Molecular Mechanism of

Zinc Uptake and Regulation in Cereals

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Dedicated to the memory of my brother "Kitti"

Though you are no longer with us, you have and always will be a source of inspiration, symbol of patience and hard work.

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Abstract

Limited availability of zinc in many soils in the world leads to zinc deficiency in plants. Some plants acquire zinc more efficiently than others but little is known about the molecular mechanism of zinc uptake/efficiency in plants especially cereals. *Oryza sativa* has been used as a model system in this study to provide an insight into the mechanism of zinc uptake and regulation in cereals. The cDNAs encoding four putative transporters *OsR06, OsE60* and *OsS13* and *OsS16* have been identified from rice based on high degree of homology to known *Arabidopsis thaliana* zinc transporters. All the transporters identified fit well into the ZIP family. *OsR06* and *OsE60* partially complement the zinc-deficient *Saacharomyces cerevisiae* mutant ZHY3 and the manganese-deficient *S. cerevisiae* mutant SLY8 while *OsS13* does not complement these mutants. *OsS16* was not tested for complementation as it is a partial length cDNA fragment. Toxicity, growth and ion content studies of ZHY3 expressing *OsR06* and *OsE60* reveal that in addition to zinc, *OsR06* transports cadmium, calcium and magnesium into the cells while *OsE60* has more specificity for zinc as the substrate. Northern analysis of RNA extracted from rice plants showed that the expression of *OsS16* and *OsR06* is induced in zinc deprived roots and shoots while the expression of *OsS13* is induced specifically in zinc-deprived roots. Uptake studies with ^{65}zinc isotope provided clear evidence for the zinc uptake activity of transporters *OsR06* and *OsE60*. The zinc uptake activity of these transporters shows distinct pH optima. Michaelis-Menten kinetics of *OsR06* and *OsE60* has been calculated. The characterisation of *in planta* expression of the rice transporters suggests regulation of the transporters at the transcriptional level by zinc availability.

Agrobacterium-mediated transformation of *Hordeum vulgare* cv. Golden Promise was carried out with a binary vector containing the cDNAs encoding the two zinc transporters *AtZIP1* and *AtZIP3* from *A. thaliana*. Primary analysis of the *AtZIP3* transformed plants revealed the presence of only the ubiquitin gene promoter but not the *AtZIP3* cDNA. Molecular analysis of *AtZIP1* transformed

plants revealed the insertion of multiple copies of *AtZIP1* stably integrated into the genome and a Mendelian pattern of inheritance of the transgenes. Overexpression of the *AtZIP1* in T_1 and T_2 generations of transgenic plants, as compared to the untransformed plants, was detected by northern analysis. Uptake experiments with ^{65}zinc showed increased zinc uptake in both roots and shoots of the overexpressing lines as compared to the untransformed line. These results suggest that overexpression of *AtZIP1* enhances zinc uptake efficiency in barley plants. Plants from overexpressing line 51-2(7) and null line 91-3(9) were deprived of zinc for three hours and then resupplied with zinc. Zinc uptake in these plants showed rapid decline in the activity of the AtZIP1 suggesting that AtZIP1 might be post-translationally regulated in response to excess zinc in barley plants.

Declaration

This work contains no material which has been accepted for the award of any other degree or diploma at any university and to the best of my knowledge and belief contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis when deposited in the University Library, being available for loan and photocopying.

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Abbreviations

At	*Arabidopsis thaliana*
aa	Amino acid
BAP	Benzyl amino purine
BPS	Batho-phenanthroline sulphonate
Cd	Cadmium
Co	Cobalt
Cu	Copper
cv	Cultivar
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EGTA	Ethylene glycol-bis (β-aminoethyl ether)- N,N,N',N'-Tetra acetic acid
EST	Expressed sequence tag
Fe	Iron
GP	Golden Promise
FW	Fresh weight
his	Histidine
hr	Hour
Kb	Kilobase
KDa	Kilo Dalton
LZM	Low zinc medium
min	Minute
Mn	Manganese
MOPS	3-[N-morpholino] propanesulfonic acid buffer
MS	Murashige and Skoog's medium
Na	Sodium
Ni	Nickel
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Sc	*Saccharomyces cerevisiae*

Ta	*Thalspi arvense*
Tc	*Thlaspi caerulescens*
μM	Micromolar
UT	Untransformed
YNB	Yeast Synthetic medium
YPD	Yeast complete medium
Zn	Zinc

Note : Throughout this thesis two letter prefix denoting the genus and species has been used for the zinc and iron transporters from *Arabidopsis thaliana* (*AtZIP1-4*, *AtIRT1* and *AtIRT2*) and zinc transporters from *Thlaspi caerulescens* (*TcZNT1*).

Chapter 1   Introduction

The world's population has grown rapidly since 1950, swelling from 2.52 billion to over 6 billion today (Graham *et al.*, 2001). Progress in agricultural sciences in the last 100 years has resulted in an enhanced capability to provide adequate food energy for the increasing population of the world (Welch and Graham, 1999). However, evidence shows that our global food systems driven by modern agriculture are failing to deliver adequate quantities of healthy, nutritionally balanced food especially to underprivileged people (Welch and Graham, 1999). An important factor in nutritionally balanced foods is micronutrients. Zinc, iron and iodine appear to be the most frequently deficient micronutrients in human nutrition (Gibson, 1994). Micronutrient malnutrition now afflicts over two billion people world wide, resulting in poor health, high rates of mortality, morbidity and permanent impairment of cognitive abilities of infants born to micronutrient-deficient mothers. In recent years, agriculture production has aimed to increase productivity, sustainability and also increase the nutritional quality of foods.

In plants, micronutrients are required in trace quantities and play an essential role in various growth and metabolic processes. Zinc is one of the essential micronutrients in plants (Berg, 1996; Keilin and Mann, 1940), involved in critical processes such as photosynthesis, respiration, protein synthesis and regulation of gene expression. Plants with insufficient micronutrient supply show various symptoms of deficiencies and reduction in growth, yield and nutritional quality of food grains. Seeds or grains with low zinc content may cause major health problems when consumed by humans especially in developing countries where cereals are major staple food (Graham and Welch, 1996; Welch *et al.*, 1993).

Zinc deficiency is very common in soils with low levels of plant available zinc, in many parts of the world (Takkar and Walker, 1993). Low availability of this micronutrient in the soils rather than low content is often the cause of zinc deficiency in plants (Mengel and Kirkby, 1982). Remedial measures to correct these deficiencies can be used but little information is available as to why some plants acquire and utilize nutrients such as zinc more efficiently than others. We now have a greater understanding of the physiological mechanisms of zinc uptake

(Kochian, 1991; Lindsay, 1972; Moore, 1972) and the molecular mechanisms (see section 1.6.4), but zinc efficiency in plants is still poorly understood (Graham, 1984; Rengel and Graham, 1995).

The importance, availability and deficiencies of micronutrients such as iron, copper, manganese, nickel, boron and molybdenum in plants and the molecular mechanisms of uptake of iron, copper and manganese in yeast and plants will be reviewed briefly in this chapter. Remainder of this chapter will focus on different aspects of zinc such as the importance, availability and deficiencies in plants and also the molecular mechanisms of uptake in both yeast and plants.

1.1   *Micronutrients limit plant growth*

The ionic forms in which the various micronutrients exist and their solubility in soil solution determines their availability to the roots of plants. Some metal ions exist in sparingly soluble forms while other metal ions exist in chelated forms and have to be displaced from ligands to which they are bound before they can be available to the roots for uptake (Marschner, 1995). The oxidation states in which they exist and their solubility in soil solutions also often limits availability of micronutrients. Most micronutrients are present in sufficient quantities in the soil yet plants exhibit deficiencies that suggest that micronutrients are unavailable in required quantities.

1.1.1   Iron

Iron is an essential nutrient for all organisms. Iron mainly exists as Fe (II) or Fe (III) ions and has a marked propensity for forming six-coordinate complexes with ligands containing oxygen, nitrogen and sulphur (Guerinot and Yi, 1994) in plants. Iron participates in a wide variety of cellular oxidation - reduction reactions due to its ability to donate and accept electrons. Depending on the ligands iron associates with, the redox state of iron varies greatly (Romheld and Marschner, 1991).

In plants, iron is a component of heme and non-heme proteins (Romheld and Marschner, 1991). Heme proteins such as cytochromes are characterized by a

heme Fe-porphyrin complex as the prosthetic group. Other heme proteins include catalase, peroxidase and leghemoglobin. In non-heme proteins (also known as the Fe-S proteins) such as ferrodoxin and superoxide dismutase, iron is coordinated to the thiol group of cysteine or inorganic sulphur as clusters (Romheld and Marschner, 1991). These proteins have functions in metabolic processes such as photosynthesis, SO_4^{2-} and SO_3^- reduction, respiration, TCA cycle and N_2 fixation.

Plants face great difficulty in acquiring iron due the insoluble nature of Fe (III), which is the most abundant form of the metal (Guerinot and Yi, 1994; Yi and Guerinot, 1996). The problem with iron is one of availability at biological pH in aerobic environments, rather than abundance. The solubility of inorganic iron in well-aerated soils is controlled by the dissolution and precipitation of Fe^{3+} oxides (Romheld and Marschner, 1986b). In calcareous soils, high concentration of HCO_3^- increases soil pH, which leads to decreased solubility of iron (Bloom and Inskeep, 1988; Moraghan and Mascagani, 1991) thereby limiting the availability of soluble iron to the roots of the plants (Guerinot and Yi, 1994; Marschner, 1995b). The availability of Fe to plant roots depends on the ability of the roots to lower pH and to reduce Fe^{3+} to Fe^{2+} in the rhizosphere. Under oxidized environmental conditions, ferric ion predominates in the soil and for uptake by most plant species has to be reduced to the ferrous form.

In order to obtain adequate Fe for plant growth, higher plants have developed at least two different mechanisms for Fe acquisition (Romheld and Marschner, 1986). Strategy I plants (Romheld, 1987) include the dicots and nongraminaceous plants while the strategy II plants (Romheld, 1987) include the graminaceous monocots. The strategy I plants reduce the Fe (III) in Fe chelates via a plasma-membrane-bound reductase system (Guerinot and Yi, 1994; Romheld, 1987). This system is capable of reducing ferric chelates accompanied by the transfer of electrons from the cytosolic NADH or NADPH across the plasma membrane to the extracellular ferric chelates (Marschner and Romheld, 1994). Strategy II plants release phytosiderophores that complex and solubilize the ferric ions so that they can be transported across the plasma membrane (Romheld, 1987). The phytosiderophores are non-proteinogenic amino acids released in response to iron deficiency and their exudation is proportional to the extent of deficiency or stress (Cakmak et al., 1994; Rengel and Romheld, 2000; Rengel et al., 1998). Phytosiderophores efficiently chelate and solubilize Fe in

soils at high pH and high concentrations of bicarbonate (Takagi *et al.*, 1988). This higher efficiency of Fe acquisition in alkaline soils favors the growth of graminaceous species (strategy II) as compared to the nongraminaceous species on bicarbonate buffered soils. Secretion of organic acids and phenolics in the swollen zones of iron starved roots have been described in strategy I plants and may aid in iron acquisition (Olsen *et al.*, 1981). These compounds could participate in iron reduction and chelation of ferric iron (Julian *et al.*, 1983; Romheld and Marschner, 1983a) in response to iron deficiency.

In leaves of all plants, the major symptom of iron deficiency is the yellow interveinal stripes on the younger leaves, followed by yellowing of the entire leaf tissue, which is a consequence of impaired formation of chloroplast pigments (Spiller and Terry, 1980; Terry, 1980). Iron deficiency thus results in sharp decline in photosynthetic activity.

1.1.2 Copper

Copper is a transition element with electron filled 3d shell and shares a number of similarities with iron such as, existing in several oxidation states, having close atomic and ionic radii numbers, similar electronegativity, formation of highly stable complexes, participation in redox reactions and easy electron transfer (Linder, 1991). Copper usually exists in Cu^{2+} form. In contrast to iron, copper containing enzymes can react directly with molecular oxygen and thus catalyze preferentially terminal oxidation processes.

Copper is an essential micronutrient for plants and functions as an important cofactor for a variety of enzymes such as cytochrome oxidase, superoxide dismutases, ascorbate oxidases, polyphenol oxidases and Rubisco (Baron *et al.*, 1995; Casimiro *et al.*, 1990; Delhaize *et al.*, 1982; Graham, 1976). Copper is an integral component of plastocyanins (Casimiro *et al.*, 1990) that play an important role in photosynthesis. Two atoms of copper and two iron atoms are present in cytochrome oxidase which is involved in mitochondrial electron transport chain while one atom of copper is present in the superoxide dismutase which is directly involved in the mechanism of detoxification of O_2^- radicals generated during photosynthesis (Halliwell and Gutteridge, 1984). Copper may also play a role in lignin biosynthesis via polyphenol oxidases although the

process is not clearly understood (Marschner, 1995). As an essential trace element copper is involved in electron transport reactions in plants.

Many soil types are low in copper. For example organic soils, peats, mucks, sand and soil from igneous rocks have low amounts of labile copper (Oplinger and Ohlrogge, 1974). Soils derived from volcanic ash and pumice and soils such as ferrallitic and ferruginous coarse textured soils are inherently low in copper (Alloway and Tills, 1984). In soils rich in organic matter, copper is complexed to organic substances and is unavailable to the roots of the plants (Alloway and Tills, 1984). As soil pH increases, copper availability declines due to stronger copper adsorption to soil particles (Cavallaro and McBride, 1984; Marschner, 1995b).

Visible symptoms of copper deficiency are stunted growth, leaf and stem twisting, distortion of young leaves (Gherardi et al., 1999), chlorosis and necrosis of apical meristems (Longnecker et al., 1993; Marschner, 1995). Impaired lignification of cell walls (Gherardi et al., 1999; Marschner, 1995; Oldenkampf and Smilde, 1966) due to depression of polyphenol oxidases and decrease in content of soluble carbohydrates during vegetative growth (Brown and Clark, 1977) occurs as a result of copper deficiency. Copper deficiency inhibits the synthesis of copper containing plastocyanin (Droppa et al., 1984) and causes thylakoid disorganization at the level of grana (Casimiro et al., 1990) thereby decreasing the photosynthetic rate. The activity of Cu-Zn-SOD is much lower with copper deficiency resulting in impairment of the detoxification system, leading to tissue damage. Copper deficiency can delay flowering, depress male fertility, cause pollen sterility (Bussler, 1981; Dell, 1981; Graham, 1975) and delay plant maturity (Longnecker et al., 1993). Unavailability of copper would thus lead to disruption of critical growth processes in plants.

1.1.3 Manganese

In plants, manganese mainly exists in Mn^{2+} form (Kochian, 1991) but it can be readily oxidized to Mn^{3+} and other higher oxidation states. Manganese therefore plays an important role in redox processes. The ionic radius of manganese lies between magnesium and calcium and therefore it can compete or substitute in reactions involving these ions (Hughes and Williams, 1988). Although manganese

can be present in many chemical forms in soil, only the Mn^{2+} form in soil solution is regarded as available for absorption to the roots of the plants. Manganese is an essential micronutrient in plants and functions as a cofactor, activating 35 different enzymes (Burnell and Hatch, 1988) that primarily catalyze the various oxidation-reduction, decarboxylation and hydrolytic reactions in plants. The existence of two manganese containing enzymes namely the water splitting enzyme in PSII (Clarkson, 1988) and superoxide dismutase (Elstner, 1982) have been well characterized in plants. The most well known function of manganese, is in the photosynthetic oxygen evolution (Hill Reaction) in the chloroplasts where electrons liberated by the water splitting enzyme S (which contains four atoms of manganese) are transferred to the photosystem II (Rutherford, 1989). Due to this key function in water splitting reaction, manganese plays an essential role in photosynthesis.

Mn-SOD is present in the mitochondria, peroxisomes and glyoxysomes. As with other superoxide dismutases, Mn-SOD plays an important role in protecting the cells against the deleterious effects of superoxide free radicals, which are formed in the various biochemical reactions in which molecular oxygen is involved (Elstner, 1982). Manganese is required as a constituent of phytoene synthetase and as an activator for kaurene synthetase in the isoprenoid pathway (Wilkinson and Okhi, 1988). It is involved in the synthesis of various other products in the isoprenoid pathway such as carotenoids, gibberellic acid, sterols and quinones. Manganese functions as a cofactor for a number of key enzymes involved in the biosynthesis of plant secondary metabolites such as phenolics, lignins, coumarins and flavonoids and thus, plays a key role in plant defense mechanisms (Burnell, 1988).

Soils derived from crystalline shale and acid igneous rocks are low in available manganese (Krauskopf, 1972). Poorly drained sandy-textured soils and highly leached tropical soils are low in available manganese (Alley et al., 1978) and plants grown in such soils are prone to manganese deficiency. In soils rich in organic matter, chelation with compounds such as carbonates limits the availability of manganese (Farley and Draycott, 1973) to the roots of the plants. Solubility of manganese decreases with increasing pH and therefore the availability of manganese in calcareous soils is limited.

In dicots, the most common symptom of manganese deficiency is interveinal chlorosis in younger leaves while in cereals, greenish gray spots occur on the basal leaves while in legumes, the 'split seed' disorder is the most common symptom (Campbell and Nable, 1988). Several physiological and anatomical changes occur as a result of manganese deficiency such as inhibition of root growth (Campbell and Nable, 1988) and lower lignin content in the roots (Brown *et al.*, 1984). Manganese deficiency decreases the concentrations of metabolites in the shikimic acid pathway and may be responsible for a decrease in disease resistance of manganese deficient plants (Burnell, 1988; Romheld and Marschner, 1991).

1.1.4 Boron

Boron is an essential micronutrient required for growth and development of vascular plants, diatoms, and some species of marine algal flagellates (Dugger, 1983; Hu and Brown, 1997). Boron possesses properties intermediate between metals and electronegative nonmetals and shows a tendency to from cationic complexes (Romheld and Marschner, 1991). Warington (1923) first reported the essentiality of boron for plant growth. Some species of cyanobacteria require boron (Bonnilla *et al.*, 1990; Mateo *et al.*, 1986) when dependent on nitrogen fixation but boron is not required by fungi and other bacteria.

The chemistry of boron is simple when compared with some other nutrient elements. Boron does not undergo oxidation-reduction reactions or volatilization reactions in soils (Goldberg, 1997). The physiological and biochemical role of boron in plant nutrition has been reviewed by Blevins and Lucaszweski (1998); Dugger (1983); Marschner (1995); Romheld and Marschner, (1991).

The major function of boron in growth and development of plants is based on its ability to form complexes with compounds having the cis-diol configurations (Dugger, 1983; Marschner, 1995; Romheld and Marschner, 1991). Boron plays a major role in the development and structural integrity of dicotyledonous plant cell walls (Goldberg, 1997). Boron has been suggested to be involved in maintenance of intactness of cell wall and its synthesis, probably by the formation of B-pectin complexes (Hu *et al.*, 1996). Boron is involved in maintaining membrane integrity and rapid boron-induced changes in the

membrane functions may be attributed to boron-complexing membrane constituents (Blevins and Lukaszewski, 1998). Boron is also thought to expedite the sugar transport across the membrane (Blaser-Grill *et al.*, 1989; Cakmak *et al.*, 1995; Ferrol *et al.*, 1993; Power and Woods, 1997; Shelp *et al.*, 1995).

Boron is a member of the metalloid group of elements existing as a weak acid at pH<7.0. Thus, in neutral or slightly acid soils, boron exists as undissociated boric acid (Raven, 1980). The factors affecting boron availability and the extent of boron adsorption in the soils are pH, soil texture, soil moisture and temperature (Goldberg, 1997). Boron availability to plants decreases with increasing soil pH, particularly in calcareous soils due to decreased solubility in the soil solution (Barrow, 1989). As soil moisture decreases, availability of boron in soil decreases due to reduced mobility in the soil solution (Goldberg *et al.*, 1993). Thus boron often becomes unavailable under drought conditions (Marschner, 1995a).

Boron deficiency is a widespread problem globally (Shorrocks, 1997). Deficiency of boron impairs cell enlargement and division in the meristamatic region, resulting in the inhibition of root elongation and growth (Dugger, 1983; Marschner, 1995b). Root growth inhibition may also be a result of disrupted ascorbate metabolism as seen in squash plants (Lucaszweski and Blevins, 1996). Typical symptoms of boron deficiency seen in shoots include discoloration and death of terminal buds or youngest leaves (Hu and Brown, 1994), shortening of internodes, interveinal chlorosis in mature leaves and increase in diameter of petioles and stem (Shelp, 1988). Boron deficiency induces male sterility in many species of monocots and dicots (Cakmak and Romheld, 1997) and impairs reproductive development early or late in the flowering or fruiting cycle (Dear and Lipsett, 1987) affecting fruit quality and reducing crop yields. Thus, boron deficiency affects plants in all stages of development. From a practical point of view, identification of the different stages in reproduction that are sensitive to boron deficiency may allow for corrective measures such as foliar spraying to be undertaken that prevent losses in yield of crops.

1.1.5 Nickel

Nickel is chemically related to iron and cobalt and forms stable complexes with cysteine and citrate (Brown *et al.*, 1987b). Nickel requirement of plants is

generally low, 1.7 nmol/g or less in dry biomass (Brown *et al.*, 1987a) but plants cannot complete their life cycle without nickel (Brown *et al.*, 1987b; Eskew *et al.*, 1984; Welch, 1981). In higher plants, urease is the only known enzyme that is activated by nickel (Alagna *et al.*, 1984; Eskew *et al.*, 1983; Krogmeier *et al.*, 1991; Marschner, 1995). Nickel is not required for the synthesis of the enzyme but is essential for the catalytic function of the enzyme (Brown *et al.*, 1987a; Brown *et al.*, 1987b; Eskew *et al.*, 1984). Nickel is involved in nitrogen metabolism in plants and in low-nickel plants supplied with urea as the sole nitrogen source the utilization of urea is impaired and often results in urea toxicity (Brown *et al.*, 1987b).

Serpentine soils are very rich in nickel and plants growing on these soils hyperaccumulate nickel (Homer *et al.*, 1991). Hyperaccumulation of nickel by plants is an attractive trait that can be used for the development of technologies aimed at the decontamination of metal polluted soils using plants (Kramer *et al.*, 1997).

There is no evidence of nickel deficiency in soil grown plants, but there is more concern about nickel toxicity in crop plants (Hooda, 1997; Marschner, 1983) especially when plants are grown on soils where sewage sludge is applied. What we know of nickel deficiency in plants is a result of studies in nutrient solutions. Nickel deficient plants show severely reduced growth (Eskew *et al.*, 1983; Walker *et al.*, 1985), have chlorotic leaves (Gerendas and Sattelmacher, 1997) and accumulate considerable amounts of urea leading to toxicity (Krogmeier *et al.*, 1989). Nickel deficiency affects the levels of amino acids such as arginine in the orinithine cycle that is important in nitrogen storage and translocation (Chou and Splittsoesser, 1972; Gerendas and Sattelmacher, 1997). Thus, nickel plays a major role in the nitrogen metabolism of plants.

1.1.6 Molybdenum

Molybdenum is a transition element and exists mainly as molybdate oxyanion MoO_4^{2-} (Marschner, 1995). The functions of molybdenum as a micronutrient are related to the valency changes it undergoes as a metal component of the enzymes. Molybdenum itself is catalytically inactive until it is complexed by a special co-factor namely the pterin in almost all organisms, with the exception of bacteria

(Zimmer and Mendel, 1999). In higher plants, only a few enzymes have molybdenum as the cofactor such as nitrate reductase which catalyzes the initial step of the assimilatory nitrate reduction (Brodrick and Giller, 1991), nitrogenase (Dilworth *et al.*, 1988), xanthine oxidase/dehydrogenase that is involved in purine catabolism (Vunkova-Radeva *et al.*, 1988) and aldehyde oxidases that catalyze the final oxidation in the phytohormone biosynthesis of indole acetic acid (Koshiba *et al.*, 1996). The functions of molybdenum are therefore mainly related to nitrogen metabolism in plants, as plants can only assimilate nitrogen with the aid of molybdenum (Lang and Kaupenjohann, 1999; Mengel and Kirkby, 1987).

Molybdenum deficiency can occur in soils with low molybdenum content or extensively weathered soils (Zimmer and Mendel, 1999). Molybdenum deficiency is widespread in acid mineral soils with a large content of iron or aluminum oxihydroxides that sequester molybdenum (Zimmer and Mendel, 1999). Decrease in pH from 6.5 to 4.5 and below results in the formation of polyanions that lead to decrease in molybdenum uptake (Bibak and Borggard, 1994; Marschner, 1995) and thus limit its availability.

In molybdenum-deficient plants, symptoms of nitrogen deficiency such as stunted growth, chlorosis or yellowing of leaves, drastic reduction in size and irregularities in formation of leaf blade are common (Bussler, 1981). Molybdenum deficiency has been observed to delay flowering, inhibit tasselling, anthesis and development of sporogenous tissues (Martin *et al.*, 1995). In spite of the low amounts of molybdenum required by the plants, it plays an essential role in the nitrogen metabolism of plants.

1.1.7 Zinc

Zinc exists as a divalent cation, Zn^{2+} which has a completely filled d shell with 10d electrons. Due to the filled d shell, Zn^{2+} has no ligand field stabilization energy, is a borderline acid, undergoes neither oxidation nor reduction reactions (Berg, 1996). Being a borderline acid, zinc can interact strongly with a number of ligand types such as sulphur from cysteine, nitrogen from histidine and oxygen from glutamate, aspartate and water. Zinc has been known as an essential trace element for 100 years but the specific biological role of zinc for the catalytic activity of carbonic anhydrase was established only in 1940 (Keilin and Mann,

1940). Since then more than 300 zinc containing enzymes have been identified (Coleman, 1992; Vallee and Auld, 1990). In contrast to iron, copper and manganese, zinc is not subject to valency changes and therefore does not take part in oxido-reduction reactions. The metabolic functions of zinc are based on its tendency to form tetrahedral complexes with nitrogen, oxygen and particularly sulphur or S-ligands (Berg, 1996; Vallee and Auld, 1990). This is in contrast to iron, which forms octahedral complexes with different ligands.

Zinc plays both functional (catalytic) and structural roles in enzyme reactions in plants (Vallee and Auld, 1990). In the enzymes where zinc is a structural component, a single atom of zinc is bound by four cysteine residues forming a tetrahedral complex that maintains the structure of the macromolecule (Vallee and Auld, 1990). In enzymes with catalytic activity, zinc is bound by three ligands (Vallee and Auld, 1990). Some of the major enzymes in plants that require zinc as an essential component of their composition are carbonic anhydrase, alcohol dehydrogenase, superoxide dismutase and DNA-dependent RNA polymerase (Marschner, 1995). In enzymes such as carbonic anhydrase and carboxy peptidase, a single zinc atom is coordinated to four ligands while in enzymes such as alcohol dehydrogenase, two zinc atoms are coordinated to S-groups of four cysteine residues (Vallee and Auld, 1990). Carbonic anhydrase plays an important role in the photosynthetic carbon dioxide assimilation in plants (Brown *et al.*, 1993; Cakmak, 2000). Low zinc conditions would impair the activity of this enzyme, thereby affecting the process of photosynthesis. Zinc is the structural component of superoxide dismutase, which is a critical enzyme responsible for prevention of oxidative damage caused by high levels of O_2^- radicals. These radicals are produced as a byproduct of oxygen consuming reactions in the mitochondria at the ubiquitone-cytochrome-b site or by membrane bound NADPH-oxidases (Cakmak and Marschner, 1988a). Majority of the dismutases in the plants are the Cu/Zn type and Cu/Zn SOD has been found in the cytosol, chloroplasts and in mitochondrial membranes.

A structurally important role for zinc in plant cells is as a component of the cell membranes (Cakmak and Marschner, 1988). Zinc helps maintain the integrity of the biomembranes by bridging the sulphydryl groups of membrane proteins. In zinc deficient conditions, the sulphydryl groups form disulphide bonds that distort the membrane, leading to destabilization and leakiness (Cakmak

and Marschner, 1988; Welch and Norvell, 1993). A number of zinc-dependent enzymes such as Fructose 1,6-biphosphate and aldolase are involved in carbohydrate metabolism in plants. Fructose 1,6 biphosphate is involved in partitioning of C_6 sugars in the chloroplast and cytoplasm while aldolase is involved in regulation of flow of metabolites via the glycolytic pathway (Marschner and Cakmak, 1989). Zinc is involved in the auxin metabolism in plants probably in tryptophan and indoleacetic acid synthesis but its mode of action is still obscure (Cakmak *et al.*, 1989). Auxins such as indole-3-acetic acid are mainly required for the elongation of stem and meristem development in the plants (Marschner, 1995).

Zinc plays a critical structural role in many proteins. Zinc is a structural component of ribosomes and is essential for their structural integrity (Prask and Plocke, 1971). RNA polymerase requires zinc during cell replication (Brown *et al.*, 1993). Several motifs found in transcriptional regulatory proteins are stabilized by zinc, including the zinc finger, zinc cluster and RING domains (Fox and Guerinot, 1998). Studies have shown that zinc is involved in DNA replication and transcription through zinc finger proteins (Coleman, 1992; Klug and Rhodes, 1987; Vallee and Falchuk, 1993). These fingers are small zinc-based domains that play a structural role in the correct recognition of the promoter region (Vallee *et al.*, 1991). The first described zinc finger protein was the TFIIIA (Vallee *et al.*, 1991). Zinc binds to the cysteines and histidines and 12-13 amino acids between the center ligands make a loop that forms the "finger". Zinc deficiency prevents proper folding of the zinc finger protein and prevents transcription from occurring.

Thus, zinc is an essential micronutrient involved in various essential processes in the plants such as photosynthesis, carbohydrate accumulation and gene regulation.

1.1.7.1 Bioavailability of zinc

The concentration of zinc in the soil solution and its rate of replenishment are important factors determining the availability of zinc to the roots of the plants. In most soils, the total zinc content exceeds the crop requirement but availability is the limiting factor for uptake by plants (Mengel and Kirkby, 1982). The factors

limiting or affecting the bioavailability of zinc to the plants can be classified into abiotic and biotic:

1.1.7.1.1 Abiotic or soil factors

Total zinc concentration in the soil varies greatly and parental material has a much greater effect on soil zinc than pedogenic factors (Moraghan and Mascagani, 1991). Basic eruptive rocks such as basalt and gabbro usually contain more zinc than metamorphic rocks or sedimentary rocks such as limestone or sandstone. Soil from siliceous rocks are inherently low in zinc when compared to soils from igneous rocks (Marschner, 1995a). Highly leached soils are also low in available zinc. Clays and soil organic matter are capable of holding zinc quite strongly (Lindsay, 1972) and thus limit the availability to the roots of the plants.

The pH of soil has a marked effect on availability of zinc to the plants. The concentration of water-soluble zinc decreases with increasing pH partly due to increased adsorption by soil constituents (Bar-Yosef et al., 1980). As soil pH increases above 5.5, hydrous oxides of aluminum and iron adsorb zinc (Kalbasi et al., 1978), limiting availability. Precipitation of specific zinc compounds at increasing pH values (Lindsay, 1972) may account for decreased zinc availability at high pH, but the nature of such compounds is not known. In alkaline soils, low availability of zinc results from the adsorption of zinc to $CaCO_3$ (Trehan and Sekhon, 1977). The diffusion coefficient in a calcareous soil is about 50-fold less than for acidic soil, leading to reduced zinc mobility and thus availability (Melton et al., 1973; Norvell et al., 1987).

Soils rich in organic matter have a higher adsorption capacity for zinc and thus there is limited availability of zinc in such soils (Shuman, 1975). Zinc interacts with the organic matter in the soil and forms both soluble and insoluble organic complexes. Highly decomposed organic matter was shown to result in high bicarbonate concentrations that immobilized zinc and reduced its availability to rice roots (Forno et al., 1975). Therefore, high bicarbonate levels lead to increase in pH and subsequently decrease availability of zinc to the plants.

The solubility of zinc decreases in soils rich in phosphorus content probably due to the formation of zinc-phosphate -$Zn_3 (PO_4)_2. 4H_2O$ and therefore (Lucas and Knezek, 1972) the application of phosphorus fertilizers to soils low in

available zinc induces zinc deficiency (Robson and Pitman, 1983). Zinc-phosphorus interactions are quite complex and high phosphorus supply is often associated with a reduction in root growth and a lesser degree of colonization of roots by arbuscular mycorrhizae (Lonergan *et al.*, 1979). Both these factors are important for acquisition of zinc by the plants. Zinc-deficient plants seem to lose the ability to down regulate the expression of phosphate transporters leading to enhanced phosphate accumulation (Huang *et al.*, 2000) suggesting that zinc plays a role in signal transduction pathways involved in regulation of genes encoding high affinity phosphate transporters. This leads to the upregulated expression of high affinity phosphate transporters in both phosphate-sufficient and deficient *H. vulgare* plants. Upregulation of the genes thus enhances phosphate accumulation, which may be toxic under phosphate sufficient conditions. This data when interpreted in light of earlier findings by Lonergan *et al.* (1979) and Cakmak and Marschner (1987) suggest that phosphorus toxicity observed in leaves of zinc deficient plants was due to the overexpression of high affinity phosphate transporters.

Plants growing in areas with cool temperatures and wet spring seasons often develop zinc deficiency (Lucas and Knezek, 1972). This may be due to restricted or impaired development of roots, which ultimately affects the volume of soil that can be explored by the roots. Increase in severity of zinc deficiency at lower temperatures, also suggests that availability of soil zinc per se is less at lower temperatures (Bauer and Lindsay, 1965). In addition at low soil temperatures arbuscular (AM) mycorrhizal infection is severely reduced (Hayman, 1974). This may lead to decreased availability of zinc, since AM mycorrhizae play an important role in acquisition of zinc from the soil.

1.1.7.1.2   Biotic or Plant factors

Zinc has limited mobility in soil solution and is mainly supplied to the roots by diffusion. When soil water is low, mechanical impedance of the soil increases, thereby limiting the availability of zinc to the roots (Marschner, 1993). Formation of depletion zones around the roots (Macay and Barber, 1985; Macay and Barber, 1987) decreases the availability of zinc to the roots unless root hairs can extend to explore areas beyond the depletion zones.

Mycorrhizal fungi such as AM are associated in a symbiotic relationship with the plants. Host plants supply carbon to the fungi and in return receive nutrients such as phosphate and zinc. The arbuscular mycorrhizae effectively increase the growth of colonized plants by improving the supply of mineral nutrients of low mobility such as zinc. The beneficial effects of AM on zinc uptake are clearly observed in soils low in extractable zinc or soils with high pH or plants with coarse root systems such as apple (Runjin, 1989) and *Lucaena luecocephala* (Manjunath and Habte, 1988). The efficiency of AM in supplying a relatively large proportion of the required zinc is important for the zinc nutrition of plants as the fungal hyphae can absorb and translocate zinc to the host from outside the depletion zone (Marschner, 1993).

Plants have evolved various adaptations to mobilize nutrients to increase availability from the soil. In dicots, root induced acidification of rhizosphere due to imbalance in cation/anion uptake rates (Hoffland *et al.*, 1989a) or excretion of organic acids is a widespread phenomenon (Hoffland *et al.*, 1989b). In members of Proteaceae and *Lupinus albus* (Grierson and Attiwill, 1989), proteoid roots release large amounts of citric acid leading to a decline in pH of the soil. Enhanced H^+ extrusion decreases the pH of the rhizosphere, leading to increased solubility of zinc in the soil solution and thus more availability. In contrast to dicots, graminaceous species respond to deficiency of micronutrients such as zinc and iron by release of non-proteinogenic amino acids called phytosiderophores (Rengel, 1997; Romheld, 1987; Takagi *et al.*, 1984; Zhang *et al.*, 1991) as explained in section 1.1.1. The exudation of phytosiderophores (PS) from roots increases under zinc deficiency. Phytosiderophore-assisted uptake of zinc is controversial. According to an earlier model, ferrated PS are transported across the plasma membrane by a transport system that does not carry large amounts of ZnPS (Ma *et al.*, 1993; Ma and Nomoto, 1993). However a more recent study has shown that ZnPS complex can also be taken up by maize roots thereby providing evidence for mobilization and uptake of zinc by PS exuded in response to zinc deficiency (Von Wiren *et al.*, 1996).

1.2 *Zinc deficiency*

Among the micronutrients, zinc deficiency is one of the most widespread nutritional disorders in crop plants in a wide range of soils (Cakmak *et al.*, 1999;

Cakmak *et al.*, 1996b; Graham *et al.*, 1992; Kalayci *et al.*, 1999; Rengel and Graham, 1995). The problem of zinc deficiency has been accentuated by intensive cultivation of high yielding varieties of crops and use of mineral fertilizers (Grewal and Graham, 1999). Most characteristic visible symptoms of zinc deficiency in monocotyledonous plants are chlorosis of leaves around the midrib, water soaked appearance and necrotic spots on the leaves (Marschner, 1995). The major visual symptoms in dicotyledonous plants are malformed leaves, interveinal chlorosis and inhibition of shoot elongation, leading to stunted growth and 'die back' of shoots (Grewal and Graham, 1997; Zhang *et al.*, 1991). At the physiological level, zinc deficiency causes changes such as increases in permeability of membranes or leakiness due to increased generation of O_2^- radicals and impaired detoxification system (Cakmak and Marschner, 1987). Zinc deficient plants have low activity of the enzyme superoxide dismutase. This results in the production of the reactive oxygen species or ROS (Cakmak, 2000; Cakmak and Marschner, 1988). Reactive oxygen species (ROS) are highly reactive oxygen molecules that interact rapidly with cell components, membrane bilayers, proteins and small molecules causing peroxidative damage (Cakmak, 2000; Cakmak and Marschner, 1988) to cells and chloroplasts, leading to loss of membrane integrity and increased permeability (Cakmak and Marschner, 1988; Thompson *et al.*, 1987).

Zinc deficiency also decreases the net rate of photosynthesis by about 70% by reducing the carbonic anhydrase activity, chlorophyll content (Brown *et al.*, 1993) and other essential photosynthetic enzymes (Rengel, 1995a). Wheat crops grown under zinc deficiency are known to produce seed with low zinc (Graham *et al.*, 1992). Such seeds when sown on Zn deficient soils can reduce early seedling vigour and lower subsequent growth and grain yields in wheat (Rengel and Graham, 1995a). Zinc deficiency thus impairs the functioning of biomembranes and affects the integrity of subcellular organelles such as chloroplasts and ribosomes. Zinc deficiency also impairs the critical processes such as photosynthesis and protein synthesis.

1.3 Zinc uptake

Metal ions are taken up and transported across the plasma membrane by different mechanisms. Three parameters namely the membrane potential (E_m), the

cytoplasmic concentration (M_{cyt}) and the soil concentration (M_{ext}) of the metal ion, determine whether an ion can enter the root passively through a channel, or whether an energy dependent transport process is essential. A simple equation known as the Nernst equation (Harold, 1986) can determine whether a metal ion is taken up passively through channels or actively through carriers or pumps. Although the external concentration of zinc (0.05 - 0.25 mmol m^{-3}) in the soil solution is known (Carroll and Loneragan, 1968), we do not know the internal cytoplasmic concentration of zinc and therefore the Nernst equation cannot be used to predict the mode of uptake of zinc.

It has been hypothesized that when the roots take up cations in unchelated form, uptake is facilitated by passive ion flux through divalent cation channels. The major component of driving force would arise from the negative membrane potential maintained across the plasma membrane of plants cells (Kochian, 1991). Zinc may be transported as Zn^{2+} ions across the plasma membrane via a divalent cation channel (Kochian, 1991). Transport through these channels is driven by the electrical potential and channels may open and close in response to a variety of cellular and environmental stimuli (Kochian, 1991) to control ion movement through these channels.

In some genotypes of *Phaseolus vulgaris*, ferric reductase involved in response to iron deficiency, has been induced under zinc deficiency (Jolley and Brown, 1991). It has been suggested that ferric reductase is involved in the opening of channel, which allows zinc to be taken up passively through the channels, along with iron. Based on these observations, a model for uptake of zinc suggests that uptake may occur via a cation channel, which is influenced by the activity of the plasma membrane ferric reductase induced in response to iron deficiency. However, to date, no channel/s involved in zinc uptake have been identified in higher plants, though in *Chara* (Reid *et al.*, 1996) the existence of more than one channel is suggested to be involved in passive transport of zinc.

In light of the above findings, it may be suggested that uptake of zinc occurs through an active process. Active transport process in contrast to the passive process involves the movement of ions or solutes from a region of lower electrochemical potential to a region of higher electrochemical potential. This process involves direct or indirect use of metabolic energy generated during the hydrolysis of ATP by ATPase, to pump the solute/ions across the membrane. In

this process, the free energy gradient generated by the primary transport process is harnessed to move other solutes or ions through the membrane via the carrier or channel proteins. Some studies support the hypothesis that zinc is taken up actively by plant roots. Steady state uptake of zinc across the barley root plasma membrane is sensitive to low temperature, anaerobiosis, DNP and azide (Schmid *et al.*, 1965). In sugarcane leaf tissue, zinc uptake was observed to be considerably reduced by low temperature and metabolic inhibitors suggesting that uptake is metabolically controlled (Bowen, 1969). In rice seedlings, DNP a metabolic inhibitor severely depressed zinc uptake from solutions containing either 0.005 or 5 mmol^{-3} zinc (Giordano *et al.*, 1974). This suggests that zinc uptake may be an active process or may be linked to active uptake.

Genes encoding zinc transporters have been identified in yeast, plants and humans (Grotz *et al.*, 1998; Palmiter *et al.*, 1996a; Palmiter *et al.*, 1996b; Palmiter and Findley, 1995; Pence *et al.*, 2000; Zhao and Eide, 1996a; Zhao and Eide, 1996b). If zinc uptake is directly linked to the hydrolysis of ATP (active process), then ATP binding sites in the transporters would be expected,;however no ATP binding sites have been identified in these proteins. This suggests that zinc is not transported across the plasma membrane by an active process, but could still be transported by a secondary active transport mechanism that relies on the proton gradient generated by the H^{+}-ATPase.

1.4 Zinc efficiency

Zinc efficiency can be defined as the ability of the plants to grow and yield well in soils too deficient for a standard genotype (Graham, 1984) or as the ratio of grain yield under zinc deficiency to ratio of grain yield under zinc application (Kalayci *et al.*, 1999). The mechanisms contributing to zinc efficiency in different cultivars of crop species are not yet understood but studies suggest that more than one process may be operating in a single genotype (Graham, 1984; Rengel and Graham, 1995). Crop species markedly differ in their tolerance to zinc deficiency. In cereals rice, sorghum and maize are classified as sensitive to zinc deficiency while barley, wheat and rye are classified as less sensitive (Viets *et al.*, 1954). Bread and especially durum wheat cultivars possess high sensitivity to zinc deficiency (Cakmak *et al.*, 1997; Cakmak *et al.*, 1998; Cakmak *et al.*, 1996;

Graham *et al.*, 1992). Studies by Graham (1984) and Cakmak *et al.* (1998) predict the order of sensitivity to zinc deficiency in cereal crops to be rye< triticale < barley < bread wheat < oats < durum wheats.

Genotypic variation exists among the genotypes of a given species in tolerance to zinc deficiency. Takkar *et al.* (1983) tested the responses of six genotypes of barley to zinc deficiency conditions in India and reported that all the genotypes were resistant to zinc deficiency. However in another study with 10 genotypes of barley, Pathak et al. (1979) observed that low soil zinc depressed yield of barley ranging from 9.0 to 55 % in glasshouse conditions. This suggests that there is considerable variability among barley genotypes in response to zinc deficiency. Graham et al. (1992) observed significant variation in response to zinc fertilization in different cultivars of barley grown in a field trial at Lameroo, South Australia in 1988. However in the following years, they found smaller responses to zinc fertilization and had difficulty in measuring efficiency similar to those measured in 1988. This fluctuation in response was attributed mainly to environmental factors such as variability in climatic factors and soil profiles over sites and years. Variation seen in the results makes it difficult to reliably assess the extent of genotypic variation in zinc efficiency in the field.

Kalayci etal, (1999) examined 37 bread wheat and 3 durum wheat cultivars for grain yield, zinc efficiency and zinc concentration in shoots and grains when grown in zinc-deficient soil with and without fertilization, in both field and glasshouse conditions. Zinc efficiency ranged from 47% to 92% and was comparable between field and glasshouse conditions. This work demonstrated that there was substantial variation in zinc efficiency among bread wheat cultivars but found no relation between zinc efficiency and zinc concentration in grain or shoots dry matter.

Seven bread wheat, three durum wheat, two rye, three barley, two triticale and one oat cultivar were investigated for their responses to zinc deficiency and zinc fertilization in nutrient solution and zinc deficient calcareous soil (Cakmak *et al.*, 1998). This study indicated that differential susceptibility of cereal cultivars of a species did not correspond well with zinc concentration in the shoot. However total amount of zinc per shoot showed a good correlation with the sensitivity of cereals to zinc deficiency. Highest total shoot content of zinc was seen in the most zinc efficient rye and triticale. High zinc efficiency in rye and triticale and some

bread wheat cultivars has been suggested to be due to capacity of these plant species to take up more zinc from the soils. High capacity for uptake of zinc could also be due to greater root growth and root surface area, which results in a more efficient use of soil zinc (Dong *et al.*, 1995). The capacity to absorb zinc and translocate zinc (Cakmak *et al.*, 1997; Cakmak *et al.*, 1996a; Cakmak *et al.*, 1998; Rengel and Graham, 1995) into shoots at higher rates is an important trait that determines the zinc efficiency under deficiency of zinc in soils. An efficient re-translocation of zinc within the plant to the sites of high zinc demand such as the apical meristems is a desirable trait that ensures the maintenance of plant growth under zinc-deficient conditions. Increased root uptake and shoot translocation of zinc by zincefficient cereals may also be due to release of phytosiderophores that are capable of mobilizing and complexing zinc in soils and plants (Treeby *et al.*, 1989). But the rates of release of phytosiderophores do not correlate with differential zinc efficiency of cereal species (Erenoglu *et al.*, 1999). Zinc-efficient and zinc-inefficient genotypes however differ in their ability to absorb Zn-phytosiderophores (Erenoglu *et al.*, 1996) or transport Zn-chelated phytosiderophores across the plasma membranes of roots (Von Wiren *et al.*, 1994).

Seed nutrient reserves play an important role during germination and early growth of seedlings especially in soils deficient in a given nutrient. Cereals grown under zinc deficient conditions generally produce seed with low zinc concentration and content (Graham *et al.*, 1992) and such seeds when resown in a zinc deficient soil, produce plants with poor seedling vigor and ultimately low yield at harvest (Rengel, 1995b; Rengel and Graham, 1995b). Increasing micronutrient content in the seed has been effective in increasing yields in plants such as wheat (Rengel and Graham, 1995a; Rengel and Graham, 1995), barley (Longnecker *et al.*, 1991) and lupin (Crosbie *et al.*, 1994). The beneficial effects of seed zinc content on the growth and yield of wheat (Rengel and Graham, 1995a; Rengel and Graham, 1995b) suggests that zinc efficiency may be sensitive to seed Zn content. Genc *et al.* (2000) studied the effect of seed zinc content on early growth of two genotypes of barley and found that severity of zinc deficiency symptoms decreased with increasing seed zinc content. A significant increase in dry matter as a result of higher seed zinc content was observed in genotypes of barley but highest dry matter production was achieved as a result of high seed

zinc and adequate zinc fertilization. High seed zinc content alone does not replace the need for zinc fertilization under certain conditions. In summary, the effect of seed zinc content on growth is an important parameter to be taken into account if developing a screening method for selecting zinc efficient genotypes based on growth in soils with varying zinc levels.

The genetic control of zinc efficiency is not very well understood when compared to other micronutrients such as iron (Weiss, 1943), boron (Wall and Andrus, 1962) and manganese (McCarthy et al., 1988) where the involvement of a single major gene with minor genes in tolerance to these micronutrient deficiencies has been suggested. In rice (Mahadevappa et al., 1981), Agrostis tenuis (Gartside and McNeilly, 1974) and soybean (Hartwig et al., 1991), genetic studies have suggested multigenic control of zinc deficiency. Multigenic control of zinc efficiency is further supported by studies of Schlegel et al. (1998) in Secale, Agropyron intermedium and Haynaldia villosa wherein individual chromosomes carry genes conferring tolerance to zinc deficiency in these species. The rye chromosomes 1R, 5R and 7R carry genes that promote the uptake of zinc under conditions of limited ion availability. In Agropyron intermedium, chromosomes L1, L4 and L7 may carry genes enhancing zinc efficiency while the chromosomes V2 and V7 of Haynaldia villosa may possess genes for enhancing zinc efficiency. These studies suggest possible multigenic control of tolerance to zinc deficiency in other cereals is likely but more studies are needed to understand the genetic mechanisms involved in tolerance to zinc deficiency.

