



# **MECHANISMS OF MN EFFICIENCY IN BARLEY**

by

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## **Declaration**

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## List of abbreviations

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A	Adenine
ATP	Adenosine 5'-triphosphate
bp	base pair
BSA	Bovine serum albumin
C	Cytosine
cDNA	complementary DNA
cpm	counts per minute
CTP	Cytidine 5'-triphosphate
dATP	2'-deoxy adenosine 5'-triphosphate
dCTP	2'-deoxy cytidine 5'-triphosphate
ddATP	2',3'-dideoxy adenosine 5'-triphosphate
ddCTP	2',3'-dideoxy cytidine 5'-triphosphate
ddGTP	2',3'-dideoxy guanine 5'-triphosphate
ddTTP	2',3'-dideoxy thymidine 5'-triphosphate
DEPC	Diethyl pyrocarbonate
dGTP	2'-deoxy guanine 5'-triphosphate
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	2'-deoxy nucleotide 5'-triphosphate
DM	Dry matter
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
dTTP	2'-deoxy thymidine 5'-triphosphate
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglyco-bis-( $\beta$ -aminoethyl ether)N'N'-tetraacetic acid
G	Guanine
HEDTA	N-hydroxyethylethylenediaminetriacetic acid

ICP	Inductively coupled plasma spectrometry
IPTG	Isopropyl $\beta$ -thiogalactopyranoside
kb	kilobase
kDa	kilodalton
LB	Luria broth
MES	2-(N-Morpholino)ethanesulfonic acid
min	minute
MOPS	3-(N-Morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
ng	nano gram
nM	nano mole
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIPES	Piperazine-N,N'-bis-(2-ethanesulfonic acid)
RNA	Ribonucleic acid
rpm	revolutions per minute
RT	Room temperature
SDS	Sodium dodecyl sulphate
Tris	Tris(hydroxymethyl)amino methane
Triton X-100	$\alpha$ -[4-(1,1,3,3-tetramethylbutyl)phenyl]- $\omega$ -hydroxypoly(oxy-1,2-ethanediyl)
Tween 20	Polyoxyethylenesorbitan monolaurate
UV	Ultraviolet
X-Gal	Bromo-(5)-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside
YEB	Youngest emerged leaf blade



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## Thesis summary

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The mechanisms of manganese (Mn) efficiency (genetic tolerance to Mn-deficient soils) in barley (*Hordeum vulgare* L.) were investigated at both physiological and molecular levels. The restriction of expression of Mn efficiency was observed in small pots. By using a pot of adequate size, genotypic differences in dry matter production and shoot Mn concentration were demonstrated in controlled conditions over a wide range of Mn supply. Thus, measurement of Mn concentrations of youngest expanded leaf blade or shoots was applied in soil-based pot screening as an index of Mn efficiency. This newly developed laboratory procedure has been proven to be robust, with low sensitivity to high seed Mn content and to variations in available soil Mn. Soil culture experiments indicate that the basis of Mn efficiency is higher Mn acquisition from soil. Mn<sup>2+</sup> absorption was investigated further in a chelate-buffered nutrient solution. The results showed that unlike the soil culture, no clear genotypic differences in Mn accumulation of shoots were detectable over a range of Mn supply and over a range of pH. However, genotypic differences in Mn concentration of roots were observed at high pH in the same nutrient solution. These results show that the mechanism of Mn efficiency is likely to be a genotype ability in Mn mobilisation from soil. Thus, genotypic differences in Cu<sup>2+</sup> and Fe<sup>3+</sup> reductions were assessed, but no genotypic difference could be observed. Thereafter, molecular aspects of Mn efficiency were explored to find genes which may be related to Mn efficiency. Two barley genes, *Ids1* and *Ids2* from Japan, which are implicated in Fe acquisition, were tested for their connection with Mn efficiency. No genetic difference in *Ids1* expression was found between Mn-efficient and Mn-inefficient cultivars, but differential expression of *Ids2* was found, which was inversely related to Mn efficiency. Attempts were made to isolate Mn efficiency related genes. A root cDNA library was constructed from a Mn-efficient genotype and differentially screened with root cDNAs from a Mn-inefficient

genotype. More than one hundred putative clones were isolated. One of these clones, *Mne-1* was characterised because it appeared to be more abundant in the Mn-efficient plant under the low Mn conditions than in the Mn-inefficient plant by RNA gel blot analysis. DNA sequencing indicated that *Mne-1* encoded a zinc finger protein, novel in higher plants, showing a possible role in Mn efficiency through Mn binding or transcriptional regulation. For further insights into the functions of *Mne-1*, Mne-1 recombinant protein was expressed in *E. coli*, and polyclonal antibodies to the recombinant Mne-1 protein were raised. Protein gel blot analysis showed that the higher accumulation of Mne-1 protein in roots of Mn-efficient plants was consistent with higher accumulation of Mne-1 mRNA. Under low Mn conditions, the higher expression of *Mne-1* at both mRNA and protein levels is correlated to greater Mn efficiency. The analysis of metal contents showed that the recombinant Mne-1 protein contained Zn but no Mn. This suggests that *Mne-1* may function as a transcriptional factor in adaptive response to low available Mn in soil to regulate genes responsible for Mn efficiency. Further applications of the Mne-1 recombinant protein and anti-Mne-1 antibodies will enable us to determine the transcriptional function of *Mne-1* gene, and thus increase the understanding of the role of the *Mne-1* in Mn efficiency. *Mne-1* is the first gene associated with differences in micronutrient efficiency traits, and a molecular marker for this gene may be useful for future breeding programs for South Australian soil conditions.

## Publications

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Huang C, Webb M J and Graham R D (1993) Effect of pH on Mn absorption by barley genotypes in a chelate-buffered nutrient solution. *In* Plant Nutrition from Genetic Engineering to Field Practice. Ed. N J Barrow. pp 653-656. Kluwer Academic Publishers, Australia. **(Results of Chapter 3)**

Huang C, Webb M J and Graham R D (1993) Effect of pH on Mn absorption by barley genotypes in a chelate-buffered nutrient solution. *Plant Soil* 155/156, 437-440. **(Results of Chapter 3)**

Huang C, Webb M J and Graham R D (1994) Manganese efficiency is expressed in barley growing in soil system but not in solution culture. *J. Plant Nutr.* 17, 83-95. **(Results of Chapter 2 and 3)**

Huang C, Webb M J and Graham R D (1996) Pot size affects expression of Mn efficiency in barley. *Plant Soil* 178(2). 178, 205-208. **(Results of Chapter 2)**

Huang C, Graham R D, Barker S J and Mori S (1996) Differential expression of *Ids2* gene in barley genotypes differing in Mn efficiency. *J. Plant Nutr.* 19, 407-420. **(Results of Chapter 5)**

Huang C, Barker S J, Langridge P and Graham R D (1995) Cloning and expression of a novel zinc finger gene associated with Mn efficiency. *In* Australian Society of Plant Physiologists 35th Annual General Meeting & New Zealand Society of Plant Physiologists 17th Annual General Meeting. Abstract 155. Published by Aust. Soc. Plant Physiol. Inc., Sydney. **(Results of Chapter 6 and 7)**

## Thesis introduction

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Manganese (Mn) deficiency in crops has been found in various areas of the world which encompass many soil types (Reuter *et al.*, 1988; Welch *et al.*, 1991). Usually, Mn-deficient soils are calcareous. There is a large area of Mn-deficient soils in South Australia. The yield loss of cereal crops is estimated at 5 million Australian dollars a year in South Australia alone. In barley, the yields without Mn fertiliser were reported to be 40% to 70% of those with Mn (Reuter *et al.*, 1973a). In a case of subclinical deficiency, it is difficult to avoid appreciable yield losses because plants do not produce visual symptoms or because deficiency symptoms appear too late for effective correction (Graham, 1984). Furthermore, the depression of root growth caused by Mn deficiency (Webb and Dell, 1990) can restrict plant root access to water if roots fail to grow into deeper soil horizons towards maturity. In addition, plants with Mn deficiency are susceptible to diseases (Graham, 1983; Huber and Wilhelm, 1988; Graham and Webb, 1991). Thus, control of Mn deficiency is important for crop production in Mn-deficient soils.

Application of Mn fertilisers is a common practice to control Mn deficiency, but the effectiveness of Mn fertilisers is temporary and costly. For soil application, a large application of  $\text{MnSO}_4$  is required at sowing, but frequently it is only partially effective as soluble Mn is rapidly converted into unavailable Mn compounds in soils with high pH (Reuter *et al.*, 1973b), and thus it has a low residual value. In conjunction with soil application, several foliar sprays are needed to prevent Mn deficiency during the crop growing season (Reuter *et al.*, 1973a) because little Mn applied to leaves moves to the growing parts of the plant (Nable and Loneragan, 1984a; Pearson and Rengel, 1994). Therefore, other solutions are needed to cope with Mn deficiency in problem soils.

Current agricultural practices have been changing from traditional alteration of the edaphic environment to match plant growth needs to genetic manipulation of plants

in adaptation to the soil environment, with fewer inputs. Breeding and growing Mn-efficient cultivars are the most effective and economic solution for coping with Mn deficiency in problem soils. For breeding Mn-efficient cultivars, two prerequisites have to be met. Genetic variation for greater efficiency must exist in germplasm and soils must have potential to supply efficient genotypes with adequate Mn. A Mn-efficient genotype, in an agronomic sense, is a genotype which is able to grow and yield well without added Mn fertiliser in a soil which is limiting in available Mn for another, standard, genotype (Graham, 1988). Considerable genotypic variation in tolerance to Mn-deficient soil is found in barley (Graham *et al.*, 1983) as an example shown in Fig. 1, and Mn efficiency in barley is likely to be under control of a single, major dominant gene (Longnecker *et al.*, 1990). In addition, Mn-deficient soils generally have a large immobile Mn reserve which can provide Mn-efficient genotypes with adequate Mn. Therefore, it is possible to breed Mn-efficient cultivars.

Plant breeding needs good screening techniques. Initially, parental materials from a world collection were screened in the field based on the symptoms of Mn deficiency and relative grain yields (Graham, 1983), but the field screening could be done in the South Australian climate only once a year. Later, a screening technique under controlled conditions was developed by Longnecker *et al.* (1990), using visual scores of Mn deficiency symptoms to screen various breeding materials. However, this technique is not consistent over time (Webb *et al.*, 1993a), and also the association of the visual symptoms with Mn efficiency is unknown. In order to develop a meaningful screening technique for breeding, knowledge of the mechanism of Mn efficiency is essential.

To gain insight into the mechanism of Mn efficiency, the following five aspects at both physiological and molecular levels were investigated.

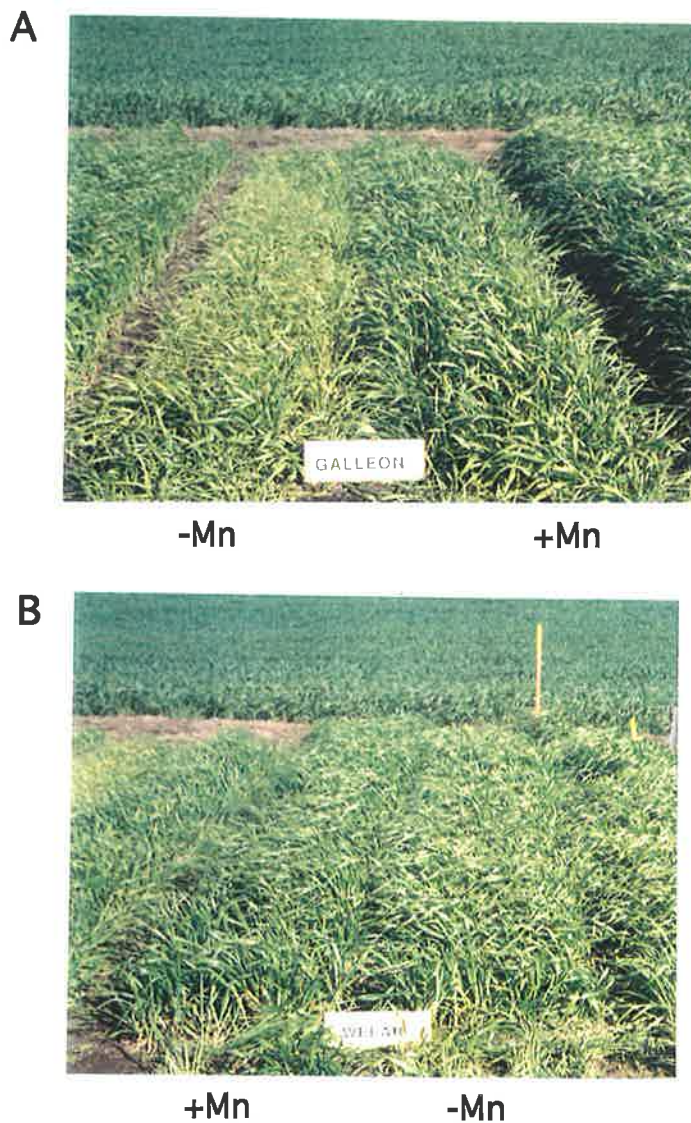
- (1) To screen for Mn efficiency in soil under controlled conditions (Chapter 2).

(2) To determine genotypic variation in Mn accumulation by plants in solution culture (Chapter 3).

(3) To assess  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  reduction by Mn-efficient and Mn-inefficient genotypes (Chapter 4).

(4) To examine differential expression of Fe deficiency-induced genes in Mn-efficient and Mn-inefficient genotypes (Chapter 5).

(5) To isolate and characterise genes related to Mn efficiency (Chapter 6 and 7).



**Fig. 1.** Genotypic difference in Mn efficiency

**A.** Mn-inefficient genotype, Galleon.

**B.** Mn-efficient genotype, Weeah.



# Chapter 1

## Literature review

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### 1.1 Introduction

This review describes current knowledge of manganese (Mn) in plants and soils, with emphasis placed on those aspects relevant to Mn acquisition and genotypic differences in Mn acquisition. Molecular studies of genes related to nutrition are also reviewed.

### 1.2 Manganese in plants

Manganese was identified as an essential element for plant growth in 1920s (McHargue, 1922). Manganese belongs to the transition metal group in the periodic table, a group featuring variable valence. Its d orbit below the valence shell contains 5 unpaired electrons like Fe. Thus, various transition states can be found from a basic divalent ion to an acidic permanganate  $7^+$  ion. Manganese is involved in redox processes such as water splitting in photosystem II and dismutating toxic superoxide radicals to  $O_2$  and  $H_2O_2$ . Moreover, Mn also shows some properties of both alkali earth cations (Ca and Mg) and transition metals (Zn and Fe). Manganese can activate a number of enzymes. In plant cells, about 5 mg/kg Mn is required for biochemical reactions. Larger amounts of Mn are bound to the cell wall or located in vacuoles (Garnham *et al.*, 1992).

#### 1.2.1 Biochemical functions

##### 1.2.1.1 Photosynthesis

Manganese is essential for light-induced water-splitting and  $O_2$  evolution in photosynthesis of green plants (Cheniae, 1970). Photosystem II, a pigment-protein complex in the thylakoid membranes of green plants, mediates electron transfer from  $H_2O$  to plastoquinone molecules (Vermaas, 1993). Four Mn ions are required for



the reaction center of photosystem II (Ghanotakis and Yocum, 1990). Under mild Mn deficient conditions, O<sub>2</sub> evolution and the electron transport activity of photosystem II are reduced (Amesz, 1983; Nable *et al.*, 1984), but the chloroplast ultrastructure and the chlorophyll concentration are not affected (Cheniae and Martin, 1968; Nable *et al.*, 1984). However, the lamellar system is broken down in severe Mn deficiency (Mercer *et al.*, 1962).

