.5 mL Eppendorf tube and boiled in a water bath for 5 min. It was then cooled on ice for a further 5 min and then added to one of the 25 mL aliquots of hybridisation solution (at 42 °C). The nylon membrane to be probed was taken and placed flat in a small plastic container so that the RNA - exposed side faced upwards. A small volume (ca. 20 mL) of 2 x SSC (100 mL 20 x SSC, 10 mL 10% (w/v) SDS and 890 mL sterile water) was poured into the container and gently agitated in order to wet the membrane. The excess solution was drained and the aliquot of hybridisation solution + salmon sperm DNA poured in. The container was sealed and the membrane and bathing solution incubated at 42 °C for 4 hr with constant shaking. A further 500 µL salmon sperm DNA was then prepared as before, and added to the second 25 mL aliquot of hybridisation solution (at 42 °C). The previous solution was then drained from the membrane and the new one added. Next, radioactive probe was added to the solution and gently mixed. Saran wrap™ was draped over the container before it was sealed. This was done in order to reduce the build up of condensation on the container lid, and thereby maximise the amount of radioactive probe remaining in the bathing solution. The container was then placed in a hybridisation oven and incubated at 42 °C overnight (ca. 16 - 18 hr) with constant shaking. After approximately 16 – 18 hr incubation, the radioactive hybridisation solution was poured off and discarded. The membrane was then subjected to a number of wash steps, the first carried out at 37 °C and the remainder at 42 °C, each being of 15 min duration with constant shaking. All solutions were pre-warmed and composed of various concentrations of SSC and 0.1% (w/v) SDS. The lower the salt concentration, the greater stringency of the wash. Checks of the membrane radioactivity levels were made after each medium and high stringency wash. This was done by passing a Geiger counter probe slowly across the upper surface of the membrane. This ensured that background signal (diffuse in pattern) was minimised whilst making sure the target signal still remained. The wash steps used are shown in Figure 2.3. After the washing steps were complete, the membrane was drained of excess solution, wrapped in Saran wrap™ and then exposed to X-ray film in a cassette at – 70 °C. The duration of exposure

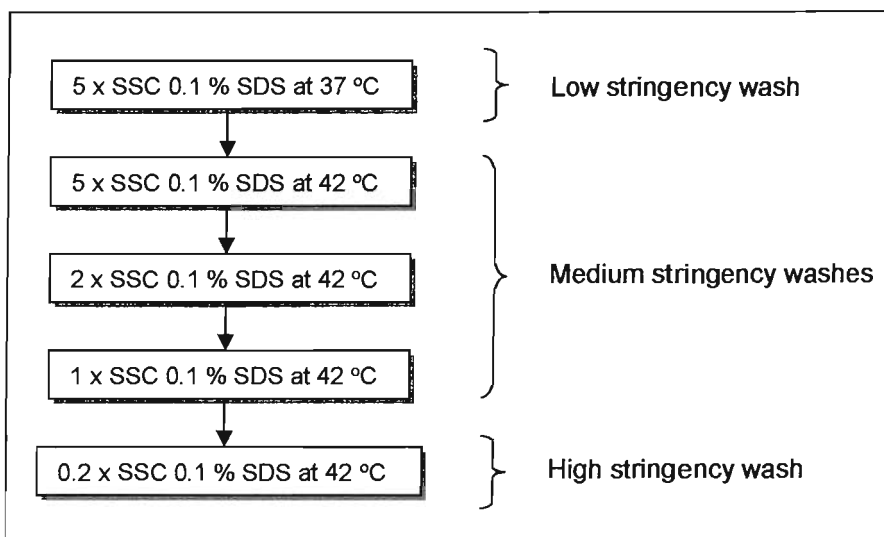


Figure 2.3 Washing procedures carried out on northern blots probed with ^{32}P - labelled cDNA. See text for details.

depended upon the intensity of radioactive signal. Intensifying screens were used in each exposure.

2.15 Western analysis

2.15.1. Extraction of membranes from plant tissue

Arabidopsis thaliana plants grown hydroponically as described above were treated with either normal nutrient solution or nutrient solution amended with either, 1 mM K₂SO₄, 1 mM ZnSO₄ or 0.8 mM MnSO₄, each at pH 5.2. Roots were cut from the plants into a container filled with clean ice-water. This was carried out whilst the plants were still supported in their hydroponic raft. After all the roots had been removed, they were agitated briefly in the ice water and gently blotted dry between several sheets of paper towel. A fresh weight measurement was taken immediately before the tissue was added to a blender containing 150 mL of ice-cold homogenisation medium (Uemura et al. 1995; 500 mM sorbitol, 50 mM MOPS-KOH pH 7.6, 5 mM EDTA, 5 mM EGTA, 1.5% (w/v) polyvinylpyrrolidone, 0.5% (w/v) BSA, 1 mM PMSF, 4 mM salicylhydroxamic acid, 2.5 mM potassium metabisulphate). The tissue was pulsed at high speed for 30 s and the homogenate poured through muslin sheets into four 50 mL polycarbonate centrifuge tubes. The tubes were kept on ice whilst the procedure was repeated for the leaf tissues. This was carried out exactly as before, except that only a small portion of the leaves were harvested. When completed the homogenates were centrifuged at 12,000 x g for 10 min at 4 °C in a Sorvall RC2B centrifuge (Kendro Laboratory Products Plc.). The pellets were retained ("low speed pellet") and the supernatants were poured carefully into 50 mL Oakridge tubes and centrifuged at 100,000 x g for 45 min at 4 °C in a Sorvall RC28S centrifuge (Kendro Laboratory Products Plc.). The supernatants from this step were discarded and the pellets retained ("high speed pellet"). The high-speed and low-speed pellets were then subjected to the same washing procedure: both were resuspended in 20 mL of resuspension buffer (250 mM sorbitol, 1.0 mM MOPS-KOH pH 7.3, 2 mM DTT), centrifuged at 100,000 x g for 45 min at 4 °C and then resuspended in the final step with minimal volumes (approximately 300 µL for the high speed and 500 µL for the low speed pellets respectively) of resuspension buffer. The extracts were then stored in liquid nitrogen until required. Membranes were extracted from yeast as described in section (2.17).

2.15.2 SDS-PAGE and protein transfer

Proteins were separated by discontinuous SDS-PAGE (Laemmli 1970), using 7.5% (w/v) separating gels, in a Bio-RAD Protean II dual slab gel with water cooling. Proteins were diluted 1:1 with sample reducing buffer (62.5 mM Tris-HCl (pH 6.8), 5% (v/v) 2-mercaptoethanol, 2% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue) and heated at 70°C for 20 min before loading. Molecular weights were determined using SDS 7B pre-stained molecular weight markers (Sigma-Aldrich Co. Ltd.). Proteins were separated at 35 mA (constant voltage) for 3 hs. Gels were equilibrated in 25 mM Tris, 192 mM glycine and 20% (v/v) methanol at 4 °C and western blotted onto PVDF membrane (Hybond P, Amersham Pharmacia Biotech Ltd.) at 100 V for 70 min. The PVDF membranes were rinsed in deionised water and air-dried before storage. To re-use, the PVDF blots were hydrated for 30 s in 100% MeOH, rinsed several times in deionised water and followed by several rinses of PBS (phosphate-buffered saline: 150 mM NaCl, 10 mM NaH₂PO₄/NaOH (pH 7.2)) or TBS (Tris-buffered saline: 150 mM NaCl, 10 mM Tris-HCl (pH 7.6)) containing 1% (v/v) Triton X-100 and incubated at 55 °C in 62.5 mM TrisHCl (pH 6.80) with 100 mM 2-mecraptoethanol and 2% (w/v) SDS, for 30 min.

2.15.3 Antibody production

Polyclonal anti-peptide antibodies were supplied for this project. AtHMA antibodies were raised to peptide stretches of between 14 and 15 amino acids in a rabbit host by Genosphere Biotechnologies, Paris, France and supplied in the initial antisera and as an affinity purified extract. Pre-immune control serum was also obtained. Anti-peptide antibodies to *Nicotiana tabacum* PMA2 (raised in rabbits) were received as a gift from Dr. M. Boutry (Louvain, Belgium).

2.15.4 Antibody binding and detection

PVDF membranes were blocked in 10% (w/v) non-fat dried milk (Marvel: Premier International Foods Ltd. Morton, UK.) in PBS (pH 7.2) or in TBS (pH 7.6) with 0.1% (v/v) Tween 20 (TBSTw) for 1 hr, and then reacted with the primary antibodies (1:100 to 1:1000 dilutions tested) in PBS or TBS with 5% (w/v) non-fat dried milk for 1 hr at room temperature or overnight at 4 °C. The blots were washed in PBS (pH 7.2) with 0.1% (v/v) Tween 20 (PBSTw) for 5 x 5 min before incubating

for 1 h in PBS or TBST with 5% (v/v) non-fat dried milk and the horseradish peroxidase (HRP)-conjugated secondary antibody (typically 1: 100,000 dilution used). The blots were washed in PBS or TBS with 1% Triton X-100 for 5 x 5 min, and reacted with an enzyme chemiluminescence kit (ECL Plus: Amersham Pharmacia Biotech Ltd.) according to the manufacturers instructions. Antibody reactions were visualised by exposure of the blots to photographic film (Amersham Pharmacia Biotech Ltd.).

2.16 Yeast strains and growth conditions.

The *Saccharomyces cerevisiae* reference strain BY4741 (*MATa;his3 Δ 1;leu2 Δ 0; met15 Δ 0;ura3 Δ 0*) was obtained from Euroscarf (Frankfurt, Germany). The transformants provided for this study were produced previously in the William's Laboratory (Southampton, UK). These included *S cerevisiae* BY4741 transformed with 1) the shuttle vector *p426* (Mumberg et al. 1994), 2) *AtHMA4* in *p426* and 3) *AtHMA1* in *p426* (Mills et al, 2003; Mills and Williams, unpublished). This vector contains the galactose-inducible promoter, GAL1 (Mumberg et al. 1994).

To prepare cells for use in radiolabelled-metal uptake assays, cells were grown in selective media (liquid) 2 days prior to the uptake assay as follows: yeast were inoculated from colonies growing on agar media into 150 mL of synthetic complete(Sc)-raffinose minus uracil containing 1.7 g/L yeast nitrogen base (YNB), 5 g/L (NH₄)₂SO₄, 1.92 g/L yeast synthetic drop-out media (Sigma-Aldrich Co. Ltd.), 20 g/L raffinose, (pH 5.5). Cultures were shaken at 30 °C for 28 hr and aliquots were taken to measure optical density. Measurements were made on diluted cultures, typically containing around 20% of the original cell suspension, and adjustments made until a solution of 0.1 OD₆₀₀ was obtained. Calculations made from these dilutions were then used to adjust 150 mL of Sc-galactose minus uracil containing 1.7 g/L yeast nitrogen base, 5g/L (NH₄)₂SO₄, 1.92 g/L yeast synthetic drop-out media (Sigma-Aldrich Co. Ltd.), 20 g/L galactose (pH 5.5), to the same OD. These fresh inoculums were then incubated under the same conditions as before, for approximately 14 hr (during which the cells were in their exponential growth phase). Growth in Sc-galactose was carried out to induce the expression of the *AtHMA* genes.

To prepare transformants for extraction of membrane protein, cells were inoculated separately into 2 x 5 mL aliquots of Sc-galactose minus uracil media. The cultures were grown overnight with shaking table at 30 °C until they had reached an

optical density (O.D.₆₀₀) of approximately 1.5 units. 10 mL of each culture was then added to a further 500 mL of fresh Sc-galactose minus uracil and grown for an additional 2 days.

2.17 Extraction of membranes from yeast

Following growth as described in the section above, the yeast suspensions were centrifuged at 4000 $\times g$ for 5 min in a Sorvall Legend RT centrifuge (Kendro Laboratory Products Plc.), before resuspending the pellet in 50 mL of buffer 1 (0.1 M Tris-HCl, pH 9.4, 50 mM 2-mercaptoethanol, 0.1 M galactose). The cells were then washed in buffer 1 with shaking for 10 min at 30 °C. Following this the cells were spun again at 4000 $\times g$ for 5 min. The pellets were then resuspended in 50 mL buffer 2 (0.9 M sorbitol, 0.1 M galactose, 50 mM Tris-MES, pH 7.6, 5 mM DTT, 0.043% (w/v) YNB, 0.25 \times Sc – uracil, 0.05% (w/v) Zymolase enzyme (Seikagaku Kogyo Co., Tokyo, Japan) and incubated with gentle shaking at 30 °C for 1h to form spheroplasts. The suspensions were centrifuged at 3,000 $\times g$ for 10 min and the pellet resuspended in 30 mL of 1 M sorbitol before being washed by centrifugation at 3000 $\times g$ for 10 min. The supernatant was carefully removed and the pellet resuspended in 20 mL of buffer 3 (50 mM Tris-MES, pH 7.6, 1.1 M glycerol, 1.5% (w/v) PVP 40,000, 5 mM EGTA, 1 mM DTT, 0.2% (w/v) BSA, 1 mM PMSF, 1 mg/L leupeptin). Once complete, the cells were ruptured in a glass homogeniser vessel. This was done in 10 mL aliquots with the use of a ceramic plunger. The procedure was standardised so that each 10 mL aliquot was homogenised with 20 thrusts of the plunger. Each yeast transformant was treated in a separate vessel and the homogenates were stored briefly on ice during processing of the samples. After this time each sample homogenate was transferred to a centrifuge and spun at 2,000 $\times g$ for 10 min at 4 °C. The pellet from this was referred to as the low speed fraction and was resuspended in resuspension buffer described below. The supernatant was poured from the pellets into separate ultracentrifuge tubes and centrifuged at 120,000 $\times g$ for 45 min at 4 °C (Sorvall Ultracentrifuge OTD 55B; Kendro Laboratory Products Plc.). The supernatants were discarded and the pellets resuspended in 25 mL of resuspension buffer (5mM Tris-MES, pH7.6, 0.3 M sorbitol, 1 mM DTT, 1 mM PMSF, 1 mg/L leupeptin), before a final spin at 120,000 $\times g$ for 45 min at 4 °C. The supernatant was discarded again, and the pellets resuspended in a minimal volume (approx. 5 mL) of resuspension buffer. Samples were then stored in liquid nitrogen prior to their use.

2.18 Protein determination

2.18.1 Bradford assay

Proteins in plant and yeast membrane samples were determined using a modification of Bradford's method (Bradford 1976), with 1% (w/v) SDS in the reagent to solubilise membranes and using BSA standards. The Bradford assay procedure was used to quantify the protein content of samples where reductant such as DTT was present in the medium. In such cases the BCA (Bicinchoninic acid) method was unsuitable.

2.18.2 BCA protein assay

Diluted yeast samples were boiled for 5 min in an equal volume of 10% (w/v) SDS. 25 μ L aliquots were taken and the protein concentration determined using a BCA protein assay kit (Pierce Biotechnology Inc. Rockford, USA). Samples and replicates were handled in 96 well plates and the colour change reaction measured using a Dynatech MR5000 plate reader.

2.19 Radiolabelled metal-uptake assay

2.19.1 Preparation of yeast cells

Following growth in Sc-galactose, each yeast culture was transferred to 3 x 50 mL falcon tubes and spun at 2,000 x *g* for 5 min in a Sorvall Legend RT centrifuge (7500 6445 swinging bucket rotor). The supernatant was discarded, and the pellets re-suspended in small volumes of ice cold 1.8% glucose (w/v) with 0.2% (w/v) galactose. Each replicate was then bulked together in one tube and made up to 30 mL with further 1.8% (w/v) glucose 0.2% (w/v) galactose. The cells were then spun at the same speed and duration at 4 °C (Sorvall Legend RT centrifuge), before the resuspension and pelleting steps were repeated. After the second wash, the cells were resuspended in 20 mL of ice cold 1.8% glucose, 0.2% galactose (w/v) and transferred to fresh pre-weighed falcon tubes. A final spin at 2,000 x *g* for 5 min at 4 °C was then carried out. The supernatant was then poured off and the tubes immediately inverted to ensure all traces of sugar solution were drained from the pellet. Fresh weights were then calculated (measurements taken in triplicate) and

ice-cold 1.8% glucose, 0.2% galactose (w/v) added so as to obtain a cell suspension at 0.1 mg/ μ L. The yeast were then stored briefly on ice and used immediately for the radiolabelled uptake assay.

2.19.2 Time-course studies

Eight hundred and eighty μ L of 4.3% (w/v) glucose with 0.48% (w/v) galactose and 1100 μ L of 20 mM MES-NaOH (pH 5.5) were pipetted into three 5 mL conical flasks and incubated in a shaking water bath at 30 °C. After 10 min incubation, 220 μ L of the vector control yeast suspension were pipetted into the first flask and mixed several times using pipette-tip suction ($t = \text{minus } 10 \text{ min}$). Two min later, cells expressing *AtHMA1* were added to the second flask. After 4 min the *AtHMA4*-transformed cells were added to the final flask. In this way, each culture was treated in an identical fashion, separated only by a two-min staggering period. Exactly ten min after the vector control cells were added to the flask, 20 μ M (2.265 MBq μmol^{-1}) of ^{65}Zn -labelled Zn or 10 μ M (10 MBq μmol^{-1}) ^{109}Cd -labelled Cd were added, and gently mixed using the pipette tip. 100 μ L of the culture was then immediately taken (time-zero sample) and pipetted on to the surface of a glass microfibre filter disc (25 mm dia., Whatman International Ltd., Maidstone, UK.), supported on a porous suction-stage. 15 mL of ice-cold wash buffer (1 mM EDTA, 1 mM NaCl, 1 mM MgSO_4 , 1 mM CaCl_2 , 1 mM KH_2PO_4 , 20 mM sodium citrate buffer pH 4.2) were carefully pipetted on to the disc, the excess solution being siphoned away to a liquid waste vessel. The disc was then removed and placed in a scintillation vial prior to counting. Two and 4 min later the same procedure was repeated for the time = zero samples of *AtHMA1* and *AtHMA4* expressing cells. Further 100 μ L samples were taken from the cultures, 6, 12, 18, 24 and 30 min after the addition of the radioactive isotope. The staggered sampling arrangement was maintained throughout the experiment until the last *AtHMA4* sample.

2.19.3 Single time-point studies

A time point of 12 min was chosen to carry out multiple replicate reactions. All reagents in the isotope-incubation culture were scaled down to 1/8th of that used on the previous experiments, with the proportions of each component being maintained exactly as before. The protocol was modified slightly in order to account for the handling of small volumes. 1540 μ L of 4.275% glucose with 0.475% (w/v) galactose and 1925 μ L of 20 mM MES-NaOH (pH 5.5) were pipetted into a 5 mL

(wide mouth) glass vial and placed in a water bath at 30 °C. 53.9 µL of ^{65}Zn was then added and mixed with a pipette. After 10 min of shaking incubation, glass vials containing 27.5 µL of vector control, *AtHMA1*- and *AtHMA4*- transformed cells were added to the water bath. At the same time a vial with 27.5 µL of vector control cells was also placed on ice. After 5 min, 251.35 µL of isotope-incubation mix was added to the 'vector ice' cells and mixed several times with the pipette. Two min later, the same was carried out for the 30 °C vector cells, and again on 4 and 6 min for *AtHMA1* and *AtHMA4* transformed cells. 12 min after addition of the isotope-incubation mix, 100 µL of the culture was taken from the relevant vessel and pipetted on to the surface of a glass microfibre filter disc (25 mm diameter; Whatman International Ltd.), supported on a porous suction-stage. The discs were treated as previously with 15 mL of ice-cold wash buffer being siphoned through, before being removed to scintillation vials for short-term storage.

2.19.4 Scintillation counting

Four mL of OptiPhase 'HiSafe'3 (Fisher Scientific Ltd.) liquid scintillation cocktail. Care was taken to ensure that each of the glass micro-fibre filter discs had been fully submerged, and no large trapped air bubbles remained in the solution. Radioactivity was counted in a Beckman LS 6500 scintillation counter.

2.20 Yeast metal-tolerance assay

S. cerevisiae BY4741 transformed with 1) the shuttle vector *p426* (Mumberg et al. 1994), 2) *AtHMA4* in *p426* and 3) *AtHMA1* in *p426* (Mills et al, 2003; Mills and Williams, unpublished) were grown in Sc-raffinose minus uracil, followed by Sc-galactose minus uracil in exactly the same manner as for the radiolabelled assay experiments. After growth in Sc-galactose, the cultures were adjusted to an O.D₆₀₀ of 0.4 units. They were then streaked onto 2% (w/v) agar plates made with the same Sc-galactose media. The agar was adjusted as required with zero, 10 µM, 30 µM and 40 µM CdSO₄. The plates were incubated for 3 days at 30 °C before the growth of the yeast was examined and recorded.

Chapter 3 Cloning, sequencing and analysis of *AtHMA4*

3.1 Introduction

The completion of the *Arabidopsis* genome-sequencing project has represented a major breakthrough in plant biology research (Zhu et al. 2001). A total of 25,948 genes have been identified by gene prediction software, but most are of unknown function (*Arabidopsis* Genome Initiative 2000). General annotations have been applied to much of the genome, but a large number of annotations are tentative and refer to hypothetical proteins or putative homologues (Brendel and Zhu 2002). This has generated large volumes of data that awaits clarification by experimental evidence. The cloning and sequencing of a particular gene from mRNA can provide an in-depth characterisation of its function(s). It may be especially useful in identifying different transcripts or splice variants that may be important under different environmental conditions. It is also necessary for the subsequent expression in heterologous systems which allow a detailed investigation of gene products in isolation from the complex network of the plant. In addition to function, biochemical analyses and explorations of structure-function relationships may also be assessed (Dreyer et al. 1999). Thus the cloning and sequencing of a gene represents the first, and arguably one of the most important steps in characterising the role of a gene in a particular organism. The use of expressed sequence tags (ESTs) have facilitated this process by providing information on various aspects of gene expression and acting as an alternative route toward the isolation of several genes (Ohlrogge and Benning 2000).

Dramatic improvements in DNA-sequencing technology have allowed the use of large-scale single-pass cDNA sequencing for the construction of large EST databases. Expressed sequence tags are fragments of cDNA (ca. 600 - 3500 bp in length) that have been amplified from mRNA extracted from specific plant tissues by a reverse transcriptase reaction. These fragments are cloned to give a cDNA library, which is catalogued to provide a resource that may be screened against with any new sequence data. The technique has proved to be a powerful tool in the discovery of novel genes and mapping of their location on chromosomes using linkage analysis. For example many enzymes involved in fatty acid modification, isoprenoid and cell-wall biosynthesis have been discovered in this way (Van de Loo et al. 1995; Lange and Croteau 1999). Despite the completion of the *Arabidopsis* genome project (*Arabidopsis* Genome Initiative 2000), ESTs can still provide invaluable information in areas such as gene splicing (Yang et al. 1997; Reddy and

Harris 1998; Reddy et al. 2000; Ogawa et al. 2000) and gene expression (Ohlrogge and Benning 2000). With their different uses in mind, it is important to consider that different cloning and sequencing strategies are the key to success in the construction and analysis of EST (cDNA) libraries. Figure 3.1 highlights some of these cloning strategies and includes possible advantages and disadvantages for each. For example, different cloning schemes are applied for construction of libraries optimised for high throughput gene identification by sequencing, expression profiling or for the isolation of full-length clones (Sterky and Lundeberg 2000). Obviously each of these different libraries have a common requirement at the outset: the isolation of mRNA and its conversion to double-stranded cDNA. This can be achieved by the use of reverse transcriptase, primed by an oligo (T)-primer, random hexamer primers or primers specific for a certain gene. The oligo (T) primer method is often used in semi-quantitative RT-PCR, a technique which can be used to build up an expression profile of a particular set of genes. Priming RNA with a gene-specific oligonucleotide is a technique utilised in 5' prime RACE-PCR.

There are many instances when an EST library may not contain the full-length sequence of a gene. As mentioned above, the library may not have been constructed with such an end-point in mind, or it may lack sequence because of incomplete first strand synthesis (Sterky and Lundeberg 2000). In instances where the full-length cDNA is required, such as for detailed sequence analysis or the expression of recombinant protein, RACE-PCR can be an effective solution. In 5' RACE reverse primers complementary to a known fragment of the gene of interest are used on mRNA to reverse transcribe the first strand of cDNA. In theory all transcripts of the gene are amplified complete to their 5' untranslated promoter regions. A cap is then applied using terminal transferase and normal PCR carried out using cap specific and gene specific primer pairs. The technique is quick and relatively successful compared to more traditional methods, which would focus on screening for longer fragments in further cDNA libraries (Sterky and Lundeberg 2000).

Finally, the last but perhaps most important benefit of all to be gained from using ESTs to isolate a particular gene is the clarification that the gene's effect is real. It therefore has some influence upon the phenotype of the organism from which it was obtained. Genes belonging to large superfamilies or indeed any gene in the *Arabidopsis* genome may be the subject of genetic redundancy (The Arabidopsis Genome Initiative 2000). With ESTs being sourced initially from RNA, the isolation of a predicted gene sequence on one or several ESTs means that it is extremely unlikely to represent a non-functional pseudogene.

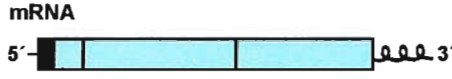



Cloning Strategy	Characteristics
 <p>mRNA 5' [cap] [exon] [intron] [exon] [poly(A)] 3'</p>	<p>The position of the clones in the strategies described are shown in relation to this mRNA molecule</p>
	<p>The fragments will be distributed over the whole transcript, but the direction of the insert is not known. The inserts often cover coding sequence, which facilitates functional classification by sequence homologies. Problems with poly(A)-tail are minimised, but full length cDNA clones will not be available.</p>
	<p>Libraries optimised to be rich in full-length sequences still contain a fraction of clones that are truncated in the 5' end. Coding sequence is therefore often obtained from this end. 3' end sequencing using PCR will be difficult due to the poly(A)-tail. Expression analysis by assembly will be incomplete due to non-overlapping sequences from the same transcript</p>
	<p>Libraries with 3' end fragments are best applied in mapping and expression analysis. Sequences from this region will overlap and an assembly will reveal the true expression profile. The 3' UTR sequences are poorly conserved and contain few introns, which helps in distinguishing between members of gene families and facilitating the generation of Short tagged sequences based on these sequences</p>

Figure 3.1 Summary of the alternative cloning strategies used to create EST libraries and their respective advantages and disadvantages. (Source; Sterky and Lundeberg 2000)

The main aims of the work described in this chapter were to clone the full-length *AtHMA4* cDNA for subsequent functional studies; to analyse the 5' untranslated region (UTR), and to analyse the sequence of *AtHMA4* and other *AtHMAs* to provide information about the structure and function of this family.

3.2 Results

The completion of the *Arabidopsis* genome sequencing project has provided the foundation for the discovery of many novel genes. Sequence for the putative metal transporter *AtHMA4* was identified in genomic sequence from BAC clone t20k24 (database accession AC002392; Williams et al. 2000). Translation of this sequence indicated that it may code for a protein with strong similarity to P-type ATPase transporters, more specifically those belonging to the HMA-type subgroup. In an attempt to isolate the full-length coding sequence for cloning and functional studies, two strategies were adopted and run concurrently. These were the isolation of the cDNA using RT-PCR and isolation through screening (data-base mining) plant-EST libraries.

3.2.1 Amplification, cloning and sequencing of *AtHMA4* from *Arabidopsis* using RT-PCR

To isolate a full-length cDNA for *AtHMA4* an RT-PCR approach was taken, using RNA prepared from *Arabidopsis thaliana* Columbia ecotype. Initially, primers HMA4for(out) and HMA4rev(out) (Table 2.4, Chapter 2), complimentary to the 5' and 3' UTRs in BAC clone AC002392 were used. These were positioned 66 and 57 bp upstream and downstream of the predicted coding region termini. PCRs using these primers with a range of reaction conditions proved unsuccessful, therefore attempts were made to amplify the gene in two halves. Primer pairs HMA4for-start/HMA4rev(nest) and HMA4for(nest)/HMA4rev(out) were used (Table 2.4). These were complimentary to the predicted start codon (HMA4for-start) and a short sequence stretch 900 bp downstream (HMA4rev(nest)) and a site 510 bp 3' of the predicted start codon (HMA4for(nest)) to the HMA4rev(out) sequence, 57 bp downstream from the predicted coding terminus. A cDNA fragment encoding the predicted N-terminus of the protein (926 bp with 86 bp of 5' UTR) and an overlapping cDNA fragment encoding the C-terminus of the protein (3006 bp with 80 bp of 3' UTR) were amplified using root cDNA and cloned into pGEM (Figure 3.2, lanes 2 and 4). These were named products 5.2 and 6.4 respectively. PCR-products of the correct predicted size (3851 and 3646bp) were also amplified from *Arabidopsis thaliana* genomic DNA using these primers (Figure 3.2, lanes 1 and 3 respectively). Primer locations are shown on the *AtHMA4* gene in Figure 3.3. The two partial cDNA fragments were cut out of the pGEM-T easy vector using *EcoRI* and were digested with *XmnI*. The latter enzyme cuts a unique site present in the

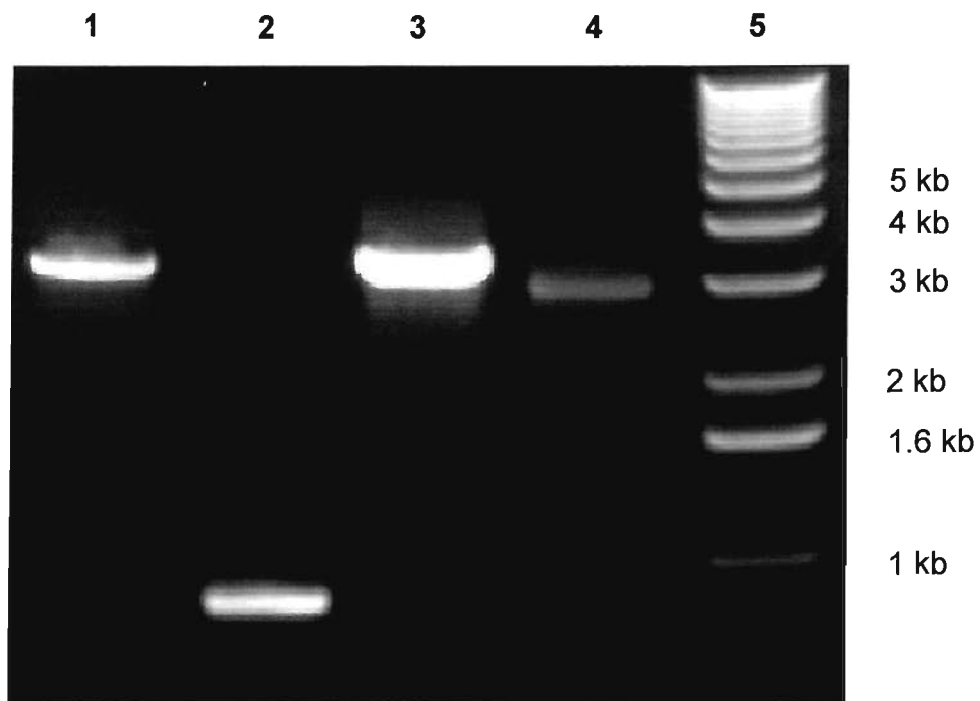


Figure 3.2 Amplification of partial-length cDNAs for *AtHMA4* from *Arabidopsis thaliana*. RT-PCR-amplified products from *Arabidopsis thaliana* were resolved on a 0.8% agarose gel and visualized with ethidium bromide. *AtHMA4* 5'- and 3' halves were amplified from root material (lanes 2 and 4, respectively). PCR-amplified genomic products for the corresponding 5' and 3' segments are also shown (lanes 1 and 3). Lane 5 shows products from a 1 Kb ladder.

AtHMA4

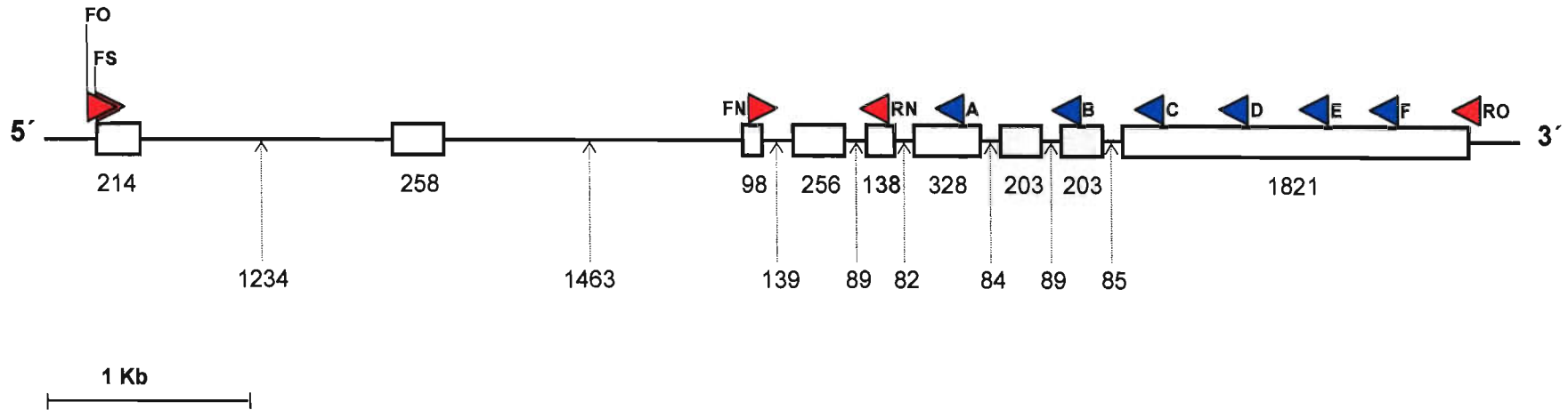


Figure 3.3 Diagram showing the location of primers (triangles) on the *AtHMA4* gene. Sizes (in bp) of introns (black lines) and exons (grey boxes) are given below the diagram in the relevant locations. Primers used for cloning and expression analysis are indicated with red triangles, those used in sequencing are indicated with blue triangles. Primer sequences are given in Table 2.4. FO = Forward(out), FS = Forward(start), FN = Forward(nest), RN = Reverse(nested), RA = Reverse A, RB = Reverse B, RC = Reverse C, RD = Reverse D, RE = Reverse E, RF = Reverse F, RO = Reverse(out).

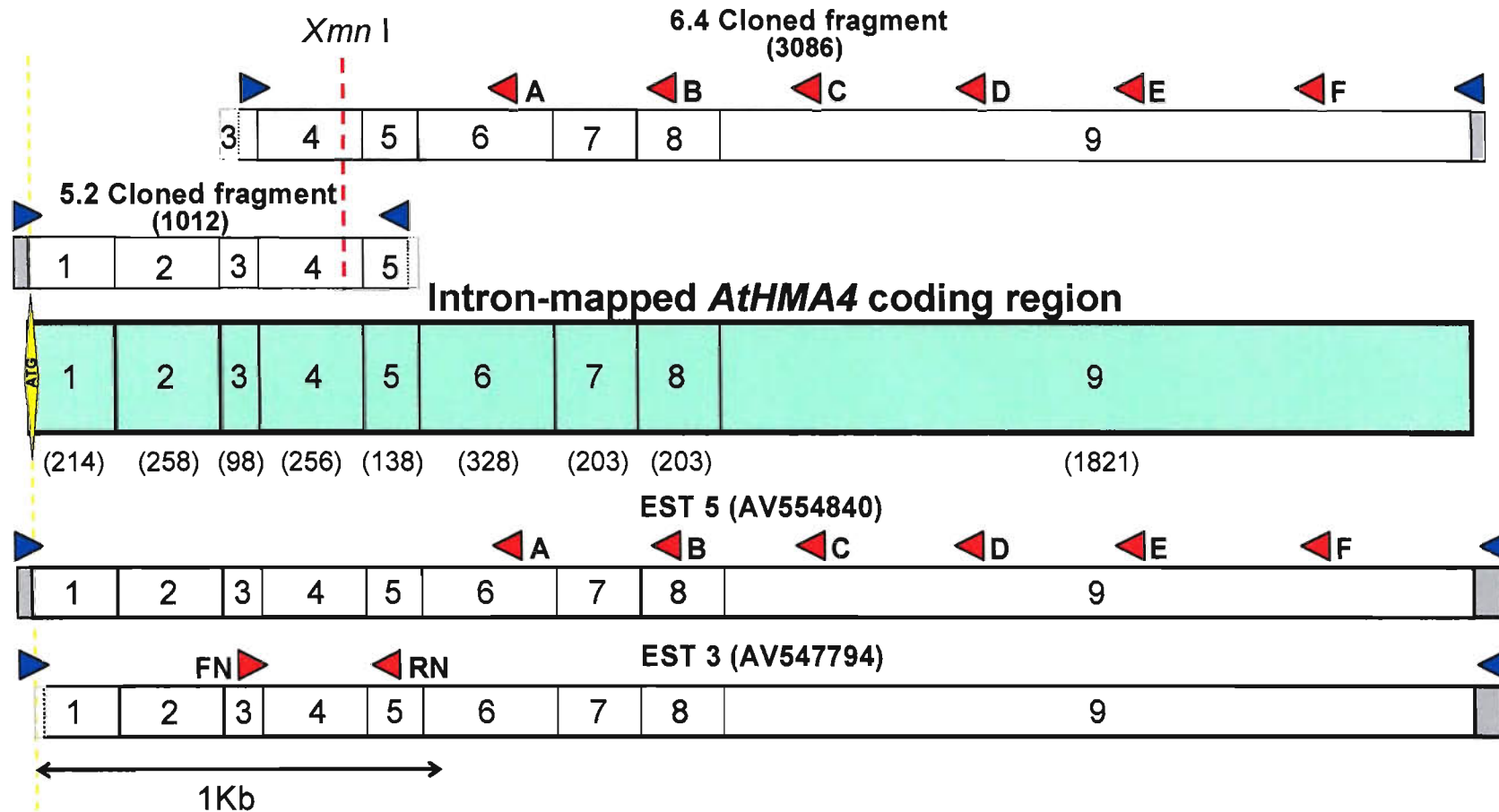


Figure 3.4 Strategy used to sequence *AtHMA4* cDNA (shown in green). Fragments cloned by RT-PCR (6.4 and 5.2) and ESTs 5 and 3 are shown in relation to the intron-mapped coding region of *AtHMA4*. Locations of primers used are indicated with arrows; blue = universal M13 primers, red = gene specific primers (see Table 2.4). Grey (shaded) boxes indicate additional sequence obtained outside of coding region. Numbered boxes (black border) with dashed-line termini indicate an incomplete exon in the respective cDNA fragments. Unshaded grey boxes indicate the extent of the complete exon (for reference purposes) in these regions. Yellow diamond indicates location of start ATG on the *AtHMA4* sequence. Dashed yellow line shows its relative position in the cDNA fragments. Dashed red line shows location of *Xmn I* site (GAANN ∇ NNTTC) used to ligate the two cloned fragments. Figure shown is to scale.

overlapping sequence of both fragments (Figure 3.4). The two digested partial-length cDNAs were ligated and the product was cloned into the *EcoRI* site of pBluescript. Sequencing was carried out on the separately cloned fragments using the dye-labelled terminator method (Materials and Methods section). Universal M13 primers were used to sequence the entire length of fragment 5.2, and termini of fragment 6.4. Gene-specific primers HMA4rev(A-F) were used at internal sites on fragment 6.4 and often produced sequence with over 200 bp of overlap. The ligated product (referred to now as *AtHMA4*_(FL) cDNA) was also sequenced in the overlapping region to check the junction for errors. The region was preserved. The *AtHMA4*_(FL) cDNA has an open reading frame of 3516 bp. This encodes a polypeptide of 1172 amino acids which is identical to that predicted for the coding sequence in the MIPS *A. thaliana* database (At2g19110). The cDNA sequence was deposited in the EMBL/GENBANK/DDBJ databases as AJ297264.

3.2.2 Isolation and sequencing of expression sequence tags

Expression sequence tags (ESTs) corresponding to *Arabidopsis HMA4* cDNA were identified in the Membrane Protein Library database, MIPS (<http://mips.gsf.de/>). PCR results using universal M13 primers on two ESTs, AV547794 (= EST3, a 3' EST) and AV554840 (= EST5, a 5' EST) obtained from the Kazusa DNA Research Institute (Japan), produced product sizes similar to that of the full-length cDNA. Sequencing of EST 3 using universal M13(F-R) primers complimentary to the pGEM border sites, revealed the cDNA was an incomplete copy of the predicted HMA4. Approximately 80 base pairs were found to be missing from the 5' coding region. For EST5, M13 forward and reverse-primed sequencing confirmed the presence of both coding termini (as predicted in the MIPS database). Subsequent sequencing reactions with gene-specific primers HMA4rev (A-F), showed the cDNA to be complete and the data obtained was used to support the results generated from the cloned fragments 5.2 and 6.4 (Figure 3.4).

3.2.3 Analysis of the 5' sequence of *AtHMA4*

Analysis and comparison of the sequence data obtained from cloned fragment 5.2 and EST5 revealed a difference in the 5' border region of the two cDNAs. EST5 displayed an additional 96 bp of 5' sequence. Comparison of EST5 with genomic DNA showed that the additional sequence was interrupted by a 494 bp intron (Figure 3.5). The intron was positioned only 3 bp upstream from the

Sequence	
Shinn et al. cDNA	ATGTTCTAAGACTTCTCTCAACCTTTATCTGATCGCACCAAACCAGTTTTTCGCATCGGCTTCTTCCTTTTGCTACTAGCTCTCCTCTCTTCTCCG Intron
EST5	ATGTTCTAAGACTTCTCTCAACCTTTATCTGATCGCACCAAACCAGTTTTTCGCATCGGCTTCTTCCTTTTGCTACTAGCTCTCCTCTCTTCTCCG Intron
RACE(2) cDNA	ATGTTCTAAGACTTCTCTCAACCTTTATCTGATCGCACCAAACCAGTTTTTCGCATCGGCTTCTTCCTTTTGCTACTAGCTCTCCTCTCTTCTCCG Intron

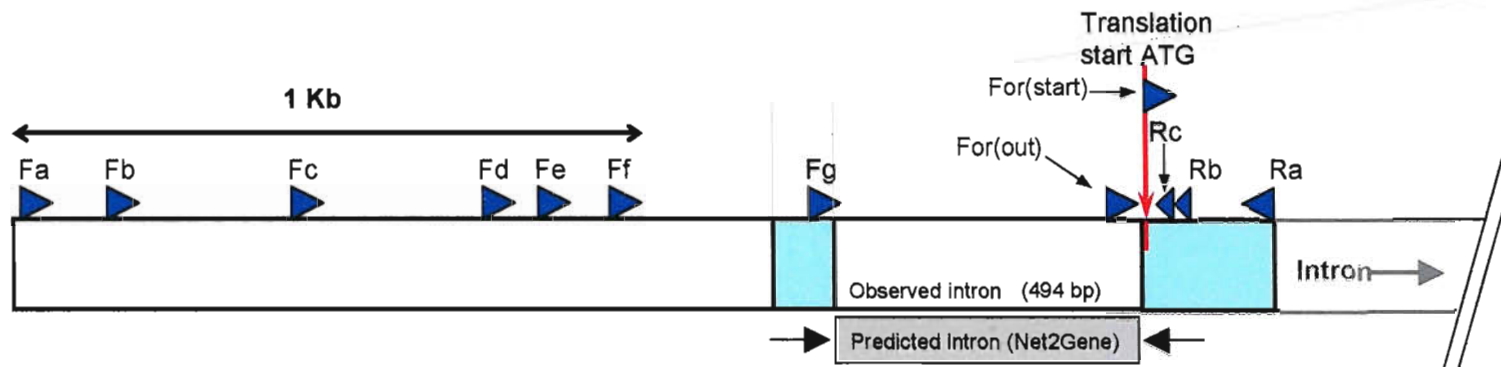


Figure 3.5 Schematic diagram of the *AthMA4* 5' region showing the location of forward (Fa-Fg; ►) and reverse (Ra-Rc; ◄) primers used in RT and RACE-PCR. Blank boxes (□) indicate intron regions and blue boxes (■) exon regions. The sequences shown correspond to the exon discovered in this work which exists 5' of the predicted start ATG. Sequences were obtained from EST5 and the RACE(2) product (this work) and cDNA generated by Shinn et al. (subsequently submitted to EMBL; accession number AY096796).

predicted start ATG of *AtHMA4* and included the region complimentary to primer HMA4-forward(out), used in the initial steps of the RT-PCR cloning strategy (Section 3.2.1). This could explain the failure of the PCR reactions using the HMA4-for(out) and HMA4-rev(out) primers. EST 5 terminated only 12 bp away from another potential ATG start site (Figure 3.5); therefore it was considered that the correct start codon for the gene maybe different from our initial predictions and that predicted from the *Arabidopsis* genome database (<http://mips.gsf.de/>). The potential ramifications of this could be extremely significant, since the protein product may be much larger and/or possess further domains involved in structural organisation (e.g. further TMDs) or enzyme function.

In order to test for the presence of additional coding sequence in the 5' region, PCR was carried out on several different batches of cDNA, prepared from reverse-transcribed RNA extracted from root tissue. Forward and reverse primers were designed against various portions of the sequence (Figure 3.5). Priming sites for forward primers Fa-g (Table 2.4, Figure 3.5) were designed approximately equidistant from one another in a 1.8 kb stretch, 5' of the M.I.P.S predicted start ATG. These were used in conjunction with reverse primers RACE-a (Ra) and RACE-b (Rb), positioned in the first exon of the confirmed coding sequence (Figure 3.5). Repeated PCR reactions carried out with all combinations of primers (forward) a-f and (reverse) RACE-a/b failed to yield any discernable or consistent products using cDNA (Figure 3.6). Products of the predicted size were obtained using these primers on gDNA (Figure 3.6) indicating that primers could bind appropriately. A variety of product sizes of the correct size were obtained with the different primer pairs using gDNA up to approximately 2 Kb, however the largest bands appeared to amplify less efficiently (Figure 3.6). PCR reactions carried out with the forward g primer, complimentary to sequence shown by EST 5 as being an untranslated exon, produced products of good yield, in all samples tested (Figure 3.6, lane Fg). The product size (approximately 250 bp) confirmed the presence of the 494 bp intron.

Experiments were conducted with root cDNA prepared from material treated with a range of Zn concentrations (Figure 3.7). Products of a similar size (approximately 250 bp) were obtained from all material indicating that this particular transcript was not altered in plants exposed to this metal (Figure 3.7).