Successful breeding of zinc efficient cultivars requires genotypic variation for a particular selectable character. There may be a number of mechanisms operating at molecular, physiological and developmental levels in the plants that confer tolerance to zinc deficiency. Some mechanisms may operate in soil environments such as differences in root architecture and capacity for mycorrhizal infection while others may involve differential uptake, translocation, release of phytosiderophores and better or efficient utilization of the micronutrient taken up from soil. The involvement of a number of factors suggests that mechanisms of tolerance to zinc deficiency are a complex process controlled genetically by a number of genes. A better understanding of the process of zinc efficiency would result in the identification of specific traits that at least partially determine the efficiency of cereal cultivars.

1.5   Yeast and its importance in molecular studies

Baker's yeast *Saccharomyces cerevisiae* has been used effectively in the recent years as a molecular model for studying many aspects of higher eukaryotic genetics and physiology. Yeast mutants can be used to efficiently screen cDNA libraries (Minet *et al.*, 1992). Complementation of yeast mutants has led to the cloning of many cDNAs encoding proteins involved in plant ion transport (Grotz *et al.*, 1998; Muchhal, 1996; Pence *et al.*, 2000; Schachtman and Schroeder, 1994; Sentenac *et al.*, 1992; Smith *et al.*, 1997; Smith *et al.*, 1995; Zhao and Eide, 1996a; Zhao and Eide, 1996b). Functional expression of plant genes in yeast has provided a valuable tool for understanding the functional mechanisms of nutrient uptake and regulation in eukaryotes.

1.6   Mechanisms of uptake of micronutrients in yeast and plants

Growth, metabolism and transport of nutrients depend on the activities of enzymes and transport proteins. These activities are regulated by various factors, some of which interact directly with the proteins while others regulate the genes, which encode these proteins (Clarkson and Hawkesford, 1993). Study of cloned genes that encode nutrient transporters has provided an insight into their structure, function and regulation.

1.6.1   Iron

Iron exists in ferric form (Fe^{3+}) and has to be reduced to its ferrous form (Fe^{2+}) in yeast and strategy I plants before it can be taken up by the cells. Iron uptake is a two-step process in which the extracellular Fe^{3+} is reduced to the more soluble Fe^{2+} by surface reductases. The reduced product is taken up by specific transport systems in many organisms such as yeast *S. cerevisiae* (Dancis *et al.*, 1990; Eide *et al.*, 1992; Lesuisse and Labbe, 1989), bacteria (Evans *et al.*, 1986; Johnson *et al.*, 1991), fungi (Ecker and Emary, 1983; Roman *et al.*, 1993) and many plants (Grusak *et al.*, 1990; Romheld and Marschner, 1983b).

The high affinity iron transporter *FTR1*, *FET3* that encodes a multicopper oxidase and low affinity iron transporter *FET4* transport iron across the plasma membrane in yeast *S. cerevisiae* (Askwith *et al.*, 1994; Askwith and Kaplan, 1998; De Silva *et al.*, 1995; Dix *et al.*, 1994; Stearman *et al.*, 1996). The first step in iron transport is the reduction of ferric iron mediated by a transmembrane electron transporter system encoded by the *FRE1* and *FRE2* (Dancis *et al.*, 1990; Georgartsou and Alexandraki, 1994). The *FET3/FTR1* transport system mediates iron uptake only in an aerobic environment, as oxygen is required to catalyze the oxidation of ferrous iron. Yeast also possesses an alternative iron transport system, mediated by the *FET4* (Dix *et al.*, 1994) that allows iron uptake under iron replete and anaerobic conditions. *FET4* is a plasma membrane protein that is also capable of transporting a number of transition metals.

The high affinity iron transport system in yeast is regulated by the gene product of AFT1 which is an iron-responsive DNA binding protein that activates transcription of the target genes (*FRE1*, *FRE2*, *FTR1*, *CCC2*) by binding to their promoters (Yamaguchi-Iwai *et al.*, 1995; Yamaguchi- Iwai *et al.*, 1996). The mechanism by which the expression of *FET4* is regulated is yet to be elucidated.

Most plants take up iron in similar way to *S. cerevisiae* (except grasses) (Eide, 1998) including acidification of the rhizosphere to increase solubilization of Fe^{3+}, reduction of Fe^{3+} to Fe^{2+} and transport of iron across the plasma membrane of roots. In *Arabidopis thaliana*, a gene encoding the metal transporter *AtIRT1* (Eide *et al.*, 1996; Korshunova *et al.*, 1999) was cloned by functional complementation of a yeast mutant deficient in iron uptake. *AtIRT1* is predicted to be an integral membrane protein with a metal binding domain and prefers Fe (II) as the substrate to Fe (III). Co^{2+}, Mn^{2+}, Cd^{2+} and Zn^{2+} are potent inhibitors of iron uptake by *AtIRT1* at 100-fold excess. *AtIRT1* is expressed in roots and is induced by iron deficiency (Eide *et al.*, 1996). *AtIRT1* also complements a mutant yeast strain defective in high affinity manganese uptake – smf1Δ (Korshunova *et al.*, 1999) and a mutant (ZHY3) defective in zinc uptake (Korshunova *et al.*, 1999). *AtIRT1* dependent manganese uptake has an apparent K_m of 9 ± 1 μM whereas *AtIRT1* dependent zinc uptake has an apparent K_m of 2.8 ± 0.6 μM and occurs only at a pH of 4.2. As addition of Fe (II) increased zinc uptake it has been speculated that either zinc is cotransported with iron or Fe (II) may allosterically regulate the ability of *AtIRT1* to transport zinc at pH 4.2. In addition to

transporting iron, zinc and magnesium, *AtIRT1* also transports cadmium (Rogers *et al.*, 2000). *AtIRT1* is thus an iron transporter with capacity to transport other divalent cations.

Recently, another gene *AtIRT2* (Vert *et al.*, 2001) involved in uptake and accumulation of iron has been cloned and characterized from *A. thaliana*. AtIRT2 shows 69% identity to AtIRT1 from *Arabidopsis* at the amino acid level. *AtIRT2* complements the growth defect of the iron uptake deficient yeast mutant *fet3fet4* and the zinc uptake deficient yeast mutant ZHY3 indicating that the protein encoded by the gene transports both iron and zinc. However unlike *AtIRT1*, *AtIRT2* does not transport manganese or cadmium. The expression of *AtIRT2* was induced in roots in response to iron deficiency though low levels of transcripts were detected in roots of iron sufficient *Arabidopsis* plants. Analysis of transgenic *Arabidopsis* plants carrying the fusion of promoter/ 5'UTR region of *AtIRT2* gene and GUS reporter gene has shown that the *AtIRT2* gene is active in the youngest part of the roots and root hairs (Vert *et al.*, 2001) under iron deficient conditions. *AtIRT2* may be a part of the physiological response of the dicots to iron deficiency as *AtIRT2* transports Fe^{2+} and its promoter activity co-localizes with two other responses of the root: rhizosphere acidification and iron reduction.

Grasses (Strategy II plants) release low molecular weight Fe (III)-specific ligands called the phytosiderophores in response to iron deficiency. These molecules are nonproteinogenic amino acids (Ma and Nomoto, 1996) called mugineic acids that efficiently chelate Fe (III) with their amino and carboxyl groups. The Fe (III)-mugineic acid complexes are thought to be internalized by specific transport systems (Ma and Nomoto, 1996). Recently a transposon tagged maize line was used to clone the gene YS1 that encodes a Fe (III)-phytosiderophore transporter (Curie et al., 2001). *YS1* might be responsible for transport of Fe (III)-phytosiderophore complex from the root surface. The cloning of *YS1* is an important step in understanding the uptake of iron in grasses and has implications for understanding the mechanisms of iron homeostasis in plants. Several homologs of the YS1 gene have been identified in *Arabidopsis*.

Another family of divalent cation transporters, the *NRAMP* (Natural Resistance Associated Macrophage Protein) family has been well studied in plants. The *AtNRAMPs* from *Arabidopsis* belong to the NRAMP family with related sequences in rat (Gunshin *et al.*, 1996), yeast (Gunshin *et al.*, 1996),

bacteria (Nelson, 1999) and humans (Gunshin *et al.*, 1997). In *A. thaliana*, six genes encode NRAMP proteins and three of the proteins AtNRAMP1, AtNRAMP3 and AtNRAMP4 have been shown to be upregulated by iron starvation in plants and involved in iron homeostasis (Curie *et al.*, 2000; Thomine *et al.*, 2000). Overexpression of *AtNRAMP1* in *A. thaliana* leads to an increase in plant resistance to toxic concentrations of iron suggesting that this gene plays a role in iron homeostasis in plants. Since *Arabidopsis* expresses *AtIRT1* (Eide *et al.*, 1996; Korshunova *et al.*, 1999) involved in iron uptake, it is hypothesized that *AtNRAMP1* might have a role in sequestration of free cytosolic iron into cellular compartments such as plastids or vacuoles. However the precise role of this gene in iron homeostasis is still not clear. Thus the study of iron transporters from *A. thaliana*, provide a starting point for molecular insight into iron transport and regulation in higher plants.

1.6.2 Copper

Transport of copper is highly regulated. Copper is found in the environment as the oxidized cupric form but is transported as the reduced cuprous form. *S. cerevisiae* cells acquire copper as Cu (I) in copper deplete conditions by the action of Cu (II)-Fe (III) reductase, encoded by *FRE1* (Dancis *et al.*, 1992; Hasset and Kosman, 1995) and two high affinity copper transporters *CTR1* (Dancis *et al.*, 1994) and *CTR3* (Knight *et al.*, 1996). *CTR1* exists as a multimer in the plasma membrane, has three potential transmembrane domains and shows structural similarity with the bacterial proteins involved in the handling of copper (Dancis *et al.*, 1994). It is suggested that *CTR3* may function in endocytic copper transport pathway based on its localization pattern (Knight *et al.*, 1996). A low affinity copper transporter *CTR2* (Kampfenkel *et al.*, 1995) was identified in yeast due to significant homology to the COPT1 (Kampfenkel *et al.*, 1995) protein involved in the uptake of copper in *A. thaliana*. However *CTR2* cannot rescue a ctr1-3 yeast mutant and does not possess a metal binding domain (Kampfenkel *et al.*, 1995). Over expression of *CTR2* confers increased resistance to copper starvation.

Expression of *CTR1* (Dancis *et al.*, 1992) is homeostatically regulated by copper availability. Copper deprivation induces and copper loading represses the transcription of *CTR1*. This regulation is mediated by binding of MAC1 protein

(Yamaguchi *et al.*, 1997) to specific sequence in the promoter of *CTR1*. Thus, MAC1 functions as a copper sensor regulator that controls the expression of surface reductase and copper uptake activity in yeast and provides homeostatic control of copper acquisition from the environment. *CRS5* (Gralla *et al.*, 1991) and *SOD1* (Culotta *et al.*, 1994) are specifically induced in response to high concentrations of copper in the environment. These three genes are involved in the protection of yeast cells from accumulating excess levels of copper and copper dependent expression of *CUP1* is mediated by the transcription factor Ace1 (Welch *et al.*, 1989). Thus, yeast appears to possess an excellent homeostatic mechanism to maintain a copper balance in the cells and prevent copper induced toxicity.

A putative copper transporter from *A. thaliana*, *COPT1* can complement the growth defect of a yeast mutant ctr1-3 that lacks high affinity copper uptake (Kampfenkel *et al.*, 1995). *COPT1* is 49% similar to *CTR1* from yeast and 56% similar to the *hCTR1* from humans (Zhou and Gitschier, 1997). *COPT1* is predicted to encode a protein with 169 amino acids that is significantly smaller than *CTR1* (406 aa) from yeast due to truncations at the N and C termini (Fox and Guerinot, 1998). However like *CTR1*, *COPT1* has three putative transmembrane domains and contains a N-terminal putative metal binding domain rich in methionine and serine residues (Fox and Guerinot, 1998). Copper deprivation induces and copper loading represses the transcription of *COPT1*.

1.6.3 Manganese

The mechanism of manganese transport is starting to be understood through studies in *S. cerevisiae* (Supek *et al.*, 1996), mammals (Gunshin *et al.*, 1997), *A. thaliana* (Curie *et al.*, 2000; Thomine *et al.*, 2000) and *Oryza sativa* (Belouchi *et al.*, 1997). The putative transporters involved in manganese transport belong to the NRAMP family originally identified in mouse (Cellier *et al.*, 1995) and the CAX gene family. A high affinity Mn^{2+} uptake gene *SMF1* (Supek *et al.*, 1996) was identified in *S. cerevisiae* due to the inability of a yeast mutant to grow in the presence of 12.5 mM EGTA. The phenotype of this mutant could be complemented by overexpression of *SMF1*. This gene encodes a highly hydrophobic protein located in the plasma membrane and resembles the Nramp

proteins from *Drosophila* (Vidal *et al.*, 1993) and mammalian macrophages (Rodrigues *et al.*, 1995). Zinc inhibited uptake of manganese by *SMF1* and it has been suggested that zinc competes with manganese transport mediated by SMF1 (Supek *et al.*, 1996). Another NRAMP homologue identified in yeast, *SMF2* (Supek *et al.*, 1996) transports Mn^{2+}, Cu^{2+}, Co^{2+}, Cd^{2+} (Liu *et al.*, 1997) and more recently has been shown to transport Fe $^{2+}$ (Chen *et al.*, 1999). The functional similarity between mammalian *NRAMP2* (Gruenheid *et al.*, 1995) and yeast *SMF1* and *SMF2* was confirmed by complementation of smf1/smf2 mutants in yeast by mouse *Nramp2* gene (Pinner *et al.*, 1997). The product of *BSD2* (Liu and Culotta, 1999) encodes an endoplasmic recticulum membrane protein, which is responsive to metal concentrations and post-translationally regulates the activity of SMF1. When the cells are exposed to high metal concentrations, BSD2 directs SMF1 to the vacuole for degradation.

Recently, five *AtNRAMP* genes have been isolated from *A. thaliana* (Curie *et al.*, 2000; Thomine *et al.*, 2000) that complement the *smf1* mutant from yeast. *AtNRAMPS* show significant homology to NRAMP gene family from bacteria, yeast, plants and animals. *AtNRAMPs* transport cadmium and *AtNRAMP3* and *AtNRAMP4* also transport iron. In *Arabidopsis*, the *AtNRAMP1, 2, 3* and *4* are expressed in both roots and shoots. The *AtNRAMP1, 3* and *4* mRNA levels are up regulated under iron starvation (Curie *et al.*, 2000; Thomine *et al.*, 2000). The *AtNRAMP3* overexpressing plants can accumulate higher levels of iron, upon cadmium treatment (Thomine *et al.*, 2000). Overexpression of *AtNRAMP1* confers resistance to toxic levels of iron (Curie *et al.*, 2000) suggesting that in addition to *AtIRT1* (Eide *et al.*, 1996; Korshunova *et al.*, 1999) and *AtIRT2* (Vert *et al.*, 2001), the *AtNRAMP* transporters may be involved in iron transport and homeostasis in plants. Disruption of *AtNRAMP3* in *Arabidopsis*, leads to an increase in cadmium resistance, whereas overexpression of this gene imparts increased cadmium sensitivity (Thomine *et al.*, 2000). This suggests that *AtNRAMP3* plays a role in the transport of cadmium. Three homologues of the *NRAMP1* have been identified and characterized in *O. sativa* (Belouchi *et al.*, 1997). The NRAMP proteins have been placed in two classes; *AtNRAMP1* from *Arabidopsis*, *OsNRAMP1* and *OsNRAMP3* from *O. sativa* represent one class while *AtNRAMP2-5* and *OsNRAMP2* represent the other class (Curie *et al.*, 2000; Maser *et al.*, 2001). The studies with yeast and plants suggest that there is a high

degree of conservation among the proteins that transport manganese in different organisms.

1.6.4 Zinc

Zinc transporters have been identified from different organisms such as yeast (Zhao and Eide, 1996a; Zhao and Eide, 1996b), mammals (Gaither and Eide, 2000; Palmiter *et al.*, 1996a; Palmiter and Findley, 1995; Wenzel *et al.*, 1997), *A. thaliana* (Grotz *et al.*, 1998) and *T. caerulescens* (Pence *et al.*, 2000). All the putative transporters involved in the influx of zinc belong to the ZIP (ZRT-IRT like protein) family. Most of the ZIP family members are capable of transporting a variety of cations such as zinc, cadmium, manganese and iron (Guerinot, 2000b). All the functionally characterized ZIP family members have eight putative transmembrane domains (TM), histidine-rich region between the TM three and four and conserved histidine and serine residues in the TM four region. The histidine and serine residues may comprise part of an intramembranous heavy metal binding site that is a part of the transport pathway (Eng *et al.*, 1998).

1.6.4.1 Genes involved in influx of zinc

In *S. cerevisiae ZRT1* (Zhao and Eide, 1996a) was the first gene encoding a zinc influx transporter to be identified due to its significant similarity to the iron regulated transporter (*AtIRT1*) from *A. thaliana* (Eide *et al.*, 1996). *ZRT1* is a member of a closely related family of transporter genes found in diverse organisms such as fungi, plants, nematodes and humans. This gene encodes a protein with 376 amino acids and has high affinity for zinc and is induced in zinc-deficient cells. Fe^{2+} and Cu^{2+} inhibit the uptake activity of *ZRT1*. *ZRT2* encodes the lower affinity zinc transporter in yeast (Zhao and Eide, 1996b) that is active in the zinc-replete cells. *ZRT2* was identified as a multicopy suppressor of a zinc-limited growth defect of the zrt1 mutant i.e. a mutant strain of yeast in which the high affinity system was disrupted. Activity of *ZRT2* is unaffected by Co^{2+}, Fe^{3+}, Mg^{2+}, Mn^{2+} and Ni^{2+} but Cu^{2+} and Fe^{2+} inhibit *ZRT2* mediated zinc uptake. A double mutant of yeast *ZHY3* in which two of the zinc transporters have been disrupted was viable and the viability of the mutant may be due to the existence of

an additional pathway for uptake of zinc (Zhao and Eide, 1996b). However the molecular identity of the additional pathway is not known.

The complementation of the yeast mutant ZHY3 has resulted in identification of zinc transporters from higher plants. Three genes encoding zinc transporters (ZIP 1-3 genes) were cloned from *A. thaliana* (Grotz *et al.*, 1998) by screening an *A. thaliana* cDNA expression library for clones that restored zinc limited growth of the *zrt1zrt2* mutant of yeast- *S. cerevisiae*. *AtZIP4* was identified from *A. thaliana* (Grotz *et al.*, 1998) during genome sequencing due to its similarity to other ZIP genes. *AtZIP4* does not complement the growth defect of ZHY3 and it has been suggested that ZHY3 mutant expressing *AtZIP4* fails to accumulate zinc due to the intracellular localization of the gene in yeast (Grotz *et al.*, 1998). Genome searches have revealed that *Arabidopsis* contains 15 ZIP genes (IRT1-3 and ZIP 1-12). ZIP genes of *A. thaliana* belong to a growing family of eukaryotic proteins with related members found in humans, bacteria, fungi, rice and nematodes (Grotz *et al.*, 1998).

Kinetics of zinc uptake of *AtZIP* genes as expressed in yeast mutant ZHY3.

GENE	APPARENT K_m, μM	V_{max}, fmol/min/10^6 cells
AtZIP1	13 ± 2	693 ± 43
AtZIP2	2 ± 0.3	107 ± 4
AtZIP3	14 ± 2	3528 ± 207

AtZIP1 and *AtZIP3* are expressed to high levels in the roots of zinc deficient plants while *AtZIP4* is also zinc responsive and expressed in both roots and shoots of zinc deficient plants. *AtZIP4* has been suggested to be targeted to the chloroplasts (Grotz *et al.*, 1998) and this is interesting given that *AtZIP4* is induced in roots of zinc deficient plants where chloroplasts are absent. *AtZIP2* mRNA was not detected in northern blots from plants grown in either zinc sufficient or zinc deficient conditions (Grotz *et al.*, 1998).

Uptake studies with ^{65}Zn isotope revealed that zinc accumulation was dependent on temperature and pH (Grotz *et al.*, 1998). No significant differences

were observed in the zinc uptake rate of *AtZIP1* and *AtZIP3* between pH 4.0 and 6.0, however in *AtZIP2* expressing strains, zinc accumulation was detected only at pH 6.0. The uptake activity of *AtZIP1*, *AtZIP2* and *AtZIP3* was observed to be saturable, concentration dependent and the K_m values were found to be similar to levels of zinc found in the rhizosphere (Welch, 1995). The ZIP genes were assayed for substrate specificities and it was observed that *AtZIP1* and *AtZIP3* had greater specificity for zinc as the substrate than *AtZIP2* that had the same specificity for cadmium as zinc (Grotz *et al.*, 1998).

The presence of multiple zinc transporters in *A. thaliana* may suggest that they are involved in different activities such as uptake of zinc from the soil and transport across the cellular and organellar membranes (Grotz *et al.*, 1998). The ZIP proteins from *Arabidopsis* are speculated to localize to different membranes similar to the *ZRT1*, *ZRT2* and *ZRT3* from yeast *S. cerevisiae* (MacDiarmid *et al.*, 2000; Zhao and Eide, 1996a; Zhao and Eide, 1996b). A heavy metal transporter *TcZNT1* was isolated from *Thlaspi caerulescens* (Pence *et al.*, 2000) through functional complementation of the yeast mutant ZHY3. This transporter has been shown to mediate high affinity zinc uptake and low affinity cadmium uptake. Uptake and transport of cadmium in plants is likely to occur through endogenous zinc transporters (Kochian, 1991) and this study provides the first direct evidence for this idea. Expression of *TcZNT1* is regulated by soil zinc status. Exposure to high zinc concentrations results in the overexpression of the *TcZNT1* gene resulting in increased zinc influx into roots of the hyperaccumulating *Thalspi* species. This study provides an insight into the molecular mechanism of accumulation of heavy metal uptake and regulation in a hyperaccumulating species. No genes encoding zinc transporters have been cloned in cereals to date.

1.6.4.2   Genes involved in efflux or sequestration of zinc

Heavy metals such as zinc are essential components in several enzyme reactions, yet are toxic at high concentrations. Genes encoding proteins involved in the detoxification of intracellular zinc have been identified in yeast (Kamizono *et al.*, 1989), *E. coli* (Rensing *et al.*, 1997), *A. thaliana* (Van Der Zaal *et al.*, 1999), mice (Huang and Gitschier, 1997), humans (Palmiter and Findley, 1995), mouse and

monkey (Wenzel *et al.*, 1997). All these transporters belong to the CDF (Cation Diffusion Facilitator) family and were first identified by Nies and Silver (1995). They are found in an array of diverse organisms such as bacteria, fungi, plants and mammals. Recently, this family has been renamed as the CE (Cation Efflux) family (Maser *et al.*, 2001) due to lack of information about the energetics and the known efflux function of the characterized transporters. All the transporters characterized to date from the CE family, have six putative transmembrane domains and a signature N-terminal amino acid sequence (Paulsen and Saier, 1997). A histidine-rich region is present in all the eukaryotic members, between the TM domains four and five (Paulsen and Saier, 1997). *ZRC1* identified from *S. cerevisiae* encodes a protein that confers resistance to zinc and cadmium in yeast (Kamizono *et al.*, 1989) but not to Cu^{2+}, Co^{2+} or Ni^{2+}. Yeast strains with multicopies of *ZRC1* can grow at high concentrations of zinc and any disruptions in the gene lead to sensitive phenotypes. Although the level of zinc and cadmium resistance is determined by the quantity of ZRC1 product (Kamizono *et al.*, 1989), the mechanism that confers resistance to high levels of zinc and cadmium has not been clearly understood. Another putative transporter characterized in yeast is the *COT1* (Conklin *et al.*, 1992) that is involved in cobalt resistance. Yeast deletion mutants show sensitivity to cobalt whereas overexpression in yeast leads to increased resistance to cobalt. Both *COT1* and *ZRC1* are localized to the yeast vacuolar membrane (Li and Kaplan, 1998), suggesting that they are involved in the efflux of cobalt, zinc and cadmium into the vacuole. *ZRT3* (MacDiarmid *et al.*, 2000) identified from yeast functions in the transport of zinc from the vacuole into the cytoplasm in zinc deplete conditions. This putative transporter has been dealt with in greater detail in section 1.7.1.3.

Zinc transporter *ZAT* from *A. thaliana* was identified during differential screening of cDNA library prepared from RNA isolated from auxin treated root cultures of *Arabidopsis* ecotype C42 (Van Der Zaal *et al.*, 1999). This transporter has six putative transmembrane domains, a histidine-rich region between the TM four and five and encodes a protein of 398 amino acids. *ZAT* may be involved in the facilitation of vacuolar sequestration of excess zinc which otherwise may lead to toxicity and cell death (Van Der Zaal *et al.*, 1999). This putative transporter represents the first full length CE family member to be identified and shown to be involved in heavy metal tolerance in plants. Recently *ZAT* has been renamed

AtMTP1 (Maser *et al.*, 2001), the two letter prefix identifying the species and MTP standing for metal tolerance protein.

1.7   Zinc homeostasis in the yeast S. cerevisiae

Cells have numerous mechanisms for solubilization and uptake of metals from the extracellular environment. However in excess concentration, metals can interfere with vital processes and can lead to toxicity and cell death. Therefore it is essential that the uptake of metal ions is tightly regulated and precise homeostatic regulation is maintained. *S. cerevisiae* has provided an excellent model system to study metal ion homeostasis. Studies in *S. cerevisiae* suggest that regulation of zinc concentration in yeast is mediated by uptake, storage, mobilization, regulation of gene expression (Conklin *et al.*, 1992; Hamer, 1986; Kamizono *et al.*, 1989; MacDiarmid *et al.*, 2000; Palmiter *et al.*, 1996; Palmiter and Findley, 1995; Rauser, 1995) and modulations at the post-translational level.

1.7.1   Zinc uptake systems

1.7.1.1 Genes involved in the influx of zinc

In yeast, two transport systems have been identified for uptake of zinc, which have been described in detail in the section 1.6.4.1. Intracellular zinc pools (Zhao and Eide, 1996a; Zhao and Eide, 1996b) regulate the activity of the transport systems. A study of mutants in which *ZRT1* expression was not repressed in zinc replete conditions led to identification of *ZAP1*-1^{up} allele (Zhao and Eide, 1997). In this allele, a cysteine was replaced by serine in the N terminal region, which caused constitutive expression of the target genes (Zhao and Eide, 1997). The wild type allele of this gene, which restored the regulation of target genes by zinc, was called *ZAP1* (Zhao and Eide, 1997), a transcriptional activator of *ZRT1* gene expression (Zhao and Eide, 1997). *ZAP1* encodes a protein of 93 kDa and has sequence similarity to other transcriptional activators. The promoter of ZAP1 has a single ZRE (Zinc responsive element). Recent information about the zinc responsive domain (ZRD) of *ZAP1* suggests that the mutation in *ZAP1*-1^{up} allele does not alter the zinc responsiveness of *ZAP1*, as it is far removed from the site to which the ZRD of *ZAP1* has been

mapped. An alternative hypothesis proposed to explain the activity of the 'up' allele is that the allele activates a normally quiescent activation domain leading to constitutive expression of the target genes. Most transcription factors are post-translationally regulated by controlling their nuclear localization, however *ZAP1* is localized to the nucleus in both zinc deplete and replete conditions (Bird *et al.*, 2000b). This fact suggests that post-translational regulation of *ZAP1* does not occur. *ZAP1* is regulated through the control of its DNA binding domains and transcriptional activation domains.

1.7.1.2 Vacuolar storage

Another important component of zinc homeostasis in yeast is the vacuole, which accumulates divalent cations such as Ca^{2+}, Co^{2+}, Fe^{2+}, Mn^{2+} and Ni^{2+} (Bode *et al.*, 1995; Dunn *et al.*, 1994; Nishimura *et al.*, 1998; Ramsay and Gadd, 1997). The vacuole has been identified as a site of zinc detoxification (Eide *et al.*, 1993; Ramsay and Gadd, 1997) and zinc is transported into the vacuole by two genes *ZRC1* (Kamizono *et al.*, 1989) and *COT1* (Conklin *et al.*, 1992) belonging to the cation diffusion facilitator or CDF /CE family of transporters (Maser *et al.*, 2001; Paulsen and Saier, 1997).

1.7.1.3 Mobilization of stored zinc

ZRT3 (MacDiarmid *et al.*, 2000) belongs to the ZIP gene family and encodes a protein with a predicted molecular mass of 55 KDa. *ZRT3* is involved in transport of zinc out of the vacuole during transition from zinc replete to zinc deplete conditions. During zinc deplete conditions, the labile pool of cytoplasmic zinc decreases. Decrease in zinc concentration results in the upregulation of the activity of *ZAP1*, leading to increase in the expression of *ZRT3*. *ZRT3* transports zinc out from the vacuole into the cytoplasm where it becomes available for use by the cell.

1.7.2 Regulation of gene expression

The expression of the genes involved in zinc uptake in yeast is regulated in response to varying zinc levels either at the transcriptional or post-translational level.

1.7.2.1 Transcriptional regulation

Zinc is transported across the plasma membrane of cells by either *ZRT2* (Zhao and Eide, 1996b) and/or *ZRT1* (Zhao and Eide, 1996a) depending on the zinc status of the cells. Zinc then enters the labile pool in the cytoplasm and is utilized by the cells. During conditions of zinc sufficiency, excess zinc is transported and stored in the vacuole by *ZRC1* (Kamizono *et al.*, 1989) and *COT1* (Conklin *et al.*, 1992). When cells encounter zinc deficient conditions, the concentration of zinc in the labile pool in the cytoplasm decreases, leading to upregulation of the activity of *ZAP1* (Zhao and Eide, 1997). *ZAP1* induces increased expression of *ZRT1, ZRT2 and ZRT3* by binding to a conserved 11 bp consensus sequence 5'- ACCTTNAAGGT -3' or the *ZREs* present in the promoters of *ZAP1, ZRT1* and *ZRT2* (Bird *et al.*, 2000a; Bird *et al.*, 2000b; Lyons *et al.*, 2000; Zhao *et al.*, 1998). Increased expression of *ZRT1* leads to enhanced uptake of zinc from the extracellular environment, whereas increased expression of *ZRT3* results in remobilization of zinc from the vacuole to the cytoplasm. In zinc replete conditions, *ZAP1* activity is repressed thereby preventing overaccumulation of zinc and maintaining metal ion homeostasis.

1.7.2.2 Post-translational regulation

ZRT1 is also regulated at the post-translational level (Gitan *et al.*, 1998) in yeast. ZRT1 is a stable N-glycosylated plasma membrane protein under low zinc conditions. On exposure to high zinc conditions, zinc limited cells expressing high levels of *ZRT1* show a rapid loss of zinc uptake activity (Gitan *et al.*, 1998). The lysine residue at position 195 in ZRT1 is ubiquitinated (Gitan and Eide, 2000) with either one or two 8 k Da ubiquitin proteins. Ubiquitinated ZRT1 accumulates in clathrin coated pits formed at the plasma membrane and is

then internalized as the endosome pinches off into the cytoplasm. These endosomes are packaged into vesicles and transported to the vacuole where the proteases degrade the transporter (Gitan and Eide, 2000). Thus on exposure to high levels of zinc, ubiquitinated ZRT1 undergoes endocytosis and subsequent degradation in the vacuole. Studies with other yeast plasma membrane proteins suggest that zinc induced inactivation of *ZRT1* is a specific response of the zinc transporter (Gitan *et al.*, 1998) to excess zinc and ubiquitin conjugation is a critical step that controls the endocytosis of *ZRT1* in response to high levels of extracellular zinc (Gitan *et al.*, 1998). Different models have been proposed to explain how the process of ubiquitination signals the endocytosis of *ZRT1* (Gitan and Eide, 2000). One model proposes that the process of ubiquitination signals endocytosis by resulting in conformational changes in the transporter that exposes internalization signals found within *ZRT1*. Such internalization signals have been known to signal endocytosis in other proteins (Kirchhausen *et al.*, 1997). The other model proposes that zinc binds directly to the transporter and exposes a hydrophobic recognition signal, which leads to conformational changes causing Lys195 to be ubiquitinated and subsequently degraded. *ZRT1* possesses a metal binding domain **HDHTHED** that is conserved in several *ZIP* family members and is located on the same transmembrane domain loop as Lys195 (Eng *et al.*, 1998).

All this evidence suggests zinc homeostasis in yeast is regulated post-translationally by ubiquitination, endocytosis and vacuolar degradation of ZRT1 on exposure to high zinc levels. The regulation of *ZRT1* prevents zinc uptake activity in cells exposed to high concentrations of zinc thereby preventing overaccumulation of potentially toxic metal. Each component involved in zinc homeostasis in yeast cells has an important role to play in regulation and maintenance of metal ion balance. Thus zinc homeostasis in yeast represents a fine balance between transcriptional and post-translational regulatory mechanisms and the transporter proteins they control.

1.8   *Zinc homeostasis in plants*

Knowledge of the mechanisms controlling zinc uptake in plants has increased rapidly with the identification of both ZIP and CE family genes in plants. Three

of the four *A. thaliana* ZIP transporters *AtZIP*1-3 (Grotz *et al.*, 1998) are predicted to play roles in zinc transport based on increased zinc uptake observed when expressed in ZHY3 yeast mutant. The fourth gene *AtZIP4* does not confer increased zinc uptake when expressed in yeast and it has been suggested that this could be due to poor expression or mislocalization of the protein in yeast. *AtZIP1* and *AtZIP3* have been implicated in uptake of zinc from the rhizosphere (Grotz *et al.*, 1998). In *A. thaliana*, the expression of *AtZIP1*, *AtZIP3* and *AtZIP4* is induced in response to zinc deficiency. This zinc responsive regulation of mRNA levels in response to zinc availability suggests that transcriptional control may be operating in plants. *AtZIP1* when expressed in yeast also shows zinc-induced inactivation on exposure to high concentrations of zinc (Guerinot, 2000b). This suggests that the expression of *AtZIP1* is post-translationally regulated in yeast, similar to the regulation of expression of *ZRT1*. However we do not know if the expression of the *AtZIP1* is regulated in a similar way in plants.

ZAT1 has been identified in *A. thaliana* (Van Der Zaal *et al.*, 1999) that is very similar to the *ZnT* genes (Palmiter *et al.*, 1996a; Palmiter *et al.*, 1996b) from mammalian cells. Overexpression of *ZAT1* resulted in enhanced zinc resistance and increased accumulation of zinc in the roots of *A. thaliana*. It has been speculated that *ZAT1* (Van Der Zaal *et al.*, 1999) might be involved in vacuolar sequestration of zinc similar to ZRCI and COT1 genes from yeast (MacDiarmid *et al.*, 2000). In the hyperaccumulator *T. caerulescens*, the expression of *ZNT1* (Pence *et al.*, 2000) is downregulated when plants are exposed to very high concentrations of zinc. This leads to reduction in root zinc uptake but the rate of accumulation is still higher than that observed in non-hyperaccumulating species *T. arvense*. This suggests that regulation of zinc transport in *Thlaspi* (Pence *et al.*, 2000) occurs through alteration in rates of zinc uptake in response to zinc status of the plant rather than an increase in zinc transporter gene expression.

Recently, three putative transporters have been identified from the nickel hyperaccumulator *Thlaspi goesingense* (Maser *et al.*, 2001) that complement the yeast strains deficient in *COT1* or *ZRC1*. These transporters confer increased resistance to cadmium, cobalt, nickel and zinc when expressed in yeast. Complementation of the yeast strains deficient in vacuolar metal sequestration

by the TgMTP proteins suggests that they play a role in metal homeostasis in plants. However the functional data on the plant MTP proteins is limited and a more detailed analysis of the family members is required to understand their role in metal homeostasis. Thus the *AtZIP* genes cloned from *A. thaliana* and *TcZNT1* from *T. caerulescens* provide a valuable starting point in understanding the mechanism of uptake of zinc across the plasma membranes in plants and also the process of zinc homeostasis.

1.9   Conclusions

Based on the studies of molecular mechanisms of various micronutrients, a clear picture of uptake and homeostatic regulation is beginning to emerge in yeast. In yeast transcription factors such as AFT1 (iron), ATH1 (iron), ZAP1 (zinc) and MAC1 (copper) activate the expression of genes such as *AtIRT1, ZRT1, ZRT2, NRAMP1-5, CTR1* and *CTR3* in metal deplete conditions enabling the cells to take up more metal ions from the extracellular environment. However the expression of manganese tranporters does not seem to be regulated by transcription factors and the mechanism of regulation is yet to be elucidated. On the other hand, in metal replete conditions the expression of these genes is repressed, thereby preventing overaccumulation of metal ions. When the cells are exposed to high concentrations of metal ions, genes such as *FTH1, ZRC1, ZRT3, CUP1, CSR5 and SOD1* regulate the concentration of metal ions by either effluxing the ions out of the cell or by sequestration into vacuoles. Also zinc, copper, and manganese transporters show post-translational regulation in yeast. When the cells are exposed to high concentrations of metal ions, the activity of metal ions is regulated by degradation of the high affinity transporter proteins in the vacuole. However no such regulation is seen for high affinity iron transport in yeast. Once the expression of the transporter is induced, exposure to high concentrations of iron does not effect a rapid reduction in the transporter activity. Therefore, it has been speculated that *S. cerevisiae* might accommodate excess iron by rapid sequestration (Askwith and Kaplan, 1997). Thus eukaryotic cells such as yeast possess an efficient mechanism for uptake of micronutrients such as zinc and also an excellent system of homeostatic regulation. However more studies that

elucidate the regulatory components of zinc homeostasis in plants are required to understand fully the molecular basis of metal ion regulation in plants.

1.10 Aim of this study

The molecular mechanisms of zinc uptake in yeast and plants are being elucidated and a much clearer picture of uptake and homeostasis is beginning to emerge from these studies. However, to date very little information is available about the molecular mechanisms of uptake and transport of zinc in cereals that are important worldwide sources of food. Thus the broad aims of this study were to identify and characterize genes responsible for zinc uptake in cereals using rice as a model system and to determine whether overexpression of zinc transporters increases zinc uptake efficiency.

1.10.1 Specific objectives

1. To identify and isolate genes encoding zinc transporters in rice.
2. To characterize the function of the encoded proteins in the yeast mutant ZHY3.
3. To characterize the expression of these genes in plants.
4. To overexpress the genes encoding the zinc transporters and study their effect on zinc uptake.

Chapter 2 Identification of zinc transporters showing similarity to *Arabidopsis* zinc transporters

2.1 *Introduction*

The cloning and the characterization of the genes encoding ion transporters in plants (Williams *et al.*, 2000) and animals (Rolfs and Hediger, 1999) has led to a major advancement in our understanding of the ion transport processes. In humans, the molecular identification of ion transporters is of great importance in understanding diseases such as anemia, Menkes's disease, Wilson's disease, Alzheimer's disease and Parkinson's disease that are thought to be related to disturbances in metal ion homeostasis (Rolfs and Hediger, 1999). In plants, complementation of yeast mutants has led to the cloning of many cDNAs encoding proteins involved in ion transport (Grotz *et al.*, 1998; Muchhal *et al.*, 1996; Pence *et al.*, 2000; Schachtman and Schroeder, 1994; Sentenac *et al.*, 1992; Smith *et al.*, 1997; Smith *et al.*, 1995; Zhao and Eide, 1996a; Zhao and Eide, 1996b). Micronutrients such as copper [Chapter 1, section 1.6.2], iron [Chapter 1, section 1.6.1] and zinc [Chapter 1, section 1.6.4] are essential for many physiological processes yet are toxic when present in excess in plants. In the last decade, major advances in our understanding of the zinc transporters and their regulation in eukaryotic organisms has been possible due to the identification of two families of transporters, the ZIP (Zrt-Irt like protein) and CDF (Cation Diffusion Facilitator) or CE (Cation Efflux) families (Gaither and Eide, 2001) that play important roles in zinc transport. The ZIP family members are involved in the influx of zinc into the cells and the zinc transporters *(AtZIPs)* from *A. thaliana* (Grotz *et al.*, 1998) were the first zinc transporters to be identified in plants, followed by zinc transporter *(TcZNT1)* from *Thlaspi careulescens* (Pence *et al.*, 2000). Study of cloned genes encoding these nutrient transporters has provided an insight into their structure such as the presence of eight potential transmembrane domains, extracytoplasmic amino and carboxy termini, presence of variable region and metal binding domains. These studies have shown that the transporters are involved in the uptake of zinc but may also transport other cations once

expressed. The expression of *AtZIP* and *TcZNT1* genes is regulated by zinc status in the external medium. The knowledge gained from these studies may eventually be used to engineer plants to serve as better dietary sources of zinc, to more efficiently remove heavy metal ions from contaminated soils or to create plants that are more zinc efficient.

The functional expression of plant genes in yeast has provided a valuable tool for understanding the molecular structure and functional mechanisms of nutrient uptake and regulation in eukaryotes. In the recent years *Saccharomyces cerevisiae* have been used in the identification of plant transporters (Frommer *et al.*, 1993; Barbier-Brygoo *et al.*, 2001). *S. cerevisiae* has proved to be a powerful tool mainly because mutant strains defective in specific transport activities are available. Functional complementation of the yeast strains defective in zinc uptake has led to the identification of plant zinc transporters (Grotz *et al.*, 1998; Pence *et al.*, 2000) while the yeast zinc transporters (Zhao and Eide, 1996a; Zhao and Eide, 1996b) were identified based on homology to the iron regulated transporter *AtIRT1* from *A. thaliana* (Eide and Guarente, 1992; Korshunova *et al.*, 1999). Functional complementation of the yeast mutants has been a highly successful method for cloning plant transporters as it relies on the functional similarities between transport mechanisms rather than the structural similarities in proteins. However functional complementation in yeast mutants may not always be successful because plant proteins might be incorrectly targeted to the wrong membrane or the transport system might require plant specific protein partners that are not available in the heterologous systems (Barbier-Brygoo *et al.*, 2001).

The genes encoding transporters can be cloned by complementation of yeast mutants or by Polymerase Chain Reaction (PCR) with degenerate primers (designed based on the conserved regions of the known genes), cDNA or genomic library screening with low stringency hybridization to heterologous probes and searching the EST (expressed sequence tags) databases for predicted protein sequences. These methods can be used to identify homologues to known genes encoding transporters.

Rice was chosen as a model system in our studies to identify the genes encoding the zinc transporters in cereals based on homology to the known transporters. The genome of *Oryza sativa* is considerably smaller than the other cereal genomes and the size of its genome is estimated at 430 megabase pairs

resulting in higher gene density relative to other cereals (Goff, 1999; Sakata *et al.*, 2002). Since a significant amount of colinearity or synteny exists among the various cereal genomes, rice has been proposed as a model for cereal genomics (Ahn *et al.*, 1993; Havukkala, 1996). The resources available for rice include:

i)   MAFF Genebank Project

The Ministry of Agriculture, Forestry and Fisheries (MAFF) started a genebank project in 1985. The MAFF Plant Genetic Resources activities consist of the Central Bank (NIAR) and the Sub-Banks located throughout Japan. There are 12 plant genetic resources research groups including one for rice (http://www.rgp.dna.affrc.go.jp). MAFF has a collection of more than 30,000 rice ESTs in its database and provides researchers with access to the DNA.

ii)   The International Rice Genome Sequencing Project (IRGSP)

This project was established in 1998 with the goal of sequencing the entire rice genome and to date >137 Mb of the genome sequence has been made available to the public domain. The database called Rice Genome Automated Annotation System (RiceGAAS) has a catalog of nearly 30,000 expressed genes, a high density linkage map composed of 2275 DNA markers, a physical map with YAC clones covering ~ 70% of the whole rice genome (Sakata *et al.*, 2000).

iii)   National Center for Biotechnology Information (NCBI)

NCBI creates public databases, conducts research in computational biology, develops software tools for analyzing genome data, and disseminates biomedical information. The ESTs from the rice genome sequencing project have been deposited in the databases at NCBI and provide a valuable tool in identification of rice genes.

In the present study, NCBI databases were searched with known zinc transporters from *A. thaliana*, to identify and clone genes of interest. In recent years, zinc transporters have been cloned from *A. thaliana* (Grotz *et al.*, 1998), *L. esculentum* (Eckhardt et al., 2000), *T. caerulescens* (Pence *et al.*, 2000) and soybean (Moreau et al., 2002).

However, apart from the genes mentioned no other genes from higher plants, especially the cereals, have been identified at the functional level. Thus the

aim of this investigation was to clone and characterize genes encoding zinc transporters from a cereal such as rice. Given the synteny between the cereal genomes, the knowledge gained from studying the rice zinc transporters can be used to clone similar transporters from other cereals such as wheat and barley. *Arabidopsis* zinc transporter protein sequences were used to search the EST database at NCBI and resulted in identification of four ESTs *OsR06, OsE60, OsS13* and *OsS16* from *O. sativa* with considerable homology to the *Arabidopsis* zinc transporters. This chapter presents the results of cloning and functional characterization of these ESTs in the yeast mutant ZHY3.

2.2   Methods

2.2.1   BLAST search for zinc transporter homologues

BLAST (Basic Local Alignment Search Tool) is a set of similarity search programs designed to explore all of the available sequence databases regardless of whether the query is protein or DNA. The BLAST programs are run by NCBI and can be accessed at the web site http://www.ncbi.nlm.nih.gov (Altschul et al., 1990). Amino acid sequences encoded by zinc transporter genes from *A. thaliana* were kindly provided by David Eide prior to publication (Grotz *et al.*, 1998) and were used in the BLAST search against the NCBI EST database with translated BLAST searches tblastn in this study.

2.2.2   Sequencing

Four ESTs from *Oryza sativa* (rice) were identified as putative zinc transporter homologues in the BLAST search and were ordered from MAFF DNA Bank in Japan (1-2, 2-chome, Kannondai, Tsukuba, Ibaraki 305, Japan). These ESTs were sequenced using the DNA Sequencing Kit (BigDye™ Terminator Cycle Sequencing Ready Reaction) from Applied Biosystems (Foster city, CA). 250 - 500 ng of the plasmid DNA was added to 8 µl of Big Dye Terminator Cycle Sequencing ready reactions. M13 primers and internal primers to a final concentration of 3.2 pmol were added to the reaction mix and volume made up to 10 µl with deionised water. Reactions were subjected to PCR (96° 10 sec, 50° 5

sec, 60° 4 min, 25X cycles) and the products were precipitated using 75% isopropanol. The sequences were obtained from the Molecular pathology sequencing laboratory at the Institute of Medical and Veterinary Science (IMVS) as ABI sequence files and the chromatograms were edited using the program *Chromas* (version 1.45; School of Health Sciences, Griffith University, Gold Coast Campus, Southport, Queensland, Australia). Sequence data was analysed using the sequence analysis package GCG (Version 8.1.0, Genetics Computer Group, Wisconsin, USA) provided by ANGIS (Australian National Genome Information Service, Sydney).

Nucleic acid and predicted amino acid alignments were carried out using the GCG tools, GAP and BESTFIT. Multiple sequence alignments at the nucleic acid or amino acid level were generated using the GCG tools PILEUP and Pretty Box programs from ANGIS. Hydropathy profiles were generated using Kyte and Doolittle's method (Kyte and Doolittle, 1982) with a window size of 19 amino acids. A dendrogram of the identified rice cDNAs was constructed using the GCG tools PILEUP, DISTANCES and GROWTREE and the results represented graphically using the Tree View (Copyright: Roderic D. M. Page, 2001) program available from the web site http:// taxonomy.zoology.gla.ac.uk/rod/rod.html.

2.2.3 Cloning into a yeast expression vector

Full-length cDNAs were digested with restriction enzymes Not1 (Boehringer Mannheim, Germany) and Kpn1 (Boehringer Mannheim, Germany) and the fragments cloned into yeast expression vector pYES2 (Invitrogen, San Diego) [Appendix 1.1]. The clones were electroporated into DH5α competent cells and plated onto LB (Table 1, Appendix 1.2) plates with ampicillin. Single colonies were inoculated into 3 ml LB with ampicillin and grown overnight. Plasmid DNA was extracted using the QIAprep Spin Mini Prep Kit from QIAGEN according to the manufacturer's instructions.

2.2.4 Transformation of Os cDNAs into *S. cerevisiae* mutants

The cDNAs cloned into pYES2 were transformed into yeast mutants ZHY3 (Zhao and Eide, 1996b), DEY 145 (Dix *et al.*, 1994) and SLY8 (Supek *et al.*, 1996). The

composition of the solutions used in this protocol is listed in Table 2 (Appendix 1.3). ZHY3 (*zrt1zrt2*) is a yeast mutant in which two genes encoding zinc transporters have been deleted rendering the mutant partially deficient in zinc uptake. In our preliminary experiments using low zinc medium (LZM) [Appendix 1.4] the mutant required a minimum of 400 μM zinc for growth. The DEY 145 (*fet3fet4*) mutant of yeast is deficient in iron uptake due to the deletion of FET4 that encodes an iron transporter and FET3 that encodes a multicopper oxidase and is a component of high affinity transport system. The SLY8 mutant has one gene encoding manganese transporter deleted rendering it deficient in manganese uptake and unable to grow on medium containing EGTA [Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-Tetra acetic acid].