Some light energy intercepted by leaves is not consumed in photosynthesis, and instead is emitted from leaf tissues as fluorescence at red wavelengths. The room temperature fluorescence of leaves is found to be correlated to Mn deficiency (Kriedemann *et al.*, 1985). Thus, the leaf fluorescence has been used to diagnose Mn deficiency in lupin and cereals (Graham *et al.*, 1985; Hannam *et al.*, 1987; Longnecker and Graham, 1990).

#### **1.2.1.2 Mn-containing superoxide dismutase (SOD)**

Superoxide dismutases can catalyse the dismutation of toxic superoxide radicals to O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub>. The SOD plays a major role in the protection of cells against the deleterious effects of superoxide free radicals generated from various enzymatic reactions (Fridovich, 1983). The generation of superoxide can be further increased by environmental stresses (Barényi and Krause, 1985; Wise and Naylor, 1987). Consequently, SOD has been considered to have an important role in plant stress tolerance. Three forms of SOD have been identified in plants, based on the metal ion present at the active site: Cu/Zn SOD, MnSOD and FeSOD. Cu/ZnSOD is normally present in the cytosol, and some plants also contain a chloroplastic isoform (see Bannister *et al.*, 1987, for a review). MnSOD is most often found in the mitochondrial matrix (Duke and Salin, 1983; Sandalio *et al.*, 1987). MnSOD exists as functional homodimers and homotetramers with a subunit molecular weight of about 23 kDa (Bannister *et al.*, 1987). Several MnSOD cDNAs have been isolated from different plant species (White and Scandalios, 1988; Bowler *et al.*, 1989; Wong-Vega *et al.*, 1991; Miao and Gaynor,

1993). There is some evidence that MnSOD overexpression can protect plants against environmental stresses. For example, the overexpression of a chloroplastic MnSOD gene in transgenic tobacco plants provides additional protection from oxidative stress (Van Camp *et al.*, 1994). Transgenic alfalfa plants expressing the chloroplastic MnSOD gene had significantly increased resistance to freezing which cosegregates with the expression of the transgene in alfalfa (McKersie *et al.*, 1993). In addition, the chloroplastic MnSOD gene in transgenic cotton provides increased chilling tolerance (Trolinder and Allen, 1994).

### 1.2.1.3 Other enzymes

Manganese can bridge ATP with the enzyme complex in phosphokinases and phosphotransferases as Mg does. Mn also activates decarboxylases and dehydrogenases of the tricarboxylic acid cycle (Mukhopadhyay and Sharma, 1991). In some cases, such as for phosphatidylinositol 4-kinase, the stimulation by Mn as an activator is stronger than that by other divalent ions (Steinert *et al.*, 1994). However, in many cases,  $Mn^{2+}$  can be replaced by  $Mg^{2+}$  as stability constants of  $Mn^{2+}$  within oxyanions and coordination number are of similar magnitude as those of  $Mg^{2+}$  (Mahler, 1961). In addition, Mn stimulates a number of key reactions involved in the biosynthesis of plant secondary metabolites (Burnell, 1988). Many intermediates of secondary metabolites are simple phenols, which are widely distributed in plants and have an important role in disease resistance (Friend, 1981; Graham and Webb, 1991). Mn deficiency decreased the production of phenols and lignins (Huber and Wilhelm, 1988).

Mn-deficient plants exhibited an increase in peroxidase and IAA oxidase activity (Taylor *et al.*, 1968; Morgan *et al.*, 1976). Considerable differences in peroxidase activity and isozyme patterns between soybean genotypes were observed under a low level of Mn supply (Leidi *et al.*, 1989). There also were differences in chloroplast peroxidase activity between wheat genotypes grown in a Mn-deficient soil (Kaur *et al.*, 1989). In addition, manganese peroxidase (MnP) is identified as one of two extracellular

peroxidases involved in lignin degradation in *Phanerochaete chrysosporium* (Kuwahara *et al.*, 1984). MnP oxidises Mn(II) to Mn(III) which, in turn, oxidises a variety of phenols and phenolic compounds (Glenn *et al.*, 1986).

## **1.2.2 Mn deficiency**

### **1.2.2.1 Critical level and symptoms of deficiency**

The critical deficiency level of Mn in most plant species is narrow, falling in the range of 10-20 mg Mn/kg DM (Hannam *et al.*, 1987), depending on plant tissues analysed, stage of plant growth when the tissues are sampled and growing conditions. The critical Mn concentrations for graminaceous species are considerably similar, ranging from 10 to 12 mg/kg DM of young expanded leaf blades, but appear to vary from 10 to 50 mg/kg DM in young leaves of other field crops and pasture species (Hannam and Ohki, 1988). In barley, the critical concentration of Mn which results in decreasing vegetative yields is 11.0-11.6 mg/kg DM, using the youngest emerged leaf blades from both controlled and field conditions, but slightly higher (12.0-12.4 mg/kg DM) using whole shoots from controlled conditions (Hannam *et al.*, 1987).

Visual symptoms of Mn deficiency appear only when plant growth is severely depressed by Mn deficiency. The symptoms vary with species and conditions. Under controlled conditions, the symptoms in barley appear first on the youngest leaf, characteristic of interveinal chlorosis, and then necrosis is developed. The necrosis usually is worse on the second youngest leaf (Longnecker and Graham, 1990; Webb *et al.*, 1993b), as the development of the necrosis takes some time. In the field, however, Mn deficiency symptoms, grey-brown necrotic spots and streaks, appear first on old leaves of barley, and eventually develop on younger leaves (Reuter *et al.*, 1973a).

### **1.2.2.2 Effect of Mn deficiency on growth and development**

Manganese deficiency depresses root growth, including total root length and number of lateral roots, but increases the number of lateral root primordia, many of

which fail to emerge (Webb and Dell, 1990). A greater abundance of small, nonvacuolated cells in the Mn-deficient roots is observed (Abbott, 1967). These results indicate that Mn deficiency impairs cell elongation to a greater extent than it does cell division. The change of the growth and development caused by Mn deficiency may be due to hormone imbalance rather than lack of carbohydrate because under conditions of ample carbohydrate supply, the growth of isolated roots still is severely inhibited by Mn deficiency, but the growth inhibition can be relieved within a few hours by resupplying Mn (Abbott, 1967).

Mild Mn deficiency in barley decreases the number of tillers, prolongs the vegetative growth, and delays the reproductive growth (Longnecker *et al.*, 1991). Severe Mn deficiency can result in death of barley plants before they develop to the stem-elongation stage (Longnecker *et al.*, 1991). Moreover, mild Mn deficiency in wheat results in the development of smaller anthers and the production of fewer, smaller and infertile pollen grains, but in contrast, there is no significant effect on ovule fertility (Sharma, 1992). As a result, mild Mn deficiency can lead to severe yield loss. These effects on the reproductive organs are superficially similar to those of Cu deficiency (Graham, 1975).

### **1.2.3 Mn absorption and translocation within plants**

#### **1.2.3.1 Mn absorption**

Manganese is generally considered to be absorbed mainly as a free  $Mn^{2+}$  ion from soil solution into plants (Page *et al.*, 1962; Geering *et al.*, 1969; Kochian, 1991). Manganese can form complexes with organic ligands of plant and microbial origin as well as with synthetic chelates. Generally, synthetic chelating agents are considered not to be absorbed by growing plants. When Mn is supplied as a synthetic chelate complex, it will be absorbed more slowly than the free cation (Barber and Lee, 1974; Webb *et al.*, 1993b). This may be due to decreasing the concentration of free  $Mn^{2+}$  ion in the solution. Recent results show that barley plants can grow well at about 5 nM of free

Mn<sup>2+</sup> supplied as 0.5 µM of MnHEDTA (Webb *et al.*, 1993b). Other Mn forms such as Mn<sup>3+</sup> complexed with transferrin can also be absorbed into human cells (Suarez and Eriksson, 1993), but it is not clear whether Mn<sup>3+</sup> complexes can be absorbed by plant roots.

Mn<sup>2+</sup> absorption by roots is characterised by biphasic uptake. An initial rapid phase of the uptake within a few minutes is reversible and nonmetabolic. Labelled Mn<sup>2+</sup> sorbed during this period is freely exchangeable with Ca<sup>2+</sup> and unlabelled Mn<sup>2+</sup>, and this uptake is independent of metabolism (Page and Dainty, 1964; Garnham *et al.*, 1992). In this phase, Mn appears to be adsorbed by cell wall constituents of root-cell apoplastic space. A second slower phase of the uptake follows, which can proceed for several hours at a constant rate. Mn absorbed in the second phase is less readily exchangeable (Maas *et al.*, 1968). Incubation in the dark, at low temperature or in the presence of metabolic inhibitors decreases Mn absorption (Maas *et al.*, 1968; Garnham *et al.*, 1992), which indicates that the Mn absorption in the second phase is dependent on metabolism. The Mn absorbed during this phase is likely to be transported into symplasm.

The plasma membrane is a barrier for Mn entry into cells. Mn enters into cells presumably via carriers or channel proteins (Garnham *et al.*, 1992; Welch, 1995). A Mn transporter complex in cyanobacteria has been isolated, which contributes to Mn absorption at a low level of Mn (<0.5 µM). This Mn transporter complex has significant sequence similarities to the members of ATP binding cassette superfamily of transporter proteins (Bartsevich and Pakrasi, 1995). In addition, divalent cation channels such as Ca channels may also contribute to Mn absorption. Ca channels in animal cells were found permeable to Mn, Co, Cd and La (Shibuya and Douglas, 1992). In plants, Ca channels in plasma membrane vesicles were also permeable to Mn<sup>2+</sup> when 1 mM Mn<sup>2+</sup> was applied (Piñeros and Tester 1995). However, the role of the carriers and the channels in Mn absorption may differ in different external concentrations. The carriers may play an essential role in Mn absorption at a low external concentration, < 0.5 µM for example, in

the cyanobacteria mentioned above (Bartsevich and Pakrasi, 1995); whereas, when the external Mn concentration is increased, the cation channels such as Ca channels may contribute significantly to Mn absorption.

Clarkson (1988) estimated by kinetic experiments in the laboratory that the rate of Mn absorption may be 100 to 1000 times greater than the needs of plants. He suggested that unlike the major nutrient ions, for which uptake is broadly regulated by growth and metabolism, the uptake of Mn is not tightly controlled. This may be due to the high capacity of ion carriers and channels in the transportation of Mn ions through the plasma membrane at a speed of several hundred ions to several million ions per second per protein molecule (Tester, 1990; Tyerman, 1992).

The effect of metabolism on  $Mn^{2+}$  absorption appears to be a secondary effect. The inhibition of metabolism would decrease ATP production, and in turn decrease the membrane potential generated by proton-translocating ATPases in the plasma membrane of root cells. The membrane potential would influence the carrier function and channel opening, leading to the impact on  $Mn^{2+}$  absorption. However, the exact dependence of Mn absorption on metabolism is not clear. Page and Dainty (1964) showed that potassium cyanide had no effect on  $Mn^{2+}$  uptake in oat roots. Yet, dinitrophenol (DNP) and azide strongly inhibited Mn uptake in excised maize roots (Maas *et al.*, 1969), and DNP inhibited Mn influx to intact maize roots (Ratkovic and Vucinic, 1990).

$Mn^{2+}$  absorption by plants is strongly affected by pH in nutrient solution (Munns *et al.*, 1963; Maas *et al.*, 1968; Vorm and Diest, 1979).  $Mn^{2+}$  absorption was completely inhibited below pH 4, and increased linearly with increasing pH from 4.0 to 6.5, but further increasing pH to neutral and alkaline ranges dramatically depressed the absorption (Munns *et al.*, 1963; Maas *et al.*, 1968). The depression of  $Mn^{2+}$  absorption at the high pH was considered to be the oxidation of  $Mn^{2+}$  and precipitation from the solution (Munns *et al.*, 1963; Maas *et al.*, 1968). In contrast, the absorption of  $Mn^{2+}$  in a single

salt solution was increased to the maximum value when the pH was over 7, and remained constant in a range of pH 7.0-8.7 (Ratkovic and Vucinic, 1990). The difference between the nutrient solution and the single salt solution may be because  $Mn^{2+}$  precipitation is much less likely to occur in the single salt solution, and there is no competition of  $Mn^{2+}$  with other cations during the absorption process. The effect of pH on  $Mn^{2+}$  absorption may be through its impact on the membrane potential.

### 1.2.3.2 Mn translocation within plants

Little is known about the processes involved in intercellular translocation and long distance translocation in either xylem or phloem. Mn is translocated by transpiration stream to shoots, presumably as a divalent ion in equilibrium with Mn complexes formed by Mn weakly bonding with organic ligands (Clarkson and Hanson, 1980; Benes *et al.*, 1983; Anderegg and Ripperger, 1989). Mn retranslocation in phloem is limited but it does occur in certain circumstances. The Mn in leaves is hardly retranslocated to other parts of plants (Nable and Loneragan, 1984a; El-Baz *et al.*, 1990; Pearson and Rengel, 1994). Little Mn was found to be translocated from the Mn-treated half root system of subterranean clover to the other half of untreated roots in split root culture (Nable and Loneragan, 1984b). However, the Mn absorbed from one single seminal root of wheat can be retranslocated to other roots. The amount of retranslocated Mn appears to depend on the plant age, the Mn status and the relative metabolic activity of the organ studied (Pearson and Rengel, 1995a). In addition, during grain development, only the Mn in roots and stems can be translocated to wheat heads (Pearson and Rengel, 1994; 1995b).

## 1.3 Mn in soils

Total Mn in soils is in a range of 20-6000 mg/kg (Krauskoff, 1972), but it predominantly occurs as insoluble forms in fractions of soil solid phases, which are not available for direct absorption by plant roots (Lindsay, 1991; Shuman, 1991). The soluble Mn in the solution of most soils is very low, and in some soils, especially in calcareous soils, it is too low to provide plants with adequate intake to meet their

metabolic requirement. Many abiotic and biotic factors affect the availability of Mn in soil. Therefore, soil analysis is not reliable as an indicator of Mn availability (Mahler *et al.*, 1992).

### 1.3.1 Mn forms in soils

Mn in soils exists as Mn(II) ions in soil solution, as Mn(II) adsorbed by organic matter and clay minerals, or coprecipitated with carbonates and inorganic oxides, as sparingly soluble oxides of Mn(III, IV), and as primary and secondary minerals (Bartlett, 1986; Norvell, 1988). Divalent Mn, often existing as various inorganic and organic complexes is soluble in soil solution, which is available for plant absorption. Mn(III) generated by several redox reactions as a reactive, unstable intermediate may also exist in soil solution though it may not stay long in soil solution possibly because of disproportionate and oxidative decomposition of the associated ligand (Norvell, 1988). Organic chelates derived from microbial activity, degradation of soil organic matter, plant residues and root exudates can form metal complexes with micronutrient cations, and thus increase the micronutrient cation solubility and mobility. During active uptake, the concentration of micronutrient cation complexes is higher in the bulk solution than at the root surface. The complexes diffuse to the root surface down a concentration gradient, and, after dissociation,  $Mn^{2+}$  ions absorbed by plants. Meanwhile, the concentration of metal-free ligands is higher at the root surface due to plant extrusion (Shi *et al.*, 1989) and the dissociation of arriving complexes, and thus the metal-free ligands diffuse away down their concentration gradient to the bulk solution, where they bind free metal cations, resulting in a decrease of concentrations of free metal cations. Consequently, additional metal ions are desorbed from mineral surface or dissolved from minerals to resupply micronutrient ions in the bulk solution. According to Tisdale *et al.* (1993), this chelate-micronutrient “cycling” is an extremely important mechanism in soil, providing available Fe for plants. The same mechanism may also be important in the provision of available Mn for plants.