3.2.4 5' - RACE

The results obtained above (Section 3.2.3) analysing the 5' region support the existence of EST5-like transcripts in *Arabidopsis thaliana*. They do not however,

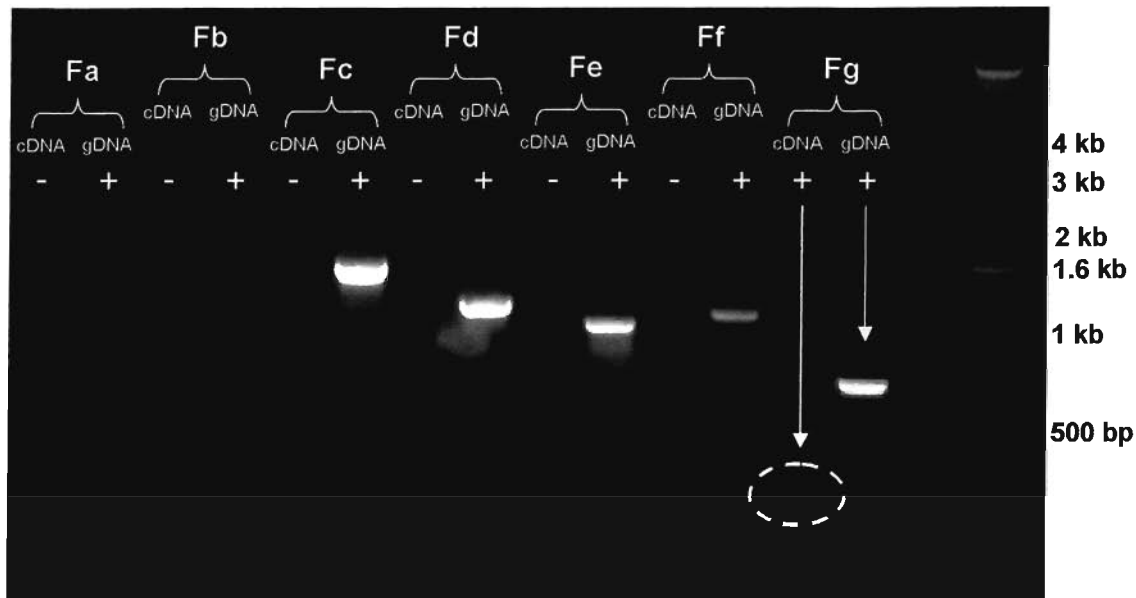


Figure 3.6 1.4 % agarose gel showing products obtained from a PCR reaction carried out using HMA4 RACE-rev A primer and a selection of 5' forward primers on cDNA (prepared from root tissues) and genomic DNA (prepared from entire plants). The gel shown was chosen as a representative example of the 5' PCR results, carried out with combinations of forward primers A-G and reverse primer RACE-rev A. Plus/minus labels are indicative of the overall result. Only products from Fg-primed reactions gave bands of the expected size (cDNA = 252 bp and gDNA = 746 bp). The dashed circle highlights the faint product band obtained from an Fg-primed reaction on cDNA.

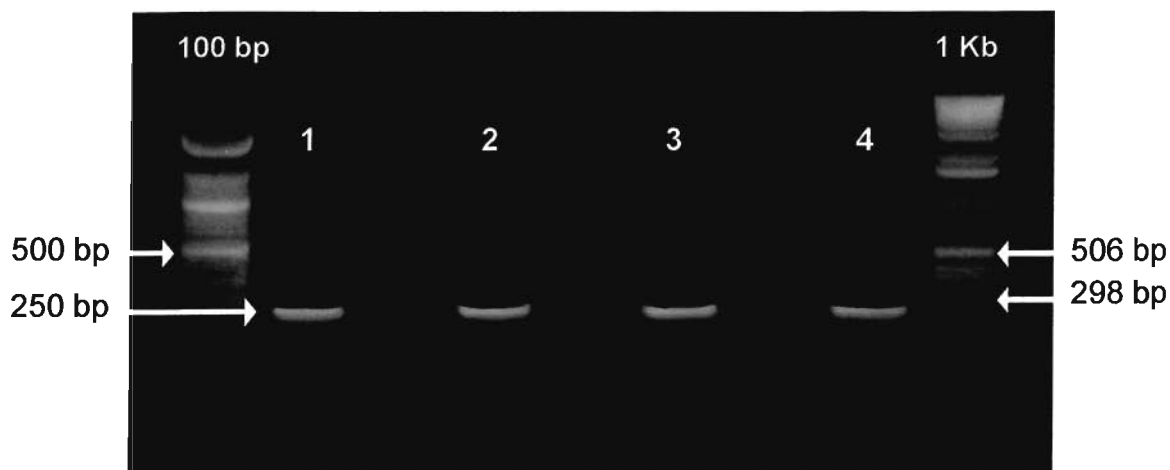


Figure 3.7 Products produced from PCR reactions carried out using the Fg (forward) and RACE a (reverse) primer combination that span the 5' untranslated intron of *AthMA4*. cDNA used in the reactions was obtained from roots of plants subjected to 0.1 mM (1), 0.35 mM (2) and 1.0 mM (3) ZnSO_4 and 1.0 mM K_2SO_4 (4) 30 hour-treatments. 100 bp (far left) and 1 Kb (far right) size markers were run for accurate determination of band sizes.

confirm if the transcripts are the most important in terms of abundance. Whilst numerous failed PCR attempts with primer *HMA4*forward(out) which primes 66 bp upstream from the predicted start codon (Table 2.4) indicate that the existence of shorter *AtHMA4-5'* fragments is unlikely, RACE techniques were employed in order to further characterise the leader sequence of *AtHMA4*. 5' RACE involves the capping of all *AtHMA4* transcripts with a homopolymeric (e.g. poly-C) tail (Frohman et al. 1988). PCR reactions are then carried out with a cap-specific (for) primer and a gene-specific (rev) primer in order to amplify the product(s). The PCR is then repeated again with further specific primers to increase yield. The product(s) may then be used in subsequent steps such as cloning or sequencing reactions.

In this study, the first RACE experiment was conducted on DNAase-treated RNA generated from a range of hydroponically grown root material either untreated or exposed to 1 mM Zn. Metal-treated roots were of interest, since expression analysis had indicated they would be particularly rich in *AtHMA4* transcripts (Chapter 5). To test for genomic contamination, control reactions were performed on both sets of RNA without the reverse transcriptase reaction. A small, smeared product of approximately 100 – 150 kb was amplified in one of the reactions (Figure 3.8a, Lane 4), but this was not indicative of gDNA. A reaction on uncapped cDNA also produced short products (Figure 3.8b Lanes 5 and 6), highlighting that potential mis-priming may occur in the absence of the cap target sequence.

Initial PCR reactions carried out using cap-specific AAP and gene-specific RACE-Rb primers (Table 2.4, Figure 3.5) produced a product from an untreated root sample of approximately 350 bp (Figure 3.8b Lane 10). Re-amplification of this product in a further PCR with primers AAP and RACE-rev c, was carried out in order to increase yield. However the product produced appeared to be much larger, around 500 bp in size (result not shown). After cloning into the pGEM easy-T vector, sequencing, revealed that the majority of the DNA was *AtHMA4*-specific, but was preceded by 104 bp of unrelated sequence (Figure 3.9). Remarkably, this sequence was present downstream of the RACE cap primer, and had been amplified whilst attached to the *AtHMA4* transcript. Database searches failed to suggest the identity or source of this DNA. Of the extra sequence that had been obtained from this RACE experiment, 339 nucleotides were found that were not present in EST 5. These were located entirely in the 'spliced out' region thought to be an intron (Figure 3.10).

For the second RACE experiment new batches of RNA were prepared from the root and leaf material of plants subjected to various Zn-concentration treatment regimes (including controls with no additional Zn). DNAase treated mRNA was

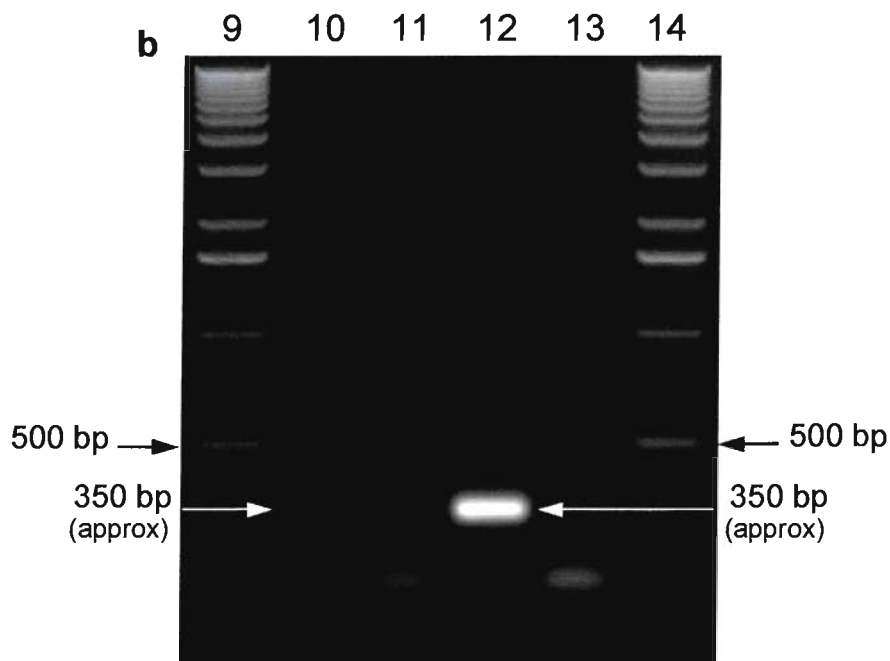
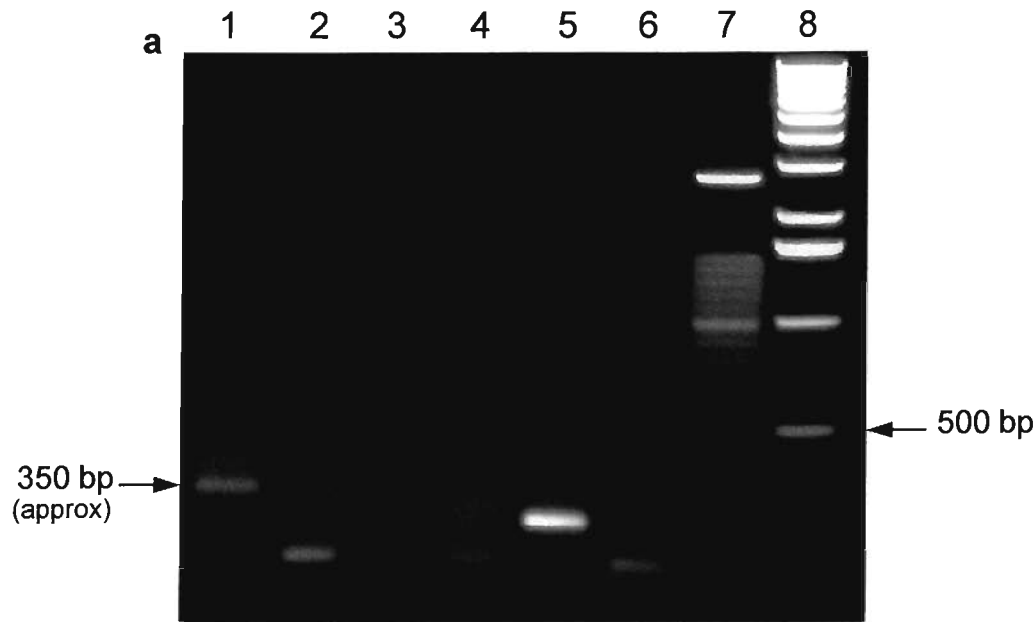


Figure 3.8a,b Products generated from *Arabidopsis* RNA using 5' RACE-PCR. RACE was carried out on RNA extracted from Zn treated (a, lanes 1-2) and untreated roots (b, lanes 10-11), or a mixture of the two (b, lanes 12-13). Products were amplified in PCR using the Cap-specific-Rb (a, lanes 1,3,5 and b, 10,12) and Fg-Rb primer pairs (a, lanes 2,4,6 and b, 11,13). Control-PCRs were performed on mixed RNA samples that were not subjected to a reverse transcription reaction (a, lanes 3-4) or cDNA that had not been capped in the RACE procedure (a, lanes 5-6). Lanes 8, 9 and 14 show products of a 1Kb ladder, and lane 7, products from a 100 bp ladder.

Genomic **TCTTACTTATCTGCCTATATAAGCAACAACCTCCCAFTTCTTGACTTCTCTATGTTCCCTAA**
 CompRACE -----

Genomic **CACTTCTCTCAACCTTATCTGATCGCACCAAAACCAGTTTTTTCGCATCGGCTTCTTCCT**
 CompRACE -----

Genomic **TTTGCTACTAGCTCTCCTCTCTTCTCCGGTATTTTTGTCTCTCTTCTTAATTCACACAGA**
 CompRACE -----

Genomic **TTTCATGATAAGTGATGATCTATAACAAGACGCTAACTCTTCTCTTGCAATTCCTGTT**
 CompRACE -----CCTGGACCAC--CCAAGCGCTN-TCGNGTGGCCGCCCTACCGA

Genomic **TTCAATTTCTTGTTACGCCAAATTTATCCCTTCAAATCATTTTTTTATGGTATAGAAAT**
 CompRACE **CG-GCGCACCCGCNCCGTAGGAGCCCTCGGGCGGGGGGTCCGCCTCACACCC---ACC**

Genomic **CCAAATAATAAGTAAAAGCTGATTTCGTCTTCTTCCACTTAACACAAGTAAGCAATGAGAG**
 CompRACE **GGAAATAATAAGTAAAAGCTGATTTCGTCTTCTTCCACTTAACACAAGTAAGCAATGAGAG**

Genomic **GGTGAAGATTTTTCTTTAGGAAAAAAAAGAGAGTGAAGATATTTTTTTGGCTTGATCTCAA**
 CompRACE **GGTGAAGATTTTTCTTTAGGAAAAAAAAGAGAGTGAAGATATTTTTTTGGCTTGATCTCAA**

Genomic **CATTATTTTTCTTAAAAGTAAACATTTTTCAATAAGAAAATACAAGACCCATACCGAAAG**
 CompRACE **CATTATTTTTCTTAAAAGTAAACATTTTTCAATAAGAAAATACAAGACCCATACCGAAAG**

Genomic **TTTCTTCTTAAAAAAAAAAAAAAAAAGGTTTTGAACTGTTTCATGATAATGATAACTAAAAAA**
 CompRACE **TCTCTTCTTAAAAAAAAAAAAAAAAAGGTTTTGAACTGTTTCATGATAATGATAACTAAAAAA**

Genomic **GTTTTTGCTTCTTGTTTTTTTTCTCCTCCGCAAAACAGTCTTAAAGTATAACCAAAAAGCC**
 CompRACE **GTTTTTGCTTCTTGTTTTTTTTCTCCTCCGCAAAACAGTCTTAAAGTATAACCAAAAAGCC**

Genomic **TATAAATCAATATAATTTGTTGTTTTGATTTACGTTTTACAGAAAATGGCGTTTACAAAAC**
 CompRACE **TATAAATCAATATAATTTGTTGTTTTGATTTACGTTTTACAGAAAATGGCGTTTACAAAAC**

Genomic **AAAGAAGAAGAGAAAAAGAAAGTGAAGAAGTTGCAAAAAGATTACTTCGATGTTCTCGGA**
 CompRACE **AAAGAAGAAGAGAAAAAGAAAGTGAAGAAGTTGCAAAAAGAT-----**

Genomic **ATCTGTTGTACATCGGAAGTTCCTATAATCGAGAATATTCTCAAGTCACTTGACGGCGTT**
 CompRACE -----

Genomic **AAAGAATATTCGTCATCGTTCCCTCGAGAACCCTGATTGTTGTTTCAGACAGTCTCCTC**
 CompRACE -----

Figure 3.9 Sequence alignment of the RACE 1 product with genomic sequence from the 5' region of *AtHMA4*. The RACE sequence shown (CompRACE) is from one sequencing reaction. Also shown is the unknown sequence stretch, attached upfront of the RACE sequence.

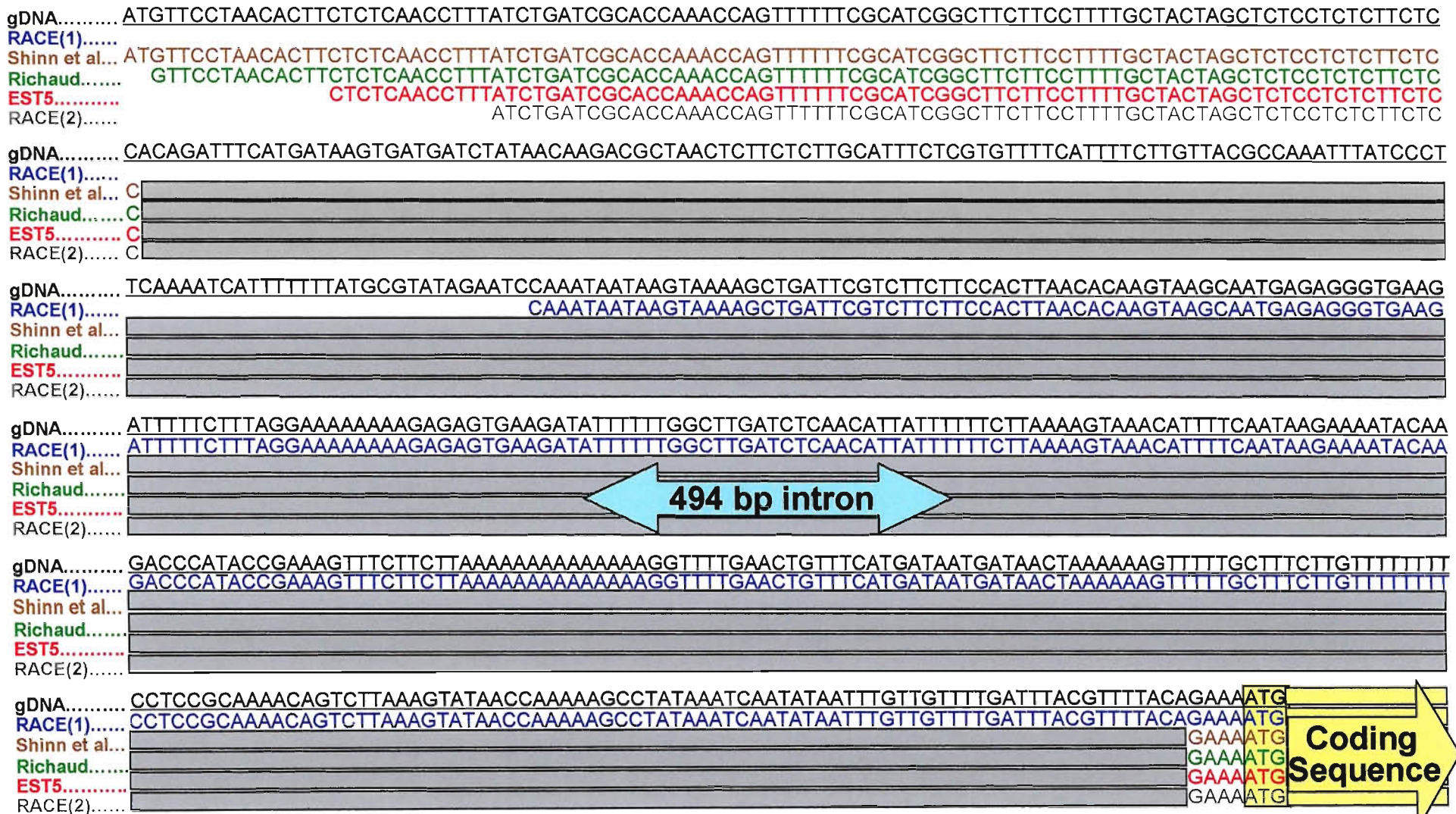


Figure 3.10 Alignment of AtHMA4 5' sequences. Sequences obtained by RACE PCR (RACE 1 and 2) were aligned manually with other sequence data from EST5 (MIPs; AV554840; <http://mips.gsf.de/>), Richaud (EMBL; AF412407; <http://srs.ebi.ac.uk/>) and Shinn et al. (EMBL; AY096796; <http://srs.ebi.ac.uk/>). Intron regions are shown with grey bars, and the M.I.Ps (<http://mips.gsf.de/>) predicted coding region in yellow.

subjected to the same protocol as before, although a slight modification to one of the latter stages was made. Primer AUAP was substituted for the AAP-RACE-rev C PCR used previously. This was carried out in an attempt to increase the specificity of the PCR and to prevent mis-priming, which may occur when using oligomers rich in deoxyinosine (Martin et al. 1985). Negative-control reactions were conducted as before and indeed no products were produced for any sample (Figure 3.11).

When the RACE samples were analysed by agarose gel electrophoresis each sample appeared to give exactly the same size product(s) of approximately 250 bp (Figure 3.11). This was regardless of the Superscript enzyme used, treatment or tissue type. A repeat of the second PCR stage with a different DNA polymerase enzyme (Takara exTaq) also produced the same but slightly weaker bands (not shown). Product yield was generally low for all of the samples, even after several PCR amplifications. This prevented the sequencing of the fragments directly and so a cloning strategy was adopted. Product yield from leaf tissues was so low that the samples were abandoned. The fragments from root were ligated into the pGEM Easy-T vector (Figure 3.12) and sequenced with the universal M13 (forward + reverse) primers. Only samples derived from root material grown in 1.0 mM ZnSO₄, yielded any significant stretches of sequence related to *AtHMA4*. In this experiment, 64 bp of sequence upstream of the predicted ATG was obtained. This corresponded to sequence in EST5 and is consistent with a 494 bp intron appearing to interrupt the sequence 3 bp before the predicted start ATG site. It did not however share the same 5' terminus; the RACE (2) transcript was initiated some 12 bp downstream of EST5 (Figures 3.5 and 3.10).

3.2.4.1 Comparison of the RACE product with EST5 and sequences subsequently deposited in the EMBL database

Since starting the RACE procedure, two additional mRNA sequences for *AtHMA4* have been submitted into the EMBL database (<http://srs.ebi.ac.uk/>). These are the sequence of Richaud (referred to now as Richaud *AtHMA4* cDNA) from the Wassilewskija ecotype (EMBL; AF412407; <http://srs.ebi.ac.uk/>) and that of Shinn *et al.* (referred to now as Shinn *et al.* *AtHMA4* cDNA) from the Columbia ecotype (EMBL; AY096796; <http://srs.ebi.ac.uk/>). An alignment of the 5' regions carried out using the ClustalW computer programme (Figure 3.10) showed that the Richaud and Shinn *et al.* *AtHMA4* cDNA sequences are slightly longer than both the EST5 and the RACE (2) sequences. They possess around 98 nucleotides, 5' of the predicted translation start site, compared to 76 and 64 bp for the EST5 and

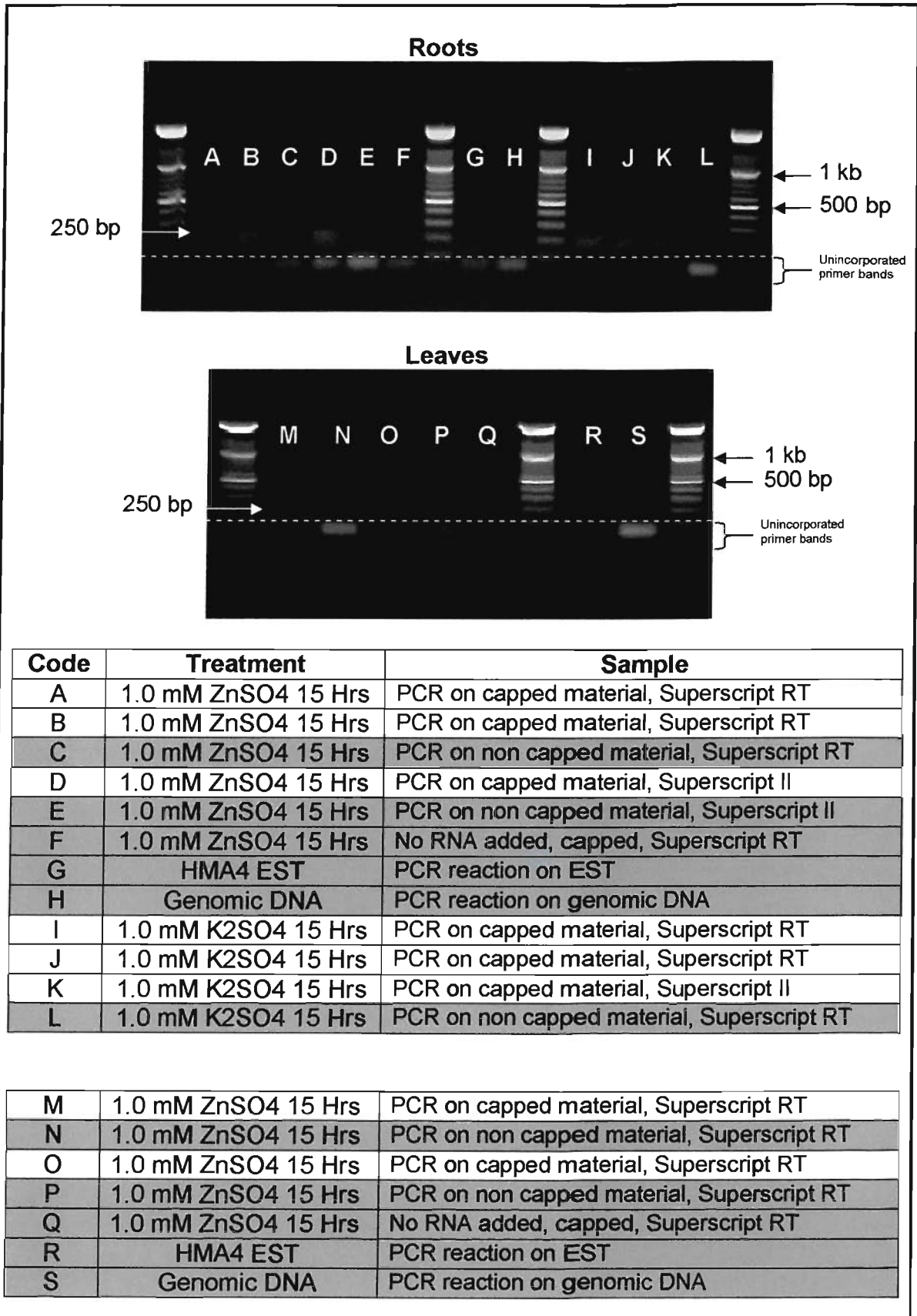


Figure 3.11 Products generated from 5' RACE using primers specific to the *AtHMA4* gene. Samples highlighted in grey in the table are negative controls.

Key	Sample	Growth on LB-amp.
B	1.0 mM ZnSO ₄ 15 Hrs PCR on capped material, Superscript RT (Ex-taq amplification)	8 colonies
B	1.0 mM ZnSO ₄ 15 Hrs PCR on capped material, Superscript RT (r-taq amplification)	3 colonies
I	1.0 mM K ₂ SO ₄ 15 Hrs PCR on capped material, Superscript RT (Ex-taq amplification)	None
K	1.0 mM K ₂ SO ₄ 15 Hrs PCR on capped material, Superscript II (Ex-taq amplification)	1 colony
K	1.0 mM K ₂ SO ₄ 15 Hrs PCR on capped material, Superscript II (r-taq amplification)	None
	negative control (water in ligation)	None

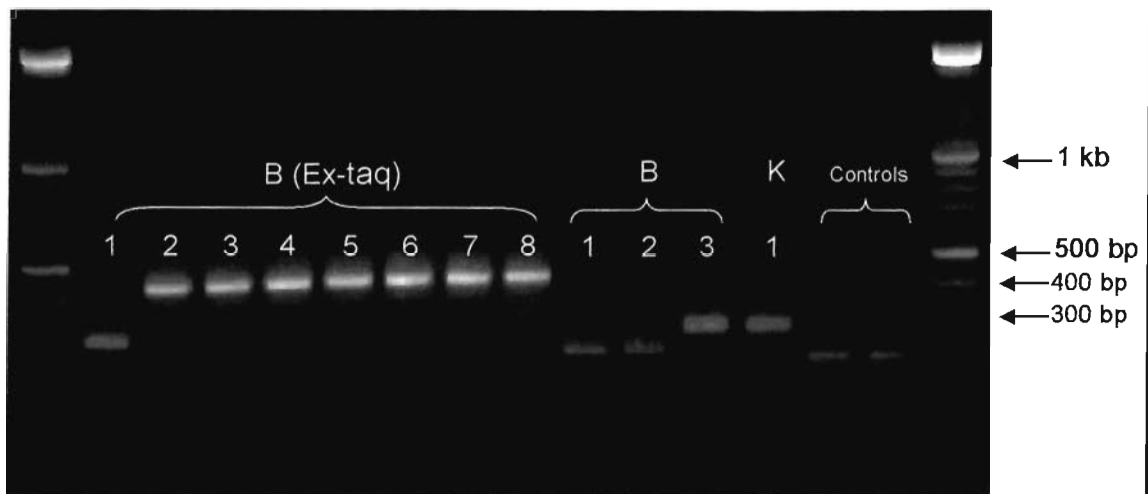


Figure 3.12 RACE products successfully ligated into the pGEM easy-T vector and subsequently cloned into *E.coli* DH α cells. Inserts were checked by performing PCR with universal M13 forward + reverse primers and selected samples sequenced using the chain termination method. Grey rows in the table indicate samples where no colonies were obtained. Negative controls are of ligations carried out without a DNA template. The key refers to the sample code given in Figure 3.9

RACE(2) sequence. All sequences except the RACE (1) and cloned fragment 5.2 sequences share a common 494 bp intron. The RACE (1) product possesses 342 bp of sequence 5' of the predicted ATG. This suggests that the first transcribed exon may be extended under certain conditions.

The presence of an additional ATG within the sequence from Shinn *et al.* is unique. This could suggest that the extra 5' sequence has the potential to code for a new or additional translation start site for *AtHMA4*. However if the sequence is analysed backward (3' to 5') from either the predicted translation start site or from the 494 bp intron start site, this particular ATG codon is not in-frame. Additionally, no sequence 5' to the Shinn *et al.* ATG has been produced. This would be an unexpected occurrence near to a translation start site, where the upstream 25 - 30 nucleotides (approximately) commonly form a transcribed promoter region in multicellular eukaryotes (Beebee and Burke 1988). All nucleotides upstream of the MIPS predicted ATG are therefore likely to form part of the promoter region and the sequence analysis described below support this.

3.2.4.2 Analysis of potential motifs in the RACE product

Analysis of the *AtHMA4* RACE and EMBL mRNA database sequences Richaud *AtHMA4* cDNA and Shinn *et al.* *AtHMA4* cDNA were carried out using the Signal Scan motif search (<http://bimas.dcrf.nih.gov/molbio/signal/>) and the NetGene2 intron-exon predictor programme (<http://www.cbs.dtu.dk/services/NetGene2/>). TATA boxes, indicative of potential transcription start sites, were found at 3 points in a 1 kb segment upstream of the predicted translation start site ATG(GCGTT) (Figure 3.13). Two of these fell within the RACE(1) product, being 245 and 39 bp upstream of the predicted translation start site and 121 and 299 downstream from the RACE(1) 5' terminus. Using data from the BAC clone (AC002392) only one other potential TATA box was identified (Figure 3.13, TATA box 1). This was positioned 29, 43, 55, and 279 bp upstream from the start of the Shinn *et al.* *HMA4* cDNA, EST5, RACE(2) and RACE(1) transcription sequences respectively. Of those sites identified, this was considered as being the most likely transcription start for the RACE(2), EST and Shinn *et al.*/Richaud *HMA4* cDNA products. Further evidence to support such a role can be derived from the presence of a complementary or 'pairing' CAAT motif, further upstream of the TATA box site (Beebee and Burke 1988). Four potential candidates were found by manual inspection of the sequence. Of these, the characteristics of the latter promoter region (Figure 3.13, CAAT box 1 TATA Box 1) appears to be in

Sequence of putative exon	
Shinn et al. cDNA	ATGTTCTAACACTTCTCTCAACCTTTATCTGATCGCACCAACCAGTTTTTTCGCATCGGCTTCTTCCTTTTGCTACTAGCTCTCCTCTCTTCTCCG Intron
EST5	ATGTTCTAACACTTCTCTCAACCTTTATCTGATCGCACCAACCAGTTTTTTCGCATCGGCTTCTTCCTTTTGCTACTAGCTCTCCTCTCTTCTCCG Intron
RACE(2) cDNA	ATGTTCTAACACTTCTCTCAACCTTATCTGATCGCACCAACCAGTTTTTTCGCATCGGCTTCTTCCTTTTGCTACTAGCTCTCCTCTCTTCTCCG Intron

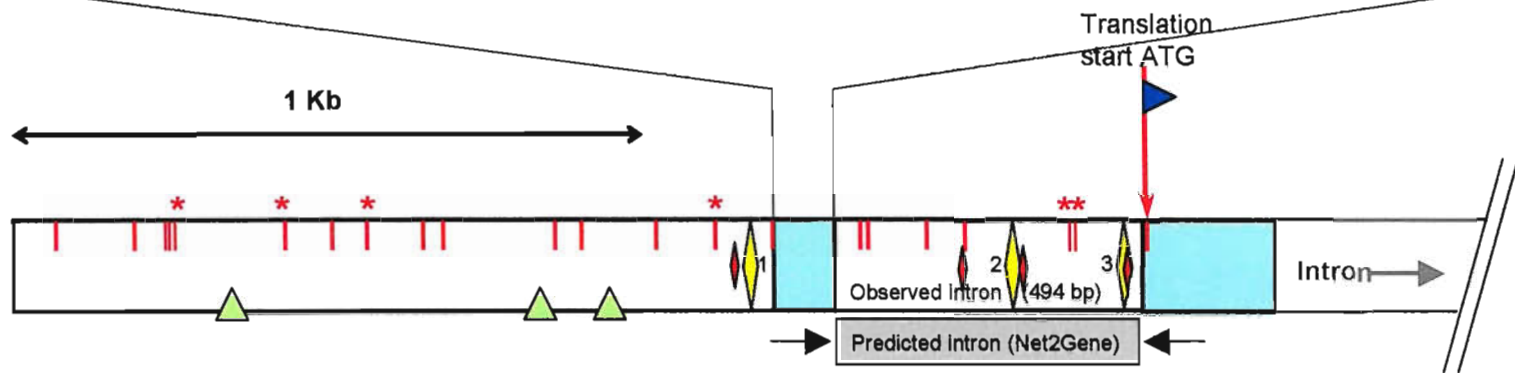


Figure 3.13 Schematic diagram of the *AtHMA4* 5' region showing the location of various putative motifs. Blank boxes (□) indicate intron regions and blue boxes (■) exon regions. Potential TATA/CAAT boxes indicated by diamonds (◇ and ◆ respectively); putative translation start sites by bars (|) and additional asterisks (*) for those in frame with the predicted ATG; poly C regions by triangles (△). The sequences shown correspond to the exon discovered in this work which exists 5' of the M.I.Ps (<http://mips.gsf.de/>) predicted start ATG. Sequences were obtained from EST5 and the RACE(2) product (this work) and Shinn et al. *AtHMA4* cDNA.

keeping with those most commonly found in higher eukaryotes (Beebee and Burke 1988). TATA sites 2 and 3 are located in the putative intron displayed by the RACE(2), EST5 and EMBL sequences. They exist in the spliced-out sequence which remains intact in the RACE(1) product. Analysis of this region, using the NetGene2 programme agreed that the presence of an intron in the putative 5' untranslated position (shown in Figure 3.13) was highly probable (data not shown). Tests on the rest of the gene showed that the programme could correctly predict the size and location of all but the most 3' intron across the *HMA4* sequence. Finally, whilst the Signal-Scan programme (<http://bimas.dcrf.nih.gov/molbio/signal/>) only identified 3 potential TATA boxes in the same locality as these upfront sequences, it is interesting to note that the RACE(1) 5' terminus was initiated along the stretch of sequence CAAATAATAA, which was not considered to be a potential transcription start site by this programme.

Whilst searching for TATA sites in the upfront sequence, efforts were also made to locate potential CpG islands. These GC-rich regions often replace TATA boxes (in similar locations) in constitutively expressed or 'housekeeping' genes. They are more common in animals, but have been shown to occur in other eukaryotes such as plants (Berg et al. 2003). Use of the CPGPLOT computer programme (<http://bioweb.pasteur.fr/seqanal/interfaces/cpgplot.html>) failed to highlight any such regions in *AtHMA4*. A comparative analysis carried out on *AtHMA1* and the constitutively expressed *Actin 2* gene (McDowell et al. 1996) also failed to identify such regions. The stringency of this rule in plants is not known, and indeed the characteristics of GC rich regions in *Arabidopsis* have not been fully investigated (Baxter et al. 2003).

3.2.5 Analysis of the confirmed *AtHMA4* coding sequence

The cloning and sequencing of *AtHMA4* has revealed that this gene has by far the largest coding sequence of the *Arabidopsis* HMAs (Table 3.1). Its 3519 bp of coding DNA is interspersed amongst 5295 bp of genomic DNA in an arrangement of 9 exons. This number appears to be fairly common amongst the family, and is mid-way between the extremes of *AtHMA5* (unconfirmed sequence) and *PAA1/AtHMA6*, which display six and seventeen exons respectively (Baxter et al. 2003; <http://plantst.sdsc.edu/>). Of those genes possessing 9 exons, *AtHMA2* shows the greatest similarity to *AtHMA4* in its structural arrangement (Figure 3.14). Both have two relatively isolated exons in the 5' region (A), a cluster of six near the centre of the gene (B) and a large single unit at the 3' end (C). This large unit accommodates

Table 3.1 Gene characteristics of *Arabidopsis* HMAs (additional data from; Baxter et al. 2003; <http://mips.gsf.de/proj/thal/db/index.html>; <http://plantst.sdsc.edu/>)

Gene Name	cDNA(bp)	gDNA(bp)	Exons	Amino acids	kDa.
AtHMA1	2460	6366	13	819	88.19
AtHMA2	2856	6325	9	951	101.98
AtHMA3	2283	5102	9	760	81.94
AtHMA4	3519	8778	9	1172	127.20
AtHMA5	2988	5455	6	995	108.32
AtHMA6(PAA1)	2850	8857	17	949	100.00
AtHMA7(RAN1)	3006	5972	9	1001	107.36
AtHMA8	2571	6593	17	856	90.82

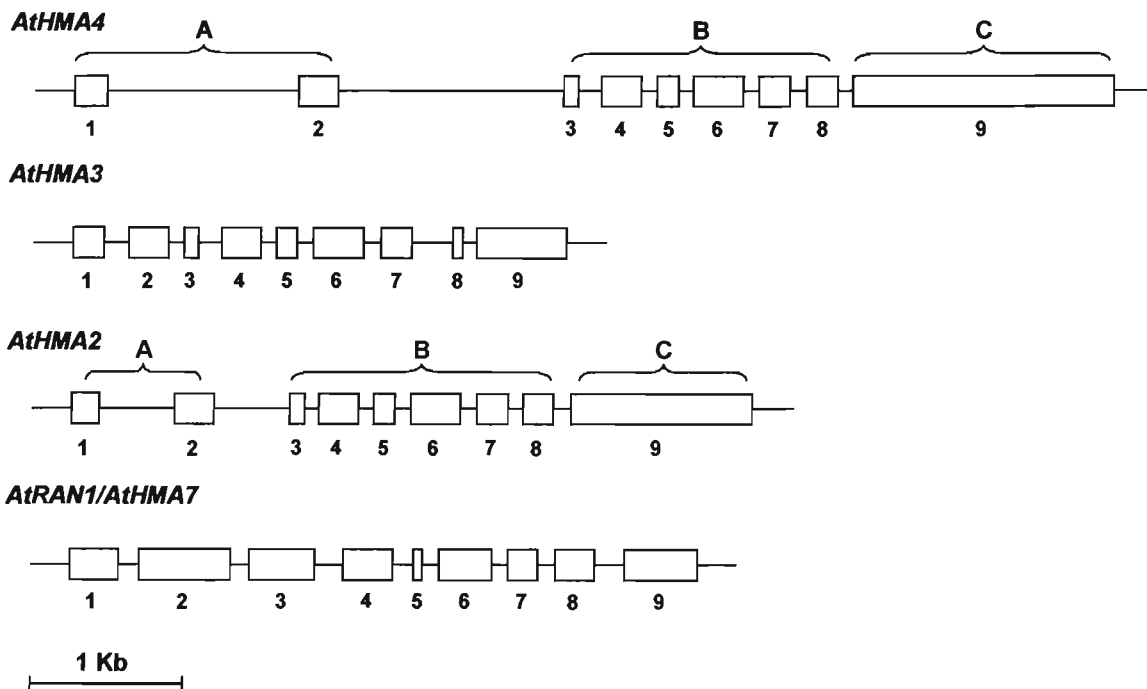


Figure 3.14 Schematic representation of gene structure in a selected group of *Arabidopsis* CPx-ATPases. Boxes and lines indicate the relative size and location of exons and introns respectively. A,B,and C refer to notes in text.

sequence encoding the large C-terminal tail seen in the respective proteins (Axelsen and Palmgren 2001).

3.2.6 Analysis of the AtHMA4 predicted protein sequence

In a standard protein-protein BLAST search (<http://www.ncbi.nlm.nih.gov/BLAST/>), proteins showing the highest score were putative heavy-metal transporting P-type ATPases from *Arabidopsis* and also various other organisms including eubacteria (*Staphylococcus*, *Streptococcus*, *Bacillus*, *Pseudomonas*, *Legionella*, and *Nostoc*) and archaea (*Halobacterium* and *Pyrococcus*). The percentage identities to AtHMA4 at the amino acid level are shown for the other *Arabidopsis* HMAs (Table 3.2). *AtHMA3* has recently been cloned and the coding region conforms to that predicted by MIPs (Gravot et al. 2004). *AtHMA2*, another *Arabidopsis* HMA found in the *Arabidopsis* genome shows highest identity (76%) whereas the others showed a relatively low percentage identity (29-36%) at the amino acid level (Table 3.2). A number of transmembrane (TM) prediction programs (Materials and Methods) based on different approaches were compared. To assess their accuracy, their ability to predict the number and location of TM domains for two previously characterised P-type ATPases, a proton pumping P-type ATPase from *Neurospora crassa* (Scarborough 2000) and a calcium transporting P-type ATPase, SERCA1, from rabbit (Toyoshima et al. 2000) were tested. There is good evidence from the crystal structures that these two P-type ATPases contain 10 TM domains and there is also evidence indicating the locations of several of the TM domains in SERCA1. None of the TM prediction programmes tested were ideal in accurately determining the number and location of the TM domains in these two proteins. Therefore, combined TM predictions from the various programmes together with the basic Kyte-Doolittle hydrophathy prediction, and information from the location of conserved sequences in the crystal structure of SERCA1 were used to try to predict the locations of TM domains in the HMAs. From this they are predicted to contain eight TM domains with a small cytoplasmic loop between TM domain 4 and 5 and a large cytoplasmic loop between TM domains 6 and 7. This differs from the ten TM domains proposed for most other P-type ATPases.

Plotting the location of the putative TM domains on the hydrophathy profiles indicates the presence of marked differences at the N- and C-termini of the HMA proteins (Figure 3.15). Comparison of AtHMA4 with a selection of other *Arabidopsis* HMAs shows that it contains a large cytoplasmic domain at the C-terminus after TM

Table 3.2 Similarities between HMA sequences of *Arabidopsis* and other organisms. The percentage similarities (top) and percentage identities (bottom) at the amino acid level were calculated using the EMBOSS programme 'Matcher' (for local alignment). Default settings for gap penalty (14) and gap extension penalty (4) were used. Protein entries were standardised; sequences shown on axis x, being compared to those on y. Accession numbers for *A. thaliana*: HMA1 = At4g37270, HMA2 = At4g30110, HMA3 = At4g30120, HMA4 = At2g19110, HMA5 = At1g63440, PAA1/HMA6 = At4g33520, RAN1/HMA7 = At5g44790, HMA8 = At5g21930, *Staphylococcus aureus* CadA (SaCadA) = P20021 *Helicobacter pylori* CadA = Q59465, *Homo sapiens* ATP7A (Menkes protein) = Q04656, *Saccharomyces cerevisiae* CCC2 = P38995, *Eschericia coli* ZntA = P37617.

		X																					
		Percentage Similarity																					
HMA1	HMA1																						
HMA2	48.9	HMA2																					
HMA3	51.3	79.3	HMA3																				
HMA4	48.4	86.3	79.7	HMA4																			
HMA5	46.7	54.7	50.1	53.6	HMA5																		
PAA1	49.8	51	48.2	50.1	56.4	PAA1																	
RAN1	45.7	53.6	50.4	52.6	67.4	55.4	RAN1																
HMA8	47.2	48.9	54	50.1	57.4	62.1	54.7	HMA8															
CadA	45.6	56.1	52.6	56	52.6	50.3	51.2	49.5	CadA														
SaCadA	51.6	58	54.8	58.5	58.2	53.3	56	53.4	57.6	SaCadA													
ZntA	49.1	50.7	49.2	50.9	55.3	54.4	52	53.1	54.6	59.1	ZntA												
ATP7A	47.3	48.2	51.5	50	58.1	52	57.4	49	50.7	52.7	50.5	ATP7A											
CCC2	44.9	52.2	49.5	50.2	56.4	56.7	55.9	53.5	51.2	53.4	50.3	54											
CCC2	27.5	29.4	29.3	29.4	35.8	37.3	36.8	33.6	28	32.1	30.9	34											
ATP7A	28.1	30.6	34.4	33.7	40	34	40	30.3	27.6	32.3	31.4	ATP7A											
ZntA	28.9	32.1	29.7	32.1	34	34.5	32.3	33.6	32.7	36.4	ZntA												
SaCadA	30.3	36.3	34.2	36.6	35.6	31.7	34	32.3	32.1	SaCadA													
CadA	27	31.2	30.3	29.1	28.2	28	28	28.2	CadA														
HMA8	29.9	31.5	33.7	32.4	36.9	43.9	36	HMA8															
RAN1	27.4	32.6	33.7	32	48.8	38.8	RAN1																
PAA1	30.2	30.8	28.6	30.6	39.1	PAA1																	
HMA5	27.2	33.3	30.3	32.4	HMA5																		
HMA4	29.6	75.6	65.7	HMA4																			
HMA3	34.2	66.5	HMA3																				
HMA2	29.5	HMA2																					
HMA1	HMA1																						
		Percentage Identity																					
		Y																					

Figure 3.15 Hydropathy plot of AtHMA4, AtHMA1, AtRAN1/AtHMA7 and AtPAA1/AtHMA6 showing approximate positions of transmembrane domains and important functional motifs. Plots were carried out using the Kyte-Doolittle algorithm (EMBOSS computer software) with a window of 9 amino acids. Positions of transmembrane domains were calculated by individual alignments with (EMBL – ClustalW; <http://www.ebi.ac.uk/clustalw/>) *H. pylori* CadA (Melchers et al. 1996) from *Helicobacter pylori*. Each plot has been aligned relative to the DKTG phosphorylation domain shown by the light green box, centre. Motifs common to all P-type ATPases are shown with blue labels and those unique to subgroup IB in orange. Putative heavy-metal binding motifs (HHH = histidine stretch, CxxC = Cysteine) are shown with red boxes and labels.