2.2.4.1 Preparation of competent cells

Competent cells were made from each yeast mutant before transformation. A 5 ml culture of each yeast strain inoculated into YPD medium (Table 4 Appendix 1.5) was grown overnight. A 1:100 dilution of overnight culture was inoculated into 100 ml of YPD medium and cells were grown to mid - log phase (OD $_{600\ nm}$ = 0.6). The cells were spun down by centrifugation at 5000 rpm in a table-top Eppendorf centrifuge for 5 minutes. The pellets were washed once in 20 ml of solution A (Table 2, Appendix 1.3) and resuspended in 2 ml of solution A. Aliquots of 200 μl were dispensed into eppendorf tubes and placed at -80° C for use in transformation.

2.2.4.2 Transformation

1-2 μg of plasmid DNA and 50 μg of salmon sperm DNA (denatured by boiling for 10 min and cooled on ice for 2 min) were added to the frozen competent cells and thawed for 5 min at 37° C. The tubes were inverted several times to mix. One ml of solution B (Table 2, Appendix 1.3) was added to the bottom of the tubes and mixed by pipetting. The tubes were incubated at 30° C for an hour and centrifuged in a table-top centrifuge at 3000 rpm for 2 minutes. The pellets were washed twice in 800 μl of solution C (Table 2, Appendix 1.3) and resuspended in

200 µl of solution C. Aliquots of 50 - 100 µl were plated onto plates with selective medium depending on the strain being transformed. The selective medium did not contain uracil and the transformants were selected with uracil as the selectable marker. The yeast strain ZHY3 transformed with the cDNAs were plated onto LZM (Low Zinc Medium-Tables 3 A and B, Appendix 1.4) plates supplemented with 1 mM zinc while the *fet3fet4* yeast strain transformed with the cDNAs was plated onto plates with LIM (Low Iron Medium) supplemented with 10 µM $FeCl_3$. The yeast strain SLY8 transformed with the cDNAs was plated on to YNB plates (Synthetic Minimal Medium, Table 5 - Appendix 1.6). The plates with ZHY3 and *fet3fet4* transformations were incubated for 3-5 days at 30° C while plates with SLY8 transformations were incubated for 5- 7 days at 30° C.

2.2.5 Complementation of yeast mutants

2.2.5.1 ZHY3 mutant

Single colonies from transformations on LZM plus 1 mM zinc plates were streaked onto LZM plus 1 mM zinc plates and grown in a 30°C incubator. The inoculum from these plates was used to grow overnight cultures in LZM medium supplemented with 1 mM zinc. These cultures were centrifuged at 3000 rpm in an Eppendorf bench-top centrifuge and the pellets washed twice in Tris-EDTA (TE pH 8.0) [Table 6, Appendix 1.7]. The pellets were resuspended in 200 µl of TE, pH 8.0. The optical density ($OD_{600 \, nm}$) of the samples was adjusted to 0.1 and 10X dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the samples were carried out. An aliquot of 5 µl of each of the dilutions was spotted onto LZM plates with either 150 µM zinc or 1 mM zinc to test for complementation of the ZHY3 mutant by the Os cDNAs. *AtZIP1* and *AtZIP3* cDNAs from *Arabidopsis thaliana* expressed in ZHY3 were used as positive controls in the experiments. The plates were incubated at 30°C for 3 days before being photographed.

2.2.5.2 DEY145

Aliquots of 5 µl of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) processed as detailed in section 2.2.5.1 (except that single colonies were obtained from the

transformations plated on 10 μM FeCl₃ LIM plates) were spotted onto YNB plates supplemented with either 80 μM BPS (Batho-phenanthroline sulphonate) and 10 μM FeCl₃ or no supplementation, to test for complementation of the DEY145· mutant by the Os cDNAs. The plates were incubated at 30° C for 3 days before being photographed.

2.2.5.3   SLY8 mutant

Serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) of the samples were generated according to the procedure detailed in section 2.2.5.1, except that the single colonies were obtained from the transformations plated on YNB plates with no added supplements. Aliquots of 5 μl were spotted onto YNB plates with no added supplements or plates with 20 mM EGTA, to test for complementation by the Os cDNAs. *AtIRT1* cDNA from *A. thaliana* (Eide *et al.*, 1996; Korshunova *et al.*, 1999) was used as positive control in the experiments. The plates were incubated at 30° C for 5 days before being photographed.

2.2.6   Toxicity experiments

Low Zinc Medium (LZM) [Tables 3 A and B, Appendix 1.4] plates with different supplements, such as zinc sulphate (0.2 mM - 1 mM), nickel chloride (0.01 mM - 3 mM), cobalt chloride (0.01 mM - 3 mM), manganese chloride (0.025 mM - 3 mM), calcium chloride (1 mM - 5 mM), magnesium chloride (5 mM - 10 mM), sodium chloride (50 mM - 200 mM) and cadmium chloride (0.01 mM - 0.2 mM) were used in the toxicity experiments. The ZHY3 cells expressing the *AtZIP1* and *AtZIP3* cDNAs from *A. thaliana* were used as positive controls in the experiment. ZHY3 cells expressing the *AtZIP* cDNAs and the *OsS13*, *OsR06* and *OsE60* cDNAs were grown in 5 ml cultures overnight in LZM supplemented with 1 mM zinc. The yeast cells were centrifuged at 3000 rpm in an Eppendorf bench-top centrifuge for 5 min and the pellets washed twice in Tris-EDTA (TE pH 8.0). The pellets were resuspended in 200 μl TE pH 8.0. The optical density (OD_{600nm}) of the samples was adjusted to 0.1 and 5 μl aliquots of serial dilutions (10^{-1}, 10^{-2}, 10^{-3}) were spotted onto plates supplemented with different concentrations of metal

ions. Each experiment was replicated twice. The plates were incubated at 30°C for 3-4 days before being photographed.

2.2.7 Growth experiments in liquid medium

Based on the results from the toxicity experiments, growth experiments with ZHY3 cells expressing the various Os cDNAs were carried out in synthetic minimal medium (YNB) [Table 5, Appendix 1.6] supplemented with chloride salts of different metal ions such as cadmium (0.01 mM-0.2 mM), zinc (0.15 mM-1 mM), calcium (1 mM-3 mM), magnesium (5 mM-10 mM) and sodium (20 mM-200 mM). All the tubes containing YNB medium supplemented with different metal ions were inoculated with overnight cultures to the same starting optical density (OD_{600nm} = 0.08), except in the growth experiments involving cadmium where the various yeast strains were grown to mid-log phase (OD_{600nm}=0.8) before the addition of indicated concentrations of cadmium. Each experiment had three replicates i.e three tubes each with 5 ml of the medium supplemented with indicated concentrations of the metal ion. The cultures were incubated at 30°C with shaking and sampled periodically for 72 hours. The optical density of each sample was measured and recorded.

2.2.8 Preparation of yeast strains for ICP analysis

The yeast strain ZHY3 expressing OsR06, OsE60 and OsS13 cDNAs and containing the empty plasmid pYES2 was grown in flasks containing 200 ml of YNB supplemented with either 0.01 mM cadmium (24 hours), 1.7 mM calcium (48 hours) and 9 mM magnesium (48 hours). Each culture was grown in three replicate flasks. The cultures were centrifuged at 3000 rpm in an Eppendorf centrifuge and the pellets washed twice TE (Tris-EDTA, pH 8.0) buffer. These pellets were dried in an oven at 80° C and then weighed. The dried samples were digested with nitric acid and analyzed by inductively Coupled Plasma Atomic Emission Spectrometry (ICPAES) at the Waite Analytical Center, University of Adelaide. The results represent the mean of three replicates.

2.2.9   5' RACE of *OsS16* cDNA

To obtain the 5' end of the partial cDNA fragment *OsS16*, 5' RACE (5' RACE kit GIBCO, BRL, version 2.0) and SMART RACE PCR (protocol adapted from SMART™ RACE cDNA Amplification Kit from CloneTech) were carried out according to the manufacturer's instructions. Oligos were designed towards the 5' end of the *OsS16* fragment (Appendix 1.14). Total RNA extracted from the zinc sufficient rice plants grown in hydroponics (Chapter 4, sections 4.2.1 and 4.2.2) was used as a starting template for the 5' RACE. Both the 5' RACE protocols use similar strategy or principles. The first strand cDNA synthesis is primed using a gene specific antisense oligonucleotide (GSP1). Following cDNA synthesis, the first strand is purified from uncorporated dNTPs and GSP1. Terminal deoxynucleotidyl transferase (TdT) is used to add homopolymeric tails to 3' ends of the cDNA, followed by PCR with a mixture of three primers: a nested gene specific primer (GSP2) which anneals 3' to GSP1; and a combination of anchor primer and adapter primer which permit amplification of the homopolymeric tail. This leads to the amplification of unknown sequences between the GSP2 and the 5' end of the mRNA. The PCR products are then run on an agarose gel, band of interest purified, cloned into a suitable vector and sequenced to confirm the full length or 5' end of the clone.

2.3   Results

2.3.1   Identification of rice ESTs

BLAST search carried out with the known *A. thaliana* zinc transporter proteins (*AtZIP* genes), identified four ESTs *OsR06, OsS13, OsE60* and *OsS16* from *Oryza sativa* (rice) in the NCBI EST database. These ESTs showed a high degree of similarity to the *AtZIP* genes from *A. thaliana*. One EST (*OsR06*) was from a root cDNA library of *O. sativa* cv. Nipponbare while the other three ESTs (*OsS13, OsE60* and *OsS16*) were from shoot cDNA libraries of *O. sativa* cv. Nipponbare.

2.3.2 Sequence Analysis

The four ESTs *OsR06, OsE60, OsS13* and *OsS16* were obtained from the MAFF DNA Bank in Japan and sequenced with universal primers and with primers designed along the length of the sequence to obtain the full length sequences. Sequence analysis of the rice ESTs suggested that the three cDNAs *OsS13, OsR06* and *OsE60* were full-length sequences while the fourth cDNA *OsS16* was a partial length fragment. *OsE60* is 1.361 kb (Appendix 1.8 A) and encodes a predicted protein of 365 amino acids (Appendix 1.8 B) while *OsR06* cDNA is 1.369 Kb (Appendix 1.9 A) and encodes a predicted protein with 352 amino acids (Appendix 1.9 B). *OsS13* cDNA is 1.333 Kb (Appendix 1.10 A) encoding a predicted protein with 362 amino acids (Appendix 1.10 B) while *OsS16* cDNA is a 0.6 Kb partial length fragment (Appendix 1.11) with an ORF (open reading frame).

Analysis of the sequences at the amino acid level revealed significant homologies to the known zinc transporters from *A. thaliana* but not to the zinc transporters from *S. cerevisiae* (Table 7). The *OsE60* and *OsS16* cDNAs show high degree of similarity to the *AtZIP1* gene from *A. thaliana* while the cDNAs *OsR06* and *OsS13* are homologous to the *AtZIP2* gene from *A. thaliana* (Table 7). Intracellular localization of the cDNAs using the PSORT1 tool (www.ExPASy.ch) suggests that the cDNAs *OsS13, OsR06* and *OsE60* are likely to encode plasma membrane proteins. Comparison of *OsR06, OsS13* and *OsE60* deduced amino acid sequences show characteristic features of the members of the ZIP gene family such as the presence of conserved histidine residues in TMs (transmembrane regions) II, IV and V (Figs. 1A and B). Conserved histidine and serine residues are present in the transmembrane IV region and a motif $(HX)_n$ where n = 3 to 4 histidine residues is present in the *OsE60* predicted protein at residues 184-189. The hydropathy profiles (Fig.1D) of the full length cDNAs *OsE60, OsR06* and *OsS13* revealed the presence of eight putative transmembrane domains with distinct hydrophobic and hydrophilic regions, which is a characteristic feature of membrane proteins. A variable cytoplasmic loop between the putative transmembrane domains III and IV is present in all the three Os cDNAs.

Based on sequence analysis of the *OsS13, OsR06, OsE60* and *OsS16* amino acid sequences, a dendrogram (Fig.1E) was constructed with the known ZIP gene family members. The dendrogram has four clades and all the four cDNAs *OsR06, OsS13, OsE60* and *OsS16*, identified from *O. sativa* fit well into the ZIP gene family. One clade is the plant Fe transporter group including the *OsS16* from *O. sativa* and the second clade is a group containing *AtZIP2* from *Arabidopsis, OsR06* and *OsS13* from *O. sativa*. Third clade contains the *AtZIP4* protein from *Arabidopsis*, which was proposed to be plastidic in nature (Grotz *et al.*, 1998) and the fourth clade contains the *AtZIP1* protein from *Arabidopsis, OsE60* from *O. sativa* and *Znt5* from *T. caerulescens*. When the ZIP sequences from yeast were used along with the plant sequences, the yeast zinc transporters formed a distinct clade from the plant zinc transporters (data not shown).

2.3.3   5' RACE of *OsS16* cDNA

Attempts with various internal primers were unsuccessful in obtaining the 5' end of the partial fragment *OsS16* cDNA. Instead the 5' RACE protocols amplified sequences homologus to the hydroxyproline rich gene from *O. sativa* or *Zea mays*.

2.3.4   Complementation of yeast mutants

2.3.4.1   ZHY3 mutant

The functional complementation of the yeast mutant ZHY3 (defective in both high and low affinity zinc uptake) was tested according to the protocol outlined in section 2.2.5.1. All the strains were expected to grow at a zinc concentration of 1 mM whereas those in which the inserted plasmids complemented the ZHY3 mutant would grow on plates with 150 µM zinc. Growth of ZHY3 cells expressing the *AtZIP* cDNAs, *OsS13, OsR06, OsE60* cDNAs and containing the empty plasmid pYES2 was observed at all the dilutions on LZM plates with 1 mM zinc (Fig. 2A). At a zinc concentration of 150 µM, ZHY3 cells expressing *OsR06* and *OsE60* weakly complemented the mutant. The cDNA *OsS13* did not complement the ZHY3 mutant at 150 µM zinc.   All the *AtZIP* cDNAs

complemented the ZHY3 mutant at 150 μM zinc. ZHY3 cells containing the empty plasmid did not grow at 150 μM zinc (Zhao and Eide, 1996b). The results of the complementation experiments with the ZHY3 mutant have been summarized in Table 8. Confirmation of the zinc uptake activity of the Os cDNAs is presented in Chapter 3.

2.3.4.2 SLY8 mutant

The functional complementation of the SLY8 mutant (defective in manganese uptake) was examined by the method outlined in section 2.2.5.3. SLY8 mutant is unable to grow on medium supplemented with EGTA (Supek *et al.*, 1996) while the yeast strain expressing *AtIRT1* cDNA can grow on medium with EGTA and has been used as a control in the experiments. All the yeast strains were observed to grow on plates with YNB medium (Fig. 2B) however, on plates supplemented with EGTA, SLY8 cells expressing the *AtIRT1* cDNA grew well, and cells expressing *OsR06* and *OsE60* cDNAs grew slightly (Fig.2B). This suggested that the cDNAs *OsR06* and *OsE60* weakly complemented the mutant SLY8. SLY8 cells expressing *OsS13* did not grow on the plates with EGTA suggesting that the cDNA did not complement the mutant. The results of the complementation experiments with the SLY8 mutant have been summarized in Table 8.

2.3.4.3 DEY145 mutant

The functional complementation of the DEY145 (*fet3fet4*) mutant (deficient in iron uptake) was examined by protocol detailed in the section 2.2.5.2. On plates with YNB medium all the yeast strains grew, however no growth of the *fet3fet4* cells expressing the Os cDNAs occurred on plates supplemented with either BPS alone or BPS and 10 μM Fe. These results suggest that the Os cDNAs do not complement the DEY145 mutant (Table 8).

2.3.5 Toxicity experiments

To determine which metal ions might be transported by the different rice cDNAs, medium was supplemented with 1 mM Zn sulphate and different divalent cations.

The absence of growth of the yeast strains containing the Os cDNAs at different concentrations of the metal ions was taken as an indicator that the metal ions were either transported by the Os cDNAs or blocked zinc uptake. The ZHY3 yeast strain expressing Os cDNAs was tested for toxicity to cadmium, nickel, cobalt, iron, manganese, zinc, copper, calcium, magnesium and sodium. In all the experiments, ZHY3 cells expressing the *AtZIP1* and *AtZIP3* cDNAs from *A. thaliana* were used as positive controls.

2.3.5.1 Manganese

The growth of ZHY3 cells expressing the *OsR06*, *OsE60*, *OsS13*, *AtZIP1* and *AtZIP3* cDNAs on LZM plates supplemented with 0.025 mM - 0.2 mM manganese chloride (Fig. 3A) was similar at all the dilutions (10^{-1}, 10^{-2}, 10^{-3}) tested. These results suggest that manganese is not toxic to the ZHY3 cells expressing the *OsR06*, *OsS13*, *OsE60*, *AtZIP1* and *AtZIP3 cDNAs*.

Table 7   Percent identity and percent similarity between putative zinc transporter proteins from O. sativa, A. thaliana and S. cerevisiae.

Zinc transporters	% Identity				% Similarity			
	OsS13	*OsR06*	*OsE60*	*OsS16*	*OsS13*	*OsR06*	*OsE60*	*OsS16*
AtZIP1	24	22	55	56	50	52	75	79
AtZIP2	50	59	51	20	70	78	19	51
AtZIP3	20	24	50	56	47	50	71	75
AtZIP4	22	22	51	50	50	53	70	73
ScZRT1	17	25	25	39	46	52	53	63
ScZRT2	23	22	35	33	50	53	54	62

At = *A. thaliana*; Sc = *S. cerevisiae*. The percent identities and similarities have been calculated using the GCG tool GAP.

Fig. 1 **Alignment of amino acid sequences to *A. thaliana* zinc transporters.** Conserved residues between the sequences are highlighted while conserved histidine residues between the sequences are marked with *. Histidine and serine residues conserved between the sequences are underlined. These alignments were generated using the GCG tools PILEUP and PRETTYBOX.

A.   *OsR06* and *OsS13* to *AtZIP2*

B.   *OsE60* to *AtZIP1*

C.   *OsS16* to *AtIRT1*

A.

```
AtZIP2  .....MALSSKTLKSTLVFLSIIFLCFSLILAHGGIDDGDE  36
OsR06   MARTMTMRVSSLLVAVVLLAA..LSFQACSGHGGINDGDG  38
OsS13   MAGGRGARA.SLHLHLAWLCAFATTAWAHGGGGGGGDSDA  39

                                         I
AtZIP2  EEETNQPPPATGTTTVVNLRSKGLVLVKIYCIIILFSTF  76
OsR06   QVDAPATPASSS.....GVRSKGLIAVKVWCLVILLVFTF  73
OsS13   DADGGGEGKP.......DLRARGLVAAKLWCLAVVFAGTL  72

                         II                    *
AtZIP2  LAGISPYFYRWNESFLLLGTQFSGGIFLATALIHFLSDAN  116
OsR06   AGGVSPYFYRWNESFLLLGTQFAAGVFLGTALMHFLADST  113
OsS13   AGGVSPYFMRWNDAFLALGTQFAGGVFLGTAMMHFLADAN  112

                    III
AtZIP2  ETFRG.LKHKEYPYAFMLAAAGYCLTMLADVAVAFV....  151
OsR06   STFKG.LTTNQYPFSFMLTCVGFLLTMLSDLVIAAVARRS  152
OsS13   ETFADLLPGTAYPFAFMLACAGYVLTMLADCAISFVVARG  152

AtZIP2  ..AAGSNNHVGASVGESREDDDVAVKEEGRREIKSGVDVS  189
OsR06   AAAGVSDNQV..SEQQQRQQAEGAVMSR.KEEEAAAVAHP  189
OsS13   GGRTEPAAAAGAGLEEGKLSSTNGNASDPPAADAAAQDHS  192

                              *IV
AtZIP2  ..QALIRTSGFGDTALLIFALCFHSIFEGIAIGLSDTKSD  227
OsR06   ..AMLVRTSSFEDAVLLIVALCFHSVFEGIAIGVSASKSE  227
OsS13   VASMLRNASTLGDSVLLIAALCFHSVFEGIAIGVAETKAD  232

              *V
AtZIP2  AWRNLWTISLHKVFAAVAMGIALLKLIPKRPFLTVVYSF  267
OsR06   AWRNLWTIGLHKIFAAVAMGIALLRMIPKRPFLMTVVYSL  267
OsS13   AWKALWTISLHKIFAAIAMGIALLRMLPDRPFLSCFGYAF  272

            VI                       VII
AtZIP2  AFGISSPIGVGIGIGINATSQGAGGDWTYAISMGLACGVF  307
OsR06   AFAVSSPVGVGIGIAIDATSQGRAADWTYAISMGLATGVF  307
OsS13   AFAVSSPVGVGIGIVIDATTQGRVADWIFAVSMGLATGIF  312

                *                   VIII
AtZIP2  VYVAVNHLISKGYKPLEECYFDKPIYKFIAVFLGVALLSV  347
OsR06   IYVAINHLIAKGYRPHHPTAADKPLFKFLAVLLGVAVMAV  347
OsS13   IYVSINHLLSKGYTPLRPVAADTPAGRLLAVVLGVAVIAV  352

AtZIP2  VMIWD.....352
OsR06   VMIWD*....352
OsS13   VMIWDTCRSZ362
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B.

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                                                           *           I
OsE60    .......M G A K K H T L Q V L P W L L L F A Q H T A A S A C D C A N T T D G   34
AtZIP1   M S E C G C F S A T . . T M L R I C V V L I I C L H M C C A S S D C T S H D D P   38


OsE60    A D R Q G . . . A M K L K L I A I A S I L A A G A A G V L V P V I G R S M A A L   71
AtZIP1   V S Q D E A E K A T K L K L G S I A L L L V A G G V G V S L P L I G K R I P A L   78

                            II                        *
OsE60    R P D G D I F F A V K A F A A G V I L A T G M V H I L P A A F D A L T S P C L K   111
AtZIP1   Q P N . D I F F M V K A F A A G V I L C T G F V H I L P D A F E R L S S P C L E   117

                            III
OsE60    R G G G D R N P F P F A G L V S M S A A V S T M V V D S L A A G Y Y H R S Q F R   151
AtZIP1   D T T A G K . . F P F A G F V A M L S A M G T L M I D T F A T G Y Y K R Q H F S   155

                                      *        *   *       *   *   *
OsE60    K A R P V D N I N V H K H A G D E R A E H A Q H I N A H T H G G H T H S H G D I   191
AtZIP1   N N H G S K Q V N V . . . . V V D E E H A G H V H I H T H A S H G H T H G S .   190

                                                       *IV
OsE60    V V C G S P E E G S V A E S I R H K V V S Q V L E L G I L V H S V I I G V S L G   231
AtZIP1   . . . . . . . . . . . T E L I R R I V S Q V L E I G I V V H S V I I G I S L G   219
                                                                    _____
                       V *
OsE60    A S V R P S T I R P L V G A L S F H Q F F E G V G L G G C I V Q A N F K V R A T   271
AtZIP1   A S Q S I D T I K P L M A A L S F H Q F F E G L G L G G C I S L A D M K S K S T   259

                    VI
OsE60    V I M A I F F S L T A P V G I V L G I A I S S . S Y N V H S S T A F V V E G V F   310
AtZIP1   V L M A T F F S V T A P L G I G I G L G M S S L G Y R K E S K E A I M V E G M L   299

              VII                                    VIII
OsE60    N S A S A G I L I Y M S L V D L L A T D F N N P K L Q I N T K L Q L M A Y L A L   350
AtZIP1   N A A S A G I L I Y M S L V D L L A T D F M N P R L Q S N L W L H L A A Y L S L   339


OsE60    F L G A G L M S M L A I W A Z 365
AtZIP1   V L G A G S M S L L A I W A . 353
```

C.

```
AtIRT1  M K T I F L V L I F V S F A I S P A T S T A P E E C G S E S A N P C V N K A K A   40
OsS16   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   0

AtIRT1  L P L K V I A I F V I L I A S M I G V G A P L F S R N V S F L Q P D G N I F T I   80
OsS16   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   0

AtIRT1  I K C F A S G I I L G T G F M H V L P D S F E M L S S I C L E E N P W H K F P F   120
OsS16   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   0

AtIRT1  S G F L A M L S G L I T L A I D S M A T S L Y T S K N A V G I M P H G H G H G H   160
OsS16   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .   0

                                                                      *
AtIRT1  G P A N D V T L P I K E D D S S N A Q L L R Y R V I A M V L E L G I I V H S V V   200
OsS16   . . . . . . . . . . . . . . . . . . . . . . . . . . P R V R E M G I V V H S V V   14

AtIRT1  I G L S L G A T S D T C T I K G L I A A L C F H Q M F E G M G L G G C I L Q A E   240
OsS16   I G L G M G A S Q N V C T I R P L V A A L C F H Q M F E G M G L G G C I L Q A G   54

AtIRT1  Y T N M K K F V M A F F F A V T T P F G I A L G I A L S T V Y Q D N S P K A L I   280
OsS16   Y G G R T R S A L V F F F S T T T P F G I A L G L A L T R V Y S D S S P T A L V   94

AtIRT1  T V G L L N A C S A G L L I Y M A L V D L L A A E F M G P K L Q G S I K M Q F K   320
OsS16   V V G L L N A A S A G L L H Y M A L V E L L A A D F M G P K L Q G N V R L Q L A   134

AtIRT1  C L I A A L L G C G G M S I I A K W A 339
OsS16   A S L A I L L G A G G M S V M A K W A 153
```
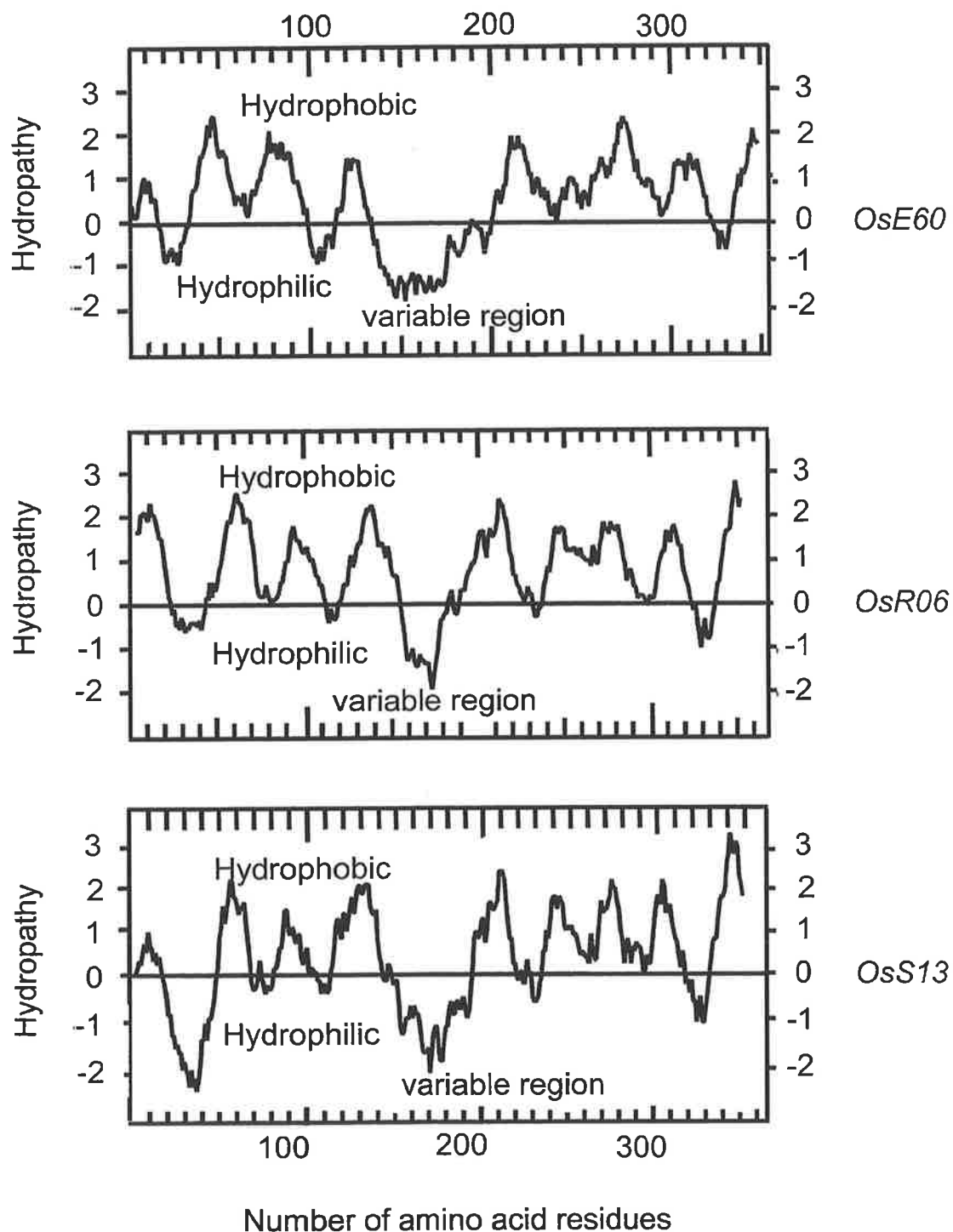
Fig. 1D   A Kyte-Dolittle hydropathy plot of the Os cDNAs (window size = 19). Eight hydrophobic regions representing the putative transmembrane domains are clearly recognized. A large hydrophilic variable region predicted to be cytosolic is present between the transmembrane domains in all the Os cDNAs.
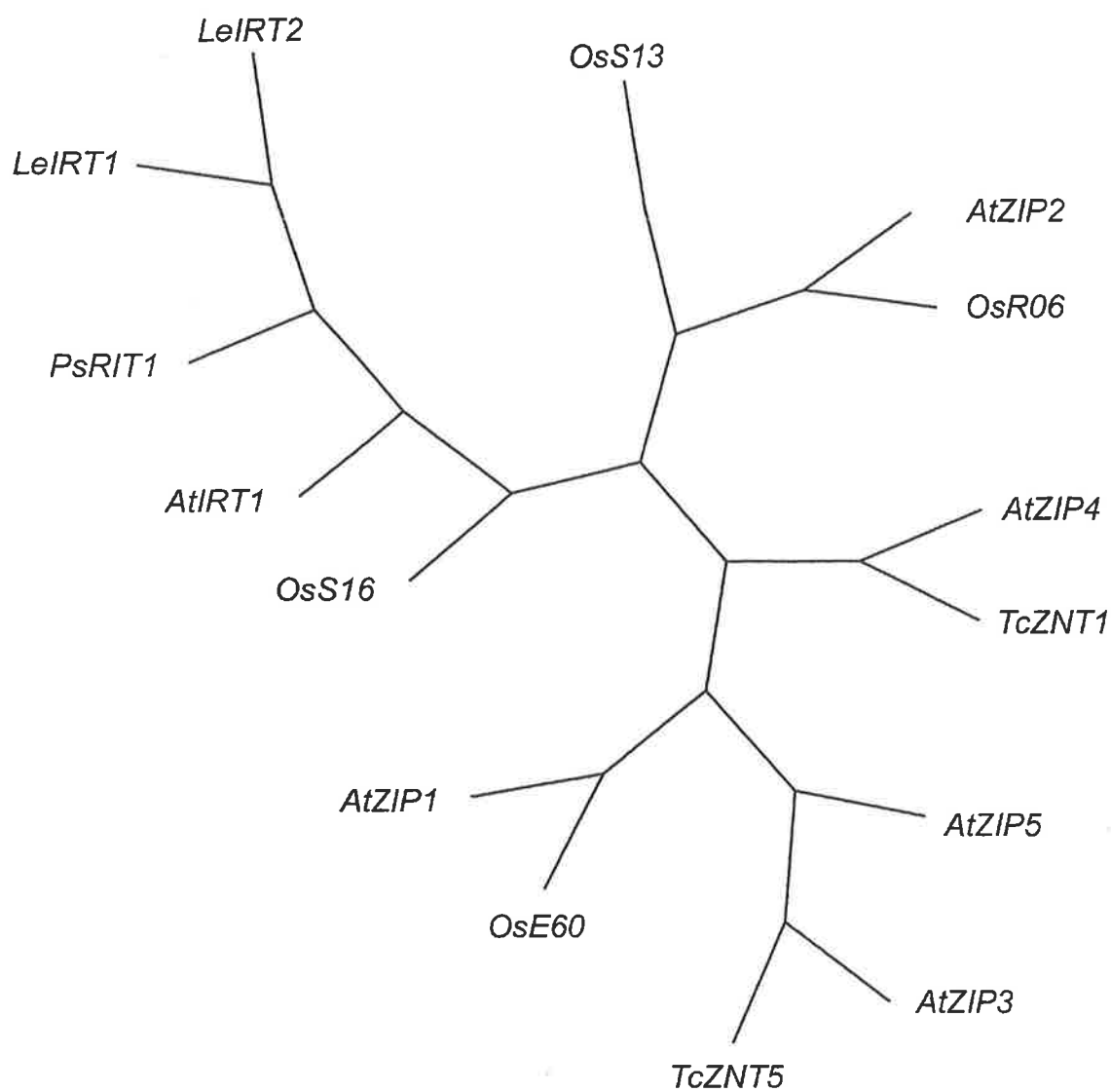
Fig. 1E Dendrogram showing the relationships at the amino acid levels among the ZIP family members from plants. Multiple sequence alignments were done using PILEUP from GCG and the figure was constructed using the Tree View program (version 1.6.5). *At = Arabidopsis thaliana*; *Os = Oryza sativa*; *Tc = Thlaspi caerulescens*; *Ps = Pisum sativum*; *Le = Lycopersicon esculentum*.

2.3.5.2   Zinc

Growth of the ZHY3 cells expressing the *OsR06*, *OsS13*, *OsE60*, *AtZIP1* and *AtZIP3* cDNAs was observed on LZM plates with 1 mM zinc at all the dilutions tested except with the *OsS13* cDNA at the dilution 10^{-3} (Fig. 3B). The cDNAs *OsR06*, *OsS13* and *OsE60* were observed to grow at 10 mM zinc in all the dilutions tested (Fig. 3B). In comparison, the dilutions 10^{-2} and 10^{-3} of the ZHY3 strains expressing *AtZIP1* and *AtZIP3* did not grow (Fig. 3B) on plates with 10 mM zinc, suggesting that zinc at 10 mM was toxic to the *AtZIP* cDNAs. These results also suggest that the *AtZIP* cDNAs may be transporting zinc at a higher rate into the cells as compared to the Os cDNAs.

2.3.5.3   Iron

The growth of ZHY3 cells expressing the *AtZIP1*, *AtZIP3* cDNAs and the Os cDNAs was unaffected (Fig.3C) by the different concentrations of iron (0.01 mM - 0.03 mM) in the medium. Iron at a concentration of 0.05 mM was toxic to all the yeast strains tested except pYES2, which grew at dilution 1.

2.3.5.4   Cobalt

The growth of the ZHY3 cells expressing the Os cDNAs was unaffected (Fig.3D) by cobalt in the medium suggesting that the *OsS13*, *OsR06* and *OsE60* cDNAs do not transport this ion into the cells. However an increase in cobalt concentration in the medium to 1 mM was toxic to the *AtZIP1* and *AtZIP3* cDNAs suggesting that the proteins encoded by these cDNAs transported cobalt into the cells.

2.3.5.5   Cadmium

0.01 mM cadmium chloride had no effect on the growth of ZHY3 cells expressing the *AtZIP* and Os cDNAs at all the dilutions tested (Fig. 3E). Increase in cadmium concentrations to 0.05 mM and 0.1 mM affected the growth of the yeast strains

A.

B.
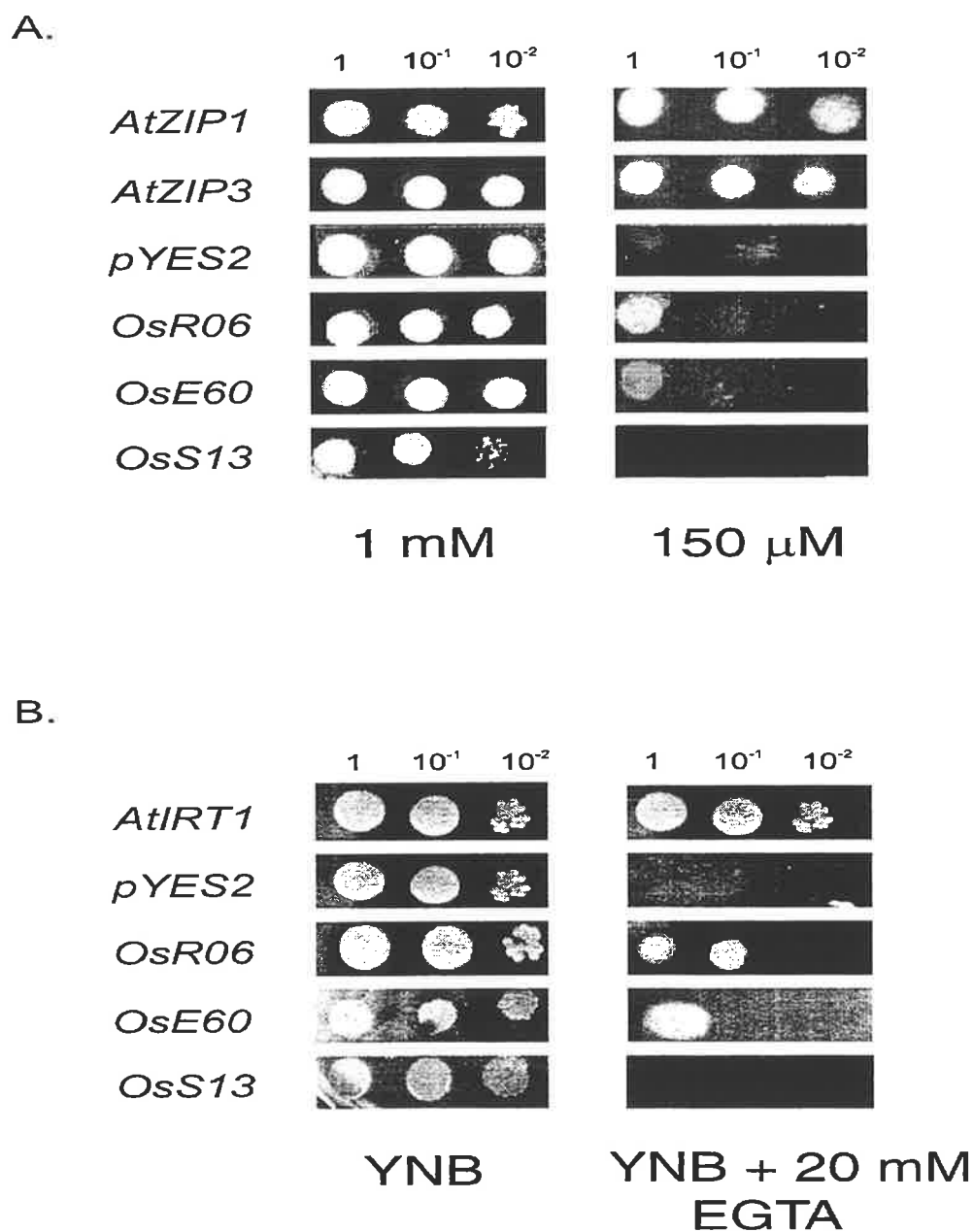
Fig. 2 Complementation of the ZHY3 and SLY8 mutants by rice cDNAs. (A) Growth of yeast mutant ZHY3 transformed with empty plasmid pYES2, Os cDNAs and AtZIP cDNAs in pYES2, on LZM medium supplemented with 1 mM or 150 μM zinc. (B) Growth of yeast mutant SLY8 transformed with empty plasmid pYES2, Os cDNAs and AtIRT1 in pYES2, on YNB medium or YNB medium supplemented with 20 mM EGTA. The optical density of all the cultures was adjusted to 0.1 and 5 μl aliquots of 10X dilutions (10^{-1}, 10^{-2}, 10^{-3}) were spotted on to the plates. The plates were incubated at 30°C for 3-5 days before being photographed.

Table 8   Rice cDNAs were tested for complementation of the yeast mutant ZHY3 (Zhao and Eide, 1996b) **deficient in zinc uptake, DEY145** (Dix *et al.*, 1994) **deficient in iron uptake and SLY8** (Supek *et al.*, 1996) **deficient in manganese uptake. The *AtZIP1, AtZIP3, AtIRT1* from *A. thaliana* and the empty plasmid pYES2 were used as controls in the experiment.**

cDNAs	ZHY3 LZM + 1mM Zinc	ZHY3 LZM + 150 μM zinc	DEY145 YNB	DEY145 YNB+BPS + 10 μM Fe	SLY8 YNB	SLY8 YNB + 20 mM EGTA
AtZIP1	+++	+++	nt	nt	nt	nt
AtZIP3	+++	+++	nt	nt	nt	nt
AtIRT1	nt	nt	nt	nt	+++	+++
pYES2	+++	-	+++	+	+++	-
OsS13	+++	-	nt	nt	+++	-
OsR06	+++	+	+++	-	+++	+
OsE60	+++	+	+++	-	+++	+

Note :   +++ = Strong growth ; +   = Weak growth; - = No growth; nt = Not tested; ; BPS = Bathophenanthroline disulphonate; EGTA = Ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'- Tetra acetic acid; LZM =   Low Zinc Medium (Eide and Guarente, 1992). LZM is prepared in the same way as LIM (Low Iron Medium) except that $ZnSO_4$ in the LIM is replaced by 10 μM $FeCl_3$ in LZM. The cDNAs from *O. sativa* (*OsR06, OsE60* and *OsS13*) were cloned in to the yeast expression vector pYES2 while the *AtZIP1, AtZIP3* and *AtIRT1* from *A. thaliana* are cloned in to the vector pFL61.

expressing the *AtZIP* and *OsR06* cDNAs (Fig. 3E). These results suggest that the *AtZIP* and *OsR06* cDNAs transport cadmium into the cells and that cadmium is toxic to these cells. The growth of ZHY3 cells containing the empty plasmid pYES2 and expressing the *OsS13* and *OsE60* cDNAs was relatively unaffected by the increase in cadmium concentrations (Fig. 3E). These results suggest that proteins encoded by the putative transporters *OsE60* and *OsS13* do not transport cadmium into the cells. Higher levels of cadmium (0.2 mM) inhibited the growth of all the yeast strains (results not shown).

2.3.5.6   Calcium

Calcium concentrations of 1-6 mM did not affect the growth of ZHY3 cells expressing the *AtZIP1*, *AtZIP3*, *OsS13* and *OsE60* cDNAs (Fig. 3F). However calcium had some effect on the growth of the ZHY3 cells expressing the *OsR06* cDNA (Fig. 3F). The proteins encoded by the putative transporters *OsE60* and *OsS13* and the *AtZIP* cDNAs did not enhance toxicity of this ion.

2.3.5.7   Copper

The growth of ZHY3 cells expressing the various cDNAs was unaffected by copper concentrations of 0.01-0.05 mM at all the dilutions tested (Fig. 3G) but 0.5 mM copper was toxic to the cells expressing *AtZIP*, *OsS13* and *OsE60* cDNAs (Fig. 3G). These results suggest that *OsS13*, *OsE60*, *AtZIP1* and *AtZIP3* cDNAs transport copper into the cells. However this concentration of copper had no affect on the growth of the ZHY3 cells expressing the *OsR06* cDNA. This suggests that the protein encoded by *OsR06* cDNA does not transport copper into the cells.

2.3.5.8   Magnesium

Different concentrations of magnesium (5-9 mM) had no effect on the growth of the ZHY3 cells expressing the *OsR06*, *OsS13*, *OsE60*, *AtZIP1* and *AtZIP3* cDNAs at all the dilutions tested (data not shown). Results suggest that the proteins

encoded by the *OsS13*, *OsR06* and *OsE60* cDNAs may not transport magnesium into the cells.

2.3.5.9 Sodium

Different concentrations of sodium (25 - 200 mM) had no effect on the growth of ZHY3 cells containing the empty plasmid pYES2 and expressing the *OsR06* and *OsE60* cDNAs (Fig. 3H) indicating that proteins encoded by the putative transporters *OsR06* and *OsE60* do not transport sodium into the cells. However sodium at concentrations of 50 and 200 mM were toxic to the growth of the ZHY3 cells expressing the *AtZIP* cDNAs and *OsS13* cDNA (Fig. 3H). These results suggest that the protein encoded by the putative transporter *OsS13* cDNA and the *AtZIP* transporters may be transporting sodium into the cells.

2.3.6 Growth experiments in liquid medium

2.3.6.1 Growth in zinc chloride

No significant differences were observed in the growth of ZHY3 cells containing the empty plasmid pYES2 and expressing the *OsS13*, *OsR06* and *OsE60* cDNAs in YNB medium supplemented with zinc concentrations of 0.15, 0.2, 0.4 and 0.6 mM (data not shown). However at a zinc concentration of 1 mM in the medium, ZHY3 cells expressing the *OsR06* and *OsE60* cDNAs grew significantly faster in the log phase (between 10-50 hours) of growth (Fig. 4A) as compared to the ZHY3 cells expressing the *OsS13* cDNA or the empty plasmid pYES2. These results suggest that the proteins encoded by the putative transporters *OsR06* and *OsE60* facilitate zinc transport as compared to the residual zinc transporters in the ZHY3 cells.

2.3.6.2 Growth in cadmium chloride

Cadmium chloride at concentrations of 0.01 - 0.1 mM was added to the medium when ZHY3 expressing the different cDNAs were in the mid-log phase of growth (OD_{600nm}= 0.8). 0.01 mM cadmium had no affect on the growth of ZHY3 cells

containing the empty plasmid pYES2, the *OsS13* and *OsE60* cDNAs (Fig. 4B). However cadmium at this concentration was extremely toxic to the ZHY3 cells expressing *OsR06* cDNA (Fig. 4B). As the concentration of cadmium in the medium was increased from 0.01 to 0.05 and 0.1 mM, cadmium did have an effect on the growth of ZHY3 strain containing pYES2, *OsE60* or *OsS13* cDNAs (Figs. 4C and D). However, the effect of increased cadmium on the growth of the ZHY3 cells expressing the *OsR06* cDNA (Figs 4C and D) was more profound. Increased cadmium concentrations inhibited the growth of ZHY3 cells expressing the *OsR06* cDNA suggesting that the protein encoded by this putative transporter is transporting cadmium into the cells. Results of this experiment support the findings from the plate experiments (section 2.3.5.5, Fig. 3E). Cadmium at a concentration of 0.2 mM was toxic to the growth of all yeast strains (data not shown).

2.3.6.3 Growth in calcium chloride

The plate experiments with calcium suggested that this ion may have some effect on the growth of ZHY3 cells expressing the *OsR06* cDNA, so growth experiments in liquid culture were done to confirm these. Growth of the ZHY3 cells containing the empty plasmid pYES2 and those expressing the *OsR06* cDNA in YNB medium with 0.7 mM $CaCl_2$ (control) was almost identical (Fig. 4E). However, the growth of the ZHY3 cells expressing the *OsR06* cDNA was highly sensitive to the increased concentrations of calcium (1-3 mM) in the medium as compared to the ZHY3 cells containing the empty plasmid pYES2. At a calcium concentration of 1.7 mM (Fig. 4E), the growth of yeast cells expressing *OsR06* cDNA was strongly inhibited, suggesting that the protein encoded by the transporter was transporting calcium into the cells. These results support observations based on the plate toxicity experiments (section 2.3.5.6, Fig. 3F).

2.3.6.4 Growth in magnesium chloride

Growth of the ZHY3 cells containing the empty plasmid pYES2 and the *OsR06* cDNA in medium with 2.0 mM $MgCl_2$ (control) was almost identical (Fig. 4F). However with an increase in concentration of magnesium to 9 mM the growth of

Fig. 3   ZHY3 cells expressing the cDNAs (*OsR06, OsE60, OsS13*) from *O. sativa* and *A. thaliana* (*AtZIP1* and *AtZIP3*) were assayed for toxicity in LZM medium supplemented with indicated concentrations of metal ions. The optical density of the samples was adjusted to 0.1 (1) and 5 µl aliquots of 10X dilutions (10^{-1}, 10^{-2}, 10^{-3}) of each sample were spotted on to the plates. The plates were incubated at 30°C for 3 days before being photographed.