### 1.3.2 Factors influencing Mn availability

The availability of Mn in soils is affected by chemical, physical and biological factors. pH and pe strongly influence the solubility of Mn according to following simple reaction scheme:



Generally, as soil pH decreases, the proportion of exchangeable Mn sharply increases (Bromfield *et al.*, 1983), and the proportion of Mn oxides and Mn bound to Fe and Mn oxides decreases (Sims, 1986). This may be due mainly to the provision of extra protons (Lindsay, 1972). Moreover, the acidification may also inhibit microbial oxidation. Conversely, as soil pH increases, chemical immobilisation of  $\text{Mn}^{2+}$  increases (Dion and Mann, 1946) and chemical auto-oxidation predominates at pH above 8.5-9.0 (Leeper, 1970; Reisenauer, 1988).

Mn-oxidising microbes are considered to be far more effective than any nonbiological system in oxidising  $\text{Mn}^{2+}$  in neutral and slightly alkaline soils (Bromfield, 1978; Uren and Leeper, 1978; Bromfield and David, 1976). Microbial reduction also may play a role in transformation of Mn forms in soil (Webb *et al.*, 1993a). Physical factors such as the crystalline structure of Mn oxide compounds and the particle sizes of Mn oxides affect Mn reactivity. Freshly precipitated Mn oxides are likely to be the most reactive as they may have a fairly high oxidation state, and are amorphous and highly hydrated (Uren, 1969). The reactivity of Mn oxides appears to be lost with increasing time. This may be due to the reversion of Mn oxides to a more ordered crystalline structure (Jones and Leeper, 1951). Temperature and moisture in soil interact with abiotic and biotic factors to influence Mn availability (Leeper, 1970; Shuman, 1980; Uren *et al.*, 1988). Low soil temperature combined with high moisture lead to severe Mn deficiency in soybean (Mederski and Wilson, 1955; Moraghan, 1985). Soil compaction also increases Mn uptake in oat and barley (Passioura and Leeper, 1963; Goldberg *et al.*,

1983). In addition, root-induced changes in the rhizosphere such as pH decrease, exudates and microbial activity can considerably change the availability of micronutrients (Marschner, 1991).

Mn deficiency is most prevalent in calcareous soils. Calcareous soils contain variable amounts of free CaCO<sub>3</sub>, and pH varies from 7.3 to 8.5 in most calcareous soils (Lindsay, 1979). pH in calcareous soils is well buffered because of the neutralising effect of carbonate materials on acidifying tendencies. CaCO<sub>3</sub> strongly influences Mn availability through its effects on pH, through surface adsorption and through precipitation or formation of manganocalcite (Jauregui and Reisenauer, 1982a).

#### **1.4 Genotypic differences in Mn efficiency**

Mn efficiency is defined as the ability of a genotype to grow and yield well in a soil which is limiting in available Mn for a standard genotype (Graham, 1984). Considerable genotypic variation in Mn efficiency exists within barley (Graham, 1988). However, the mechanisms of Mn efficiency have not been fully understood. This has hindered progress in the improvement of screening techniques and breeding programs for Mn efficiency.

##### **1.4.1 Genotypic variation and genetic control of Mn efficiency**

In calcareous soil, available Mn is low, but total Mn is often high. This ecosystem may have led to evolution of plant genomes in adaptation to such soils with low available Mn. Genotypic variation in tolerance to Mn-deficient soils was recognised by 1920 (see Graham, 1988, for a review). Since then, cereal species have been categorised by their Mn efficiency: rye > triticale > barley > wheat > oat (Gallagher and Walsh, 1943; Nyborg, 1970; Graham *et al.*, 1983; Marcar, 1986). In addition to the interspecific differences, the variation is also found within species such as shown for oat (Gallagher and Walsh, 1943; Ryan, 1958; Toms, 1958; Vose and Griffiths, 1961; Nyborg, 1970), and wheat (Gallagher and Walsh, 1943; Nyborg, 1970; Marcar and Graham, 1987).

Considerable variation in Mn efficiency was also reported among barley genotypes (Graham *et al.*, 1983). On the basis of intensity of Mn deficiency symptoms and relative grain yields, a world barley collection was classified into three primary classes with respect to Mn efficiency, and an association of Mn efficiency was found with different sources of germplasm (Graham *et al.*, 1983). Mn-efficient genotypes were derived from old English lines and Mn-inefficient genotypes were derived from CI3576, a line originating from Egypt. McCarthy *et al.* (1988) reported that Mn efficiency in barley is likely to be controlled by a single, major gene, using the data from two crosses, Weeah (Mn-efficient)  $\times$  WI2585 (most Mn-inefficient) and Weeah  $\times$  Galleon (Mn-inefficient). Weeah is derived from the old English cultivar, Plumage Archer, that is thought to have originated from calcareous soils of south eastern England (Graham *et al.*, 1983).

Mineral nutritional traits of plants in some cases are under control of a single gene, especially for micronutrient efficiency traits (Epstein, 1972; Graham, 1984; Blum, 1988). Genetic control of iron efficiency in soybean was found to be a single locus (Weiss, 1943). Boron efficiency in celery was also reported under a single gene control (Pope and Munger, 1953), and copper efficiency was governed by a single gene located on the long arm of chromosome 5 in rye (Graham, 1984; Schlegel *et al.*, 1993).

However, in other cases, more complicated genetic systems may be involved in micronutrient efficiency characters (Duncan and Baligar, 1990). For example, Fehr (1982; 1983) found that a major gene governed uptake, and additional genes with quantitative inheritance and additive gene action contributed to Fe efficiency in soybean. Coyne *et al.* (1982) reported two genes control iron efficiency in bean. Römheld and Marschner (1990) and Von Wirén *et al.* (1994) found that phyto siderophore biosynthesis and membrane transporters for Fe phyto siderophore complexes are under different genetic control. As more becomes known about mechanisms of nutrient acquisition, additional genetic input may be found contributing to nutritional traits.

#### 1.4.2 Screening techniques for Mn efficiency

Principles of screening techniques for micronutrients have been discussed by Graham (1984). Field screening is useful for identifying efficient genotypes from a large number of cultivars or lines, and is necessary to assess yields and quality of suitable lines from advanced materials in a breeding program prior to release as commercial cultivars. The genotypic variation in Mn efficiency, initially was found in field trials (Graham *et al.*, 1983). Screening in controlled environment also is very useful in selecting early generation materials from a segregating population, based on seedling bioassay for nutrient efficiency. The advantages of screening in controlled environment are that genotypes tested can be kept in a uniform condition to decrease the effects of interactions between gene and environment, and nutrient stress can be adjusted to provide the optimum screening results.

In controlled environment, two methods, soil culture and solution culture, are often used for screening. As mechanisms of controlling nutrient efficiency vary, each method can be used for certain cases. Solution culture generally can be used for efficiency characters operating from the root surface to the plant interior, such as nutrient reduction at root plasma membranes, absorption rate, translocation and efficiency of nutrient utilisation. Soil culture generally can be applied to efficiency characters operating at the root-soil interface, such as mobilisation of unavailable nutrients from rhizosphere and root system geometry (Graham, 1984).

From a practical and economic point of view, screening for Mn efficiency requires a rapid seedling-based bioassay. Uren (1981) observed cleared zones around plant roots, when the roots of intact plants were cultured on filter paper impregnated with brown  $MnO_2$ , which demonstrated reduction of  $MnO_2$  by plant roots growing along the wetted paper. Differences among species were identified by this technique (Uren, 1981). Marschner *et al.* (1982) also found cleared zones of reduction by the roots of various species, using the nutrient agar containing  $MnO_2$  precipitate. However, using the agar

technique, Harbard (1992) could not find differences in Mn efficiency among barley genotypes. Longnecker *et al.* (1990) screened a range of diverse genotypes and F1, F2 and F3 populations derived from Mn-efficient × Mn-inefficient crosses by using small pots containing a Mn-deficient soil under controlled conditions to simulate the field environment. They found that the scores of the deficiency symptoms were more useful than the leaf Mn concentration, Mn content or leaf fluorescence in the selection of Mn efficiency. The genotype ranking in Mn efficiency by using the visual scores was similar to that in the field studies.

However, some limitations were found associated with the visual score procedure. The soil used to induce Mn deficiency symptoms must contain very low available Mn, which is difficult to manage because many factors could influence the availability of Mn in the soil. Consequently, screening results were inconsistent over the time (McCarthy *et al.*, 1988; Webb *et al.*, 1993a). Moreover, seeds with low Mn content are required to produce the symptoms, which restricts the seed sources for the screening. In addition, the association of the visual scores with the mechanism of Mn efficiency was not understood. Therefore, a meaningful screening technique under controlled conditions was required. This work was carried out and reported in Chapter 2 of this thesis.

### 1.4.3 Mechanism of Mn efficiency

Several possible mechanisms of Mn efficiency have been proposed by Graham (1988), which include either better internal utilisation of Mn already existing in tissues or better ability to take up Mn. If Mn-efficient genotypes have better utilisation, they may have a lower internal requirement for Mn or different redistribution between tissues. However, in fact, critical Mn concentrations in a wide range of plant species are observed within a narrow range, 10-20 mg/kg (Hannam *et al.*, 1987). Moreover, the redistribution of Mn between tissues is rather low in dicotyledons (see Loneragan, 1988 for a review), and is usually the case in monocotyledons (Pearson and Rengel, 1995a, b). Therefore, the better internal utilisation is unlikely to be the mechanism for Mn efficiency. A

possible mechanism is better ability of Mn-efficient plants to acquire Mn from soil (Graham, 1988; Marschner, 1988), which may involve reduction of Mn oxides, mobilisation and subsequent absorption.

#### 1.4.3.1 Reduction of Mn oxides

Possible mechanisms of Mn(III, IV) reduction in rhizosphere and apoplasm have been proposed by Marschner (1988) and Welch (1995). The reduction processes may involve rhizosphere acidification via H<sup>+</sup> efflux from root cells, reducing substances possibly including electrons generated from various chemical reactions in the rhizosphere and apoplasm, and reductases associated with the plasma membrane.

Plant roots modify rhizosphere pH by H<sup>+</sup> release, which strongly affects the concentration of available Mn. However, the H<sup>+</sup> release of graminaceous plants such as barley is far less than dicots (Gardner *et al.*, 1982; Elgala and Amberger, 1988; Youssef and Chino, 1990). In a Mn-deficient calcareous soil, barley roots could not significantly decrease pH in their rhizosphere (Marcar, 1986). Moreover, no clear genotypic difference in H<sup>+</sup> efflux was found in the roots of different barley genotypes pretreated with and without Mn (Harbard, 1992). In addition, dissolution of MnO<sub>2</sub> was related not only to pH change, but also to other factors. For example, in soybean, an Fe-efficient cultivar had a larger capacity of lowering rhizosphere pH than an Fe-inefficient cultivar, but dissolved less MnO<sub>2</sub> and solubilised more Fe<sub>2</sub>O<sub>3</sub> in the rhizosphere (Youssef and Chino, 1990).

Plant roots also release a considerable amount of various organic substances into rhizosphere (Whipps and Lynch, 1986). Some of these substances are reductants which can reduce MnO<sub>2</sub> in soils. Root exudates from vetch dissolved MnO<sub>2</sub> by reduction, but oat root exudates were much less active in the dissolution of MnO<sub>2</sub> than the vetch exudates (Bromfield, 1958a, b). The major components of root exudates for reduction of MnO<sub>2</sub> in grasses, are organic acids such as malic acid in wheat (Jauregui and Reisenauer,

1982b). In addition, certain phenolics are highly effective in reduction of Mn oxides (Stone and Morgan, 1984). However, MnO<sub>2</sub> reduction by reductants is pH dependent. Godo and Reisenauer (1980) found that the rate of MnO<sub>2</sub> reduction by root exudates sharply decreased with substrate pH increasing from 5 to 6. Thus, MnO<sub>2</sub> reduction by reductants seems to play a limited role in the pH range of calcareous soils where the expression of Mn efficiency is found.

Activities of dissimilatory Mn oxide reductase have been found in the membrane fraction of some anaerobic bacteria such as *Bacillus* sp. and *S. putrefaciens* MR-1, and both Mn(IV) and Fe(III) reductase activities share some common properties (Nealson and Saffarini, 1994). However, no information of Mn(III, IV) reductases is available for aerobic organisms. Considerable information of Fe(III) reductases in plants is available. Fe (III) reductases which exist in the plasma membrane of higher plants can reduce external electron acceptors such as Fe(III) chelates and ferric cyanide (Moog and Brüggemann, 1994). As the Fe(III) reductases also appear to be involved in the reduction of Cu(II) to Cu(I) (Welch *et al.*, 1993), it has been proposed that the Fe(III) reductases may play various roles in the regulation of micronutrient cation absorption by root cells (Welch *et al.*, 1993; Welch, 1995). However, it is unknown whether the Fe(III) reductases are involved in Mn(III, IV) reduction, and contribute to genotypic differences in Mn efficiency. Therefore, the observation on the Fe(III) reductase activities was made in barley genotypes differing in Mn efficiency (Chapter 4).

#### **1.4.3.2 Mobilisation of Mn in rhizosphere**

Phytosiderophores are released by the roots of the Gramineae in soils of high pH (Marschner *et al.*, 1986), and they can effectively mobilise Fe as well as other micronutrient cations from calcareous soils, though the degree of Mn mobilisation varies in different soils (Takagi *et al.*, 1988; Treeby *et al.*, 1989; Singh *et al.*, 1992; Zhang, 1993). Less pH-dependent chelates (possibly Mn<sup>3+</sup>-complexation) are considered to be important for Mn mobilisation in calcareous soils (Warden and Reisenauer, 1991a). Any

specific chelates for Mn, however, have not been identified yet. Phytosiderophores are the only known chelates of plant origin which could increase Mn solubility and mobility in calcareous soils. In addition, there is some evidence that Fe acquisition may affect Mn acquisition. For example, an Fe-efficient oat could take up more Fe, Mn, Zn and Ni, than an Fe-inefficient oat from soil (Mench and Fargues, 1994). Mn and Fe uptake by soil-grown grass plants was also found to be positively interrelated (Warden and Reisenauer, 1991b). Therefore, in order to explore the effect of Fe acquisition on Mn efficiency in a calcareous soil, the expression of two genes, *Ids1* and *Ids2* which are likely to be involved in Fe acquisition, was examined in Chapter 5 of this thesis.

In addition, root-microbial associations in rhizosphere may affect Mn mobilisation. Unfortunately, little is known about the mechanisms involved and the relative importance of root-associated microbes. Timonin (1946) found greater numbers of Mn-oxidising bacteria in the rhizosphere of Mn-inefficient cultivars of oat, and thus assumed that this was the basis of genotypic differences in Mn uptake. However, Harbard (1992) could not find any convincing evidence of a rhizosphere change in microbial ecology for two barley genotypes differing greatly in Mn efficiency. It is to be expected that it would be rather difficult to produce consistent results from interactions among microbes, root exudates and environmental conditions if Mn efficiency were related to microbial colonisation of the rhizosphere.

#### **1.4.3.3 Mn absorption**

As available Mn concentration is very low in a Mn-deficient soil, a higher rate of absorption (higher ion-carrier affinity or higher influx rate through ion channel, or greater numbers of transport proteins) can provide more Mn for plant growth, and also a high rate of absorption can remove  $Mn^{2+}$  more efficiently from rhizosphere, which will favour further reduction of Mn oxides by root exudates (Uren, 1982) and Mn mobilisation. Hence, genetic control of absorption may be important for Mn efficiency both in Mn absorption from low concentration and in the mobilisation of Mn oxides (Graham, 1988).