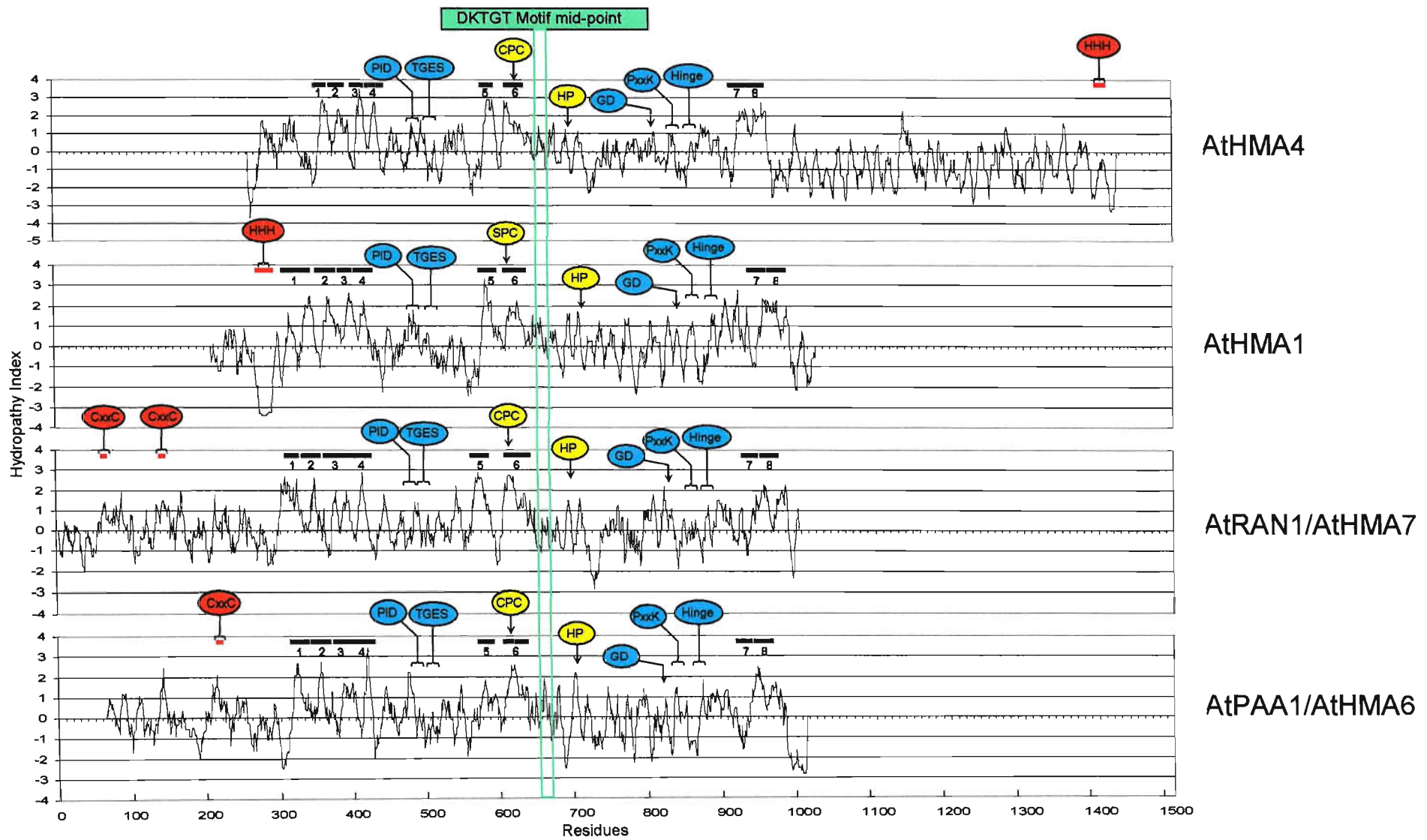


Figure 3.15 Hydropathy plot of AtHMA4, AtHMA1, RAN1 and PAA1 from *Arabidopsis* (see opposite page for legend)

domain 8. This is in stark contrast to AtPAA1 and AtRAN1, which both contain an extended N-terminus before the first TM domain. AtHMA1, a related transporter studied in the following chapters, seems to be mid-way between the two extremes. Whilst it has a longer N-terminal region, neither termini are particularly extensive (Figure 3.14). Analysis of the topology of homologues of the HMA Cad A from *Helicobacter pylori* (Melchers et al. 1996, 1999) and *S. aureus* (Tsai et al. 2002) using PhoA and LacZ fusion proteins also predicted eight transmembrane domains. Alignment of the Cad A sequence with that of a selection of *Arabidopsis* HMAs indicated that the TM domains were in comparable locations (Figure 3.15).

3.2.6.1 Sequence alignments and conserved domains

AtHMA4 contains the conserved motifs found in all P-type ATPases (Solioz and Vulpe 1996) and also motifs that are characteristic of heavy-metal ATPases (Figure 3.16). The latter includes a CPC motif in the predicted sixth TM domain (C₃₅₇PC) and an HP locus in the predicted large cytoplasmic loop (H₄₄₁P). Both the pfam (www.sanger.ac.uk/Software/Pfam/) and PROSITE (<http://ca.expasy.org/prosite/>) databases recognise a “heavy-metal-associated domain” in the N terminus of AtHMA4. However, in AtHMA4 the putative Cu-binding motif GMxCxxC is not present and its position in this domain is represented by the sequence GITCTSE. This could indicate that the Cu-binding domain is degenerate and non-functional in AtHMA4. As indicated in Figure 3.16, AtHMA4 contains a long stretch of histidines close to the C-terminus that may be involved in metal binding; it also contains multiple cysteine pairs, serine pairs and glutamate pairs distributed throughout the putative long cytoplasmic domain at the C-terminus. Multiple serine pairs are also observed in the putative N-terminal cytoplasmic domains of AtPAA1 and AtRAN1. They are not present to any real extent in the termini of AtHMA1, although there are several SxS motifs and a block of four serines in the short C terminus.

3.2.6.2 Phylogenetic analysis

A phylogenetic tree was constructed from a Clustal W alignment of 111 HMAs (Figure 3.17). This analysis includes far more representatives of this family than previously reported (Rensing et al. 1999) and is up-to-date for the 8 heavy metal ATPases that are now predicted to occur in *Arabidopsis* (Baxter et al. 2003). This shows two major clusters. The first group includes pumps for which there is

PAA1	MESTLSAFSTVKATAMARSSGGPSLPLLTISKALNRHFTGARHLHPLLARCSPSVRRLG	60
AthMA8	MASNLLRFP-----LPPSSLHIR---	19
AthMA5	MAT--KLLSLTCIRKERFSEERYPLVRKHLTRSROGGGSSSETAAFEIDDPIS---RAVF	55
RAN1	MAPSRDLQLTPVTGGSSQISDMEEVGLLDSYHNEANADDILTKIEGRDVSGLRKIQV	60
AthMA2	MAS-----KKMTKS-----	9
AthMA4	MALQNKKEEKKKVKLQKS-----	19
AthMA3	MAEG---EESKKMNLQTS-----	15
AthMA1	MEPATLTRSSSLTRFPYRRG-----	20
	*	
PAA1	GFHGSRFTSSNSALRSLGAAVLPVIRHRECLSSSSPSFRSISSSGGSGFGGNGGGSGGG	120
AthMA8	-----PISNSVEIST-----QSFESTESSIESVKSI TS-----	47
AthMA5	QVLGNTCSACAGSVEKAIKRLPGIHDVVIDALNNRAQILFPNSVDVET-----	104
RAN1	GVTGNTCAAACSNVEAALMNVNMGVFKASVALLQNRADVVDPNLVKEED-----	109
AthMA2	-----	
AthMA4	-----	
AthMA3	-----	
AthMA1	-----	
PAA1	GGGSGSESGDKSKLGANASDGVSPSSDIIILDVGGNTCCGGCSASVKKIIESQPOVASAS	180
AthMA8	-----DTP-----ILLDVSGMCCGGQVARVKSVLMSDDRVASAV	81
AthMA5	---IRETIEDAGFEASLIENEANERSRQVCRIRINGNTCTCSSTIERVLSQVNGVQRAH	161
RAN1	---IKEAIEDAGFEAEIILAEEQTQAT-LVGFQFTIGGNTCAAQVNSVEGILRDLPQVKRAV	165
AthMA2	-----YFDVLGICCTSEVPLIENIINSMDGVKKEYS	39
AthMA4	-----YFDVLGICCTSEVPIIENIILKSLDGVKEYS	49
AthMA3	-----YFDVVGICCSSESVIVGNVIRQVDGVKKEYS	45
AthMA1	-----LSTLRLARVMSFSLPPKTLIRQKPLRISAS	51
	*	
PAA1	VNLITETAIVWPVPEAKSVPDWQKSLGETLANHLTNCGFQSTPRDL--VTFNFFKVFET	237
AthMA8	VNMLTETAIVKFKPEVEVTADT---AESLAKRLTESGFQAKRRVSGMGVAENVKWKEM	137
AthMA5	VALAIEEAEIHYDPRLLS-----YDRLLLEEIENAGFEAVLISTGEDVSKIDLKIDGE	213
RAN1	VALSTSLGEVEIDFNVIN-----KDDIVNAIEDAGFEGLVQS-NQODKLVLRVDGI	216
AthMA2	VIVPSRTVIVVHDTLLIS-----QFQIVKALNQAQLEANVRVT	77
AthMA4	VIVPSRTVIVVHDSLLIS-----PFQIAKALNEARLEANVRVN	87
AthMA3	VIVPSRTVIVVHDTLLIS-----PLQIVKALNQAARLEASVRPY	83
AthMA1	VNLPPRSIRLRAVEDHHHDDHHHDE-QDHHHHHHHHHGHGCSVELKAE SKPQKMLFGFA	110
	:	
PAA1	KTKDKQARLKEGRELAVSWALCAVCLVGHLTFLG-----	273
AthMA8	VSK-KEDLLVKSRRNVAFAWTLVALCCGSHTSHILHSLGIH-----	177
AthMA5	LIDESMKVIERSLEALPGVQSVESISHTGDKISVLYKPDVGTGPRNFQVIESTVFGHSGHI	273
RAN1	LNELDAQVLEGLITRLNGVRQFRDRISGELEVVFDPEVVSRRSLVDGIEEDGFG---KF	273
AthMA2	---GETNFKNKWPSPFAVVSGLILLLSFFKYLVSF	109
AthMA4	---GETSFKNKWPSPFAVVSGLILLLSFLKFLVYSF	119
AthMA3	---GETSLKQWSPFAIVSGLVLLVLSFFKYLVSF	115
AthMA1	KAIGWVRLANYLRREHLHLCCSAAAMFLAAAVCPYLAP-----	147
	M1 M2	
PAA1	-----	VNAPWI 279
AthMA8	-----	IAEGGIW 184
AthMA5	KATIFSEGGVGRESQKQGEIKQYKSFVLSLVFTVPVFLTAMVFMIPIGDKDLLMEFKVIN	333
RAN1	KLRVMSF-YERLSSKDTGEASNMFRRFISLVLVSIPLFFIQVICPHIALFDALLVWRCG-	331
AthMA2	-----	
AthMA4	-----	
AthMA3	-----	
AthMA1	-----	
PAA1	HAIHSTGFHVSCLITLLGPRKILVLDGIKSLKGSFNMNTIVGLGALSSESFVS SLAAMI	339
AthMA8	DLLHNSYVKGGLAVGALLGPRELLFDGIKAFGKRSPNMNSIVGLGSMAAFSISLISLVN	244
AthMA5	MLTVGEIIRCVLATPVQFVI GWRFTYGSYKALRRGSANMDVIALGTNAAFFYSLYTVLR	393
RAN1	PFMMGDWLKVALVSVIQFVIGKRFVVAAWRALRNGSTNMMDVIVALGTSAS YFYSVGALLY	391
AthMA2	---FRWLAVA AVVAGIYPILAKAVASLARFRIDINIIVVVTVGATIGMOD---	156
AthMA4	---LRWLAVA AVVAGIYPILAKAFASIKRPRIDINIIVII TVIATLAMOD---	166
AthMA3	---LEWLAVA AVVAGVFPILAKAVASVTRFRIDINAVTLLAVIATLCMOD---	162
AthMA1	-EPYIKSLQNAFMIVGFPLVGVVSASILDALMDIAGGKVNIIHVMLAALAFASVFMGN---	201
	M2 M3 M4	
PAA1	PKLG---WKT-FFEEPVMLIAFVLLGRNLEQRAKIKATSDMTGILSVLP SKARLLLDGDL	395
AthMA8	PELE---WDASFFDEPVMLLGFVLLGRSLEERAKIQASTDMNELSLISTQSRLVITSSD	301
AthMA5	AATSPDFKGVDFEFTSAMLISFIIIGKYLEVMAGKTSQAIAKMNLAPDTAILSLIDKE	453
RAN1	GAVT-GFWSPTYFDASAMLI TFVLLGKYLESLAGKTS DAMKKIVQLTPATAILLTEGKG	450
AthMA2	-----YTEAAVVVFLFTAEWLQSRASYKASAVMQSLSLAPQKAVIAETG---	202
AthMA4	-----FMEAAAVVFLFTISDWLETRASYKATSVMQSLSLAPQKAVIAETG---	212
AthMA3	-----FTEAATIVFLFSVADWLESSAAHKASIVMSSLSLAPRKAVIDADTG---	208
AthMA1	-----ALEGGLLAMFNLAHIAEEFFTSSRSMDVVKELKESNPD SALLIEVHNG	249
	: : : M4 : : : * : : :	
PAA1	QNS-----TVEVPCNSLSVGDLVVILPDRVPADGVKSRSTIDESSETGEPL	444
AthMA8	NNTFVDSVLSSDSICINVSVDDIRVGDLSLLVLPGETTFVDGSLAQRVVDESMLTCESL	361
AthMA5	GNVTG---EEEIDGRLIQKNDVIKIVPAKVASDGYIWDGQSHVNESMITGEAR	504
RAN1	GKLVG-----EREIDALLIQPGDTLKVHPGAKIPADGVVWQSSYVNESMVTGESEV	501
AthMA2	---EEVEVDELKTNITVAVKAGETIPIDGVVDCNCEVDEKTLTGEAF	247
AthMA4	---EEVEVDEKVDVTVAVKAGETIPIDGIVDCNCEVDEKTLTGEAF	257
AthMA3	---LEVDVDEVGINTVVSVKAGESIPIDGVVDCSCDVDEKTLTGESE	253
AthMA1	NVPNIS----DL SYKSVPVHSEVGVSYVLVGTREIVVDCEVQGSATITIEHLTGEV K	304
	: : : * : : : * : : : * : : : * : : : *	

PAA1 PVTKESGSSQVAAGSILNLTFTVEVHRSGGETAVGDIIRLVREASREAPVQLVQKLVAG 504
AthMA8 PVFKEEGCWSAGTIWDPLRKASSTGSNSTISKVIRMDAGNAAPVQRLADAIAG 421
AthMA5 PVAKRKGDTIGGTLNENVLHVKVTRVGSSEALAQIVRLVLSAQLAKAPVQKLAIRISK 564
RAN1 PVSKEVDSPIGGTIRMHGMALHMKATKVGSDAVLSQIISLVTAOMSKAPIQKFAIVVAS 561
AthMA2 PVPKPKDSTWAGTINLNXYITVNTTALAEDCVVAKMAKLYEAKNSKTETKRFIRKCSK 307
AthMA4 PVPKQDSTWAGTINLNXYICVKTTSLAGDCVVAKMAKLYEAKNSKTETKRFIRKCSQ 317
AthMA3 PVSQKRESEMAATILNXYIKVKTTLALARDCVVAKTKLYEAKNSQTKTRFIRKCSR 313
AthMA1 PLEAKAGDRPPGGARLDRMIVKATKAWNDSTLNKIQLTVEASHSNPKLQKRWLEFGE 364
* *

PAA1 RFTYGVMAISAATFFWNLF-AHVLPALHN-----GSPMSIALQLSCSVLVAFCFA 557
AthMA8 PFVYTIMLSAMTFAPFYVVG-SHIPDVDLINDIAGPDGDALALSILKLAVDVLVSVCFCA 480
AthMA5 FFVPLVIFLFSFTWLAWFLAGKLHWYVESWIPS-----SMDSFELALQFGISVMVIAFCFA 620
RAN1 IFVPVVI TLALFTLVGWSIGGAVGAYPDEWLPE-----NGTHVFVLSMFISVVMVIAFCFA 617
AthMA2 YYTPAIIILSICFVAIPFALK-----VHNLKHWVHLALVVLVSVACFCFC 350
AthMA4 YYTPAIILVSACVAIVPVIMK-----VHNLKHWVHLALVVLVSGFCFC 360
AthMA3 YYTFAVVVSAACFAVIVPVLK-----VODLSHWVHLALVVLVSGFCFC 356
AthMA1 NYSKVVVLSLALAFILGPFLEKWPFLS-----TAACRGSVYRALGLVAASFCA 413
* * * * * M5 * * * * * M6: * * * * *

PAA1 LGLATPTAMLVGTSLGARRGLLRGGDIIEKFSLVDTVVFDKTGTITKCHPVVTEVIPE 617
AthMA8 LGLATPTAILIGTSLGAKRGYLRGGDVERLASIDCVADKGTGTEGRPVSASGL 540
AthMA5 LGLATPTAVMVGTVGASQGVVIGKQALERAHKVNCCIVFDKTGTITMGKPVVVKLLK 680
RAN1 LGLATPTAVMVAIVGVAATNGVLIKGGDALEKAKKVKVIFDKTGTITOGKATVTTTKVFS 677
AthMA2 LILSTPVATFCALTKAATSGLLIKGADYEETLAKIKIVAFDKTGTITRGEFIVMDFQSLS 410
AthMA4 LILSTPVATFCALTKAATSGLLIKSADYEDTSLKIKIVAFDKTGTITRGEFIVDFKSL 420
AthMA3 LILSTPVATFCALTKAATSGLLIKTGDCYEETLAKIKIVAFDKTGTITRGEFMVDFSRLS 416
AthMA1 LAVAFLAYATAISSARKGILKGAQVLDALASCHTIAFDKTGTITTEGLTCKAIEPIY 472
* * * * * M6 * * * * * : * : * : * : *

PAA1 NPRH-----NLNDTWSEVEVIMLAAAVSMTTHVVGKAI VKAARARN----- 659
AthMA8 -----YEEQEVILKMAAAVKTATHTAKAIVNEAESLN----- 573
AthMA5 -----NMVLRREFVLVAATVNSEHLAKAIVYAKKCFR----- 714
RAN1 -----EMDRGEFLTLVASAAASSEHLAKAIVAYARHFHFDESTEDGET 722
AthMA2 -----EDISLQSLLYVWVSSSTSKSSHPMAAAVVDYARSVS----- 445
AthMA4 -----RDINLRSLLYVWVSSVSKSSHPMAATIVDYARSVS----- 455
AthMA3 -----PSINLHKLLYVWVSSITCKSSHPMAALIDYARSVS----- 451
AthMA1 GHQGGTNSSVITCCIPNCEKALAVAAAMRGTTHVIGRAVVHDSVSKD----- 521
* * * * * : * : * : * : *

PAA1 -----CQTMKAEDGTFTTEEVSVAIVNKKRVTVGTLEWVKRHGATGN-----SLLA 707
AthMA8 -----LKTPETR-QOLT-EQDFTLAEIDGRFVAVGSLEWVSDRFLKKNDSDDMVKLES 625
AthMA5 ---DDEENPAWP-EACDFVSIIEKQVKATVKGREIMVGNKLNMDHKVITP-----DDAE 766
RAN1 NKNKDLQNSGWLIDTSDFLSALPKKCIQCLVNEKMLIVGNRKLMSENAINIP-----DHVEK 777
AthMA2 -----VEPKPEAVEYQNFDPGEIYKIDGKEVYIGNKRIASRAGLSV-----PD 491
AthMA4 -----VEPREEVEYQNFDPGECIYKIDGNDIFIGNKRIASRAGCSTV-----PE 501
AthMA3 -----VEPKPDIVENFQNFDPGEIYGRIDGDIYIGNKRIASRAGCLIT-----494
AthMA1 -----LPSIFVESFEYFDRRLTATVNGVKTVAEESRLRKASLGSIEFITSLEFKSED 573
* * * * * : * : * : * : *

PAA1 LEEHEINNQS-----VVYIGVDN-TLAAVIRFEDKVRDAAQVVENLT-RQGIDVVM 757
AthMA8 LLDHKLSENTSRTSRYSKTVVYVGRGEGIGAIASDCLRQDAEFTVARLQ-EKGIKTVM 684
AthMA5 LLADSEDAQ-----TGILVSYNSSEIIGVLSVSDPLKPSAREAISILK-SMNIKSDM 817
RAN1 FVEDLEE SGG-----TGVIVAYNGKLVGVMGIADELKREAAVVEGLL-RMGVRPDM 828
AthMA2 IDVDTKGGKT-----IGYVYVGETLAGVFNLSDACRSVGVQAQMKELK-SLGIKIAM 541
AthMA4 IEVDTKGGKT-----VGIVYVGERLAGFFNLSDACRSVGVQAQMAELK-SLGIKTAM 551
AthMA3 -----GIQTAM 500
AthMA1 ESKQIKDAVNASSYG-KDFVHAALSVDQKVTLIHLEDPQPRPGVSGVLAELKSWARLRVMM 632
* * * * * : * : * : * : *

PAA1 LSGDKRNAAANYVASVGVINHERVIAVGVKPAEKKNFINELQKKN-KIVAMVGDGINDAAAL 816
AthMA8 LSGDREGAVATVAKNVGKSESTNYSLSPKKKFEFISNLQSSG-HRVAMVGDGINDAPSL 743
AthMA5 VTCGDNWGTANSIAREVGIIDS-VIAEAKPEKAEKVKELQAAAG-HVVMVGDGINDSPAL 874
RAN1 VTCGDNWRITARAFAKEVGIIDS-VRAEVMKAGKADVIRSLQKDG-STVMVGDGINDSPAL 885
AthMA2 LTCGDNHAAAMHAQEQLGNAMDIVRAEDIPDKSEI IKQLKREE-GPTAMVGDGLNDAPAL 600
AthMA4 LTCGDNAAAAMHAQEQLGNLDVVHGDLLPEDKRSRI IQEPKKE-GPTAMVGDGLNDAPAL 609
AthMA3 LTCGDNQDAAMSTOEOLENALDIVHSELLPDKARI IDDFKIQ-GPTAMVGDGLNDAPAL 558
AthMA1 LTCGDNSSAWRVANAVGITE-VYCNLKPEDKLNHVKNIAREAGGLIMVGEINDAPAL 690
* * * * * : * : * : * : *

PAA1 ASSNVGVAMG--GGAGAVEVSPVVMGNRLTQLLDAMELSRQTMKTVMQVLLWWAFGYNI 874
AthMA8 AOADVGIALKIEAQENASNAASVIVRNKLSHVVDALSLAQATMSKVYQNLAWAIAYNV 803
AthMA5 AAADVGMIAIG--AGTDIIEAADIVMKNLEDVITADLSRRTFSRI RLNVVWALGYNL 932
RAN1 AAADVGMIAIG--AGTDVIEAADIVMRNNLEDVITADLSRRTLRIRLVYVAFAMAYNV 943
AthMA2 ATADIGISMG-VSGSALATETGNI IIMSNDIRRIPQAVKLAKRARRKVVENVVVISITMKG 659
AthMA4 ATADIGISMG-ISGSALATQTGNI IIMSNDIRRIPQAVKLARRARRKVVENVVLSITLKA 668
AthMA3 AKADIGISMG-ISGSALATETGDI IIMSNDIRRIPKGMRLAKRSHKKVENVVLSVSTIKF 617
AthMA1 AAATVHIVLA-QRASATAVADVILLRDNIITGVFPFCVAKSRQTTSLVKNVALALTSIF 749
* * * * * M7: * * * * *

PAA1 VGIPIAAGVLLPLTGTMLTPSMAGALMGVSLGVMTNSLLLRVYRFNSRNRDNKVKPEPKE 934
AthMA8 ISIPIAAGVLLPQYDFAMTPTSLSGGLMALSLIFVVSNSLLL----- 844
AthMA5 MGIPIAAGVLLPGRFRRLPFPWIAAGAMAASVSVVCCSLLLKN----- 975
RAN1 VSIPIAAGVFFPVLRVQLPPWAAGACMALSVSVVCCSLLLRR----- 986
AthMA2 AIALAFAGHPLIWAAVLADVGTCLLVILNLSMILLSDKHKTGN-KCYRESSSVVLIAEK 718
AthMA4 GILALAFAGHPLIWAAVLVDVGTCLLVIFNLSMILLREKCKKIGNKKCYR--ASTSKLNGRK 726
AthMA3 AIMVLGFVGYPLVWAAVLADVGTCLLVILNLSMILLRDEREAVS-TCYR--ASTASP--VK 672
AthMA1 LAAALPSVLGFVPLWLTVLLHEGGTLLVCLNIVRGLNDPSSWSK----- 792
* * * * * M8: * * * * *

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PAA1      GTKQPHETRWKQSS----- 949
AtHMA8    ---QLHKSETSKNSL----- 856
AtHMA5    -YKRPKKLDHLEIREIQVERV----- 995
RAN1      -YKRPRLTTVLKITTE----- 1001
AtHMA2    LEG--DAAGDMEAGLLPKISDKHKCKPGCGGTKTQEK--AMKP-AKASSDHSHSGCGETK 772
AtHMA4    LEGDDYVVDLEAGLLTKSGNGQCKSSCGDKKNQENVVMKPSSKTSSDHSHPGCGGDK 786
AtHMA3    LEE--DEVEDLEVGLLQK-SEETSKKSSCS-----GCGSGP 705
AtHMA1    -QDIVHLINKLRSQEPTSSSSNSLSSAH----- 819

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    QKDNVT-VVKKSCCAEPVDLGHG-----HDSGCCGDKSQ----- 805
AtHMA4    KEEKVKPLVKDGCCSEKTRKSEGDMVSLSSCKSSHVKHDLKMKGSGCGASKNEKGKEV 846
AtHMA3    KDNQQK----- 711
AtHMA1    -----

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    ---QPHQHEVVQQSCHN-----KPSGLDSG 828
AtHMA4    VAKSCCEKPKQQVESVGDCKSGHCEKKQAEDIVVPVQIIGHALTHVEIELQTKETCKTS 906
AtHMA3    -----
AtHMA1    -----

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    CGGGK-----SQPHQHELQQSCHDK----- 849
AtHMA4    CDSKEKVKETGLLLSENTPYLEKVLIKDEGNCKSGSENMGTVKQSCHEKGCSDEKQT 966
AtHMA3    -----
AtHMA1    -----

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    -----PSGLDIGTGEKHEGSSTLV 868
AtHMA4    GEITLASEEETDDQDCSSGCCVNEGTVKQSFDEKKHSVLVEKEGLDMETGFCCDAKLVCC 1026
AtHMA3    -----
AtHMA1    -----

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    N-LEGDAKEELKVLVNG--FCSS----- 888
AtHMA4    GNTEGEVKEQCRLEIKGEEHCKSGCGEEIQTGEITLVSEEETESTNCSTGCGVDKEEVT 1086
AtHMA3    -----
AtHMA1    -----

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    ---PADLAITSLKVKSDSHCKS-----NCSSRERCHHGSNC 921
AtHMA4    QTCHEKPASLVVSGLEVKKDEHCESSHRAVKVETCCKVKIPEACASKCRDRAKRHSGKSC 1146
AtHMA3    -----
AtHMA1    -----

-----
PAA1      -----
AtHMA8    -----
AtHMA5    -----
RAN1      -----
AtHMA2    CRSYAKESCSHDRHHTRAHGVGTLKEIVIE 951
AtHMA4    CRSYAKELCSRRRHHRRHHRRHHRRVSA----- 1172
AtHMA3    -----
AtHMA1    -----

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Figure 3.16 Alignment of *Arabidopsis* HMAs carried out using ClustalW (<http://www.ebi.ac.uk/clustalw/>). Black, indicates motifs common to all P-Type ATPases; Red, motifs found in HMAs; Green, potential metal binding domains; Purple, areas with conserved residues; Blue, region immediately following CPx motif; Yellow, areas of high similarity between HMA 2, 3 and 4. Location of transmembrane domains constructed using an alignment of AtHMA4 with CadA (Melchers et al. 1996).

Figure 3.17 For phylogenetic analysis of HMAs, sequences from various organisms were obtained from a protein families database (<http://www-biology.ucsd.edu/~ipaulsen/transport/index.html>), a P-type ATPase database (<http://biobase.dk/~axe/Patbase.html>) and by searching general databases with P-type ATPase and HMA-specific domains. Sequences were aligned using Clustal W 1.82 and a phylogenetic tree was created using the Phylip 3.6a3 neighbour-joining method with the data set bootstrapped 1000 times (Felsenstein 2002). The tree was rooted at the midpoint and displayed using Treeview 1.6.6 (Page 2001). The PROSITE (Falquet et al. 2002, <http://ca.expasy.org/prosite>) and Pfam databases (www.sanger.ac.uk/software/pfam) were used to search for conserved motifs/domains. Proteins where evidence of a transport function has been presented are denoted with rectangles (■) for Zn/Cd/Pb/Co and with circles (●) for Cu/Ag. The organisms and accession numbers for these sequences are: 1. *Lactococcus lactis* (P94888), 2. *Listeria monocytogenes* (Q60048), 3. *Bacillus firmus* (P30336), 4. *Staphylococcus aureus* (P37386), 5. *Staphylococcus aureus* (P20021), 6. *Synechocystis* PCC6803 (Q59997), 7. *Bacillus subtilis* (O31688), 8. *Synechocystis* PCC6803 (Q59998), 9. ***Arabidopsis thaliana* (Q9SW66)**, 10. *Chlamydia trachomatis* (O84732), 11. ***Arabidopsis thaliana* (O64474)** 12. *Methanobacterium thermoautotrophicum* (O26511), 13 *Bacillus subtilis* (O32219), 14. *Helicobacter pylori* (Q59465), 15. *Mycobacterium tuberculosis* (Q10866), 16. *Proteus mirabilis* (O33448), 17. *Escherichia coli* (P37617), 18. *Rhodobacter capsulatus* (O68082), 19. *Mycobacterium tuberculosis* (O69710), 20. *Mycobacterium tuberculosis* (O53160), 21. *Mycobacterium tuberculosis* (P96875), 22. *Helicobacter felis* (Q9RQB4), 23. *Pyrococcus abyssi* (Q9V060), 24. ***Arabidopsis thaliana* (Q9SZW4)**, 25. ***Arabidopsis thaliana* (Q9SZW5)**, 26. *Deinococcus radiodurans* (Q9RZ81), 27. *Vibrio cholerae* (Q9KT72), 28. *Pseudomonas aeruginosa* (Q9HXV0), 29. *Halobacterium* sp. (Q9HSN5), 30. *Streptomyces coelicolor* (Q9ZBF3), 31. *Streptomyces coelicolor* (Q9RJ01), 32. *Chlamydia pneumoniae* (Q9Z738), 33. *Aeropyrum pernix* (Q9Y8R2), 34. *Pseudomonas aeruginosa* (Q9I147), 35. *Ureaplasma urealyticum* (Q9PQU0), 36. ***Arabidopsis thaliana* (Q9C594)**, 37. *Helicobacter pylori* (P55989), 38. *Helicobacter felis* (O32619), 39. *Haemophilus influenzae* (P77868), 40. ***Arabidopsis thaliana* (Q9SZC9)**, 41. *Escherichia coli* (Q59385), 42. *Enterococcus hirae* (P32113), 43. *Synechococcus* PCC7942 (P37385), 44. *Treponema pallidum* (O83999), 45. *Synechocystis* PCC6803 (P74512), 46. *Mycobacterium tuberculosis* (P77894), 47. *Mycobacterium leprae* (P46840), 48. *Mycobacterium tuberculosis* (Q10876), 49. *Mycobacterium tuberculosis* (Q10877), 50. *Mycobacterium leprae* (P46839), 51. *Schizosaccharomyces pombe* (O59666), 52. *Proteus mirabilis* (Q59688), 53. *Methanobacterium thermoautotrophicum* (O27578), 54. *Archaeoglobus fulgidus* (O29777), 55. *Bacillus subtilis* (O32220), 56. *Synechocystis* PCC6803 (P73241), 57. *Synechococcus* PCC7942 (P37279), 58. *Listeria monocytogenes* (P77881), 59. *Saccharomyces cerevisiae* (P38995), 60. *Homo sapiens* (Q04656), 61. *Cricetulus griseus* (P49015), 62. *Mus musculus* (Q64430), 63. *Rattus norvegicus* (P70705), 64. *Rattus norvegicus* (Q64535), 65. *Homo sapiens* (P35670), 66. *Mus musculus* (Q64446), 67. *Ovis aries* (Q9XT50), 68. ***Arabidopsis thaliana* (Q9S7J8)**, 69. *Caenorhabditis elegans* (O17737), 70. *Saccharomyces cerevisiae* (P38360), 71. *Helicobacter pylori* (O26033), 72. *Aquifex aeolicus* (O67432), 73. *Bradyrhizobium japonicum* (Q59207), 74. *Rhodobacter capsulatus* (O30733), 75. *Sinorhizobium meliloti* (P18398), 76. *Rhizobium leguminosarum* (O33533), 77. *Methanobacterium thermoautotrophicum* (O26849), 78. *Archaeoglobus fulgidus* (O30085), 79. *Aquifex aeolicus* (O67203), 80. *Enterococcus hirae* (P05425), 81. *Rhizobium leguminosarum* (Q9X5V3), 82. *Salmonella typhimurium* (Q9ZHC7), 83. *Thermoplasma acidophilum* (Q9HJ30), 84. *Vibrio cholerae* (Q9KPZ7), 85. *Neisseria meningitidis* (Q9JZ27), 86. *Deinococcus radiodurans* (Q9RRN5), 87. *Sinorhizobium meliloti* (Q9X5X3), 88. *Pseudomonas aeruginosa* (Q9HX93), 89. ***Arabidopsis thaliana* (Q9SH30)**, 90. *Drosophila melanogaster* (Q9VYT4), 91. *Campylobacter jejuni* (Q9PNE0), 92. *Campylobacter jejuni* (Q9PND4), 93. *Candida albicans* (Q9UVL6), 94. *Thermotoga maritima* (Q9WYF3), 95. *Halobacterium* sp. (Q9HN90), 96. *Halobacterium* sp. (Q9HRH2), 97. *Aeropyrum pernix* (Q9YBZ6), 98. *Neisseria meningitidis* (Q9JZI0), 99. *Pseudomonas aeruginosa* (Q9I3G8), 100. *Vibrio cholerae* (Q9KS24), 101. *Candida albicans* (Q9P458), 102. *Escherichia coli* (Q59369), 103. *Escherichia coli* (Q59370), 104. *Anabaena* sp. (Q8ZS90), 105. *Bacillus halodurans* (Q9K5Q2), 106. *Ralstonia metallidurans* (Q9AKR5), 107. *Oscillatoria brevis* (Q8L158), 108. *Oceanobacillus iheyensis* (BAC12232), 109. *Streptococcus agalactiae* (AAN00131), 110. *Geobacillus stearothermophilus* (Q93GJ9), 110. OsHMA1, *Oryza sativa* 1980.t00006, Chromosome 6, 111. OsHMA2, *Oryza sativa* 3411.t00009, Chromosome 7, 112. OsHMA4, *Oryza sativa* 3408.t00005, Chromosome 6, 113. OsHMA5a, *Oryza sativa* 5495.t00009, Chromosome 4, 114. OsHMA5b, *Oryza sativa* 2540.t00009, Chromosome 2, 115. OsHMA6, *Oryza sativa* 5118.t00002, Chromosome 3, 116. OsPAA1, *Oryza sativa* 3120.t00014, Chromosome 8, 117. OsRAN1a, *Oryza sativa* 4218.t00029, Chromosome 2, 119. OsRAN1b, *Oryza sativa* 1952.t00005, Chromosome 6.

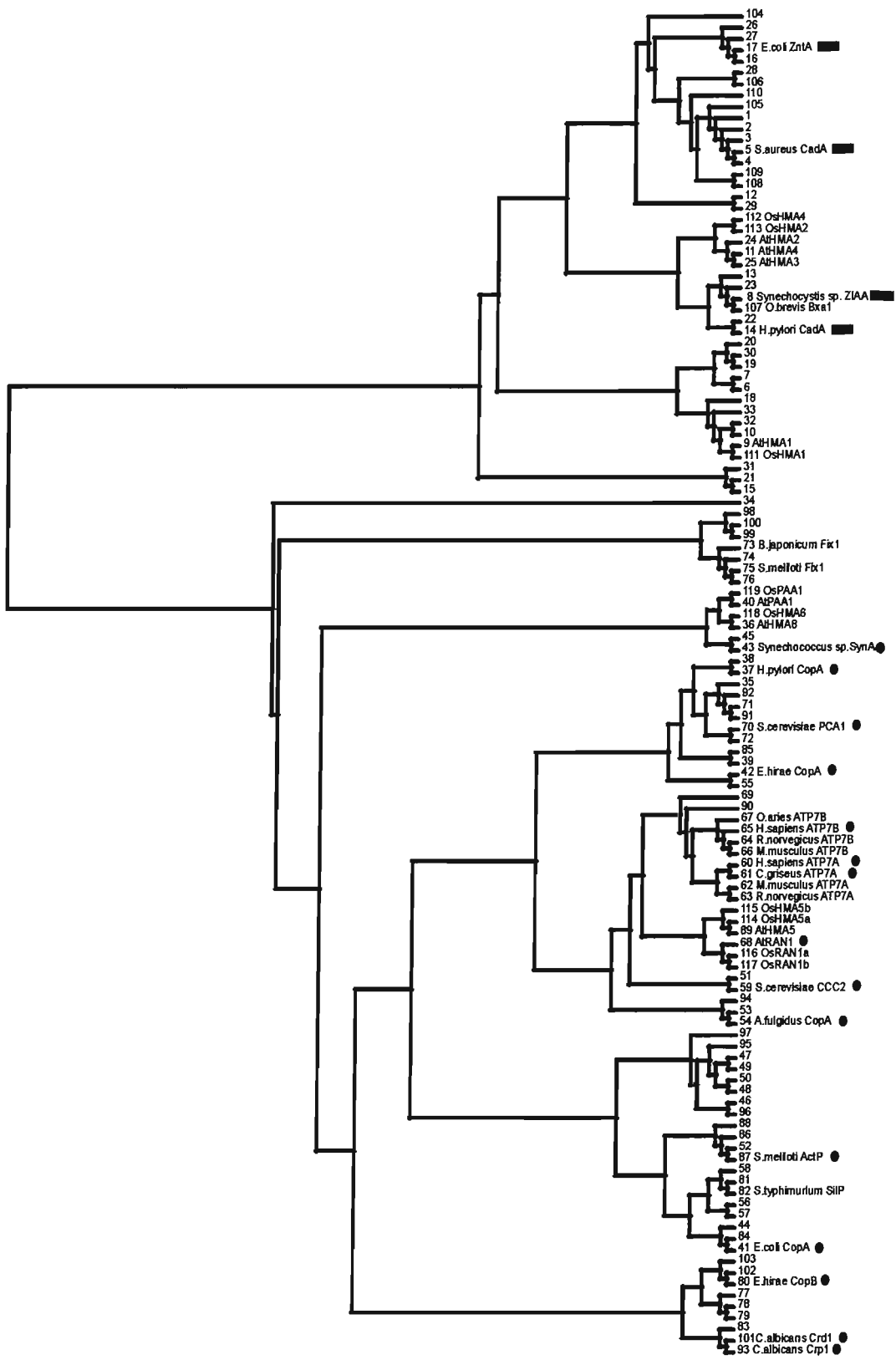


Figure 3.17 Phylogenetic tree of HMAs from a range of organisms. The tree was constructed from alignments of full-length amino acid sequences (see opposite page for organism and protein details).

experimental evidence of a role in Cu^+ and/or Ag^+ transport (highlighted by circles). The second group contains pumps which have been shown to have a role in the transport of one or more of the following: Zn^{2+} , Co^{2+} , Cd^{2+} and Pb^{2+} . AtHMA4 falls into the latter group and clusters closely with AtHMA2 and AtHMA3, two other AtHMAs found in the *Arabidopsis* genome. Recent work on AtHMA3 has added further support to this classification, with evidence produced to suggest that it may be involved in the movement of Cd and/or Pb (Gravot et al. 2004). Another *Arabidopsis* HMA falling in this cluster is AtHMA1. Although this pump still exists within the main Cd/Pb/Zn/Co grouping it appears to be more distantly related to the other *Arabidopsis* HMAs. What significance this may have in terms of how it functions is unclear. Divergence of the AtHMAs is also observed in the Cu/Ag cluster. AtRAN1/AtHMA7 and AtHMA5 appear in a different subgroup to that of AtPAA1/AtHMA6 and AtHMA8. These differences are interesting as they appear to have little to do with substrate specificity (e.g. between Cu and Ag). Both AtRAN1/AtHMA7 and AtPAA1/AtHMA6 have been shown to have roles in copper transport, although they appear to be in different locations within the plant cell (Hirayama *et al.* 1999; Shikanai *et al.* 2003).

3.3 Discussion

Several types of heavy-metal transporters have now been cloned from plants and efforts are underway to determine their physiological role and mechanism of action (Pence *et al.* 2000, Thomine *et al.* 2000). This chapter has focussed on *AtHMA4*, a member of a specific subgroup of the P-type ATPase superfamily. *AtHMA4* is a previously uncharacterised heavy metal ATPase of *Arabidopsis thaliana*. The work undertaken to clone and sequence this gene has provided the initial steps that will allow further characterisation of its functions. Some of the results of this and following chapters are reported in Mills *et al.* (2003).

The results reported here are important in confirming that *AtHMA4* is expressed in *Arabidopsis*. The amplification of *AtHMA4* from ESTs and reverse-transcribed RNA has confirmed the expression of this gene. Together with the RACE results, they have shown that *AtHMA4* is expressed in leaf and root tissues of healthy plants, when grown in soil, or hydroponically. This suggests that the translated protein is required, and indeed present under normal conditions. For *AtHMA5*, another member of the family, such evidence is still lacking, despite there being knowledge of its genomic sequence for some time (Baxter *et al.* 2003). The gene belongs to a large super-family of transporters in which there are a number of strikingly similar members (e.g. *AtHMA2*, *AtHMA3*). Some may be non expressed pseudogenes or it may be that there are cases of functional redundancy (Mäser *et al.* 2001). For *AtHMA4* and *AtHMA2*, some functional/genetic redundancy has recently been suggested (Hussain *et al.* 2004; see general discussion).

Two separate and independent methods were used to obtain information about the coding sequence of *AtHMA4*. Sequencing of RT-PCR amplified material was carried out together with sequencing of ESTs. Of the two full-length *AtHMA4* sequences that were generated, both were in agreement with the predicted coding sequence in the MIPS *Arabidopsis thaliana* database. The intron-exon boundaries were also in agreement, and the results revealed that the gene structure is similar to the closely related *AtHMA2*. Less related was the pattern shown by *AtHMA3*. The *AtHMA3* gene shares the same number of exons, with the 9th being the largest overall; however this gene does not display the same grouping of coding regions along its genomic sequence as *AtHMA2* and *AtHMA4*. A striking difference is also observed in the size of *AtHMA3*'s first 2 introns; these are much smaller than those of *AtHMA2* and 4. Cobbett *et al.* (2003) have highlighted the similarity between *AtHMA4* and *AtHMA2* in their analysis of BAC clone sequences. However, using a different approach, they also mention that *AtHMA3* does show some similarity to

AtHMA2 and *AtHMA4* (although still less related than the two to each other) when the introns are analysed with respect to their position within the coding sequence (Cobbett et al. 2003).

The full-length, 3516 bp coding sequence of *AtHMA4* was obtained by using oligonucleotide primers designed to cover predicted start and stop codons of the gene. PCRs carried out prior to this with primers to leader and trailer regions did not yield any products suggesting that there was no additional coding sequence beyond one or both of these sites. RACE PCR has shown however that *AtHMA4* may possess either an additional or extended 1st exon, 5' to the predicted start codon. Two products were obtained by this method, one had 339 bp of sequence directly upstream of the predicted start ATG, whilst the other had 69 bp of sequence interrupted by a 494 bp intron. Evidence suggests that this upfront region is non-coding, but the presence of multiple fragments could suggest that there may be alternative start codons leading to the existence of modified forms of the *AtHMA4* protein. There are a number of potential start codons upfront of the predicted ATG start site. Two of these found in the RACE 1 product (which suggests an extended 1st exon) are in frame with the predicted start ATG and only 86 and 92 bp away from a putative TATA box (Figure 3.13). Another potential start site exists 66 bp downstream of the predicted start ATG. Its presence is made more significant, by the fact the EST 3 5' terminus is 18 bp downstream from this point. Thus EST 3 could be a differentially spliced product and provide evidence that such a phenomenon occurs in the transcriptional processing of *AtHMA4*. Further work is needed to clarify this, as EST 3 may also be a partially amplified product. If real however, these additional start sites would lead to a protein only slightly smaller or larger than that resulting from the confirmed coding sequence. It can be anticipated that basal/core functions such as transport, ATP binding, phosphorylation would be unaffected, but it is conceivable that targeting of the protein or regulation may be influenced in some way. The N-terminal region has been shown to play an important part in the regulation of plant Ca²⁺ ATPases (Geisler et al. 2000; Malmström et al. 2000) and the localisation of some mammalian HMAs (Goodyer et al. 1999). Recent evidence suggests that the presence of untranslated introns could be crucial to this by allowing the production of different splice variants (Navarro-Aviñó and Bennett 2003).