A.   Manganese

B.   Zinc

C.   Iron

D.   Cobalt

E.   Cadmium

F.   Calcium

G.   Copper

H.   Sodium chloride

A.

$$0.025 \text{ mM Mn}^{2+} \qquad 0.1 \text{ mM Mn}^{2+} \qquad 0.2 \text{ mM Mn}^{2+}$$

B.

$$0.15 \text{ mM Zn}^{2+} \qquad 1 \text{ mM Zn}^{2+} \qquad 10 \text{ mM Zn}^{2+}$$

C.

AtZIP1
AtZIP3
pYES2
OsR06
OsE60
OsS13

1   10^{-1} 10^{-2} 1 10^{-1} 10^{-2} 1 10^{-1} 10^{-2}

0.01mMFe^{2+} 0.03 mMFe^{2+} 0.05mMFe^{2+}

D.

AtZIP1
AtZIP3
pYES2
OsR06
OsE60
OsS13

1   10^{-1} 10^{-2} 10^{-3} 1 10^{-1} 10^{-2} 10^{-3} 1 10^{-1} 10^{-2} 10^{-3}

0.01 mMCo^{2+} 0.1 mMCo^{2+} 1 mMCo^{2+}

E.

AtZIP1
AtZIP3
pYES2
OsR06
OsE60
OsS13

0.01 mM Cd $^{2+}$ 0.05 mM Cd $^{2+}$ 0.1 mM Cd $^{2+}$

F.

AtZIP1
AtZIP3
pYES2
OsR06
OsE60
OsS13

1 mM Ca $^{2+}$ 3 mM Ca $^{2+}$ 6 mM Ca $^{2+}$

G.

0.01 mM Cu^{2+} 0.05 mM Cu^{2+} 0.5 mM Cu^{2+}

H.

25 mM Na$^+$ 50 mM Na$^+$ 200 mM Na$^+$

the ZHY3 cells expressing *OsR06* cDNA was inhibited (Fig. 4F). This result suggests that the protein encoded by the putative transporter *OsR06* transports magnesium into the cells. This is in contrast to the results observed in the toxicity experiments (section 2.3.5.8).

2.3.7   Ion content analysis/ICP analysis

2.3.7.1   Cadmium

ZHY3 cells expressing the *OsR06* cDNA accumulated 3.5 times more cadmium in the cells as compared to the ZHY3 cells containing the empty plasmid (Tables 9 and 10, Appendix 1.7 B and C). These results show that the protein encoded by the putative transporter *OsR06* transports cadmium into the cells. These results also support the results obtained in the earlier toxicity and growth experiments. ZHY3 cells expressing the *OsE60* cDNA accumulated similar amounts of cadmium as the cells containing the empty plasmid pYES2 (Tables 9 and 10, Appendix 1.7 B and C).

2.3.7.2   Calcium

ZHY3 cells expressing the *OsR06* cDNA accumulated three times more calcium than the ZHY3 cells containing the empty plasmid pYES2 or expressing the *OsE60* cDNA (Table 10, Appendix 1.7 C). These results support the observations made in the toxicity and growth experiments.

2.3.7.3   Magnesium

ZHY3 cells expressing the *OsR06* cDNA accumulated 1.7 times more magnesium than ZHY3 cells containing the empty plasmid (Tables 9 and 10, Appendix 1.7 B and C) in the cells.   These results support the results obtained in the liquid growth experiments with magnesium (section 2.3.6.4, Fig. 4F). The ZHY3 cells expressing the *OsE60* cDNA contained 1.5 times more magnesium than the ZHY3 cells containing the empty plasmid pYES2 (Tables 9 and 10, Appendix 1.7 B and C).
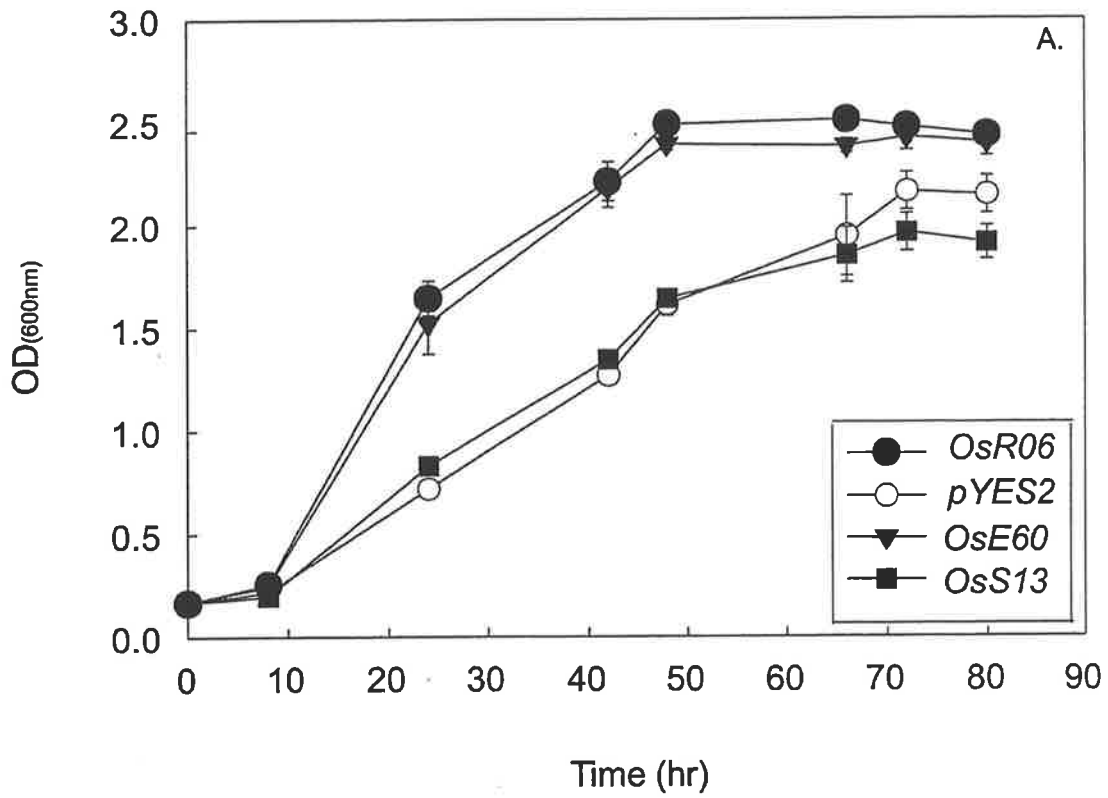
Fig. 4 A   Growth of ZHY3 transformed with the Os cDNAs and the empty plasmid pYES2 in YNB supplemented with 1 mM zinc chloride. Data represents the mean and standard error of three replicates. Error bars smaller than symbols are not visible.
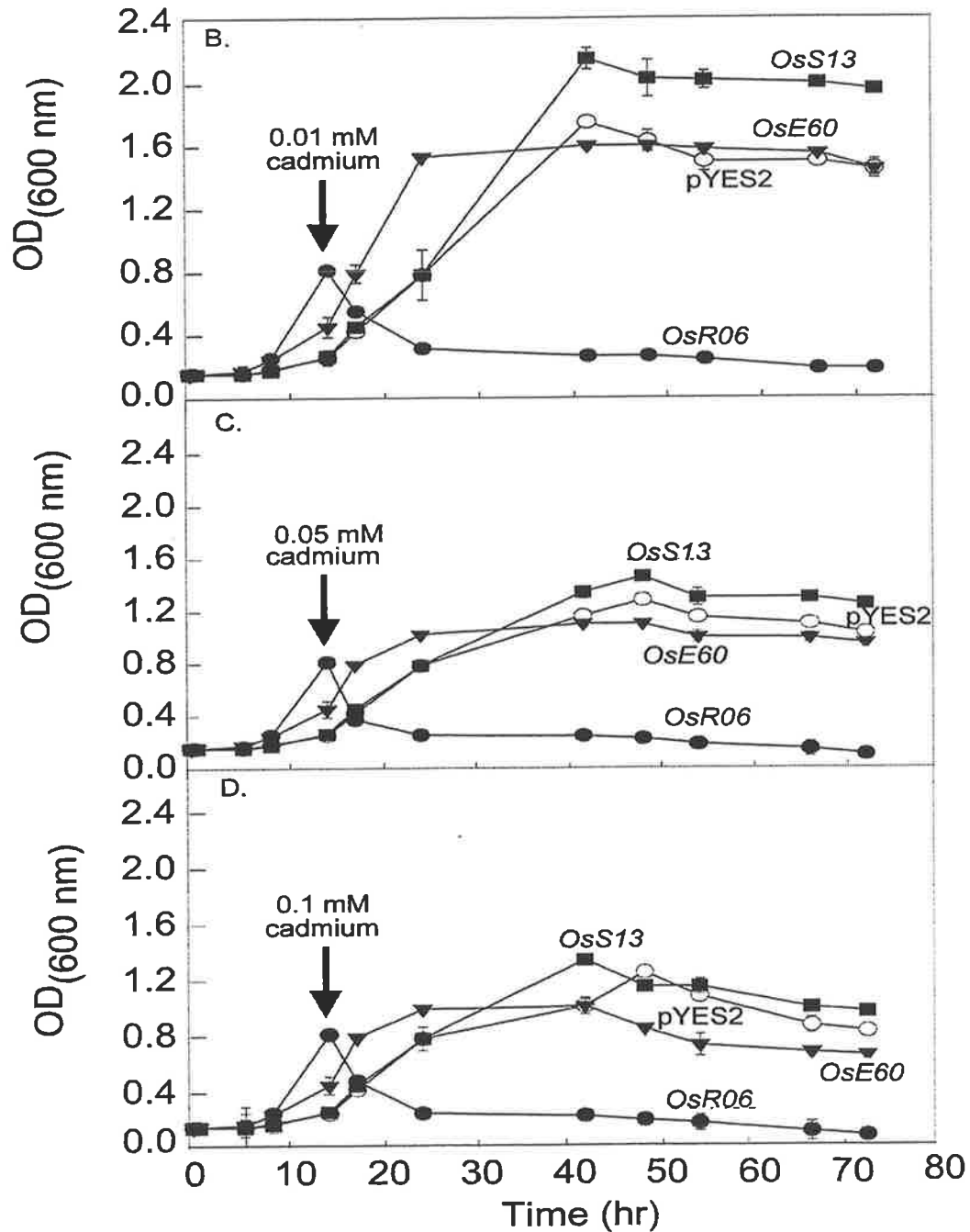
Fig. 4 **Effect of cadmium chloride addition on the growth of ZHY3 cells expressing the *OsR06*, *OsE60* and *OsS13* cDNAs from *O. sativa* and the empty plasmid pYES2 in YNB medium.** Cadmium chloride was added to the cells at the mid log phase of growth ($OD_{600\ nm}$ = 0.8) at the indicated concentrations (B, C and D). Data represents the mean and standard error of three replicates. Error bars smaller than symbols are not visible.
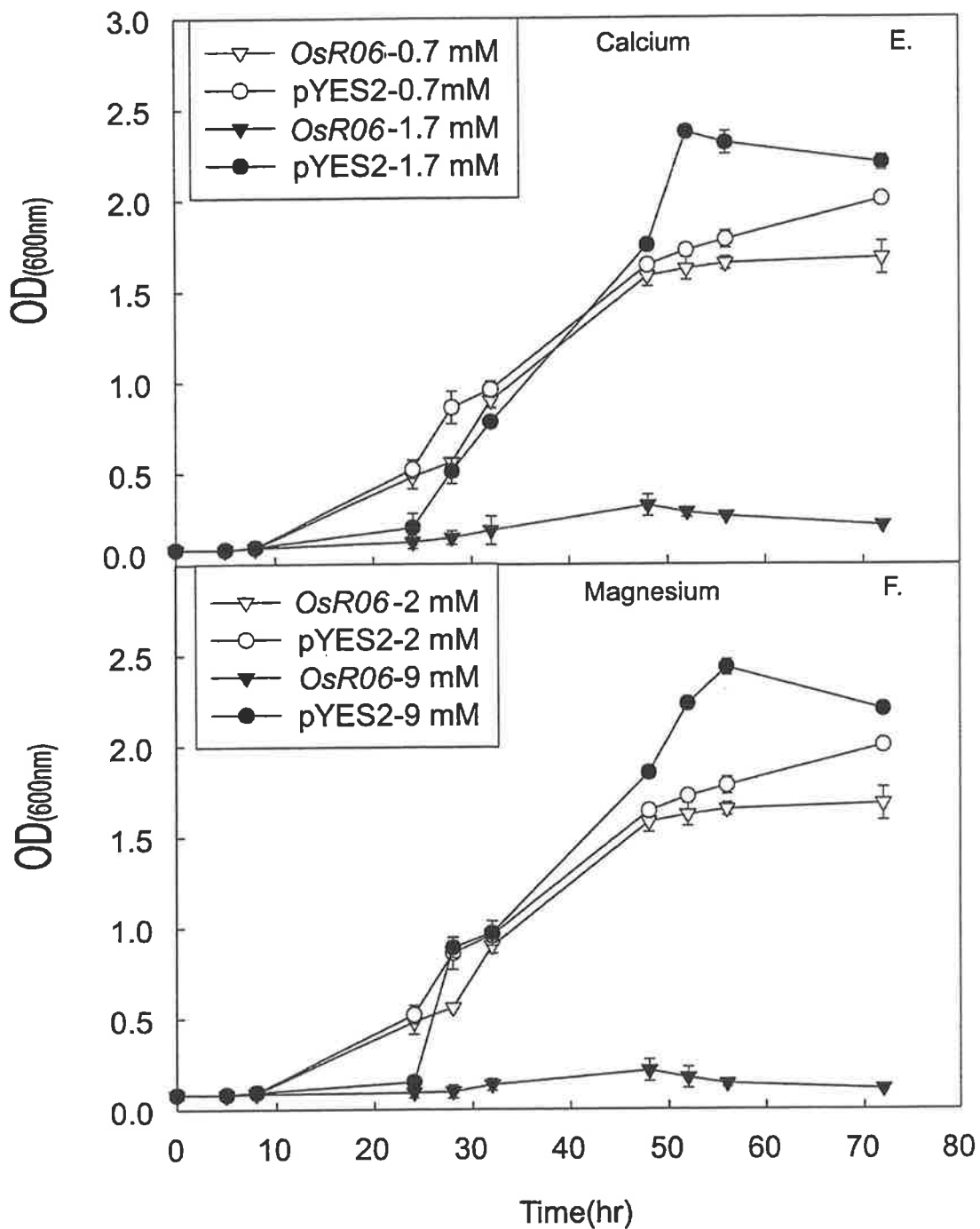
Fig. 4 **Effect of calcium chloride (E) and magnesium chloride (F) on the growth of ZHY3 cells containing the empty plasmid pYES2 and expressing *OsR06* cDNA in YNB medium supplemented with the indicated concentrations of metals.** Data represents the mean and standard error of three replicates. Error bars smaller than symbols are not visible.

The cells expressing the *OsS13* cDNA accumulated similar amounts of cadmium, calcium and magnesium when compared to the cells containing the empty plasmid pYES2 (data not shown). In summary, ion content analysis (ICP-AES) results of the cDNAs *OsR06, OsE60* and *OsS13* support the findings from the toxicity and growth experiments.

2.4 *Discussion*

Three full length cDNAs encoding putative zinc transporters *OsR06, OsE60* and *OsS13* and one partial length cDNA *OsS16* more closely related to previously characterized iron regulated transporters were identified from *O. sativa* in this study.

2.4.1 Sequence Analyses

Sequencing analysis results (section 2.3.2) indicate that the full-length cDNAs *OsR06, OsE60* and *OsS13* identified from *O. sativa* encode proteins containing 352, 365 and 362 amino acids, respectively. Most of the ZIP family members identified to date, encode proteins ranging from 309-476 amino acids in length (Guerinot, 2000a). All four putative transporters sequences identified from *O. sativa* show a high degree of identity (Table 7, section 2.3.2) to the ZIP family of transporters from *A. thaliana* (Grotz *et al.*, 1998) which include both zinc and iron transporters. In eukaryotes, the ZIP family of transporters has been found to play a major role in zinc uptake and transport of zinc into the cytoplasm. The ZIP transporters are also involved in mobilizing stored zinc from within an intracellular compartment into the cytoplasm (MacDiarmid *et al.*, 2000). The putative transporters *OsR06, OsS13* and *OsE60* have eight predicted transmembrane domains (Fig. 1 D, section 2.3.2) similar to the ZIP proteins (Guerinot, 2000b). The hydropathy plots of the *OsR06, OsS13* and *OsE60* show a large hydrophilic region between the transmembranes III and IV (Fig. 1 D, section 2.3.2), which is a variable region in most ZIP proteins (Guerinot, 2000b). Differences in the length of the complete predicted protein sequences is due to the

length of this variable region whose sequence is the least conserved among the family members.

2.4.2 ZIP signature sequence

Based on multiple sequence alignments of fifteen ZIP proteins, a signature sequence for the ZIP family was derived (Eng *et al.*, 1998). This sequence is: [LIVFA] [GAS] [LIVMD] [LIVSCG] [LIVFAS] H [SAN] [LIVFA] [LIVF MAT] [LIVDE] G [LIVF] [SAN] [LIVF] [GS].

According to this model the most conserved regions of the ZIP family of proteins occur in and adjacent to the transmembrane IV. In the fourth transmembrane domain of the deduced rice proteins OsR06, OsE60 and OsS13, a highly conserved region containing the ZIP sequence signature is present (Eng *et al.*, 1998). This sequence is suggested to have some functional or structural significance among the ZIP family members. Sequence analysis indicates the presence of a conserved histidine residue and polar residue serine in the potential transmembrane region IV of the putative transporters identified from *O. sativa* (Figs. 1A and B). This type of conservation in the transmembrane IV region is seen in most of the ZIP family members and it is predicted that the conserved residues lie on the polar face of amphipathic α-helices, suggesting a possible role in metal ion transport (Gaither and Eide, 2000; Guerinot, 2000b). Mutation of the conserved histidines or adjacent polar/charged residues in transmembrane IV and V of AtIRT1 eliminated its transport function (Rogers *et al.*, 2000) confirming a role for the residues in metal ion transport. It has also been suggested that the histidine and serine residues may be a component of an intramembranous heavy metal binding site that is part of the transport pathway (Stearman *et al.*, 1996). It is interesting to also note the presence of a conserved histidine residue with an adjacent polar residue lysine at the beginning of transmembrane V in the multiple sequence alignments of OsR06 and OsS13 predicted proteins (Fig. 1A). These residues are also conserved in many ZIP proteins (Eng *et al.*, 1998). Thus it has been proposed that both transmembrane domains IV and V in part comprise the transmembrane aqueous channel through which the substrate metal ion passes (Eng *et al.*, 1998). The putative transporters OsR06, OsE60 and OsS13 identified from *O. sativa* also show conserved histidine residues in the potential

transmembrane regions II and V. A conserved histidine residue is also present in both *OsR06* and *OsS13* in the potential transmembrane region VII (Fig. 1A). The partial fragment *OsS16* from *O. sativa* shows significant homology with the *AtIRT1* from *A. thaliana* (Eide and Guarente, 1992; Korshunova *et al.*, 1999). Detailed sequence analysis was not undertaken as the cDNA was partial length, however it does appear to contain the conserved histidine and serine residues in the putative transmembrane region IV (Fig. 1C).

2.4.3   Metal binding sequence

In the variable region between transmembranes III and IV in the OsE60 predicted protein (Fig. 1 B), a sequence **HTHSHG** is present. Similar sequence HXHXHX or $(HX)_n$ predicted to be potential metal binding domains have been found in many of the ZIP family members such as *AtIRT2* (Vert *et al.*, 2001), *AtIRT1* (Eide et al., 1996; Korshunova *et al.*, 1999), *AtZIP1* and *AtZIP4* (Grotz *et al.*, 1998), *ZRT1* and *ZRT2* (Zhao and Eide, 1996a; Zhao and Eide, 1996b), *TcZNT1* (Pence *et al.*, 2000) and *hZIP2* (Gaither and Eide, 2000). Potential metal binding domains have also been found in the efflux proteins belonging to the cation diffusion facilitator (CDF) family (Paulsen and Saier, 1997) in the variable region between transmembranes III and IV. In a number of *Caenorhabditis elegans* ZIP proteins (Guerinot, 2000a) potential metal binding domains are present in regions other than the variable region. The presence of a metal binding domain and its conservation in many ZIP proteins suggests a role in metal ion transport or its regulation (Guerinot, 2000b). The predicted protein sequences of *OsR06* and *OsS13* do not possess a motif or potential metal binding domain in the variable region. Both these putative transporters have a single histidine residue (Fig. 1A) in the variable region. These results are similar to that observed for *AtZIP2* and *AtZIP3* proteins from *A. thaliana* (Grotz *et al.*, 1998). This histidine residue in the variable region is proposed to be involved in the formation of an intramembranous heavy metal binding site involved in the transport pathway (Eng *et al.*, 1998; MacDiarmid *et al.*, 2000; Rogers *et al.*, 2000).

2.4.4   Phylogenetic analysis

To examine the phylogenetic relationship of the putative rice transporters, a dendrogram (section 2.3.2) of the known plant zinc and iron transporters was constructed (Fig. 1E).   The putative transporters identified from rice and *A. thaliana* involved in the transport of Zn^{2+} are closely related to the (IRT) transporters involved in Fe^{2+} transport in plants. The four distinct clades (section 2.3.2) observed in the dendrogram of rice proteins and other ZIP family members belonging to plants, have also been reported in the phylogenetic analysis of *LeIRT* transporters in tomato (Eckhardt *et al.*, 2001).

2.4.5   Yeast complementation

Yeast complementation has been successful in characterizing many of the plant ZIP genes (Grotz *et al.*, 1998). When introduced into zinc uptake deficient yeast mutant zrt1zrt2, *OsR06* and *OsE60* cDNAs from rice could partially restore growth on zinc limited media (section 2.3.4.1) in contrast to *AtZIP1*, *AtZIP2*, *AtZIP3* from *A. thaliana* (Grotz *et al.*, 1998) and *TcZNT1* from *T. caerulescens* (Pence *et al.*, 2000) that complemented the growth defect of the yeast mutant very well. This result suggested that *OsR06* and *OsE60* might encode zinc transporters. Both the cDNAs also partially complemented the mutation in *Smf1* yeast mutant (section 2.3.4.2) suggesting a role in manganese transport. These results are not surprising considering the broad substrate specificity of the iron regulated and zinc regulated transporters involved in the transport of divalent cations, such as *AtIRT1* (Korshunova *et al.*, 1999), *AtZIP1*, *AtZIP2* and *AtZIP3* from *A. thaliana* (Grotz *et al.*, 1998). The cDNAs *OsR06* and *OsE60* failed to complement the *fet3fet4* yeast mutant suggesting that these cDNAs are not involved in iron transport. The *OsS13* cDNA from rice failed to complement the *zrt1zrt2*, *Smf1* or *fet3fet4* mutants which could be due to mis targeting of the protein to the plasma membrane or due to the low expression levels we measured when expressed in yeast (Chapter 4).

2.4.6 Specificity of metal ion transporters

Based on the results of complementation experiments with different yeast mutants, experiments were carried out to determine if *OsR06* and *OsE60* transporters were involved in the uptake of metal ions other than zinc. Results of the toxicity, growth and accumulation experiments (ICP-AES) suggest that *OsR06* is involved in the transport of cadmium, zinc, calcium and magnesium (section 2.3.5 to 2.3.7), which suggest that this protein is a transporter with broad substrate specificity. *AtZIP2*, which is most similar in terms of amino acid sequence to *OsR06*, is also reported to have a high affinity for cadmium along with zinc. Other plant ion transporters such as *AtIRT1* (Guerinot, 2000b; Korshunova *et al.*, 1999), *AtZIP1*, *AtZIP2*, *AtZIP3* (Grotz *et al.*, 1998), *PsRIT1* (Cohen *et al.*, 1998), *LeIRT1* and *LeIRT2* (Eckhardt *et al.*, 2001), *TcZNT1* (Pence *et al.*, 2000) and *AtNRAMP3* (Thomine *et al.*, 2000) are known to transport cadmium in addition to zinc. No biological function is known for highly toxic metals such as cadmium except in marine diatoms (Lane and Morel, 2000). Thus it seems that cadmium enters the cells through transporters with broad specificity for cations and is then toxic to cells and whole organisms. Similar studies with *OsE60* suggested that this putative transporter is involved in the transport of zinc and magnesium ions but is not permeable to cadmium and calcium (sections 2.3.5 to 2.3.7).

2.4.7 Potential applications

This study has identified putative transporters from rice implicated in the uptake of zinc and other divalent cations. The molecular characterization of these transporters provides an insight into the structure of the predicted proteins, which eventually lead to understanding of their functions. This knowledge can be used to identify and characterize similar transporters from other cereals such as wheat and barley.

Recent advances in the area of plant ion transporters have increased our understanding of the mechanisms of metal ion homeostasis. One of the questions that arise from these studies is about the specificity of the metal ion transporters. Studies involving point mutation of residues that reduce, block or alter the transport of toxic ions have been carried out in the recent years (Ichida *et al.*,

1999; Nakamura *et al.*, 1997; Rubio *et al.*, 1995) in order to understand the question of cation selectivity and transport. Recently, a study was carried out with *AtIRT1* from *A. thaliana* to investigate the residues involved in metal recognition and transport (Rogers *et al.*, 2000). *AtIRT1* is an excellent model system for such a study because it is a cation transporter with broad specificity for metal ions, transporting zinc, manganese and cadmium in addition to iron and can be expressed in different yeast mutants such as *smf1* and *zrt1zrt2* and *fet3fet4* (Korshunova *et al.*, 1999). Rogers *et al.* (2000) showed that replacement of key aspartate residues (D 100 and 136) with alanine in AtIRT1 converted the transporter to a form capable of taking up only zinc. The D 136A mutant also lost its sensitivity to cadmium. The rice transporter *OsR06* has two conserved aspartate residues in the transmembrane region IV and has broad specificity for uptake and transport of cations including cadmium. These features suggest a relationship between the two proteins AtIRT1 and OsR06. Study of these transporters could give an insight into the residues conferring specificity to metal transport and how substitutions could alter the transporter activity. Also, the potential application would be to create a more selective transporter that might be helpful in reducing the uptake of toxic minerals such as cadmium.

In conclusion, sequence analysis, yeast complementation, growth and accumulation studies have revealed that the deduced rice proteins are members of the large family of ZRT/IRT-like proteins and belong to the subfamily I of eukaryotic ZIP proteins (Gaither and Eide, 2001; Guerinot, 2000b).

Since *A. thaliana* has 15 ZIP genes, it is likely that additional ZIP genes exist in the rice genome. Further work will involve careful analysis of the rice genome sequence that is now available.

Chapter 3   Uptake assays with ^{65}zinc isotope

3.1   Introduction

Uptake assays with radioactive tracers provide a short term method for measuring uptake and can be used to rapidly gather data on the functional parameters of ion transport. These studies provide information about the transport kinetics (Km and Vmax) such as concentration dependence of uptake rate from which can be derived transporter affinity (Km) and the maximum velocity (V_{max}) of transport. The kinetic values allow for the classification of transporters into different affinities for a particular substrate and provide an indication of how fast the transport process proceeds. It is also possible to indirectly study the ionic specificity of transporters through the addition of ions that might compete for uptake with the radioactive tracer.

Our understanding of zinc transport in plants has increased with the identification of both ZIP and CDF family members in many plant species. The transport kinetics of the plant zinc transporters (ZIP genes) identified from *A. thaliana* (Grotz *et al.*, 1998) and hyperaccumulator *T. caerulescens* (Pence *et al.*, 2000) have been studied by uptake assays with ^{65}zinc isotope. Three of the zinc transporters from *A. thaliana AtZIP1*, *AtZIP2* and *AtZIP3* showed a high rate of ^{65}zinc accumulation when expressed in the yeast strain ZHY3 (~270, ~19 and 270 fmol/min/10^6 cells respectively). The uptake activity of these transporters was pH dependent, concentration dependent and saturable. Inhibition of *AtZIP* dependent zinc uptake by other metal ions revealed unique sensitivities to metal ions and differences in substrate specificities of the transporters (Chapter 1, section 1.6.4.1). Radiotracer studies of whole plants *T. caerulescens* and *T. arvense* (non hyperaccumulator) suggested that the zinc uptake is controlled by regulating the number of active zinc transporters in the membrane (Lasat *et al.*, 1996). The V_{max} of the transporter from hyperaccumulating *T. caerulescens* was almost five fold higher than the non-hyperaccumulating *T. arvense*, but there was no difference in their K_m (Lasat *et al.*, 2000). Uptake studies in yeast mutant ZHY3 with zinc

transporter *TcZNT1* from *T. caerulescens* (Lasat *et al.*, 2000) suggests that this protein is involved in the uptake of both zinc and cadmium.

Radiotracers have also been used in studying the zinc efficiency of different cultivars in cereals such as wheat, rye and barley. In uptake studies with rye, bread wheat and durum wheat, ^{65}Zn uptake was higher in rye when compared to either bread or durum wheats and most likely contributed to higher zinc efficiency of rye (Erenoglu *et al.*, 1999). Root uptake has been reported to vary between rice cultivars. The high zinc requiring cultivars exhibited higher root uptake rates (Bowen, 1969). Similar results have been obtained in studies with bread and durum wheat cultivars wherein the bread wheat cultivars that are more efficient, show consistently higher root uptake rates (Hart *et al.*, 1998). Thus, the radioactive uptake studies provide a powerful tool for studying the zinc transport characteristics of proteins encoded by cloned genes and in whole plants that differ in zinc accumulation and efficiency. The aim of this chapter was to use uptake assays with ^{65}zinc isotope to obtain direct evidence for the ability of the cDNAs *OsR06, OsS13 and OsE60* to transport zinc when expressed in yeast and also to study the transport characteristics of these putative transporters from *O. sativa*.

The results obtained from uptake assays with ^{65}zinc isotope are presented in this chapter. These studies revealed that the putative transporters *OsR06* and *OsE60* transported zinc and in addition *OsR06* also transported cadmium and magnesium. The K_{ms} of both these transporters were in the micromolar range. Both the putative transporters showed pH optima. Very little zinc uptake was detected for the putative transporter *OsS13* when expressed in yeast.

3.1 *Methods*

3.2.1 Yeast Strains

Yeast strain ZHY3 expressing the three cDNAs *OsR06, OsE60 and OsS13* from *O. sativa* (prepared as described in chapter 2) were used in the uptake experiments with the ^{65}zinc isotope.

3.2.2 Yeast growth conditions

Yeast cells were grown in low zinc medium - LZM (Eide and Guarente, 1992) (Tables 3A and B, Appendix 1.4) All the yeast strains were inoculated into 100 ml of LZM supplemented with 1 mM zinc chloride at an optical density $OD_{600\ nm}$ = 0.05. Cells were grown to mid-log phase ($OD_{600\ nm}$= 0.6-0.8) before being harvested for uptake experiments.

3.2.3 Zinc uptake assays

Zinc uptake assays were performed as described by Eide *et al*. (1992) except that $^{65}ZnCl_2$ (NEN Life Science Products) and LZM - EDTA were substituted for $^{59}FeCl_3$ and LIM-EDTA. Exponentially growing cells were centrifuged at 3000 rpm for 5 min at 4°C. The pellets were washed twice in ice-cold assay buffer LZM-EDTA i.e. LZM prepared without EDTA and resuspended in 0.01 of the original volume culture in assay buffer. Cell suspensions were kept on ice before use. Uptake assay solutions were prepared by diluting $^{65}ZnCl_2$ to the specified concentrations in uptake assay buffer (LZM-EDTA). To measure uptake, 50 µl of cell suspension was added to 450 µl of uptake assay buffer containing the isotope. Cell suspensions were transferred to a heating block at 30°C and incubated for different lengths of time, depending on the experiment. Tubes containing the cells were vortexed every 2-3 minutes. After specific intervals of time, the samples were vortexed and vacuum filtered through nylon membranes. These membranes were washed with 10 ml of ice-cold SSW (Table 11, Appendix 1.12) Cell associated ^{65}Zn was measured with a Beckman Scintillation Counter (Beckman, LS 380). Background was determined by addition of 50 µl of cell suspensions to 450 µl of LZM - EDTA, followed by filtration and washing. Uptake rates were expressed as pmol Zn $\cdot 10^6 cells^{-1} \cdot min^{-1}$.

3.2.4 Zinc uptake rates

Zinc uptake rates of the yeast cells expressing the rice cDNAs were studied using the protocol outlined in section 3.2.3 with respect to change in pH, addition of

competing cations and zinc concentration dependence. Cell associated ^{65}Zn uptake levels were measured after 25 min incubation according to the protocol in section 3.2.3 for all the experiments unless mentioned otherwise. All the experiments were repeated three times with fresh cultures on separate days. In the results, means represent the average of three separate experiments with nine replicates for each data point unless stated otherwise. The effect of pH (4.0, 4.7 and 6.0) on zinc uptake by the yeast strain ZHY3 expressing the Os cDNAs was studied by adjusting the pH of the uptake assay buffer by the addition of either 0.1 N HCl or 0.1 N NaOH to the medium. The concentration of the zinc isotope used in these experiments was 6.8 µM. Zinc uptake rates were expressed in pmol/10^6 cells and cell number was calculated based on the optical density of the cells.

For substrate specificity studies the stock solutions of the competing metal ions were prepared by dissolving the chloride salts of cobalt, nickel, calcium, magnesium and cadmium in deionised water at a concentration of 100 mM. Metal ions were added to a final concentration of 68 µm to the tubes containing the uptake assay buffer with the cell suspensions. The concentration of radioactive zinc chloride was 6.8 µM. Zinc uptake rates were calculated in pmol·10^6 cells^{-1}·min^{-1} and expressed as percentage of control, control being the zinc uptake rate with no added competing metal ion.

For zinc concentration dependence studies, zinc uptake rates in the yeast cells expressing rice cDNAs were measured over a range of zinc concentrations (1 µM - 46.8 µM). The lower concentrations of zinc (1 µM – 6.8 µM) were obtained by addition of appropriate volumes of ^{65}zinc. To obtain zinc concentrations of 16.8 µM, 36.8 µM and 46.8 µM in the uptake assay buffer (LZM-EDTA), 6.8 µM of ^{65}ZnCl$_2$ and 10 µM, 30 µM or 40 µM of cold ZnCl$_2$ was used. The non-radioactive ZnCl$_2$ solution was prepared at a concentration of 10 mM in 0.02 N HCl. Points were fitted to the Michaelis-Menten equation using the GraphPad Prism software and kinetic values V_{max} and K_m were derived.

The effect of sodium chloride on ^{65}zinc uptake was studied in the ZHY3 cells expressing the *OsR06* and *OsE60* cDNAs. The uptake assay buffer (LZM-EDTA) was prepared without sodium and pH of the medium was adjusted to 4.7 for the yeast strain *OsR06* and 6.0 for the yeast strain *OsE60*. Cells were harvested and processed according to the protocol outlined in section 3.2.3. Sodium chloride was added to a final concentration of 0.1 mM, 1 mM or 10 mM

(with 6.8 μM zinc isotope) to the tubes containing the uptake assay buffer. Cell-associated ^{65}Zn was measured after 25 minutes. Zinc uptake rates were expressed in pmol/10^6 cells.

3.3 Results

3.3.1 Zinc uptake by ZHY3 cells expressing the Os cDNAs

The ability of the *OsR06* and *OsE60* cDNAs to partially complement the ZHY3 mutant suggests that the proteins encoded by the cDNAs may transport zinc (Chapter 2, section 2.3.4.1). To test this hypothesis, ^{65}Zn accumulation in ZHY3 cells expressing the cDNAs was studied at pH 4.7. The *OsR06* dependent zinc uptake had a lag phase of 10 min but the uptake was linear between 15 and 30 minutes at a zinc concentration of 6.8 μM. The ZHY3 cells expressing the *OsR06* transporter had a zinc uptake rate of 0.33 pmol Zn·10^6 cells^{-1}·min^{-1} at pH 4.7 (Fig. 5A) as compared to no uptake by the cells expressing *OsS13*, *OsE60* and pYES2 (Fig. 5A). Inset in the graph shows zinc uptake by the ZHY3 cells expressing *AtZIP1 (1.1* pmol Zn/10^6 cells/min), used as a control in the experiment and it is interesting to note the absence of a lag phase in the zinc uptake in this case.

As no zinc uptake was detected in the cells expressing *OsS13* and *OsE60* cDNAs at pH 4.7, the pH of the medium was increased to 6.0. At this pH, *OsE60* dependent zinc uptake was linear after a 15 min lag phase and the transporter had an uptake rate of 0.19 pmol Zn·10^6 cells^{-1}·min^{-1} (Fig. 5B). At this pH, OsR06 dependent zinc uptake was 0.14 pmol Zn·10^6 cells^{-1}·min^{-1} while the *OsS13* dependent zinc uptake was 0.06 pmol Zn·10^6 cells^{-1}·min^{-1} (Fig. 5B). No zinc uptake activity was detected in the ZHY3 cells containing the empty plasmid pYES2. The zinc transport activity associated with *OsS13* was significantly different from that exhibited by yeast strain containing pYES2 alone ($P<0.05$) at pH 6.0. These results demonstrated that the proteins encoded by the *OsR06*, *OsE60* transport zinc and that *OsS13* may exhibit some zinc transport activity.

3.3.2 Change in pH of the medium

Study of ^{65}zinc uptake by the ZHY3 cells expressing the *OsR06 and OsE60* cDNAs showed that the zinc uptake was sensitive to changes in pH, therefore additional experiments were carried out to study the effects of pH on ^{65}zinc uptake. At pH 4.0, ZHY3 cells expressing the *OsR06* cDNA (Fig. 6A) had a zinc uptake rate of 0.21 pmol Zn·10^6 cells^{-1}·min^{-1}, while the ZHY3 cells containing the empty plasmid or expressing the *OsE60* cDNA showed no activity at this pH (Fig. 6A). The ^{65}zinc uptake by the *OsR06* cDNA was linear and the cells showed a small lag phase at pH 4.0. With increase in pH to 5.0, the zinc uptake rate of ZHY3 cells expressing the *OsR06* cDNA (Fig. 6B) increased to 0.3 pmol Zn·10^6 cells^{-1}·min^{-1}, however there was a lag phase of 10 min before linear zinc uptake was observed. The ZHY3 cells expressing the *OsE60* cDNA and containing the empty plasmid (Fig. 6B) took up very little zinc at pH 5.0. The zinc uptake rate of ZHY3 cells expressing the *OsR06* cDNA (Fig. 6C) decreased by 50 % (0.15 pmol Zn·10^6 cells^{-1}·min^{-1}) as the pH of the medium was increased to 6.0. Interestingly, an increase in lag phase to 15 min was observed when compared to 10 min lag phase at pH 4.0 and pH 5.0. The ZHY3 cells expressing the *OsE60* cDNA (Fig. 6C) accumulated 0.18 pmol Zn·10^6 cells^{-1}·min^{-1} at pH 6.0 while the ZHY3 cells containing the empty plasmid did not take up zinc. The activity of the putative transporters clearly shows a dependence on pH.

3.3.3 Substrate specificity and metal competition

To assess whether other divalent cations are substrates for the putative transporters *OsR06* and *OsE60*, different divalent cations were tested for their ability to inhibit zinc uptake mediated by these proteins. A ten-fold excess (68 μM) of Ca, Co and Ni reduced the ^{65}zinc uptake mediated by the putative transporter *OsR06* to 60%, 57% and 45% respectively (Fig.7). Cadmium and magnesium reduced zinc uptake to 10% of the control while non-radioactive zinc reduced uptake of ^{65}zinc to 38% of the control (Fig.7). These results suggest that the two divalent cations cadmium and magnesium were either being transported

into the ZHY3 cells expressing the *OsR06* transporter or may be competing for zinc binding sites.

Zinc uptake mediated by the *OsE60* was not inhibited by a ten-fold excess (68 μM) of Co, Ni and Cd (Fig. 7). Calcium reduced the ^{65}zinc uptake rate to ~62% of the control (Fig. 7) suggesting that it had an effect on the uptake activity of the *OsE60* transporter. Non-radioactive zinc was the most potent competitor reducing ^{65}zinc uptake to 20% of the control (Fig.7). This result supports the hypothesis that *OsE60* has a greater specificity for zinc as its substrate. Magnesium reduced ^{65}zinc uptake to 40% of the control (Fig.7), suggesting that this metal may be one of the substrates being transported by the putative transporter *OsE60*. Zinc uptake mediated by *OsE60* was not sensitive to inhibition by cadmium (Fig. 7). This is in contrast to strong inhibition of *OsR06* zinc uptake by cadmium.

3.3.4 Effect of sodium on ^{65}zinc uptake

Experiments were carried out to study whether zinc uptake mediated by OsR06 and OsE60 was stimulated by sodium ions. Increasing concentrations of sodium in the medium did not stimulate the zinc uptake by either of the transporters (Figs. 8A and B). However Na^+ did inhibit zinc uptake by the putative transporters *OsR06* and *OsE60* at concentrations of 0.1, 1 and 10 mM. These results suggest that zinc is not cotransported with Na^+ into the yeast cells.

3.3.5 Concentration dependence on uptake rate

When assayed over a range of zinc concentrations (1-46.8 μM), the uptake activity of the yeast strain ZHY3 expressing the putative transporters *OsR06* and *OsE60* was concentration-dependent and saturable (Figs. 9A and B). Zinc uptake for the putative transporter OsR06 was measured at pH 4.7 while uptake for *OsE60* was measured at pH 6.0. The kinetic values derived from the data are described in Table 13.

Table 13   Vmax and Km of *OsR06* and *OsE60* transporters in yeast

Gene	Apparent K_m, μM	V_{max}, fmol/10^6cells/ min
OsR06	16.1 ± 3.6	184.3 ± 17.2
OsE60	18.5 ± 2.1	80.7 ± 3.9

3.4   Discussion

3.4.1   Functional characterization of putative zinc transporters in yeast

The cDNAs *OsR06* and *OsE60* appear to encode zinc transporters because when expressed in the yeast mutant ZHY3, they restore the ability of the cells to transport zinc. Uptake experiments with ^{65}Zn radioisotope provided direct evidence for the zinc uptake activity of the proteins encoded by the rice cDNAs. Zinc transport activity mediated by *OsR06* and *OsE60* (Figs. 6 A-C) was pH dependent. Similar pH dependence of zinc uptake activity was observed for the *AtZIP2* (pH optimum = 6.0) from *A. thaliana* (Grotz *et al.*, 1998) and human zinc transporter *hZIP2* (Gaither and Eide, 2000). OsS13 mediated uptake activity was not detected at pH 6.0 or pH 4.7, but OsE60 mediated uptake was detected at near neutral pH of 6.0 (Fig. 6 C). Sequencing analysis results (section 2.3.2, Table 7, Chapter 2) suggest that *OsR06* has a high degree of homology to *AtZIP2* from *A. thaliana* (Grotz *et al.*, 1998). The results of uptake experiments with yeast cells expressing *OsR06* suggest that inspite of this high degree of homology *OsR06* has a different pH optimum for zinc transport activity.

The biophysical mechanism of zinc transport by ZIP family members is still not clear. The yeast zinc uptake by the transporters *ZRT1* and *ZRT2* (Zhao and Eide, 1996a) showed strict energy dependence in contrast to zinc uptake by human *hZIP2* transporter, which was found to be energy independent (Gaither and Eide, 2000). Zinc uptake by *hZIP2* was stimulated HCO_3^- and it was suggested that hZIP2 functions *in vivo* by a Zn^{2+}/HCO_3^- symport mechanism. Alternatively, zinc uptake by these proteins may be driven by the concentration gradient of the metal ion substrate (Gaither and Eide, 2001). Studies by Zhang and Allen (1995) suggest that the negative-inside membrane potential found in cells may also be
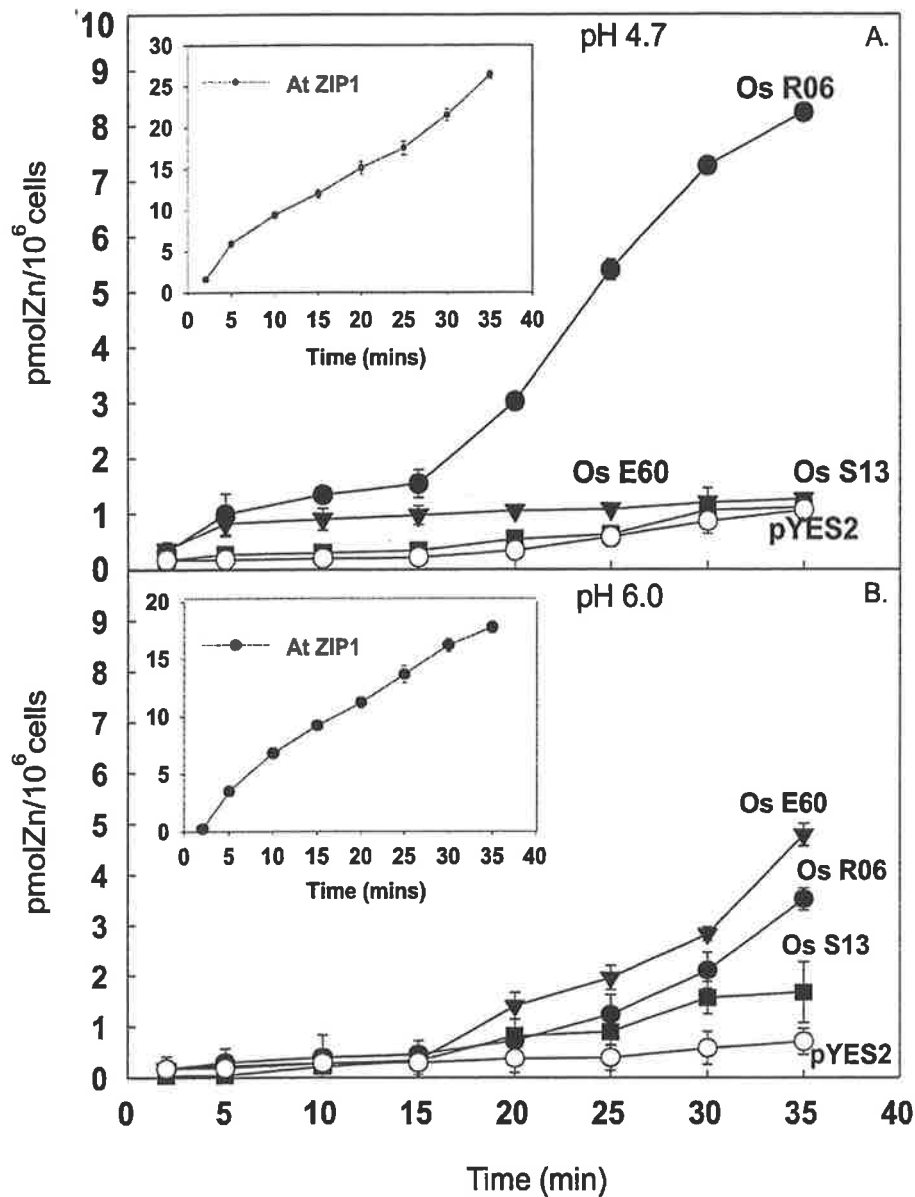
Fig. 5 **Time dependent zinc uptake by the ZHY3 cells expressing the Os cDNAs.** **(A) Zinc uptake at pH 4.7. (B) Zinc uptake at pH 6.0.** Cells were assayed with 6.8 μM ^{65}Zn. The inset graph shows linear zinc uptake by ZHY3 cells expressing the *AtZIP1* cDNA from *A. thaliana*. Data represents the mean and standard error of three separate experiments with a total of nine samples for each mean. Error bars smaller than symbols are not visible.
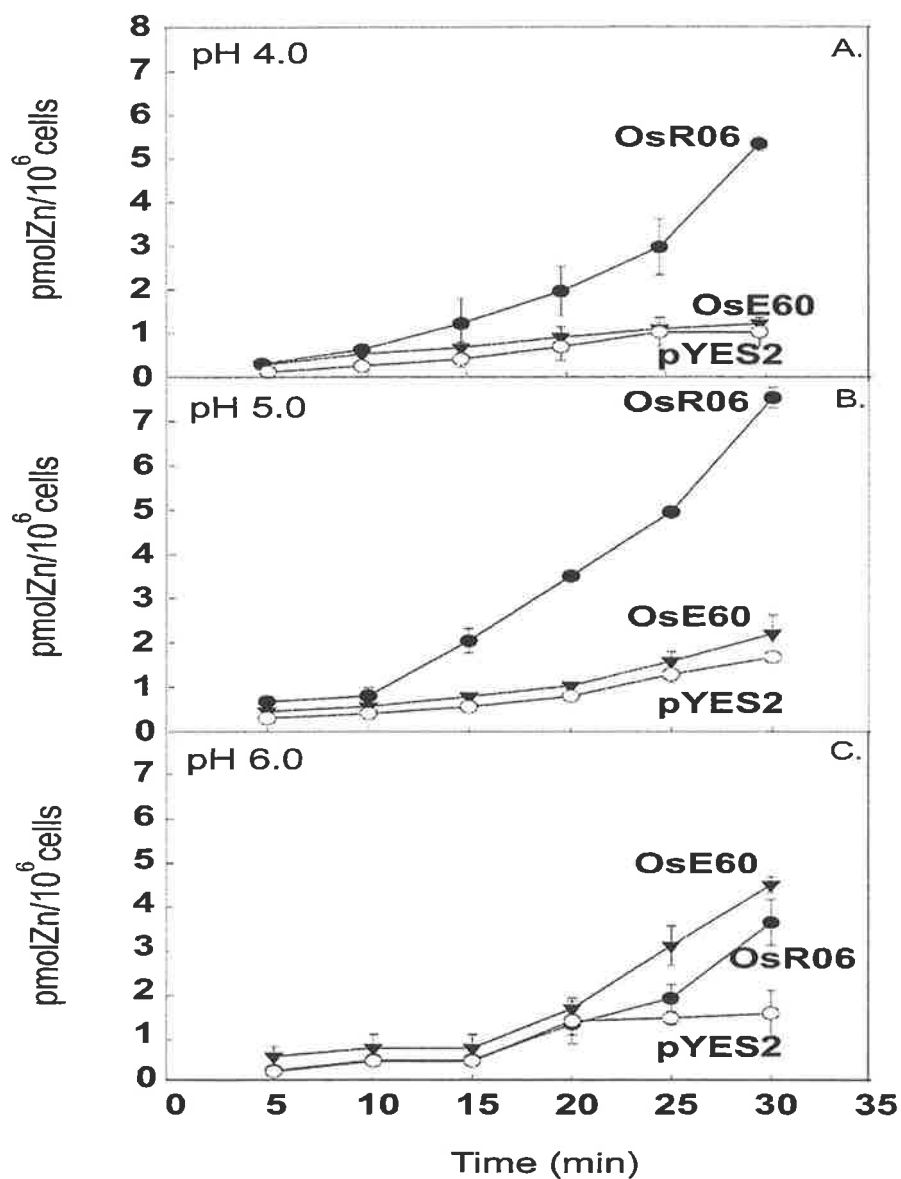
Fig. 6   Zinc uptake by the ZHY3 cells expressing the Os cDNAs show a pH optima. Zinc uptake was measured at pH 4.0 (A), 5.0 (B) and 6.0 (C). Cells were assayed with 6.8 μM of ^{65}Zn. Data represents the mean and standard error of three separate experiments with a total of nine replicates for each point. Error bars smaller than symbols are not visible.
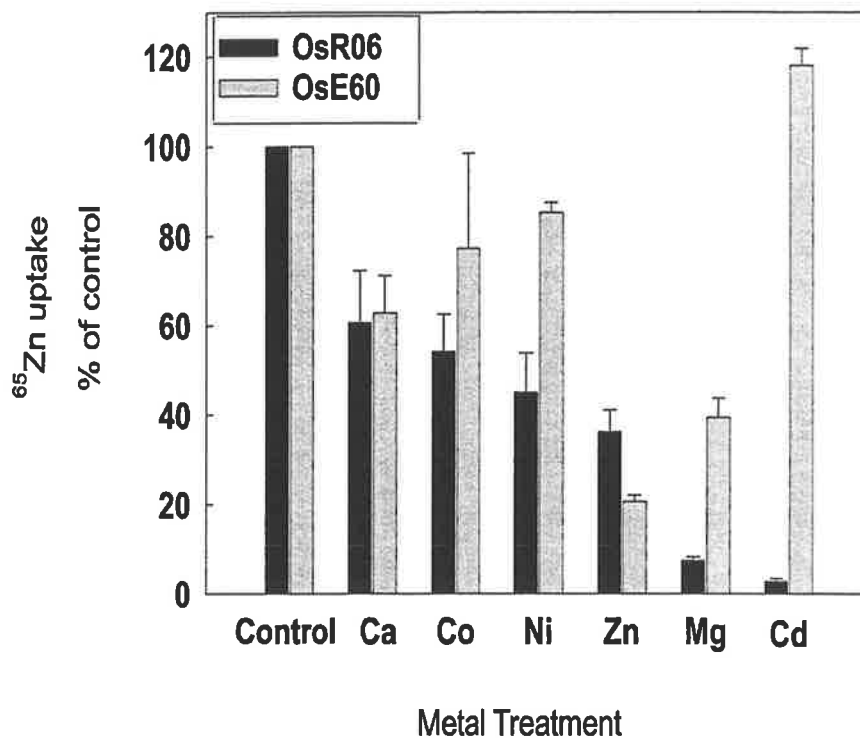
Fig. 7 **Inhibition of** *OsR06* **and** *OsE60* **dependent uptake in yeast by divalent cations.** Cells were assayed for zinc uptake with 6.8 μM ^{65}Zn in the absence (control) or presence of 68 μM of chloride salts of the indicated concentrations of metal ions. Data represents the mean and standard error of three separate experiments. Each experiment had nine replicate points. Error bars smaller than symbols are not visible.