Considerable genetic variation in rates of Mn uptake by oat and soybean was found in solution culture (Munns *et al.*, 1963; Brown and Jones, 1974; Ohki *et al.*, 1980), and in the critical external concentrations (Asher, 1987). However, interpretation of results from conventional solution culture should be made with care because of the difficulty of controlling and maintaining a low Mn concentration in such systems, and as well, some micronutrient cations may be precipitated or adsorbed by precipitates in the apoplasm of root cells (Welch, 1995). Recent advances include metal speciation computer programs, such as GEOCHEM, which can calculate ion activities in aqueous solutions. Thus, it is possible (see Chapter 3) to design absorption experiments with a range of micronutrient metal activities in nutrient solutions similar to that normally found in soil solutions (Chaney *et al.*, 1989; Norvell, 1991; Parker *et al.*, 1992b). Investigation of Mn<sup>2+</sup> absorption rate by Mn-efficient and Mn-inefficient barley genotypes from a chelate-buffered solution is the subject of Chapter 3 of this thesis.

Another way to increase absorption rate is to increase root surface area. As root interception is considered important in the absorption of immobile or slow diffusing nutrients in soil, such as P, Cu, Zn and Mn (Caradus, 1979; Bergmann, 1992), better root geometry and more lateral roots and root hairs can lead to better interception of Mn from soil, and thus more Mn can be absorbed from a low concentration of Mn. However, there was no clear difference in root surface area between barley genotypes with different Mn efficiency (Harbard, 1992). Furthermore, Cu efficiency in wheat was increased by transfer of the long arm of chromosome 5R from rye to wheat, but this translocation did not cause obvious change in root system although rye develops extensive roots with long hairs at a high density (Graham, 1988). Therefore, it is doubtful that the root morphology is involved in Mn efficiency.

Association of barley roots with vesicular arbuscular mycorrhiza (VAM) is a possibility for increasing root interception. The association of roots with VAM hyphae is considered to greatly increase root surface area for interception of soil-immobile

nutrients, and the hyphae can extend outside of the root depletion zone in the rhizocylinder (Tinker, 1984). VAM association increases P uptake in P-deficient soil (Tinker and Gildon, 1983; Baon, 1994). As Mn mobility is similar to P in calcareous soils (Bergmann, 1992), mycorrhizal association may improve Mn uptake by a similar mechanism to that for P. Unfortunately, little information is yet available for this aspect. However, two factors could strongly influence the association of barley roots with mycorrhiza. First, in Mn-deficient soils, P supply often is adequate, which leads to substantial decrease of mycorrhizal infection (Hall, 1978; Baon *et al.*, 1992). Second, low temperature and wet seasons do not favour mycorrhizal growth (Baon *et al.*, 1994), but large genotypic differences in Mn efficiency are often observed under those environmental conditions (Passioura and Leeper, 1963; R D Graham, personal comm.). In addition, there are several reports that infection of roots with VAM decreases the concentration of Mn in the shoots and roots of several crops, thus protecting VAM plants from Mn toxicity (Biermann and Linderman, 1983; Pacovsky 1986; Arines *et al.*, 1989; Kothari *et al.*, 1991). Therefore, the role of the mycorrhizal association in Mn efficiency is questionable.

## **1.5 Molecular studies of nutritional traits**

Improvement in a plant's ability to cope with nutrient stress depends on an understanding of biochemical, physiological and genetic aspects of nutritional traits. Recent advances in molecular biology provide a powerful tool to investigate these aspects at molecular level. The molecular knowledge of genes related to nutritional traits is essential for the effective manipulation of these genes to improve plant ability in adaptation to nutrient stress.

### **1.5.1 RFLP markers for nutritional traits**

Restriction fragment length polymorphisms (RFLPs) as genetic markers have been widely applied to determine the genetic map location of various useful traits. RFLPs have several advantages over conventional markers. An RFLP marker is a DNA marker

which is tightly linked to the gene with a useful trait. Therefore, in principle, any gene associated with an RFLP marker can be isolated using chromosome walking or landing techniques. Moreover, unlike many phenotypic genetic markers which are affected by environmental conditions, RFLPs are stable attributes of DNA itself, and thus RFLP analysis is not affected by environmental conditions. This is particularly important for genetic studies of nutritional traits because they are strongly influenced by various environmental factors. Thus, selection precision can be considerably improved by using RFLP analysis. Besides, the number of potential RFLP sites (effectively unlimited) is much greater than the number of morphological and biochemical markers. DNA sequence variation even within coding regions may generate RFLPs, but may not generate any other observable phenotypic variation. RFLP marker loci can theoretically cover the entire genome, and in fact highly saturated RFLP marker maps have been developed for several plant species such as arabidopsis, tomato, maize, rice and barley (Goodman *et al.*, 1994; Tanksley, 1994a, b; Coe and Gardiner, 1994; Kleinhofs and Kilian, 1994). With a high enough RFLP density, it should be possible to associate any heritable phenotypic trait with a particular DNA sequence.

RFLP analysis only uses a small amount of tissues from any part of the plant at any stage of life cycle to assay genotypes. Once linkage to the desired trait is established, DNA markers can be used as a probe to follow the inheritance of genes in breeding materials. DNA markers will be an important tool for breeding programs of the future. RFLP analysis has great potential in nutritional trait studies in the future (Sussman and Gabelman, 1989; Clark and Duncan, 1991). For example, RFLPs have been used to study the genetic basis of tolerance to low-phosphorus stress in maize. A number of loci was identified in a population segregating for low-P stress (Reiter *et al.*, 1991). A salt-tolerance gene of rice was tagged by a RFLP marker (Zhang *et al.*, 1995).

### 1.5.2 Isolation of genes related to nutritional traits

A number of strategies have been used to isolate genes related to nutritional traits. The first one is a protein-based strategy. By isolating the protein of interest, a DNA sequence can be generated from the available amino acid sequence. Once the DNA sequence is available, the gene can be isolated by designing a synthetic oligonucleotide probe to screen a cDNA library. Alternatively, a purified protein can be used to raise an antibody, and then using the antibody to screen an expression vector library, the corresponding gene can be isolated. The protein-based strategy in conjunction to a DNA-based strategy (see below) was successful, for example, in the isolation of a full-length cDNA of H<sup>+</sup>-ATPase from *Arabidopsis thaliana* (Harper *et al.*, 1989). However, a sufficient amount of purified protein has to be obtained for protein sequencing by using this strategy, and this limits its usefulness to rare proteins, such as membrane transporter proteins (Sussman, 1994).

The second is a DNA-based strategy, which uses the fragments of heterologous DNA from other plant species or other organisms known to encode proteins related to the trait of interest, for example, transport proteins, in order to isolate related genes from a specific plant. This will work if the heterologous probes share sufficient homology to give a detectable signal in library screening. This strategy was successful in the isolation of the proton pump (H<sup>+</sup>-ATPase) genes from *Arabidopsis thaliana* (Harper *et al.*, 1989; 1990; Pardo and Serrano, 1989) by using a partial-length cDNA of oat. Using the arabidopsis cDNA, P-type H<sup>+</sup>-ATPase genes of potato were isolated and characterised (Harms *et al.*, 1994). Such a DNA-based strategy is likely to be successful in much future work.

The third one is a differential screening approach if no protein and DNA information are available. The basis of this approach depends on the different concentrations of mRNA present in control cells and the cells exposed to certain treatments or differing in their genotypes. cDNAs corresponding to genes whose

expression is altered can be detected by differential hybridisation. Many genes have been cloned using this approach. Plants respond to abiotic environmental stress such as nutrient stress, by actively turning on metabolic pathways or by modifying gene expression. The genes involved in these processes can be identified by differential screening. Differential screening has been widely used in the isolation of genes related to nutritional traits. At least three genes of barley which are induced specifically under Fe deficiency, have been isolated and characterised (Okumura *et al.*, 1992; 1994; Nakanishi *et al.*, 1993). Thirteen classes of cDNA clones representing mRNA increased under phosphate deficiency in a green alga were found and sequenced (Dumont *et al.*, 1993). Five different cDNA clones from wheat root tips have been identified, which are related to Al toxicity (Snowden and Gardner, 1993). Two characterised cDNA clones from cultured tobacco cells were induced not only by Al toxicity but also by P starvation (Ezaki *et al.*, 1995). A novel cDNA clone of alfalfa has been acquired from salt stressed plants (Winicov and Deutch, 1994). As no information was available for Mn efficiency at either protein or DNA levels when this project began, and no Mn-efficient isogenic lines or Mn-inefficient mutants were available, the differential screening approach was used to isolate genes related to Mn efficiency from two genotypes greatly differing in Mn efficiency (Chapter 6).

Finally, complementation by plant DNA of known function mutant strains of yeast or bacteria also can be used to identify plant genes related to nutrient absorption. Two potassium channel genes (KAT1 and AKT1) from a cDNA library of arabidopsis are identified by complementation of K<sup>+</sup> uptake-defective yeast strains (Anderson *et al.*, 1992; Sentenac *et al.*, 1992), and the cyanobacterial genes for a Mn transporter complex were identified by using a fragment of wild-type chromosomal DNA containing three closely linked genes to complement a mutant cyanobacterial strain (Bartsevich and Pakrasi, 1995). The complementation strategy also was used to isolate sulphate transporters at the plasma membrane from yeast (Smith *et al.*, 1995), and then the

sulphate transporters of yeast can be used for the isolation of sulphate transporters from plants (Smith *et al.*, 1993).

### 1.5.3 Analysis of gene functions

Gene functions can be analysed by functional expression of cDNAs. Large, inwardly rectified K<sup>+</sup>-selective currents were recorded when KAT1 was expressed in *Xenopus* oocytes (Schachtman *et al.*, 1992), which confirms that KAT1 is an inwardly rectified K<sup>+</sup> channel gene. Functional expression of K<sup>+</sup> channel mutants in K<sup>+</sup> uptake-defective strains of yeast has been used to investigate the effect of mutation in the K<sup>+</sup> channel to elucidate molecular structure and function of K<sup>+</sup> channels (Anderson *et al.*, 1994). Another way to analyse gene functions is to transform plants using sense or antisense constructs of target genes under control of a strong promoter, such as CaMV 35S to obtain the enhanced or reduced expression of transgenic plants. However, no reports are available for transgenic plants with genes related to nutritional traits. A preliminary functional characterisation of one gene related to Mn efficiency was described in Chapter 7.

## Chapter 2

### Screening for Mn efficiency in soil under controlled conditions

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#### 2.1 Introduction

Screening barley genotypes for Mn efficiency was initially conducted in the field (Graham *et al.*, 1983), but field screening can be performed only once a year under South Australian conditions. Later, soil-based pot screening procedure under controlled conditions was developed using scores of Mn deficiency symptoms (Longnecker *et al.*, 1990). However, the soil-based pot screening was not consistent over time (Webb *et al.*, 1993a). Many factors such as temperature, moisture, microorganisms and length of soil storage time can affect Mn availability in soil (Leeper, 1970; Uren *et al.*, 1988; Webb *et al.*, 1993a), so as to influence the development of Mn deficiency symptoms. Thus, improvement of soil-based pot screening methodology became an objective. In nutrient-disease interaction studies in this laboratory, there has been a tendency to use smaller volumes of soil in order to increase pot numbers. This trend was extended to genotype testing as well, since the area of growth chamber required for experimentation was also decreased, but any effect of small pots on Mn nutrition was not addressed at that time. Pot size affects root growth and plant development (Peterson *et al.*, 1984), and changes the uptake of some nutrients such as phosphate (Füleky and Nooman, 1991). Pot size could be a factor in the inconsistency of past results. Therefore, the effect of pot size on the expression of Mn efficiency in barley was examined.

Two experiments were conducted under controlled conditions. Experiment 2A investigated the effect of pot size on the expression of Mn efficiency with a view to improving the screening procedure. Experiment 2B was to test the effectiveness of the new screening procedure developed in Experiment 2A.

## 2.2 Materials and methods

### 2.2.1 Experiment 2A: Effect of pot sizes on the expression of Mn efficiency

Two barley genotypes, Mn-efficient Weeah and Mn-inefficient Galleon (Longnecker *et al.*, 1990) were used in the experiments on pot size. Seeds of each genotype were similar in size, and contained the same, low Mn content (0.12 µg Mn per seed).

Two sizes of pot were used. The small pot was a white tapered tube (2.5 cm max. diameter × 16.5 cm) containing 50 g dry soil. The large pot was a clear plastic sample jar (6.5 cm diameter × 15 cm) covered with aluminium foil and lined with a plastic bag containing 450 g dry soil.

A calcareous sand with approximately 80% CaCO<sub>3</sub> and pH 8.5 (Wilhelm *et al.*, 1988) was used in the experiment, which was collected from a severely Mn-deficient site at Wangary, South Australia in 1991. The topsoil (0-10 cm) and subsoil (10-20 cm) were air-dried, sieved through a 1-mm stainless steel screen and stored in plastic bags, separately. The topsoil and subsoil (1:1, w/w) were well mixed with a small volume of MnSO<sub>4</sub> solution as required and water added to 20% (w/w). Then the soil was incubated in a low temperature regime (15°C day/10°C night) for three weeks. Such preincubation was required to decrease available Mn released from endogenous Mn sources during the soil drying process and storage (Uren *et al.*, 1988). Before sowing, the following nutrients were added and mixed well with the incubated soil: (mg/kg soil) Ca(NO<sub>3</sub>)<sub>2</sub>·4H<sub>2</sub>O, 918; K<sub>2</sub>SO<sub>4</sub>, 114; KH<sub>2</sub>PO<sub>4</sub>, 144; MgSO<sub>4</sub>·7H<sub>2</sub>O, 140; FeSO<sub>4</sub>·7H<sub>2</sub>O, 17.2; H<sub>3</sub>BO<sub>3</sub>, 5.6; ZnSO<sub>4</sub>·7H<sub>2</sub>O, 26.4; NaCl, 12.8; CuSO<sub>4</sub>·5H<sub>2</sub>O, 9.0; CoSO<sub>4</sub>·7H<sub>2</sub>O, 0.9; H<sub>2</sub>MoO<sub>4</sub>·H<sub>2</sub>O, 0.9.

Barley seeds were sterilised in sodium hypochlorite (1% Cl) solution for 15 min, rinsed with water and then soaked in aerated water overnight in the dark. The



germinating seeds were sown one to a small pot or five to a large pot. On day 7, the seedlings in the large pots were thinned to three per pot. Plants were grown in a controlled environment chamber with a 10-hour light/14-hour dark photoperiod at 15°C day/10°C night. The photon flux density at the surface of the pot was 500  $\mu\text{mol}/\text{m}^2/\text{s}$ . Pots were watered daily; every second or third day they were watered by weight to return the moisture content as near as possible to 20% (w/w). After four weeks, the plants were harvested, separated into shoots and roots, oven-dried (70°C), digested in 70% nitric acid and analysed for mineral elements by ICP spectrometry (Zarcinas *et al.*, 1987).

The experiments on pot size were conducted in two runs sequentially in time under the same conditions. At the first run, three levels of Mn (0, 20 and 80 mg/kg soil) were applied in the small pots, and two levels of Mn were used in the large pots (0 and 20 mg/kg soil). At the second run, four levels of Mn (0, 5, 10 and 20 mg/kg soil) were applied in the small pots, and six levels (10, 15, 20, 30, 40 and 100 mg/kg soil) was in the large pots. In order to have an overall picture, data of the two runs were combined as in Fig. 2.1. The dry matter data of two runs were combined by normalising one set to the other based on the yield of the common 20 mg Mn/kg soil treatment. The average adjustment for the two genotypes was 21% for small pots and 9% for large pots. The data presented are means of 4 replicates for the small pots and 3 replicates for the large pots.