To date, no regulatory mechanisms have been conclusively proved for any of the plant HMAs (Baxter et al. 2003). Studies in simple organisms such as bacteria and yeast have highlighted the importance of transcriptional control in genes involved in metal homeostasis. For plants, similar systems may also operate

(Wintz et al. 2002; Bereczky et al. 2003; Burleigh et al. 2003 Mills et al. 2003; Becher et al. 2004) and as a result it is pertinent to analyse the potential promotor/operator region of *AtHMA4*. The production of two contrasting sequence products in the 5' region of *AtHMA4* is a rather interesting discovery. Initially, the RACE technique was used to confirm if the nucleotide stretch **ATGGCG** used as a forward primer site during RT-PCR amplifying and cloning strategy, was the location of the *AtHMA4* translation start site as had been predicted from initial sequence analysis of BAC clone AC002392. The data generated suggested that this was the case, but it also highlighted that the 5' untranslated sequence could be subjected to alternative splicing events. As with many eukaryotic genes, the regulatory sequence for *AtHMA4* transcription may reside in this region. Alternative splicing could control transcript levels, or it may be a mechanism that initiates the production of modified transcripts for different functions (Reddy and Harris 1998; Harris 2000). Possibly in this case such events could be controlled by the metal status of the cell or tissue and link in with a host of other homeostatic mechanisms. No metal-dependent differences in the 5' region have been found in this work, although the indication that this area may be a point of regulation suggests that it should be subjected to further investigation under different metal conditions.

In humans, alternative splicing in the genes *ATP7A* and *ATP7B* coding for the Menkes and Wilsons disease proteins (Cu-ATPases), has been the subject of a number of studies. A Menkes transcript coding for a 103-residue protein has been found to be translated from the (in frame) 5' ATG immediately preceding the start site of the full-length gene (Reddy et al. 2000). The truncated variant contains only one HMA domain from *ATP7A*, and additional amino acid residues suggestive of a nuclear localisation sequence (NLS) (Reddy et al. 2000). A somewhat larger transcript was found in an earlier study (Reddy and Harris 1998). In this instance a 57-59 kDa protein was produced from a 1.9 kb cDNA product, independently of full-length *ATP7A* expression. Sequence analysis showed that exons 3-15 (out of 23) were spliced out to produce a protein that essentially lacked the 'core' of the HM-ATPase enzyme: HMA domain 1 (at N terminus) was fused directly to transmembrane domains 7 and 8 (at C terminus) (Reddy and Harris 1998). In these cases, the production of metal-binding proteins with low molecular weights, poor transmembrane anchorage and predicted water solubility, has lead to the suggestion that they may act as chaperones allowing the guided transport of ions across the cytoplasm to specific targets (Reddy and Harris 1998). To conclude that possible *HMA4* splice variants may have a similar role would be conjectural.

Analysis of the 5' UTR of *AtHMA4* has revealed several potential TATA (and

accompanying CAAT) boxes and an absence of any CpG island(s). This is indicative of a gene that is constantly regulated via specific transcription factors (e.g. Metal Regulatory Elements), and is in contrast somewhat to the copper transporters *ATP7A* and *ATP7B* which appear to act more constitutively as housekeeping genes (Tümer and Horn 1999; Cox et al. 1999). The fact that there are several such TATA boxes in the 5' region of the *AtHMA4* gene, could be indicative of different splice variants or alternatively that there are several sites involved in the induction of a single transcript. Such a mechanism has been found to operate in the β -Tubulin gene (*tubB1*) of soybean, where different TATA boxes regulated expression under different environmental conditions (Doyle and Han 2001).

Despite some of the contrasts that have been highlighted between *AtHMA4* and the Cu-ATPase *ATP7A*, there are some similarities with the RACE(2) sequence. This could support the occurrence of splicing in the plant HMA. In both cases the first exon is untranslated, whilst the second exon contains further untranslated sequence and the ATG translation start site. In the Menkes gene splice-variant, the first intron is interrupted by an additional and separate exon (exon 1b), which appears to be expressed in low amounts in several tissues (Tümer and Horn 1999). If the RACE(1) sequence is considered, then it appears that a similar situation may arise in the 5' region of *AtHMA4*. However, it must be clarified that the RACE(1) only appears to extend the second exon in the 5' direction rather than form a new and discrete unit upstream. The fact that the 5' intron/exon boundary appears as a potential area of regulation in *AtHMA4* is intriguing.

3.3.1 Sequence analysis

An alignment at the amino acid level of *Arabidopsis* HMAs shows that there may be low overall homology between certain members of the group (Table 3.2, Figure 3.16). Despite this, each one possesses motifs characteristic for all P-type ATPases (Figure 3.16). There are two conserved regions of sequence that are characteristic of the P-type ATPases superfamily. One is the haloacid dehydrogenase-like hydrolase domain characterized by a region including the DKTGT motif (contains the phosphorylated aspartate residue) and the GDGXNDXP motif (the putative Mg-binding sites of the hinge domain (Okkeri et al. 2002)). Two of the residues that are mutated in Wilson's disease protein (*ATP7B*) are present in the latter motif (GDGXNDx) indicating its importance for HMA function (Aravind et al. 1998). The other characteristic region is the E₁-E₂ domain, which includes the Px₁D and TGE motifs as well as the conserved proline (this can form part of the CPx motif

in the HMAs). A partial loss-of-function mutant *ran1-1* has been isolated (Hirayama et al. 1999) with a single amino acid change (Thr₄₉₇ of TGES is changed to Ile). Evidence suggests that this motif is essential for function as the *ran1-1* mutant was unable to functionally complement the yeast *ccc2Δ* deletion mutant (Hirayama et al. 1999).

AtHMA4 also contains a number of motifs that are characteristic of heavy metal ATPases. These include the conserved CPx motif (cysteine, proline and either cysteine, histidine or serine) (Solioz and Vulpe 1996). AtPAA1, AtRAN1 and AtHMA4 possess a CPC motif, whilst AtHMA1 is rather unusual in having SPC at this location. The proline in this trio is conserved in all P-type ATPases (see above) and evidence suggests it exists in the TM domain preceding the large cytoplasmic loop 43 amino acids N-terminal to the phosphorylatable aspartate (Moller et al. 1996; Catty et al. 1997; Cox et al. 1999). The amino acids surrounding this proline vary with different classes of P-type ATPases (Williams et al. 2000). In the HMAs, there is usually a cysteine to the amino-terminus and either a cysteine, histidine or serine to the C-terminus, hence the classification as CPx-ATPases. Despite the diversity of the third amino acid in different members of the CPx group, the residue is crucial to enzyme function. Mutation of CPC to CPA in a Cu-ATPase transformed into yeast meant it could no longer tolerate high Cu (Yoshimizu et al. 1998). This region is thought to play an important role in the translocation of heavy metals (Solioz and Odermatt 1995) but despite attempts no relationship between ion specificity and CPx sequence variation has been established. The HP locus is also a conserved motif in HMAs that is not found in other P-type ATPases. In AtHMA4, this locus is found 40 amino acids C-terminal to the phosphorylated aspartate residue in the large cytoplasmic domain. Mutation of either the H or the P of this motif in ZntA from *E. coli* results in poor phosphorylation by both ATP and inorganic phosphate and also in slow dephosphorylation rates (Okkeri et al. 2002). This has led to the suggestion that this site may be involved in nucleotide binding, but there is no consensus of agreement. Work on CopB from *E. hirae*, has suggested the converse (Bissig et al. 2001) revealing that the K_m for ATP of the CopB protein, was unaffected by a mutation introduced at the H480 site (Bissig et al. 2001).

Other unique features of HMAs are the presence of one or several putative metal-binding domains in the N- and/or C-termini. There have been a number of studies addressing the properties and function of these putative metal-binding domains, mainly with respect to ATP7A (Menkes disease protein), ATP7B (Wilson disease protein) and ZntA (*E. coli* HMA) (Hou et al. 2001, DiDonata et al. 2002). The most commonly discussed motif is a domain referred to as the "heavy-metal

associated (HMA) domain (Jordan et al. 2001), which typically includes a GMxCxxC sequence. This is present in six copies in both human ATP7A and ATP7B, but only one or two are present in bacterial or yeast orthologues (DiDonato and Sarkar 1999 book). The GMxCxxC motif is predicted, together with the presence of a leucine close by, to confer substrate specificity for Cu (Gitschier et al. 1998). An interesting study in which the entire amino-terminal domain of ATP7B or only its sixth GMxCxxC motif replaced the amino-terminal domain of ZntA resulted in chimeric proteins which still conferred resistance to Pb, Zn and Cd salts (the usual substrates of ZntA) but not to Cu and Ag (the usual substrates of ATP7B) (Hou et al. 2001). However, the chimeras were 2-3 fold less active than ZntA. Hou et al. (2001) concluded that the amino-terminal domain of these ATPases cannot alter metal specificity determined by the transmembrane segment but may interact with the rest of the transporter in a metal ion-specific manner to allow full catalytic activity. This is supported by a previous study in which a *zntA* mutant lacking its amino-terminal domain was shown to be fully capable of ATP-dependent metal transport with the same specificity as the intact protein, but with slightly lower activity (Mitra and Sharma 2001). At present, we can only speculate on possible metal-binding motifs in AtHMA4. AtPAA1/AtHMA6, AtRAN1/AtHMA7 and AtHMA4 all contain "heavy-metal-associated" domains in their N termini. In contrast to AtPAA1 and AtRAN1, AtHMA4 does not contain the characteristic GMxCxxC motif but instead the sequence GICCTSE. It is not known whether this can function as a metal-binding site. AtHMA4 has a long histidine stretch in the C-terminal region, which is interesting as histidine residues are commonly found in Zn-binding motifs. HRA1 and HRA2 of *E. coli* and CopB of *Enterococcus hirae* contain methionine and histidine-rich sequences that may function in metal-binding (Solioz and Vulpe 1996). Histidine-rich motifs are present on other Zn transport proteins including members of the ZIP and CDF families (Guerinot 2000, Gaither and Eide 2001). These putative-metal binding motifs could function in metal-regulated protein-protein interactions. In addition, the C-terminal region of AtHMA4 has multiple pairs of cysteines that could be involved in metal binding (Tong et al. 2002). Metallothioneins also contain CC and, more frequently, CXC motifs. Metallothioneins are small proteins involved in Cd detoxification that are thought to function by chelation of Cd to -SH groups of cysteines, and /or by scavenging of free radicals produced by Cd-induced stress (Palmiter 1998).

Work confirming the determinants of HM-ATPase substrate specificity has long been anticipated. Even now, the characteristics of potential metal-ion-specific binding sites are still being speculated upon. However recent work is now beginning

to highlight potential areas of interest in these proteins. Early predictions that varying CPx, CxxC or histidine stretches/repeats were the sole factor influencing specificity are now seen as oversimplified. Many exceptions have arisen and these suggest that larger portions of the protein, rather than individual binding sites are important (Hou et al. 2001). For example, Zn pumps exist (e.g. ZntA) that possess CxxC motifs commonly associated with Cu transport (Rensing et al. 1997; Okkeri et al. 2002). Similarly, there are Cu-specific ATPases in which this motif is absent and replaced by histidine (CopB) (Solioz and Stoyanov 2003). A rather recent finding has confused matters further by providing evidence that a histidine-rich HMA may transport either Cu, Ag, Zn or Cd (Tong et al. 2002). This is important as it was previously believed that heavy metal ATPase specificity was partitioned separately between the groups of Cu/Ag and Zn/Cd/Pb/Co/Hg. The characteristics of HMA4, suggest that it falls in the latter grouping (see phylogenetic analysis section), but it does share some similarities with the multi-substrate protein Bxa1.

In their attempt to explain the substrate specificity, Tottey et al. (2001) considered that a small region C-terminal of the CPx motif was important (blue shading in Figure 3.16). They noted that copper transporters generally have the extended motif; **CPCALGATP**, whilst those transporting Zn tended to share the sequence **CPCALVISTP**. Although this may be true for a number of transporters in the group, including AtPAA1/AtHMA6 and AtRAN1/AtHMA7, it provides no real help in assessing potential substrates of HMA1 or 4. HMAs 2, 3 and 4 each possess the motif **CPCGLILSTP**, whilst HMA1 appears to have a hybrid of the two designator sequences; **CPCALAVA *P** (Figure 3.16). Considering the volume of structural and functional studies carried out on other P-Type ATPases, Argüello (2003) has recently considered HMA substrate specificity with regard to findings made on those that transport Ca, Na and K. By looking at homologous regions in the HMAs, sequences were found in transmembrane domains 6, 7 and 8 that could be used to assign metal specificity to most of the 234 HMAs tested. Again, there appeared to be a few exceptions to the rule, but the analysis has highlighted the possibility of new substrate-specific clusters, one of them possibly being involved in the transport of new and undefined metal ions (Argüello 2003). For HMAs 2,3 and 4 this new analysis adds nothing new. Their similarity to each other and substrate specific grouping is confirmed, but these can be seen from sequence alignment (Figure 3.16) and phylogenetic analysis (Figure 3.17).

3.3.2 Hydropathy analysis

The basic organisation of the four HMAs (shown in Figure 3.15) cloned from *Arabidopsis* (AtHMA4, AtHMA1, AtPAA1/AtHMA6 and AtRAN1/AtHMA7) predicted from hydropathy plots is similar to the topology determined experimentally for CadAs from *Helicobacter pylori* (Melchers et al. 1996) and *Staphylococcus aureus* (Tsai et al. 2002). There are 8 predicted TM domains with a small cytoplasmic loop between TM domains 4 and 5 and a large cytoplasmic loop between TM domain 6 and 7 (Figure 3.15). These house motifs that appear to be important in the mechanistic action of the ATPase (Xu et al. 2002) and exist in a region that has been referred to as the 'conserved core' of the protein (Solioz 1998). The alignment performed in Figure 3.15 demonstrates this characteristic and it also highlights the variability often observed at the termini of the proteins. For example whereas AtPAA1/AtHMA6 and AtRAN1/AtHMA7 possess a large cytoplasmic domain in their N-terminus prior to TM domain 1, AtHMA4 has a large cytoplasmic domain at the C-terminus after TM domain 8. In general HMAs are distinctive in that they have a highly polar N-terminal domain of variable length. AtHMA4 is rather unusual in this respect with a long polar C-terminal domain (472 amino acids after end of last predicted TM domain compared with AtRAN1/AtHMA7 which has 19 and AtPAA1/AtHMA6 which has 35). This data is based purely on computer predictions and requires experimental confirmation, but it is interesting that as a family, the P-type ATPases have been shown to have regulatory regions in either N- or C-termini (Axelsen and Palmgren 2001). In ATP7A in particular N – terminal sites are thought to be involved in the regulation of protein translocation (Strausak et al. 1999a,b). There are also interesting differences in the organisation of these domains in sub-families between species. For example, in the type P_{2B} Ca²⁺ -ATPases there is evidence that in the mammalian pumps the calmodulin-binding regulatory region is at the C-terminus whereas in plants this regulatory domain is thought to occur at the N-terminus (Geisler et al. 2000; Malmström et al. 2000). For the HMAs, little work has been conducted on the forms of protein regulation that exist in any organism. Considering their function alone, it is obvious that metal ions will have an important role. The fact that the HMA protein termini are the location of different putative metal binding sites, suggests that these regions may be involved (Baxter et al. 2003). Evidence that the putative metal binding site CxxC could have such a function has been presented (Vanderwerf et al. 2001). In the Wilson's disease protein (ATP7B), two phosphorylation sites were found. One positioned in the C-terminal half of the protein displayed 'basal activity', whilst another (that required the presence of a

functional N – terminal domain) responded to increasing copper concentrations. The response was rapid, specific and reversible and so proposed to act as a regulatory mechanism that may tie in with other cellular processes (Vanderwerf et al. 2001). In AtHMA3, the C-terminal region has been highlighted as a potential point of regulation (Gravot et al. 2004). Work with GFP fusion protein suggested that when tagged at its C-terminus, the pump lost its ability to enhance the Pb tolerance of yeast. Gravot et al. (2004) suggested that GFP-tagging may perturb metal recognition and/or loading in some way.

3.3.3 Phylogenetic analysis

Phylogenetic analysis has indicated that there are two distinct clusters within the P_{1B} sub-group (Rensing et al. 1999, Sharma et al. 2000, Axelsen and Palmgren 2001). It has been suggested that the family divides according to their substrate specificity and it encompasses the Cu⁺/Ag⁺ and the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ clusters. *Arabidopsis* has an unusually large suite of HMAs for the size of its genome, with an equal number (4) belonging to each cluster (Axelsen and Palmgren 2001; Baxter 2003). AtHMA4 falls into the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ subgroup, suggesting that it may transport one or more of these metals. Support for this has come very recently with the finding that the closely related AtHMA3 protein has the ability to transport Cd and Pb (Gravot et al. 2004). The prominence of this grouping in eukaryotes is quite unusual, so far it appears that ATPases from the Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ cluster are poorly represented in all non-plant species (Axelsen and Palmgren 2001; Mills et al. 2003). Of course the phylogenetic profile could reflect other factors such as the subcellular localisation of the protein (Paulsen and Saier 1997). However, in the case of AtRAN1/AtHMA7 and AtPAA1/AtHMA6 at least this appears not to be strictly true. Both are known to be copper transporters that are localised in different parts of the plant cell (Himmelblau and Amasino 2000; Shikanai et al. 2003), but they are still clustered in the same sub-grouping. Nevertheless, there is some divergence between the two, so perhaps the localisation of the protein is a secondary factor that can be masked by the overriding influence of substrate specificity.

Phylogenetic analysis of proteins on the basis of putative metal-binding domains is not a new idea (Jordan et al. 2001), but the analysis of TM domains 6, 7 and 8 in such a way, has yielded some interesting results. The results of Argüello (2003) appear to have an improved 'resolution' over the standard full-length protein analysis (shown here) and the large Cu⁺/Ag⁺ and Zn²⁺/Co²⁺/Cd²⁺/Pb²⁺ clusters are broken down to suggest different/newer substrates. Of the *Arabidopsis* HMAs,

AtHMA1 falls into a new grouping; Argüello (2003) has speculated a role in Co^{2+} transport. This is based on its similarities with the CoaT protein from *Synechocystis* (including the SPC motif), which transports cobalt (Rutherford et al. 1999).

Chapter 4 Functional characterisation of AtHMA1 and 4

4.1 Introduction

The heterologous expression of plant genes is an important technique for determining gene-product function (Frommer and Ninnemann 1995). Heterologous expression systems have been greatly optimised since first introduced, but they still consist of two basic elements. A vector is required to ferry ectopic coding-DNA into a host organism, which provides the machinery required for correct protein synthesis (Giga-Hama and Kumagai 1999). There are number of different vector types available today such as plasmids, lambda phage, cosmids, phagmids or artificial chromosomes from bacteria, yeast or humans. Each has characteristics that may warrant consideration for certain tasks and they may be integrating or extrachromosomal (capable of autonomous replication). Generally, expression vectors possess the following attributes: an origin of replication-sequence that allows propagation in one or more organisms, a homologous promotor and terminator for efficient transcription of the foreign gene, selection marker(s) that allows maintenance of the vector in the host, and a polylinker to simplify the insertion of the heterologous/ectopic DNA (Romanos et al. 1992; Rai and Padh 2001; Figure 4.1). Obviously, much of the vector sequence, depends on the intended host(s). Artificial chromosomes require further host sequences coding for the centromeres and telomeres. Yeast and bacteria are eukaryotic and prokaryotic systems respectively that are commonly used as host organisms. *E. coli* is by far the most widely employed organism. It can maintain high growth rates with high levels of protein expression on relatively simple media and there is a vast body of knowledge about its genetics, physiology and completed genome. Its use has a number of drawbacks however and these tend to centre around the translation of DNA sequence, and the post-translation modification of proteins. In contrast, the eukaryotic secretory pathway is a high-fidelity system containing an array of foldases and chaperones to aid in correct protein folding and assembly (Smith and Robinson 2002).

Yeast is the favoured alternative host for expression. Again, yeast have the ability to grow rapidly on relatively simple media and much is known about their genetics. This has allowed the tailoring of certain species/strains in order to overcome limitations arising from their use (Brachmann et al. 1998; Müller et al. 1998). The budding yeast, *S. cerevisiae* is perhaps one of the best characterised yeast species (André 1995; Wittekindt et al. 1995; Catty et al. 1997; Daum et al. 1998; Winzeler et al. 1999; Van Belle and André 2001; Gomes et al. 2002). It has

been used for many years in research, industry and medicine. Completion of the genome in 1996 (Goffeau et al. 1996) paved the way for the construction of a variety of deletion strains that were auxotrophic for a number of nutrients. In many instances the yeast auxotrophy was compatible with existing vectors that could complement the mutation by possessing an identical and functional gene. This has allowed the selection, and maintenance of heterologous yeast cultures by growing them on minimal media lacking the relevant nutrient. Strain BY4741, produced by Brachmann et al. (1998) is auxotrophic for histidine, leucine, methionine and uracil (Brachmann et al. 1998). It contains non-reverting mutant alleles to give low background rates when carrying out transformations and has been mutated in order to reduce plasmid integration events (Brachmann et al. 1998). BY4741 is an ideal host for the vector p426 designed by Mumberg et al. (1994). This episomal vector has the (compatible) *URA3* marker gene and a 2 μ origin of replication (ORI) sequence which allows a high copy rate of the plasmid (Figure 4.1). It also possess the yeast *GAL1* promoter and *CYC1* terminator which provide strong and tight-regulation of the heterologous gene (Mumberg et al. 1994; Mumberg et al. 1995). These elements are normally involved in the regulation of galactokinase, an enzyme involved in galactose metabolism (Romanos 1992; Mumberg et al. 1994) and iso-1 cytochrome c, a small electron transport-protein of the inner mitochondrial membrane (Parrish et al. 2001). The promoter facilitates the docking of RNA polymerase to the DNA, whilst the terminator sequence initiates its detachment after a particular gene has been copied. This prevents the formation of long mRNA transcripts and allows maximal expression (Romanos 1992).

4.1.1 Responses of yeast to metals

The yeast *S. cerevisiae* has proven a powerful model for the investigation of metal uptake systems (Eide 1997; Radisky and Kaplan 1999; Gomes et al. 2002). Around 270 of its genes are predicted to code for established membrane transporters (Van Belle and André 2001). Many of these are similar to those variants found in other eukaryotes, such as plants and mammals and operate as part of similar mechanisms (André 1995; Mumberg et al. 1995; Winzeler et al. 1999). For example, the uptake and delivery of iron requires the involvement of ferric reductases and ion transporters (e.g. Nramps) as it does in mammalian cells (Yuan et al. 1995; Bassett et al. 1996; Eide 1997), whilst ZIP transporters have been implicated in the movement of Zn in both yeast and plants (Eide 1997; Guerinot and Eide 1999). In fact, yeasts appear to possess rather well developed mechanisms for

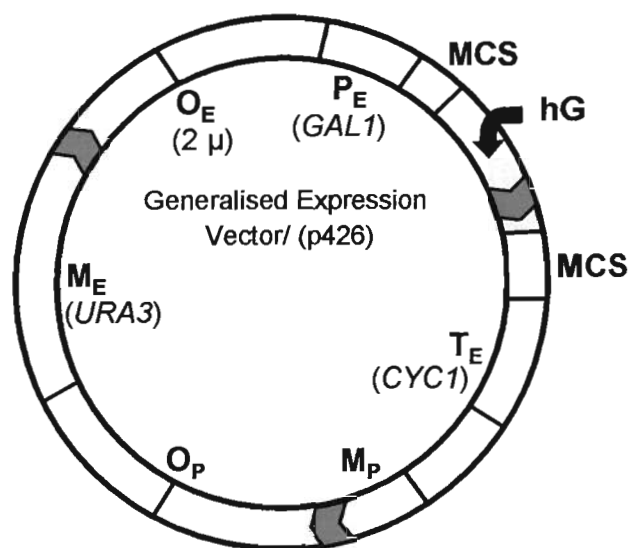


Figure 4.1 Schematic diagram of a typical shuttle vector for heterologous gene expression, with details given (bracketed) in italics for the p426 vector used in this work. MCS = multiple cloning sequence; O_p = bacterial origin of replication; M_p = marker gene for selection in bacteria; M_E = marker gene for selection in eukaryotes (*URA3* = *URA3* marker gene coding for uridine 5' -monophosphate); O_E = eukaryotic origin of replication (2μ = 2 micro origin of replication); P_E = eukaryotic promoter sequence (*GAL1* = promoter of galactokinase enzyme); T_E = eukaryotic terminator sequence (*CYC1* = terminator sequence for iso-1 cytochrome c protein); hG = heterologous gene for expression (Adapted from Rai and Padh 2001).

metal homeostasis (Gomes et al. 2002). They may resist elevated metal concentrations in a variety of ways such as repression of metal transporter-gene transcription and/or proteolytic degradation of metal transporters; chelation with intracellular ligands; and compartmentation of the metal-ion complexes. The presence of metal efflux systems analogous to those found in bacteria, have long been suspected to occur in yeast, however there have been few investigations into such phenomena (Krogh et al. 1998; Shiraishi et al. 2000, see below).

The yeast *S. cerevisiae*, strain BY4741 is an ideal candidate for studying the metal uptake characteristics of AtHMA1 and 4. Its genome codes for 16 P-type ATPases, but only two of these belong to the P_{1B} subgroup (Catty et al. 1997). CCC2 and PCA1 are related genes that bare strong resemblance to the human copper ATPases, ATP7Ap and ATP7Bp (Rad et al. 1994; Yuan et al. 1995). In yeast, these non-essential pumps have been associated the movement of Cu in pathways important to respiration (Yuan et al. 1995; Catty et al. 1997). The proposal that CCC2 mediates Cu import into an intracellular compartment has been supported by indirect evidence (Fu et al. 1995). Work on a mutated variant of PCA1, which occurs naturally in the X3382-3A strain of *S. cerevisiae* has shown that it may be located at the plasma membrane and involved in metal-ion efflux (Shiraishi et al. 2000). Rather interestingly this variant, known as CAD2, was shown to have altered substrate specificity and conferred resistance to Cd by reducing intracellular levels of this ion (Shiraishi et al. 2000). It is not clear if the mutations present in CAD2 affect localisation of the protein, but this work gives the greatest indication yet as to one of the possible roles undertaken by PCA1. In terms of the movement of metal ions across yeast membranes, no work has been presented to date, proving that either PCA1 or CCC2 function leads to appreciable reductions in the cytosolic content of any metal. This fact, coupled with the absence of dedicated Zn/Cd/Pb/Co HMAs in the *S. cerevisiae* genome, means that there are no primary heavy metal pumps present in this organism that may complicate the study of similar heterologous proteins from *Arabidopsis thaliana*.

AtHMA4 and *AtHMA1* cDNA were cloned into p426 a yeast/*E. coli* shuttle vector and transformed into the wild-type yeast reference strain BY4741 (Mills et al. 2003; Mills et al. in preparation). The yeast transformants were supplied for the experiments described in this chapter. The aim of the work here was to determine whether AtHMA4 and AtHMA1 could transport Cd and Zn. The approach taken was to compare the net uptake of radiolabelled Zn and Cd in *AtHMA*-transformants with that occurring in vector-transformed controls.

4.2 Results

4.2.1 The effect of Cd on the growth of *AtHMA1* and *AtHMA4* transformed yeast

Previous studies in this laboratory using the metal inhibition-zone method showed that of a range of metals screened (Cu, Zn Co, Ni, Mn, Cd), the most dramatic differences between the *AtHMA*-transformants and vector-only transformants was observed with Cd (Mills et al. 2003; Mills et al. in preparation). To support these results, similar experiments were carried out here but instead of using the metal-inhibition zone method (Persans et al. 2001), yeast transformants were spread on agar plates supplemented with different Cd concentrations. Vector transformed yeast was used as a control. The growth of transformed yeast was monitored on agar media supplemented with various levels of CdSO₄ (Figure 4.2). After two days incubation at 30 °C, similar levels of growth were observed for each of the transformants on media with no additional Cd (Figure 4.2). As the Cd additions increased the *AtHMA*-transformants and vector controls responded differently. Vector controls appeared to grow normally at 10 µM Cd but no growth was observed at higher levels. Cells transformed with *AtHMA1* were unable to grow on any of the Cd-supplemented media even at 10 µM Cd, where vector control cells grew well (Figure 4.2). Of all transformants tested *AtHMA4* transformants were most tolerant to Cd. Growth was observed over the entire Cd range tested (Figure 4.2).

4.2.2 Uptake of radiolabelled Cd and Zn in *AtHMA*-transformed yeast

The differential effects of Cd on growth observed in the previous experiment could be due to different transport characteristics of the *AtHMAs*. To investigate this, uptake of radiolabelled Cd and Zn was investigated in the *AtHMA*-transformants. Initial experiments were conducted to examine the growth of the different transformants which were to be used in the metal uptake assays. Nutrient requirement and metabolite production change significantly over a life cycle and so the growth phase must be carefully considered whenever microorganisms are used for heterologous gene expression (Romanos 1992). In accordance with this, the growth of the yeast transformants and gene induction was standardised throughout this work so the results generated were comparable. Since expression of heterologous proteins can be toxic to the host cell (Rai and Padh 2001) an examination of yeast growth was performed after HMA gene induction (Figure 4.3). The results showed that whilst slight variations in cell yield may occur after 8 hours

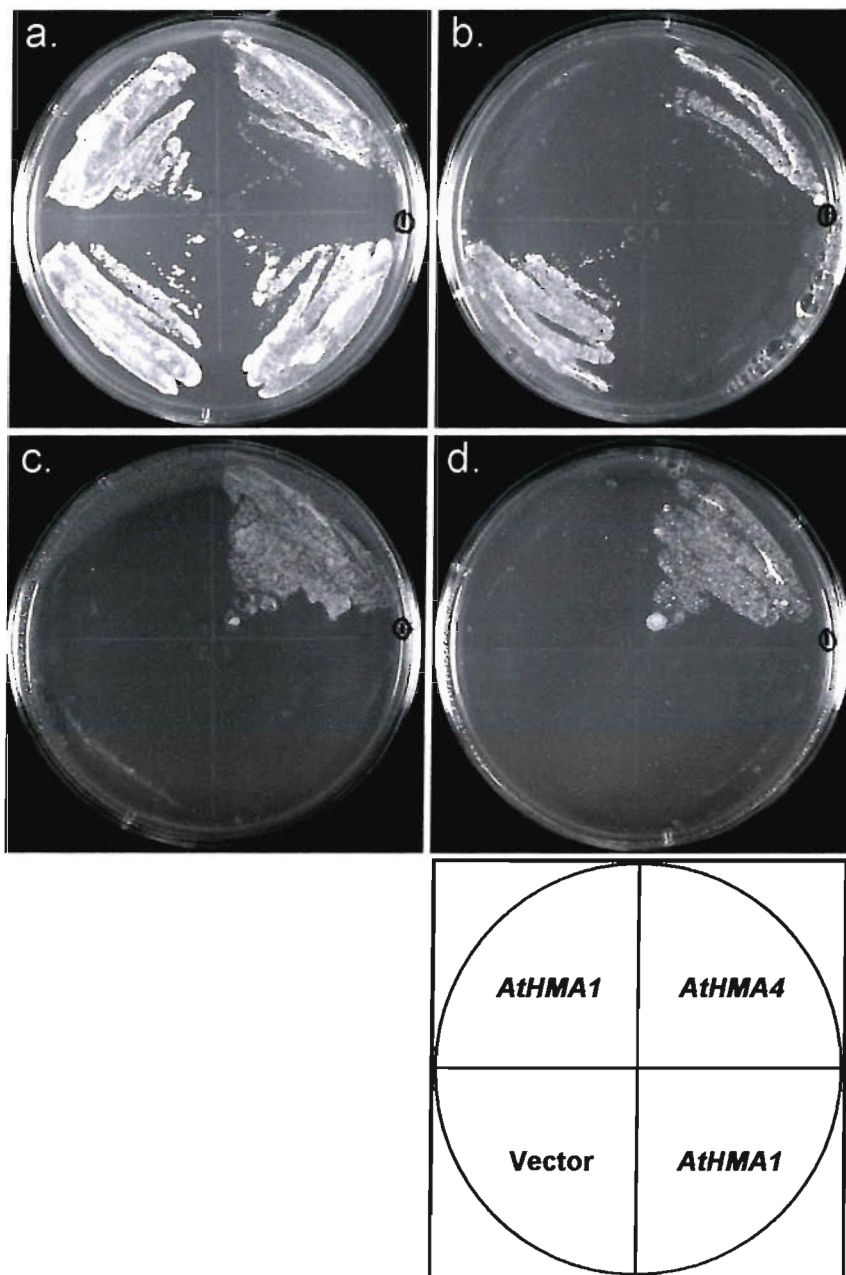


Figure 4.2 The effect of Cd on the growth of *AtHMA*-yeast transformants.

Plates containing a range of Cd concentrations (supplied as the sulphate salt) were streaked with BY4741 transformed with the p426 vector (vector control), *AtHMA1/p426* or *AtHMA4/p426*. a. 0 CdSO₄, b. 10 μM CdSO₄, c. 30 μM CdSO₄, d. 40 μM CdSO₄. Yeast were photographed after 2 days incubation at 30 °C. Results shown are a representative plate of four replicates.

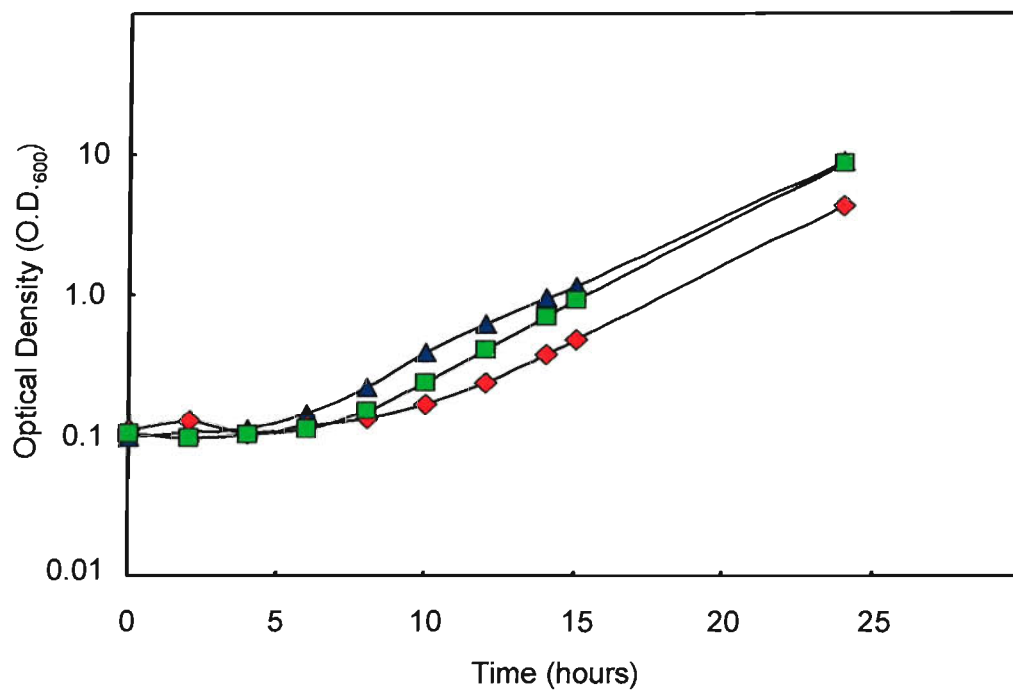


Figure 4.3 Growth of *AtHMA*-transformed yeast in Sc-galactose over 24 hours. Time course for the growth of *AtHMA4/p426* (♦), *AtHMA1/p426* (▲) and vector control-transformed (■) BY4741 yeast. Results are expressed as optical density measured at 600 nm. Data are the means of 2 replicates.

growth, the growth rate and life-cycle timing was fairly uniform between *AtHMA1*, *AtHMA4* and vector-only transformants (Figure 4.3). At 24 hours, none of the cells appeared to have entered their stationary phase, suggesting that nutrient supply and growth conditions were still optimal. At 8 - 10 hours each transformant had entered the logarithmic phase of growth. It is during this stage that cell division, metabolic activity and heterologous protein expression (production) are likely to be at their maximal rate (Romanos 1992; Rai and Padh 2001). Considering this, the 12 hour time point was chosen as the harvesting point for cells used in the radiolabelled uptake assay. Any differences in total cell yield were corrected for prior to use.

4.2.2.1 Time course for radiolabelled Cd and Zn uptake in *AtHMA*-transformants

The time dependent uptake of Cd and Zn observed for vector-transformed yeast and *AtHMA*-transformants is shown in Figures 4.4 and 4.5 and the uptake rates for each are shown in Figures 4.6 and 4.7. For Cd, the time dependent uptake was nearly identical for *AtHMA1* and vector control cells when expressed on a fresh weight or protein basis (Figure 4.4a,b). In contrast, the uptake pattern displayed by *AtHMA4*-transformants in these experiments was markedly different, with lower accumulation levels at all time points. This was significant ($P < 0.05$) at the 12 to 30 minute time points when expressed on a protein or fresh-weight basis (Figure 4.4a,b).

For Zn, differences were observed in the time-dependent uptake when the results were expressed as fresh weight or on a protein basis (Figure 4.5a,b). When expressed on a cell weight basis, the uptake patterns observed were quite similar to those for Cd; vector and *AtHMA1*-transformants showed similar accumulation at each time point, whilst the *AtHMA4*-transformants displayed a lower accumulation (Figure 4.5a). At 12 minutes and above the levels of Zn uptake by *AtHMA4*-transformants were notably less than the other transformants although the differences were not significant ($P > 0.05$). When expressed on a protein basis *AtHMA4* accumulation appeared more similar to the vector-only controls. Although at the later time points the values were lower, they were not significantly different ($P > 0.05$) (Figure 4.5b). For *AtHMA1*, results expressed on a protein basis showed Zn accumulation to be higher than the vector control and at some points significantly so ($P < 0.05$). In order to test the differences observed more rigorously, a single time point was chosen and metal accumulation studied with greater repetition of sampling. Analysis of metal uptake was carried out using relevant data from the time

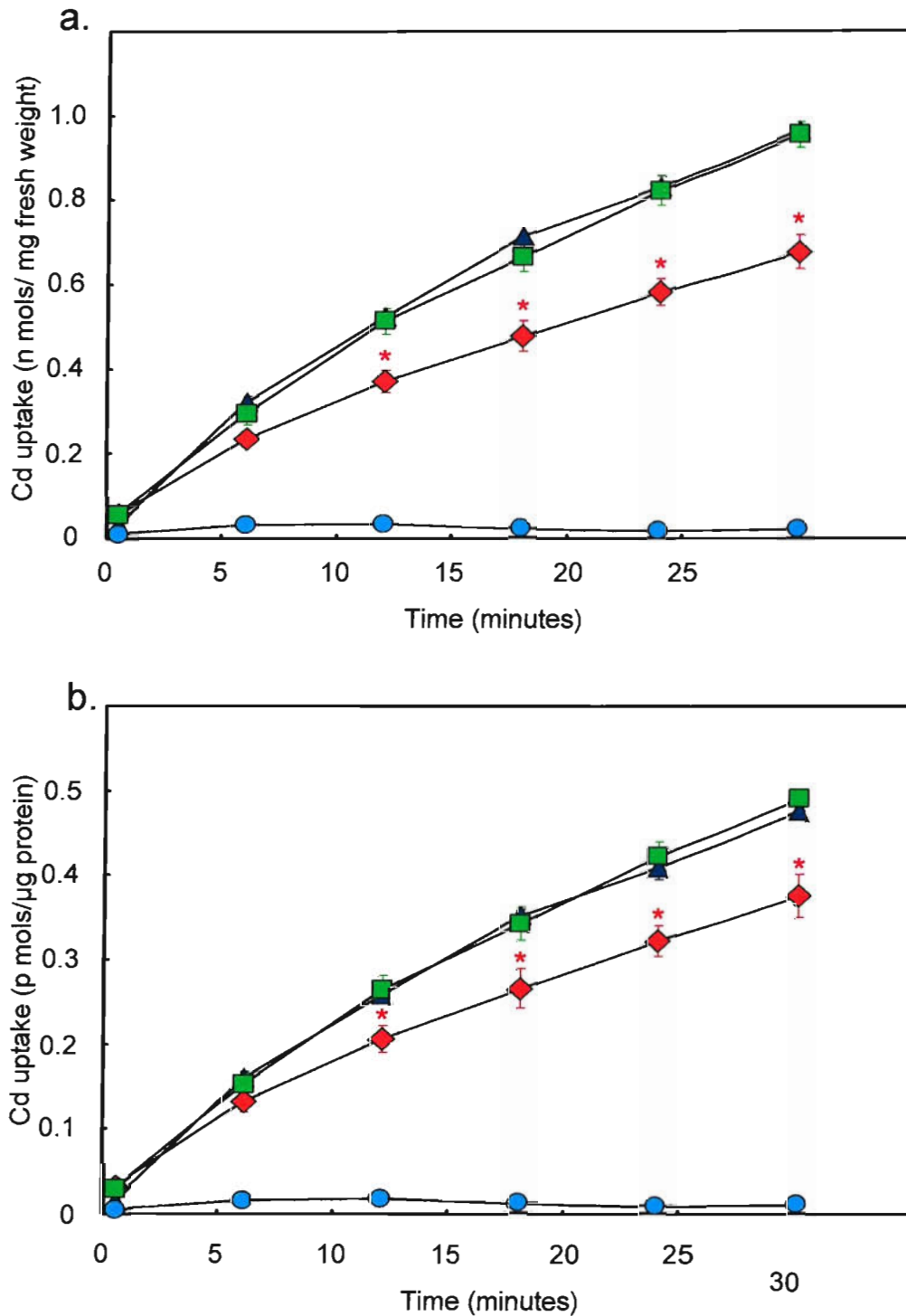


Figure 4.4 Uptake of Cd in *AtHMA*-transformed yeast

Time course for the uptake of radiolabelled Cd (^{109}Cd) in *AtHMA4/p426* transformed BY4741 yeast (♦), *AtHMA1/p426* transformed BY4741 yeast (▲), vector control-transformed BY4741 (■) and vector control-transformed BY4741 on ice (●). Results are expressed on a fresh weight basis (a) or on a protein basis (b). Data are the means \pm SE of 3 replicated experiments. Students t-test, * indicates significant difference from the vector-transformed controls ($P < 0.05$).

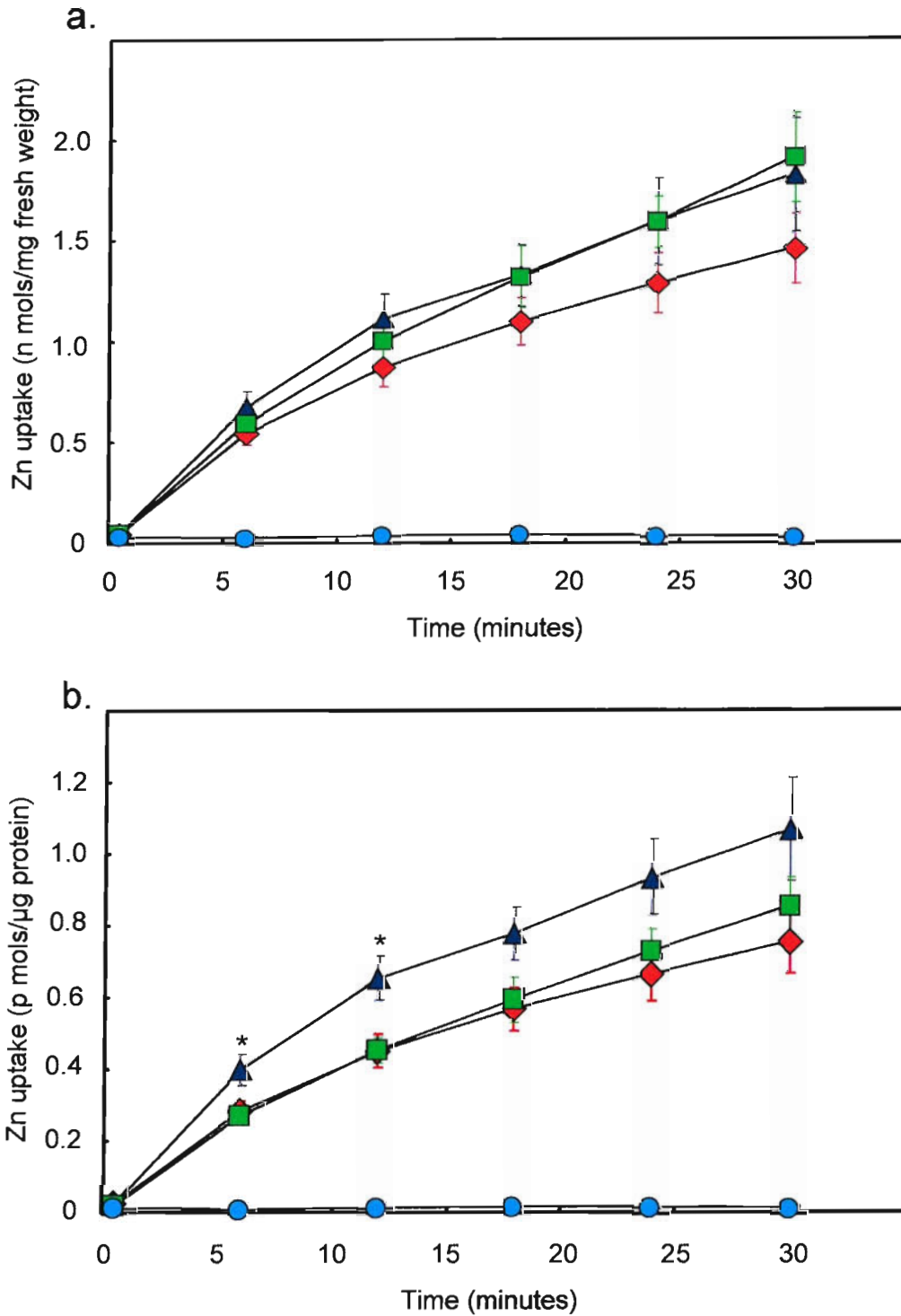


Figure 4.5 Uptake of Zn in *AtHMA*-transformed yeast

Time course for the uptake of radiolabelled Zn (^{65}Zn) in *AtHMA4/p426* transformed BY4741 yeast (\blacklozenge), *AtHMA1/p426* transformed BY4741 yeast (\blacktriangle), vector control-transformed BY4741 (\blacksquare) and vector control-transformed BY4741 on ice (\bullet). Results are expressed on a fresh weight basis (a) or on a protein basis (b). Data are the means \pm SE of 4 replicated experiments. Students t-test, * indicates significant difference from the vector-transformed controls ($P < 0.05$).

course work (Figures 4.4 and 4.5) plus additional data generated by further experiments measuring levels at 12 minutes. With increased replication, Cd accumulation by *AtHMA4*-transformants was again significantly lower ($P < 0.05$) than the vector-only controls, when expressed as either fresh weight or a protein basis (Figure 4.6). There was no significant difference in the accumulation of Cd in vector controls and *AtHMA1*-transformants although the accumulation in *AtHMA1*-transformants was slightly higher. For Zn, the data expressed both on a fresh weight and protein basis showed that *AtHMA4*-transformants accumulated significantly less Zn ($P < 0.05$), than the vector transformed controls (Figure 4.7a,b). *AtHMA1*-transformants accumulated significantly more Zn than vector control cells when expressed on both a fresh weight and protein basis ($P < 0.05$).