A.
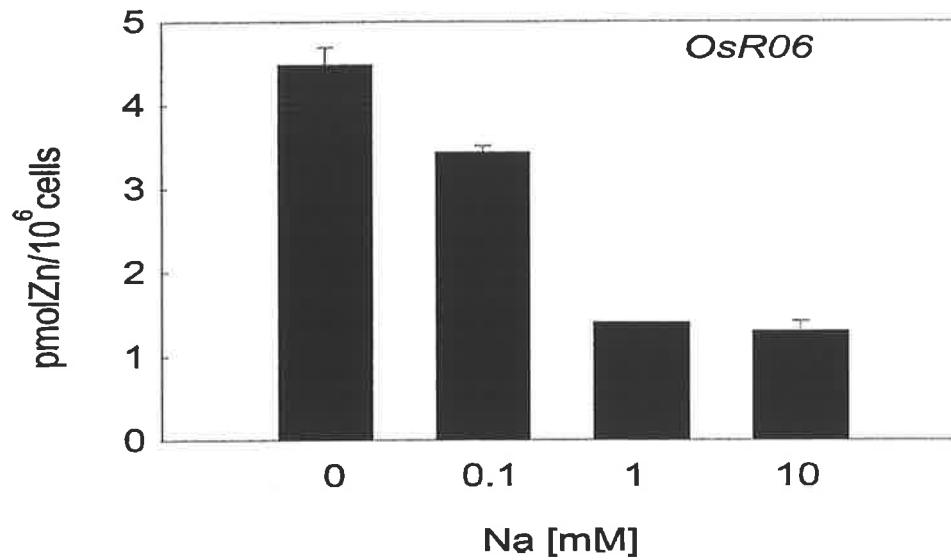
B.
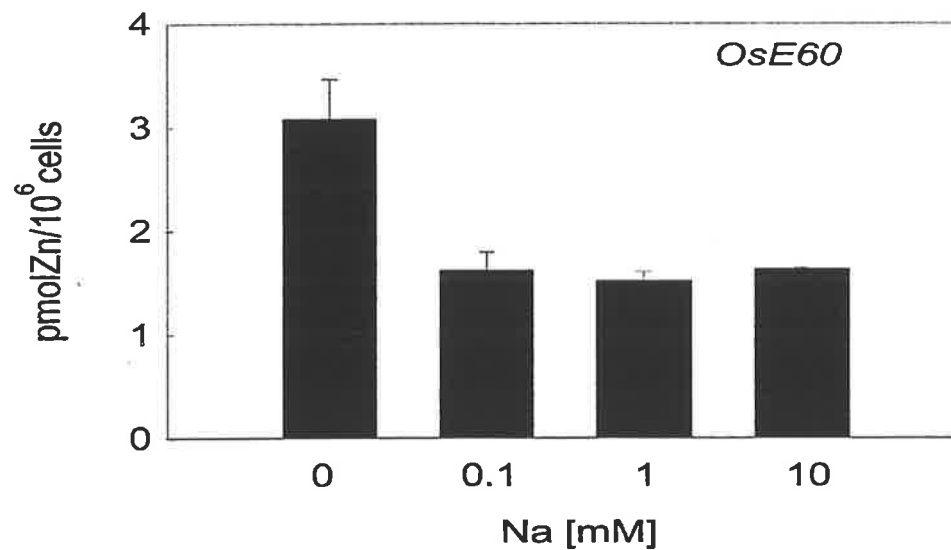
Fig. 8   Effect of Na^+ concentrations on zinc uptake by ZHY3 cells expressing the Os cDNAs. (A) Zinc uptake mediated by *OsR06* at pH 4.7. (B) Zinc uptake mediated by *OsE60* at pH 6.0. Cells were assayed for zinc uptake with 6.8 μM ^{65}Zinc. Data represents the mean and standard error of three separate experiments with nine replicates for each point. Error bars smaller than symbols are not visible.

A.

B.
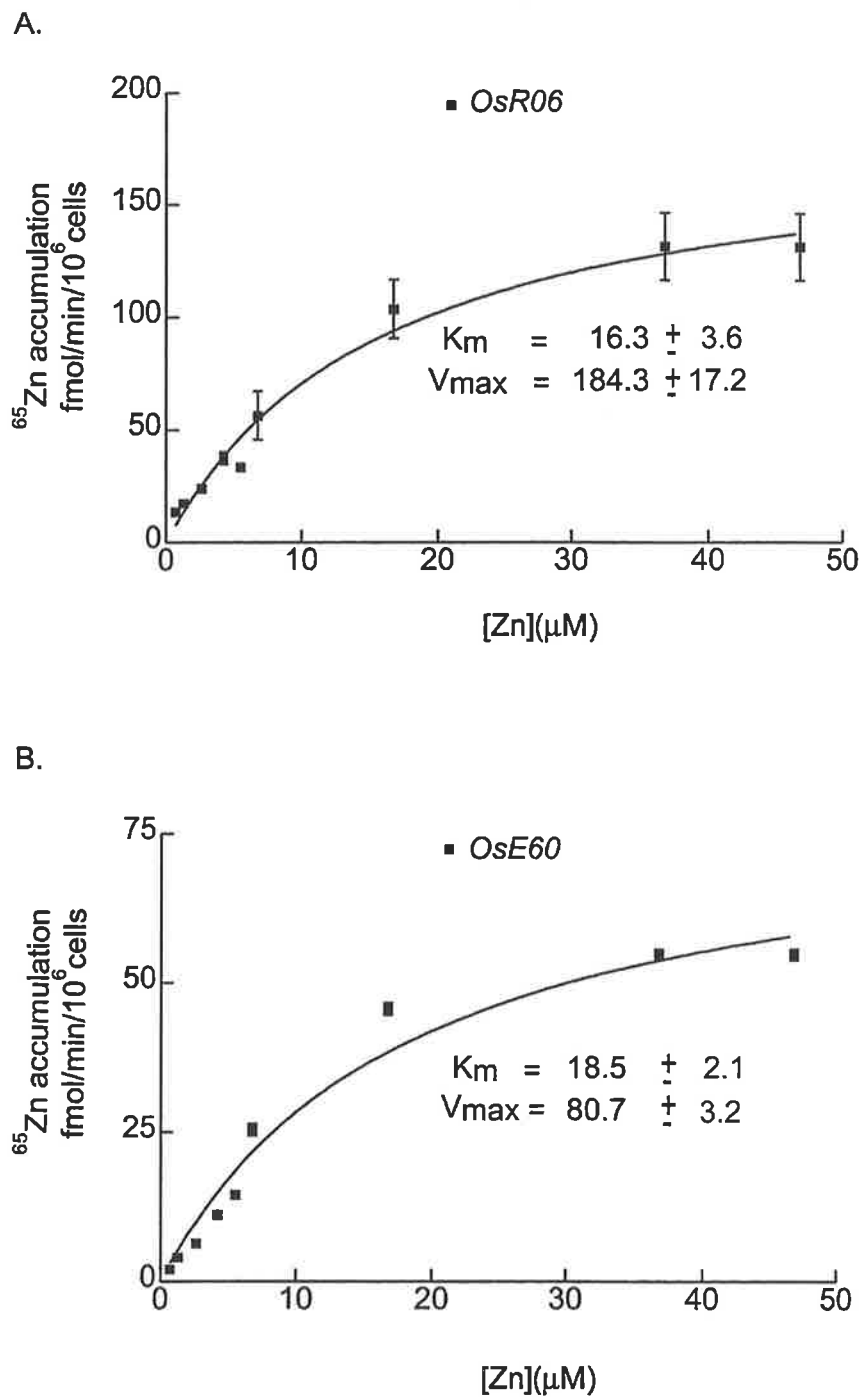
Fig. 9   Concentration dependence of zinc uptake by ZHY3 cells expressing *OsR06* (A) and *OsE60* (B) cDNAs. Zinc accumulation was measured for 25 min over a range of substrate concentrations. Data fitted to the Michaelis-Menten equation using the GraphPad Prism. K_m and V_{max} derived from the fitted curves. Data represents the mean and standard error of four separate experiments with a total of twelve replicates for each data point. Error bars smaller than symbols are not visible.

the driving force for uptake of this cation. In this study, zinc uptake activity mediated by the proteins encoded by the rice cDNAs may be energized by protons or by sodium. A Zn^{2+}/H^+ cotransport in the case of *OsR06* is possible since the optimal activity was measured at acidic pH (Fig. 6 A). OsE60 mediated zinc uptake is unlikely to be Zn^{2+}/H^+ cotransport as OsE60 mediated zinc uptake occurs at near neutral pH 6.0 (Fig. 6 C) with almost no activity at more acidic pH. The uptake of zinc does not appear to be via a Zn^{2+}/Na^+ symport as theaddition of Na^+ (Figs. 8 A and B) to uptake buffer did not stimulate OsR06 and OsE60 mediated zinc uptake

To test the substrate specificity of *OsR06* and *OsE60* transporters, divalent metal ions were tested for their ability to inhibit zinc uptake. OsR06 mediated zinc uptake was strongly inhibited by high concentrations (68 µM) of cadmium, magnesium and zinc and to a lesser extent by nickel, calcium and cobalt (Fig. 7). On the other hand, OsE60 mediated zinc uptake was strongly inhibited by high concentrations (68 µM) of zinc and magnesium and to a lesser extent by calcium (Fig. 7). Cadmium had no effect on the zinc uptake activity mediated by OsE60. AtZIP2 mediated zinc uptake in *A. thaliana* is inhibited by cadmium and zinc (Grotz *et al.*, 1998) similar to that seen in OsR06 mediated uptake. It is unlikely that inhibition of zinc uptake by cadmium observed in this study is a result of metal toxicity to the native yeast strain because incubation of yeast cells with as much as 500 µM of cadmium or cobalt over a period of 5-15 min did not impair the viability of the cells (Dix *et al.*, 1994). Zinc uptake inhibition of OsR06 protein by cadmium suggests that this transporter is involved in the transport of cadmium. Cadmium has no essential function in any living system and is probably taken up by cell due to its chemical similarities to zinc or calcium. Therefore there may exist common mechanisms of absorption and transport of zinc and cadmium as observed in *T. caerulescens* (Baker *et al.*, 1994). Studies in soybean (Cataldo *et al.*, 1983) and *Lupinus albus* (Costa and Morel, 1993) have shown that zinc competitively inhibits cadmium uptake in plant roots. *AtIRT1* (Eide *et al.*, 1996) and *AtNRAMP3* (Thomine *et al.*, 2000) are regulated by iron deficiency and also transport cadmium. These transporters are likely to also be involved in the accumulation of cadmium in plants (Eide *et al.*, 1996; Rogers *et al.*, 2000; Thomine *et al.*, 2000). Inhibitory effects of cadmium on iron transport mediated

by *AtIRT1* (Guerinot M.L. unpublished), *LeIRT* (Eckhardt *et al.*, 2001), *PsRIT1* (Cohen *et al.*, 1998), FET4 (Dix *et al.*, 1994) and toxic effects of cadmium on yeast expressing *AtIRT1* have also been reported (Rogers et al., 2000). In all of these studies including those presented here with the rice transporters, it appears that cadmium may be transported across the plasma membrane via native zinc and iron transport systems.

AtZIP2 mediated zinc uptake was not affected by nickel or cobalt but AtZIP3 mediated zinc uptake was inhibited by cobalt (Grotz *et al.*, 1998). Nickel and cobalt inhibited OsR06 mediated zinc uptake to a lesser extent than zinc (Fig. 7) while OsE60 mediated zinc uptake was not inhibited by cobalt or nickel. Inhibitory effects of nickel on *LeIRT* mediated iron uptake (Eckhardt *et al.*, 2001) have also been observed in tomato. Nickel is an essential micronutrient in plants (Zonia *et al.*, 1995) but to date, no transporters involved in nickel uptake have been identified from higher plants. Nickel uptake was impaired in the presence of high concentrations of magnesium (Eitinger *et al.*, 2000) in the fission yeast mutant Nic1p. It is possible that OsR06 protein may transport nickel, and cobalt when zinc is not present as a substrate.

Magnesium caused strong inhibition of OsR06 mediated zinc uptake while it affected the OsE60 mediated zinc uptake to a lesser extent. OsE60 mediated zinc uptake was also inhibited to a lesser extent by calcium than by zinc. The inhibitory effect of calcium on OsR06 mediated zinc transport observed in the uptake experiments was small in contrast to the large effects seen in the growth experiments (Fig. 4E, Chapter 2). The ion content experiments (ICP-AES) also showed that ZHY3 cells expressing *OsR06* accumulated more calcium in the cells than the ZHY3 cells with empty plasmid (Tables 9 and 10, Chapter 2). All these results suggest that the transporter *OsR06* is permeable to calcium. The plasma membrane in plants has multiple pathways for influx of calcium. Studies have found that yeast cells expressing wheat *LCT1* were sensitive to elevated extracellular millimolar calcium concentrations (Clemens *et al.*, 1998). Similar results have been obtained in the present study wherein yeast cells expressing *OsR06* were sensitive to increasing concentrations of calcium in the growth medium (section 2.3.6.3, Chapter 2). The weak inhibitory effect of calcium on Zn^{2+} uptake observed in the current studies may be due to low concentrations of

calcium used (1-3 mM). The calcium transport mediated by *OsRO6* may be more significant at higher concentrations.

In summary, this study has identified a transporter *OsRO6* that shows broad substrate specificity for a number of divalent cations while the other transporter *OsE60* appears to be more selective for zinc and is not permeable to toxic cadmium.

3.4.2   Observation of a lag phase in zinc uptake

Zinc uptake mediated by the rice cDNAs showed a lag phase of 10 min at pH 4.0 and 5.0 and a lag phase of 15 min at pH 6.0, followed by linear uptake of zinc. Temperature is one of the parameters that influences membrane transport processes (Sandermann, 1978). Zinc uptake by *ZRT1*, *ZRT2* and *AtZIP1-3* is temperature dependent. No uptake was detected at low temperatures - 0°C while linear uptake was detected at 30°C in yeast suggesting that uptake is strictly energy dependent. The lag phase detected in zinc uptake in this study may have been due to the time it took the cells to reach 30°C from 0°C in the heating block. However, no lag phase was observed during zinc uptake by *AtZIP1* control. This suggests that the observed lag phase in this study is not due to the time taken to adjust the temperature. Alternatively, it could be that the transporters require an induction time in the order of minutes before uptake can occur in yeast cells. In studies with *S. tuberosum*, it was suggested that a lag phase in uptake might in part be due to membrane depolarization (Hawkins and Lips, 1997) of plant cells during experimental manipulations. Another explanation for lag phase observed during uptake could be due to the requirement of cells to lower the pH of the medium. To test this hypothesis, pH of the medium was measured over a period of 30 min after the addition of ZHY3 cells expressing *OsRO6* and *OsE60*. No reduction of pH of the medium was measured suggesting that the lag phase in uptake was not due to pH changes in the medium by the ZHY3 cells expressing *OsRO6* and *OsE60* cDNAs (data not shown).

3.4.3   Kinetic analysis

The uptake activity dependent on the expression of *OsRO6* and *OsE60* was concentration dependent and saturable (Figs. 9 A and B). The saturable

component of uptake indicates a transporter-limited process that exhibits Michaelis-Menten enzyme kinetics. The apparent K_m values for Zn^{2+} transport by the *OsR06* and *OsE60* measured in yeast were in the micromolar range (Table 13). These values are comparable but slightly higher than the apparent K_m value of the *AtZIP*1 and *AtZIP*3 transporters from *A. thaliana* (Grotz *et al.*, 1998). Differences in K_m values could be due to weak or partial complementation of the ZHY3 mutant by *OsR06* and *OsE60* cDNAs (section 2.3.4.1, Chapter 2). The apparent K_m values derived for the *OsR06* and *OsE60* transporters are also similar to the levels of zinc commonly found in the rhizosphere (Welch, 1995). Physiological studies in the hyperaccumulator *Thlaspi caerulescens* have demonstrated that zinc uptake into roots shows a similar K_m when compared with the non-hyperaccumulator *Thalspi arvense* but has a 4.5 fold higher V_{max} (Lasat *et al.*, 1996). Interestingly the K_m of the TcZNT1 mediated zinc uptake (Pence *et al.*, 2000) when expressed in yeast cells lies in the range of the K_m of TcZNT1 mediated zinc uptake into the whole plant *T. caerulescens* (Clemens, 2001). These results suggest that the data from kinetic studies in yeast are well comparable to kinetic data obtained from whole plant studies.

In conclusion, two novel metal ion transporters have been identified from *O. sativa* and their uptake activity has been characterized in yeast. Both transporters show a high affinity for zinc although *OsR06* transports a broad range of divalent cations similar to *AtIRT1* from *A. thaliana* (Korshunova *et al.*, 1999). It also shows unique properties in being permeable to calcium and transporting cadmium. Further studies will be needed at the whole plant level to determine the precise role of *OsR06* and *OsE60* in metal ion transport and to understand if they function as divalent cation transporters or specific zinc transporters in response to varying substrate levels in plants and pH of the rhizosphere.

Chapter 4   Zinc transporter expression in rice

4.1 Introduction

Plants respond to a wide array of stimuli in the external environment through change in gene expression and consequent alterations in the biochemical and physiological pathways. These stimuli could range from pathogen interactions, wound responses to changes in nutrient conditions. Changes in availability of nutrients such as phosphate (Leggewie *et al.*, 1997; Liu *et al.*, 2001; Raghothama, 1999), iron (Eide *et al.*, 1996; Korshunova *et al.*, 1999) and zinc (Grotz *et al.*, 1998; Pence *et al.*, 2000) lead to the changes in the expression levels of genes. In white lupin and potato, phosphate deficiency strongly induces the expression of the phosphate transporters (*LaPT1* and *StPt1*) in the roots and stem, while the expression of the iron transporter *AtIRT1* is strongly induced in roots in response to iron deficiency in *Arabidopsis*. Zinc deficiency strongly induces the expression of the zinc transporters *AtZIP1, AtZIP3, AtZIP4* in *A. thaliana* (Grotz *et al.*, 1998) and *TaZNT1* in *T. arvense* (Pence *et al.*, 2000). This induction of gene expression in response to changes in environmental conditions is indicative of a transcriptional regulation mechanism existing in plants. Such regulation of nutrient uptake and transport is necessary because plants must be able to deploy a set of transporters either to cope with lack of nutrients which lead to deficiencies or an excess in availability of these nutrients which may lead to toxicity. The nutrient uptake in yeast is regulated through a transcriptional control mechanism (Bird *et al.*, 2000a; Bird *et al.*, 2000b; Lyons *et al.*, 2000; Zhao *et al.*, 1998; Zhao and Eide, 1997) and by post translational modification (Gitan and Eide, 2000; Gitan *et al.*, 1998) wherein the zinc transporter *ZRT1* undergoes endocytosis and subsequent degradation in the vacuole in response to high levels of zinc in the medium (Chapter 1, sections 1.7.3.1 and 1.7.3.2).

Our understanding of zinc transport and regulation in plants has advanced in recent years with the characterization of ZIP genes from *A. thaliana* (Grotz *et al.*, 1998), *T. arvense* (Lasat *et al.*, 2000) and *T. caerulescens* (Pence *et al.*, 2000). An important model system has been yeast and in that system the zinc transporters *ZRT1* and *ZRT2* are regulated at the transcriptional level by the activity of

transcription factor ZAP1 (section 1.7.2.1, Chapter 1). The activity of ZRT1 is also regulated post-translationally in response to excess zinc by endocytosis and degradation (section 1.7.2.2, Chapter 1). Among the four transporters from *Arabidopsis*, *AtZIP1* is expressed predominantly in roots while *AtZIP3* and *AtZIP4* could be detected in both roots and shoots. These three transporters show zinc responsive regulation of mRNA levels in response to zinc availability. *ZNT1* is expressed at a low level in *T. arvense* and regulated by zinc availability. In contrast, in *T. caerulescens TcZNT1* expression is unaffected by zinc availability. These studies show that the level of expression of genes varies depending on plant species with changes in external zinc availability. Thus, the aim of this chapter was to characterize the expression of the transporters from *O. sativa* with respect to tissue specificity and zinc responsiveness.

The results of the northern analysis of the *OsR06, OsE60, OsS13* and *OsS16* transcripts from *O. sativa* in response to external zinc supply are presented in this chapter. The mRNA encoding transporters *OsR06, OsE60* and *OsS16* were detected in both roots and shoots while that encoding the *OsS13* transporter was detected predominantly in roots. The expression of the transporters *OsR06, OsS13* and *OsS16* was zinc responsive.

4.2 Methods

4.2.1 Growth of rice plants in hydroponics

Seeds of rice (*Oryza sativa* cv. Jarrah) were surface sterilized with 1% commercial bleach for 15 min, followed by 2 min in 70% ethanol and then rinsed in sterile deionised water. Sterilized seeds were germinated on Whatman filter paper discs in Petri plates in darkness. Seedlings with emerging plumules and radicles were placed in foam rubber plugs and roots were placed in hydroponic growth medium (Table 12, Appendix 1.13). Five plastic tanks with a capacity of 50 litres were used to grow the rice seedlings. All solutions were made in nanopure water and the tanks contained reverse osmosis (RO) water. The growth media in all the tanks was changed every 5 days. Plants 15-18 cm high were deprived of zinc for 0, 12, 24, 48 and 96 hours. Shoots and roots were snap frozen

in liquid nitrogen and these samples were stored at -80° C and used for RNA extraction.

4.2.2   Isolation of total RNA and Poly A⁺ RNA

4.2.2.1   *O. sativa* plants

Total RNA was extracted from roots and shoots of rice plants grown in hydroponics (section 4.1.1) using the RNeasy Plant Mini Kit from QIAGEN as per the manufacturer's instruction. RNA samples were stored at -80°C until further use.   The principle involved in the extraction of total RNA is as follows. Plant tissues (upto 100 mg) are lysed and homogenized in the presence of 450 μl of guanidinium isothiocyanate buffer with 0.1 % β-mercaptoethanol. Ethanol is added to the samples to provide appropriate binding conditions and the samples are applied to the RNeasy spin columns which contain silica-gel based membranes to bind the total RNA The contaminants are washed away and high quality RNA is eluted. Poly A⁺ RNA was extracted from roots and shoots of rice plants grown in hydroponics (section 4.2.1) using PolyATract mRNA Isolation System I (Promega) as per the manufacturer's instructions. This system uses magnetic bead technology for RNA isolation. A biotinylated oligo(dT) primer hybridizes at high efficiency to the 3' Poly A⁺ region present in most eukaryotic mRNA species. The hybrids are captured and washed at high stringency using streptavidin coupled to paramagnetic particles. The Poly A⁺ RNA is eluted from the solid phase by the addition of ribonuclease free water.

4.2.2.2   Isolation of total RNA from yeast
S. cerevisiae

Yeast strain ZHY3 expressing the cDNAs *OsR06*, *OsE60* and *OsS13* were grown in LZM supplemented with 1 mM zinc sulphate to logarithmic phase in overnight cultures. The cells were harvested and processed for the extraction of total RNA using the QIAGEN RNeasy Mini Kit (section 4.2.2.1) according to the manufacturer's instructions.

4.2.3   Northern Blots

Aliquots of 4 µg RNA (5 µg in case of total RNA from yeast cells) were heated at 65°C for 10 min in 3 volumes of MOPS buffer (20 mM MOPS, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) containing 70% (v/v) deionised formamide, 8.9% (v/v) formaldehyde and bromophenol blue. The samples were chilled on ice for 5 min before being loaded on to a denaturing gel. A 4 µl aliquot of RNA marker (Promega) was treated as above and loaded onto the gel. Denatured RNA samples were run on a 1.2 % agarose gel containing formaldehyde and MOPS buffer. The RNA samples were transferred from the gel by capillary action (0.05 N NaOH) onto a Hybond N$^+$ membrane (Amersham) for at least 16 hours. The membrane was then briefly rinsed in 2X SSC. RNA was fixed by UV- cross-linking using a UV Stratalinker (model no. 1800, Stratagene). The RNA marker was cut from the gel, stained in ethidium bromide and photographed under UV light.

4.2.3.1   Probe isolation and preparation

The cDNAs *OsR06, OsE60, OsS13* and *OsS16* were excised from vector pBluescript by digestion with the restriction enzymes Kpn I and Not I (Boehringer Mannheim). The digested samples were run on 0.8% agarose gel in 0.5 X TBE [90 mM H$_3$BO$_3$, 2 mM EDTA, 90 mM Tris-HCl (pH 8.0)] and insert fragments cut out of the gel. The DNA fragments were purified from the gel slices using QIAquick Gel Extraction Kit (QIAGEN) according to the manufacturer's instructions. The purified fragments were used to synthesize probes for use in northern analysis with RNA from plants. A mixture of Os cDNAs *OsR06, OsS13* and *OsE60* was used as template for synthesizing the probe used for northern analysis of yeast total RNA. A Gigaprime DNA labelling kit (Bresatec) was used in the preparation of the radioactive probes from the purified DNA fragments. The principle involved here is that random decanucleotide primers anneal to the denatured single strand of the DNA that is to be radiolabelled. The large Klenow fragment of *E. coli* DNA polymerase I is used to add nucleotide residues to the 3'-OH termini of the decanucleotides which act as primers for DNA synthesis using the strands of the DNA as template. Reactions containing 100 ng of the denatured

template DNA and 5 µl of α- ^{32}P-dATP (10 mCi/ml; 37 MBq, Geneworks) were incubated at 37°C for 45 min. Unincorporated radionucleotides were removed by passing through a microspin™ S-200 HR columns (Amersham Pharmacia, Biotech). The probes were denatured at 95°C for 8 min, followed by cooling on ice for 5 min before use.

4.2.3.2 Hybridization and washing of the membranes

The membrane containing RNA samples were hybridized in modified Denhardt's buffer (50X Denhardt's reagent, 25% Dextran sulphate, 20X SSPE, 10% SDS, deionised formamide and 5 mg/ml Salmon sperm DNA) at 42°C after the addition of the denatured probe for at least 16-18 hours. The membrane was washed twice in 2 x SSC (150 mM NaCl, and 15 mM tri-sodium citrate, pH 7.0) and 0.1% SDS (w/v) for 10 min at 65°C, and once in 0.1 X SSC and 0.1% SDS for 15 min at 65°C. Membrane was then exposed to Kodak film (Biomax) with intensifying screen at -80°C. Autoradiographs were developed after 5 days (total RNA) or after 30 min (Poly A$^+$).

4.3 Results

4.3.1 Analysis of total RNA levels of putative transporters expressed in yeast

Due to the consistently low uptake rates in the ZHY3 cells expressing *OsS13* cDNA, northern analysis of the total RNA extracted (section 4.2.2.2) from the ZHY3 cells containing the *OsR06, OsE60, OsS13* and the empty plasmid pYES2 was used to determine the expression levels of the putative transporters. This analysis revealed that *OsR06* and *OsE60* were expressed at high levels in the ZHY3 cells while the expression of *OsS13* in ZHY3 cells was low (Fig. 10). No background expression was seen in the lane containing the total RNA from the cells containing only the empty plasmid pYES2. These results correlate well with those seen in uptake studies with ^{65}Zn isotope, wherein the yeast strain expressing the putative zinc transporters *OsR06* and *OsE60* accumulated higher levels of zinc when compared to the yeast strain expressing the putative transporter *OsS13* (Fig.

5) and suggest that low expression levels may have hampered studies designed to determine the functional characteristics of the putative transporter *OsS13*.

4.3.2 Analysis of total RNA levels of putative transporters expressed in plants

Northern analysis was carried out with the RNA isolated from the roots and shoots of rice plants grown in hydroponics and deprived of zinc for different times, in order to investigate the expression of *OsR06, OsE60* and *OsS13* and *OsS16*, in response to zinc deprivation. *OsS16* (~1400 bp) and *OsE60* (~1360 bp) transcripts could be detected in the northern analysis using total RNA. *OsR06* (~1370 bp) and *OsS13* (~1330 bp) transcripts were detected by northern analysis with poly A^+ RNA. The size of the transcripts were estimated from the autoradiographs using the RNA marker loaded on to the gel.

Maximum levels of the *OsS16* transcript were detected at 12 hours in zinc deficient roots (Fig. 11 A). The expression levels were significantly reduced at 24 and 48 hours with no transcript visible at 96 hours. A low level of the *OsS16* transcript was detected in zinc sufficient shoots with maximum levels of the transcript detected at 96 hours in zinc deficient shoots. The expression of *OsS16* thus could be detected in shoots of zinc deficient plants and only transiently in roots (Figs. 11 A and B). *OsE60* transcript was detected in both the roots and shoots of zinc sufficient and zinc deficient plants (Figs. 11 A and B). In zinc deficient shoots slightly higher levels of transcript appeared to be present at 96 hours, suggesting a slight induction in response to zinc deprivation. Expression of *OsE60* in roots did not change in response to zinc deficiency. Expression of the *OsR06* transcript was detected in both roots and shoots of zinc deficient plants (Fig. 12, upper panel) with maximum levels of the transcript seen at 96 hours in zinc deficient roots. Expression of the *OsS13* transcript was weak and was detected predominantly in the roots of zinc deficient plants (Fig. 12, middle panel). It appeared that the gene was also expressed in shoots.

Expression of the *OsS16* and *OsR06* transcripts was induced in zinc deficient roots and shoots (Figs. 11 A and B and 12, upper panel) suggesting that the expression of these transcripts is regulated in response to changes in external zinc in the medium. *OsS13* transcript levels were higher in the zinc deficient roots

at 24 and 96 hours (Fig. 12, middle panel) and very low or background levels of the transcript were observed in zinc deficient shoots (Fig. 12, middle panel) at time points 24 and 96 hours. These results suggest that *OsS13* is weakly expressed in the roots in response to zinc deficiency. The *OsE60* transcript was detected in both zinc sufficient and deficient roots at all the time points studied (Fig. 11 A), suggesting that the expression of this gene is not specifically induced in the roots in response to zinc deprivation.

In summary, it appears that the genes encoding transporters *OsR06*, *OsS16* and *OsS13* are responsive to changing zinc status in the external environment and

Fig. 10 Expression of Os cDNAs in yeast mutant ZHY3. The yeast cells expressing the Os cDNAs from *O. sativa* were grown in LZM supplemented with 1 mM zinc chloride overnight and total RNA extracted from them. A mixture of cDNA probes including *OsR06, OsE60* and *OsS13* were hybridized to a northern blot containing 5 µg of total RNA extracted from yeast cells. Ethidium bromide stained gel is shown as loading control.

their expression is induced in zinc deficient conditions. However, the expression pattern of the transporter *OsE60* seems to be unaffected by changing zinc status in roots.

4.4 Discussion

Northern analysis indicated that the putative transporters identified from rice *OsS16*, *OsR06*, *OsS13* and *OsE60* were expressed in different tissues. The expression of *OsR06* in both zinc deficient roots and shoots is suggestive of its role in zinc uptake. Further, evidence supporting this conclusion comes from the fact that *OsR06* partially complements the yeast mutant ZHY3 (section 2.3.4.1, Chapter 2) and shows zinc uptake at pH 4.7 when expressed in yeast mutant ZHY3 defective in zinc uptake (section 3.3.1, Chapter 3). *OsR06* is most similar to *AtZIP2* from *A. thaliana* (Grotz *et al.*, 1998) but the expression patterns of *AtZIP2* are unknown. *OsS13* was detected in zinc deficient roots in a pattern similar to what has been found for *AtZIP1* and *AtZIP3* (Grotz *et al.*, 1998) from *A. thaliana*. When expressed in yeast mutant ZHY3 deficient in zinc uptake, *AtZIP1* and *AtZIP3* increased the zinc uptake of the mutant. However, in the present study *OsS13* mediated zinc uptake was not detected when expressed in the yeast mutant ZHY3 (section 3.3.1, Chapter 3). Failure to complement the yeast mutant ZHY3 (section 2.3.4.1, Chapter 2) could be due to the low levels of expression of *OsS13* observed in northern analysis of total RNA extracted from yeast cells (section 4.3.1). *AtZIP4* from *A. thaliana* (Assuncao *et al.*, 2001; Grotz *et al.*, 1998) that is also expressed in both the roots and shoots of zinc deficient plants does not complement the ZHY3 mutant. *OsE60* was expressed in roots and shoots under sufficient or deficient zinc and at all the time points tested. The conclusion that *OsE60* is a zinc transporter is supported by the fact that the cDNA partially complements the yeast mutant ZHY3 (section 2.3.4.1, Chapter 2) and transports zinc when expressed in yeast mutant ZHY3 at pH 6.0 (section 3.3.1, Chapter 3). Transient expression of *OsS16* was detected in the zinc deficient roots. A similar report of transient expression of the *AtIRT2* transporter in iron deficient roots has been made in *A. thaliana* (Vert *et al.*, 2001). The results from
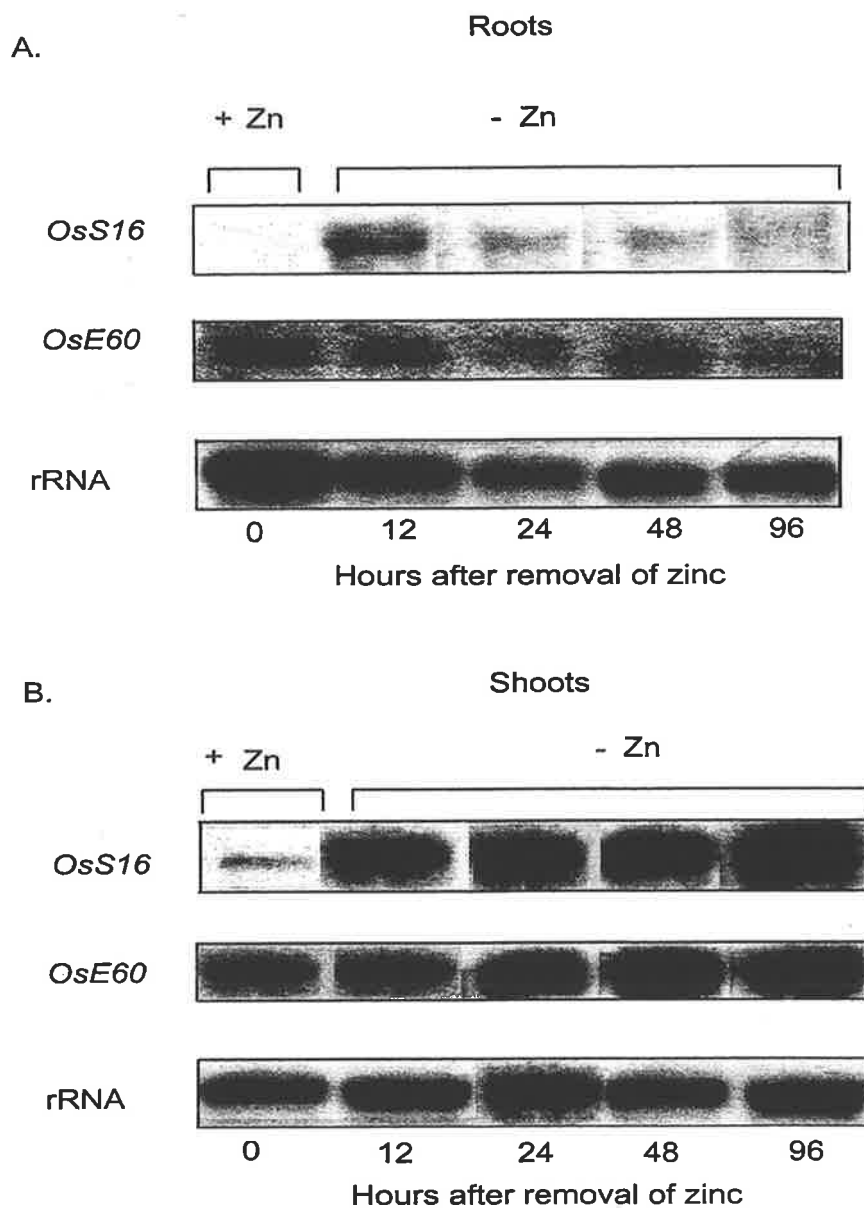
Fig. 11   Regulation of *OsS16* and *OsE60* transcripts by zinc availability. An aliquot of 4 μg of total RNA in each lane was extracted from the roots (A) and shoots (B) of rice plants grown in complete hydroponic solution and deprived of zinc for 0-96 hours. Northern blots were probed with *OsS16* and *OsE60* cDNA probes. Membranes were stripped and probed with ribosomal RNA fragment as loading control.
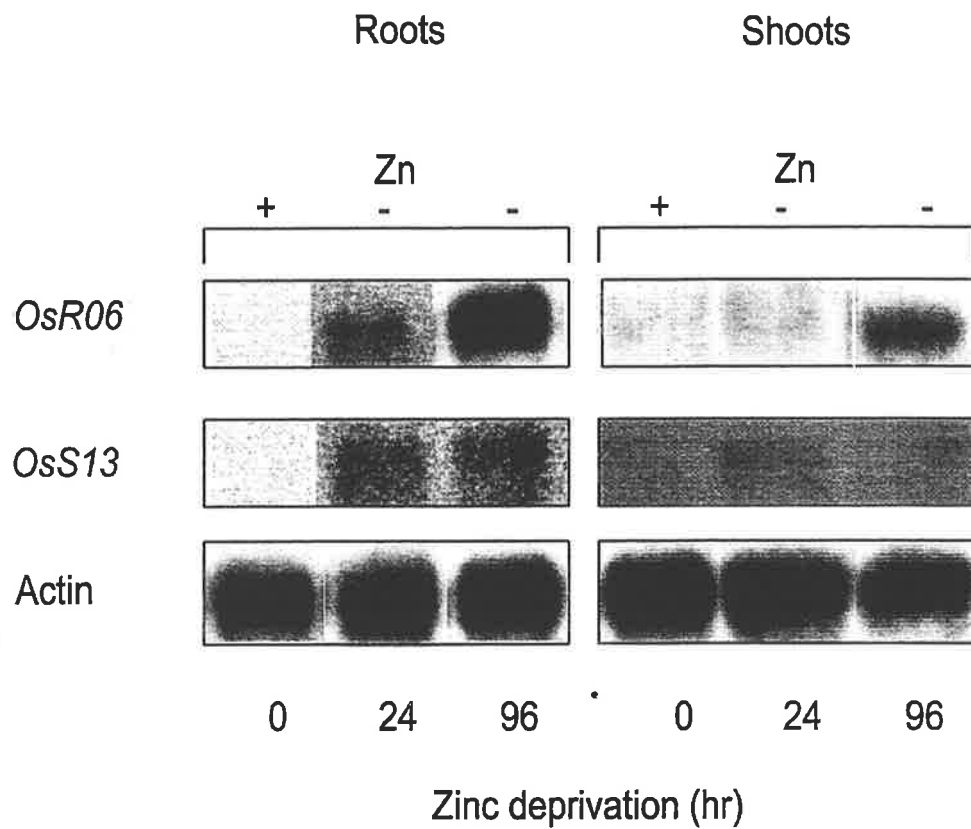
Fig. 12 **Regulation of *OsR06* and *OsS13* transcripts by zinc availability. Four micrograms of Poly A⁺ RNA was loaded in each lane.** The RNA was extracted from the roots and shoots of rice plants grown in complete hydroponic solution and deprived of zinc for 0-96 hours. Northern blots were either probed with the *OsR06* or *OsS13* cDNA probes. Membranes were stripped and probed with rice actin fragment as loading control.

northern analysis suggest a role for *OsS16* in zinc uptake but yeast complementation or uptake assays were not carried out, because we were unable to obtain the full length *OsS16* cDNA (section 2.3.3, Chapter 2).

The mRNA levels of three putative transporters *OsS16*, *OsR06* and *OsS13* identified from rice, were responsive to change in zinc status of the external medium while the expression of one putative transporter *OsE60* was not sensitive to change in zinc status of the external medium (section 4.3.2). Tissue specific and zinc responsiveness expression of the rice transporters suggests that they might play different roles in distribution of metal ions in the plant. *OsS13* identified from rice may be involved exclusively in zinc uptake in the roots similar to *AtZIP1* and *AtZIP3* (Grotz *et al.*, 1998) from *A. thaliana* while *OsR06* transporter may be involved in uptake and transport of zinc into the shoots. Constitutive expression of *OsE60* suggests that this transporter might play a function/role in uptake when zinc concentrations in soils are sufficient. Eckhardt et al., (2001) reported that *LeIRT2* from tomato is expressed in roots and is not affected by the iron status of the plants. This suggests that *LeIRT2* might play a role in iron uptake in metal replete conditions.

Metal ions such as zinc are taken up by the transporters into the roots and then distributed throughout the plant. Some of the metal ions are transported into the shoots while excess may be sequestered in the vacuole. Zinc is an essential nutrient yet in excess may lead to toxicity. Genes that encode proteins involved in zinc uptake, vacuolar sequestration and remobilization/efflux of zinc have been identified in plants and yeast (Grotz *et al.*, 1998; MacDiarmid *et al.*, 2000; Van Der Zaal *et al.*, 1999; Zhao and Eide, 1996a; Zhao and Eide, 1996b) and these genes are components of a regulatory mechanism to maintain zinc homeostasis. In yeast, the expression of zinc transporters is regulated at both the transcriptional and post-translational level (Bird *et al.*, 2000a; Bird *et al.*, 2000b; Lyons *et al.*, 1999; Lyons *et al.*, 2000; MacDiarmid *et al.*, 2000; Zhao *et al.*, 1998; Zhao and Eide, 1997). Regulation of mRNA levels in response to zinc availability in *A. thaliana* (Grotz *et al.*, 1998) and rice suggests that in plants zinc transporters are also controlled at the level of transcription. The *AtZIP1* gene from *A. thaliana* also undergoes regulation at the post-translational level when expressed in yeast (Guerinot, 2000b), but nothing is known as to how zinc transporters are regulated at the post-translational level in plants. In this study results shown in Chapter 6,

where a zinc transporter was overexpressed in plants provides new data suggesting plants also regulate zinc transport at the post-translational level. Thus a more detailed study of plant zinc transporters (Eckhardt *et al.*, 2001; Grotz *et al.*, 1998; Pence *et al.*, 2000) needs to be carried out to understand the regulatory mechanisms that control the activity of the transporters in response to zinc and provide an insight into mechanism of metal ion homeostasis.

In summary, the northern analyses of the rice transporters have provided us with clues to their functions or roles based on expression in response to external stimuli and in different tissues. Further analysis of these transporters will be necessary to understand the mechanisms of regulation in response to zinc in cereals.

Chapter 5   Overexpression of *AtZIP1* and *AtZIP3* cDNAs in *H. vulgare* cv. Golden Promise

5.1   Introduction

A major constraint to crop production throughout the world and especially in soils of South Australia and Western Australia is the low availability of micronutrient zinc. Some plants are more efficient at taking up available zinc from the soil than others. Work has been done to elucidate the physiological mechanism that governs zinc efficiency but not much is known at the molecular level (Chapter 1, section 1.4). A number of techniques such as introgression of foreign genes using transformation or selection and breeding of efficient cultivars using plant breeding techniques can be used to increase zinc efficiency in cereal crops. Overexpression of transporters in the plant roots was used in this study to investigate whether increasing the number of ion transporters in roots increases uptake of nutrients, thereby enhancing zinc uptake efficiency in plants. In whole plant studies with *T. caerulescens* and *T. arvense* (Lasat *et al.*, 1996), it has been shown that zinc uptake can be increased by regulating the number of active zinc transporters in the membrane.