### **2.2.2 Experiment 2B: Application of the results of Experiment 2A to a new screening procedure for Mn efficiency**

The effectiveness of screening a number of genotypes grown together in a large tray, such as is often used by breeders, was tested with six genotypes grown in two different soils. The six genotypes were Amagi Nijo, WA73S276, Weeah (efficient), Schooner (intermediate) and Galleon and WI2585 (inefficient), representing a wide range of Mn efficiency as defined in field studies. Seeds with high Mn content ( $\mu\text{g}$  per seed) were used in this experiment: 1.1 in Amagi Nijo, 0.9 in WA73S276, 0.7 in Weeah, 1.6

in Schooner, 1.1 in Galleon and 1.5 in WI2585. Seeds with a high Mn content were chosen because seeds from early-generation segregating populations usually contain a high Mn content.

Four plants of each of the six genotypes were grown together in a large tray (42 × 25 × 15 cm), which contained 15 kg of Wangary soil or 17.5 kg of Marion Bay soil. The large tray not only provided more soil volume for each plant, but also allowed large numbers of plants to be screened under the conditions that would be ideal for screening early-generation segregating materials. Wangary soil was the same as that used in Experiment 2A, and Marion Bay soil (calcareous sand, pH 8.3, and soil bulk density, 0.85, g/cm<sup>3</sup>) was collected from a moderately Mn-deficient site at Marion Bay, South Australia, in 1992. 30% water content (w/w) was used in the Marion Bay soil. Both soils were supplied with 10 mg Mn/kg soil. This level of Mn supply was chosen after consideration of both the high content of seed Mn used in this experiment and the optimum range of Mn supply (10-30 mg/kg soil) in the large pot of Experiment 2A (genotypic differences in dry matter production and Mn concentration).

The conditions for plant growth were the same as described in Experiment 2A. To obtain similar plant growth in each soil, shoots of plants (cut at the soil surface) were harvested after five weeks in Wangary soil, and after seven weeks in Marion Bay soil. Slower growth in Marion Bay soil was possibly due to the low soil bulk density, leading to poor root contact with soil. Plant samples were oven-dried, digested in 70% nitric acid and analysed for mineral elements by ICP spectrometry (Zarcinas *et al.*, 1987).

## **2.3 Results**

### **2.3.1 Experiment 2A**

In the small pot, dry matter of shoots in the two barley genotypes increased with increasing Mn applied from 0 to 20 mg/kg soil (Fig. 2.1a) (see Appendix A, Table A1 for analysis of variance table). When Mn applied was below 20 mg/kg soil, the symptoms

of Mn deficiency appeared after two weeks and Mn concentrations in shoots were below 10 mg/kg DM (Fig. 2.1b) (see Appendix A, Table A2 for analysis of variance table), which is below the critical level (Hannam *et al.*, 1987). Further increasing Mn supply to 20 mg/kg soil and beyond substantially increased the Mn concentrations in shoots, but had little effect on the dry matter (Fig. 2.1a, b). Genotypic differences in dry matter production appeared only at 10 mg Mn/kg soil (Fig. 2.1a). When plants were grown in the large pots, genotypic differences in the dry matter of shoots were observed over a wider range of Mn supply, that is, between 0-30 mg Mn/kg soil (Fig. 2.1d) (see Appendix A, Table A4 for analysis of variance table). If genotypic differences were assessed by the Mn concentration of shoots, the range was even wider, from 10 to 100 mg/kg soil (Fig. 2.1e) (see Appendix A, Table A5 for analysis of variance table).

In comparison with the large pots, the plants in the small pots had lower shoot dry matter at the highest (Mn adequate) (Fig. 2.1a, d), and higher Mn concentrations in shoots (Fig. 2.1b, e).

Root/shoot ratios of the two genotypes were low (about 0.2) in both pot sizes at zero added Mn, but increased with increasing Mn supply up to 20 mg/kg soil (Fig. 2.1c, f) (see Appendix A, Table A3 and A6 for analysis of variance table). High root/shoot ratios appeared only in the small pots at Mn supply above 20 mg/kg soil. Genotypic differences in root/shoot ratios were found at 10 mg/kg soil in the small pots, and between 10-40 mg/kg soil in the large pots, which may be a result of the relative depression in root growth by Mn deficiency (Webb and Dell, 1990). Lower Mn uptake by the Mn-inefficient plants could result in lower root/shoot ratios. However, at the higher rates of Mn, similar root/shoot ratios in the two genotypes were found in both pot sizes (Fig. 2.1c, f).

The critical Mn concentration in shoots for deficiency in shoot growth was calculated as the concentration corresponding to 90% of the plateau value, namely 16

mg/kg DM. The critical Mn concentration was not affected by pot size and was also independent of genotype (Fig. 2.2). The Mn concentration in roots of the two genotypes were increased with increasing Mn supply in the large pots (Fig. 2.3). No genotypic difference in Mn concentration of roots was found (Fig. 2.3) (see Appendix A, Table A7 for analysis of variance table).

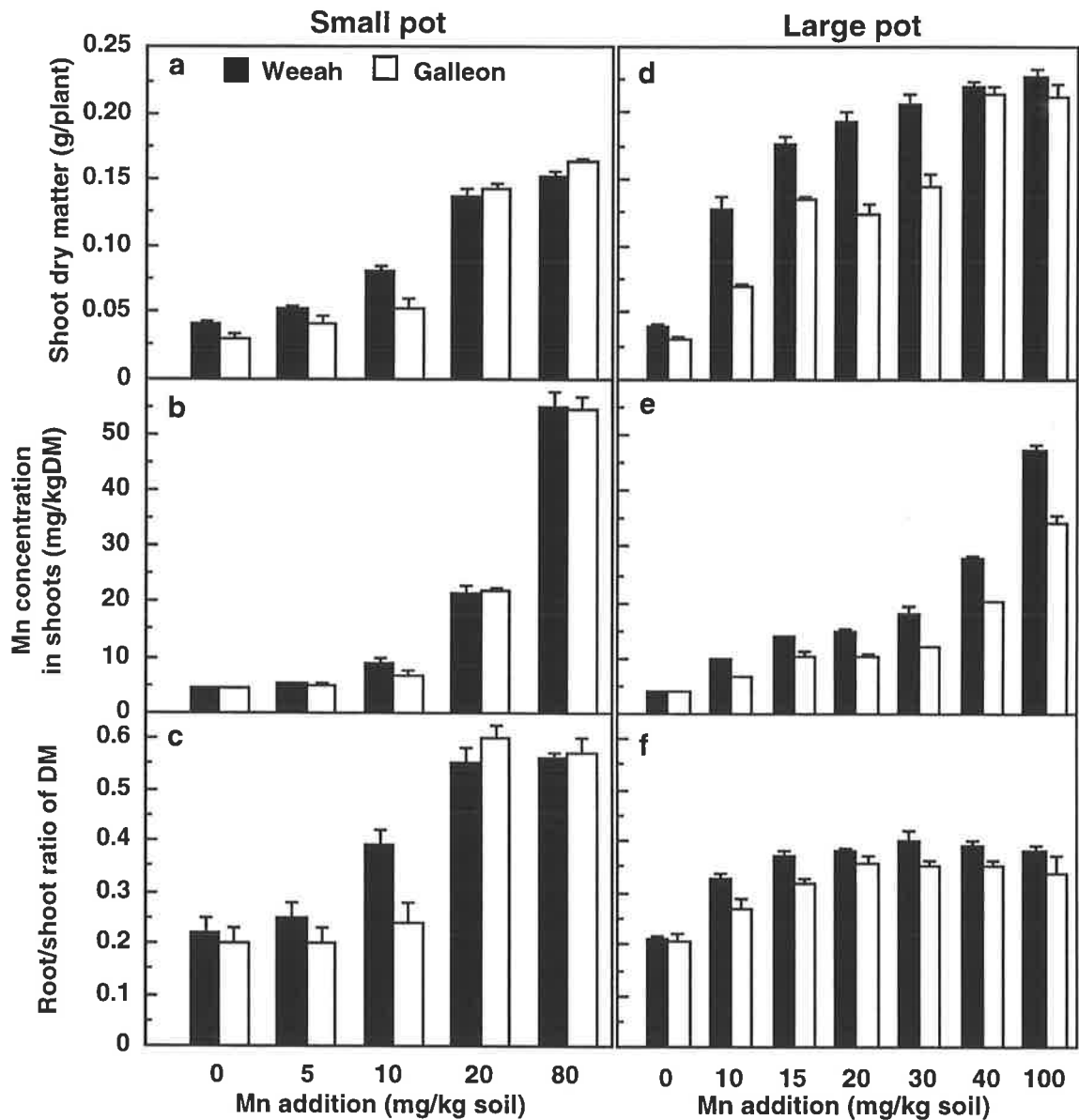
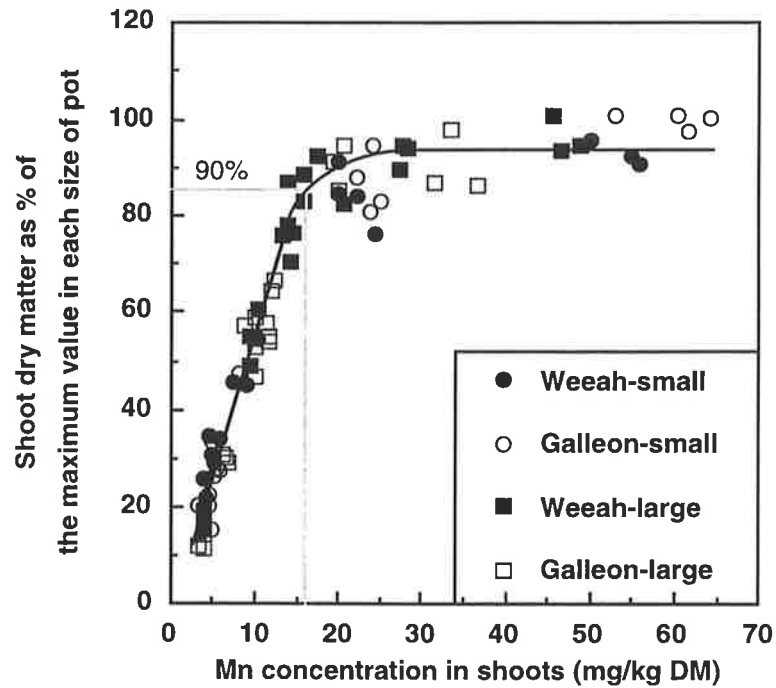
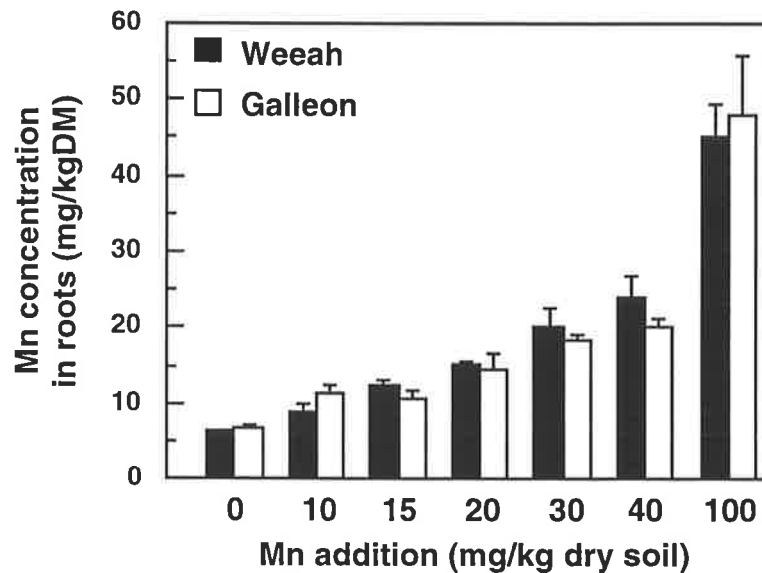


Fig. 2.1. Effect of Mn supply in two sizes of pot on the dry matter of shoots (a and d), Mn concentrations in shoots (b and e) and root/shoot ratios (c and f) for two barley genotypes. Standard errors are shown as vertical bars.



**Fig. 2.2.** Relationship between shoot dry matter and Mn concentration of shoots in two sizes of pot and two genotypes. Figure is plotted with the individual data using percentage of the maximum value of shoot dry matter in each size of pot.



**Fig. 2.3.** Effect of Mn supply in the large pot on Mn concentrations in roots of two barley genotypes. Standard errors are shown as vertical bars.

### 2.3.2 Experiment 2B

In the severely Mn-deficient Wangary soil supplied with 10 mg Mn/kg soil, the dry matter production was high in Mn-efficient genotypes, WA73S276 and Weeah, but decreased with decreasing Mn efficiency, Schooner > Galleon > WI2585 (Fig. 2.4a). Amagi Nijo, a Mn-efficient genotype in the field (R D Graham, unpublished data) had high variation of dry matter production among plants because of variable seedling vigour. Mn concentrations in the youngest expanded leaf blade (YEB) were high in the Mn-efficient genotypes, Amagi Nijo and Weeah, which were just over the critical level (Hannam *et al.*, 1987), slightly lower in WA73S276, and declined further as Mn efficiency decreased progressively, Schooner > Galleon > WI2585 (Fig. 2.4c). The genotype ranking of Mn efficiency in terms of Mn concentrations of YEB was similar to that of Mn concentrations in whole shoots (Fig. 2.4b, c).

In the moderately Mn-deficient soil from Marion Bay supplied with 10 mg Mn/kg soil, similar shoot growth was found in all genotypes except WI2585 which was slightly higher (Fig. 2.4d). The Mn concentrations in the YEB of all six genotypes (Fig. 2.4f) were above the critical level (Hannam *et al.*, 1987) and thus the growth of all genotypes was not restricted by Mn supply. However, the large genotypic differences in Mn concentrations of shoots or YEB still appeared (Fig. 2.4e, f). The ranking of the six genotypes in Mn efficiency was similar in terms of Mn concentrations in YEB or shoots to that in the more severely Mn-deficient soil from Wangary (Fig. 2.4b, c, Fig. 2.4e, f).