4.2.2.2 The effect of Zn on Cd-accumulation in *AtHMA4*-transformed yeast

Previous work in this laboratory indicated that expression of *AtHMA1* and 4 in wild-type yeast has no effect on the susceptibility of yeast to high concentrations of Zn (Mills et al. 2003, Mills et al. in preparation). In addition, neither *AtHMA1* or 4 rescued the zinc-sensitive phenotype of the *zrc1* mutant (Mills unpublished). However competition experiments conducted using the metal-inhibition zone method indicated that when Zn was supplied with Cd, it completely abolished the *AtHMA4*-conferred resistance to Cd and the *AtHMA1* conferred sensitivity of wild-type yeast (Mills et al. in preparation). In order to investigate this further in the yeast system, uptake of radiolabelled Cd by *AtHMA4*-transformants was monitored in media supplemented with various levels of Zn (Figure 4.8). Cd accumulation was determined after 30 minutes incubation and the results compared to vector control cells. Results expressed on a fresh weight and protein basis, showed that the effect of increasing concentrations of Zn was nearly identical in each case (Figure 4.8a,b). In both instances, increasing Zn in the media substantially reduced the amount of Cd accumulated by the *AtHMA4*-transformants and the vector control cells. Zinc reduced the accumulation of radiolabelled Cd, and at the concentrations tested the levels of Cd uptake were very similar in *AtHMA4* and vector-only transformed yeast (Figure 4.8).

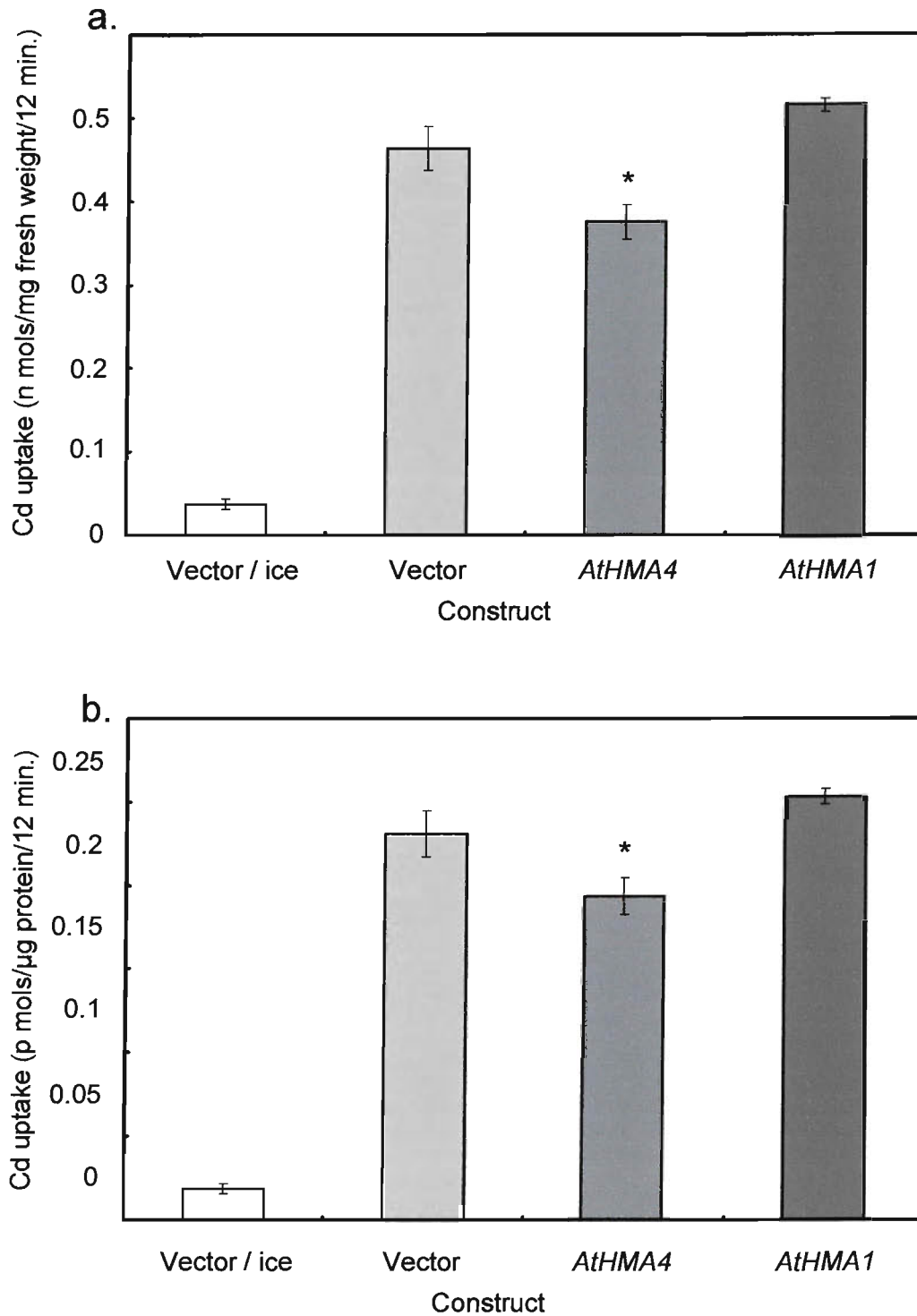


Figure 4.6 Cd uptake rates in *AtHMA*-transformed yeast

Uptake rate of radiolabelled Cd (^{109}Cd) in *AtHMA4/p426*-transformed BY4741 yeast (*AtHMA4*), *AtHMA1/p426*-transformed BY4741 (*AtHMA1*), vector control-transformed BY4741 (vector) and vector control-transformed BY4741 on ice (vector/ice); data are the means \pm SE of at least 4 replicated experiments. Students t-test, * indicates significant difference from the vector-transformed controls ($P < 0.05$).

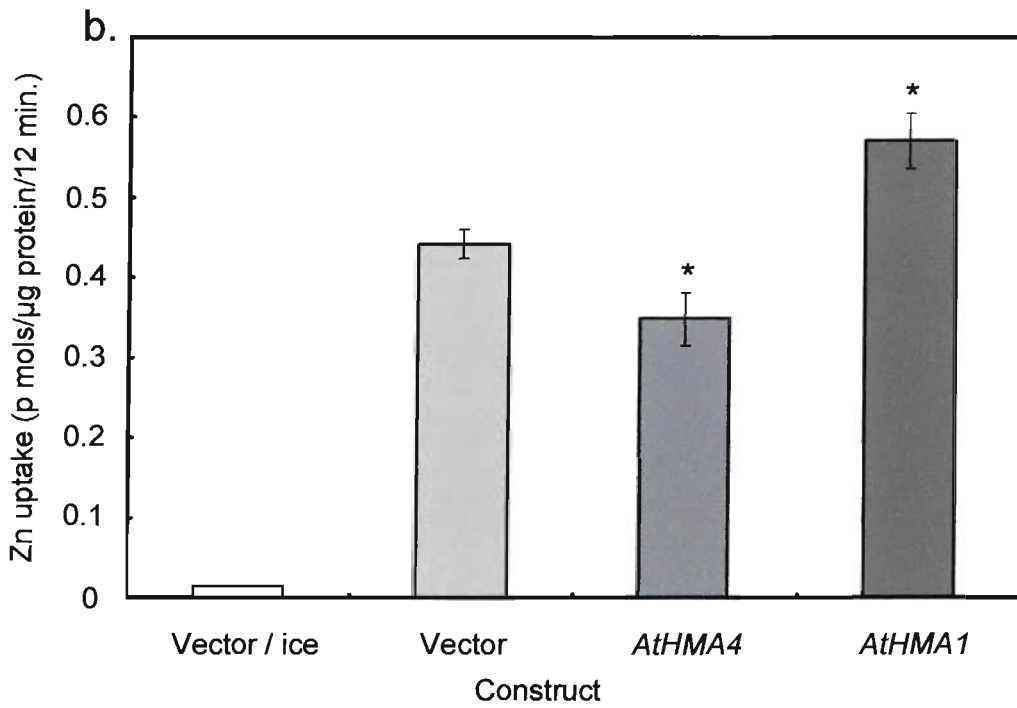
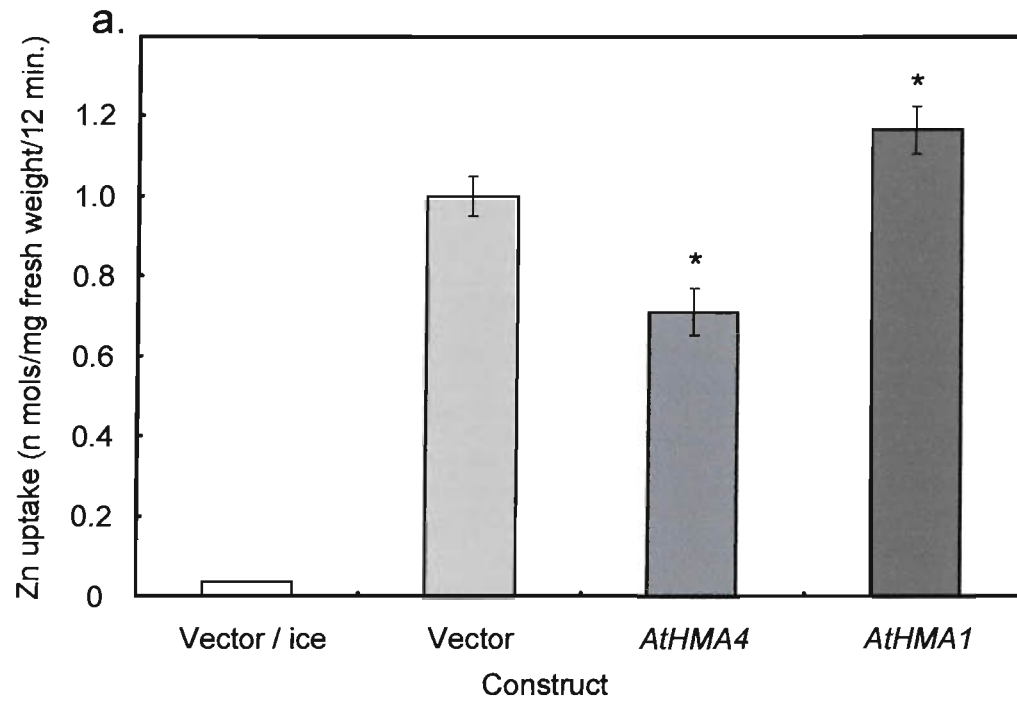


Figure 4.7 Zn uptake rates in *AtHMA*-transformed yeast

Uptake rate of radiolabelled Zn (^{65}Zn) in *AtHMA4/p426*-transformed BY4741 yeast (*AtHMA4*), *AtHMA1/p426*-transformed BY4741 (*AtHMA1*), vector control-transformed BY4741 (vector) and vector control-transformed BY4741 on ice (vector/ice); data are the means \pm SE of 7 replicated experiments. Students t-test, * indicates significant difference from the vector-transformed controls ($P < 0.05$).

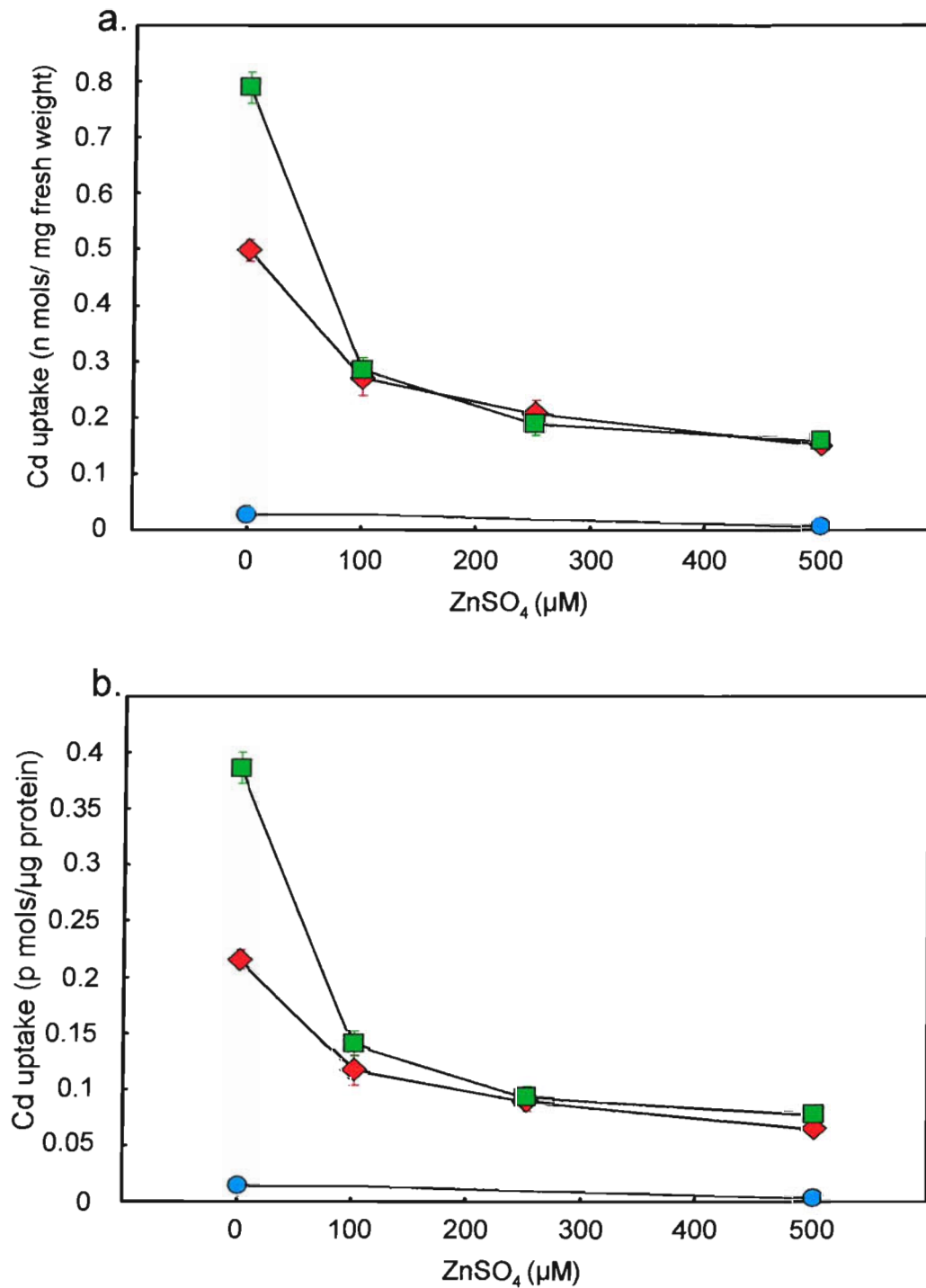


Figure 4.8 The effect of Zn on the uptake of Cd in *AtHMA*-transformed yeast.

Uptake of radiolabelled Cd (¹⁰⁹Cd) measured at 30 minutes in *AtHMA4/p426* transformed BY4741 yeast (♦), vector control-transformed BY4741 (■) and vector control-transformed BY4741 on ice (●) Uptake was measured in the presence of ZnSO₄ concentrations indicated. Results are expressed on a fresh weight basis (a) or on a protein basis (b). Data are the means ± SE of 3 replicate samples.

4.3 Discussion

The results presented in this chapter suggest that the heterologous expression of *AtHMA1* and *AtHMA4* has a significant impact on metal ion homeostasis in the host organism *S. cerevisiae*. In general, *AtHMA4* transformants accumulated less Cd and Zn than vector controls. *AtHMA1* transformants generally had slightly higher levels of Cd and Zn than vector controls but the values were not always significant. Four different metal homeostatic mechanisms have been shown to operate in yeast. These are the regulation of uptake and efflux across the plasma membrane, internal compartmentation and/or sequestration with ligands such as metallothionein or phytochelatins (Shirashi et al. 2000). Since the chelation of metal ions within the cell only affects the free-ion concentration and not the burden of the cell *per se*, it seems likely that the *AtHMAs* are exerting their effect(s) by modifying or contributing to one or more of the other homeostatic mechanisms.

4.3.1 The effect of *AtHMA4* expression on yeast metal homeostasis

The fact that *AtHMA4* promotes lower uptake of radiolabelled Cd into yeast suggests that this protein may be localised at the plasma-membrane and involved in metal ion efflux. The increased tolerance of *AtHMA4*-yeast transformants to Cd on metal-supplemented media supports this theory. The results with Zn are less conclusive. *AtHMA4* failed to confer Zn resistance to yeast, although higher concentrations were used compared to Cd (Mills et al. 2003). However Zn uptake rates were lower for *AtHMA4* transformants and this was significant in the 12 minute uptake experiments. The discovery by Mills et al. (in preparation) that Zn can abolish the Cd resistance of *AtHMA4*-transformed yeast could suggest that the metals compete for association with the transporter protein. The competition-study carried out as part of the radiolabelled uptake assay (Figure 4.8) shows that increasing Zn concentrations in the external medium reduces the amount of radiolabelled Cd accumulated in yeast. This is in contrast somewhat with the yeast tolerance assay of Mills et al. (in preparation) as reduced Cd uptake would be expected to increase resistance, not abolish it as was found on the Zn/Cd adjusted agar. The reasons for these effects are unclear. There have been a number of reports on the binding of *HMA*s with different metal ions. Some have been shown to bind with appreciable affinity to metals not thought to be the primary substrate (DiDonato et al. 1997) and others, have indicated that transport of a range of metal ions sometimes differing in ionic charge, can occur (Havelaar et al. 1998; Riggle and Kumamoto 2000). The

transport capabilities of individual HMAs for both Cd^{2+} and Zn^{2+} are more well known (e.g. *ZntA* of *E. coli*: Sharma et al. 2000), so it is possible some competing effect may have occurred. In this case the results do not appear to be HMA-specific, since reductions in Cd uptake was observed in both *AtHMA4* and vector-only transformants. The impact of Zn also appears to be on a site of uptake in yeast. If *AtHMA4* participated in efflux at the cytoplasm (as proposed), competition of Zn with Cd would be expected to increase levels of cellular Cd, not the reduction seen. Perhaps differences in the levels of metals used in the radiolabelled uptake and yeast tolerance assay work of Mills et al. (in preparation) can explain the variation in the results. Other aspects of the growth medium may also be important, and it is possible that ZnSO_4 may cause precipitation of Cd in the radiolabeled growth medium and have reduced uptake by influencing the availability of the free metal to other uptake systems.

Despite the intricacies of the Zn competition data, the radioactive uptake assays show that the levels of Cd and Zn were both reduced in the transformed yeast. As mentioned previously, this provides strong evidence for the efflux function of *AtHMA4*. To date no evidence has been produced to show a plasma-membrane efflux mechanism for either Zn or Cd in wild type yeast (Krogh et al. 1998; Shiraishi et al. 2000). Regulation of cytoplasmic levels appears to be by shuttling of the ion (e.g. ZRC1 and Zn/Cd movement), or ion-chelate in the case of Cd (e.g. movement of Cd-GSH by the ABC transporter, Ycf1), into internal compartments (Ortiz et al. 1992; Ortiz et al. 1995; Rea 1999). In plants, similar systems operate, but again there is little evidence to suggest that plasma membrane efflux is an important mechanism of metal ion homeostasis (Chardonens et al. 1999; Clemens 2001; Briat and Leburn 1999). Van Hoof et al. (2001) have pointed to the existence of a potential ATP-driven, Cu efflux pump in plant root cells so the possibility exists for a compatriate that functions in the transport of Cd/Zn.

4.3.2 The effect of *AtHMA1* expression on yeast metal homeostasis

For *AtHMA1*, the results appear to directly contrast those of *AtHMA4*. On metal-supplemented agar *AtHMA1* transformants were more sensitive to Cd than vector control cells at all concentrations tested. Zn failed to have an effect on growth when tested on *AtHMA1* transformants using the zone inhibition method (Mills et al. in preparation). Much higher concentrations of Zn are required to observe growth inhibition of wild-type cells, indicating that Cd is much more toxic than Zn. This is supported by tests on *AtHMA1* transformants. The radiolabelled assay demonstrated

that only Zn was accumulated at significantly higher levels than wild-type control cells, yet in tolerance tests only Cd inhibited the growth of *AtHMA1* transformants.

When *AtHMA1*-transformants were analysed over a time course, the accumulation of Zn was similar or greater than the vector control cells depending on how the results were expressed. When expressed on protein basis, Zn accumulation was higher from the 6 to 30 minute time points, but the differences were only significant at 6 and 12 minutes (Figure 4.5b). For Cd, no differences were observed, and the results were very similar to the vector controls. With increased replication of the 12 minute sampling, both the protein corrected and fresh weight data showed that accumulation of Zn was significantly greater than the vector controls. In the case of Cd, the uptake rate was slightly higher in the *AtHMA1*-transformed cells but not significantly more than the vector controls.

The increased sensitivity to Cd and higher influx of radiolabelled Zn and Cd in transformants suggests that *AtHMA1* mediates either uptake into the cytoplasm across the plasma membrane or into an internal compartment or intracellular organelle where Cd is toxic. Evidence from a wide variety of organisms suggests that the HMAs are unlikely to be involved in the uptake of metals across the plasma membrane (LaGier et al. 2001; Argüello 2003), so less emphasis can be placed on this scenario. Metabolically-dependent uptake of both Zn and Cd has been observed in wild type yeast (Gomes et al. 2002; Krogh et al. 1998), but transport of these ions is more likely to be mediated by proteins such as the ZIPs and Nramps (Chen et al. 1999; Portnoy et al. 2000; Gomes 2002). These transporters are perhaps more suited to an influx function since they appear to be less restricted in their choice of substrate ion and they may be energetically favoured in locations (such as the plasma-membrane) where there are H⁺ pumps to stimulate secondary active-transport processes (Guerinot and Eide 1999; Thomine 2000; Mäser et al. 2001).

AtHMA1 has a putative mitochondrial-targeting signal at its amino terminus (PSORT analysis; psort.nibb.ac.jp/). It is possible that this may be functional within the yeast (Heins et al. 1994), but correct localisation to this organelle appears less likely than it would elsewhere in the cell (Frommer and Ninnemann 1995). Heterologously expressed proteins can often become trapped inside internal membranes such as golgi vesicles or endoplasmic reticulum (Dreyer et al. 1999; Luo et al. 1999). The tonoplast also serves as a common default location when the targeting fails in yeast (Frommer and Ninnemann 1995). If incorrectly targeted, *AtHMA1* expression may indeed result in slightly higher uptake as observed here. Concentrations of Zn and Cd in the cytosol/cytoplasm would be reduced, and further uptake at the plasma membrane stimulated. Plants that hyperaccumulate metals

commonly use the vacuole as an intracellular storage site (Chardonens et al. 1999; Hall 2002) and yeast also use it in the detoxification of certain metals (Ortiz et al. 1992; Ortiz et al. 1995). If this was the case however, then it may be expected that the AtHMA1-transformants would be more tolerant to increased levels of Zn or Cd. However, as they are more sensitive, it therefore appears that mitochondrial targeting may be correct.

If AtHMA1 is normally located at the mitochondrial membrane, then the radioactive uptake suggests that it may function in the loading of this compartment with certain metals. If so, AtHMA1 would be one of a small number of HMAs that have been found to perform a metal import function. Generally, most HMAs appear to be involved in the efflux of metals out of the cytoplasm (LaGier et al. 2001), but there are a growing number that have been assigned import functions. Zn is imported at the plasma membrane in *Bacillus subtilis* by the transporter ZosA and appears to be induced by oxidative stress (Gaballa and Helmann 2002). In *Synechococcus* PCC 7942 Cu influx occurs at both the plasma and thylakoid membranes by the function of HMA proteins ctaA and pacS (Kanamaru et al. 1994; Phung et al. 1994; Tottey et al. 2001). This organism has demonstrated the importance of HMAs in the supply of metals to organelles where they may be required as cofactors for essential enzymes or pigments (Tottey et al. 2001). In eukaryotes a similar function has been assigned to PAA1. PAA1 has recently been found to transport copper into the chloroplasts of *Arabidopsis thaliana* (Shikanai et al. 2003), so HMA organelle import-proteins do exist in this organism. Interestingly AtHMA1 is similar to PAA1 in that it possesses its putative metal binding domains in the N-terminus of the protein. It is not known if this has any impact on the orientation of the polypeptide within the membrane, but it is worth pointing out that AtHMA4 which is thought to have an efflux function, has its metal binding domains in the C-terminal domain. At present, the metal ion transported and the direction of transport cannot be predicted from the sequence of a HMA transporter (Tottey et al. 2001).

Over-accumulation of metals in mitochondria could disrupt the function of enzymatic pathways involved in yeast respiration. In the plant, increased levels of heavy metals in the mitochondria are also likely to present toxicity problems, but there is a requirement for certain metal ions as catalytic centres for proteins in both organisms. Zn is specifically required in the Cu/Zn-superoxide dismutase enzyme (SOD) present in the chloroplast and mitochondria of both yeast and plants (Emig et al. 1998; Sturtz et al. 2001). These enzymes are crucial in protecting tissues against the oxidative damage caused by superoxide anions, generated in abundance at sites such as the chloroplasts, mitochondria and peroxisomes (McKersie et al. 1993;

Alscher et al. 2002). Work on SOD1 in yeast has highlighted the importance of correct metal supply in mitochondria (Sturtz et al. 2001). When expression of the CCS copper chaperone localised in the intermembrane space of mitochondria was suppressed, SOD1 accumulation was also reduced (Sturtz et al. 2001). Both Zn and Cu deficiency affect the levels of SOD1 activity in plants, and lead to increased oxidative damage (Yu and Rengel 1999). The uptake results of the transformed yeast could suggest that AtHMA1 has a similar role, loading the Zn component of this enzyme in mitochondria.

The fact that Zn, but not Cd was accumulated at significantly higher concentrations than control cells (12 minute time period; Figure 4.7) could be a result of experimental conditions. In order to avoid problems of toxicity, Cd was used at half the concentration of Zn. In order to test for the preferred substrate of AtHMA1, experiments to examine the K_m of the two metal ions would be necessary. It may be postulated that movement of Zn would be favoured, since transport of Cd ions into the mitochondria is likely to be detrimental to metabolism, but this may not necessarily be the case. The ATP-dependent uptake of toxic metal ions is known to occur in organisms (e.g. Park et al. 2003) and the ability of HMAs to transport such ions is well known. Work by Tottey et al. (2002) has confirmed that toxic metal ions may indeed be taken up by import HMAs. Deletion of *pacS* in *Synechococcus* PCC 6803 conferred Cu sensitivity but also Ag resistance, consistent with uptake of the toxic metal (Tottey et al. 2002).

4.3.3 Heterologous expression and protein targeting

The use of heterologous expression systems assumes that the basic principles of protein expression and function are similar in all organisms (Frommer and Ninnemann 1995). The principles of targeting seem to be conserved between organisms (Bennett and Scheller 1993), but when factors necessary for the correct assembly of the heterologous protein are absent or different in yeast, mis-targeting can occur (Frommer and Ninnemann 1995).

There are a number of examples where the targeting of plant proteins appears to be consistent when expressed in yeast. These include polypeptides that are normally targeted to the plasma membrane (Shibagaki and Grossman 2004), the vacuole (e.g. Yamaguchi et al. 2003), and post-golgi vesicles (Hirayama et al. 1999). Indeed some ectopic proteins even appear to be correctly regulated in response to their normal stimuli. Such was found to be the case for the Zn-dependent ZIP1 transporter protein (Guerinot and Eide 1999). For other proteins there has been less

success. In a similar study of the Zn uptake transporters *AtZIPs 1-4*, *ZIP 4* was the only member not to confer Zn uptake activity in yeast (Grotz et al. 1998). The cause of this was believed to be a result of improper targeting, since *ZIP 4* is the only member known to possess a putative chloroplastic targeting sequence (Guerinot and Eide 1999). These sequences are obviously unrecognisable in yeast, an organism that does not possess this organelle. However it is possible that *AtZIP 4* is not a Zn transporter. For the *Arabidopsis ZAT1* CDF transporter, expression in yeast was problematic for different reasons. The protein was believed to have localised correctly to the vacuole, but it could not function optimally (Bloß et al. 2002). Different proton gradients and substrate concentrations occurring in the yeast cell were thought to be causative factors (Bloß et al. 2002). For ATPases such as the *AtHMAs*, these issues are likely to be less important since transport does not rely on cation/anion gradients.

The incorrect processing of ATPase proteins has also been reported in the past. Palmgren and Christensen (1994) cloned the *Arabidopsis* H⁺ ATPase *AHA2* into *S. cerevisiae* and reported that a large proportion of the functional protein remained trapped within the endoplasmic reticulum. This species of yeast is known to have a golgi apparatus that has poor morphological organisation (Giga-Hama and Kumagai 1999). Nevertheless, the correct targeting and function of *AtHMA3*, a HM-ATPase closely related to *AtHMA4* has recently been demonstrated in this yeast species (Gravot et al. 2004).

The evidence presented in this chapter suggests that *AtHMA4* acts as an efflux pump and is located at the plasma membrane in yeast. Therefore it may be that this transporter is involved in the removal of toxic levels of Cd and Zn from the plant cell to the exterior. Under normal physiological conditions *AtHMA4* could help maintain a healthy balance of Zn ions, however under adverse edaphic conditions, it would preferentially remove the more toxic, Cd ions. These may enter the cells via unspecific transport mechanisms or as a result of membrane damage. For *AtHMA1*, the Cd-sensitivity results support the theory that this transporter may be located at a sensitive compartment and the targeting motif would suggest localisation to the mitochondria. Zn may be the physiological substrate under normal conditions. *AtHMA1* could function in supplying important enzymes such as Cu/ZnSOD, which is known to exist in plant mitochondria (Emig et al. 1998). It would be especially interesting to test Cu/ZnSOD activity in the *AtHMA1* knock-out mutants of *Arabidopsis thaliana*.

Chapter 5 Analysis of *AtHMA4* and *AtHMA1* gene expression

5.1 Introduction

Many aspects of plant metabolism and development are precisely coordinated and regulated through transcription and translation of different gene products in each cell (Zhu et al. 2001). Metal ion homeostasis is no exception and the levels of various essential free ions have to be delicately balanced within a limited optimal range. As a result, the mechanisms of control have to be subjected to the strictest regulation. Often, this regulation occurs at the most basic level, via the transcriptional control of gene expression (Xu and Rosen 1999; Clemens 2001; Donson et al. 2002). Since it is claimed that mRNA transcript levels correlate with the protein level for most genes (Zhu et al. 2001) initial assessments into gene function can often be carried out by utilising expression analysis techniques (Zhu et al. 2001). Methods such as cDNA-AFLP, and more recently cDNA microarrays have been used to screen expression patterns of large numbers of genes simultaneously (transcription profiling) in an attempt to pinpoint genes that may be important in different tissues, at different time points, or in response to different stimuli (Aharoni and Vorst 2002; Clemens 2001; Heidenreich et al. 2001; Zhu et al. 2001; Donson et al. 2002). For putative HMAs, where a transport function has already been implied, expression analysis can be used to gain a more specific insight into their role. For example, specific expression patterns may hint at associations with particular organs or tissue types, whilst responses to certain metal treatments may highlight potential substrates of transport. Of course, information gained in this way should be treated with a certain level of caution. Expression of transporters can be affected by ions that are not true substrates (Stoyanov and Brown 2003) and there are numerous facets of protein regulation, many of which we are only beginning to understand (Donson et al. 2002).

Whilst the control of gene expression at the level of transcription is the dominant form of regulation in bacteria, it is also very common in eukaryotes such as plants (Dong et al. 1999; Lloyd et al. 2001; Donson et al. 2002). This is demonstrated in mutated organisms, which display severe developmental abnormalities or disease as a result of defective transcriptional control mechanisms (Binet and Poole 2000; Gill 2001). The transcriptional control of gene expression can be achieved at two main points in the process: by regulating transcription initiation or transcript elongation (Lloyd et al. 2001). A third, and closely related mechanism, whereby mRNA stability is modified can also be considered (Lloyd et

al. 2001). This falls under the definition of post-transcriptional control, but can be identified in many expression analysis techniques, such as RT-PCR.

In most instances, the control of transcription initiation will probably have the largest influence on transcript abundance within an organism. Metals can affect this process in three main ways (Blanchard and Cousins 1997). The first two mechanisms are general, whereby metals influence either the DNA binding properties of various proteins, or the activity of certain enzymes. The third is more specific whereby occupancy of a *trans*-acting protein modulates transcription of a specific gene (Blanchard and Cousins 1997). These proteins, known as metalloregulatory proteins have the ability to bind to DNA as well as bind a limited number of ions at conserved metal-binding domains (Xu and Rosen 1999). Metal binding initiates a conformational change, which alters the property of the protein and triggers a response. This may have a positive or negative effect on the transcription process (Lloyd et al. 2001) depending on the protein(s) and gene(s) concerned. A general summary of their action is given in Table 5.1.

An important aspect in the regulation of genes coding for HMAs is the types of metal ions influencing expression. Obviously if transcriptional regulation is important, the substrate ion(s) is likely to play an important part in this. The HMAs may participate in a number of mechanisms involving the influx or efflux of metal ions. Failure to respond to fluctuating levels of substrate could therefore be detrimental, as adverse conditions could promote deficiency or toxicity symptoms. However, the regulatory proteins that influence expression can often react with a number of different ions (Sun et al. 2001). In some instances the expression of metal transporter genes can be influenced by ions that they do not transport (Binet and Poole 2000; Stoyanov and Brown 2003). The work in the following chapter aims to create a profile of expression for *AtHMA4*, *AtHMA1* and some additional HMAs in order to gain a clearer insight into their function in plants. The distribution of transcripts in specific tissues is examined along with their responses to different metal/metal-chelator treatments. Protein expression is also investigated in an effort to determine the influence of post-transcriptional regulatory mechanisms. Together, these approaches may help highlight the potential significance of plant HMAs and the specific conditions in which they may be required.

Table 5.1 Summary of the types and modes of action of various metalloregulatory proteins in different organisms.

Type of regulator	Mode of action on target gene	Effect	Example
Metalloactivator	Protein binds to DNA in promoter region and conformation change makes it more accessible to RNA polymerase*	Enhances expression	CueR regulation of <i>CopA</i> in <i>E. coli</i> (Stoyanov et al. 2001)
Metallorepressor	Protein binds to DNA in promoter region preventing access to RNA polymerase	Reduces expression	CopY regulation of Cu-ATPases in <i>E. hirae</i> (Solioz and Stoyanov 2003)
Combined function regulator	Protein normally attached to promoter region acting as a repressor. Metal binding changes conformation, so protein acts as an activator	Dual regulation - dependent on metal status	MerR regulation of Mer operon in various gram negative bacteria (Brown et al. 2003)

*Metalloactivators may also work by contacting with the RNA polymerase enzyme and recruit docking at the promoter. Other mechanisms are also thought to occur although they are not fully understood.

5.2 Results

In order to assess certain aspects of HMA regulation, transcript and protein abundance will be examined in different plant organs taken from plants grown under different conditions of metal supply. Transcript abundance will be monitored using two techniques: semi-quantitative RT-PCR and northern analysis. *AtHMA4* and *AtHMA1* will be examined further by probing western-blot with protein-specific antibodies. The range of techniques chosen are well established, and used in conjunction have the ability to detect HMA regulation at three levels: transcription, translation and protein-degradation.

5.2.1 Analysis of *AtHMA4* and *AtHMA1* expression using semi-quantitative RT-PCR

5.2.1.1 Primer selection

Oligonucleotide primer pairs with suitable characteristics were designed for use in RT-PCR based expression analysis. Primers with a high target-gene specificity, low self-complementarity and compatible annealing temperatures were desired along with the ability to yield cDNA and gDNA products of different size. For *AtHMA1*, previous work in the laboratory had demonstrated the successful use of primers *AtHMA1*-forward(out) and *AtHMA1*-reverse(nested). These are complimentary to regions in 5' UTR and exon 5 and are predicted to amplify a product of 936 bp in cDNA and 1461 bp when the sequence is interrupted by the 4 introns occurring in genomic DNA (Figure 5.1). Selection of suitable primer pairs for use in the amplification of *AtHMA4* was carried out by testing various different combinations on *Arabidopsis* cDNA. Initially the forwards-nested/reverse-out primers were highlighted as potential candidates for use in expression studies (Figure 5.2) however several later repeats on soil-grown leaf cDNA indicated that these primers had a tendency to produce inconsistent results (data not shown). Subsequent PCRs with the previously untested forward-nested/reverse-nested primer pair revealed that this combination gave successful reactions when tested on a number of different templates (Figure 5.3). The primer binding sites, located in exons 3 and 5 of *AtHMA4* are separated by 2 introns in the 594 bp genomic DNA sequence allowing it to be easily distinguished from the 362 bp cDNA product when separated electrophoretically (Figure 5.3, Lanes 2 and 5).

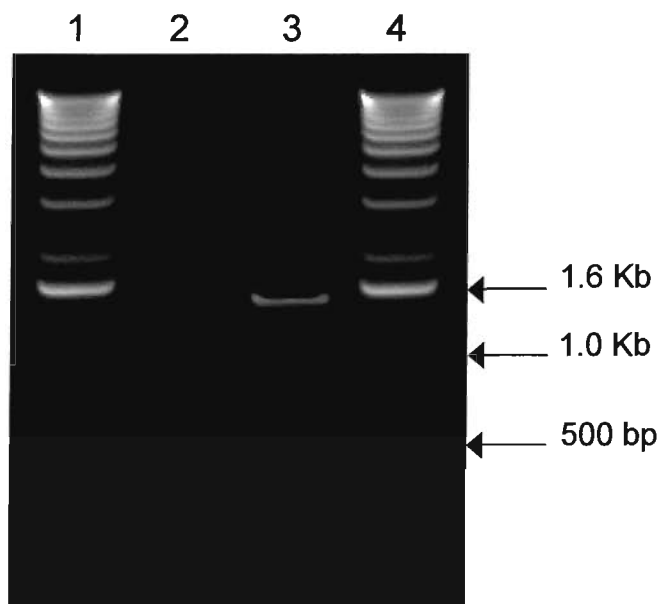


Figure 5.1 Products generated from PCR using the *AtHMA1*-forward(out)/*AtHMA1*-reverse(nested) primer pair. The gene specific primers are shown tested on two templates: Lane 2, cDNA from plant root material; Lane 3, gDNA. Lanes 1 and 4 = 1Kb DNA Ladder. Primer details in Materials and Methods.

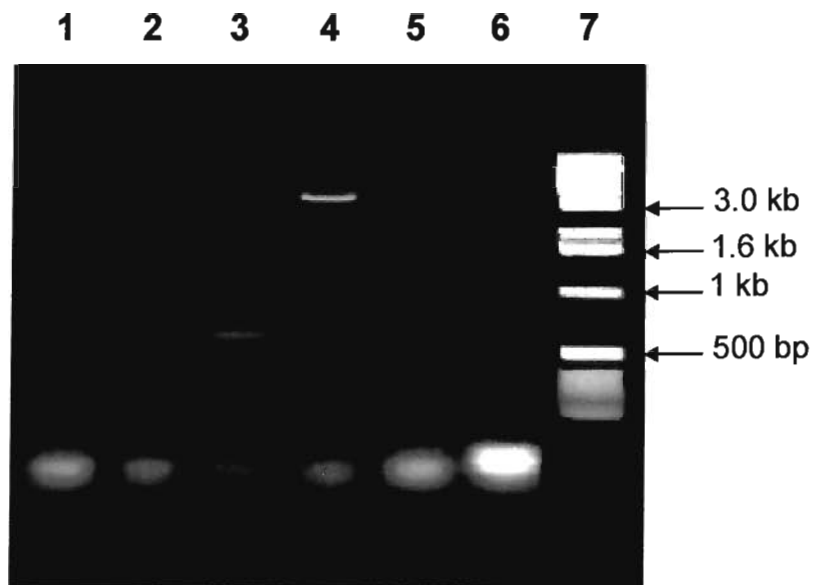


Figure 5.2 PCR products generated from *Arabidopsis thaliana* gDNA using various combinations of *AtHMA4* specific primers. Lane 1, Forward(out)/Reverse D; Lane 2, Forward(out)/Reverse E; Lane 3, Forward(out)/Reverse F; Lane 4, Nested(for)/Reverse(out); Lane 5, Forward(out)/Nested(rev); Lane 6, Forward(out)/Reverse(out); Lane 7, 1 Kb DNA ladder. Primer details in the Materials and Methods.

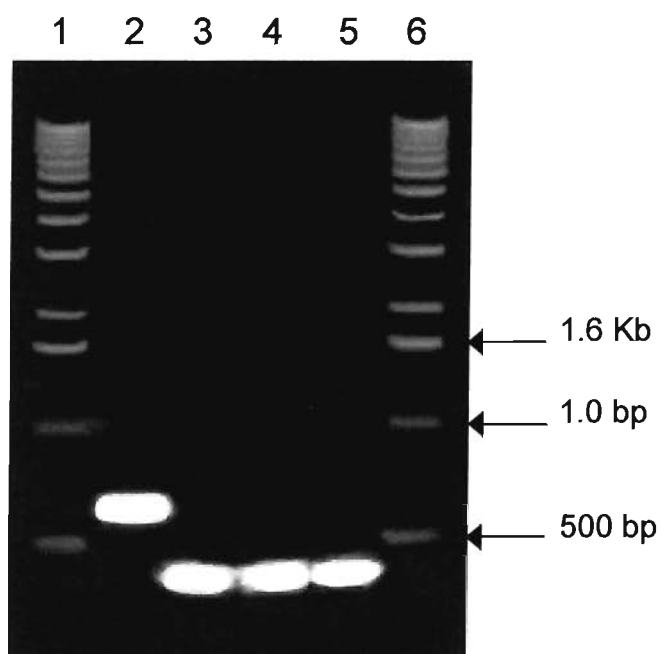


Figure 5.3 Products generated from PCR using the *AtHMA4* nested forward/nested reverse primer pair. The gene specific primers were tested on various templates: Lanes 1 and 6, 1 Kb ladder; Lane 2, Genomic DNA; Lane 3, EST 5; Lane 4, cDNA from root material treated with 1 mM ZnSO₄ for 30 hr; Lane 5, cDNA from untreated root material.

The *Actin 2* gene was used as a 'loading control' in the expression studies. This gene codes for a ubiquitous component of the plant cytoskeleton and is constitutively and strongly expressed in nearly all *Arabidopsis* tissues (McDowell et al. 1996). Genes from the actin family have been used as constitutively expressed loading controls in other studies, notably by Robinson et al. (1999) in the study of Fe uptake and by Bovet et al. (2003) when examining *AtMRP*-transcript induction by Cd. In the latter study, cDNA microarrays and semi-quantitative RT-PCR showed that *Actin 2* expression was unaffected by Cd treatment as was a further control gene *S16*, which codes for the 40S ribosomal protein in *Arabidopsis* (Bovet et al. 2003). Tests carried out to examine the expression of *Actin 2* showed that this gene had no particular sensitivity to a number of different metal ions, although its expression did appear to be affected consistently by Cu treatment. Comparison of RT-PCR products revealed that the expression pattern was very similar to *S16* (Figure 5.4), which was also affected by Cu treatment. Copper is extremely phytotoxic and is known to affect the stability of RNA (Murphy and Taiz 1995). Thus, these responses were deemed to be a result of plant toxicity or accelerated degradation of Cu-treated RNA preparations.

5.2.1.2 PCR optimisation

Various PCR conditions were tested in order to optimise the amplification of gene-specific products. Amplicons were run on ethidium bromide-stained gels and quantified using densitometry. A trial of different annealing temperatures revealed 58 °C to be optimal in the amplification of *AtHMA4* from plant cDNA and EST5 (result not shown). Previous work using the *AtHMA1*-specific primers also showed that 58 °C was optimal, whilst for *Actin 2* a slightly higher temperature of 60 °C was necessary. Semi-quantitative RT-PCR requires selection of the correct number of PCR amplifications to ensure that the products yielded originate from a non-saturated, logarithmic phase of the reaction. This highlights minute differences in gene copy number that occur as a result of differential expression. Multi-cycle PCRs carried out on cDNA from untreated plant root material produced the 'standard' sigmoidal pattern of amplicon yield for each gene tested (result not shown). Analysis of graphical evidence indicated that 28, 26 and 34 cycles of PCR were optimal for amplifying products of *AtHMA4*, *AtHMA1* and *Actin 2*. This cycle number was standardised for all subsequent PCRs, including those conducted on cDNAs from

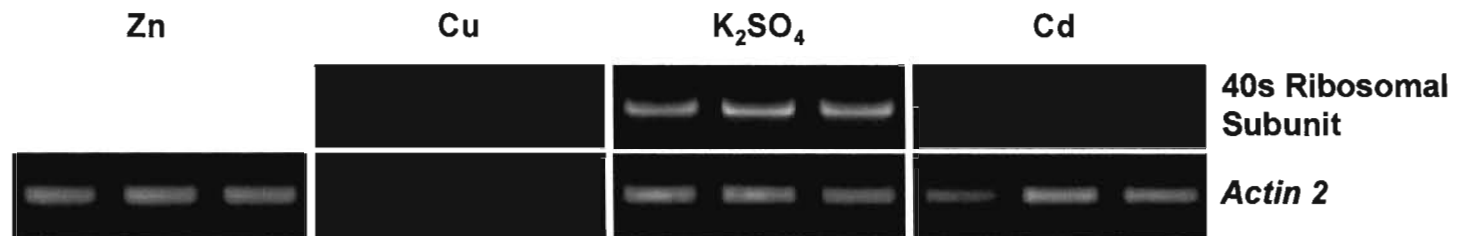


Figure 5.4 Expression of *Actin 2* and *S 16* (coding for the the 40s Ribosomal subunit in *Arabidopsis thaliana*) in metal treated roots analysed by RT-PCR. RNA was extracted from root material exposed to 1.0 mM ZnSO₄ (for *Actin 2* only), 0.5 mM CuSO₄, K₂SO₄ and CdSO₄ for 30 hours and analysed by semi-quantitative RT-PCR using gene-specific primers.

different plant tissues and different metal treatments. This ensured that all results were comparable.