Overexpression studies can also be used to understand the process of metal regulation and homeostasis in plants. The high expression of zinc transporters in *T. caerulescens*, irrespective of zinc availability has been suggested to be the major reason for enhanced zinc uptake in this species (Assuncao *et al.*, 2001; Lasat *et al.*, 2000; Pence *et al.*, 2000). The *ZAT* gene (Van Der Zaal *et al.*, 1999) was isolated from *A. thaliana* and is homologous to the mammalian zinc transporters *ZnT2* (Palmiter *et al.*, 1996a) and *ZnT3* (Wenzel *et al.*, 1997) involved in vacuolar sequestration and ZnT4 (Huang and Gitschier, 1997) which is involved in zinc transport into milk. Transgenic *Arabidopsis* that overexpressed the *ZAT* gene, exhibited enhanced zinc resistance and increased zinc content in the roots of plants when grown with high external zinc. These results suggested that *ZAT* encodes a protein that is involved in the internal compartmentation of zinc in plants (Van Der Zaal *et al.*, 1999).

The broad aim of this chapter was to investigate the effects of overexpression of zinc transporters on zinc uptake in cereals such as *Hordeum vulgare* cv. Golden Promise (Barley) and to determine if overexpression of zinc transporters can increase whole plant zinc efficiency. Zinc transporters *AtZIP1* and *AtZIP3* from *A. thaliana* were overexpressed in barley (*Hordeum vulgare* cv. Golden Promise) in the present study. Molecular and physiological analyses of the transformed plants were carried out. Barley was chosen as a model cereal because an efficient in vitro system exists for regeneration from immature scutellar tissues. (Cho *et al.*, 1998; Tingay *et al.*, 1997). This system allowed us to obtain a large number of shoots during regeneration with increased regenerability and decreased albinism in comparison to other published reports of barley transformation (Jiang *et al.*, 1998; Kott and Kasha, 1984; Wan and Leamux, 1994). Zinc transporters from *A. thaliana AtZIP1* and *AtZIP3* were chosen for the overexpression studies in *H. vulgare* cv. Golden Promise because in 1999 when the project was started these transporters were the only zinc transporters that had been well characterized.

In addition to the work done by the author of this thesis (Sunita Ramesh), others contributed to the production and care of the transgenics plants. Those contributions are acknowledged below.

Name	Contribution
Daniel Schachtman (DPS) PhD supervisor and Group leader	Moved the *AtZIP1* cDNA construct into binary vector pWVec8. Transformed AtZIP1 construct into *H. vulgare* cv. Golden Promise embryos (section 5.2.1).
Vicki Barret (Technical assistant)	Culture of *AtZIP1* and *AtZIP3* transformed plants. Care of transgenic plants (To) transformed with *AtZIP3* (section 5.2.4).
Steve Choimes (Technical assistant)	Maintainance of *AtZIP1* transgenic plants in generations To, T_1 and T_2 (section 5.2.4). Extraction of genomic DNA from the primary transformants T_0 of *AtZIP1* transformed plants (5.2.5.1).

5.2 Methods

5.2.1 Cloning of *AtZIP* genes into binary vectors

The *AtZIP* cDNAs from *A. thaliana* (Grotz *et al.*, 1998) were a gift from David Eide (Nutritional Sciences Program, University of Missouri-Columbia, Columbia). The *AtZIP1* and *AtZIP3* cDNAs were excised from the vector pFL61 by digestion with restriction enzyme Not1 (New England, Biolabs), blunt ended and directionally cloned into the Sma I site downstream of the ubiquitin promoter into the pWUbi.tml vector (Fig. 13 A) (Wang *et al.*, 1997b). The ubiquitin promoter (Christensen and Quail, 1996), *AtZIP* cDNAs and tml terminator cassette was excised from the vector with Not1 (Boerhinger Mannheim) restriction enzyme. This construct was directionally cloned into the binary vector pWVec8 (11.26 Kb) (Wang *et al.*, 1997a), which contained the 35S promoter to drive the expression of the selectable marker gene encoding hygromycin resistance (Fig. 13B). The cloning strategy is shown in Fig. 14.

5.2.2 *Agrobacterium*-mediated transformation

The *AtZIP3* and *AtZIP1* binary constructs were electroporated into *Agrobacterium tumefaciens* strain AGLO (Lazo *et al.*, 1991). The transformed cells were plated onto LB plates supplemented with spectinomycin (50 µg / µl) and incubated at 28°C for two days. DNA was extracted from the *Agrobacterium* cells using the Melane method (Appendix 1.15) and digested with Not1 restriction (Boehringer Mannheim) enzyme to confirm the presence of *AtZIP3* / *AtZIP1* cDNA inserts. Glycerol stocks were made from the constructs and stored at -80°C.

5.2.3 Southern Analysis of *Agrobacterium* DNA

Aliquots of 10 µg of the *Agrobacterium* DNA were digested with Not1 (Boehringer Mannheim) enzyme. The digested samples were size-fractionated on a 1 % agarose gel by electrophoresis. The DNA was transferred to positively charged Hybond N^+ membrane (Amersham) according to the manufacturer's

instructions. The membrane was briefly rinsed in 2X SSC and DNA fixed by UV-cross-linking using UV Stratalinker (model no. 1800, Stratagene). *AtZIP3/AtZIP1* cDNA constructs (*AtZIP3/AtZIP1* cDNA + Ubi. Promoter + tm1 terminator) were used to probe the membranes. Probes were synthesized according to the protocol described in section 4.2.3.1 and hybridization and washing of the membranes was done according to the protocol described in section 4.2.3.2. The membrane was exposed to Kodak (Biomax) film with an intensifying screen at -80°C. Autoradiograph was developed after two days exposure.

5.2.4 Transformation of the *AtZIP* binary constructs into *H. vulgare* cv. Golden Promise

5.2.4.1 Growth of *H. vulgare* cv. Golden Promise plants

Plants of *H. vulgare* cv. Golden Promise were grown under controlled conditions in the growth cabinets at 18°C during a 16 hr light period and 13°C for an 8 hr dark period. Plants were fertilized with osmocote (long life, Scotts, Castle Hill, Australia) at the time of planting and fertilized weekly with Aquasol (Hortico Pty. Ltd., Laverton North, Australia).

5.2.4.2 Preparation of immature embryos for transformation

Ten to twelve spikes of barley were harvested from *H. vulgare* cv. Golden Promise plants (section 5.2.4.1) with the size of the immature embryos ranging between 1.5 mm to 2.5 mm. The developing caryopses were surface sterilized for 10 min in a solution containing sodium hypochlorite (Marvo-Linn Bleach) containing 1% w/v chlorine and two drops of Tween 20 with constant shaking (Tingay *et al.*, 1997). The sterilized seeds were rinsed four times in sterile nanopure water. The seeds were then dissected to excise the immature embryos (1-2 mm) aseptically, under a dissection microscope in the laminar flow. The embryonic axis was excised from the embryos and the explants were placed scutellum side up, on BCI medium (Wan and Leamux, 1994).

5.2.4.3 Transformation of the immature embryos

The transformation of the *AtZIP* binary constructs was carried out at Jake Jacobsen's lab, CSIRO Plant Industry, Canberra. The *A. tumefaciens* cultures transformed with the *AtZIP* binary vectors were grown overnight in MG/L medium (Garfinkel and Nester, 1980). An aliquot of 350 μl of the *A. tumefaciens* culture was added to the plate with immature embryos and swirled around to cover all the embryos. The embryos with scutellar surface facing downwards were wiped across the plate to remove excess of *Agrobacterium* culture and transferred to new plates containing fresh BCI medium (Wan and Leamux, 1994). The plates were incubated in dark for 3 days at 20°C. After co-cultivation, the embryos were transferred to callus induction medium BCI-DM (Appendix 1.16 A, Table 13) supplemented with 2.5 mg/L Dicamba (Sigma), 50 mg/L hygromycin (Sigma) and 150 mg/L timentin (SmithKline Beecham, Australia). The plates were incubated in dark at 24°C for two weeks.

5.2.4.4 Culture and growth of transformed embryos

The transformed embryos were cultured on the callus induction medium (BCI-DM, Appendix 1.16 A, Table 13) in the dark at 24°C and subcultured at an interval of two weeks. Embryogenic callus lines resistant to hygromycin were transferred to FHG medium (Tingay *et al.*, 1997) supplemented with 1 mg/L 6-BAP (Sigma) and 20 mg/L hygromycin (Sigma) [Appendix 1.16 B, Table 14] and incubated at 24°C under 16 hr light and 8 hr dark cycle. The embryogenic calli were subcultured onto the same media until shoots regenerated. The regenerating shoots were transferred to hormone free medium (BCI) supplemented with 50 mg/L hygromycin (Sigma) and placed in magenta jars. After development of the root system, the plantlets were transferred to soil and placed in growth cabinets set at 10°C with 16 hr light and 8 hr dark cycle for two weeks for acclimatization. The plants were transferred to a containment glasshouse set to 18°C, 16 hr day and 13°C 8 h night cycle, grown to maturity and self pollinated. The seeds were harvested from mature plants and used for further analysis.

5.2.5   Analysis of transgenic plants

5.2.5.1   Isolation of DNA

Genomic DNA was extracted from the shoots of putative transgenic plants by using standard phenol-chloroform method (Appendix 1.17). The DNA samples were stored at -20° C until further use.

5.2.5.2   PCR analysis of putative transgenic plants

100 ng of each genomic DNA sample (isolated as described in the section 5.2.5.1) along with oligonucleotide primers (Figs. 15 A and B) designed to the 3' ends of the *AtZIP1* and *AtZIP3* cDNAs were used in the polymerase chain reaction (PCR). The expected amplification products were 183 bp for the *AtZIP1* cDNA and 174 bp for the *AtZIP3* cDNA.

5.2.5.3   Southern hybridization

Genomic DNA was extracted from youngest expanded leaves of the primary transformants T_0 using standard phenol - chloroform method (Appendix 1.17). Ten micrograms of the genomic DNA extracted from three putative transgenic lines of barley transformed with the *AtZIP3* binary construct and 17 putative transgenic lines of barley transformed with the *AtZIP1* binary construct were used in the Southern analysis. Genomic DNA from the putative *AtZIP3* lines was digested with the restriction enzymes EcoR V or Hind III (single restriction site in the gene construct) [Boehringer Mannheim]. The genomic DNA from the putative *AtZIP1* lines was digested with restriction enzyme Bgl II (single restriction site in the gene construct) from Boehringer Mannheim. The digested samples were size-fractionated on 1 % agarose gel by electrophoresis. The DNA was transferred to positively charged Hybond N^+ membranes (Amersham) according to the manufacturer's instructions. The membranes were briefly rinsed in 2X SSC and DNA fixed by UV-cross-linking using UV Stratalinker (model no. 1800, Stratagene). The membranes containing the genomic DNA of *AtZIP1* transformed plants were probed with the full length *AtZIP1* cDNA (Fig. 15 D).

The membranes containing the genomic DNA of *AtZIP3* transformed plants were probed with either the full length *AtZIP3* cDNA or fragments of *AtZIP3* cDNA construct (Fig. 15 C). Probes were synthesized according to the protocol described in the section 4.2.3.1 and the membranes were washed according to protocol described in section 4.2.3.2. The membranes were exposed to the Kodak (Biomax) films with intensifying screens at -80°C. Autoradiographs were developed after 3-5 days exposure.

5.2.5.4 Analysis of expression of the transgenes and identification of T_2 overexpressing lines for zinc uptake experiments

Northern analysis was done to investigate the expression of the transgenes in the T_1 and T_2 generations of the putative transgenic plants and to identify overexpressing T_2 lines. Three putative transgenic lines of barley transformed with the *AtZIP1* binary construct (Lines 25, 51 and 91) were selected for northern analysis and three putative transgenic lines of barley transformed with the *AtZIP3* binary construct (Lines 3, 5 and 17) were chosen for northern analysis. T_0 and T_1 seeds were surface sterilized in 1% bleach for 8 min followed by three rinses in sterile nanopure water. Twenty siblings per line from the sterilized T_0 seeds and twenty plants per sibling per line from the T_1 sterilized seeds were grown in a glasshouse. The T_1 plants were harvested and the roots and the shoots of the siblings were bulked before total RNA extraction. In the T_2 generation, roots from 20 plants of each sibling were bulked while the shoots from 20 plants of sibling numbers 1, 3, 5, 7 and 9 were bulked. Total RNA was extracted from the bulked roots and shoots by standard phenol-chloroform method (Appendix 1.18). Northern analysis was carried out according to the protocols outlined in the section 4.2.3 except that the full-length *AtZIP3* and *AtZIP1* cDNAs were used to synthesize the probes used in the experiments.

A.
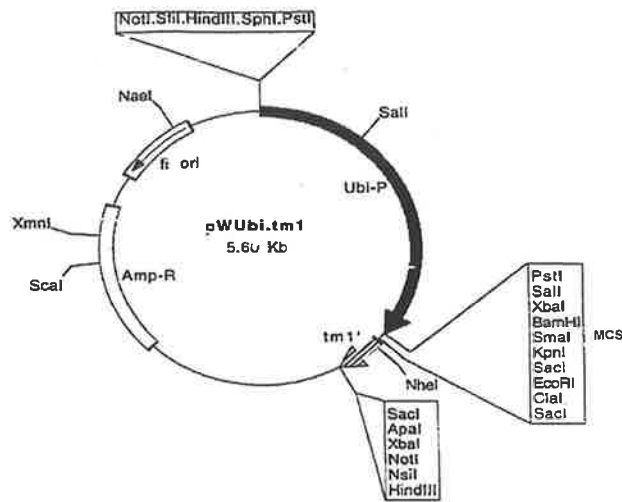
B.
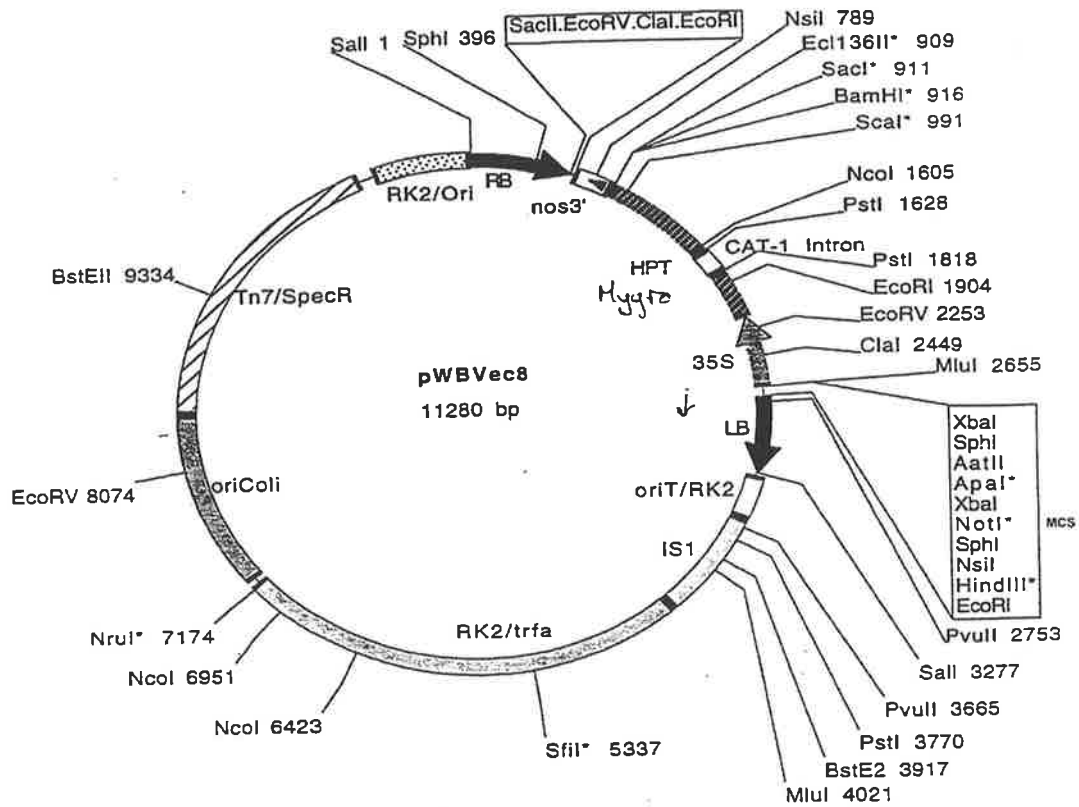
Fig. 13 **Vectors used in the transformation of** *AtZIP* **cDNA constructs into** *Agrobacterium* **strain AGLO** (Lazo *et al.*, 1991). (A) Vector pWUbi.tml (Wang *et al.*, 1997a). (B) Binary vector pWVec8 (Wang *et al.*, 1997b). MCS= Multiple cloning site.
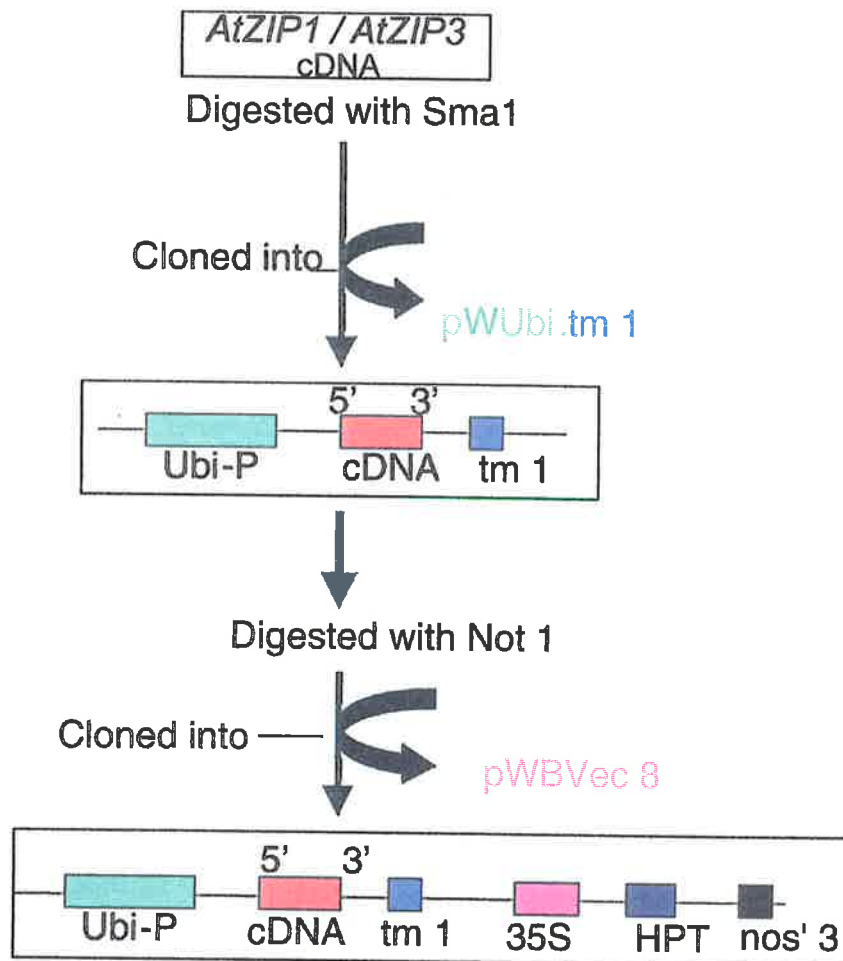
Fig. 14 **Cloning strategy for the *AtZIP* cDNAs into the binary vector pWVec8.** Ubi P = Ubiquitin promoter; tm1 = terminator; 35S = promoter; HPT = Hygromycin phosphotransferase gene; nos3' = terminator.

5.2.5.5 Transmission of the transgenes in the T_2 progeny

Mature T_1 seeds were surface sterilized according to the protocol detailed in the section 5.2.5.4. Twenty plants per sibling from each of the three *AtZIP1* lines 25-1, 51-2 and 91-3 were grown from the sterilized seeds in the glasshouse for 10 days. The transmission of the transgenes in the T_2 progeny was tested using a leaf antibiotic resistance assay method (Wang and Waterhouse, 1997). A 1cm piece of leaf from the growing axis and the ligule was excised and placed on plates with MS medium supplemented with 50 µg/ml hygromycin (Sigma) and 1 mg/L BAP (Sigma). The plates were incubated at 25°C for seven days under fluorescent light and then scored for resistance / sensitivity to hygromycin. The leaf explants that were bleached were scored as sensitive to the antibiotic hygromycin while the explants that did not show bleaching were scored as resistant to the marker hygromycin. Based on the resistance /sensitivity ratio exhibited by the siblings in a line, the segregation ratios were calculated. A chi square analysis (χ^2) was done using Sigma Plot, Scientific Graphing Software, Version 4.00.

5.3 Results

5.3.1 Hybridization analysis of *A. tumefaciens* transformed with *AtZIP3* binary construct

DNA was extracted from the *A. tumefaciens* strain AGLO (Lazo *et al.*, 1991) according to the method described in the section 5.2.2. Southern analysis was done according to the protocol described in the section 5.2.3 to check for the presence of the *AtZIP3* binary construct in the AGLO strain of *A. tumefaciens*. The *AtZIP3* cDNA probe hybridized to a 3.2 kb fragment (Fig. 16) in all the lanes confirming that the *A. tumefaciens* strain was transformed with the *AtZIP3* cDNA construct. Daniel Schachtman did similar analysis for *AtZIP1* binary construct (data not shown).

5.3.2 Growth of the putatively transformed barley plants

Transformation of the immature embryos of barley was carried out according to the protocols described in the section 5.2.4. The culture and growth of the barley embryos transformed with the *AtZIP1 / AtZIP3* binary construct is shown in the figure 17 (A-H). The immature embryos were transformed by co-cultivation with *A. tumefaciens* and the explants were cultured on callus induction medium BCI-DM containing timentin (150 mg/l) to inhibit bacterial growth. Callusing of the embryos was initiated all over the surface of the embryos under these conditions but most vigorously around the scutellar tissue (Fig. 17 A). After 2 weeks in culture, the embryos had expanded in size and development of smooth embryogenic callus was evident (Fig. 17 B). The callus was cut into smaller pieces and allowed to proliferate on BCI-DM with hygromycin (20 µg/ml). Proliferating callus was subcultured on this medium for 4 weeks. During subculture, brown callus and non-embryogenic calli were discarded. After two passages in culture (four weeks) the embryogenic calli were transferred to regeneration medium FHG supplemented with BAP (1 mg/ml) and hygromycin (20 µg/ml) and the cultures were grown at 24°C in a 16:8 hr light to dark cycle. The transformed embryogenic callus turned green on this medium (Fig. 17 C and D). Over time, regeneration of shoots was observed on this medium (Fig. 17 E and F) and shoots were subcultured on FHG medium and allowed to grow (Fig. 17 G). During rounds of subculture, brown callus and albino shoots were discarded (Fig. 17 H). Shoots when 2-3 cm high were transferred to hormone free medium BCI with 50 µg/ml hygromycin for rooting in majenta jars. Roots were initiated after 3 days in culture (Fig. 17 I) and continued to grow, giving rise to 2-3 roots after 1 week in culture (Fig. 17 J). A well-rooted plantlet was obtained after two weeks in culture (Fig. 17 K). The rooted plantlets were transferred to growth chambers for acclimatization and then to glasshouse for further growth. Three putative transgenic lines with 15 plants were obtained from transformation with the *AtZIP3* binary construct while 50 lines with 200 putative transgenic plants were obtained from the transformation with the *AtZIP1* binary construct (see section 5.2.2 and 5.2.3). A single line was considered to be the plants derived from a single embryo. These plants were used for further molecular analysis.

5.3.3 Molecular analysis of the primary transformants

5.3.3.1   PCR of the T_0 plants transformed with *AtZIP3* binary construct

Polymerase chain reaction (PCR) was carried out to check for the presence of the *AtZIP3* cDNA insert in the putatively transformed lines of *H. vulgare* cv. Golden Promise. PCR was carried out with the oligos designed to the 3' end or the promoter region of the *AtZIP3* construct to check for the presence of the *AtZIP3* cDNA. PCR with the primer pairs 1+2 and 3+4 (Fig. 15 A) designed to the 3' end of the *AtZIP3* cDNA, resulted in the amplification of a 174 bp (Fig. 18 A) and 212 bp product respectively in both the putatively transformed and untransformed Golden Promise lines (Table 15). These results suggest the amplification of the endogenous barley gene. The primer pair targeted to the ubiquitin promoter (5+6) [Fig. 15 A and Table 15], resulted in the amplification of a 960 bp product in the putatively transformed lines only. These results suggest the presence of the ubiquitin promoter in the putatively transformed lines. PCR reactions with primer combination of 7+8 (Fig. 15 A and Table 15) resulted in the amplification of multiple products in the putatively transformed lines, however no amplification was observed in the untransformed line of barley. Overall, the PCR reactions did not show the presence of the *AtZIP3* cDNA in the putatively transformed plants though the presence of ubiquitin promoter was detected in the transformed lines.

5.3.3.2   PCR of the T_0 plants transformed with *AtZIP1* binary construct

Genomic DNA from seventeen putative transgenic lines (25 plants) was used for polymerase chain reaction (PCR) to check for the presence of the *AtZIP1* cDNA insert. PCR reactions carried out with the oligos (Fig. 15 B) designed to the 3' end of the *AtZIP1* cDNA, resulted in the amplification of a 183 bp product (Fig. 18 B) in all the 17 putatively transformed lines tested. No product was amplified in the untransformed Golden Promise line. These results suggest that all the 25 plants of barley tested were transformed and contained the *AtZIP1* cDNA.

5.3.3.3 Southern analysis of primary transformants T₀

5.3.3.2.1 Plants transformed with the *AtZIP3* binary construct

Southern analysis was carried out on the three primary transformant lines (T₀) to check for the presence and copy number of the *AtZIP3* cDNA insert. Different length fragments of either the cDNA or the construct (Fig. 15 C) were used as probes in the southern analysis. All the probes except probes 5 and 6 hybridized to similar size fragments in both the putatively transformed and untransformed lines of barley (data not shown). When the probes designed to the ubiquitin promoter (Fig. 15 C, no. 5) or a part of the ubiquitin promoter and 407 bp (Fig. 15 C, no. 6) of the 5' end of the cDNA were used, they hybridized to 2.1 kb and 2.4 kb fragments respectively in the putatively transformed lines only (data not shown). No hybridization of the latter two probes to the membrane was seen in the untransformed barley genomic DNA. These results suggest the presence of the ubiquitin promoter in the putatively transformed lines of barley. Similar results were obtained using PCR (section 5.3.3.1).

5.3.3.3.2 Plants transformed with the *AtZIP1* binary construct

The full length *AtZIP1* cDNA fragment was used to probe the membranes containing 10 µg of genomic DNA per lane from the 17 putatively transformed lines. Variation in the signal intensity between the lanes was observed (Figs. 19 A and B) which may be indicative of a varying number of copies of the T-DNA being present in the individual transformants (Abedenia *et al.*, 1997). The banding pattern observed indicated that the different lines contained between 1 and up to 3 copies of the promoter cDNA cassette (Figs. 19 A and B, Table 16) in the transformed lines. The banding pattern seen in different lanes in the hybridization experiment suggests that a total of fourteen independent transformation events might have occurred to give rise to the transgenic plants in the primary transformants analyzed. Single transformation events occurred to give rise to

A.

Ubi.P 5' 8 AtZIP3 cDNA 3'

5 6 7 3 1 2 4

B.

Ubi.P 5' AtZIP1 cDNA 3'

1 2

C.

Ubi.P 5' AtZIP3 cDNA 3'

1
5 2
3
4
6

D.

Ubi.P 5' AtZIP1 cDNA 3'

1

1000 bp
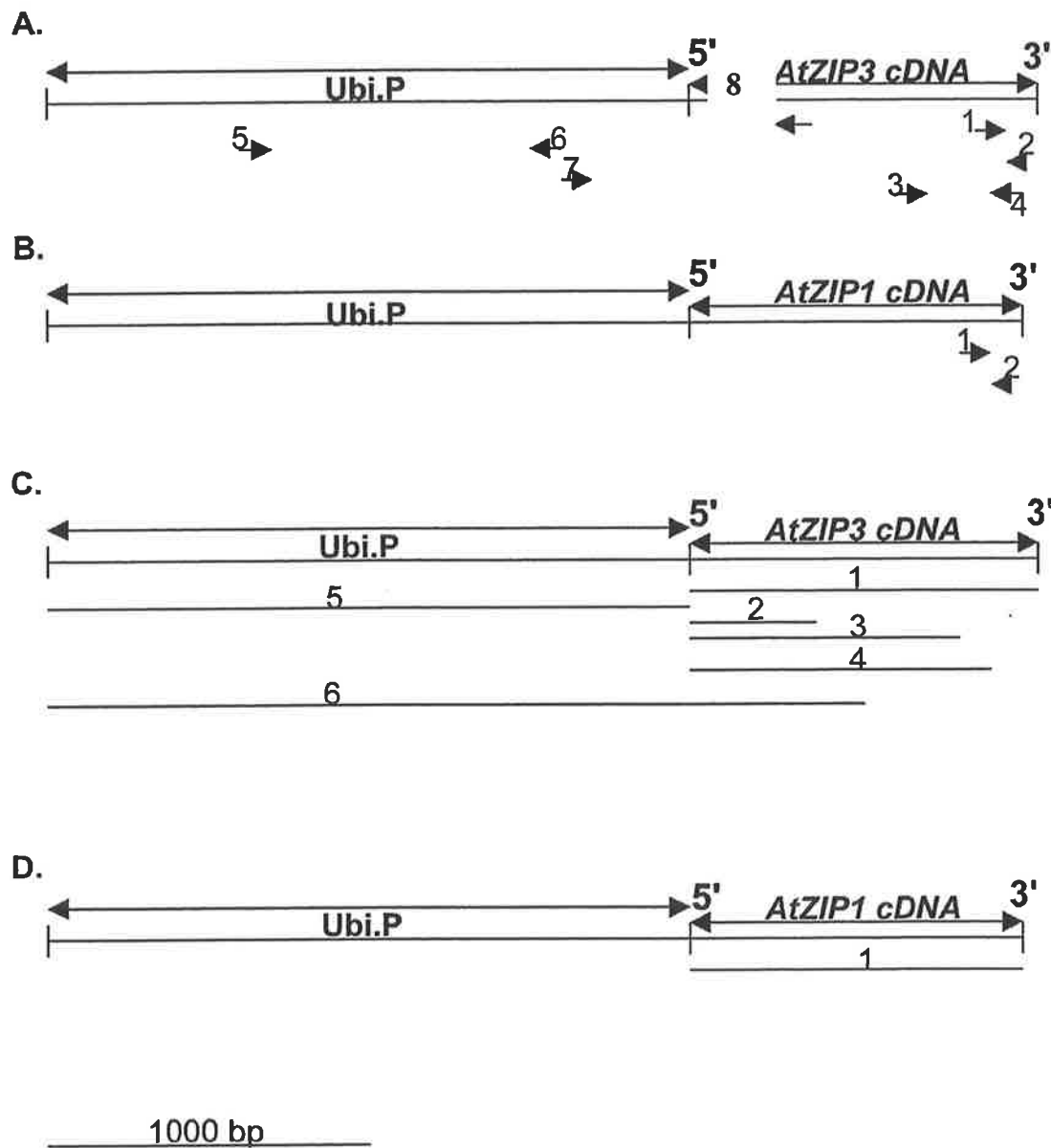
ig. 15   Schematic diagram of primers and probes designed to the *AtZIP* binary constructs used in PCR and southern analysis. (A and B) Primers used in the PCR analysis of the plants transformed with *AtZIP3* or *AtZIP1* binary constructs respectively. Numbers refer to the position (in base pairs- bp) where the primers were designed on the constructs. 1=1034, 2= 1208, 3=914, 4=1126, 5=601, 6=1561, 7= 1562 and 8 =349 on the *AtZIP3* cDNA construct. 1=1021 and 2=1204 on the *AtZIP1* cDNA construct. (C and D) Probes used in the southern analysis of the plants transformed with *AtZIP3* or *AtZIP1* cDNA constructs respectively. Numbers refer to the length of the probe (in base pairs- bp). 1=1249, 2=322, 3=842, 4=927, 5=2100 and 6=2507 for the *AtZIP3* binary construct. 1=1215 for the *AtZIP1* binary construct. Ubi.P = Ubiquitin promoter.

possibly identical progeny in the lines 25, 74 and 91 (Figs. 19 A and B, Table 17). In line 51, different transformation events in the same embryo appear to have given rise to the different progeny L 51-1 and L 51-2 (Figs. 19 A and B, Table 17). Line 95 was observed to have an intensely hybridizing band suggesting that perhaps multiple copies of the gene have been inserted at the same locus (Fig. 19 B).

5.4   Inheritance in the T_2 generation

Segregation of hygromycin resistance was determined in the T_2 progeny of three transgenic *AtZIP1* lines (91-3, 25-1 and 51-2) using the leaf test method described in the section 5.2.5.5. In all the progeny of the siblings of line 91-3 segregated in a 3:1 ratio (Table 18) confirming the presence of a single copy of the gene (Tables 16 and 17). In the line 25-1, hygromycin resistance was a 3:1 ratio in the progeny of four siblings (2, 3, 5, 7 and 8) while the segregation ratio for the progeny of sibling 6 was lower than a 3:1 ratio (Table 16). In the progeny of all the siblings of line 51-2 hygromycin resistance segregated with a ratio of of 1:0 (Table 18), which is in accordance with the 2-3 copies of the cDNA evident from the Southern blot analyses (Table 16, Fig. 19 B). All untransformed *H. vulgare* cv. Golden Promise tested were susceptible to the antibiotic (Table 18). This analysis also identified a putative homozygous line 25-1(1) that showed complete resistance to the marker hygromycin (Table 18). Two siblings from the lines 91-3(9) and 25-1(9) did not express the hygromycin marker in the T_2 plants (Table 18) suggesting they were nulls.

5.5   Northern analysis of plants from T_1 generation

5.5.1   Plants transformed with the *AtZIP3* binary construct

This investigation was carried out to investigate the expression levels of the *AtZIP3* cDNA in the T_1 progeny. Similar levels of hybridizing transcripts were detected in both the roots and shoots of the putatively transformed plants and the untransformed Golden Promise plants (GP) [Fig. 20 A]. These results suggest that the probe hybridizes to the endogenous barley gene. Together with the results
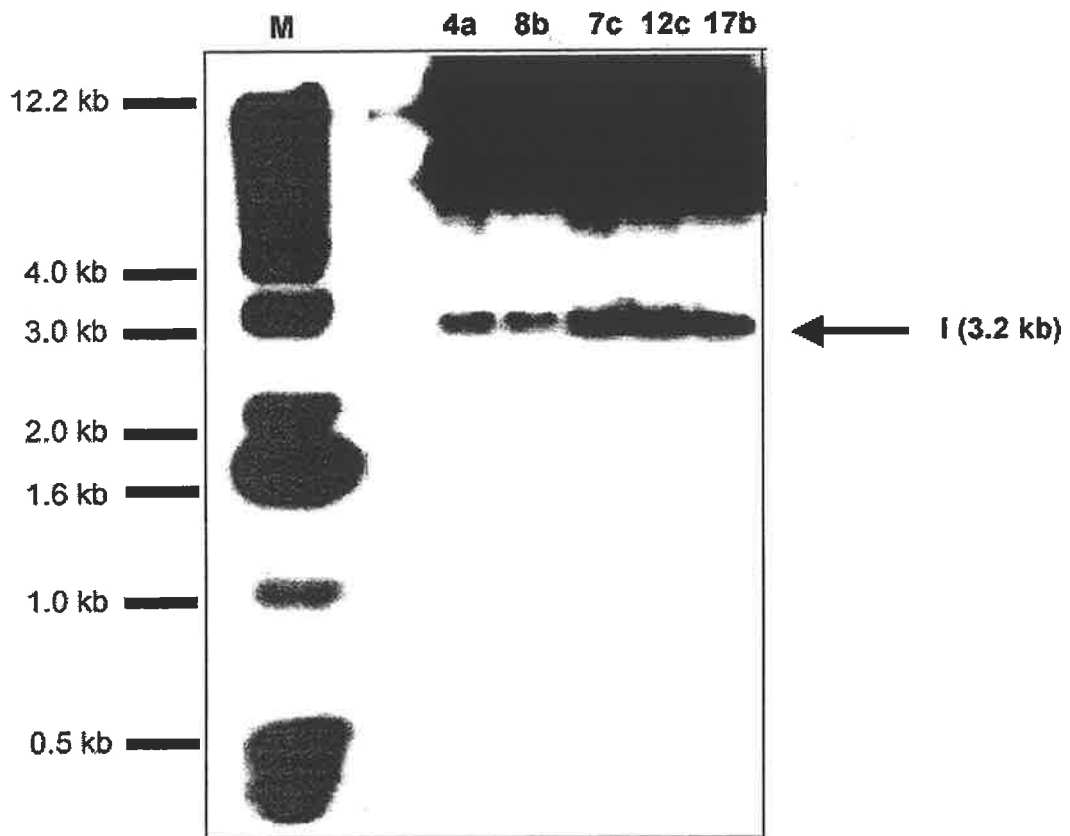
Fig. 16   Southern analysis of the *Agrobacterium* DNA to confirm the presence of the *AtZIP3* cDNA construct. Aliquots of 15 μg of DNA was digested with Not I restriction enzyme, separated on a 1% agarose gel, blotted onto Hybond N⁺ (Amersham) membrane and hybridized with the construct containing *AtZIP3* cDNA + ubiquitin promoter and tml terminator. I = insert which is the construct containing *AtZIP3* cDNA + ubiquitin promoter and tml terminator.
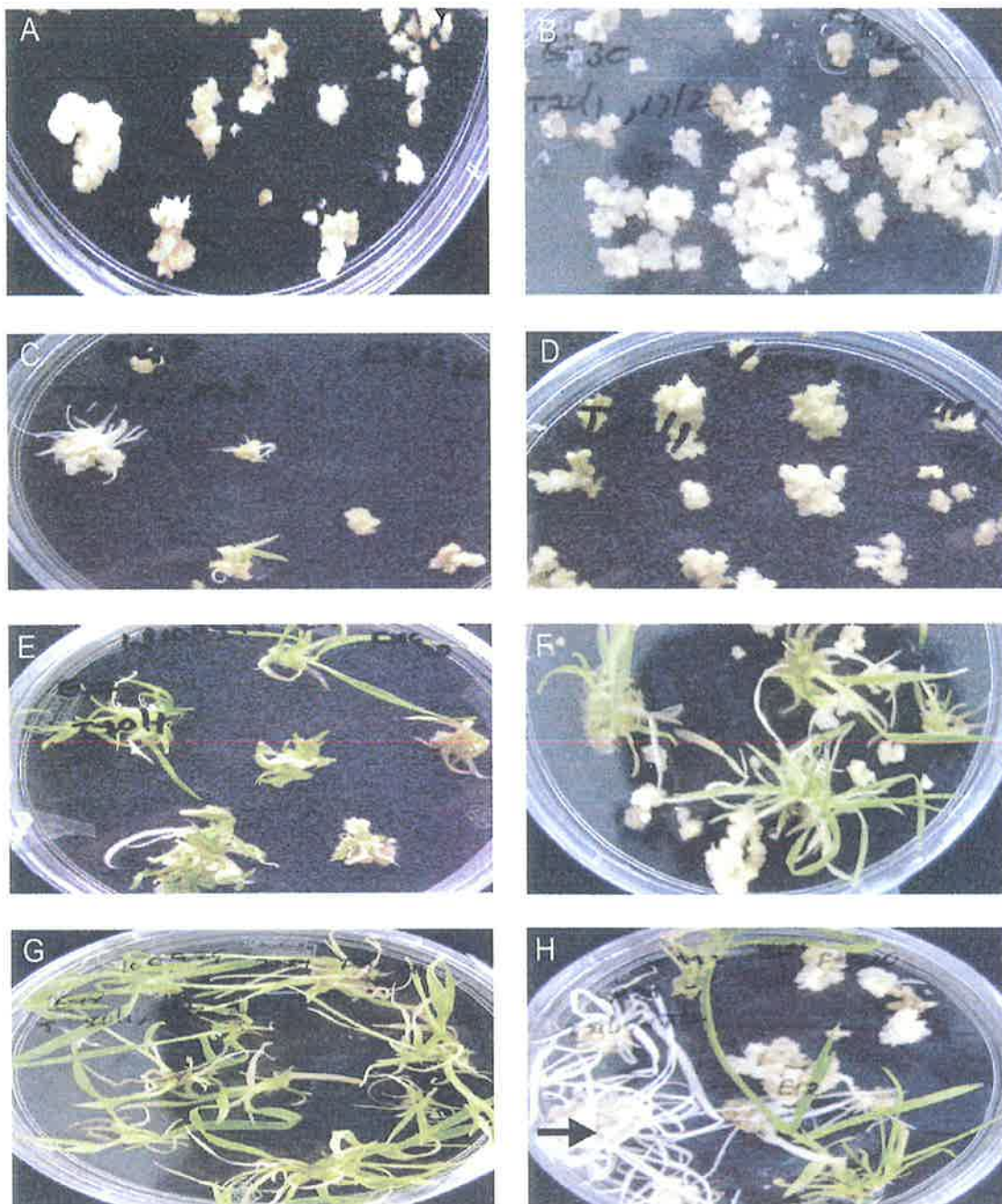
Fig. 17 Stages in the production of transgenic plants from *H. vulgare* cv. Golden Promise. (A) *Agrobacterium* infected immature barley embryos callusing on selection medium. (B) Proliferation of embryogenic callus on selection medium. (C and D) Greening of embryogenic callus on selection medium (E and F) Regeneration of shoots on selection medium. (G) Transgenic barley plants on selection medium. (H) Plate showing selection of transgenic plants with hygromycin. The bleached plantlets are not transformed or were chlorophyll mutants.
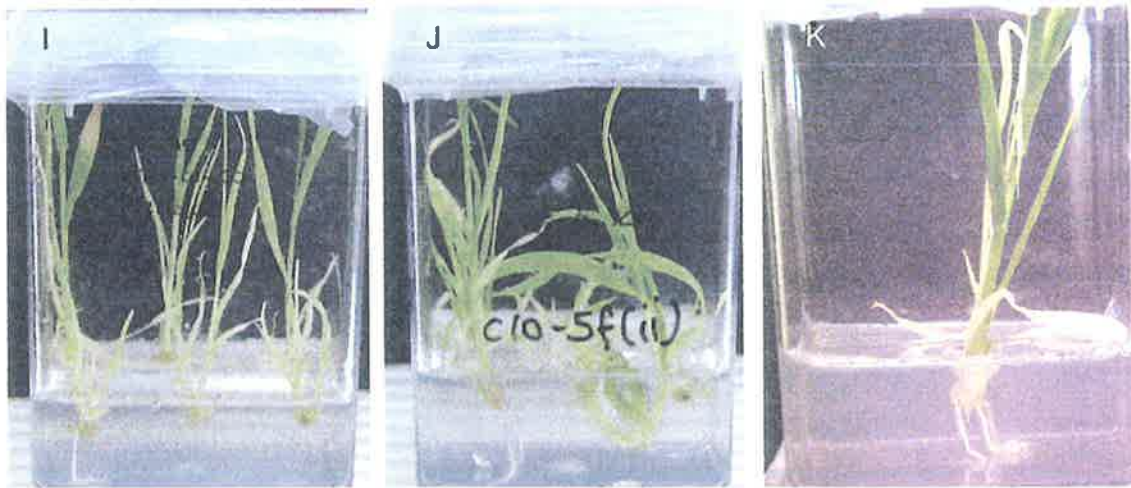
Fig. 17   Stages in the production of transgenic plants from *H. vulgare* cv. Golden Promise. (I) Initiation of roots on BCI medium with 50 µg/L hygromycin. (J) Two day old plantlets with roots. (K) Well rooted plant.
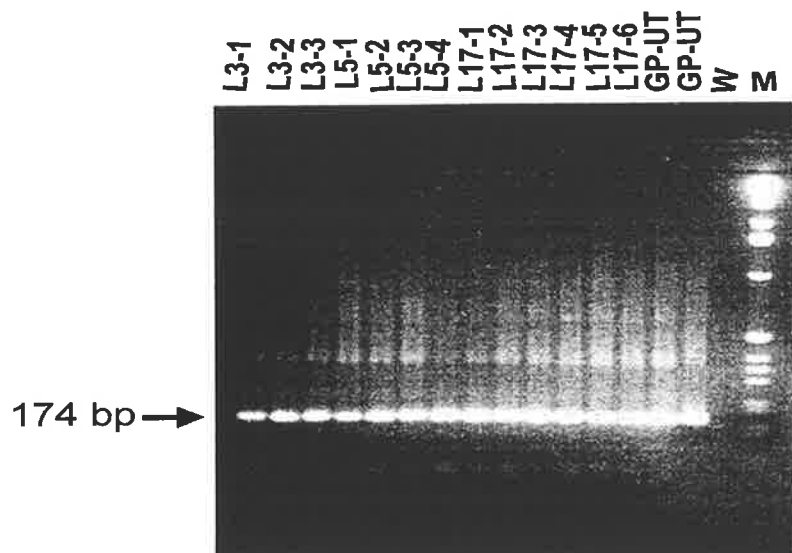
Fig. 18 A   Analysis of putative transgenic plants transformed with the *AtZIP3* binary construct by polymerase chain reaction (PCR). Genomic DNA isolated from the primary transformants was used in amplification of a 174 bp fragment by PCR. PCR products from DNA of various lines and untransformed barley Golden Promise are also shown. W = water used instead of DNA and M = 1 Kb marker (Gibco, BRL) as size standard.

Table 15   PCR analysis of the putative transgenic barley plants transformed with the *AtZIP3* binary construct.

Primer combination	Expected product size(bp)	Putative transformed lines	Untransformed Golden Promise
1+2	174	+	+
3+4	212	+	+
5+6	960	+	-
7+8	860	+ * multiple bands were also observed	-

+ presence of the expected product.

- absence of the expected product.
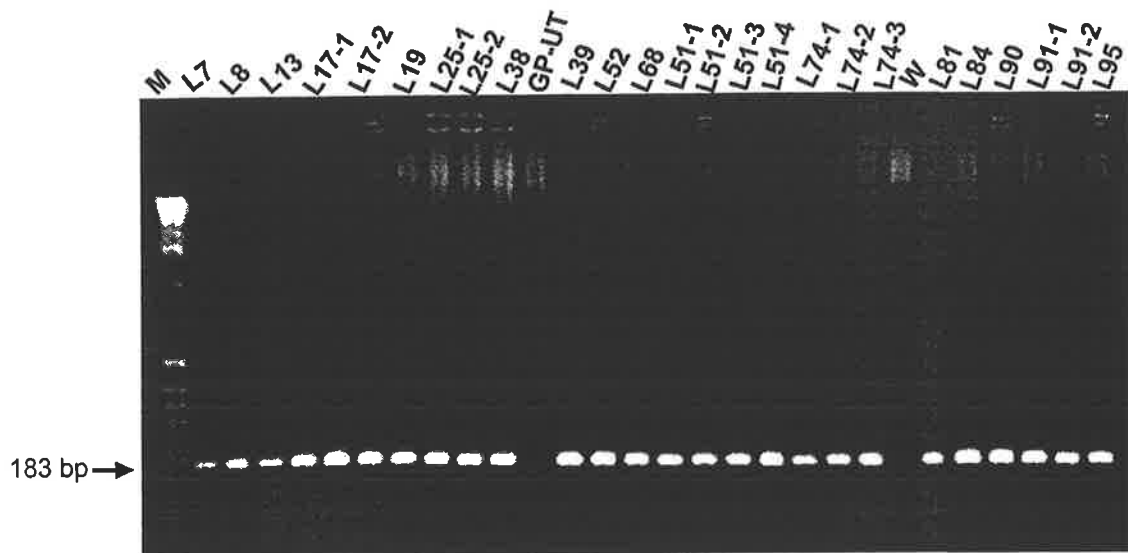
Fig. 18 B **Analysis of putative transgenic plants transformed with the *AtZIP1* binary construct by polymerase chain reaction (PCR).** Genomic DNA isolated from the primary transformants was used in amplification of a 183 bp fragment by PCR. PCR products from DNA of various lines and untransformed barley Golden Promise are shown. W = water was used instead of DNA and M = 1 Kb marker (Gibco, BRL) as size standard.

from the PCR analysis (section 5.3.3.1) and Southern analysis (section 5.3.3.2) they indicate that the transgenic plants do not contain or are not overexpressing *AtZIP3*.

5.5.2 Plants transformed with the *AtZIP1* binary construct

Northern analysis with total RNA extracted from the progeny of the T_1 generation of three barley lines 91-3, 25-1 and 51-2 transformed with the *AtZIP1* binary construct, showed high levels of the expression of the *AtZIP1* transcript in both the roots and shoots (Fig. 20 B). These results together with those of PCR and Southern analyses suggest that the progeny of the three lines 91-3, 25-1 and 51-2 contain and are overexpressing the *AtZIP1* cDNA. In contrast, lower transcript levels were observed in the untransformed Golden Promise (GP) (Fig. 20 B).