## 2.4 Discussion

The expression of Mn efficiency was affected by pot size. In the small pots, Mn efficiency could be expressed at only one, low rate of Mn application (10 mg/kg soil). Below that rate, the growth of the Mn-efficient genotype was affected by severe Mn deficiency, so that no genotypic differences could be detected. When Mn supply was

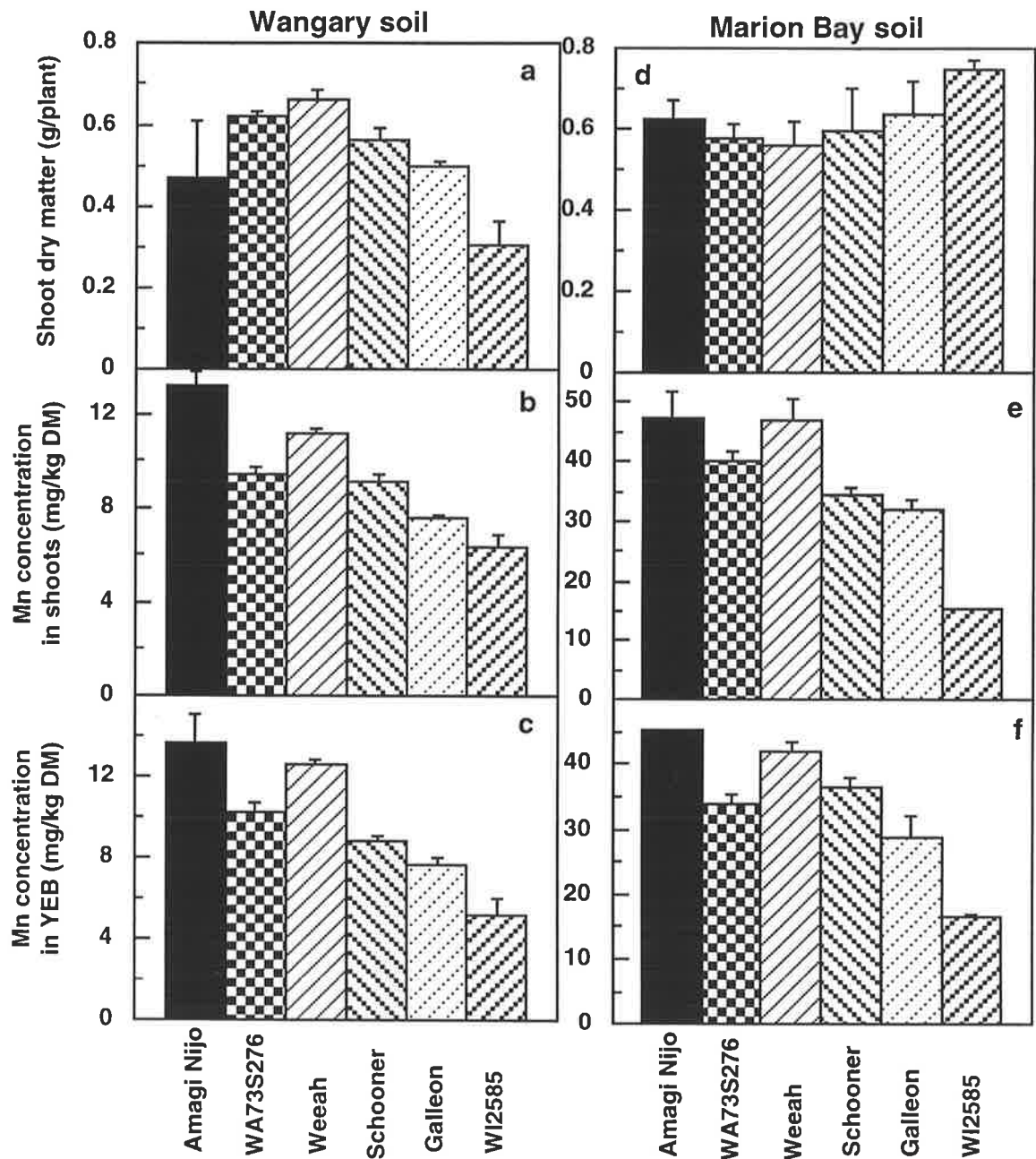


Fig. 2.4. Dry matter of shoots (a and d), Mn concentrations in shoots (b and e) and YEB (c and f) for six genotypes grown in two soils in a large tray. Standard errors (n=4) are shown as vertical bars.

beyond 10 mg/kg soil, high Mn concentrations of shoots were achieved in both genotypes compared with those in the large pots, so as to eliminate the genotypic differences. However, the critical value of Mn for deficiency in shoot growth was not affected by the small pots. Thus, factors other than the critical value for Mn deficiency could be involved. Plants in the small pots strongly responded to root restriction by decreasing yields of shoots and increasing root/shoot ratios and Mn concentrations of shoots (Fig. 2.1). Lower yields caused by small pots (Peterson *et al.*, 1984) may be partly responsible for the higher Mn concentration, but the main factor may be higher Mn mobilisation, resulting from higher root/shoot ratios and possibly higher root exudation stimulated by root restriction (Barber and Gunn, 1974). The higher Mn concentration of shoots achieved by both Mn-efficient and Mn-inefficient genotypes at lower rates of Mn supply, therefore, eliminated the genotypic difference in dry matter production because no Mn deficiency affected the plant growth of the two genotypes, and the genotypic difference in Mn concentration of shoots no longer existed.

In the large pots, any effect on plant growth due to the restriction of pot size was expected to be diminished. Mn efficiency was expressed over a wider range of Mn supply. A practical outcome of these results is that by using an adequate size of pot, measurement of Mn concentration of shoots provides an effective index of Mn efficiency. Moreover, the optimum rates of Mn fertiliser for screening Mn efficiency can be more safely chosen since in the large pots, the rate is less critical, bearing in mind soil variability from batch to batch and in the period of storage (Webb *et al.*, 1993a). This means that the effect of variation in Mn availability, caused by abiotic and biotic factors, on screening results can be substantially decreased.

In order to apply the results in Experiment 2A to screening, the effectiveness of the newly developed screening procedure needed to be applied to selection in systems such as are used by plant breeders. Where screening for a trait is possible in soil in a glasshouse, breeders often use a large tray with many genotypes grown together. Such



screening is never done with replication because of the large numbers of genotypes they handle, and in any case, it is often impossible: for example, F<sub>2</sub>s cannot be replicated. Thus, six genotypes with a high content of seed Mn were grown in two soils to rank their Mn efficiency, but in doing so, the implications of Experiment 2A were extrapolated to trays from the pots of that study; the trays, being larger still, were more suited to testing large numbers of segregants in a future breeding program. The ranking of six genotypes was consistent with the field results (Longnecker *et al.*, 1990) in terms of Mn concentrations in YEB or shoots, except that WA73S276 was relatively lower in Mn efficiency than that established in the field results. The expression of Mn efficiency in terms of Mn concentration in shoots or YEB was reproducible in two different soils, and even when using seeds with high Mn content. This indicated that reproducible results for screening could be generated by using the new procedure. In addition, in the large trays, the mixed root systems of Mn-efficient and Mn-inefficient genotypes do not seem to interact to affect their ranking in Mn efficiency, which is in agreement with earlier observations of J Lush (undergraduate research project report, University of Adelaide, 1987) and B Dinkelaker (personal comm., 1989). This suggests that any change in rhizosphere, which may be mediated by the Mn-efficient plants, is limited to a narrow spatial zone, so that it does not confer any cross-advantage to the Mn-inefficient plants. The mixed culture has a practical value for screening different genetic materials together with standard genotypes in one pot. Large trays appear to be a suitable means of screening large numbers of genotypes from early-generation segregating material, quickly and under controlled conditions. If assessment is done by using Mn concentration in YEB, together with a non-destructive, visual score of vigour and colour, the best selections can be grown on for seed.

The results also showed that although the expression of Mn efficiency was different between the genotypes in the different sizes of pot, the Mn critical concentration for deficiency in shoot growth was independent of genotype (Fig. 2.2), indicating that internal requirements for Mn are not different in the two genotypes differing in Mn

efficiency. Moreover, the Mn concentration in roots was similar in the two genotypes, but the Mn concentration in shoots was higher in the Mn-efficient genotype than in the Mn-inefficient genotype. This indicates that the low shoot Mn content of the Mn-inefficient genotype grown in calcareous soil is not due to poor translocation from roots to shoots. The high Mn accumulation in shoots of the Mn-efficient genotype must be due to Mn mobilisation and uptake. In addition, higher Mn accumulation in shoots of the Mn-efficient genotypes was not suppressed even at the highest level of Mn in the experiments, which was high enough for both Mn-efficient and Mn-inefficient genotypes to obtain more than adequate Mn. This shows that the operation of any mechanism of Mn efficiency in roots under soil conditions is not suppressed by Mn adequacy in shoots. Thus, the expression of Mn efficiency appears to be controlled by factors in roots.

## Chapter 3

### Genotypic variation in Mn accumulation by plants in solution culture

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#### 3.1 Introduction

Mn-efficient genotypes can acquire more Mn than Mn-inefficient genotypes from calcareous soils (Chapter 2). However, little is known about the actual process of Mn acquisition by Mn-efficient genotypes. Two possibilities are most likely to be involved. One is that Mn-efficient genotypes may have a higher rate of Mn absorption from available Mn in soil solution than Mn-inefficient genotypes. The other is that Mn-efficient genotypes may have a higher rate of Mn mobilisation from insoluble forms of Mn in soil. In this chapter, Mn absorption by plants of different genotypes was investigated in solution culture because it is difficult to separate Mn absorption from Mn mobilisation in soil.

As roots of graminaceous species can maintain an adequate intake of Mn into plants when exposed to external concentrations less than 1  $\mu\text{M}$  in Mn (Robson 1988; Webb *et al.*, 1993b), a low concentration of Mn supply is needed to be maintained in nutrient solution. However, it is rather difficult to maintain such low concentration by using conventional methods because of depletion and contamination (Uren *et al.*, 1988). Besides, Mn deficiency is most common in calcareous soil with high pH. To apply high pH in nutrient solution, precipitation of some nutrients such as Fe, P, Ca and Mn has to be prevented.

A new chelate-buffering technique has been developed (Bell *et al.*, 1991a). Using this technique, micronutrient cations in the nutrient solution are complexed with synthetic chelates so that the concentrations of free ions are very low and constant. In these two important respects, it simulates soil conditions and avoids depletion. Another

advantage of this technique is that risks of precipitation of some nutrients in solutions are reduced, especially at higher pH. This technique has recently been used to study absorption of Fe by plants (Bell *et al.*, 1991a), Zn (Parker *et al.*, 1992a; Norvell and Welch, 1993; Welch and Norvell, 1993) and Cu (Bell *et al.*, 1991b), as well as Mn (Chaney *et al.*, 1989; Webb *et al.*, 1993b).

Therefore, using the chelate-buffered nutrient solution, possible genotypic differences in rate of Mn accumulation by barley plants were investigated over a range of Mn supply as  $Mn^{2+}$  ions (3A), and over a range of pH levels (3B).

### **3.2 Materials and methods**

#### **3.2.1 Experiment 3A: Mn accumulation in plants of three genotypes with three rates of Mn supply**

Three barley cultivars, Weeah, Schooner and Galleon, were used in the experiments. Seeds with similar, low Mn content ( $\mu\text{g}$  Mn per seed): 0.12  $\mu\text{g}$  for Weeah, 0.13  $\mu\text{g}$  for Schooner and 0.12  $\mu\text{g}$  for Galleon, were used in the experiment to minimise any possible confounding effects of endogenous seed Mn with absorption of exogenous Mn from low Mn media.

A chelate-buffered nutrient solution was used in the experiment. Experimental procedures and basal nutrient solutions were similar to those described by Webb *et al.*, (1993b). The basal nutrient solution contained the macronutrients (in mM):  $KNO_3$  1.5,  $Ca(NO_3)_2$  1.0,  $NH_4H_2PO_4$  0.2 and  $MgSO_4$  0.25, and the micronutrients (in  $\mu\text{M}$ ): KCl 50,  $H_3BO_3$  12.5,  $H_2MoO_4$  0.1, FeHEDTA (N-hydroxyethylethylenediaminetriacetic acid) 20, ZnHEDTA 10, CuHEDTA 1.0, NiHEDTA 0.1 and  $K_3HEDTA$  25. 5 mM MES buffer was used to buffer the nutrient solution to pH 6.0. Three concentrations (4 nM, 40 nM and 400 nM) of MnHEDTA were applied in the experiment as treatments, which covered the range from deficiency to adequacy for barley grown in this system (Webb *et al.*, 1993b). The activities of these three levels of  $Mn^{2+}$  were calculated using a computer program GEOCHEM-PC (Parker *et al.*, 1992b), and when expressed as the

negative logarithm of  $Mn^{2+}$  activity ( $pMn^{2+}$ ), these three levels were 10.2, 9.2, and 8.2, respectively (Appendix B, Table B1, B2 and B3, respectively). Precautions were taken against contamination and entry of light into the rooting medium (Norvell and Welch, 1993). All equipment used to grow plants was washed in 10% nitric acid. High purity water (18 Mohm-cm resistivity) was used throughout the experiment.

Plants were grown in a controlled environment chamber with a 10-hour light/14-hour dark photoperiod at 15°C day/10°C night. The photon flux density at the surface of the pot was 500  $\mu\text{mol}/\text{m}^2/\text{s}$ .

On day 0 (D0), barley seeds were sterilised in sodium hypochlorite (1% Cl) solution for 15 min, rinsed with water and then soaked in aerated water overnight in the dark. Five germinating seeds were placed on polyethylene mesh which was located on the bottom of a seedling cup, and the cup was filled with black polyethylene beads to block light and provide support to seedlings. Four seedling cups were located in the lid of each black polyethylene pot containing 900-ml nutrient solution. Each treatment was replicated four times. On D7, seedlings in each pot were thinned to one cup containing 4 seedlings and the other three cups containing 3 seedlings each, and solutions were renewed. In order to minimise depletion of nutrients, plants were transferred to fresh solution in newly washed pots every 3 or 4 days. At each transfer, the plant roots, pot lid, air tube and cups were rinsed with high purity water. At subsequent harvests on D11, D18, D25, D31 or D37, the cup containing 4, 3, 3 or 3 seedlings, respectively was removed from each pot and replaced with a new cup containing black beads to prevent light infiltration. The roots of plant samples were washed in high purity water for approximately 10 seconds, and excess water was blotted on fresh laboratory tissues. Then, plants were separated into different parts as required, oven-dried (70°C), digested in 70% nitric acid and analysed for mineral elements by ICP spectrometer (Zarcinas *et al.*, 1987). Accumulation rates of Mn between harvests were calculated using the formula of Williams (1948).

### 3.2.2 Experiment 3B: Effect of pH on Mn accumulation in plants of three genotypes

Mn absorption is strongly affected by pH through its effect on Mn availability in soil and on external pH of root plasma membranes. In soil, Mn absorption by plants decreases with increasing pH because of a decrease in Mn availability (Marschner, 1988). On the other hand, in nutrient solutions, Mn absorption increases linearly with increasing pH from pH 4.2-6.5 (Munns *et al.*, 1963; Vorm and Diest, 1979), but was dramatically suppressed in the neutral and alkaline ranges because of Mn oxidation and precipitation as suggested by Munns *et al.* (1963) and Maas *et al.* (1968). Thus, in order to reliably study Mn absorption from nutrient solutions with high pH, it is essential to prevent possible precipitation of Mn and other nutrients. A chelate-buffered nutrient solution was used in this experiment, in which predicted precipitation at high pH can be avoided with help of computer programs such as GEOCHEM-PC (Parker *et al.*, 1992b).

The formation constants of Tris complexes with  $H^+$ ,  $Ni^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$ ,  $Ca^{2+}$  and  $Mg^{2+}$  obtained from Smith and Martell (1989) were included in the GEOCHEM data base. Because GEOCHEM-PC predicted that Ca and P would precipitate at higher pH, the concentrations of  $Ca(NO_3)_2$  and  $NH_4H_2PO_4$  were reduced to 250  $\mu M$  and 100  $\mu M$ , respectively. Similar activity of each micronutrient cation at each pH was achieved by adjusting the total nutrient-chelate supply (Table 3.1). The activities of Mn, Zn, Cu and Ni expressed as negative logarithm of activity (pMetal) were 9.0, 10.5, 14.3 and 15.0, respectively (Appendix B, Table B4, B5 and B6, respectively). The level of Mn supply was just below the level predicted as adequate for barley growth in this system at pH 6.00 (Webb *et al.*, 1993b). Fe activity was adjusted to pFe 18.8 at pH 6.00 and 6.85, but to avoid precipitation only pFe 19.7 at pH 7.70. To ensure adequate Fe status in all treatments, plants were grown at pFe 17.5 and pH 6.0 until pH treatments were imposed on D11 (Appendix B, Table B7). The maximum pH that could be attained without further compromising nutrient solution composition and still avoiding

precipitation, was pH 7.70. The three pH levels were buffered with 5 mM of different combinations of MES-Tris, which were 3.61 mM MES and 1.39 mM Tris at pH 6.00, 2.71 mM MES and 2.29 mM Tris at pH 6.85, and 2.32 mM MES and 2.68 mM Tris at pH 7.70, respectively.