5.2.1.3 Organ-specific expression

RT-PCR was conducted on individual RNA preparations made from the roots, stems, leaves, flowers and siliques of hydroponically grown plants. Analysis of the PCR product yields by agarose gel electrophoresis showed that there was variation in the tissue specific expression between the three genes. The expression of *Actin 2* appeared uniform in all tissues, supporting the choice of this gene as a 'loading' control. *AtHMA4* was also expressed in all tissues, but it was found at higher levels in the roots (Figure 5.5). Expression of *AtHMA4* in the aerial portions of the plant was generally much weaker, although the transcript levels were fairly high in the leaves. Analysis of the same plant material for *AtHMA1* showed that transcripts for this gene were similarly ubiquitous, however their abundance appeared to be highest in the leaves, relative to other tissues. Further RT-PCR reactions carried out on the leaves and roots of numerous different plant samples have confirmed that the expression profile of *AtHMA1* in these two organs is opposite to that seen for *AtHMA4*, namely that expression occurs at a higher level in leaves than in roots.

5.2.1.4 Expression upon metal stress

5.2.1.4.1 General metal screen

In light of organ-specific results, metal induced expression studies were carried out on both root and leaf material. Plants growing in hydroponic culture were exposed to a range of metals and metal-chelators. RT-PCR was used to determine broad changes in expression in both tissues following a 30 hour exposure to metals. Three repeated PCR reactions were carried out on individual cDNA samples reverse transcribed from RNA extracted from 5 plants. Tests with *Actin 2* primers showed that the expression of this gene did not vary with metal treatments except for plants treated with 0.5 mM CuSO₄, which showed a marked difference from the control (Figure 5.4). *Actin 2* was therefore used as a 'loading' control, with the HMA results being expressed relative to those of *Actin 2*. Results of Cu treated leaves were included in this experiment as a comparison. The effects of Cu on *Actin 2* expression in leaves were not as dramatic as those seen in the root (result not

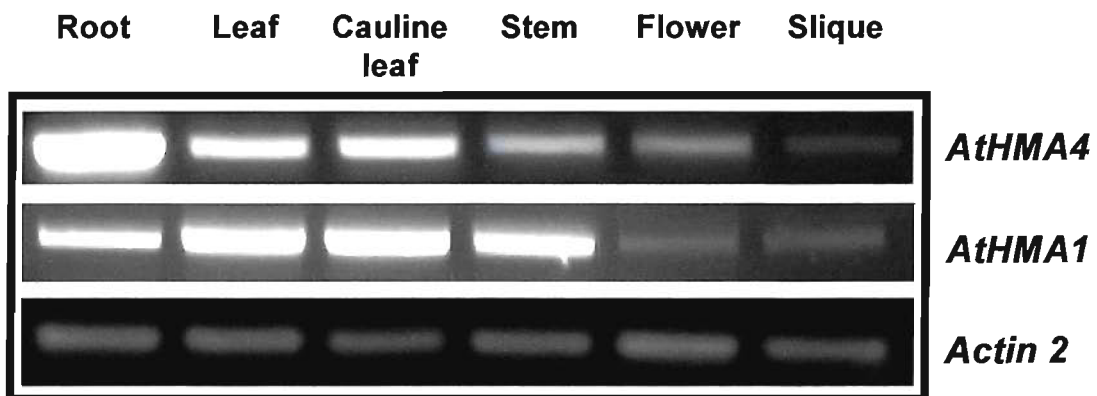


Figure 5.5 Expression of *AtHMA4* and *AtHMA1* in different *Arabidopsis* tissues analysed by RT-PCR. RNA was extracted from plants grown hydroponically in nutrient solution and subjected to semi-quantitative RT-PCR using gene specific primers. The results shown are representative of 2 experiments.

shown). The results shown for this preliminary screen (Figures 5.6 and 5.7) are the means from three replicate PCR reactions. Generally the trends were the same (i.e. metal-dependent up-regulation or down-regulation) between replicated runs. The actual IDV values did vary between the replicated runs by as much as four-fold in some cases but this was due to the analysis of gel bands using different size images. This did not affect the trends of the expression data, which remained the same in most instances. Zn and Mn treatments caused a marked increase in expression of root *AtHMA4*, whilst Cd exposure resulted in reduced expression (Figure 5.6a). For *AtHMA1* in roots only ferrozine and to a lesser extent Co, appeared to cause any discernable increase in expression relative to the potassium sulphate control. NaEDTA did reduce *AtHMA1* transcript abundance in roots, however the effect was not marked (Figure 5.6b). In leaf tissues harvested from the same plants, ferrozine and Co again tended to cause an up-regulation of *AtHMA1* expression, in line with the results for roots, however in these tissues both increases were smaller than that induced by Mn (Figure 5.7b). Induced gene expression by Co treatment appeared to be a common response in the samples, with induction of *AtHMA4* by this element also being evident in the leaves (Figure 5.7a). Indeed similarities in the expression profiles of *AtHMA1* and *AtHMA4* were observed in the leaf tissues, notably Fe, Cu and Zn all appeared to reduce gene expression in this preliminary screen (Figure 5.7a,b).

5.2.1.4.2 Responses to selected treatments and different exposure periods

Results from the general metal screen, indicated that the responses of *AtHMA1* and *AtHMA4* to Zn, Mn, Cd and Co should be examined in more detail. Further tests on Cu were also performed since it was not possible to isolate good quality RNA from treated material, particularly the roots. Treatment concentrations for Cd and Cu were lowered from 0.5 mM to 0.1 mM. Initially, additional root samples were analysed for differences in *AtHMA4* expression, since the general metal screen showed that this gene displayed a wide variation in transcript abundance with metal/chelator treatment. *AtHMA1* was also examined. Repeated RT-PCRs on RNA prepared from different plant material identified a few obvious trends in the expression of the two genes (Figure 5.8a,b). Cd caused down regulation of both *AtHMA1* and *AtHMA4*. Cobalt similarly reduced expression of *AtHMA4*, although there was no discernable affect on *AtHMA1*. As before, Cu treatment again appeared to affect the quality of the RNA extracted. As a result, the treatment appeared to induce the down-regulation of both genes when agarose gels

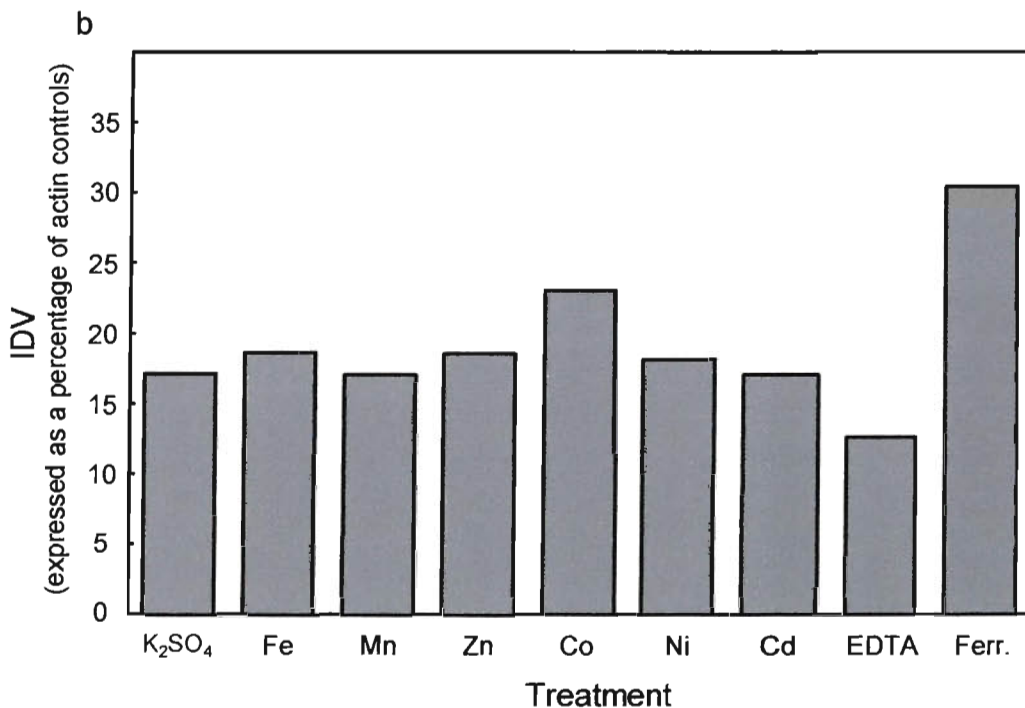
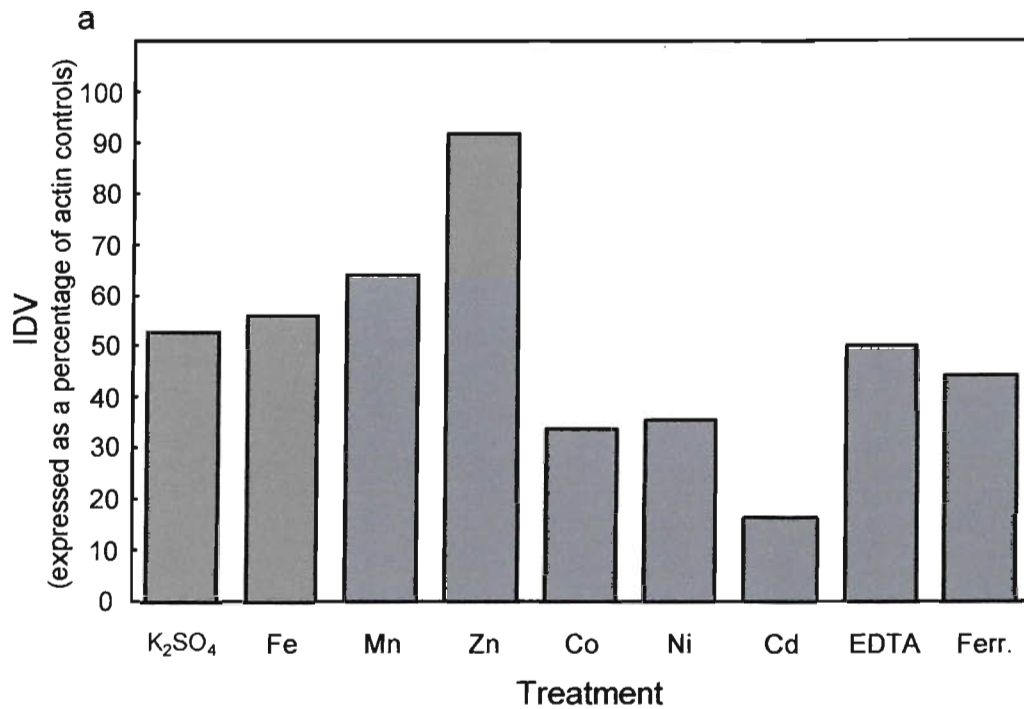


Figure 5.6 Expression of *AtHMA4* (a) and *AtHMA1* (b) shown as mean IDV values expressed as a percentage of the actin IDV values in root material from plants exposed to various different ion and ion-chelator treatments. Hydroponically grown plants were exposed to modified nutrient solutions for 30 hours. RNA was extracted from a bulked sample consisting of 5 plants. One RT-reaction was performed for each treatment followed by replicated PCR reactions. Results shown are the mean IDV values expressed as a percentage of the actin control (*AtHMA4* $n = 3$, *AtHMA1* $n = 2$). Although variability was observed between replicated runs, this did not affect the trends which were similar in most cases. Treatments added in addition to the normal nutrient solution were: K₂SO₄ – 1.0 mM, FeSO₄ – 20 mM, MnSO₄ – 0.8 mM, ZnSO₄ – 1.0 mM, CoSO₄ – 0.5 mM, NiSO₄ – 0.5 mM, CdSO₄ – 0.5 mM, NaEDTA – 5.0 mM, Ferrozine – 1.0 mM. Note: No RNA was obtained from roots treated with 0.5 mM CuSO₄.

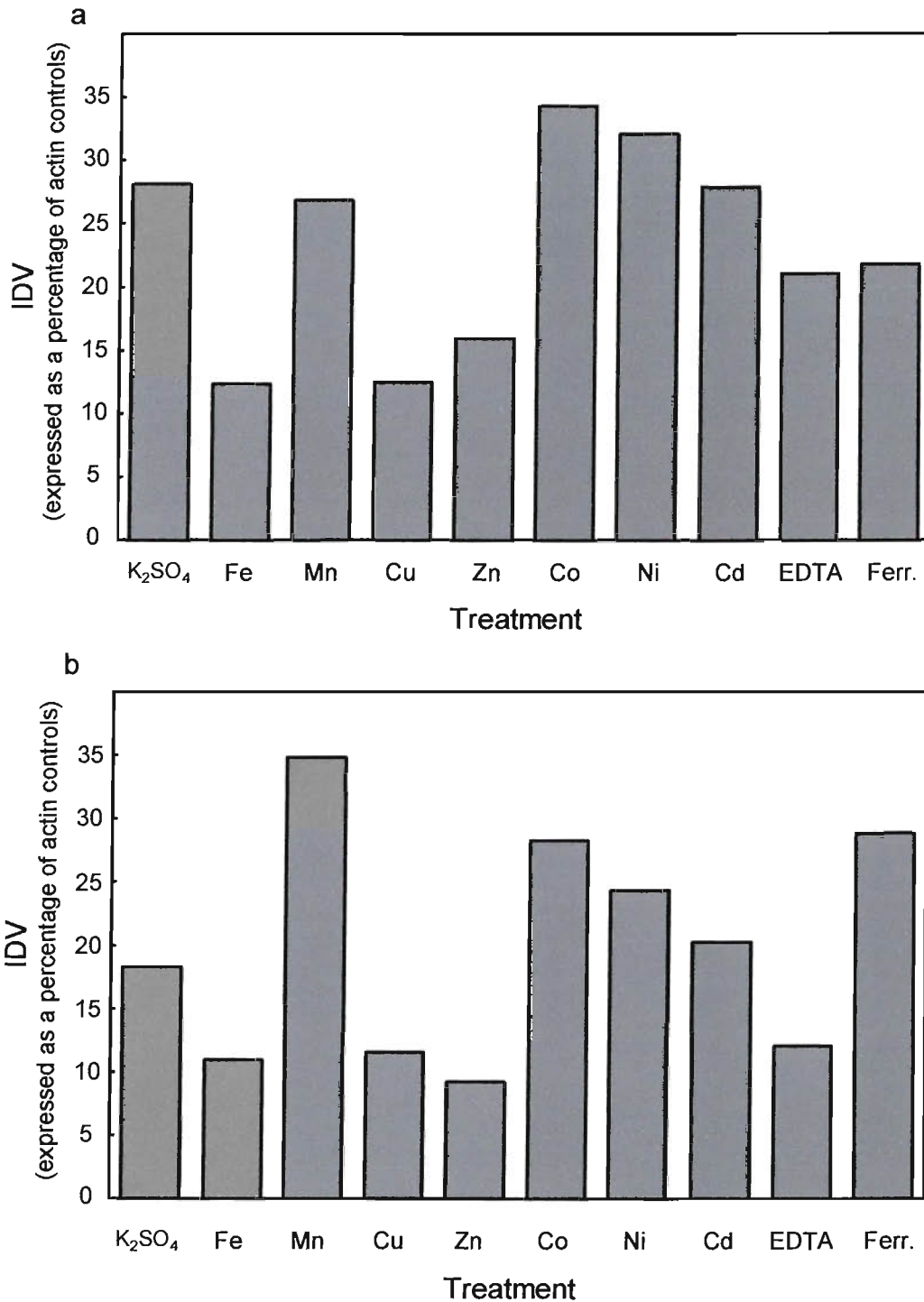


Figure 5.7 Expression of *AtHMA4* (a) and *AtHMA1* (b) shown as mean IDV values expressed as a percentage of the actin IDV values in leaf material from plants exposed to various different ion and ion-chelator treatments. Hydroponically grown plants were exposed to modified nutrient solutions for 30 hours. RNA was extracted from a bulked sample consisting of 5 plants. One RT-reaction was performed for each treatment followed by replicated PCR reactions. Results shown are the mean IDV values expressed as a percentage of the actin control (*AtHMA4* $n = 3$, *AtHMA1* $n = 3$). Although variability was observed between replicated runs, this did not affect the trends which were similar in most cases. Treatments added in addition to the normal nutrient solution were: K₂SO₄ – 1.0 mM, FeSO₄ – 20 mM, MnSO₄ – 0.8 mM, CuSO₄ – 0.5 mM, ZnSO₄ – 1.0 mM, CoSO₄ – 0.5 mM, NiSO₄ – 0.5 mM, CdSO₄ – 0.5 mM, NaEDTA – 5.0 mM, Ferrozine – 1.0 mM.

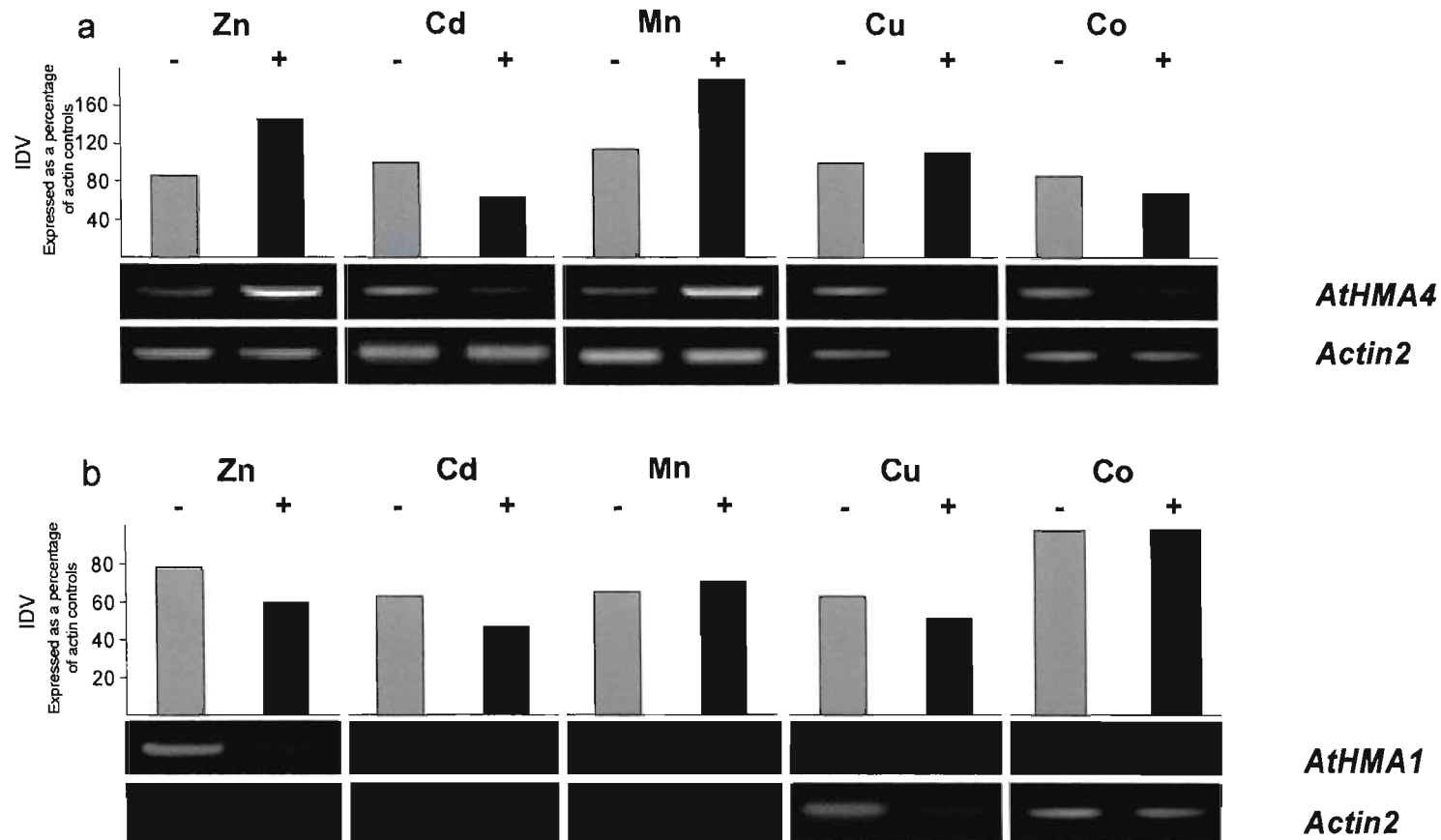


Figure 5.8 Expression of *AtHMA4* (a) and *AtHMA1*(b) in metal-treated plant-root material analysed by RT-PCR. Plant roots were exposed to metals (+); 1.0 mM ZnSO₄, 0.1 mM CdSO₄, 0.8 mM MnSO₄, 0.1 mM CuSO₄, 0.5 mM CoSO₄ and an equivalent concentration of K₂SO₄ (-) for 30 hours in hydroponic nutrient solution. RNA was extracted from harvested tissue and analysed by semi-quantitative RT-PCR using gene specific primers. Graphs show levels of expression as integrated densitometry values (IDV). The results shown are from one experiment with three replicates. A single replicate is shown although similar trends were observed for each sample.

were first inspected (product bands in Figure 5.8a,b). The *Actin*-corrected data revealed a different trend, indicating that Cu may in fact enhance the expression of *AtHMA4* (Figure 5.8a graphical data). Problems with EDTA-treated material also tended to obscure the results (data not shown). This metal-ion chelator appeared to cause slight down-regulation of *AtHMA1* and *AtHMA4*, but the results could not be confirmed in further tests as PCR reactions proved unreliable. RNA yields appeared to be affected in plants exposed to periods greater than 30 hours so toxicity of the treatment may have caused these inconsistencies. Zn and Mn treatments produced clear and consistent responses in the roots for both genes. *AtHMA4* was strongly up-regulated by Zn, whilst *AtHMA1* was down-regulated. For Mn both genes showed appreciable up-regulation, although increases in *AtHMA4* expression were far greater (Figure 5.8a,b).

The results for roots suggest that the expression of *AtHMA1* and *AtHMA4* are dependent upon the metal species and concentration. Considering that the metal burden of leaf tissues may be controlled by the rates of xylem loading/unloading, expression was studied in these aerial tissues by examining expression over different exposure periods. Fifteen, 30 and 60-hour time points were chosen to create a profile of the expression pattern for Zn, Mn and Co. In Zn treated leaf material *AtHMA4* expression was higher at all time points than in control plants. At 15 and 60 hours, the differences were negligible, but there was a discernable peak at the 30 hour mid-point (Figure 5.9a). For *AtHMA1* in the same material expression was raised at the earlier time points (15 and 30 hr), but was reduced to a level just below control plants at 60 hours (Figure 5.9b). Exposure to Mn appears to have provoked similar expression profiles for *AtHMA1* and *AtHMA4* in the leaves. Both genes display a profile where expression is lower at 15 hours, higher at 30 hours and lower at 60 hours than the control plants over the complete time course (Figure 5.10a,b). In the case of Co, only *AtHMA1* data is available. At the 15-hour time point, expression is similar in treatment and control plants but thereafter Co tends to reduce levels of expression (Figure 5.11).

5.2.1.4.3 The effect of different metal concentrations on *AtHMA4* and *AtHMA1* gene expression

The effect of metal exposure on gene expression was examined over a concentration range. Zn, the metal used at the highest concentrations in initial exposures was examined at lower levels to examine if the expression pattern of genes was influenced accordingly. Expression of a number of genes were studied

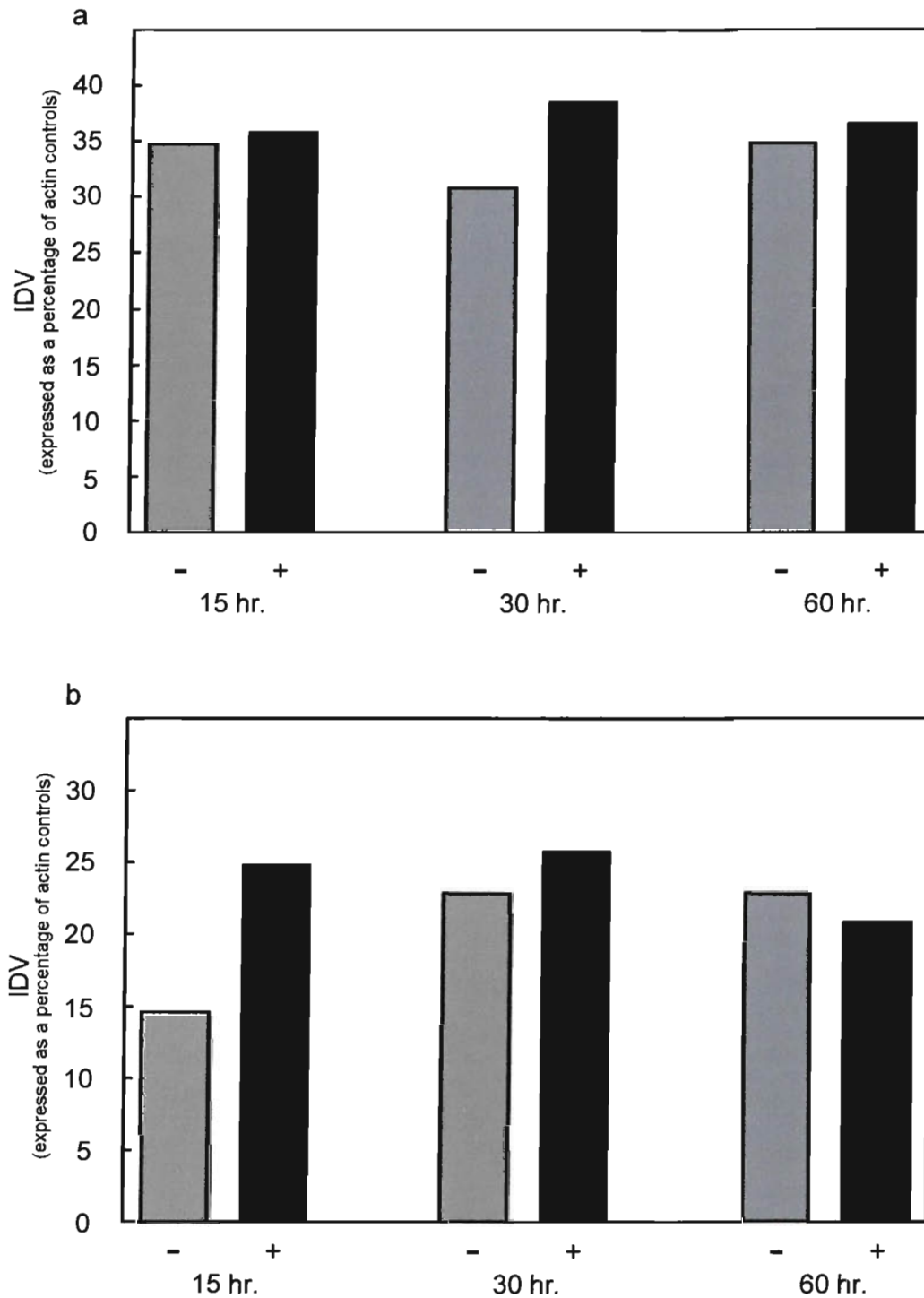


Figure 5.9 Time-dependent expression profiles of *AtHMA4* (a) and *AtHMA1* (b) in leaves exposed to Zn. Hydroponic plants supplied with 1.0 mM additional $ZnSO_4$ (+) or an equivalent level of K_2SO_4 (-) in the rooting medium were exposed to elevated ion concentrations for periods of 15, 30 and 60 hours. Leaf tissues were analysed using semi-quantitative RT-PCR for transcripts of *AtHMA4* (a) and *AtHMA1* (b) using gene-specific primers. Expression was quantified using densitometry on ethidium bromide-stained agarose gels. Results are expressed relative to *Actin 2* and are from one experiment.

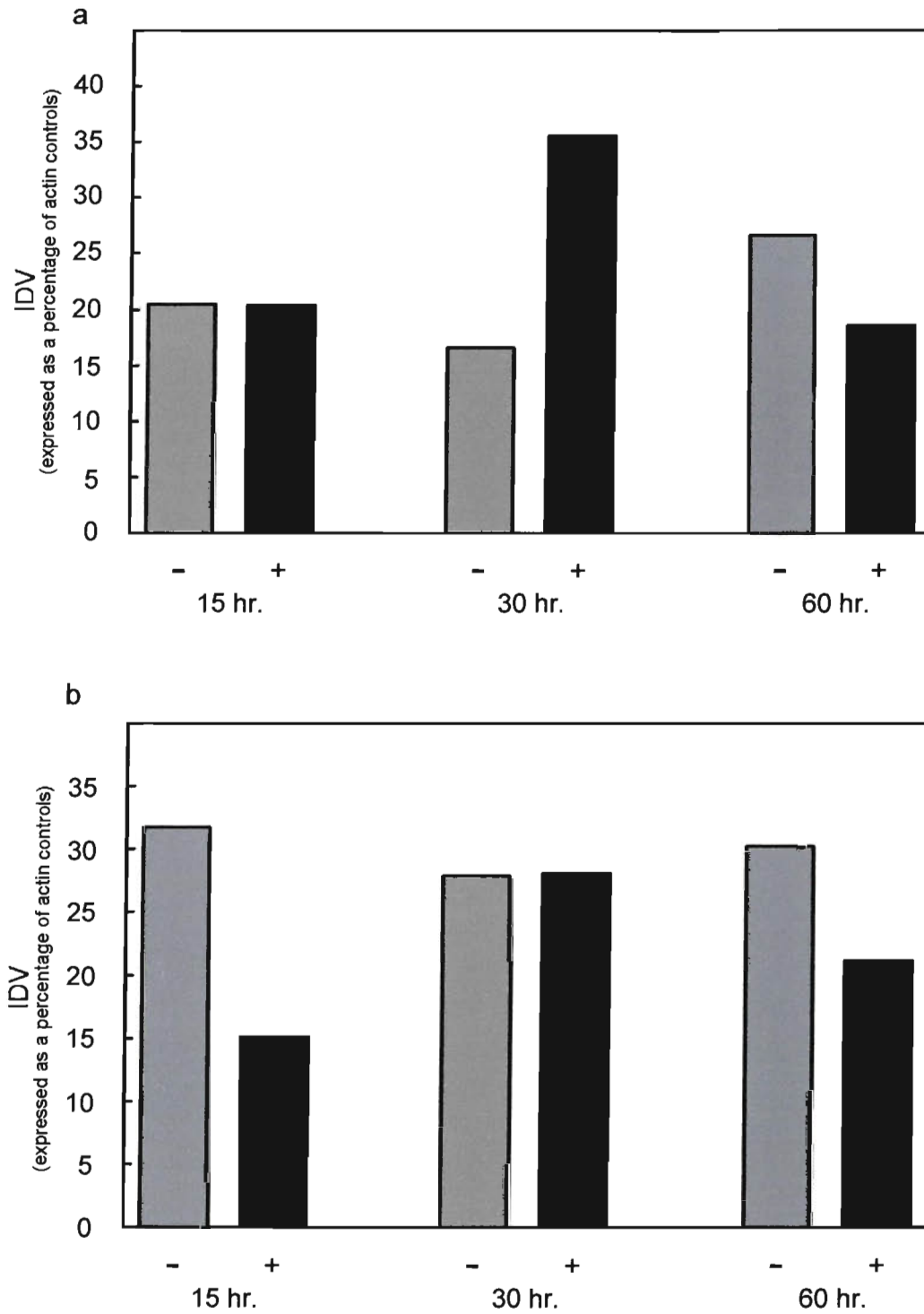


Figure 5.10 Time-dependent expression profiles of *AtHMA4* (a) and *AtHMA1* (b) in leaves exposed to Mn. Hydroponic plants supplied with 0.8 mM additional MnSO_4 (+) or an equivalent level of K_2SO_4 (-) in the rooting medium were exposed to elevated ion concentrations for periods of 15, 30 and 60 hours. Leaf tissues were analysed using semi-quantitative RT-PCR for transcripts of *AtHMA4* (a) and *AtHMA1* (b) using gene-specific primers. Expression was quantified using densitometry on ethidium bromide-stained agarose gels. Results are expressed relative to *Actin 2* and are from one experiment.

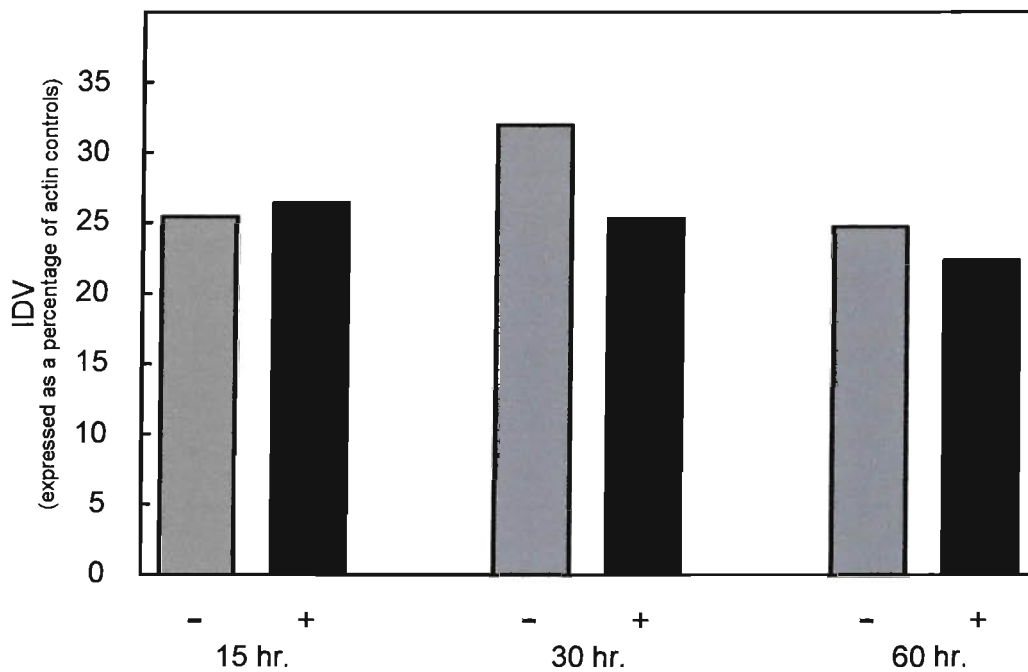


Figure 5.11 Time-dependent expression profile of *AtHMA1* in leaves exposed to Co. Hydroponic plants supplied with 0.5 mM additional CoSO₄ (+) or an equivalent level of K₂SO₄ (-) in the rooting medium were exposed to elevated ion concentrations for periods of 15, 30 and 60 hours. Leaf tissues were analysed using semi-quantitative RT-PCR for transcripts of *AtHMA1* using gene-specific primers. Expression was quantified using densitometry on ethidium bromide-stained agarose gels. Results are expressed relative to *Actin 2* and are from one experiment.

independent of the *Actin 2* gene, which was also examined in the same material for reference purposes. Exposure periods of 15, 30 and 60 hours were studied and root material from each experiment produced near identical results. Only PCRs from 30-hour exposures are shown. For *AtHMA4*, increasing Zn concentrations in the nutrient solution resulted in an increase in gene expression (Figure 5.12). Changes in gene expression were higher between the lower Zn concentration increments (0 – 0.1 and 0.1–0.35 mM additional ZnSO₄), and appeared to level off at 1.0 mM ZnSO₄, although higher concentrations were not tested. For leaf tissues, low RNA yields permitted only a limited number of PCR reactions and restricted analysis to *AtHMA4*. In these tissues, *AtHMA4* also displayed up-regulation when exposed to additional Zn (Figure 5.13). Levels of expression were much lower than in the roots, but relative increases upon Zn exposure were similar. The expression profile was also different compared to the roots, with levels peaking at 0.1 mM Zn and being slightly lower at 0.35 and 1.0 mM Zn (Figure 5.13). For *AtHMA1* in roots, all levels of additional Zn resulted in reduced expression. Reductions were small but consistent across the concentration range and slightly greater between the 0 – 0.1 mM and 0.1 mM – 0.35 mM Zn increments (Figure 5.14).

5.2.1.4.4 Effects on additional members of the HMA transporter family

Gene-specific oligonucleotide primers were designed complementary to the coding regions of *AtHMA2*, *AtHMA3* and *AtHMA5* and used in optimised PCR reactions. Tests on root material revealed that each gene displayed a different response to increasing Zn concentrations, with the trends being consistent over the entire concentration range (Figures 5.15 – 5.17). *AtHMA2* showed no discernable response to Zn, with levels of expression being similar at each concentration (Figure 5.15). In contrast, *AtHMA3* had a more dramatic response to elevated Zn (Figure 5.16). Transcript abundance appeared unaffected at 0.1 mM additional Zn, but a large reduction was established at 0.35 mM Zn and larger still at 1.0 mM Zn. In total, expression was around 50 percent lower than control plants when exposed to 1.0 mM Zn (Figure 5.16). An opposite and more subtle response was observed in the case of *AtHMA5*. Expression was increased at each additional Zn concentration (Figure 5.17).

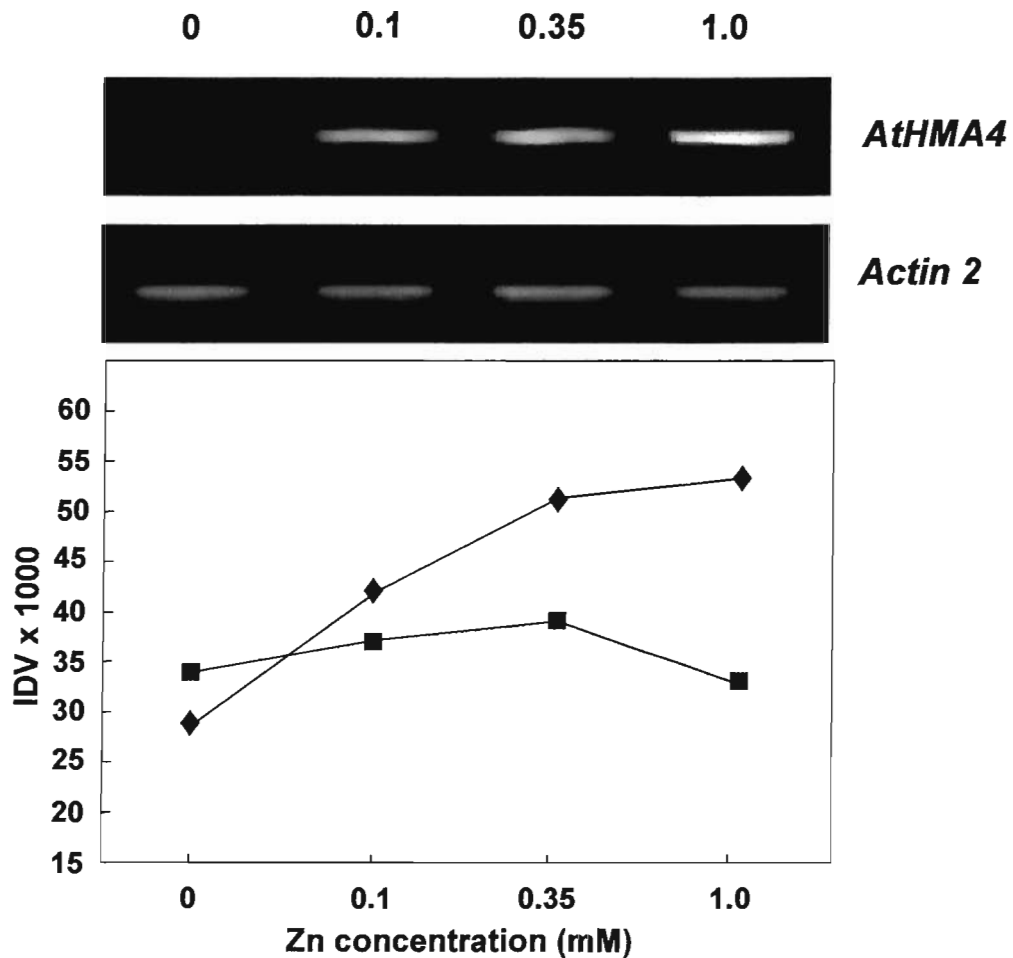


Figure 5.12 Expression of *AtHMA4* (◆) and *Actin 2* (■) in root tissues following the exposure of plants to different levels of $ZnSO_4$. RNA was extracted from hydroponically-grown plants exposed to varying concentrations of $ZnSO_4$ for a period of 30 hours. Semi-quantitative RT-PCR was conducted using gene specific primers and the products generated analysed for integrated densitometry values (IDV) on ethidium-bromide-stained agarose gels. Results are from one experiment, with three replicates. A single replicate is shown although similar trends were observed for each sample.

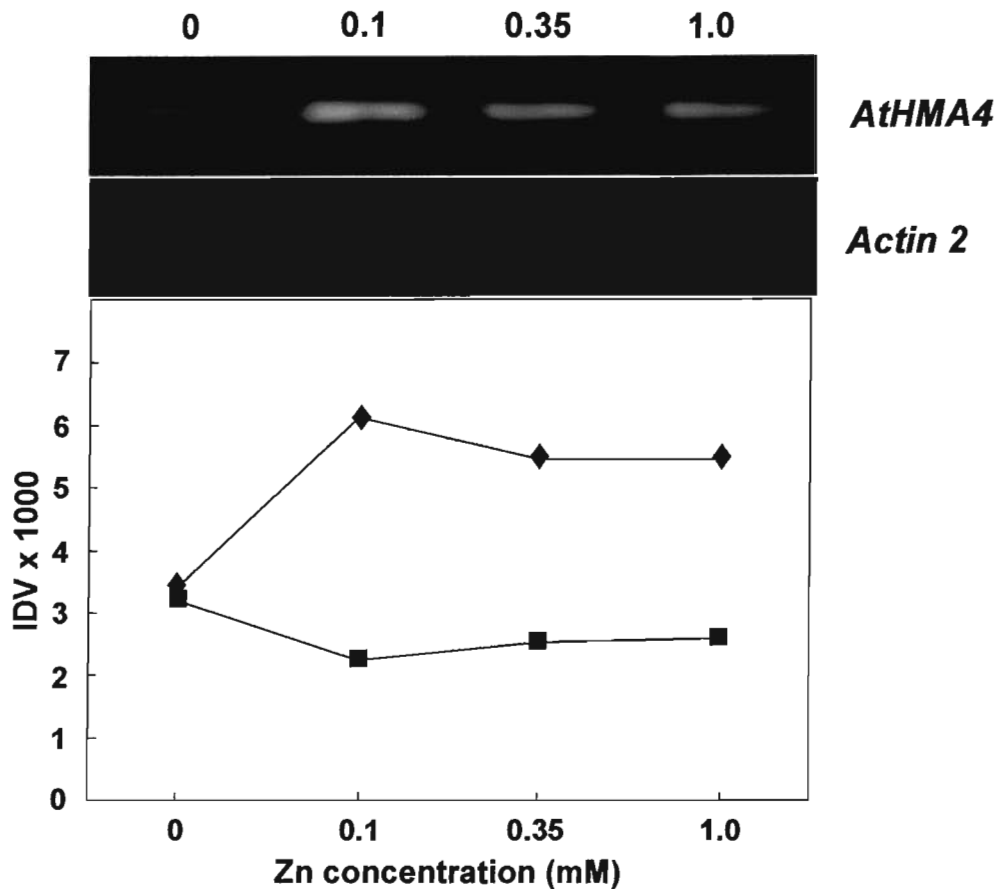


Figure 5.13 Expression of *AtHMA4* (◆) and *Actin 2* (■) in leaf tissues following the exposure of plants to different levels of ZnSO_4 . RNA was extracted from hydroponically-grown plants exposed to varying concentrations of ZnSO_4 for a period of 30 hours. Semi-quantitative RT-PCR was conducted using gene specific primers and the products generated analysed for integrated densitometry values (IDV) on ethidium-bromide-stained agarose gels. Results are from one experiment.

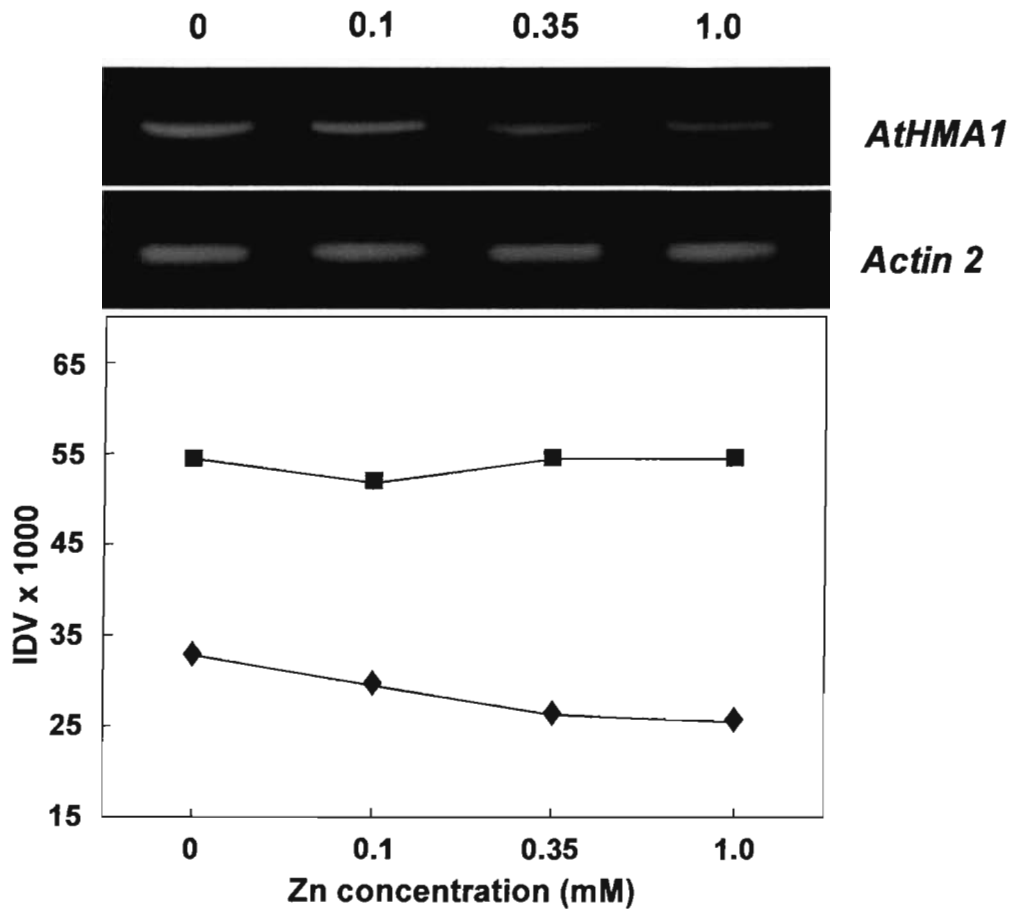


Figure 5.14 Expression of *AtHMA1* (◆) and *Actin 2* (■) in root tissues following the exposure of plants to different levels of ZnSO_4 . RNA was extracted from hydroponically-grown plants exposed to varying concentrations of ZnSO_4 for a period of 30 hours. Semi-quantitative RT-PCR was conducted using gene specific primers and the products generated analysed for integrated densitometry values (IDV) on ethidium-bromide-stained agarose gels. Results are from one experiment.