5.6 *Identification of overexpressing T_2 lines*

Northern analysis was done with total RNA from the siblings of the three *AtZIP1* transformed lines. High levels of the transcript were detected in the roots (R) and shoots (S) of the siblings of line 91-3 except in the samples labelled 9R and 9S (Fig. 21 A). These results suggest that the *AtZIP1* cDNA is overexpressed in the siblings except in 9R and 9S in the T_2 generation. Lack of expression observed in 9R and 9S is in agreement with the leaf hygromycin resistance tests (Table 18) where 91-3(9) was seen to be susceptible to hygromycin. Increased levels of the transcript or overexpression was observed in both the roots (R) and the shoots (S) of the siblings of the line 25-1 with the exception of the samples marked 9R and 9S (Fig. 21 B). This result is in agreement with leaf hygromycin resistance tests (Table 18) where 25-1(9) was observe to be susceptible to hygromycin. Northern analysis of the siblings from line 51-2 indicated that both roots and the shoots consistently overexpressed the *AtZIP1* cDNA in the T_2 generation (Fig. 21 C). Expression pattern of the cDNA was similar in both the roots and the shoots. The results suggest that the transgene is stably inherited. Three lines that overexpress
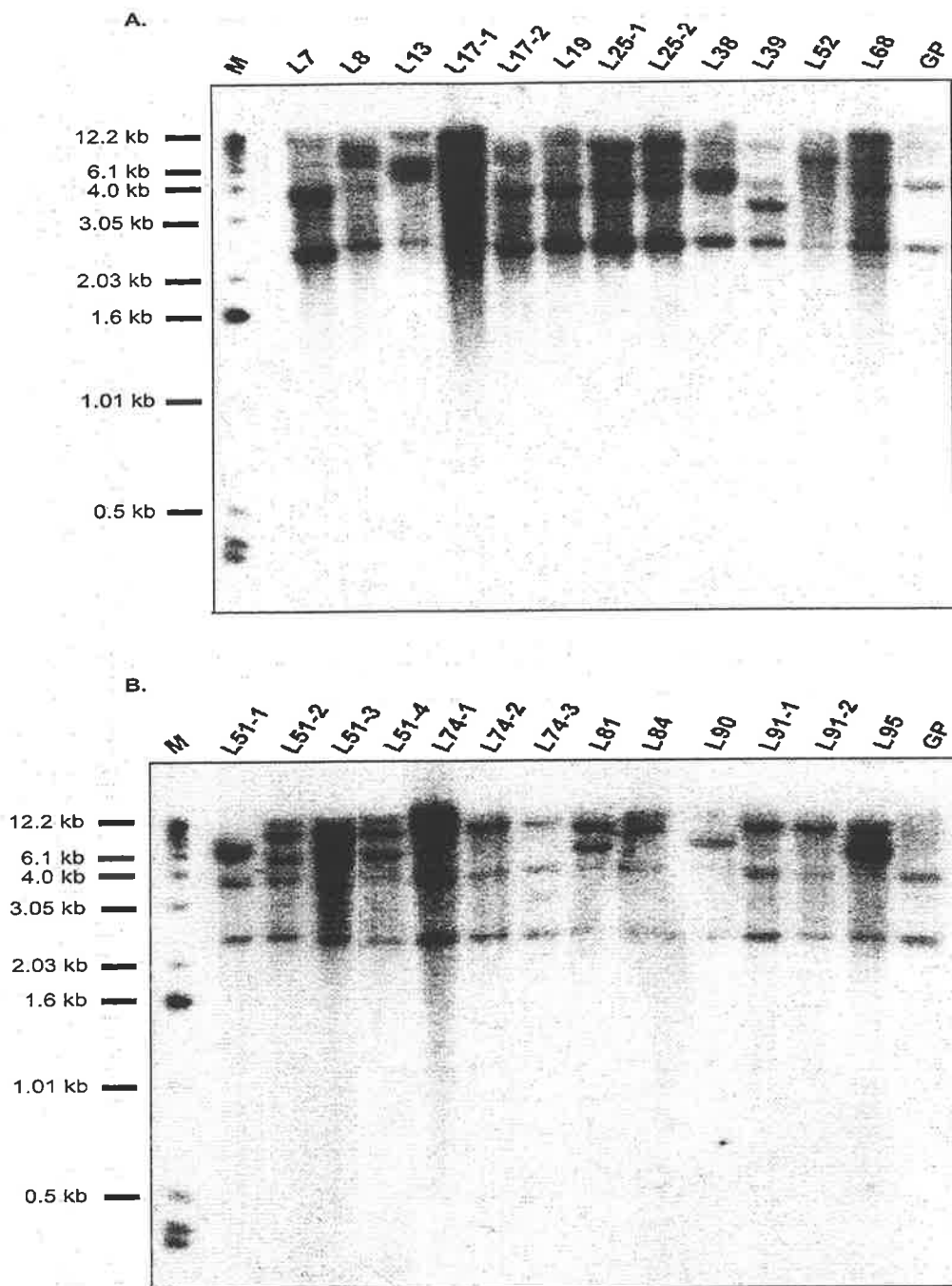
Fig. 19 A and B **Southern analysis of genomic DNA of *AtZIP1* transformed *H. vulgare* cv. Golden Promise lines provides an indication of transgene number.** (A and B) Genomic DNA extracted from the putative transgenic lines (indicated by code numbers) were digested with Bgl II, separated on 1% agarose gel, blotted onto a Hybond N+ (Amersham) membrane and hybridized with ^{32}P labelled *AtZIP1* cDNA. GP = Untransformed Golden Promise; M = molecular weight marker.

Table 16   Copy number of the *AtZIP1* cDNA estimated from Southern analysis of primary transformants.

Line No.	Number of siblings tested	Copy no. per genome
7	1	1
8	1	2
13	1	2
*17	2	2
19	1	1
25	2	1
38	1	1
39	1	2
**51	4	3
52	1	1
68	1	2
74	3	1
81	1	2
84	1	2
90	1	1
91	2	1
95	1	2***

*   L17-1 may have1-2 copies of the gene.
**   51-1 has only 2 copies of the gene.
*** Presence of an intense or bright band suggesting the integration of more than one copy of the cDNA at the same locus.

Table 17   Transformation events apparently occurring in a single embryo. This data is based on the results of the southern analysis of the primary transformants.

Line number	Number of transformation events
25	One
51-2	Two
74	One
91	One

Table 18   **Transmission of transgenes in the *AtZIP1* T₂ progeny.** This data represents the results of the leaf test assay (Wang and Waterhouse, 1997) to determine the hygromycin resistance of the T₂ progeny derived from the transgenic lines. The leaf explants that were bleached were scored as sensitive while the explants that did not show bleaching were scored as resistant to the marker hygromycin.

Line No.	No. of plants analysed	Resistant	Susceptible	Segregation Ratio
51-2(1)[a]	18	18	0	1:0
51-2(2)	16	16	0	1:0
51-2(3)	16	16	0	1:0
51-2(4)	14	13	1	1:0
51-2(5)	20	20	0	1:0
51-2(6)	20	20	0	1:0
51-2(7)	17	16	1	1:0
51-2(8)	18	18	0	1:0
51-2(9)	20	20	0	1:0
51-2(10)	20	20	0	1:0
25-1(1)	20	20	0	1:0
25-1(2)	17	17	3	5.6:1*
25-1(3)	20	14	4	3.5:1*
25-1(4)	16	9	5	1.8:1*
25-1(5)	19	15	4	3.7:1*
25-1(6)	18	5	13	0.38:1
25-1(7)	19	16	3	5.3:1*
25-1(8)	15	8	6	1.3:1*
25-1(9)	17	0	17	Susceptible
25-1(10)	18	18	4	4.5:1*
91-3(1)	15	11	4	2.7:1*
91-3(2)	19	13	6	2.1:1*
91-3(3)	20	14	6	2.3:1*
91-3(4)	20	15	5	3:1*
91-3(5)	18	14	4	3.5:1*
91-3(6)	19	14	5	2.8:1*
91-3(7)	19	15	4	3.7:1*
91-3(8)	19	13	6	2.1:1*
91-3(9)	19	0	19	Susceptible
91-3(10)	17	11	6	1.8:1*
GP-UT	4	0	4	Susceptible
GP-UT	5	0	5	Susceptible
GP-UT	6	0	6	Susceptible

Note: GP-UT refers to the untransformed barley plant (*H. vulgare* cv. Golden Promise) used as a control in the experiments.

*Analysis using the χ^2-test indicated that the segregation ratios of T₂ progeny were not significantly different from 3:1 (at $\alpha = 0.05$).

[a] Line designation e.g. 51-2(1) = Transgenic line 51-2, first sibling plant
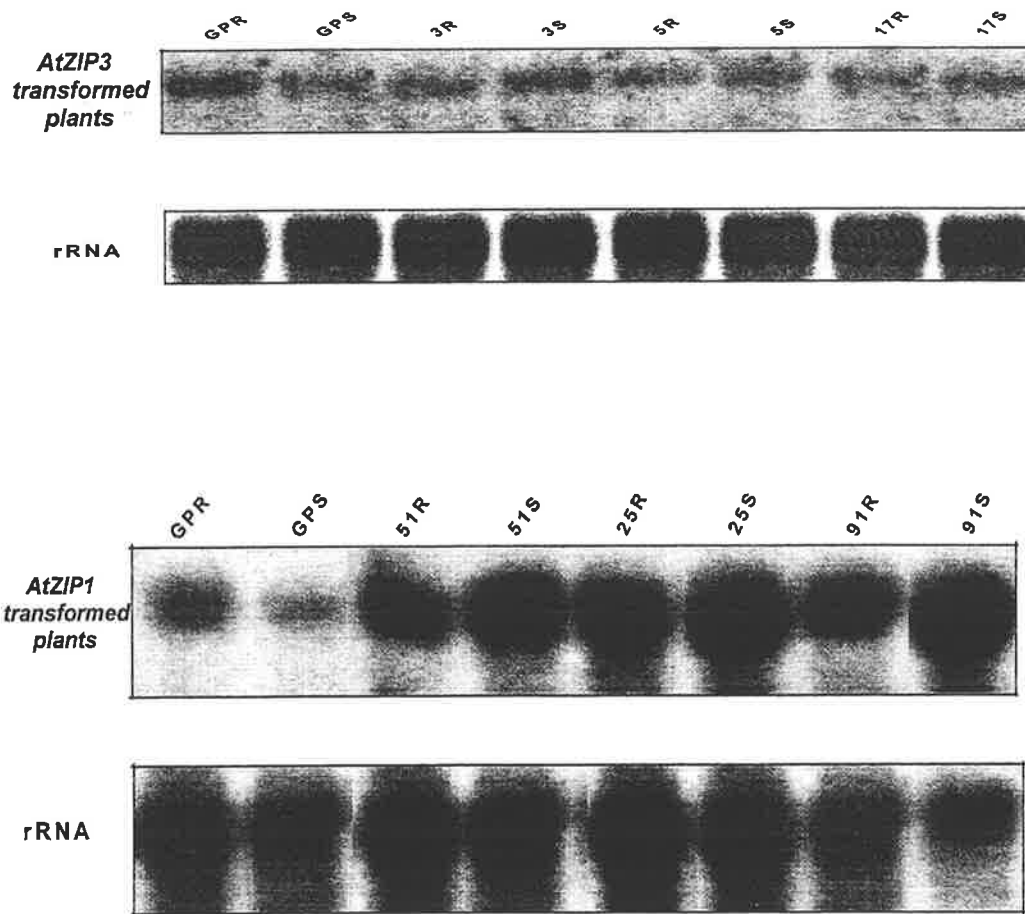
Fig. 20 Northern analysis of total RNA extracted from T₁ generation of putatively transgenic barley plants transformed with AtZIP3 and AtZIP1 cDNA constructs. Aliquots of 10 µg of total RNA extracted from the roots and shoots of T₁ generation plants (referred to as *AtZIP3* and *AtZIP1*) was separated by electrophorosis on a denaturing gel, transferred on to Hybond N⁺ membrane and probed with ³²P labelled *AtZIP3* or *AtZIP1* cDNAs. Membranes were stripped and probed with ribosomal RNA (rRNA) fragment as loading control. R = roots, S = shoots and GP = Untransformed Golden Promise. (A) Level of hybridizing transcripts in T₁ plants transformed with *AtZIP3* binary construct. (B) Level of hybridizing transcript in T₁ plants transformed with *AtZIP1* binary construct.
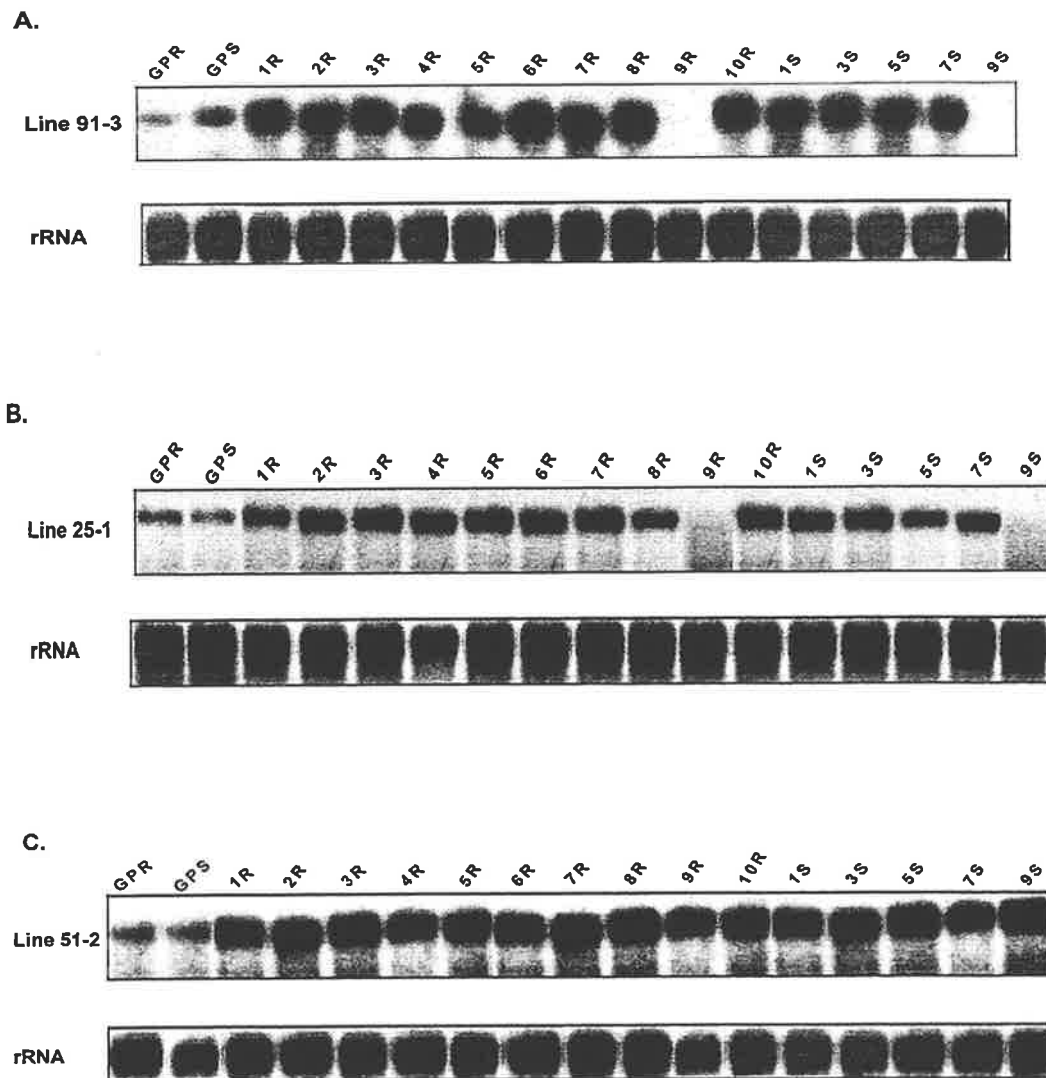
Fig. 21 **Northern analysis of total RNA extracted from T₂ generation of putatively transgenic barley plants transformed with *AtZIP1* binary construct.** Aliquots of 10 µg of total RNA extracted from the roots and shoots of T₂ generation plants was separated by electrophorosis on a denaturing gel, transferred on to Hybond N⁺ membrane and probed with ³²P labelled *AtZIP1* cDNA. Membranes were stripped and probed with ribosomal RNA (rRNA) fragment as loading control. R = roots, S = shoots and GP = Untransformed Golden Promise used as control. (A) Transgene expression in T₂ plants of line 91-3. (B) Transgene expression in T₂ plants of line 25-1. (C) Transgene expression in T₂ plants of line 51-2.

the *AtZIP1* cDNA were identified by northern analysis and the siblings of these lines can be used to determine how overexpression affects zinc uptake.

5.7 Discussion

5.7.1 Transformation

The introduction of new tools of biotechnology have made introgression of heterologous genes into plants relatively easy. Foreign genes can be integrated into plants by direct DNA transfer using particle bombardment techniques or through *Agrobacterium* mediated transformation. Dicotyledonous plants were relatively easy to transform with *Agrobacterium* but the monocotyledonous plants were initially difficult to transform using *Agrobacterium* due to their recalcitrance to in vitro regeneration, resistance to *Agrobacterium* infection and low level of activity of the promoters used in the transformations. Recently, *Agrobacterium* mediated transformation has been successfully applied to the monocots (Hiei *et al.*, 1997; Hiei *et al.*, 1994; Ishida Y. *et al.*, 1996; Smith and Hood, 1995; Tingay *et al.*, 1997) due to the development of efficient in vitro regeneration systems and use of efficient promoters. Plants regenerated after *Agrobacterium*-mediated transformation have been reported to have lower copy number integration of the transgene, relatively precise mode of DNA transfer, a predictable pattern of integration, reduced problems with cosuppression and instability over generations and high efficiency of transformation (Hiei *et al.*, 1994; Zambryski, 1988). In comparison, the direct DNA delivery systems have been reported to lead to the integration of multiple genes at the same loci, rearrangement of transgenes, silencing of transgene and reduced fertility in both dicotyledonous and moncotyledonous transgenic plants (Christou *et al.*, 1991; Dai *et al.*, 2001; Finnegan and McElroy, 1994; Flavell, 1994; Kohli *et al.*, 1998; Pawlowski and Somers, 1996; Somers *et al.*, 1992; Vasil *et al.*, 1992). Thus *Agrobacterium*-mediated genetic transformation of plants is an attractive alternative to direct DNA delivery methods and has been successfully used in the genetic engineering of various cereal crops.

Transformation of barley cultivar Golden Promise has become a standard procedure (Tingay *et al.*, 1997; Wan and Leamux, 1994). In *Agrobacterium*-mediated transformation of barley, factors crucial for delivery of transgenes such as type of growth regulator, duration of culture period, plasmolysis and wounding have been evaluated (Trifonova *et al.*, 2001). Several promoters that direct constitutive expression of the transgenes in monocot plants have been used in genetic transformation. These include the maize ubiquitin promoter (Christensen and Quail, 1996), rice actin 1 promoter (McElroy *et al.*, 1990) and cauliflower mosaic virus (CaMV) promoters (Kay *et al.*, 1987; Vain *et al.*, 1996). However studies have found that in cereals, the maize ubiquitin promoter consistently directed significantly higher levels of transgene expression than other promoters used (Able *et al.*, 2001; Chair *et al.*, 1996; Christensen and Quail, 1996; Hill-Ambroz *et al.*, 2001; Li *et al.*, 1997; Rooke *et al.*, 2000; Tingay *et al.*, 1997; Upadhyaya *et al.*, 2000; Vain *et al.*, 1996). In the present study the maize ubiquitin promoter drove the expression of *AtZIP1* and *AtZIP3* cDNAs.

5.7.2 Expression of *AtZIP* genes in Barley

Agrobacterium-mediated transformation of barley was first reported by Tingay et al. (1997). In the present study, *H. vulgare* cv. Golden Promise was genetically engineered with expression cassettes containing the *A. thaliana* transporters *AtZIP1* and *AtZIP3* by *Agrobacterium*-mediated transformation. Fifteen putative transgenic plants (T_0) transformed with *AtZIP3* binary construct were analyzed by PCR to check for the presence of the *AtZIP3* cDNA insert and only the primer combination 7+8 gave a positive result (results, section 5.3.3.1). All other tests (results, sections 5.3.3.1 and 5.3.3.3) done on these plants failed to show that the plants were transformed with the *AtZIP3* cDNA. The advantage of *Agrobacterium*-mediated transformation is the accuracy of T-strand excision and integration into the plant genomic DNA. Usually the whole T-DNA is integrated together with residual border sequences into the plant genome (Mayerhoefer *et al.*, 1991), but sometimes the integrated T-DNA copy may be truncated or rearranged or may contain vector DNA sequences from outside the T-DNA borders (Cluster *et al.*, 1996; Mayerhoefer *et al.*, 1991). The results of the PCR analysis suggest that a truncation of the T-DNA might have occurred during

integration into the plant genome. Fifty-five transgenic lines of barley transformed with *AtZIP1* binary construct from *A. thaliana* were obtained using *Agrobacterium*-mediated transformation (results, section 5.3.2).

Molecular analysis of the transgenic plants by PCR revealed that the plants were transformed with the *AtZIP1* cDNA. 1-3 copies of *AtZIP1* cDNA were detected in southern hybridization analyses of 25 plants from 17 lines. The results of the southern analysis suggested random insertion of transgenes into the barley chromosomes which is consistent with the patterns of single and multiple integration events that have been reported when using *Agrobacterium* mediated transformation of cereal species (Cheng *et al.*, 1997; Tingay *et al.*, 1997; Zhang *et al.*, 1997). Northern analysis of T_1 and T_2 plants showed that the *AtZIP1* transcript was abundant in both roots and shoots suggesting that the transgenic plants were overexpressing the *AtZIP1* cDNA.

5.7.3 Stability of transgene expression

In the present study, the transgene was inherited in a simple mendelian ratio of 3:1 (Table 18, section 5.4) in almost all the *AtZIP1* T_2 progeny. A 3:1 segregation ratio for transgene expression indicates a single site of transgene integration in the plant genome (Cho *et al.*, 1999). In the line 25-1, progeny number 6 had a lower segregation ratio than 3:1. This deviation in segregation ratio could be due to loss or low rates of physical transmission of the transgene(s) to the progeny (Cho *et al.*, 1999; Pawlowski and Somers, 1996; Pawlowski *et al.*, 1998). The progeny of the line 51-2 had skewed expression ratios higher than 3:1 suggesting that two-three genes could be integrated into different chromosomes in the transgenic plants. Similar reports of deviation from Mendelian ratios have been made in studies with oat (Cho *et al.*, 1999), barley (Wan and Leamux, 1994) and rice (Li *et al.*, 1997). The in vivo expression of the transgenes can be affected by factors such as transgene-locus number (Elmayan and Vaucheret, 1996; Jorgensen *et al.*, 1996; Matzke *et al.*, 1994), the genomic context of the integrated loci or position effects and abnormal configuration of the integrated T-DNA in the plant genome. All these factors may lead to variation in expression between plants with different integration patterns. The consistent expression of *AtZIP1* cDNA in T_0, T_1 and T_2 in all the progeny studied suggests the stable integration of the transgene into the

plant genome (sections 5.3.3.3, 5.5.2 and 5.6). The influence of transgene copy number on the level of gene expression is complex (Dai *et al.*, 2001). Multiple gene copies frequently lead to co-suppression and gene silencing (Vaucheret *et al.*, 1998) and transgene copy number can be positively or negatively associated with transgene expression (Hobbs *et al.*, 1993). In the present study, silencing of the transgenes was not observed in the progeny of all the generations of transgenic plants studied. All the three transgenic lines 25-1, 51-2 and 91-3 showed overexpression of the *AtZIP1* cDNA (sections 5.5.2 and 5.6). In the northern analysis, the expression levels of the transgenes did not correlate with the copy number. Line 91-3 which had a single copy of the transgene showed similar levels of expression of the transgene to line 51-2 that had 3 copies of the transgene (sections 5.5.2 and 5.6, Figs. 21 A, B and C). Line 25-1 that had two copies of the transgene showed lower levels of expression when compared to line 91-3 (sections 5.5.2 and 5.6, Figs. 21 A, B and C). However, variation in gene expression levels in genetically modified plants is a general phenomenon and similar reports of lack of correlation between copy number and expression have been made in studies with barley (Tingay *et al.*, 1997; Trifonova *et al.*, 2001). In many cases copy number, position effects and the organization of a given insert account for highly variable levels of expression displayed by the transgenic plants (Cervera *et al.*, 2000).

In conclusion, in this study *H. vulgare* cv. Golden Promise plants transformed with *AtZIP1* cDNA from *A. thaliana* and overexpressing the cDNA stably across different generations were produced.

Chapter 6   Zinc uptake studies with transgenic *H. vulgare* cv. Golden Promise plants overexpressing *AtZIP1*

6.1   *Introduction*

Zinc deficiency is a worldwide nutritional constraint for crop production and is widespread in cereals growing in calcareous soils and in all climates (Graham *et al.*, 1992). Selecting and breeding crops with high zinc efficiency is one way of overcoming reductions in yield and growth due to zinc deficiency. Zinc efficiency has been defined as the ability of plants to grow and yield well in soils too deficient for a standard genotype (Graham, 1984) or as the ratio of grain yield under zinc deficiency to ratio of grain yield under zinc sufficiency. Shoot dry weight under zinc deficient conditions is also considered to be a good efficiency indicator (Rengel and Graham, 1995a). The mechanisms governing zinc efficiency are not fully understood and it has been suggested that more than one factor may be involved in defining the efficiency of a particular cereal crop (Cakmak *et al.*, 1997; Cakmak *et al.*, 1998; Graham, 1984; Kalayci *et al.*, 1999; Rengel and Graham, 1995b; Schlegel *et al.*, 1998). Therefore a combination of these factors provides the most reliable method to determine whether a genotype is zinc efficient (Streeter *et al.*, 2001).

Overexpression of transporters in plant roots is one approach, which can be used to investigate whether increasing the number of ion transporters in roots increases uptake and thereby enhances zinc efficiency in plants. Work done in the previous chapter identified three lines of *H. vulgare* cv. Golden Promise that were overexpressing the zinc transporter *AtZIP1* from *A. thaliana*. One of the aims of this chapter was to investigate if overexpression of zinc transporters increased zinc uptake in the transgenic lines of *H. vulgare* cv. Golden Promise (Barley). The other aim of this chapter was to determine if the activity of overexpressed zinc transporter is regulated post-translationally in response to exposure to high levels of zinc in plants.

6.2   Methods

6.2.1   Growth of transgenic barley in hydroponics

Seeds of untransformed barley cv. Golden promise (GP) and overexpressing transgenic lines 51-2(7), 51-2(8), 91-3(3), 91-3(6) and null [91-3(9)] were surface sterilized and germinated on Whatman filter paper discs in Petri plates in darkness. Seedlings with emerging plumules and radicles were placed in foam rubber plugs and roots were placed in 1/4th strength Hoagland's solution (Table 19, Appendix 1.19). Two plastic tanks with a capacity of 50 litres were used to grow the barley seedlings. All solutions were made in nanopure water and the tanks contained reverse osmosis (RO) water. The nutrient solutions in the tanks were changed every 5 days. The plants were grown under a 16 h light and 8 h dark cycle.

6.2.2   Zinc treatments of barley plants

Twleve to fifteen day old barley plants (GP-UT, 51-2(7), 51-2(8), 91-3(3), 91-3(6) and null [91-3(9)] were subjected to zinc deprivation treatments. Plants were deprived of zinc for 0, 0.5, 1, 3, 12 and 24 hours in 1/4th strength Hoagland's nutrient solution minus zinc in smaller plastic containers. All the nutrient solutions were made in nanopure water. For resupply experiments, overexpressing line 51-2(7) and null line 91-3(9) were deprived of zinc for 3 hours in 1/4th strength Hoagland's nutrient solution minus zinc and zinc was resupplied to the plants for 0, 0.5, 1 and 2 hours by placing them in 1/4th strength Hoagland's nutrient solution containing 0.8 μM ZnCl$_2$. Zinc uptake was measured with ^{65}Zn.

6.2.3   Uptake assays with ^{65}Zn isotope

Zinc uptake assays were performed using a modified protocol according to Hart et al. (1996). All the uptake experiments were done under a (400 watt) halogen light with a light intensity of 150 μmol m^2 / sec measured using a light meter (Delta T AP4). Stomatal conductance of the plants was measured with a Delta T porometer

AP4 and ranged from 193 mmol m^2/sec to 279 mmol m ^{2}/sec under experimental conditions. During the course of the experiments, the solutions were agitated gently at intervals of 5 min each. Zinc deprived plants were placed in uptake assay buffer containing 2 mM MES-Tris, pH 6.0, 0.05 mM CaCl$_2$ minus zinc for 10 min. An aliquot of 6 μl of 100 mM ZnCl$_2$ (final concentration = 10 μM) per plant was added to the container 1 min before the addition of radioactive ^{65}Zn isotope. Uptake was measured for 20 min after the addition of 0.069 μM ^{65}Zn (NEN Life Science Products) in case of zinc accumulation experiments. Uptake was measured for only 5 min after the addition of the isotope in case of resupply experiments. At the end of the uptake period, plants were transferred to a container with desorption solution (100 μM ZnCl$_2$ / 5 mM CaCl$_2$ / 2 mM MES-Tris, pH 6.0) containing 60 ml solution/ plant, for 15 minutes. Plants were harvested after desorption, roots were blotted dry and the fresh weights of the roots and shoots measured. Weighed roots and shoots were placed in scintillation vials, scintillation fluid added and ^{65}Zn in the tissue was measured with a Beckman Scintillation Counter (Beckman, LS 380). Background was determined after addition of 200 μl of uptake assay buffer to 4 ml of scintillation fluid and measured with a Beckman Scintillation Counter (Beckman, LS 380). Specific activity was measured by taking an aliquot of 200-250 μl of the uptake assay buffer with the isotope and adding 4 ml of the scintillation fluid. Three replicates/plants per line were used in the experiment and experiments were repeated on two separate days. Zinc uptake rates have been expressed as nmol Zn /gFW roots/ hr and nmol Zn /gFW shoots/ hr. Results represent the average of six plants.

6.3   *Results*

6.3.1 ^{65}Zinc accumulation studies

Zinc uptake into the roots and transport to shoots was measured in the transgenic barley lines [51-2(7), 51-2(8), 91-3(3), 91-3(6)], null line [91-3(9)] and untransformed Golden Promise (GP-UT) under control conditions and when subjected to zinc deprivation treatments for different lengths of time. No

differences were observed in the zinc uptake or accumulation in zinc sufficient plants in the various lines studied (Figs. 22 A and 23 A). When the plants were deprived of zinc for 0.5 hours, the overexpressing transgenic lines [91-3(3), 91-3(6), 51-2(7), 51-2(8)] accumulated 2-3 fold more zinc in both roots and shoots in comparison to the null line and GP-UT. Three hours after zinc deprivation, GP-UT had a zinc uptake rate of 194 nmol/ gFW /hr into the roots while the various overexpressing lines had a zinc uptake rate ranging from 261 to 289 nmol/ gFW /hr in the roots (Fig. 22 C). Zinc transport to shoots 3 hours after zinc deprivation in GP-UT was 8.2 nmol/gFW /hr in comparison to the overexpressing lines which showed zinc transport rates ranging from 14.1 to 19.5 nmol/ gFW /hr (Fig. 23 C). Zinc uptake rate decreased in both the roots and shoots of GP-UT with increase in zinc deprivation time to 12 hours (Figs. 22 D and 23 D). In comparison, the zinc uptake rate of the overexpressing plants continued to increase in both roots and shoots and reached a maximum of 354 nmol/gFW /hr and 31.6 nmol/ gFW /hr respectively after 12 hours zinc deprivation. When the plants were deprived of zinc for 24 hours, a lower zinc uptake rate was observed in the roots and shoots of GP-UT and null plants compared with other plants (Fig. 22 D and 23 D). The zinc uptake rate was lower in the roots and shoots of plants from the overexpressing lines compared with rates after 12 hr zinc deficiency but the uptake rate was still higher (2-3 fold) when compared to the GP-UT or null line tested (Fig. 22 D and 23 D). Thus the overexpressing transgenic lines of barley had a higher zinc uptake rate in both roots and shoots when compared to GP-UT and the null line after 3-24 hours zinc deprivation.

6.3.2 Resupply experiments

Zinc uptake rates in an overexpressing line 51-2(7) and null line 91-3(9) were measured after resupplying the plants with zinc after 3 hours of zinc deprivation to investigate whether the activity of an overexpressed zinc transporter (*AtZIP1*) is regulated post-translationally in plants. Zinc uptake rate in the roots of line 51-2(7) and null 91-3(9) were 283 nmol /gFW /hr and 235 nmol /gFW /hr three hours after zinc deprivation. When the plants deprived of zinc for three hours were exposed to zinc for different lengths of time, a more rapid decrease in the zinc

uptake activity was observed in the overexpressing line 51-2(7) as compared to the null line 91-3(9) (Figs. 24 A and B). The decrease in the zinc uptake rate in the line 51-2(7) was rapid in the first 30 min and reached the basal rate of ~50 nmol/ gFW /hr previously measured under zinc sufficient conditions (Fig. 22 A). These results suggest that when plants with a high level of the *AtZIP1* zinc transporter activity were exposed to zinc, zinc-induced loss of activity occurred in these plants.

6.4   Discussion

6.4.1   Overexpression of transporters and their effect on zinc uptake

Differences in zinc uptake have been studied in a number of cereals (Graham *et al.*, 1992; Rengel and Graham, 1996) as increased zinc uptake into the roots and shoots is one of the factors involved in determining zinc efficiency. In this study, 3 hours after zinc deprivation large differences in zinc uptake rates into the roots were observed in both transformed and untransformed barley plants (Fig. 22 C) as compared to uptake rates by zinc sufficient plants (Fig. 22A). These results suggest the induction of endogenous zinc transporters in response to zinc deprivation account for higher zinc uptake rates in untransformed controls and partially contribute to the higher uptake rates in barley plants overexpressing *AtZIP1*. Plants overexpressing the *AtZIP1* transporter showed higher uptake rates after 3, 12 and 24 hours zinc deprivation when compared to the untransformed control. These results show that both zinc deprivation and overexpression of *AtZIP1* lead to increased zinc uptake in the transgenic barley plants.

Increased zinc uptake and accumulation in response to zinc deficiency has been observed in the roots and shoots of wheat and chickpea respectively (Khan *et al.*, 1998; Nable and Webb, 1993; Rengel and Graham, 1996). Zinc efficient rye had the highest rate of root to shoot translocation of zinc when compared to bread and durum wheat cultivars (Erenoglu *et al.*, 1999) in zinc deficient conditions. In studies with the hyperaccumulator *T. caerulescens* it has been hypothesized that increased zinc uptake in to the roots was due to an increased abundance of zinc transporters in root cell plasma membranes (Lasat *et al.*, 1996; Lasat *et al.*, 2000)
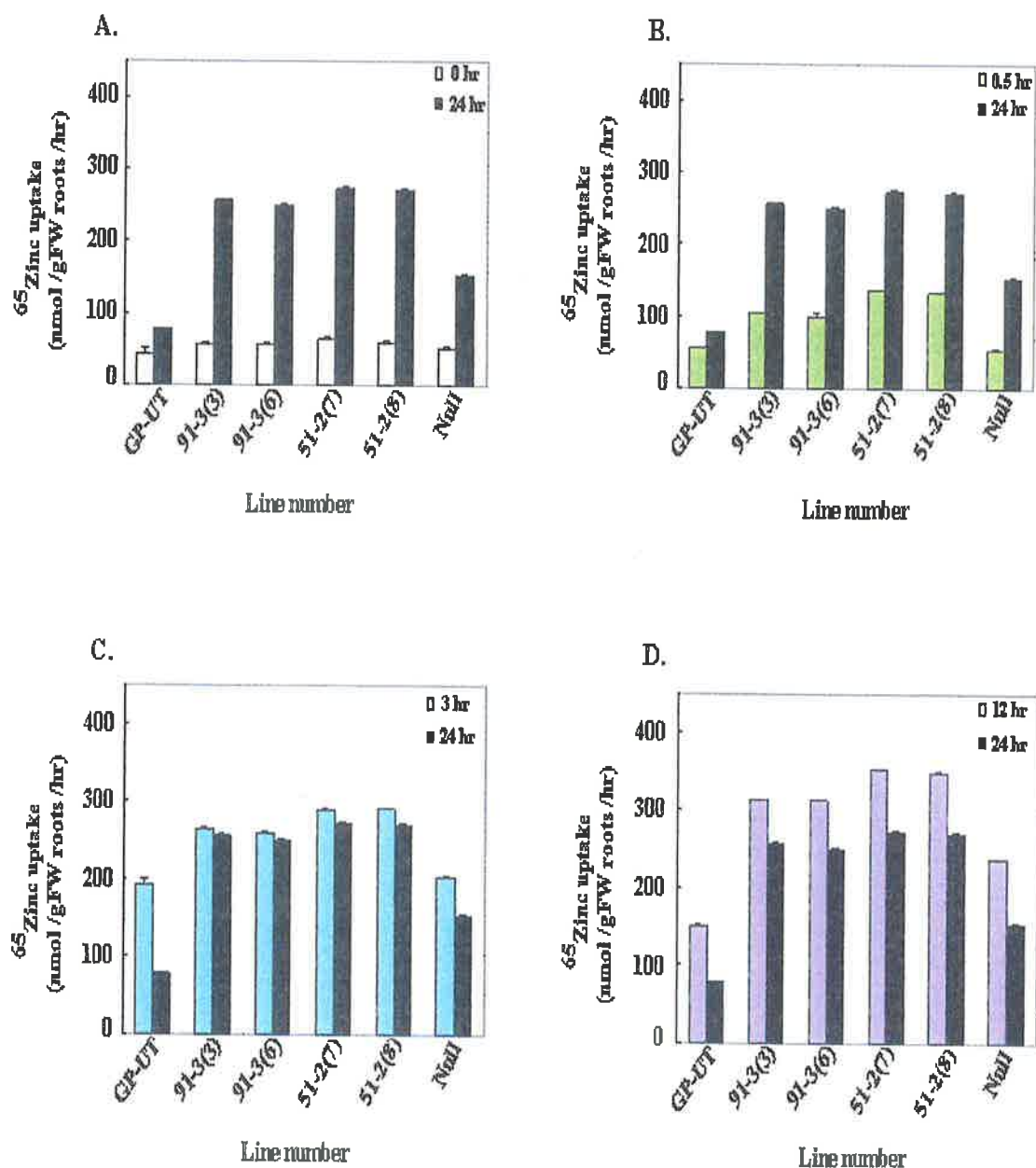
Fig. 22 Comparison of zinc uptake rates in the roots of transgenic vs. non transgenic *H. vulgare* Golden Promise plants. Plants from Golden Promise (GP-UT), transgenic lines 51-2(7), 51-2(8), 91-3(3), 91-3(6) and null line 91-3(9) were grown in 1/4th strength Hoagland's nutrient solution for 12-15 days. Zinc uptake was measure in both the transgenic and untransformed Golden Promise plants in zinc sufficient conditions (A, 0 hr) and when plants were deprived of zinc for different lengths of time (B-D, 0.5, 3, 12 and 24 hr) in 1/4th strength Hoagland's nutrient solution. The uptake assay buffer contained 10.1 μM $ZnCl_2$. Error bars represent the standard error calculated from two separate experiments with a total of six plants. Error bars smaller than symbols are not visible.
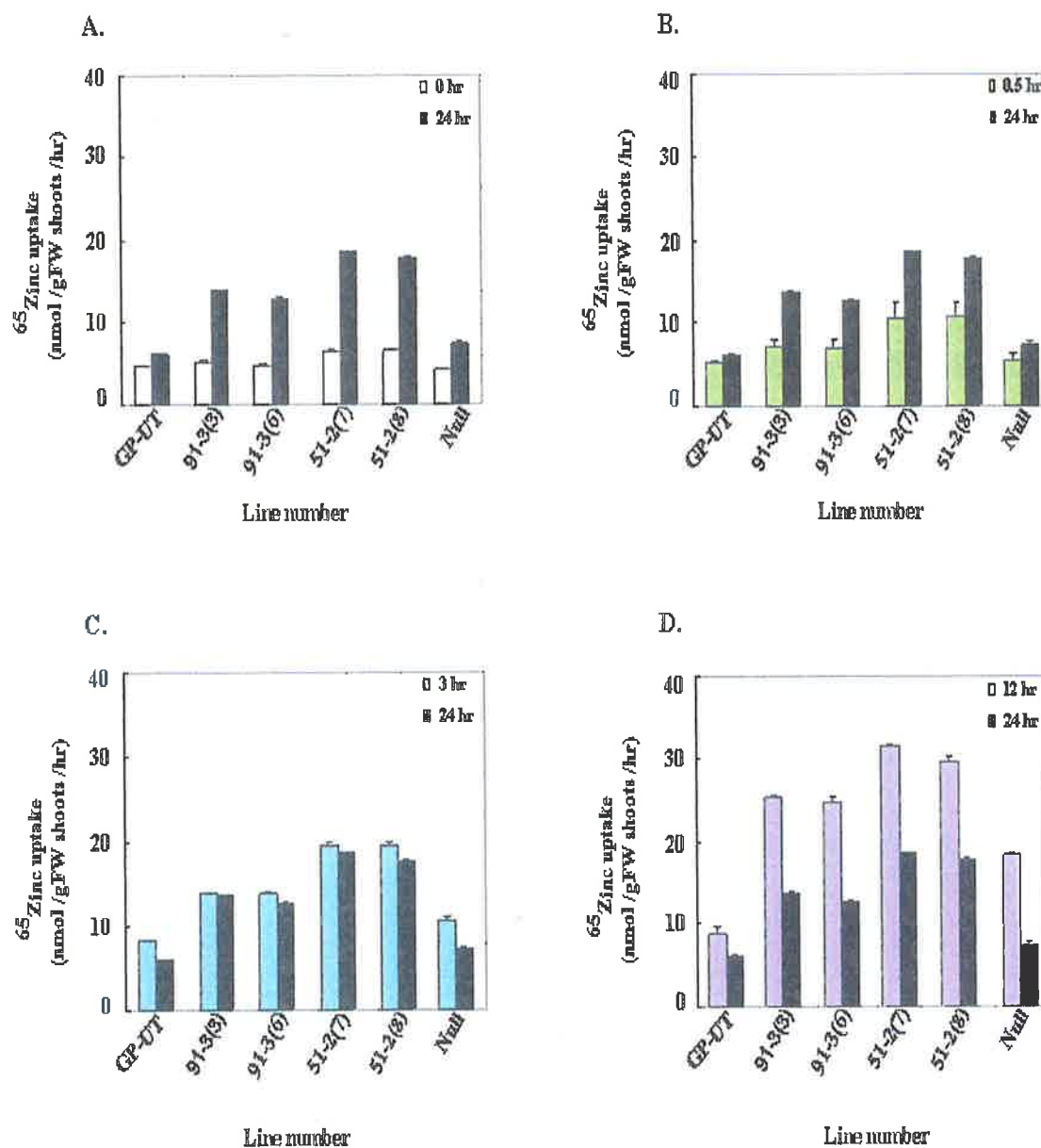
Fig. 23 **Comparison of zinc uptake rates in the shoots of transgenic vs. non transgenic** *H. vulgare* **Golden Promise plants.** Plants from untransformed Golden Promise (GP-UT), transgenic lines 51-2(7), 51-2(8), 91-3(3), 91-3(6) and null line 91-3(9) were grown in 1/4th strength Hoagland's nutrient solution for 12-15 days. Zinc uptake was measure in both the transgenic and untransformed Golden Promise plants in zinc sufficient conditions (A, 0 hr) and when plants were deprived of zinc for different lengths of time (B-D, 0.5, 3, 12, and 24 hr) in 1/4th strength Hoagland's nutrient solution. Plants were placed in uptake assay buffer with 10.07 μM $ZnCl_2$ and assayed for ^{65}zinc uptake activity. Error bars represent the standard error calculated from data obtained from two separate experiments with a total of six plants. Error bars smaller than symbols are not visible.
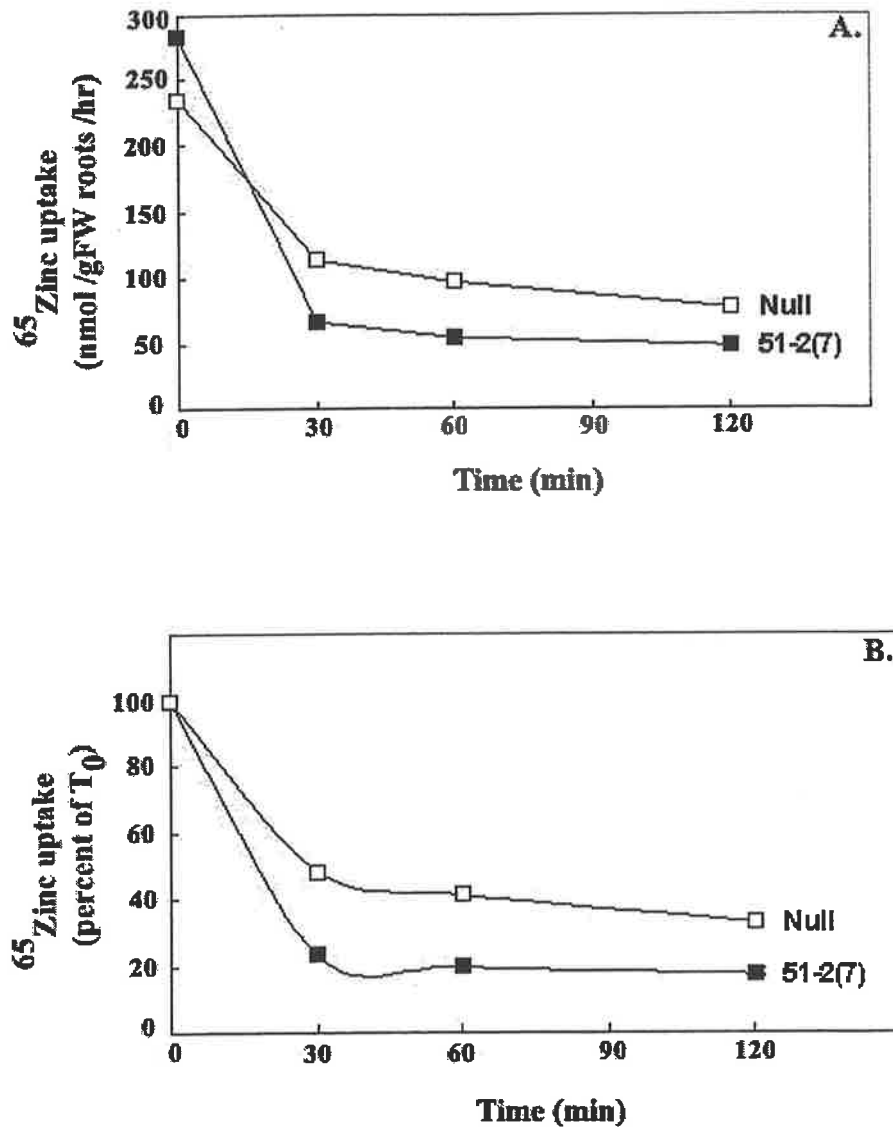
Fig. 24 Comparison of uptake rates in zinc deficient overexpressing transgenic line vs. null line on exposure to zinc. (A) Plants from line 51-2(7) [closed squares] and null -91-3(9) [open squares] were grown in 1/4th strength Hoagland's solution for 12 days and deprived of zinc for 3 hours. Zinc was reintroduced to the nutrient solution containing plant roots for indicated lengths of time. The plants were placed in uptake assay buffer with 10.07 µM $ZnCl_2$ and assayed for [65]zinc uptake rate. (B) The data presented in (A) is replotted as a percentage of uptake rate at time zero for each line. Error bars represent the standard error calculated from data obtained from two separate experiments with a total of six plants. Error bars smaller than symbols are not visible.

In long term experiments under field conditions and in short- term experiments under controlled conditions zinc efficient wheat genotypes had higher zinc uptake than the zinc inefficient genotypes (Cakmak *et al.*, 1997; Graham *et al.*, 1992; Rengel and Graham, 1996; Rengel *et al.*, 1998). Zincefficient wheat cultivars were observed to have a higher maximum net uptake rate than the zinc inefficient cultivars under zinc deficiency (Rengel and Wheal, 1997) though in both similar K_m values were measured. Studies on concentration dependent kinetics of root zinc uptake in two different wheat species found that zinc efficient bread wheat cultivar had a higher zinc influx rate than the inefficient cultivar (Hart *et al.*, 1998).