Three barley cultivars, Weeah, Schooner and Galleon were used in this experiment, which was the same as those used in Experiment 3A. The experimental procedures and plant growth conditions were the same as described in Experiment 3A. On D1, germinating seeds were transferred to three seedling cups located in each pot. The three seedling cups contained 6, 5 or 5 germinating seeds, respectively. On D7, seedlings were thinned to 4, 3 and 3 seedlings per cup for the harvests on D11, D15 and D19, respectively, and the solution was renewed. On D11, the cup with 4 seedlings was removed from each pot and replaced with a new cup containing black beads to prevent light infiltration. The solution was changed and then pH treatments were imposed. All treatments were replicated four times.

**Table 3.1. Compositions of pretreatment and pH treatments.**

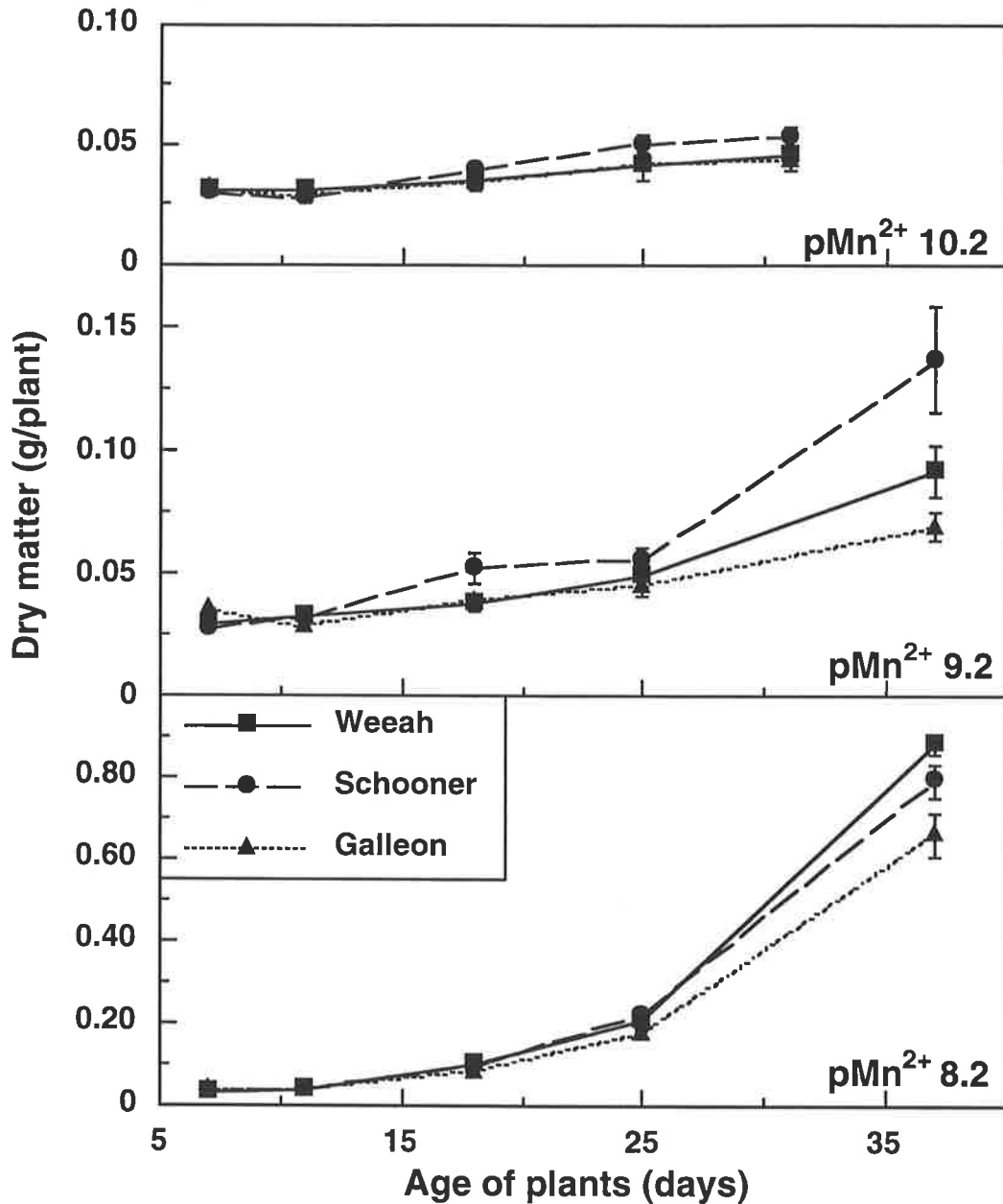
Treatment	Nutrient solution composition ( $\mu\text{M}$ )				
	Fe	Mn	Zn	Cu	Ni
Day 1-Day 11 pH 6.00	50.0	0.215	10.0	1.0	0.1
Day 11-Day 19					
pH 6.00	2.5	0.215	10.0	1.00	0.1
pH 6.85	20.0	0.250	11.5	1.15	0.1
pH 7.70	20.0	0.260	12.0	1.20	0.1

### 3.3 Results

#### 3.3.1 Experiment 3A

At extremely low Mn supply (Fig. 3.1,  $\text{pMn}^{2+}$  10.2), dry matter production of the three genotypes increased only slightly from D11 to D31, and any genotypic difference was small. With an increase in Mn supply, the dry matter production increased dramatically. Although there were still no consistent genotypic differences in dry matter production within 25 days at either  $\text{pMn}^{2+}$  9.2 or 8.2, the differences appeared

by D37 at  $\text{pMn}^{2+}$  9.2 and  $\text{pMn}^{2+}$  8.2 (see Appendix A, Table A8 for analysis of variance table). At  $\text{pMn}^{2+}$  9.2, the dry matter of Schooner was higher than that of Weeah and Galleon, and the difference between Weeah and Galleon was not large, compared with the difference in the soil-based pot experiment (see Fig. 2.1d). The differences at adequate supply ( $\text{pMn}^{2+}$  8.2) may be related to genotypic potential in dry matter production in solution culture as no nutrients were limiting.



**Fig. 3.1.** Effect of three levels of  $\text{Mn}^{2+}$  activity on the dry matter production of three barley genotypes grown in a chelate-buffered nutrient solution. Standard errors ( $n=4$ ) are shown as vertical bars.



With increasing plant age, Mn concentrations of shoots decreased at  $pMn^{2+}$  10.2 and 9.2, but greatly increased at  $pMn^{2+}$  8.2 (Fig. 3.2). Mn concentrations of roots were very low and hardly changed at  $pMn^{2+}$  10.2, but greatly increased at  $pMn^{2+}$  9.2, and were nearly constant at a level above 6 mg/kg DM at  $pMn^{2+}$  8.2 (Fig. 3.3). However, there were no consistent genotypic differences in either Mn concentrations of shoots or roots at any of these levels of Mn supply (Fig. 3.2; 3.3).

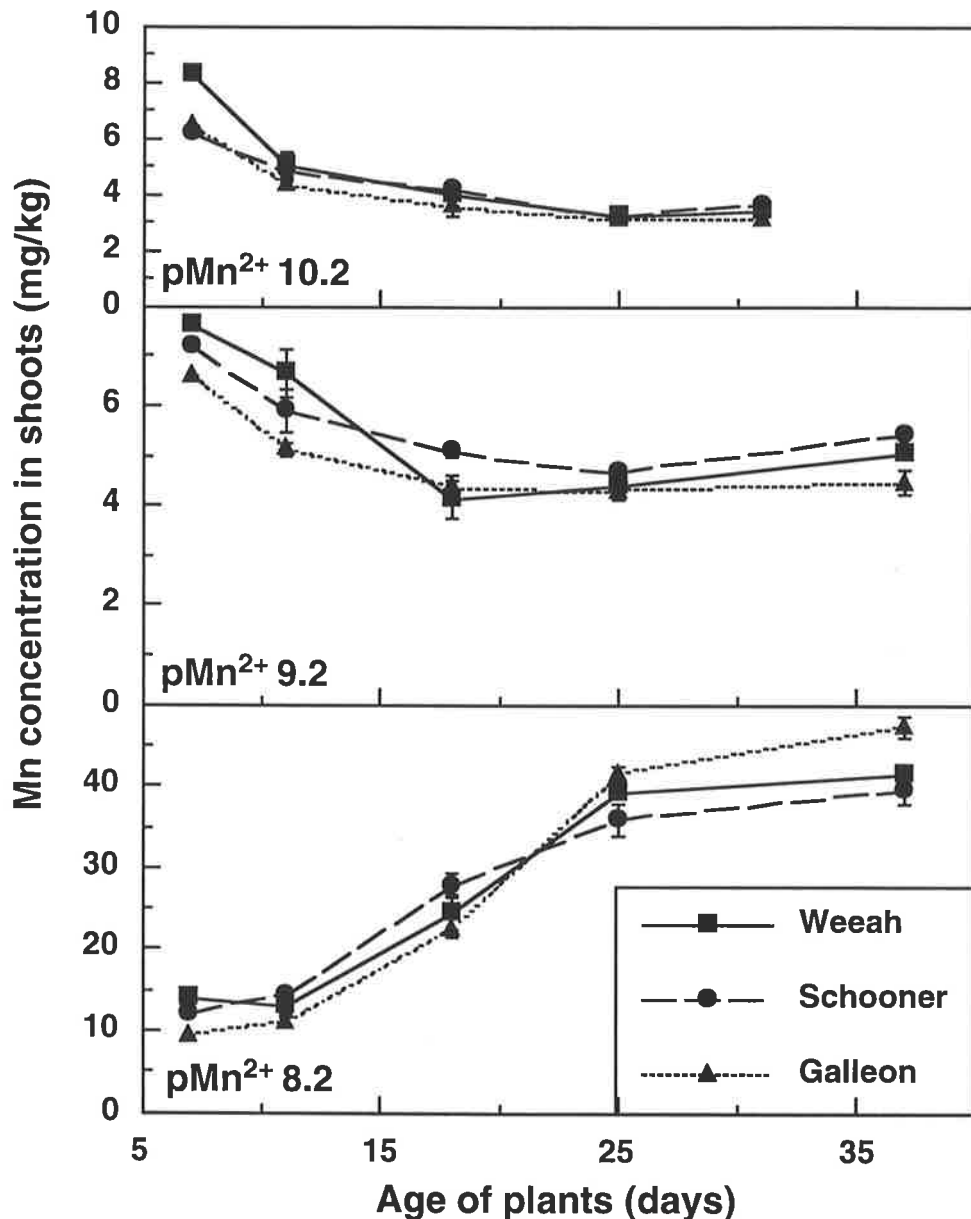
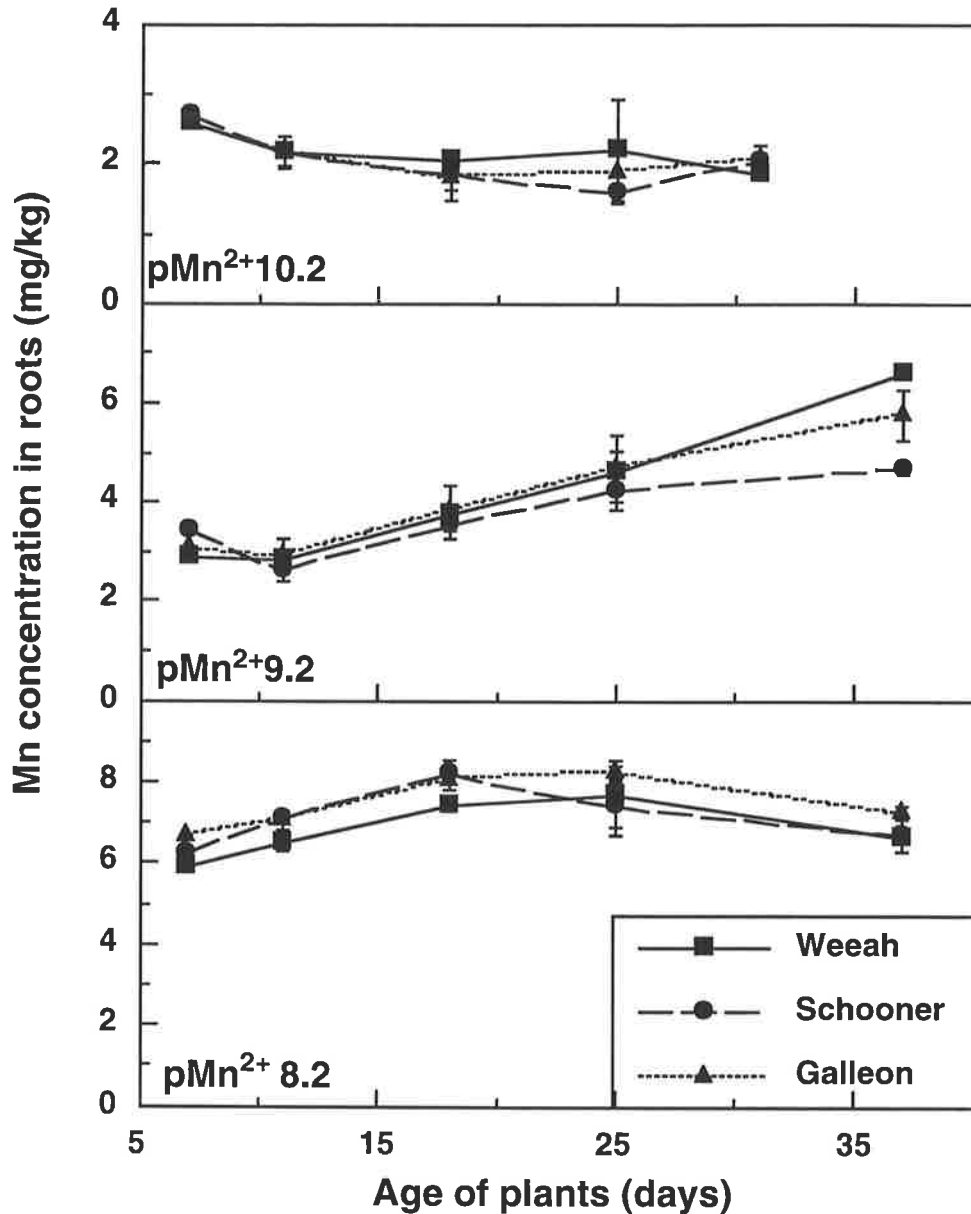
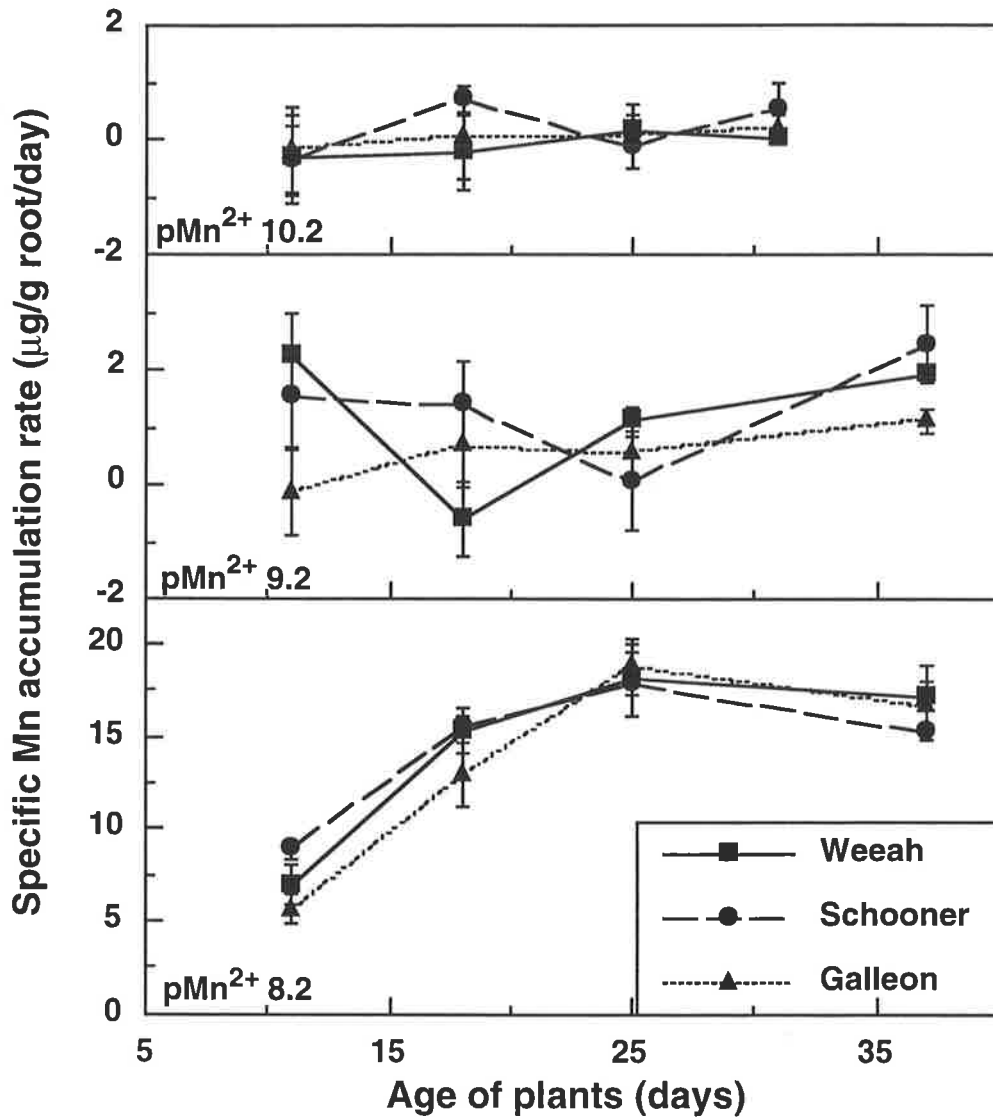