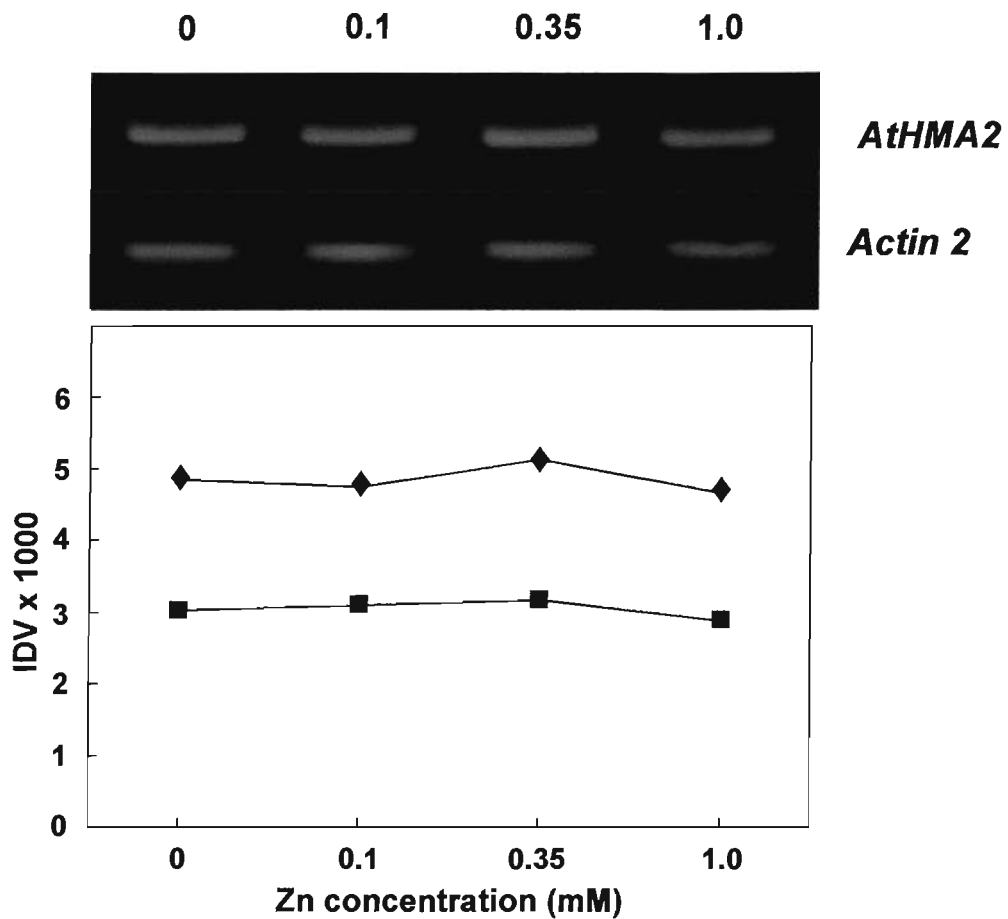


Figure 5.15 Expression of *AthMA2* (◆) and *Actin 2* (■) in root tissues following the exposure of plants to different levels of $ZnSO_4$. RNA was extracted from hydroponically-grown plants exposed to varying concentrations of $ZnSO_4$ for a period of 30 hours. Semi-quantitative RT-PCR was conducted using gene specific primers and the products generated analysed for integrated densitometry values (IDV) on ethidium-bromide-stained agarose gels. Results are from one experiment.

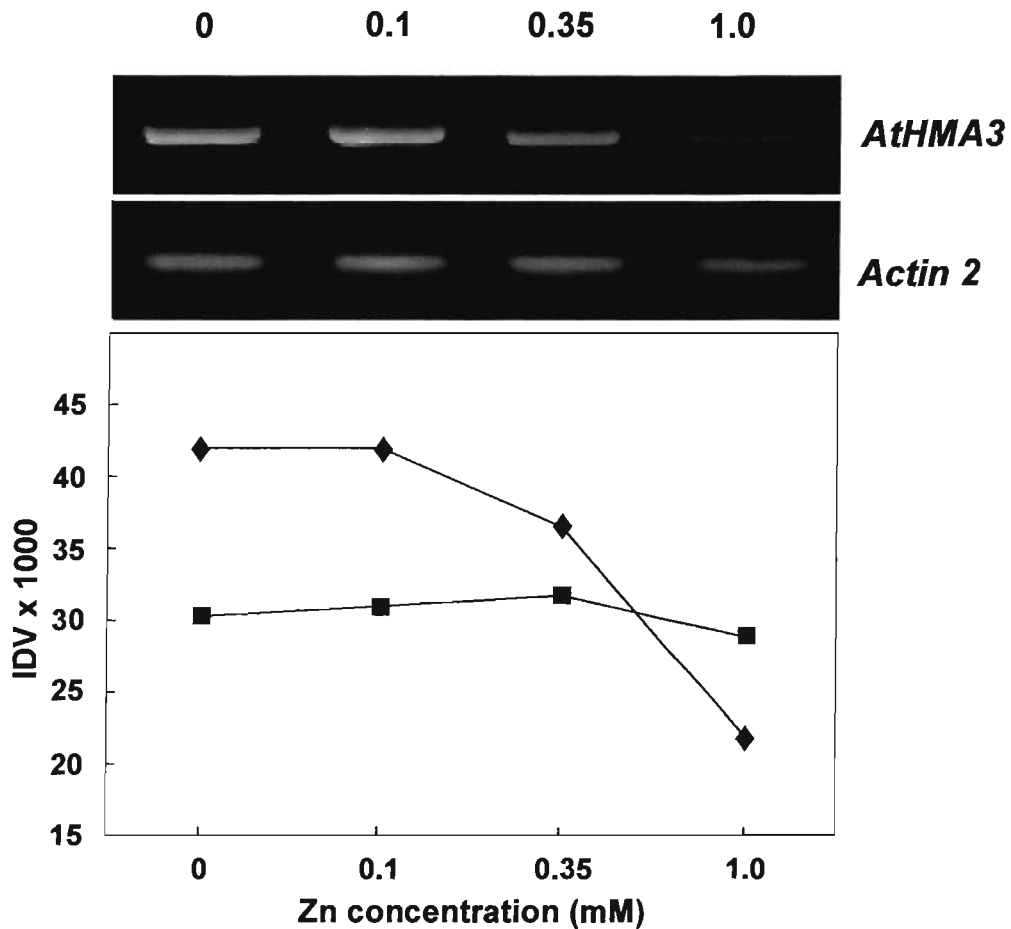


Figure 5.16 Expression of *AtHMA3* (◆) and *Actin 2* (■) in root tissues following the exposure of plants to different levels of $ZnSO_4$. RNA was extracted from hydroponically-grown plants exposed to varying concentrations of $ZnSO_4$ for a period of 30 hours. Semi-quantitative RT-PCR was conducted using gene specific primers and the products generated analysed for integrated densitometry values (IDV) on ethidium-bromide-stained agarose gels. Results are from one experiment.

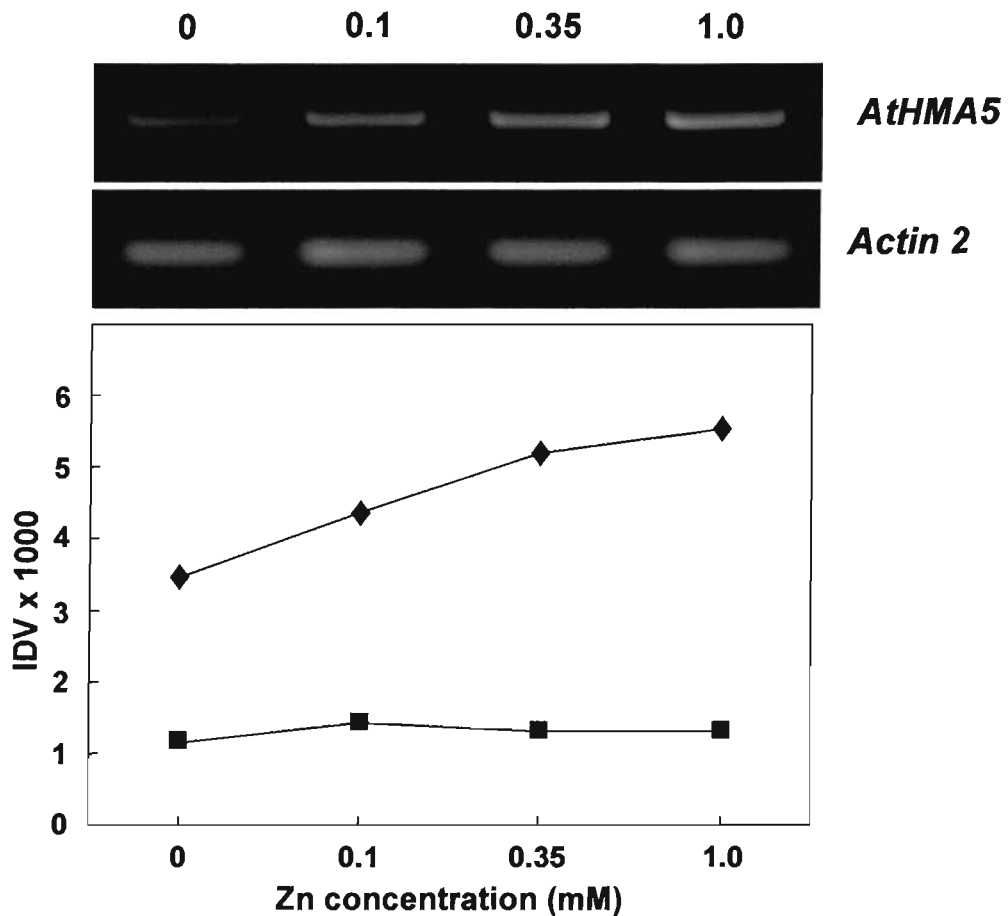


Figure 5.17 Expression of *AtHMA5* (◆) and *Actin 2* (■) in root tissues following the exposure of plants to different levels of ZnSO_4 . RNA was extracted from hydroponically-grown plants exposed to varying concentrations of ZnSO_4 for a period of 30 hours. Semi-quantitative RT-PCR was conducted using gene specific primers and the products generated analysed for integrated densitometry values (IDV) on ethidium-bromide-stained agarose gels. Results are from one experiment.

5.2.2 Northern analysis

5.2.2.1 Molecular probe specificity dot-blots

Preliminary experiments were conducted to ensure gene specific probes had been successfully generated for use in expression studies. ³²P-labelled probes complementary to a full-length clone of *AtHMA1* and a 362 bp internal fragment of *AtHMA4* were made as described in the Materials and Methods section. Neither a full-length clone of *AtHMA4*, nor sequence from the 5' untranslated region had been obtained at the time of probing, so the *AtHMA4*-specific sequence amplified from the nested forward/nested reverse primer pair was used. A preliminary check of the sequence with the TAIR BLAST computer programme (<http://www.arabidopsis.org/>) was used to determine the potential specificity of the probe. Digoxigenin (DIG)-labelled probes complementary to the same sequence stretches were also made and tested.

High stringency washes indicated that there was no significant cross-hybridisation between the ³²P and DIG-labelled probes with a selection of different DNA templates (Figures 5.18 and 5.19). Both methods appeared highly sensitive in short exposure tests, with 20 ng of DNA being easily detectable by the X-ray (³²P) and photographic (DIG) film used in conjunction with these methods. Due to the greater longevity of radiant emission by ³²P over digoxigenin, probes labelled with the former were chosen for use in northern analysis. This allowed small quantities of RNA to be probed over longer time periods and ensured that RNA preparations of low yield could be included in the analysis. Unfortunately at the time of these experiments no plasmids for *AtHMA2* or *AtHMA3* were available to check cross-hybridisation.

5.2.2.2 Northern analysis of RNA extracted from metal-treated plants

Northern analysis was carried out to determine the pattern of expression for *AtHMA1*, *AtHMA4* and *Actin 2* using total RNA isolated from metal-treated tissues. Root and leaf material was harvested after 30 hours exposure to Zn or Mn. Gene-specific probes were hybridised separately with the total RNA and washed at high stringency conditions. After probing, the RNA blots were stained with methylene blue and examined in a light cabinet to ensure equal loading of RNA for each sample preparation. Results from the hybridisations showed that *AtHMA4* in root tissues showed the largest response to Zn and Mn treatment where it was greatly

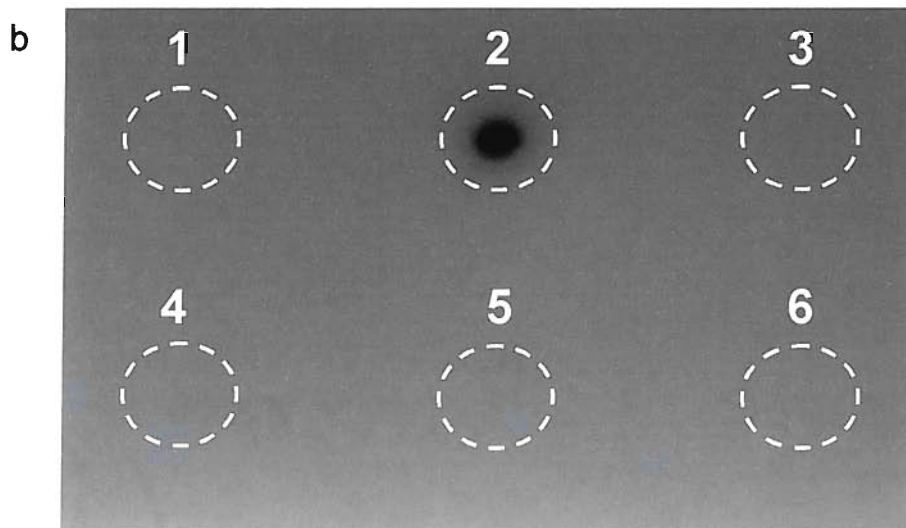
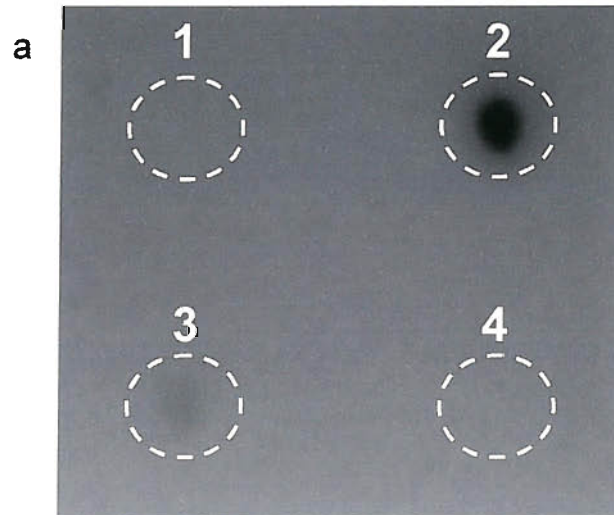


Figure 5.18 Dot blot analysis of the *AtHMA4* (a) and *AtHMA1* (b) ^{32}P - labelled probes. The dot blot was prepared using 200 ng of the cDNAs of interest except, *AtHMA4* on blot a = 20 ng, *AtHMA1* on blot b = 20 ng and gDNA on blot b = 1 μg . The membrane was incubated with the probe and washed in exactly the same manner as the plant RNA-derived samples. 1: ZAT2, 2: HMA1, 3: HMA4, 4: gDNA, 5: pAVA, 6: p426.

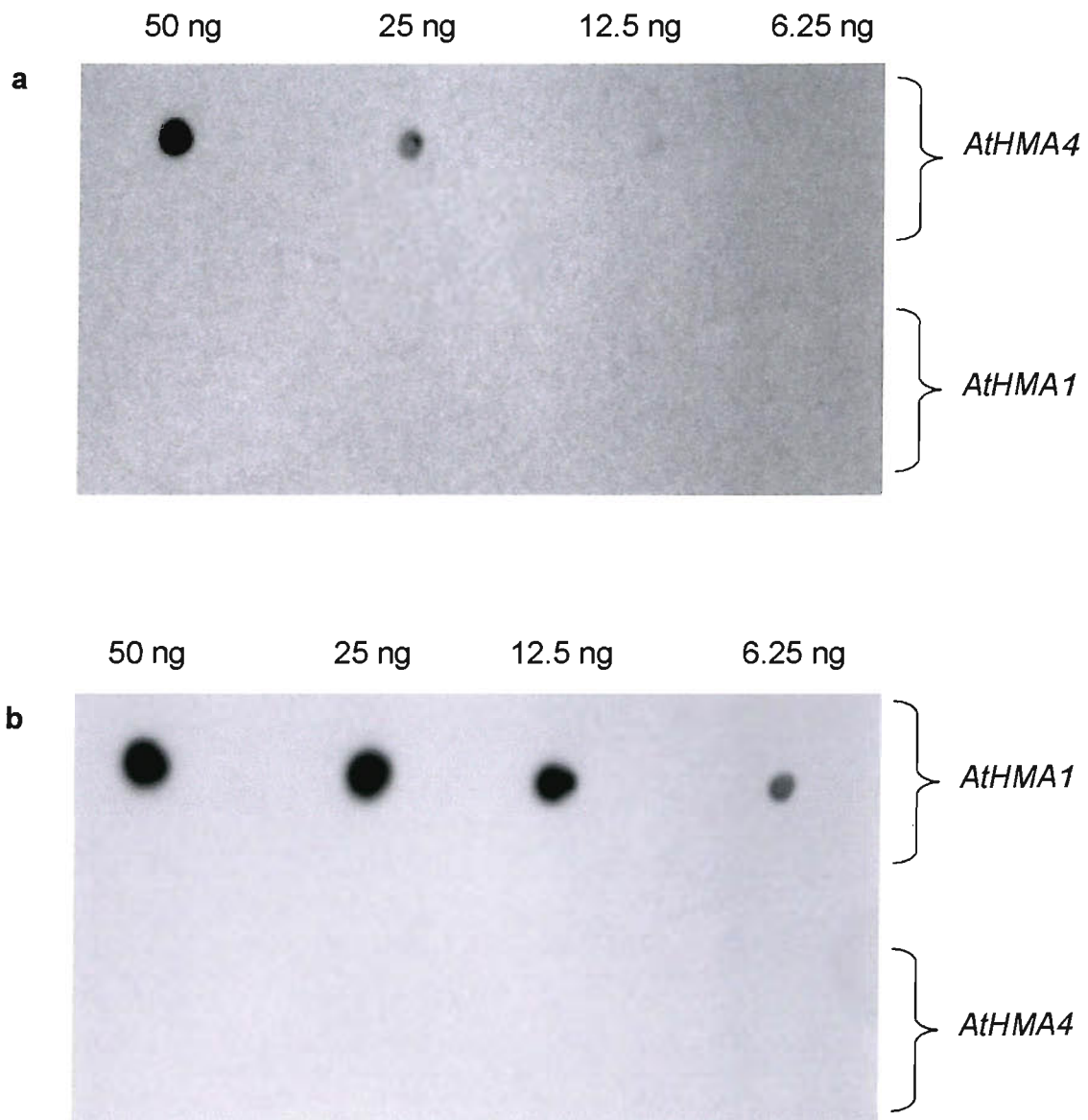


Figure 5.19 Dot blot test determining the sensitivity and specificity of digoxigenin-labelled probes trialled for use in northern hybridisations. (a) *AtHMA4* probes and (b) *AtHMA1* probes were tested on for varying amounts of *AtHMA4* and *AtHMA1* cDNA. Amounts of cDNA spotted onto the blot are shown above the film image.

up-regulated in the exposed tissue (Figure 5.20a,b). Analysis of plants exposed for different time periods also produced the same results with blots for 15 and 60 hours for Zn and 15 hours for Mn being near identical (results not shown). RNA extracted from 60-hour Mn exposures appeared degraded and could explain the absence of a response in this treatment (results not shown). In leaves *AtHMA4* was barely detectable and no conclusive trend could be identified for either treatment (Figure 5.20a,b). Expression of *AtHMA1* was more prominent in the leaves, with films exposed to leaf preparations giving darker bands than those exposed to roots for the same time period (Figure 5.20a,b). Responses of this gene in leaves to metal treatment appeared to be small, with no major differences picked up for plants exposed to either Zn or Mn (Figure 5.20a,b). Slight differences in RNA loading may have obscured a trend showing down regulation of *AtHMA1* in Zn treated leaf material at 30 hours (Figure 5.20a). A greater amount of total RNA was detected by methylene-blue staining in the Zn-treated leaf sample. This was seen to increase the intensity of the Zn-treated *Actin 2* band, and so *AtHMA1* may have been similarly affected. In plant leaves exposed to Zn for 15 and 60 hours there appeared to be slight down regulation of *AtHMA1*, where RNA loading was more equal (results not shown). In roots *AtHMA1* showed little metal-dependent variation.

5.2.3 Western analysis

Polyclonal antibodies raised in a rabbit host were supplied for this project and used to probe plant and yeast protein preparations for the presence of *AtHMA4* and *AtHMA1*. Antibodies were raised to peptide stretches of between 14 and 15 amino acids located at various positions within the protein. Three antibody probes were made for *AtHMA1*, corresponding to the N and C termini and an internal site, and one was raised to the C-terminus of *AtHMA4* (Figure 5.21). An antibody raised to the *Nicotiana tabacum* PMA2 H⁺-ATPase (Morsomme et al. 1996) was also used as a comparison (Figure 5.22). Previous studies indicate that this H⁺ ATPase runs at around 95-100 KDa (Morsomme et al. 1996). When used here distinct single bands were seen which were enriched in the high speed fraction as expected, although the molecular weight (118 KDa) was slightly higher than previously reported. Initial incubations with the HMA antibodies showed that each HMA antibody had failed to react specifically with protein bands of the predicted size (*AtHMA4* = 128 kDa, *AtHMA1* = 88 kDa; Figures 5.23 and 5.24). The *AtHMA1* and *AtHMA4* C-terminal antibodies, were seen to react with a diffuse band around 130 KDa in the Zn and Mn treated low speed extractions. These appeared absent from

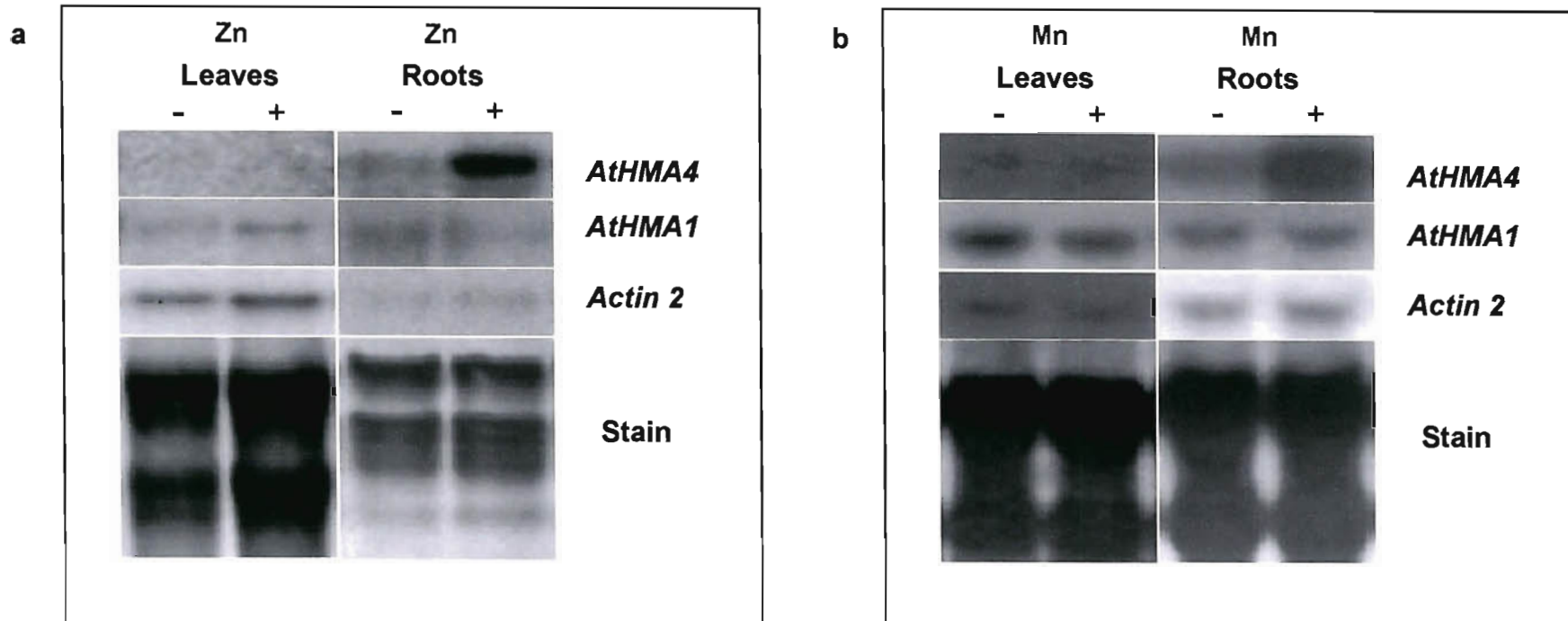


Figure 5.20 Expression of *AtHMA4*, *AtHMA1* and *Actin 2* in metal exposed plants as analysed by northern hybridisation. RNA was extracted from the roots and leaves of plants exposed to additional metals (+) or an equivalent concentration of K_2SO_4 (-) for 30 hours. Shown are the results from plants exposed to 1.0 mM additional $ZnSO_4$ (a) and 0.8 mM additional $MnSO_4$ (b). Northern hybridisations were carried out by transfer of total RNA onto nylon membrane before incubating with gene-specific probes labeled with ^{32}P . The efficiency of total-RNA transfer is shown by methylene-blue staining of the nylon membrane (stain) below the gene specific hybridisations. The results shown are from one experiment.

other treatments, but the specificity of binding was low and difficult to differentiate from background signals (Figures 5.23 and 5.24). Similar problems were encountered with the AtHMA1-internal and AtHMA1-N-terminus blots. Diffuse bands of around 80 - 90 KDa were evident on these blots, and perhaps more so in the Zn and Mn samples probed with the AtHMA1 N-terminal antibody, but the results were still far from conclusive. Various root material was tested, but in general no striking differences were found between the treatments (Figures 5.23 and 5.24).

Modifications to the incubation and blocking solutions, antibody dilutions and incubation conditions were made in an attempt to detect HMA-specific products. No products of the expected size were detected, but background signals were reduced by optimising the reaction conditions. Further protein blots were made from new material and probed under optimised conditions. Protein was extracted from leaves and roots of Zn and Mn-treated plant material and yeast transformed with *AtHMA4*, *AtHMA1* and the *p426* vector. No significant products were detected in any of the leaf samples, nor root samples treated with Mn (results not shown). Very few products were detected on the blots probed with the AtHMA1 or AtHMA4-C antibodies and bands that were detected did not appear to be of the correct size (Figure 5.25). Detection of the proteins in transformed yeast also appeared unsuccessful. The AtHMA4 C-terminal antibody reacted unspecifically with proteins of around 53 and 32 KDa (Figure 5.25) whilst the AtHMA1 C-terminal antibody produced smeared bands around 53 KDa (Figure 5.25). In addition to this, the AtHMA1-C-terminal antibody was also observed to highlight a product of around 130 KDa (Figure 5.25). This was observed most strongly in *AtHMA4* transformed yeast and to a lesser extent in those transformed with *AtHMA1*. Absence of a reaction with the *p426* control cells, could indicate that the antibody had reacted incorrectly with ectopically expressed AtHMA4.

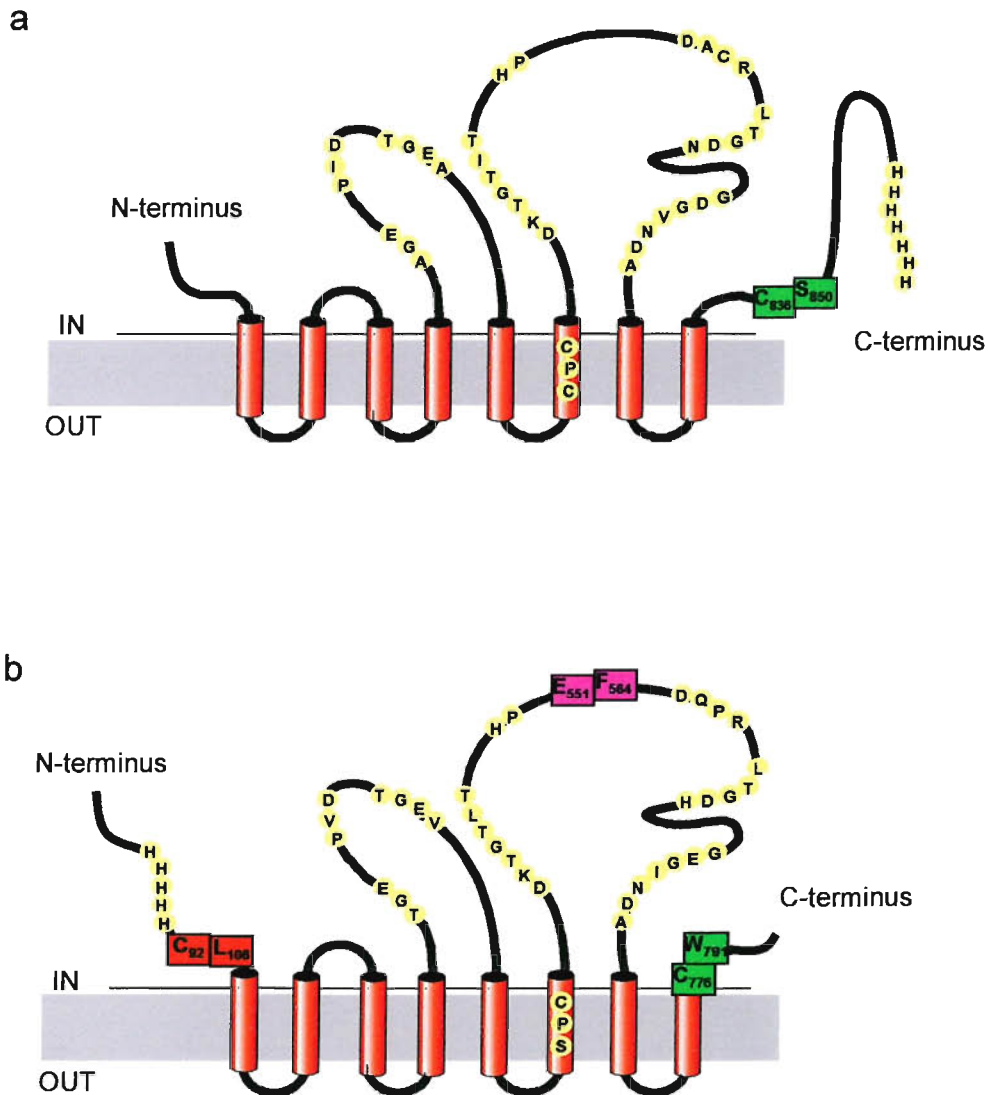


Figure 5.21 Schematic diagram showing the locations of epitopes used to create polyclonal antibodies for western blotting. The two terminal amino acids of the epitope sites are shown within rectangles (with amino acid number in subscript) on a simple 2-dimensional model of the HMAs. a; shows the C-terminal site (green rectangles) used for the single AtHMA4 antibody and b; the N-terminal (red rectangles), internal (purple rectangles) and C-terminal (green rectangles) sites used for AtHMA1. Also shown (yellow circles) are salient motifs of the two HMAs.

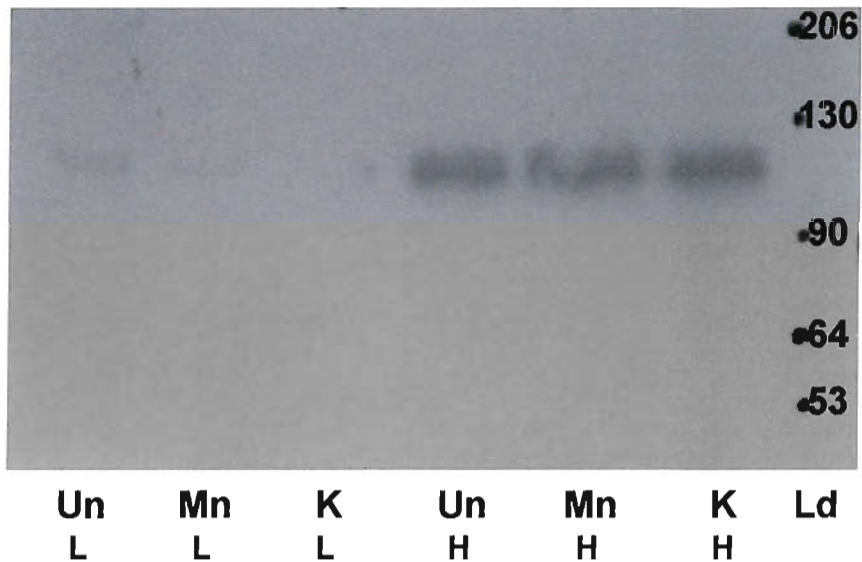


Figure 5.22 Western blot of *Arabidopsis thaliana* membrane fractions probed with the PMA2 H⁺-ATPase antibody. Leaf membranes were prepared from plants grown in nutrient solution (Un) or exposed to 0.8 mM MnSO₄ (Mn) or 1.0 mM K₂SO₄ (K) for 30 hours. Low Speed (L) and High Speed (H) fractions were subjected to SDS-PAGE before transfer onto PVDF membrane. Membranes were subsequently probed with the polyclonal H⁺ ATPase antibody raised to tobacco PMA2. Protein ladder (Ld) size markers are shown.

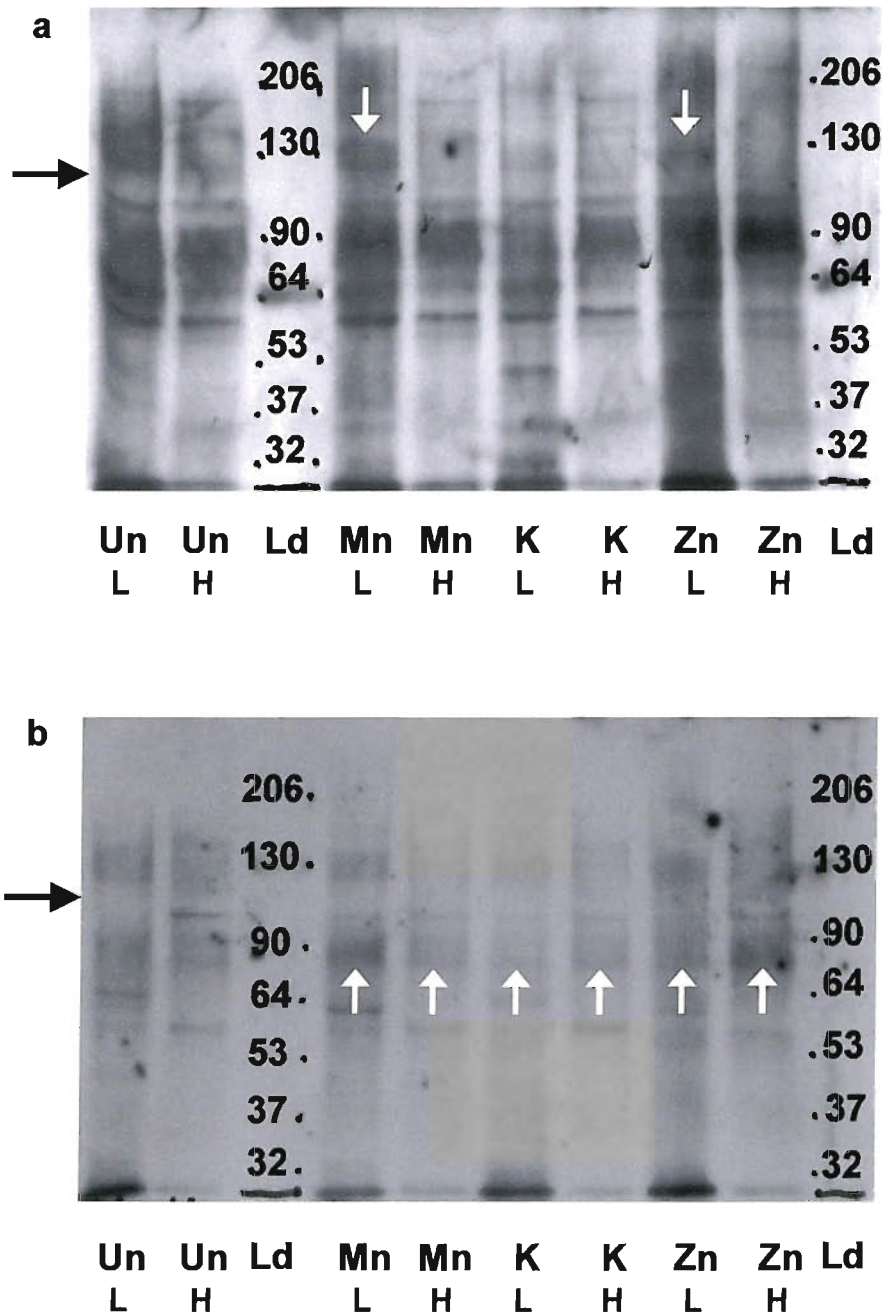


Figure 5.23 Western blots of plant membrane fractions probed with AtHMA-specific antibodies. Root membranes were prepared from plants grown in nutrient solution (Un) or exposed to 0.8 mM MnSO_4 (Mn), 1.0 mM K_2SO_4 (K) or 1.0 mM ZnSO_4 (Zn) for 30 hours. Low Speed (L) and High Speed (H) fractions were subjected to SDS-PAGE before transfer onto PVDF membrane. Membranes were subsequently probed with polyclonal antibodies raised to peptides in the (a) AtHMA4 C-terminal domain and (b) AtHMA1 N-terminal domain. Protein ladder (Ld) size markers are shown, with the approximate relative location of the H^+ ATPase bands determined following re-probing with the PMA2 H^+ -ATPase antibody (dark arrows).

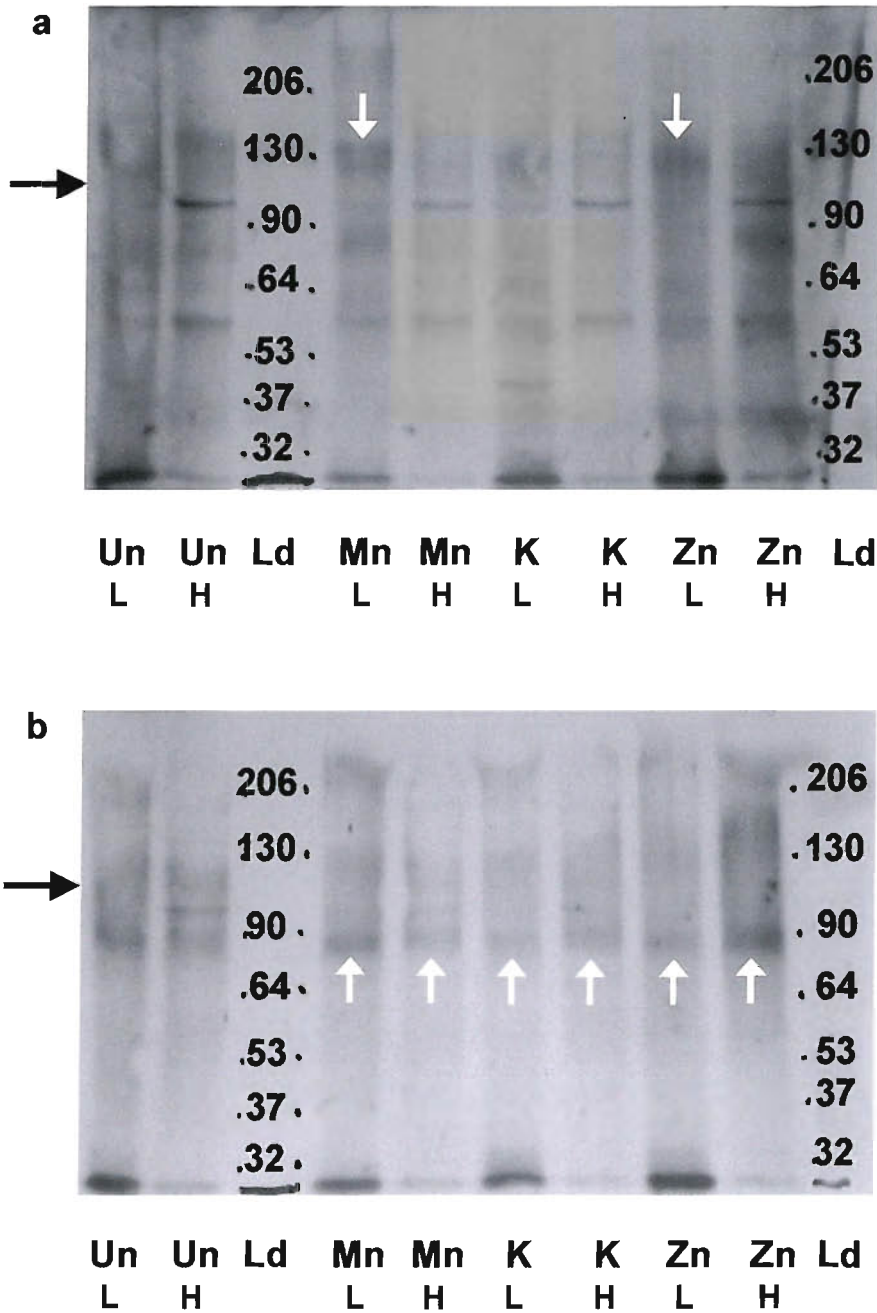


Figure 5.24 Western blots of plant membrane fractions probed with AtHMA-specific antibodies. Root membranes were prepared from plants grown in nutrient solution (Un) or exposed to 0.8 mM MnSO₄ (Mn), 1.0 mM K₂SO₄ (K) or 1.0 mM ZnSO₄ (Zn) for 30 hours. Low Speed (L) and High Speed (H) fractions were subjected to SDS-PAGE before transfer onto PVDF membrane. Membranes were subsequently probed with polyclonal antibodies raised to peptides in the (a) AtHMA1 C-terminal domain and (b) AtHMA1 internal domain. Protein ladder (Ld) size markers are shown, with the approximate relative location of the H⁺ ATPase bands determined following re-probing with the PMA2 H⁺ -ATPase antibody (dark arrows).

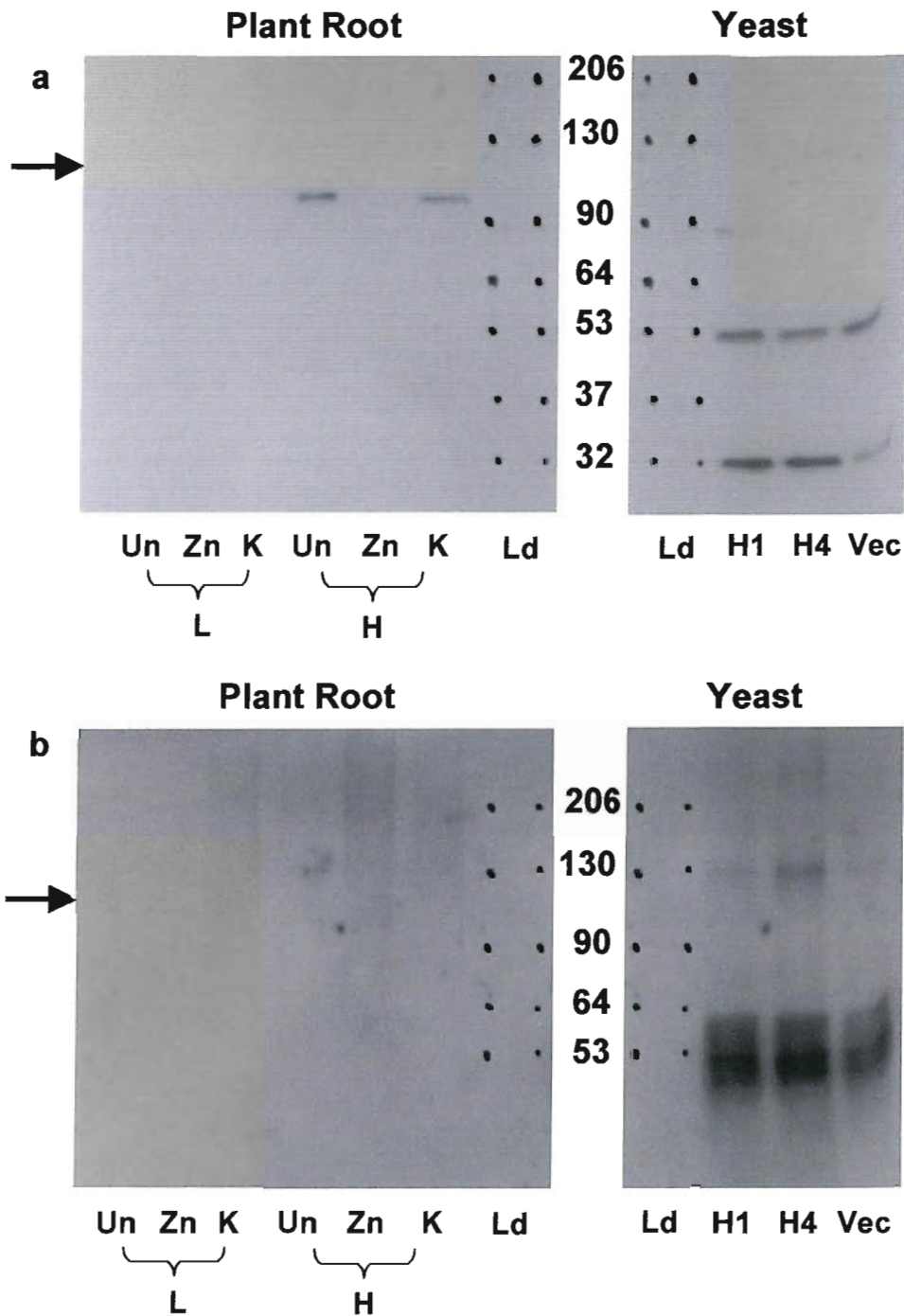


Figure 5.25 Western Blots of plant and yeast membrane fractions probed with AtHMA-specific antibodies. Root membranes from plants grown in nutrient solution (Un) or exposed to 0.8 mM MnSO_4 (Mn), 1.0 mM K_2SO_4 (K) or 1.0 mM ZnSO_4 (Zn) for 30 hours were extracted alongside preparations from transformed yeast. Low speed (L) and high speed (H) fractions were subjected to SDS-PAGE before transfer onto PVDF membrane. Membranes were subsequently probed with polyclonal antibodies raised to peptides in the (a) AtHMA4 and (b) AtHMA1 C terminal domains. Protein ladder (Ld) size markers are shown, with the approximate relative location of the H^+ ATPase bands determined following re-probing with the H^+ ATPase antibody (dark arrows).