The results presented here extend previous results and show that overexpression of *AtZIP1* increased zinc uptake by the roots and enhanced translocation into the shoots in response to zinc deprivation. The time course observed for increased zinc uptake after deprivation in this study was very rapid (<3 hours). Overexpression of zinc transporter genes leading to increase in mRNA abundance has been suggested to be responsible for increased uptake in the root cells of hyperaccumulator *T. caerulescens* (Lasat *et al.*, 2000; Pence *et al.*, 2000). Our time course studies also showed increased uptake by the roots and this was presumably due to an increased abundance of the transporters in the roots. Molecular evidence in support was obtained from northern analysis (Figs. 20 and 21, Chapter 5), where it was evident that *AtZIP1* mRNA abundance was higher in both roots and shoots of transgenic barley plants as compared to the untransformed barley plants. Increased translocation of zinc to the shoots was also observed in the time course studies. However, it is not yet clear whether increased uptake is driving the increased translocation to shoots or if the overexpression of zinc transporters in specific cells that control xylem unloading is driving the increased translocation to shoots.

Thus, overexpression of *AtZIP1* led to the increase in zinc uptake and translocation capacity in barley plants in the short term under zinc deficient conditions. This increase in zinc uptake and translocation in overexpressing plants might be quite significant; however growth experiments must be conducted over the long term to determine how overexpression of *AtZIP1* affects plant growth under zinc deficient and sufficient conditions.

6.4.2 Are zinc transporters in plants regulated at the post-translational level?

Our knowledge of zinc homeostasis in eukaryotes comes from studies in *S. cerevisiae* (Gaither and Eide, 2001). Zinc is an essential micronutrient but can be toxic if accumulated in excess amounts. Thus with changes in zinc levels in the external medium, cells must regulate the supply of intracellular zinc. The activity of high affinity and low affinity zinc transporters *ZRT1* and *ZRT2* is regulated by the transcription factor ZAP1 (section 1.7.1, Chapter 1). ZRT1 is a stable plasma membrane protein in zinc-limited cells, but exposure to high levels of extracellular zinc (1 µM-2 mM) triggers the endocytosis and degradation of *ZRT1* (Gitan *et al.*, 1998) [section 1.7.3.2, Chapter 1]. This zinc-induced endocytosis is specific to *ZRT1*, occurs specifically in response to zinc and is a specific regulatory system that shuts off zinc uptake activity in yeast cells exposed to high levels of zinc to prevent toxicity. Zinc as well as copper has been observed to stimulate the endocytosis of the Prion protein (*PrP^c*) in mammalian cells (Pauly and Harris, 1998). Regulated endocytosis of plasma membrane proteins in response to changing nutrient availability has been observed with other plasma membrane proteins in yeast such as endocytosis of maltose permease by glucose (Medintz *et al.*, 1996; Riballo *et al.*, 1995) and of galactose permease by GAL2 (Chiang *et al.*, 1996). The role of ubiquitin in endocytosis has been shown in the case of yeast STE2 α-factor mating-pheromone receptor (Hicke and Riezman, 1996), STE3 α-factor receptor, the STE6 mating-factor transporter and FUR4, MAL61 and GAP1 nutrient transporters (Galan *et al.*, 1996; Kolling and Hollenberg, 1994; Roth and Davis, 1996; Springael and Andre, 1998).

The activity of the zinc transporter *AtZIP1* from *A. thaliana* (Grotz *et al.*, 1998) is regulated post-translationally when expressed in yeast (Guerinot, 2000) but nothing is known about its regulation in plants. In this study a rapid reduction in zinc uptake was observed in plants overexpressing *AtZIP1* and the control in response to resupply of zinc. This result is in agreement with the previous work done with the *ZRT1* transporter from yeast (Gitan *et al.*, 1998). Interestingly the reduction of zinc uptake in plants overexpressing *AtZIP1* was more rapid when compared to the control. The more rapid decrease in the activity of AtZIP1 in the overexpressing line suggests that perhaps degradation proceeds at a more rapid

rate in these plants. This may be because the ubiquitination system may already be operating in the transgenic plants to regulate the production of excess protein due to high levels of constitutive expression. This regulation may be very important for the success of overexpression strategies so that even under zinc sufficient conditions the overexpressing lines will not accumulate toxic levels of zinc.

The results presented in this chapter suggest that in barley plants overexpressing *AtZIP1*, the activity of the transporter is regulated at the post-translational level in response to extracellular zinc. This finding is exciting as it suggests that the post-translational mechanism to maintain zinc homeostasis observed in *S. cerevisiae* is also operating in plants and that these processes are evolutionarily conserved. Inactivation of a zinc transporter from *Arabidopsis* when expressed in barley also suggests that the inactivation mechanism is conserved between plant species. In studies with *ZRT1*, it was suggested that one mechanism of zinc sensing might involve direct binding of zinc to the transporter at the potential metal binding domain HDHTHED and this binding could induce endocytosis of the protein (Gitan *et al.*, 1998). This metal binding domain is located in the same inter-membranous domain loop as the ubiquitinated lysine residue (Gitan and Eide, 2000) that is involved in the process of endocytosis. It is interesting to note that *AtZIP1* also has a potential metal binding domain HGHTHG in the same inter-membranous loop in which lies a homologous ubiquitinated lysine residue in *ZRT1*. This provides further evidence for evolutionary conservation of homeostatic regulation of zinc concentrations in eukaryotes. However more studies will be needed with *AtZIP1* and other plasma membrane transporters to understand the mechanisms of metal-regulated endocytosis in post-translational regulation in plants.

Chapter 7   General discussion

Zinc is an essential micronutrient involved in various growth and metabolic processes of plants. Factors such as limited mobility in soil solution, pH and alkalinity affect the bioavailability of zinc to plants. Zinc uptake by plants is also determined by genetic differences, which is based on observation that some plants are more zinc efficient than others. The mechanisms of zinc uptake and regulation are beginning to be understood in yeast and dicotyledonous plants but little is known about the mechanism of zinc uptake, regulation and zinc efficiency in monocotyledonous plants such as cereals. Thus this study was aimed at understanding:

a)   the molecular basis of zinc uptake;

b)   the mechanism of regulation of zinc;

c)   the effect of zinc transporter overexpression on the zinc uptake efficiency in cereals using rice and barley as model systems.

Zinc transporters from *A. thaliana* (Grotz *et al.*, 1998) cloned by complementation of the zinc deficient yeast mutant ZHY3 provide the starting point for understanding the molecular mechanism of zinc uptake in higher plants. In this thesis work, three full length and one partial length cDNA clones from *O. sativa* (Chapter 2) have been identified which show high degree of sequence homology to the known *Arabidopsis* zinc transporters. Recent analyses have shown that the ZIP family members are not restricted to eukaryotes but are also found in archeae and eubacteria (Gaither and Eide, 2001) suggesting a very ancient origin for this family. The ZIP family was subdivided into four subfamilies and the rice transporters belong to a single subfamily (Gaither and Eide, 2001).

In this study, the expression of three rice ZIP transporters *OsR06*, *OsS13* and *OsS16* were induced in response to zinc deprivation (Chapter 4) and thus can be classified as zinc responsive. The fourth rice ZIP transporter *OsE60* was constitutively expressed in roots and shoot which is a new finding in plants. This *OsE60* transporter gene is also more selective for zinc as the substrate (Chapters 3 and 4). The data from characterization of *OsE60* suggests that this gene might be involved in a housekeeping function in zinc accumulation. This fact might be

quite significant in mineral nutrition as most of the plant ZIP genes identified to date, transport a broad range of divalent cations. It might be interesting to study the role of *OsE60* in zinc nutrition by knocking out the expression of this gene and characterizing the knockouts to gain an insight into the role of this transporter as well as the integrated roles of different rice zinc transporters in planta.

The zinc responsive nature of the gene encoding the rice transporter *OsS16* is surprising as this transporter clusters with the iron-regulated transporters from plants (Chapter 2). The iron-regulated transporters from *A. thaliana* (Eide *et al.*, 1996; Korshunova *et al.*, 1999; Vert *et al.*, 2001) and *L. esculentum* (Eckhardt *et al.*, 2001) complement the zinc deficient mutant when expressed in yeast suggesting that these transporters transport both iron and zinc. However nothing is known about the expression of these iron-regulated transporters in response to zinc deficiency. It would be interesting to obtain the full-length cDNA of *OsS16* and test it for complementation in different yeast transport mutants. A different approach would be to grow the plants under different iron regimes and study the expression of this gene in response to iron deficiency. Together this data would provide insight into aspects such as substrate specificity and metal ion homeostasis.

Data from the functional (Chapter 3) and expression analysis (Chapter 4) of the zinc regulated rice transporter *OsR06* suggests that this transporter has broad substrate specificity as is seen with other ZIP transporters *AtZIP1–3* (Grotz *et al.*, 1998) and *AtIRT1* (Eide *et al.*, 1996; Korshunova *et al.*, 1999). *OsR06* transports cadmium and confers sensitivity to this toxic ion when expressed in yeast. The activity of this transporter is upregulated in response to zinc deficiency and thus it might be responsible for transport and accumulation of cadmium in plants. Similar cadmium transport activity has been observed with the iron-regulated transporters *AtIRT1*, *LeIRT1* and *LeIRT2* in plants. It has been hypothesized that cadmium enters the plants via roots due to uptake by native iron/zinc transporters. Further work with *OsR06* would involve mutation studies in the conserved region or the metal binding domain wherein the amino acids could be replaced with residues such as alanine. The resulting site directed mutants could be transformed into yeast mutants and tested for complementation. This would provide us with an insight into the residues conferring substrate specificity to metal ion transport. It would also be possible to use this knowledge

to engineer plants to selectively transport cadmium and this would have a good application in the phytoremediation of contaminated soils.

The data from chapters 3 and 4 shows that low expression levels of *OsS13* in yeast hampered the functional characterization of this zinc-regulated transporter.

The common theme that emerges from the study of the already identified plant ZIP transporters and the rice transporters in this study is the involvement of multiple transporters in the uptake and transport of a single cation. The question that arises from all these studies is why do plants require so many transporters and is there a redundancy in their function? Since metal ions need to be taken up and transported across the different organellar and cellular membranes, it is possible that the multiple transporters are localized to and expressed in different cell types. Evidence for this comes from the studies with the iron-regulated transporters from *A. thaliana* wherein both *AtIRT1* and *AtIRT2* were expressed in root tissue but *AtIRT2* localized to the primary and secondary root subapical root zones (Vert *et al.*, 2001). Thus it is possible that the zinc-regulated rice transporters expressed in the root would localize to different cell types. Specific cellular localization of the iron-regulated transporter *AtIRT2*, which is involved in rhizosphere acidification and iron reduction, also argues against the redundancy in function of the transporters. It is also hypothesized that the expression of multiple transporters might be modulated by additional stimuli such as light, phytohormones and nutritional status (Vert *et al.*, 2001) to cater to different developmental and physiological requirements of the plants. *OsR06*, *OsS13* and *OsS16* might be active in metal deplete conditions while *OsE60* might be active in metal replete conditions. Thus, in order to elucidate the precise role of multiple transporters in uptake and transport of a single cation, it would be necessary to carry out tissue and cell specific localization of these transporters.

Plants that are more efficient in uptake of zinc grow better in zinc deficient soils. The exact mechanism of zinc efficiency is not yet known, but we do know that zinc efficient plants show increased uptake and accumulation of zinc in the roots and increased translocation into the shoots (Cakmak and Romheld, 1997; Erenoglu *et al.*, 1999; Khan *et al.*, 1998; Rengel and Graham, 1996). A transgenic approach was used in this thesis to investigate the effect of overexpression of *Arabidopsis* zinc transporters on zinc uptake in barley plants (Chapter 5). The

overexpression of *AtZIP1* in *H. vulgare* led to increased uptake of zinc in the roots and increased translocation to the shoots as compared to controls (Chapter 6). The shoots of overexpressing plants took up more zinc after 3-24 hours zinc deprivation in comparison to controls. Increased translocation of zinc into shoots is important in maintaining growth of plants when growing in zinc deficient soils. As the transgenic plants in the present study had enhanced zinc uptake into roots and increased translocation into shoots, it suggests that overexpression of transporters could lead to enhanced zinc uptake efficiency. However the overexpressing transgenic plants will need to be tested to evaluate the long term effects of enhanced zinc uptake and translocation on the growth of plants in soils with differing zinc concentrations. Plants could be evaluated in the field for characteristics such as yield, zinc concentration / accumulation in the roots and shoots all of which are indicators of zinc efficiency. It will also be important to measure the kinetic parameters such as the maximum net uptake rate and the K_m values between the overexpressing transgenic and untransformed lines. Together these studies might provide more information on role of increased zinc uptake in determining the zinc efficiency in cereals.

In order to maintain the concentration of essential metals within physiological limits and to minimize the detrimental effects of nonessential metals, plants have evolved a complex network of homeostatic mechanisms that serve to control the uptake, accumulation, trafficking and detoxification of metals (Clemens, 2001). The main components of metal homeostasis are transport, chelation and sequestration processes. Their regulated activities ensure the proper delivery and distribution of metal ions at the cellular and at the organismal level, resulting in a basic level of metal tolerance. Zinc is an essential nutrient for all living organisms and plays a critical role in various biochemical and physiological processes. Although zinc is essential, it can be toxic in excess and therefore cells must control intracellular levels when exposed to high zinc concentrations. *S. cerevisiae* possesses a system consisting of transcriptional and post-translational control for regulating the uptake of zinc (section 1.7, Chapter 1). In *A. thaliana*, there is evidence that expression of zinc transporters *AtZIP1*, *AtZIP3* and *AtZIP4* are metal responsive suggesting regulation by a transcriptional control mechanism. *OsR06*, *OsS13* and *OsS16* rice transporters identified in this study (Chapter 4) are zinc responsive suggesting a transcriptional control of these

transporters in response to changing zinc levels in the external medium. Further proof for transcriptional regulation can be obtained by constructing promoter/5'UTR-GUS reporter gene constructs and introducing these fusion into rice plants and analyzing the GUS activity in transformed lines grown under zinc deficient and sufficient conditions. Similar work with *AtIRT2* proved that the expression of this transporter is regulated at the transcriptional level in response to iron status (Vert *et al.*, 2001). The zinc transporter *AtZIP1* from *A. thaliana* (Guerinot, 2000) undergoes post-translational regulation in response to excess zinc levels when expressed in yeast but nothing was known about its activity in plants. In the present study, when the zinc-limited *H. vulgare* plants overexpressing *AtZIP1* transporter were exposed to excess levels of zinc, there was a rapid decline in the activity of *AtZIP1* (Chapter 6). This finding suggests that the activity of *AtZIP1* transporter from *A. thaliana* is post-translationally regulated in barley plants. This result points to the fact that there may exist a similar mode of regulation and homeostasis of zinc that is conserved in both yeast and plants. However further studies will be needed with other plants transporters to determine if the same mechanism of post-translational control as seen in yeast operates in higher plants. Preparation of specific antibodies to actually look at the changing levels of protein might be a good avenue for future research.

In summary, this work provides a starting point for understanding the molecular mechanisms of zinc uptake and the regulation of zinc transport in cereals. At least two different zinc transporters in rice were identified which establishes the beginning for future advances in understanding how zinc transport is controlled at the molecular level. The overexpression of a zinc transporter in barley proved that short term zinc uptake rates can be increased using genetic engineering and that the activity of transporters is post-translationally regulated in plants in response to zinc sufficiency. Even though the zinc transporter activity was down regulated in transgenic plants under zinc sufficient conditions, the increased zinc uptake under zinc deficient conditions by overexpression of transporters, could be exploited for creating zinc efficient cereals. Zinc efficient cereals would yield more on soils with low zinc and could potentially result in increased zinc content of grains. Future advances will come from additional studies on the regulation of zinc transport and from a thorough understanding of where specific transporters are expressed.

Appendix
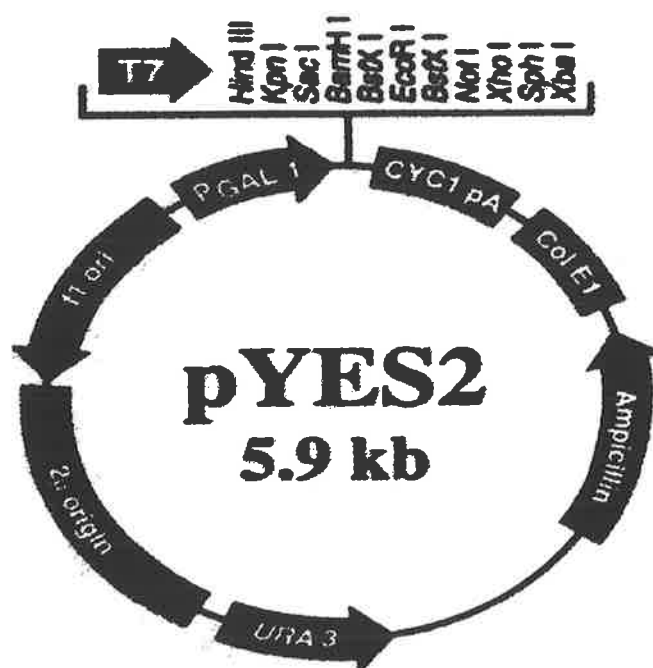
1.1   Yeast expression vector pYES2.

1.2

Table 1   Composition of Luria-Bertani medium.

Medium	Component	Composition
LB	Bacto-tryptone Bacto-yeast extract Sodium chloride Bacto-agar Distilled water	10 g 5 g 5 g 15 g to 1000 ml

Autoclave for 20 min at 121°C and cool to 50°C before pouring the plates. For liquid medium, do not add agar.

1.3

Table 2   Composition of solutions used in the transformation of *S. cerevisiae*

Solution A

Chemical	Final
Bicine, pH 8.35	10 mM
Sorbitol	1 M
Ethylene glycol	3 %

Solution B

Chemical	Final
Bicine pH 8.35	200 mM
PEG 1000	40 %

Solution C

Chemical	Final
Bicine, pH 8.35	10 mM
NaCl	150 mM

1.4

Table 3A   Composition of Low Zinc Medium (LZM)

	Chemical	*Stock concentration*	*Final concentration*	*Volume/L*
Macronutrients	Sodium-EDTA	0.5 M	1 mM	2 ml
	Magnesium sulphate.4H$_2$O	0.5 M	5 mM	10 ml
	Sodium chloride	0.1 M	1 mM	10 ml
	Calcium chloride.2H$_2$O	0.1 M	1 mM	10 ml
Amino acids	As per Table 5b			100 ml
	Ammonium sulphate	3.8 M	38 mM	10 ml
	Potassium dihydrogen phosphate	0.1 M	1 mM	10 ml
	Tri-sodium citrate.2H$_2$O	1 M	20 mM	20 ml
Carbon source	Galactose	2%		20 g
Vitamins	d-Biotin	16 µM	0.016	195 µl
	Calcium pantothenate	1.7 mM	1.7 µM	202 µl
	Myo-inositol	10 mM	10 µM	0.5 ml
	Pyridoxine hydrochloride	2 mM	2 µM	1 ml
	Thiamine Hydrochloride	1 mM	1 µM	1 ml
Micronutrients	Boric acid	0.1 M	10 µM	100 µl
	Copper sulphate	2 mM	0.2 µM	100 µl
	Potassium iodide	5 mM	0.5 µM	500 µl
	Manganese chloride	0.25 M	25 µM	100 µl
	Sodium molybdate	0.01 M	1 µM	10 µl
	Ferric chloride	0.1 M	10 µM	100 µl
	Zinc sulphate	0.1 M	Varied	As required

Note: Low iron medium *(LIM)* is the *same as LZM except that 10 µM ferric chloride is replaced by 10 µM zinc sulphate.*

1.4

Table 3 B **Composition of the amino acid stock used in the low zinc medium**

Amino acid	10X stock (mg/l)	Final concentration (mg)
L-Tryptophan	20	200
L-Histidine	20	20
L-Leucine	30	300
L-Lysine	30	300
L-Methionine	20	200
L-Tyrosine	30	300
Adenine	10	100

1.5

Table 4 **Composition of the complete medium (YPD) used for growth of the yeast strains**

Medium	Component	Composition
YPD	1%Bacto-yeast extract	10 g
	2% Bacto-peptone	20 g
	2% Galactose	20 g
	2% Bacto-agar	20 g
	Distilled water	to 1000 ml

1.6

Table 5   *Composition of the synthetic medium (YNB) used in the growth of yeast strains. YNB medium was made up from dehydratedDIFCO Bacto Yeast nitrogen base (without amino acids and ammonium sulphate) powder.*

Medium	Component	Composition
YNB	Bacto-yeast nitrogen base	1.7 g
	Ammonium sulphate	5.0 g
	2% Bacto-agar	20 g
	Distilled water	1000 ml

The following compounds are present in the YNB powder.

Vitamins	Biotin	20 µg
	Calcium pantothenate	2 mg
	Folic acid	2 µg
	Inositol	10 mg
	Niacin	400 µg
	p-Aminobenzoic acid	200 µg
	Pyridoxine	400 µg
	Riboflavin	200 µg
	Thiamine hydrochloride	400 µg
Compounds supplying trace elements	Boric acid	500 µg
	Copper sulphate	40 µg
	Potassium iodide	100 µg
	Ferric chloride	200 µg
	Manganese sulphate	400 µg
	Sodium molybdate	200 µg
	Zinc sulphate	400 µg
Salts	Potassium phosphate monobasic	850 mg
	Potassium phosphatedibasic	150 mg
	Magnesium sulphate	500 mg
	Sodium chloride	100 mg
	Calcium chloride	100 mg

100 ml of amino acid stock (Table 3B) per litre was added to the medium after autoclaving.

1.7 A

Table 6 Composition of the Tris-EDTA buffer

Tris-EDTA, pH 8.0 (TE)

Chemical	Stock concentration	Final concentration
Tris, pH 8.0	1 M	10 mM
EDTA, pH 8.0	0.5 M	1 mM

1.7 B

Table 9   Ion content of ZHY3 cells expressing the *OsR06* and *OsE60* cDNAs and the empty plasmid pYES2.
ZHY3 cells expressing the Os cDNAs and containing the empty plasmid pYES2 were grown in YNB medium supplemented with the indicated concentrations of cadmium chloride (for 24 hours), calcium chloride and magnesium chloride (for 48 hours). Cultures were centrifuged and dried pellets were analyzed with ICP-AES. Control refers to growth of the yeast cells in YNB medium containing 0.7 mM $CaCl_2$, 2 mM $MgCl_2$ and 0 mM $CdCl_2$. Concentrations of the metal ions in the first row in the table represent the additional amount of cations added to the medium.

Values of ions in mg/g DW

Strains	Calcium content of cells grown in control	Calcium content of cells grown in 3.0 mM $CaCl_2$	Magnesium content of cells grown in control	Magnesium content of cells grown in 7.0 mM $MgCl_2$	Cadmium content of cells grown in control	Cadmium content of cells grown in 0.01 mM $CdCl_2$
ZHY3/pYES2	0.02 ± 0.003	0.05 ± 0.002	0.13 ± 0.017	0.41 ± 0.001	-	0.04 ± 0.001
OsR06/pYES2	0.02 ± 0.003	0.15 ± 0.002	0.14 ± 0.093	0.70 ± 0.002	-	0.14 ± 0.004
OsE60/pYES2	0.02 ± 0.004	0.05 ± 0.004	0.12 ± 0.020	0.61 ± 0.003	-	0.05 ± 0.0003

Values represent the mean and standard errors of three individual experiments: n = 3.

1.7 C

Table 10   **Comparison of the ion content of the ZHY3 cells expressing *OsR06* and *OsE60* cDNAs in pYES2 with ZHY3 transformed with empty plasmid pYES2.** ZHY3 cells expressing the Os cDNAs and containing the empty plasmid pYES2 were grown in YNB medium supplemented with indicated concentrations of calcium (for 48hr), magnesium (for 48 hr) or cadmium chloride (for 24 hr). Cultures were centrifuged and dried pellets were analyzed with ICP-AES. Ratios were calculated by dividing the ion contents of ZHY3 cells expressing the Os cDNAs with the ion content of ZHY3 cells expressing the empty plasmid pYES2. Concentrations of the metal ions in the first row in the table represent the additional amount of cations added to the medium

Metal Ion Content	Ratio of ion content in ZHY3 cells expressing *OsR06* to cell with empty plasmid pYES2	Ratio of ion content in cells expressing *OsE60* to cell with empty plasmid pYES2
Calcium chloride (3.0 mM)	3.0	1.0
Magnesium chloride (7.0 mM)	1.7	1.5
Cadmium chloride (0.01 mM)	3.5	1.3

1.8 A   Nucleotide sequence of the putative transporter OsE60 from O. sativa.

```
   1    CCACGCGTCC  GACATCGTAT  CACAGGGAAA  TAATCAACAT  AATCAGCAAC
  51    AATGGGAGCC  AAGAAGCATA  CCTTGCAAGT  GCTTCCATGG  CTACTGCTCT
 101    TTGCGCAGCA  CACTGCAGCC  AGTGCCTGCG  ACTGTGCTAA  CACCACAGAC
 151    GGGGCAGACA  GACAGGGTGC  AATGAAGCTA  AAGCTCATTG  CCATTGCATC
 201    CATCCTTGCA  GCTGGGGCGG  CTGGTGTCTT  GGTGCCAGTG  ATTGGCCGCT
 251    CCATGGCTGC  GCTGCGCCCT  GATGGTGACA  TCTTCTTTGC  TGTCAAGGCA
 301    TTTGCAGCCG  GCGTCATCCT  TGCCACTGGC  ATGGTGCACA  TTCTTCCAGC
 351    GGCGTTTGAT  GCGCTCACAT  CTCCATGCCT  CAAAAGGGGT  GGTGGGGATA
 401    GGAATCCCTT  CCCCTTTGCG  GGCCTTGTTT  CAATGTCTGC  TGCAGTGTCC
 451    ACAATGGTGG  TGGACTCATT  GGCTGCTGGA  TACTATCACC  GGTCTCAATT
 501    CAGGAAGGCA  CGCCCAGTTG  ACAACATCAA  CGTACACAAG  CATGCTGGAG
 551    ACGAGAGGGC  TGAACATGCA  CAACACATAA  ATGCGCACAC  ACATGGAGGA
 601    CATACACATT  CACATGGTGA  TATAGTGGTC  TGTGGCTCAC  CAGAGGAGGG
 651    TTCAGTAGCT  GAATCAATTC  GACATAAGGT  AGTATCTCAG  GTTCTTGAGC
 701    TGGGAATCTT  GGTGCATTCA  GTTATCATTG  GAGTATCCTT  AGGTGCATCT
 751    GTGAGGCCAT  CTACTATCAG  GCCACTAGTT  GGTGCCCTCA  GCTTCCACCA
 801    GTTCTTTGAA  GGTGTAGGTT  TGGGTGGTTG  CATTGTTCAG  GCTAATTTTA
 851    AGGTAAGGGC  AACCGTCATT  ATGGCAATAT  TTTTCTCCCT  GACTGCACCT
 901    GTGGGCATCG  TGCTAGGAAT  TGCAATTTCA  TCGAGCTACA  ATGTGCATAG
 951    CTCAACTGCC  TTCGTCGTTG  AAGGAGTCTT  CAACTCAGCC  TCAGCAGGAA
1001    TTTTGATCTA  CATGTCCCTA  GTTGACCTTC  TAGCAACAGA  TTTCAATAAC
1051    CCGAAGCTAC  AGATTAATAC  AAAGCTTCAG  CTCATGGCAT  ACCTTGCACT
1101    TTTCCTAGGT  GCTGGACTGA  TGTCAATGCT  TGCCATATGG  GCATAGATCT
1151    TCAGAATAAC  AGAGGATCTT  ATTTCCCAGA  TAGGCTTGAT  TTGTATTAAT
1201    CTATGAGTTT  TCTATCTTCG  CAAAATAACA  TACTCTTGGT  GGAGTATTTA
1251    TTGTATCCAT  CCTAGATTTT  CTAAAACCAA  AGCTTATCTC  AGGTGATCTC
1301    TTTGTACTTT  ATGATATGAT  AATACATGAT  GACTTAACTT  GTTCTTAAAA
1351    AAAAAAAAA  A
```

1.8 B   Predicted amino acid sequence of OsE60 from O. sativa.

```
   1    MGAKKHTLQV  LPWLLLFAQH  TAASACDCAN  TTDGADRQGA  MKLKLIAIAS
  51    ILAAGAAGVL  VPVIGRSMAA  LRPDGDIFFA  VKAFAAGVIL  ATGMVHILPA
 101    AFDALTSPCL  KRGGGDRNPF  PFAGLVSMSA  AVSTMVVDSL  AAGYYHRSQF
 151    RKARPVDNIN  VHKHAGDERA  EHAQHINAHT  HGGHTHSHGD  IVVCGSPEEG
 201    SVAESIRHKV  VSQVLELGIL  VHSVIIGVSL  GASVRPSTIR  PLVGALSFHQ
 251    FFEGVGLGGC  IVQANFKVRA  TVIMAIFFSL  TAPVGIVLGI  AISSSYNVHS
 301    STAFVVEGVF  NSASAGILIY  MSLVDLLATD  FNNPKLQINT  KLQLMAYLAL
 351    FLGAGLMSML  AIWAZ
```

1.9 A Nucleotide sequence of the cDNA encoding putative transporter OsR06 from O. sativa

```
   1  CCCCTCGAGG TCGACCCACG CGTCCGCTGG CCCAGAAGTC GATCGATCGA
  51  TCGTGACGGC GACGGCGCGA GCCACGACCA TGGCCAGGAC GATGACGATG
 101  AGGGTTTCTT CGCTCCTTGT CGCCGTGGTC CTCCTCGCCG CGCTCTCGTT
 151  CCAGGCGTGC AGCGGCCATG GCGGCATCAA CGACGGCGAC GGGCAGGTCG
 201  ACGCCCCGGC AACGCCTGCG TCGTCGTCCG GCGTGCGGTC CAAGGGGCTG
 251  ATCGCCGTGA AGGTGTGGTG CCTGGTGATC CTGCTGGTGT TCACCTTCGC
 301  CGGCGGCGTC TCCCCCTACT TCTACCGGTG GAACGAGAGC TTCCTCCTCC
 351  TCGGCACCCA GTTCGCCGCC GGCGTCTTCC TCGGCACCGC GCTGATGCAC
 401  TTCCTCGCCG ACTCCACCTC CACCTTCAAG GGCCTCACTA CCAACCAGTA
 451  CCCGTTCTCC TTCATGCTCA CCTGCGTCGG CTTCCTGCTC ACCATGCTCA
 501  GCGACCTCGT CATCGCCGCC GTCGCGCGGA GGAGCGCCGC CGCCGGCGTT
 551  AGCGACAACC AGGTCAGTGA GCAGCAGCAG CGGCAGCAAG CCGAGGGGGC
 601  GGTGATGAGC CGCAAGGAGG AGGAGGCGGC GGCGGTGGCG CACCCGGCGA
 651  TGCTGGTGAG GACATCGTCG TTCGAGGACG CCGTGCTGCT CATCGTCGCG
 701  CTCTGTTTCC ACTCCGTCTT TGAAGGGATC GCCATTGGTG TCTCAGCGAG
 751  CAAGAGCGAG GCGTGGAGGA ACCTGTGGAC GATCGGGCTG CACAAGATAT
 801  TCGCGGCGGT GGCGATGGGA ATCGCGCTGC TCCGGATGAT CCCCAAGCGC
 851  CCCTTCCTCA TGACCGTCGT CTACTCCCTC GCCTTCGCCG TCTCCAGCCC
 901  CGTCGGCGTC GGCATCGGCA TCGCCATCGA CGCCACCTCC CAGGGCCGCG
 951  CCGCCGACTG GACCTACGCC ATCTCCATGG GCCTCGCCAC CGGCGTCTTC
1001  ATCTACGTCG CCATCAACCA CCTCATCGCC AAGGGCTACC GCCCCcACCA
1051  CCCCACCGCC GCCGACAAGC CGCTCTTCAA GTTCCTCGCC GTCCTCCTCG
1101  GCGTCGCCGT CATGGCTGTC GTCATGATCT GGGACTGATC CATCCATCCT
1151  AGTATTACAC TGCTTCATTT CTTCAATTCC TGCGCCCATG TATGTATGCT
1201  TAATTTGGCT TGTTTAATTA ACTGTATGTA CACATGCTGC ATGCATGTTC
1251  GATTTCTATC ACCCCATTCA TTAATCGTGG CCGAAACAGT TCATGTCCAC
1301  TAATGATTGC TGTATCTATC TACATATATC AAACCGTGAA TAAAAGTTGT
1351  TATAAAAAAA AAAAAAAA
```

1.9 B Predicted amino acid sequence of OsR06 from O. sativa

```
   1  MARTMTMRVS SLLVAVVLLA ALSFQACSGH GGINDGDGQV DAPATPASSS
  51  GVRSKGLIAV KVWCLVILLV FTFAGGVSPY FYRWNESFLL LGTQFAAGVF
 101  LGTALMHFLA DSTSTFKGLT TNQYPFSFML TCVGFLLTML SDLVIAAVAR
 151  RSAAAGVSDN QVSEQQQRQQ AEGAVMSRKE EEAAAVAHPA MLVRTSSFED
 201  AVLLIVALCF HSVFEGIAIG VSASKSEAWR NLWTIGLHKI FAAVAMGIAL
 251  LRMIPKRPFL MTVVYSLAFA VSSPVGVGIG IAIDATSQGR AADWTYAISM
 301  GLATGVFIYV AINHLIAKGY RPHHPTAADK PLFKFLAVLL GVAVMAVVMI
 351  WD*
```

1.10 A   Nucleotide sequence of the cDNA encoding putative transporter OsS13 from O. sativa

```
   1  ACCGGGCCCC CCCTCGAGGT CGACCCACGC GTCCGCCGTC ATCGGTCGAA
  51  CACCACACCT GCGTCCATGG CGGGAGGCAG GGGAGCCCGC GCCAGCCTCC
 101  ACCTCCACCT CGCCTGGCTC TGCGCCTTCG CGACCACCGC GTGGGCGCAT
 151  GGCGGTGGCG GCGGCGGGGG CGATTCTGAC GCCGACGCCG ACGGCGGCGG
 201  CGAGGGGAAG CCGGACCTGC GGGCGCGGGG GCTGGTGGCG GCGAAGCTGT
 251  GGTGCTTGGC GGTGGTGTTC GCCGGGACGC TGGCCGGCGG CGTGTCCCCC
 301  TACTTCATGC GGTGGAACGA TGCGTTCCTG GCGCTGGGCA CGCAGTTCGC
 351  GGGGGGAGTC TTCCTCGGCA CCGCCATGAT GCACTTCCTC GCCGACGCCA
 401  ACGAGACTTT CGCCGACCTC CTCCCCGGCA CCGCCTACCC CTTCGCGTTC
 451  ATGCTCGCCT GCGCCGGCTA CGTCCTCACC ATGCTCGCCG ACTGCGCCAT
 501  CTCCTTCGTC GTCGCCCGCG GCGGCGGCCG GACCGAACCC GCCGCCGCCG
 551  CCGGTGCAGG GCTGGAGGAG GGTAAGCTGA GCAGCACAAA TGGCAACGCC
 601  TCTGACCCAC CAGCAGCTGA TGCGGCGGCG CAAGACCACT CCGTGGCGTC
 651  GATGCTGCGG AACGCGAGCA CGCTCGGCGA CAGCGTGCTG CTCATCGCCG
 701  CGCTCTGCTT CCACTCCGTC TTCGAGGGCA TCGCCATCGG AGTCGCCGAG
 751  ACGAAGGCTG ACGCATGGAA GGCGCTGTGG ACGATCAGCC TGCACAAGAT
 801  CTTCGCGGCC ATCGCCATGG GCATCGCGCT GCTCCGGATG CTGCCGGACC
 851  GGCCGTTCCT CTCCTGCTTC GGCTACGCCT TCGCCTTCGC CGTCTCCAGC
 901  CCCGTCGGCG TCGGCATCGG CATCGTCATC GACGCCACCA CGCAGGGCCG
 951  GGTGGCCGAC TGGATCTTCG CCGTCTCCAT GGGCCTCGCC ACCGGCATCT
1001  TCATCTACGT CTCCATCAAC CACCTCCTCT CCAAGGGCTA CACCCCGCTG
1051  AGGCCCGTCG CCGCCGACAC GCCGGCGGGG AGGCTGCTCG CCGTCGTCCT
1101  CGGCGTCGCC GTCATCGCCG TCGTCATGAT TTGGGACACC TAATGCCGCT
1151  CATGATTTCG GACGTTTGTT GGTTCTTGCA GGAATGTGTG TGTGTAGTAA
1201  GTTCGTCGTG AAATTAGCAT GTGTAAAGTT TGTGCCACTC CAACCAAAGT
1251  TTCAGGATTA TTTATAGCTT TGAATATATC ATCAACGCTG GGGCCCCAAA
1301  AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA AAA
```

1.10 B   Predicted amino acid sequence of OsS13 from O. sativa.

```
   1  MAGGRGARAS LHLHLAWLCA FATTAWAHGG GGGGGDSDAD ADGGGEGKPD
  51  LRARGLVAAK LWCLAVVFAG TLAGGVSPYF MRWNDAFLAL GTQFAGGVFL
 101  GTAMMHFLAD ANETFADLLP GTAYPFAFML ACAGYVLTML ADCAISFVVA
 151  RGGGRTEPAA AAGAGLEEGK LSSTNGNASD PPAADAAAQD HSVASMLRNA
 201  STLGDSVLLI AALCFHSVFE GIAIGVAETK ADAWKALWTI SLHKIFAAIA
 251  MGIALLRMLP DRPFLSCFGY AFAFAVSSPV GVGIGIVIDA TTQGRVADWI
 301  FAVSMGLATG IFIYVSINHL LSKGYTPLRP VAADTPAGRL LAVVLGVAVI
 351  AVVMIWDTCR SZ
```

1.11 Partial nucleotide sequence of the cDNA encoding putative transporter OsS16 from O. sativa

```
  1   CCACGCGTCC GCGAGATGGG CATCGTGGTG CACTCAGTGG TGATCGGGCT
 51   GGGCATGGGG GCGTCGCAGA ACGTGTGCAC GATCCGGCCG CTGGTGGCGG
101   CGCTGTGCTT CCACCAGATG TTCGAGGGGA TGGGGCTCGG CGGCTGCATC
151   CTGCAGGCGG GGTACGGCGG GAGGACGAGG TCGGCGCTGG TCTTCTTCTT
201   CTCCACCACG ACGCCGTTCG GGATCGCGCT GGGGCTCGCG CTGACCAGGG
251   TGTACAGCGA CAGCAGCCCG ACGGCGCTGG TCGTCGTCGG CCTGCTCAAC
301   GCGGCGTCGG CGGGGCTGCT GCACTACATG GCGCTGGTGG AGCTCCTCGC
351   CGCCGATTTC ATGGGGCCCA AGCTGCAGGG CAACGTCCGT CTCCAGCTCG
401   CCGCGTCCCT CGCCATCCTC CTCGGCGCCG GCGGCATGTC CGTCATGGCC
451   AAGTGGGCGT GAGCGTGACC GTGACCGGCG TCAGGGTCGA AGATATGCAT
501   GAAGTATAGG TGAAGGTGAC ACCGTATGTG TTGAGTTGTG ATCCAAATTC
551   ATTTGCACTT AATAAAGACC AGCTTCTGCC GTAAAAAAAA AAAAAAAAA
```

1.12

Table 11   Composition of the SSW solution used in the uptake assays

Chemical	Final concentration
EDTA	1 mM
Tri-sodium citrate	20 mM
Potassium dihydrogen phosphate	1 mM
Calcium chloride	1 mM
Magnesium sulphate	5 mM
Sodium chloride	1 mM

1.13

Table 12 Composition of the hydroponic medium used for growth of O. sativa plants

Chemical	Stock concentration	Final concentration
Calcium chloride	1 M	1.25 mM
Potassium sulphate	1 M	1.25 mM
Ammonium nitrate	1 M	1.25 mM
Sodium dihydrogen phosphate	1 M	1.25 mM
Magnesium sulphate	1 M	1.25 mM
Boric acid	-	2.86 g/l
Copper sulphate	-	0.079 g/l
Manganese sulphate	-	1.015 g/l
Sodium molybdate	-	0.90 g/l
Fe-EDTA	-	10.5 g/l

A stock of 1 M zinc sulphate was made up in 0.1 M HCl. Zinc was added to a final concentration of 0.012 mM to the 50 litre tanks as required.

1.14 Gene specific oligos designed for 5' RACE of OsS16 cDNA fragment

Note: GSP refers to gene specific oligos used in the 5' RACE.

1 = GSP1 5' AGA AGA CCA GCG CCG ACC TCG TCC TCCC 3'
2 = GSP2 5' TCC CCT CGA ACA TCT GGT GGA AGC ACA GCG 3'

1.15 Extraction of DNA from Agrobacterium cells (Melane method)

1. Inoculate 4 x 3 ml MGL + spec (or LB + 0.1 % Glucose) with four single *Agrobacterium* colonies.

2. Transfer 2 ml of overnight cultures to 2 ml eppendorf tubes.

3. Spin for 2 minutes and discard the supernatant.

4. Resuspend cells in 1 ml of 0.5 % Sarkosyl and vortex well.

5. Spin for 2min, discard supernatant and resuspend the pellets in 400 μl of resuspension buffer (50 mM glucose, 25 mM TE, pH 8.0, 10 mM EDTA, pH 8.0).

6. Add 10 μl Rnase (10 mg/ml) and vortex for 5-10 seconds.

7. Add 400 μl of fresh lysis solution (200 μl 10N NaOH, 1 ml 10 % SDS, 8.8 ml H_2O) and 300 μl of cold 7.5 M NH_4OAC, pH 7.6.

8. Mix well by inversion, stand on ice for 5 min and spin for 10 minutes.

9. Add 900 μl of supernatant to 0.6 volumes (550 μl) of isopropanol and mix well by inversion.

10. Incubate at room temperature for 10 min, spin for 10 minutes and discard the supernatant.

11. Add 1 ml of 70 % ethanol, spin for 5 min and discard the supernatant.

12. Dry the pellet in a speedy vac for 5 minutes.

13. Resuspend the DNA pellet in 25 μl TE, pH 8.0.

1.16 A

Table 13   Medium used for callusing of barley embryos BCI-DM

	Stock solution (Grams/L)	Volume of stock/L of BCI-DM
MACRO (20xstock)		
Ammonium nitrate	16.5	
Potassium nitrate	19.0	
Calcium chloride.$2H_2O$	4.4	
Magnesium sulphate.$7H_2O$	3.7	50ml
Potassium dihydrogen phosphate	1.7	
MICRO (1000xstock)		
Boric acid	0.62	
Manganese sulphate.H_2O	1.56	
Zinc sulphate.$7H_2O$	0.86	
Potassium iodide	0.08	1ml
Sodium molybdate.$2H_2O$	0.03	
Copper sulphate.$5H_2O$	0.003	
Cadmium chloride.$6H_2O$	0.003	
IRON (200xstock) Ferrous sulphate. $7H_2O$	1.86	5ml
EDTA Disodium EDTA. $2H_2O$	7.45	5ml
Maltose	-	30g
Thiamine-HCL	0.001	1ml
myo-Inositol	-	0.25g
Casein hydrolysate	-	1.0g
Dicamba (1mg/ml)	1.0	2.5ml
Proline	-	0.69g
Phytagel	-	3.5 g

pH of the medium was adjusted to 5.9 before autoclaving.

1.16 B

Table 14   Medium used for regeneration of shoots from embryogenic calli of barley -FHG (Hunter,1988)
FHG medium is modified MS medium. This medium has low concentration of ammonium nitrate and slightly higher concentration of manganese sulphate.

	Stock solution (Grams/L)	Volume of stock/L FHG medium
MACRO (10xstock)		
Ammonium nitrate	1.7	
Potassium nitrate	19.0	100ml
Calcium chloride.2H$_2$O	4.4	
Magnesium sulphate.7H$_2$O	3.7	
Potassium dihydrogen phosphate	1.7	
MICRO (1000xstock)		
Boric acid	0.62	
Manganese sulphate.H$_2$O	1.69	
Zinc sulphate.7H$_2$O	0.86	
Potassium iodide	0.08	10ml
Sodium molybdate.2H$_2$O	0.03	
Copper sulphate.5H$_2$O	0.003	
Cadmium chloride.6H$_2$O	0.003	
Thiamine-HCl (1mg/ml)	-	1ml
IRON (200xstock) Ferrous sulphate. 7H$_2$O	1.86	5ml
EDTA Disodium EDTA. 2H$_2$O	7.45	5ml
Inositol	-	0.1
Glutamine	-	0.73
Maltose	-	62g
Phytagel	-	3.5 g

pH of the medium was adjusted to 5.9 before autoclaving.

Genomic DNA isolation using standard phenol-chloroform method

1. Collect a 10 cm long healthy barley leaf, snap freeze in liquid nitrogen and crush to a fine powder using a pestle and mortar.

2. Add 600 µl of extraction buffer and homogenise the tissue.

3. Add 600 µl of phenol/chloroform/iso-amylalcohol (25:24:1), mix and place on ice. Place all the samples on an orbital mixer for 10 -60 minutes.

4. Spin 6-10 samples at a time for 10 min and transfer the aqueous phase to a tube
   containing: 60 µl   3M NaOAC
   600 µl   Isopropanol
   Mix by inversion.

5. Allow the DNA to precipitate for 2 min at room temperature.

6. Pour off supernatant and rinse the pellet in 1 ml of 70 % ethanol.

7. Dry briefly and resuspend in 30 µl of R40 overnight in a refrigerator.

8. Store the genomic DNA at -20°C.

Composition of extraction buffer

Chemical	Amount per litre
1 % sarkosyl	10 g
100 mM Tris - HCl	12.1 g Trizma base
100 mM sodium chloride	5.8 g
100 mM EDTA	3.2 g sodium EDTA
2 % PVPP	20 g

Adjust the pH to 8.5 with concentrated hydrochloric acid (HCl).

R40 is 40 µg/ml of RNase A (boiled) in TE buffer.

1.18 Extraction of total RNA from the transgenic barley plants

1.   Rinse polytron with water and sonicate in phenol and 1 % SDS.

2.   Mix 25 ml of RBA solution and 25 ml of phenol and use 20 mls of this solution per a gram of plant tissue.

3.   Place approximately 2 g of frozen plant tissue in the above solution.

4.   Grind the tissue with polytron for several minutes till the tissue is homogenised.

5.   Transfer the homogenate to a 50 ml falcon tube and spin at 15 K for 15 min in a Beckman centrifuge.

6.   Transfer the upper clear/green (in case of shoots) phase to a new oakridge tube with a 10 ml disposable pipette.

7.   Add 1/3 rd volume 8 M LiCl (lithium chloride, final concentration 2 M), mix well and precipitate overnight at 4°C.

8.   Spin down the solution with precipitate at 15 K for 15 min at 4°C in a Beckman centrifuge.

9.   Discard the supernatant carefully and wash the pellet twice in 5 ml cold 80 % ethanol by spinning at 4°C for 15 min at 15 K in a Beckman centrifuge.

10.   Discard the supernatant and air dry the pellet.

11.   Resuspend the pellet in 1 ml 0.1 mM EDTA and transfer to a 1.5 ml eppendorf tube.

12.   Store the RNA at -80°C.

Composition of RBA solution used in the extraction.

Chemical	Stock concentration	Final concentration (mM)	Volume of stock/100ml
Lithium chloride (LiCl)	8 M	100 mM	1.25 ml
Sodium Dodecyl sulphate (SDS)	10 %	1 %	10 ml
Tris-Cl (pH7.5)	1 M	100 mM	10 ml
DiSodium salt of Ethylene diamine tetraacetic acid (EDTA)	0.5 M	100 mM	20 ml
β-mercaptoethanol (βME)	Stock chemical	1 %	1 ml
Sterile nanopure water	-	-	57.75 ml

Table 19   *Hoagland's nutrient solution (1/4th strength*) for growth of barley plants. * The macronutrients were used* at 1/4th strength while the micronutrients and iron source were used at full strength.

Chemical	Stock solution	Final concentration	Volume/L
Potassium nitrate	1 M	0.25 mM	0.25 ml
Calcium nitrate	2 M	0.25mM	0.125 ml
Magnesium sulphate.7H$_2$O	2 M	0.125 mM	0.05 ml
Potassium dihydrogen phosphate	1M	0.05 mM	0.05 ml
Ferrous EDTA	-	0.03 g/L	-

The following micronutrients were added to the final solution.

Chemical	Grams/L
Boric acid	28.5 g/L
Manganese chloride.4H$_2$O	18.1 g/L
Zinc sulphate. 7H$_2$O	2.2 g/L
Copper sulphate.5H$_2$O	0.8 g/L
Sodium molybdate.2H$_2$O	0.5 g/L

All the solutions were made in nanopure water.

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