5.3 Discussion

The work presented in this chapter has highlighted that the control of *AtHMA* transcript abundance is likely to play a key role in the regulation of these transporters and in turn the movement of their substrate ions. The precise nature of the regulatory process has not been defined, but it has been shown that *AtHMA* gene expression does not follow a constitutive pattern and that individual members can respond differently to different stimuli. Regulation at the protein level could not be clearly determined, and so the importance of translational or post-translational mechanisms are unclear.

Under standard conditions of nutrient supply, unchallenged by elevated levels of metal ions, *Arabidopsis thaliana* plants were shown to express *AtHMA4* and *AtHMA1* in all of the tissues tested. This indicates that these genes are required for normal homeostatic mechanisms of plant nutrition. However there were differences in their tissue-specific expression. *AtHMA4* was expressed at considerably higher levels in roots, intermediate levels in the leaves and lower levels in the stems, flowers and siliques. For *AtHMA1*, apart from flowers and siliques, the pattern was reversed with leaf and stem expression being more prominent than in the roots. These results have been confirmed in further studies carried out subsequent to this work. A slightly less detailed analysis of different plant organs by Gravot et al. (2004) using RT-PCR showed that roots displayed the highest levels of *AtHMA4* expression. Leaves showed the second highest transcript abundance, however the levels were slightly lower than that seen in this study, being almost equal to that found in the stem. For *AtHMA1*, two later studies have commented upon the relative levels in the leaves and roots. The microarray analysis of Becher et al. (2004) and undisclosed method of Kim and Lee (2004, meeting abstract) both revealed higher levels in the leaves. The difference in the distribution of the two expressed genes is a good indicator of the discrete but possibly inter-linked roles of these transporters.

Further experiments in this project have also confirmed higher expression in the root for *AtHMA2* (results not shown), whilst work on *AtHMA3* by Gravot et al. (2004) has shown that this gene displays a more constitutive expression pattern. Thus differential expression of the HMAs is not necessarily a family-wide phenomenon. In the case of *AtHMA4* and *AtHMA1*, their differential expression patterns can be used in an attempt to glean information on localisation of the functional proteins within the plant.

AtHMA4 is obviously prominent in the roots but it is also important in the leaves and stem. This indicates that *AtHMA4* is located in cells that are present in all three organs. Hussain et al. (2003) have shown with the GUS reporter gene that *AtHMA4* expression is concentrated in the locales of both the xylem and phloem tissues. It may be that *AtHMA4* participates in xylem/phloem unloading/loading. *AtHMA1* has greatest expression (in order) in the leaves, stem, then roots. From this profile it is difficult to envisage a specific location of the transporter within cells or cell types. Work using promotor-GUS constructs for *AtHMA1* or immunolocalisation of *AtHMA1* protein would clarify this, since other than the tissue-specific expression data presented here, nothing more detailed appears to have been published on the localisation of this gene or gene products. Obviously, with expression occurring in the roots it is unlikely that *AtHMA1* is involved in transport at the chloroplast as has been shown for PAA1 (Shikanai et al. 2003), a related Cu/Ag transporting ATPase. Similarly if associated with the mitochondria, it may be expected to be expressed at similar levels in roots and shoots, although there does appear to be little evidence on the abundance of these organelles in different plant organs. Specific predictions of protein targeting are not possible with the expression data and it is acknowledged that only suggestions can be made with the results of this chapter. However they are discussed in light of the predicted mitochondrial-targeting signal that has been found in *AtHMA1*.

One of the important aspects of tissue-specific expression revealed in this work has been the evidence produced to show that transcripts of *AtHMA4* and *AtHMA1* respond differently to various stimuli in different organs. In the first instance the expression of both genes has been shown to be metal responsive. This indicates that these particular HMAs are likely to play an active and important role in plant metal homeostasis. Not all members of this gene family appear to possess this character (Gravot et al. 2004; Eren and Argüello, meeting abstract 2004). In addition to this several metals have been shown to evoke what appears to be a specific response. Throughout this work Zn and Mn exposed plants have produced the most consistent results. Mn up-regulates transcripts of both genes in the roots of plants, but appears to down regulate *AtHMA1* and have a variable impact on *AtHMA4* in the leaves. The up *versus* down regulation in roots and leaves for *AtHMA1* appears unusual and is difficult to explain. It may be the result of a secondary effect since Mn is not a predicted substrate of any of the *At*HMAAs. Mn, predominating in the plant as Mn^{2+} , shares many similarities with the 'hard metal' ions such as Mg^{2+} and Ca^{2+} , which are normally transported by the larger 'calcium-type' ATPases. In other organisms, Mn may also be shuttled by ABC transporters, or because of additional

similarities it shares with Fe^{2+} , members of the Nramp family of proteins (Jakubovics and Jenkinson 2001).

Whilst very few HMA transporters use Mn as a substrate, all are thought to require Mg (as Mg-ATP) for completion of their reaction cycle. Little work appears to have been carried out on the aspects of Mg requirement or interaction with metal transporting HMAs, but evidence has been produced in animal cells to show that its presence is required for full activity of a Zn-transporting ATPase (Wang et al. 2001). If this is the case then it is quite conceivable that Mn may substitute for Mg, when present at high concentrations. The two are known to be interchangeable in different chelate structures (Jakubovics and Jenkinson 2001), so perhaps Mn-ATP inhibits the transport reaction upon association with the HMA-ATPase. The coding gene could then respond to this inhibition by increased expression and by production of more transporter protein. In the leaves reduced expression of *AtHMA1* could be due to a secondary effect of Mn on the true substrate. Metal ions are known to compete for translocation in the xylem and the presence of one ionic species may reduce the leaf contents of another. If the true substrate of *AtHMA1* was, as suggested by sequence analysis, Zn, then high levels of Mn may induce Zn deficiency. In plant leaves treated with 1.0 mM additional ZnSO_4 for 30 hours *AtHMA1* was up-regulated, so Zn deficiency (induced by Mn) could conceivably provoke an opposite response.

In plants treated with additional Zn, *AtHMA1* also displayed different responses in root and leaf tissues. However, in contrast the responses induced by Zn were exactly opposite to those provoked by Mn. Zn down regulated this gene in roots and tended to up-regulate in the leaves. This makes it more difficult to formulate any theories that can explain the results. Assuming that Zn is the primary substrate, and that Mn effectively competes with Zn for uptake into the plant cytoplasm, then the following scheme appears to be true. In the roots: Zn increase = down-regulation of *AtHMA1*, Zn decrease (Mn induced) = up-regulation of *AtHMA1*. In the leaves: Zn increase = up-regulation of *AtHMA1*, Zn decrease (Mn induced) = down-regulation of *AtHMA1*. So based on these assumptions it does appear that there is differential regulation in these organs, negative in roots and positive in leaves. To clarify this generalisation of the data, it must be stressed that increases in *AtHMA1* transcript abundance were not observed in the first leaf-metal screen. An important point to make from the time course data is that the response of *AtHMA1* to Zn does appear to diminish with increased exposure periods. At 15 hours, the greatest induction of this gene was observed, however by the 60 hour time point the induction had transformed into a mild cessation with levels being slightly lower in the

treated plants. Interestingly, subsequent work on *Arabidopsis* exposed to 0.1 mM (100 μ M) $ZnSO_4$ for 4 days has revealed that treated plants exhibited slightly lower levels of *AtHMA1* transcripts than controls (Becher et al. 2004).

For *AtHMA4*, the Zn response is perhaps easier to explain. There was up-regulation found in both leaf and root tissues. Initial results suggested that the increase in root transcripts was of a greater magnitude than leaves, but later experiments carried out over a concentration range of Zn exposure indicated that the level of increase may be similar. This was evident despite the overall levels being much lower in the leaves. Further investigation has been started to study this response in more detail, by increasing the levels of Zn in the leaves with foliar applications. This aims to test if any differences observed are related to the amount of Zn entering these tissues. A similar idea has been tested by Bovet et al. (2003), in their study of the ABC transporter *AtMRP3* and its regulation by Cd^{2+} . These workers originally found an increase in *AtMRP3* root transcripts upon exposure to (root-applied) $CdCl_2$, but no change in the leaves. In later experiments $CdCl_2$ was fed directly to the shoots by excising the roots and placing the stems (plus some of the primary root) in $CdCl_2$ adjusted solution. In these plants *AtMRP3* induction was shown to be greater in the leaves than roots, when expressed relative to *Actin 2*. Thus in this case at least, gene induction was found to be higher in roots solely because they were subjected to increased levels of inducing ions in intact plants (Bovet et al. 2003). The shoots displayed lower expression levels as they were protected by a 'buffering capacity' of the roots (Bovet et al. 2003).

Despite a recent increase in the volume of published work on HMA transporters, there are very few other reports on the metal-induced expression patterns of *AtHMA4*. Bernard et al. (2004) briefly studied *AtHMA4* transcript levels in response to 10 μ M $CdSO_4$ exposure as part of a study on a related transporter in *Thlaspi caerulescens*. Using northern analysis they found no significant change in expression in roots and could not detect any transcripts in the leaves (Bernard et al. 2004). Northern analysis carried out to look at Zn and Mn exposure in this work, also showed that leaf transcript levels were difficult to detect. However, RT-PCR of plant root material exposed to 0.1 mM (100 μ M) Cd showed that this metal could in fact reduce the expression of *AtHMA4*. The consistent up-regulation of *AtHMA4* in roots exposed to 1.0 mM additional $ZnSO_4$ has been supplemented by additional data from the concentration gradient experiments (observed at 15, 30 and 60 hour exposures: only 30 hour data shown). These have shown that the *AtHMA4* response is proportional to the amount of Zn applied to the roots. The regulation of plant membrane transporters by Zn has not been reported widely. *ZIP2* from

Medicago truncatula is thought to transport Zn across the plasma membrane of xylem tissues and this was thought to be Zn regulated (Burleigh et al. 2003). The responses observed for *AtHMA4* provides good evidence that the transcriptional up-regulation is specific to Zn.

When expression of the other HMAs were tested over a Zn concentration range various different responses were reported for each, although the trends observed were consistent at each increment. For *AtHMA1*, down regulation was in agreement with earlier results (this work) and a very slight down regulation found in roots treated for 4 days with 0.1 mM (100 μ M) ZnSO₄ by Becher et al. (2004). Work by Kim and Lee (2004, meeting abstract), opposes these results, since they have reported up-regulation under elevated Zn conditions (conditions unreported). Of the remaining three genes tested, each displayed a different response over the Zn concentration course. Zn had no discernable effect on the expression of *AtHMA2*, a result that appears to agree with the unpublished work of others (Erin and Argüello 2004, meeting abstract). The reasons for this are unclear but it may be speculated that the gene codes for a HMA that is not part of an active homeostatic mechanism. Alternatively it is possible, that the transport protein resides in a compartment unaffected by additional Zn. There are two reports that point to the involvement of *AtHMA2* in the movement of Zn. Hussain et al. (2004) have shown with mutant plants that *AtHMA2* plays a role in the Zn translocation pathway and is located in vascular tissues. Leaf tissues in *athma2/athma4* double mutants were deficient in Zn, supporting a role for *AtHMA2* in metal uptake (Hussain et al. 2004). Conversely, the work of Erin and Argüello (2004, meeting abstract) has apparently suggested that *AtHMA2* is involved in the efflux of Zn²⁺ out of plant cells. It was also claimed that removal of *AtHMA2* transcripts results in greater Zn accumulation in the plant (Erin and Argüello 2004, meeting abstract). In either case, it is hard to see why *AtHMA2* expression is not modified when the need arises. If part of an uptake mechanism, expression of *AtHMA2* may be expected to be reduced at high Zn (thus avoiding toxicity) or alternatively increased, if involved in metal ion efflux. Perhaps its function is redundant in this respect, with other HMAs (for example *AtHMA4*) taking on this role. Constitutive expression of a Zn transporter has been reported in *Arabidopsis thaliana* previously. Van der Zaal et al. (1999) have shown that CDF *ZAT2* expression was unaffected by increased levels of the substrate ion. Despite this however, they found that plants overexpressing the gene had enhanced Zn resistance and accumulation in root tissues, over the wild-type controls (van der Zaal et al. 1999). This suggested that the protein, which is purported to be located

at vesicular or vacuolar membranes did have a tangible impact on Zn homeostasis, even though expression of the gene was unresponsive.

The expression of *AtHMA3* was shown to be unaffected by low levels of Zn, but depressed rather severely at higher concentrations. Recent work on this transporter has provided evidence that it is localised at the vacuole, where it may import Cd^{2+} and/or Pb^{2+} ions (Gravot et al. 2004). No evidence has been obtained for Zn, but from its sequence it would be expected to be a potential substrate. If Zn was not a substrate of *AtHMA3*, then high concentrations of this ion could swamp the gene and prevent regulation by its true substrate (e.g. Cd or Pb), potentially switching off the mechanism by which it is induced.

There still appears to be no published data for *AtHMA5*. Results from microarray experiments shown on the PlantsT website (<http://plantst.sdsc.edu>), indicate that this gene is expressed mainly in pollen and lateral roots, with little found in the shoots. No data is presented however to show the gene's response to metal treatments (<http://plantst.sdsc.edu>). The data presented here clearly show up-regulation of *AtHMA5* in roots. The response appears similar to that of *AtHMA4* with a peak at 1.0 mM additional ZnSO_4 but the rate of its induction is less dramatic, being on a lower scale. It is interesting to note that *AtHMA5* is predicted to transport Cu or Ag ions and resides in a different subgroup to *AtHMA4* when subjected to phylogenetic analysis (Chapter 3), yet both appear to be up-regulated by Zn. It will be interesting to test whether this transporter is regulated by other metals.

As has been suggested above for the other HMAs investigated in this work, Zn may compete with the true substrates of other transporters and affect expression indirectly. Alternatively, it is now evident (from studies in bacteria) that HMAs may exist that have the ability to transport ions that traditionally split members into Cu/Ag and Zn/Cd/Pb/Co-transporter subgroupings. Early work with animal lysosomes demonstrated HMAs that could transport the divalent ions of Cd, Cu and Ag (Havelaar et al. 1998), but the relevance of these experiments is questionable. Cu and Ag are believed to exist in their monovalent forms within the cell and are unlikely to behave in the same manner as their divalent cations (Rosen 1999; Rensing et al. 2000; Silver 2003; Solioz and Stoyanov 2003). More recent findings have provided the first evidence of a HMA that appears to move both monovalent and divalent cations (Tong et al. 2002). *Bxa1* from *Oscillatoria brevis* conferred resistance on *E.coli* to Zn^{2+} , Cd^{2+} , Cu^{1+} and Ag^{1+} and was found to be induced by each of these ions in the host (i.e. parent) organism (Tong et al. 2002).

Finally, a last but extremely important phenomenon that may have a significant bearing on the work of this chapter is the finding that some HMA-coding

genes can be directly induced by metals that are not substrates of transport (Binet and Poole 2000; Stoyanov and Brown 2003; Stoyanov et al. 2003). There appears to be no indication of how wide-spread this may be but work on *ZntA* and *copA* of *E. coli* has shown that induction of expression may be relatively high and sometimes higher than that caused by a true substrate (Stoyanov and Brown 2003). The reasons for this are probably due to unspecific binding of the metal ion with the protein that regulates expression of the HMA gene (Stoyanov and Brown 2003). At present our understanding of metal regulatory proteins in *Arabidopsis* is lacking. Metal-dependent regulation of transport proteins is also little understood.

It is disappointing that the HMA antipeptide polyclonal antibodies failed to specifically detect products of predicted size in the western blot hybridisations. It is possible that the HMA transporter proteins may differ in size from that envisaged, especially if they undergo some form of modification in the protein secretory pathway. However the blots were of an appearance consistent with general background binding of the antibodies. The failure of the immuno-detection method may have occurred for a number of different reasons. P-Type ATPases have a rather complex tertiary structure (Zhang et al. 1998; Toyoshima et al. 2000) and it may be that the epitope sites are sequestered upon protein folding. In addition, the association of the protein with the plasma membrane could also cause problems in this way. In future, it may be advisable to try alternative epitope sites, preferably with monoclonal antibodies. These are beneficial as they usually have greater specificity for the antigen and their use can reduce problems of low sensitivity that can occur with polyclonal antisera (Catty 1988). Immuno-labelling of a successful antibody could provide some important answers as to the role(s) of AtHMA4 and AtHMA1. This technique can be used *in-situ* and would allow the proteins to be localised under different metal conditions. If the acquisition of reliable antibodies appears difficult, the creation of artificial epitopes (epitope tagging) is another avenue for exploration. The procedure is rather laborious, as it requires the creation of tagged DNA constructs, transforming agrobacteria, and then inoculating and screening for successfully transformed plants. The immuno-detection steps would likely be more robust however, since the specificity of antibody for antigen (artificial epitope) would be known.

In light of the comments on metal regulation of gene transcription (discussed above) the potential importance of protein regulation in metal transporters must be noted. The degradation of several metal transport proteins in response to elevated metal levels has been demonstrated in yeast (Radisky and Kaplan 1999). These include the copper transporter CTR1, which appears to be degraded at the plasma-

membrane by a surface bound protease (Ooi et al. 1996), Znt1, a zinc transporter that is degraded in the vacuole via endocytosis (Gitan et al. 1998; Gitan and Eide 2000), and Smf1 an Nramp with variable metal specificity that is degraded in the vacuole after modification at the endoplasmic reticulum (Liu and Culotta 1999). Whilst mechanisms of transporter regulation may be different in yeast, protein regulation has been shown to occur in plants (Curie et al. 2000). In their work on the iron-specific ZIP transporter IRT1, Connolly et al. (2002) showed that whilst Zn had no effect on *IRT1* transcript abundance, it could influence the amount of protein expressed. Fe could also influence protein levels independent of gene expression, but it could also influence transcript abundance. Since Fe is the proposed true substrate of this transporter, it was not known if the effects of Zn were direct, or if they were mediated by an influence on the levels of Fe (Connolly et al. 2002). Nevertheless, the work of Connolly et al. (2002) demonstrates the importance of protein expression studies, and reinforces the suggestions made here for further work.

Chapter 6 General discussion

The aim of this study was to characterise the *Arabidopsis* HMAs, *AtHMA4* and *AtHMA1*, and to investigate their roles in plant-metal homeostasis. Part of this work has been dedicated to the sequencing and cloning of *AtHMA4* and its comparison with *AtHMA1* (also cloned by this laboratory) and a further six related HMA transporters in *Arabidopsis* (Baxter et al. 2003). Functional studies of *AtHMA4* and *AtHMA1* in yeast have been used to identify potential substrates of these transporters. Expression analysis carried out on *Arabidopsis* tissues has indicated that transcription of these genes is regulated in a metal-specific manner. Results presented in each chapter have highlighted a number of observations that point to a Zn/Cd transport function of the two HMAs. Using this evidence, together with recent published work, it is now possible to speculate on their possible roles *in planta*.

6.1 Sequence analysis

Sequencing of *AtHMA4* has revealed that this gene has a coding region of 3.5 Kb, comprising 9 exons. It is somewhat different to *AtHMA1*, which has 2.4 Kb of coding sequence within 13 exons. These are arranged differently within the gene; exons of *AtHMA4* can be segregated into 3 clusters (Figure 3.14), whilst those of *AtHMA1* follow no particular pattern (not shown) (<http://plantst.sdsc.edu/>; Baxter et al. 2003). Phylogenetic analyses using the protein sequences show that whilst both proteins fall within the Zn/Cd/Pb/Co subclass of the HMA transporter group (Mills et al. 2003), *AtHMA4* clusters more closely with *AtHMA2* and *AtHMA3*, whereas *AtHMA1* appears more distantly related (Figure 3.17). The significance of this divergence is not clear. Phylogenetic analyses may highlight differences in HMA substrate specificity (Axelsen and Palmgren 1998; Fox and Gueriot 1998; Baxter et al. 2003), and indeed there appears to be a clear segregation of Cu/Ag pumps from those which transport Zn/Cd/Pb/Co (Axelsen and Palmgren 2001; Baxter et al. 2003; Cobbett et al. 2003; Mills et al. 2003). However other factors may also be important and have some influence, such as localisation within the cell (Paulsen and Saier 1997). Targeting sequences do exist within the HMA proteins; *AtHMA1* has a putative mitochondrial targeting signal (identified as LRAVED by Psort prediction; <http://psort.nibb.ac.jp/form.html>), and *AtHMA6* (PAA1), a chloroplastic targeting signal, with chloroplast localisation recently confirmed by Shikani et al. (2003). There is also evidence that the closely related proteins *AtHMA2*, *AtHMA3* and

AtHMA4 are all plasma-membrane-bound (Eren and Argüello 2004, meeting abstract; Gravot et al. 2004; Hussain et al. 2004).

6.2 Protein Motifs

AtHMA4 and AtHMA1 possess a number of motifs common to all P-type ATPases (including the DKTGT motif containing the phosphorylated aspartate residue and the GDGXNDXP motif in the putative hinge domain (Solioz and Vulpe 1996; Williams et al. 2000) but they also possess certain other features that may be relevant to their association with heavy metal ions. Whilst some are consistent between certain members of the group others appear unique. AtHMA5,6,7 and 8 each possess one or more CxxC motifs in their N-terminal domain. These are common, but not exclusive to Cu transport proteins (Wernimont et al. 2000; Jordan et al. 2001; Opella et al. 2002). AtHMA2, 3 and 4 share CC repeats in their C-terminal region, with AtHMA2 and 4 having additional histidine stretches. Histidine-rich sequences are found in zinc-finger proteins and CC/CxC motifs in metallothioneins have been implicated in the binding of metals such as Zn and Cd (Goldsbrough 2000; Englbrecht et al. 2004). AtHMA1 is a rather unusual member of the AtHMA transporter group. Phylogenetic analyses (Figure 3.17) has shown that it forms a general cluster with AtHMA2,3 and 4, (transporters suspected to transport Zn/Cd/Pb/Co) but is less related. In terms of its shared similarities, AtHMA1 displays a rather large (discontinuous) histidine stretch and one CC repeat (Figure 3.15). What is unique to this protein is the location of the metal-binding domains, as both reside in the N-terminal domain. Additional variation in AtHMA1 structure is evident in the CPx motif. At this location AtHMA1 possesses an SPC motif. Comparison with HMAs from a wide range of organisms shows that this is not as rare as once thought with TPC, APC and other SPC-containing HMAs being found in a recent analysis (Argüello 2003). Nevertheless AtHMA1 is the only *Arabidopsis* HMA to possess this particular CPx variant. The CPx motif is known to be an essential component of the enzyme. Mutation of this region has been shown to prevent enzyme function (Yoshimizu et al. 1998; Bissig et al. 2001), possibly because of an inhibition to metal-dependent phosphorylation (Fan and Rosen 2002). The functional significance of the particular variant is not clear but it is known that amino acids surrounding the proline of this motif vary with different classes of P-type ATPases (Solioz and Vulpe 1996; Williams et al. 2000), and may indicate the metal ion transported. The amino acids VPE exist in Na⁺K⁺-ATPases and IPE in the Ca²⁺-ATPases (Solioz and Vulpe 1996). Considering the substrate specificities of other

HMAAs that possess an SPC-motif, Argüello (2003) proposed that AtHMA1 is likely to transport Zn. Cobalt is also a potential substrate since CoaT from *Synechocystis* shares this domain (Rutherford et al. 1999) but it has no other putative metal-binding domains analogous to the histidine stretch of AtHMA1.

6.3 Organ-specific expression of AtHMA4 and AtHMA1 and regulation by metals

AtHMA4 and *AtHMA1* expression has been found in all tissues of *Arabidopsis* plants when grown under standard nutrient conditions (Chapter 5). This indicates that *AtHMA4* and *AtHMA1* are required for homeostatic mechanisms that are a part of normal metabolism. *AtHMA4* was expressed highest in roots, followed by the leaves, whilst *AtHMA1* expression was dominant in the leaves, followed by the stem and root tissues. This variation in organ-specific expression provides good evidence that the two transporters have different functions within the plant, and their responses to different metal treatments supports this. This work has shown that the localisation of transcripts for both HMA genes varies within the plant but also that they respond differently to metal treatments. In general, Zn and Mn appeared to produce the largest and most consistent responses in transcript levels. Zn up-regulated *AtHMA4* expression in leaves and roots, and did so in a concentration dependant manner (Figures 5.12 and 5.13). Burleigh et al. (2003) claimed that up-regulation of transporter genes upon Zn addition is quite a rare phenomenon. For *AtHMA1*, Zn exposure lead to reduced transcript levels in roots, but tended to increase levels in the leaves (Figures 5.14 and 5.10b). When treated with Mn, the opposite response was true for *AtHMA1*; leaves responded with a general down-regulation while roots showed up-regulation. *AtHMA4* transcripts also increased in roots upon Mn addition, but the response in leaves was variable (Figures 5.8 and 5.10a). The results from expression analysis have shown clearly that Zn appears to regulate the transcript abundance of *AtHMA4*. This is consistent with a transporter enzyme being regulated by the availability of its substrate (see later). Effects of Mn on expression of *AtHMA4* are also apparent, and the reasons for this can only be speculated upon. It is important to point out that the regulation of transporter gene expression can be influenced by metal ions that are not the transport substrates (Stoyanov and Brown 2003).

Unfortunately the work with anti-peptide antibodies carried out as part of Chapter 5 failed to detect AtHMA4 and AtHMA1 proteins in a range of metal-treated tissues. This may have failed for a number of reasons, but failure to detect protein in

a rather simple background (transformed yeast cells) could indicate a lack of cross reactivity. If successful, this work could have given a general idea as to the membrane location of the proteins within different tissues, under different metal conditions.

6.4 Functional studies and implications for physiological roles

In order to obtain more conclusive evidence for the transport substrates of these pumps, wild-type yeast were transformed with the respective cDNAs under the influence of the galactose inducible promoter, *gal1* (Mumberg et al. 1994; Mumberg et al. 1995). Yeast are the preferred expression system for plant genes (Frommer and Ninnemann 1995) and are well suited to flux measurements that allow the determination of substrate specificity (Dreyer et al. 1999). However, the presence of numerous different transport systems and problems of protein mis-targeting should be considered. Alternative expression systems could be used and indeed expression of *AtHMA4* in a bacteria, *E.coli* mutant was carried out as part of the work reported by Mills et al. (2003). *AtHMA4* was able to rescue the Zn-sensitive *ZntA* mutant from high concentrations of this ion in the growth medium, suggesting a role in efflux (Mills et al. 2003).

The results presented in Chapter 4 have used the yeast transformants described in Mills et al. (2003) to conduct further experiments. Using metal tolerance assays, which measured the zone of yeast growth inhibition around discs supplemented with metals, *AtHMA4* and *AtHMA1* were observed to make yeast more resistant or more sensitive respectively to CdSO_4 , but not ZnSO_4 (Mills et al. 2003). In this study the Cd-resistance conferred by *AtHMA4* and the Cd-susceptibility conferred by *AtHMA1* was confirmed by growth experiments where yeast transformants were grown on metal-supplemented agar. Importantly, work reported here measuring radiolabelled metal uptake has shown that *AtHMA4* and *AtHMA1* have a discernable and sometimes significant impact on yeast-metal homeostasis, since transformation with these cDNAs was observed to alter patterns of metal accumulation. The data show that the accumulation of both Zn and Cd were effected in the HMA-transformants compared to vector controls. The data presented showed that *AtHMA1* transformants tended to accumulate radiolabelled Zn compared to vector control cells, although significant differences with Cd were not observed. *AtHMA4* transformants accumulated lower levels of Cd and Zn than vector controls. This could be explained by the proteins functioning in different ways and/or being localised differently within yeast. *AtHMA4* could be targeted to the

plasma membrane and participate in metal extrusion from the cell, which could explain the lower accumulation of radiolabelled Cd and the resistance to Cd observed in the growth assays. AtHMA1 could be targeted to the plasma membrane and function in influx into the cell, or perhaps into an organelle, leading to an overall increase in yeast metal content. Generally HMAs have been reported to function in efflux at the plasma-membrane rather than influx although HMAs with an influx function have been described in a few organisms (LaGier et al. 2001; Nies 2003). Evidence for HMA-mediated metal uptake at the plasma membrane has been observed for Cu and Zn in various prokaryotes (Phung et al. 1994; Gaballa and Helmann 2002), whilst uptake of Cu into photosynthetic organelles mediated by HMAs has been observed in cyanobacteria and plants (Kanamaru et al. 1994; Tottey et al. 2001; Shikanai et al. 2003). The movement of toxic ions by influx HMAs has also been shown to occur by these mechanisms with Ag accumulation or transport being demonstrated. (Tottey et al. 2002). Considering its putative mitochondrial targeting sequence, it is possible that AtHMA1 may localise to yeast mitochondria as predicted in the plant. The correct targeting of proteins within yeast has been observed in a number of experiments including those that target to the mitochondria and plasma membrane (Zhao et al. 2003; Shibagaki et al. 2004), but there have been cases where the correct processing of the protein has proved unsuccessful (Axelsen and Palmgren 1994).

The increased sensitivity of *AtHMA1* yeast transformants to Cd could be accounted for by higher accumulation of this metal ion, although in the radiolabelled uptake assays accumulation compared to vector controls was only significant for Zn and not Cd (Figure 4.6 and 4.7). The sensitivity could be explained by AtHMA1 directing the metal ion to a particularly sensitive organelle. This could perhaps rule out localisation of AtHMA1 to the yeast vacuole, as this compartment is often used to compartmentalise excess toxic metals. Here metal ions are stored in an inert form, bound as organic complexes as part of a normal homeostatic mechanism (Ortiz et al. 1992; Ortiz et al. 1995). Whilst the targeting of AtHMA1 to the yeast vacuolar membrane may not explain the Cd sensitivity observed in the transformants, it must be pointed out that targeting of AtHMA1 or AtHMA4 to this site could still occur and may not necessarily lead to increased metal susceptibility or resistance. A study on a putative Zn/Cd/Co/Pb HMA from *Arabidopsis thaliana*, AtHMA3, localised the heterologous protein at the vacuolar membrane (Gravot et al. 2004). Despite this however, increased tolerance to Cd was not seen in wild-type cells, although it was observed in *ycf1* mutants, which lack the vacuolar ABC transporter, YCF1.

There appears to be little work on the sensitivity of mitochondria *in vivo* to metal toxicity. In plants, mitochondria have been described as being relatively insensitive to metal ions (Ernst 1999), but there is evidence to show that they can be affected by increasing metal concentrations (Davies et al. 1995). It is not hard to envisage that cations with high affinity for sulphhydryl groups (e.g. Cd) could disrupt the mitochondrial membrane, or disrupt charge distribution. This is a possible explanation of the observed results for AtHMA1. In the plant, *AtHMA1* is expressed in leaves and roots, consistent with an organelle that is present in both tissues (Figure 6.1A and B). The fact that relatively higher transcript levels are found in the leaves is difficult to explain, since one would expect the number of mitochondria (and thus levels of *AtHMA1*) in the respiring root tissues to be equally high. However a greater presence of *AtHMA1* at leaf mitochondria may be required because of a greater demand for the Cu-ZnSOD enzyme. In the plant Zn could be the true physiological substrate and AtHMA1 could donate Zn to Cu-ZnSOD. In the leaves there may be a greater demand for Cu-ZnSOD since there may be higher levels of oxygen radical formation in the photosynthesising tissues. A notable result from the expression analysis experiments has been the contrasting responses of *AtHMA1* to Zn and Mn in root and leaf tissues. It is not known if these ions have a specific affect on the expression of this gene, but it is possible that one metal may compete for uptake into the leaves with the other and affect expression indirectly. Indeed the levels and response of ions in the leaf tissues to metal treatments is unknown and may be completely different to that observed in the roots. Interestingly Zn and Mn are both components of superoxide dismutase (SOD) enzymes found in mitochondria and chloroplasts. CuZnSOD is found in mitochondria and chloroplasts, whilst MnSOD is located primarily in mitochondria (Wu et al. 1999). If AtHMA1 was involved in Zn influx into mitochondria then it is possible that excess Mn may disrupt this process by accumulating in the same position prior to movement by its true transporter. In leaves the interaction between Zn and Mn may be different to roots, because of the presence of chloroplasts that may act as an additional sink for Zn. Mitochondria have been observed to cluster closely with chloroplasts in the leaves and it has been suggested that components may be exchange between the two organelles (Logan and Leaver 2000). Of course the reason why *AtHMA1* expression appears to be regulated differently in the root and leaf tissue by Zn is not clear from this work, and is only speculated upon. It is also possible that AtHMA1 does not have a mitochondrial location *in planta* and that it functions in influx of Zn at the plasma-membrane in plants. Protein localisation studies are therefore essential to resolve this.

For *AtHMA4* transformants the increased tolerance to Cd in yeast growth assays, is consistent with the finding that such cells accumulate significantly less of this metal ion in tracer studies. Efflux of the metal at the plasma membrane is thought to be an explanation for these results and is consistent with the usual functioning of HMAs from this sub-class. It could be argued that *AtHMA4* reduces uptake by transporting a form of chelate to the exterior, reducing the concentration of the free ion in the medium, but this is incongruous with their usual mode of action. Unpublished work cited in Gravot et al. (2004) claims that in *AtHMA4*-transformed *ycf1* yeast mutants of *Saccharomyces cerevisiae*, reduced levels of Cd accumulation were observed, consistent with a metal-ion efflux mechanism. This finding is in agreement with the results presented here for work carried out in wild-type yeast cells. Bernard et al. (2004) recently published data that contradicts these findings. Working with the *AtHMA4* homologue, *TcHMA4* from the Cd hyperaccumulator *Thlaspi caerulescens*, showed that *S. cerevisiae* BY4141 transformed with this gene were more sensitive to elevated Cd than the vector control cells. Paradoxically however this study also found that *TcHMA4* cells accumulated less Cd. These authors failed to explain properly the inconsistency of their results. It may be that the Cd-supplemented media was not buffered sufficiently to maintain the pH in replication of the control environment and hence increased acidity led to reduced tolerance of the yeast. The pH of growth media was rigorously monitored for all experiments presented in this report. The addition of metal solutions was found to decrease pH, and control media was adjusted accordingly. Despite their conflicting results, Bernard et al. (2004) considered it possible that *TcHMA4*, as suggested here for *AtHMA4*, may function as a plasma-membrane efflux pump. In order to ascertain the function of *AtHMA4* it would be pertinent to carry out localisation studies within yeast. In future work, it would be advisable to study yeast grown in media with a range of different metal concentrations, and with different levels of protein expression (i.e. driven by different promoters). Both of these factors could have an impact on the localisation of the expressed protein and this could affect the uptake results.

Recent work by Hussain et al. (2004) has reported on tissue expression of *AtHMA4* within plants grown under normal nutrient supply. Using plants transformed with the *AtHMA4* promotor linked to GUS, Hussain et al. (2004) detected a predominance of expression in the vascular tissue of roots, leaves and stems. In these organs, GUS activity was apparently detected in both the xylem and phloem, although the distinction between the two was hard to determine. Based on this evidence, and Zn deficiencies found in the shoots of *hma4* and *hma2/hma4*

knockout mutants, these authors suggested a primary role for AtHMA4 in the loading of Zn in xylem tissues (Hussain et al. 2004). In addition, with transcripts being localised in the phloem they also considered it to have a possible role in the mobilisation of Zn from shoot to root.

Work from our laboratory has shown that *hma4*-knockout plants additionally have reduced tolerance to elevated levels of Cd and Zn showing an inhibition of root and shoot growth (Mills et al. in preparation). This suggests that in addition to a role in heavy metal uptake (as put forward by Hussain et al. 2004), AtHMA4 may also participate in heavy metal detoxification, should the need arise at higher metal concentrations. The potential roles of this transporter and how it may achieve a dual function is intriguing. It is possible that AtHMA4 may simply function to load the vascular tissues in all metal conditions. In this case, AtHMA4 could have a nutritional role in the normal growing environment, but still aid to protect the cells at high metal concentrations. It is conceivable that the up-regulation of root *AtHMA4* upon exposure to additional Zn may be part of a mechanism that protects root tissues from metal overload. AtHMA4 could function at the xylem parenchyma to flood the xylem with additional Zn, where an abundance of chelates such as citrate and nicotianamine (White et al. 1981; von Wirén et al. 1999) could bind the ions and reduce toxicity symptoms. Nicotianamine was originally implicated in the transport of Fe (Pich et al. 2001), but evidence has shown that it is also involved in the movement of other metals including Zn (Takahashi et al. 2003). A recent study on *Arabidopsis halleri* has shown that this Zn hyperaccumulator expresses nicotianamine synthase at a level 70 times more than the non hyperaccumulating species *Arabidopsis thaliana* (Webber et al. 2004).

An alternative theory to account for the observations of Mills et al. (in preparation) and Hussain et al. (2004) could involve the relocalisation of AtHMA4 under conditions of metal excess, in order to stimulate metal efflux. This could be achieved by modification of the existing protein (e.g. cleavage, methylation) or it may involve the modification or production of alternative gene transcripts. The RACE work in Chapter 3 has highlighted the possibility of alternative splicing in the 5' UTR of *AtHMA4*. Perhaps this could alter targeting of the transcribed product or maybe it is a transcript from a different tissue type. It still remains to be seen if the increase in AtHMA4 transcript numbers occurring in Zn treated roots is due to increased expression in the vascular tissues. Hussain et al. (2004) localised transcripts under normal conditions, but no work has been produced for plants grown in conditions with elevated metal ions. Perhaps the transcripts are induced in

epidermal cells under such conditions leading to metal efflux from root tissues to the rhizosphere and a reduction in toxic effects.

Clearly, it would be advantageous for future studies to localise AtHMA4 protein in control and metal-treated plants. Hussain et al. (2004) have localised *AtHMA4* transcripts in the vascular tissue of untreated plants, but it is important to recognise that the phloem cells of the vascular tissue may be involved in the long distance transport of mRNA (Kim et al. 2001) and that accumulation of transcripts in this location may be related to this fact. Whilst transcriptional regulation has been considered as part of a dual function mechanism for AtHMA4, it is worth considering that protein movement within the cells could lead to modification of metal homeostasis in tissues. A dual function has been shown for the Menkes protein from humans (Petris et al. 1996; Petris et al. 1998; Strausak et al. 1999a, b). This transporter normally participates in the shuttling of Cu to the trans-Golgi network, but at high Cu levels, the protein re-localises to the plasma-membrane to participate in metal efflux. Site-directed mutagenesis has showed that cysteine residues in specific CxxC motifs and a dileucine motif, both situated in the N-terminus are crucial to this mechanism (Petris et al. 1998; Strausak et al. 1999b). The cysteine residues appear to be important for the metal-induced relocalisation, whilst the dileucine motif is important in steady-state targeting to trans-Golgi network (Petris et al. 1998; Strausak et al. 1999b). Although AtHMA4 does not possess any putative metal-binding domains at its N-terminus, it does possess a number of potential binding sites (CC and SS repeats and a histidine stretch), along with a dileucine motif in its C terminus (Mills et al. 2003). Whilst other sites have now been implicated in the targeting of Menkes protein (Petris et al. 1998), the similarities between AtHMA4 and this dual function transporter could lead to the suggestion that protein relocalisation may occur for this plant HMA. Whether changes in localisation occur upon metal supply remains to be investigated. If this is an important mechanism it would seem likely that other transporters may be involved. If increased Zn supply caused the AtHMA4 protein to relocalise within the xylem parenchyma in roots, it is possible that it may switch from loading the xylem to loading the apoplast of the stele. In this way it could reduce metal export to the leaves but it would cause the concentration of ions in the stele to rise. Thus it may be necessary to have an additional export mechanism at the endodermis.

In the leaves, it is somewhat difficult to predict the function of AtHMA4 and although located in the vascular tissue the cellular localisation has not been determined. It is possible that AtHMA4 could be involved in phloem loading (via companion cells) in the leaf tissues. Under normal conditions AtHMA4 would be



required for correct distribution of metal (Zn) ions but when subjected to high concentrations it could accelerate export to the phloem thereby protecting the mesophyll cells. As has been suggested for the xylem, greater concentrations of chelates in phloem cells for example nicotianamine (Takahashi et al. 2003), could act to protect the plant. Perhaps chelate production would be stimulated as part of this response. This has already been demonstrated with histidine in the xylem (Kramer et al. 1996).

Considering the expression data (Chapter 5) and the work of Hussain et al. (2004), it seems likely that AtHMA4 exports Zn to the phloem in leaves. However, Bernard et al. (2004) found that *HMA4* expression in leaves was greater in *Thlaspi* compared to *Arabidopsis*. If considered to be involved in shoot-root transfer via the phloem, then exaggerated expression of leaf *HMA4* may be expected to lead to reduced levels of Zn/Cd in the leaves and not the Cd hyperaccumulation trait observed in *Thlaspi*. Clearly there must be other functions of AtHMA4. This work has shown that the gene is expressed in flowers and siliques. The work of Hussain et al. (2004) has confirmed this data, and has provided additional evidence to suggest that AtHMA4 may be involved in the loading of Zn to developing flowers. They demonstrated that the anthers of an *Arabidopsis thaliana hma2/hma4* knock-out mutant were unable to produce pollen, and that the flowers were sterile (Hussain et al. 2004).

6.5 Suggestions for future work

This study has provided evidence that suggests possible roles for AtHMA4 and AtHMA1 in Zn/Cd homeostasis in *Arabidopsis thaliana* (discussed above). It has also indicated areas pertinent for further investigation. The RACE work of Chapter 3 has shown differential splicing of the AtHMA4 5' UTR which could suggest that different transcripts may be important in the regulation of expression, or in tissue-specific distribution. Initially additional work could focus on the sequencing of these various transcripts by repeating the 5' RACE procedure. Alternatively PCR could be carried out with a range of oligonucleotide primers that have been designed to various sites within the region of interest highlighted by this work. The functional significance of these transcripts could then be ascertained further by promoter-GUS and antisense methodologies.

Localisation of AtHMA4 and AtHMA1 protein at the cellular level would be crucial to further investigations on these HMA transporters. At present, there is only evidence on the transcript localisation of these pumps. Whilst protein levels may

follow that of the transcripts, protein regulation of plant metal transporters does occur and so predictions on AtHMA4 and AtHMA1 function would be far more conclusive than those made with the current information. Localisation of the proteins would perhaps be most useful in *Arabidopsis* tissues, but localisation in yeast would also be helpful by complementing the tolerance assay and radiotracer studies of this work. GFP has been used in separate studies to localise AtHMA2 in plant tissues (Hussain et al. 2004) and AtHMA3 in yeast cells (Gravot et al. 2004). Interestingly the use of green fluorescent protein (GFP) to localise AtHMA3 in yeast, by Gravot et al. (2004), has also produced evidence to reveal a potential regulatory domain in the protein. These authors found that the GFP fusion had an impact on the tagged protein. Their evidence suggested that metal loading, or recognition by the HMA protein was perturbed, as the movement of one of the enzymes normal substrates appeared to be inhibited (Gravot et al. 2004). Since it has been proposed that substrate specificity may involve the co-operative action of a large part of the protein (Argüello 2003), it is possible that GFP may have a 'global' effect on the protein. If GFP does have the ability to transmit subtle changes along the length of the protein, then it may be possible that other functions could be affected. For example, effects on protein targeting are conceivable and so the use of anti-peptide antibodies in future localisation studies should not be discounted. Indeed for AtHMA4 it has been suggested (Chapter 6) that the protein may undergo re-localisation at different metal levels within the cell. If this aspect of the protein were to be studied, then perhaps the use of anti-peptide antibodies would be more suitable, since GFP-tagging could hamper relocalisation or sensing of metal ions.

The cloning of *AtHMA4* (this work) and *AtHMA1* by this laboratory will allow future studies on the purified proteins. Reconstitution of proteins in proteoliposomes or everted membrane vesicles is a rather laborious task but has the potential to answer many questions on the transport kinetics of the two pumps. Specifically, information on the rates of transport and affinities for Zn and Cd could be tested along with further studies on additional ions such as Mn, and Cu/Ag, which would not be expected to undergo transport by these HMAs. One aspect that has also been mentioned in this work, has been a possible competition of Mn with the ATPase co-factor Mg. This could also be investigated.

The widening use of microarrays and plant-gene knock-out libraries naturally lend themselves to the study of the *Arabidopsis thaliana* HMAs. Chapter 6 mentioned the possible involvement of other metal transporters in a homeostatic mechanism that was proposed for AtHMA4. The capacity of microarrays to study 'global' gene expression could reveal interactions with transporters from other

families and enzymes from particular metabolic pathways. In future, microarray analysis of the HMA knockouts may reveal the impact of the mutations on other genes.

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