Fig. 3.2. Effect of three levels of  $Mn^{2+}$  activity on Mn concentrations in the shoots of three barley genotypes grown in a chelate-buffered nutrient solution. Standard errors ( $n=4$ ) are shown as vertical bars.



**Fig. 3.3.** Effect of three levels of  $\text{Mn}^{2+}$  activity on Mn concentrations in the roots of three barley genotypes grown in a chelate-buffered nutrient solution. Standard errors ( $n=4$ ) are shown as vertical bars.

The specific accumulation rates of Mn calculated between harvests for each of the three genotypes are shown in Fig. 3.4. Generally no net accumulation occurred at  $\text{pMn}^{2+}$  10.2. There was a low rate of accumulation (about  $1 \mu\text{g Mn/g root/day}$ ) when Mn was supplied at  $\text{pMn}^{2+}$  9.2, and a high rate of accumulation at  $\text{pMn}^{2+}$  8.2 for all three genotypes. Again, no clear genotypic difference in the accumulation rate was apparent in the chelate-buffered nutrient solution at pH 6.0.

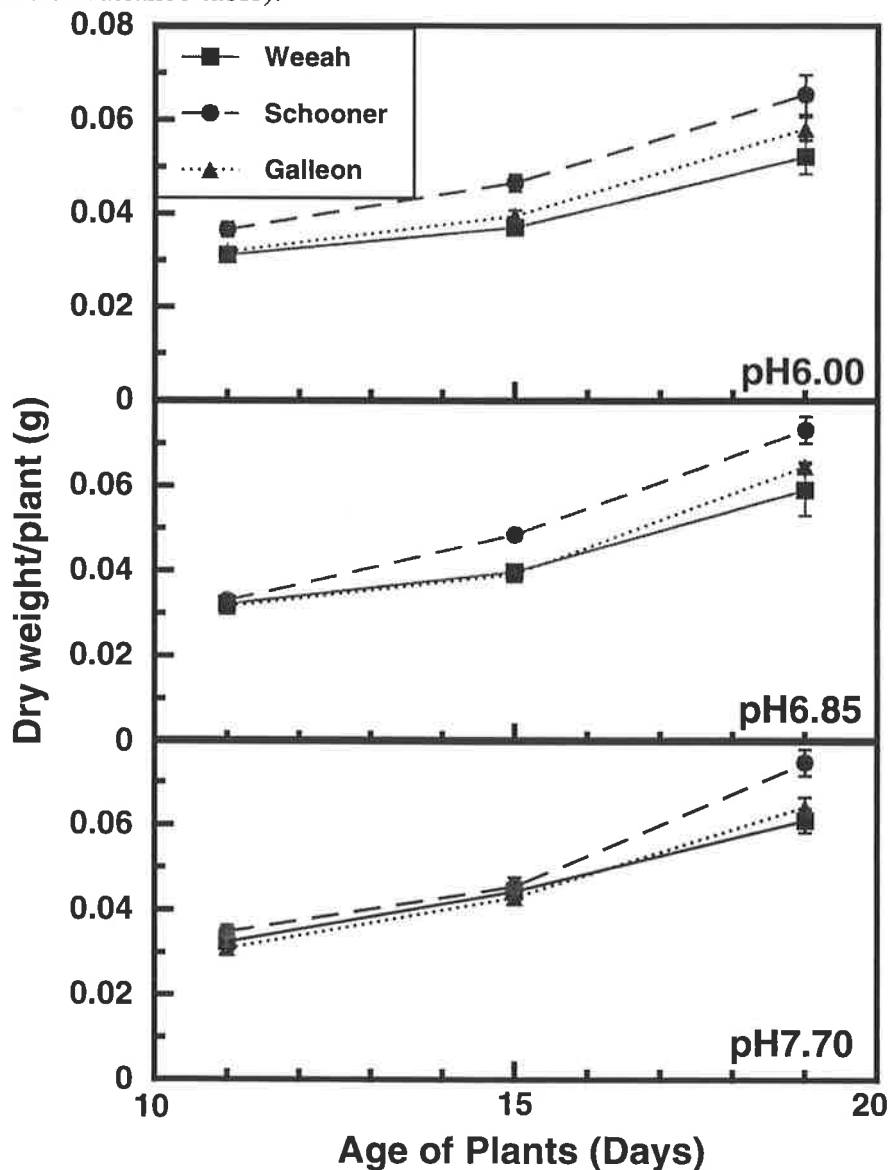


**Fig. 3.4.** Effect of three levels of  $Mn^{2+}$  activity on specific accumulation rate of Mn by the roots of three barley genotypes grown in a chelate-buffered nutrient solution. Plant age refers to age at the end of each successive accumulation period. First absorption period began on D7. Mn accumulation rates were calculated from two harvests. Standard errors ( $n=4$ ) are shown as vertical bars.

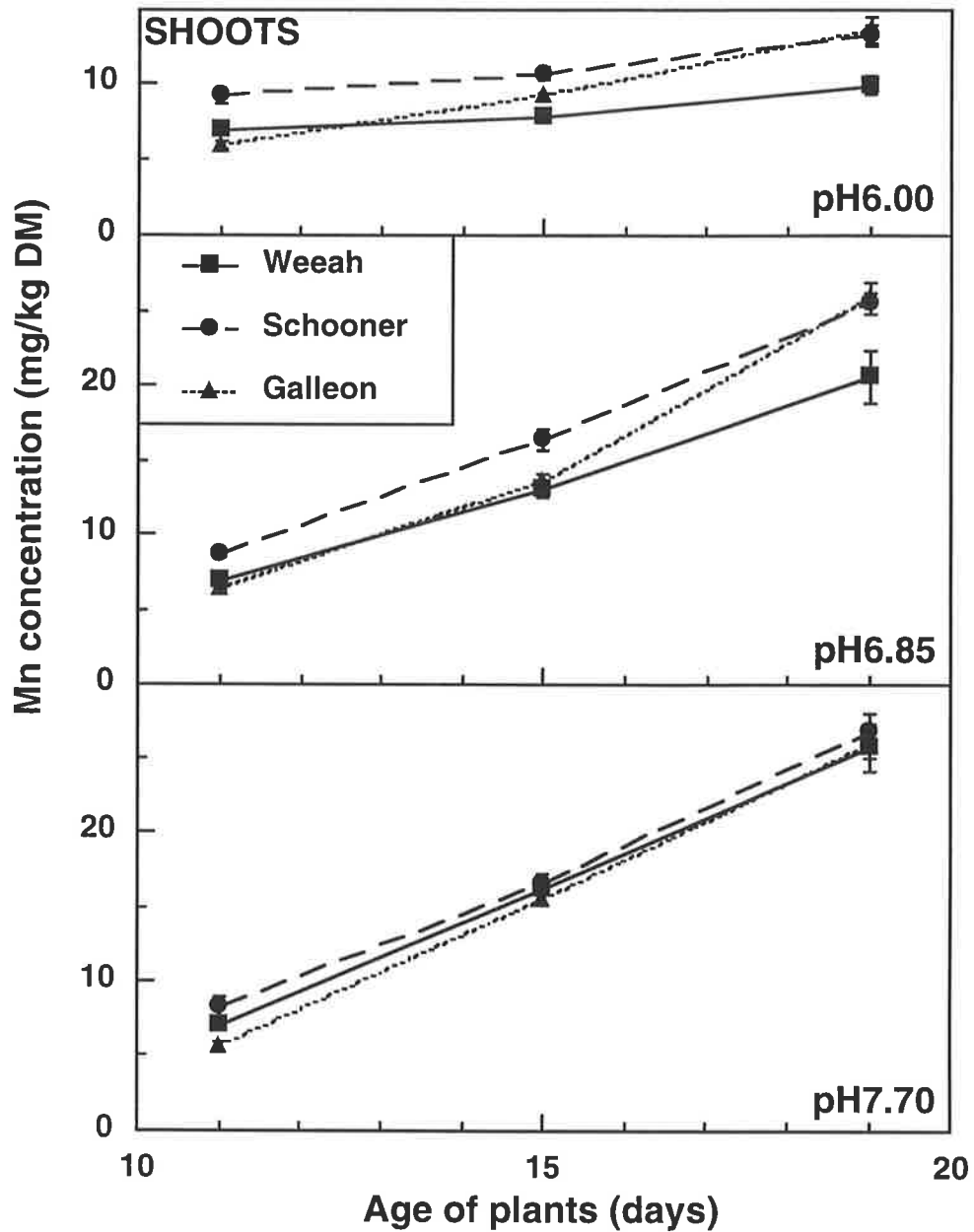
### 3.3.2 Experiment 3B

At  $pMn^{2+}$  9.0, dry matter production of all three genotypes slightly increased as pH rose (Fig. 3.5), and no genotypic differences in dry matter production were found between Weeah and Galleon, but there were differences between Schooner and Weeah or Galleon (see Appendix A, Table A9 and A10 for analysis of variance table). Mn concentrations in shoots of all three genotypes increased with increasing pH from 10-14 mg/kg DM at pH 6.00 to 21-27 mg/kg DM at pH 6.85 and 7.70 (Fig. 3.6). At pH 6.00

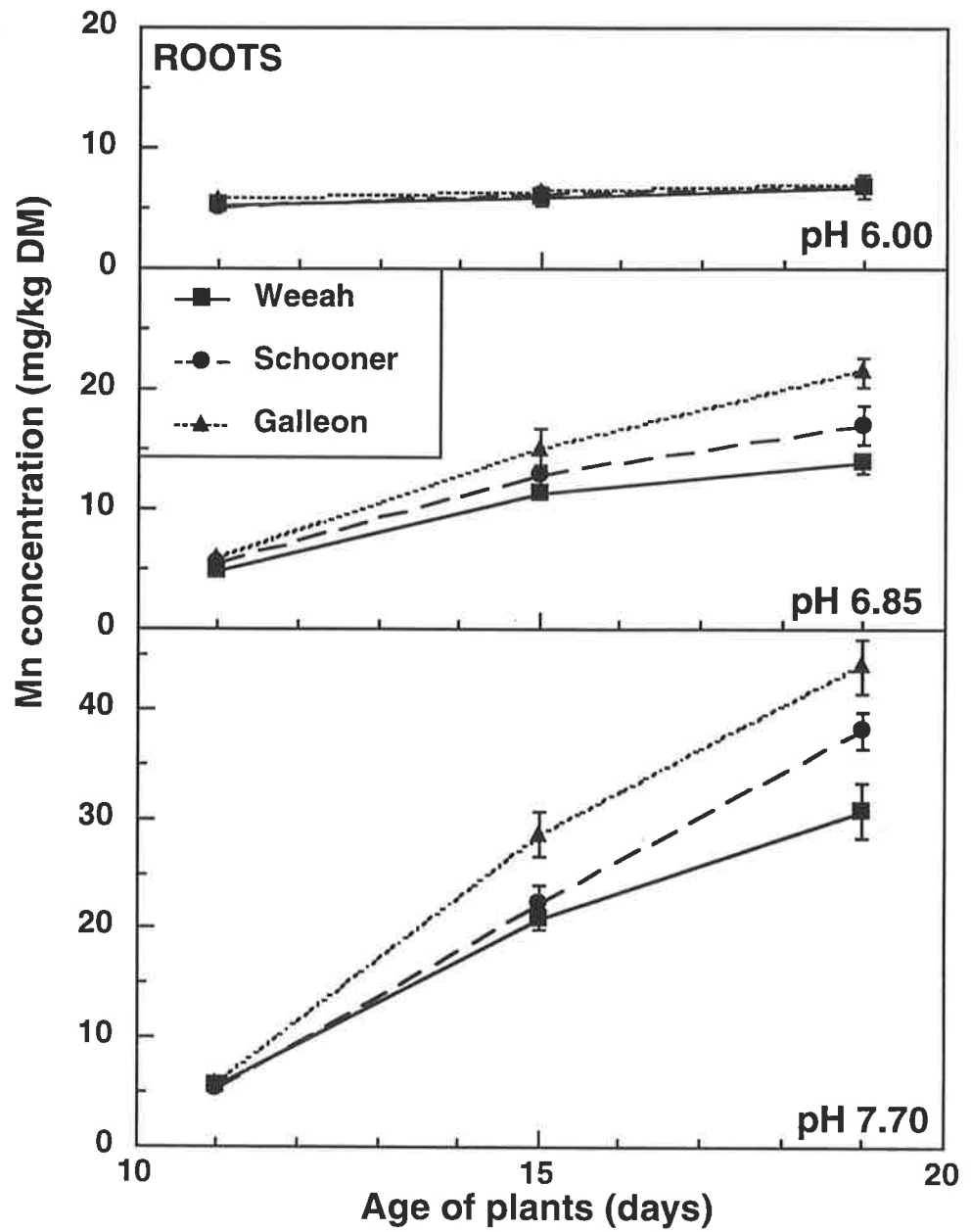
and 6.85, Mn concentrations in shoots of Weeah were lower than those of Galleon and Schooner, but no differences were observed at pH 7.70 (see Appendix A, Table A11 and A12 for analysis of variance table). Mn concentrations in roots of all three genotypes were low at pH 6.00, but increased substantially over time with increasing pH (Fig. 3.7). Moreover, genotypic differences in Mn concentrations of roots were found at both 6.85 and 7.70. On D19, Mn concentrations in roots were higher than those in shoots after 8 day treatment at pH 7.70 (see Appendix A, Table A13 and A14 for analysis of variance table).



**Fig. 3.5.** Effect of three levels of pH on the dry matter production of three barley genotypes grown in a chelate-buffered nutrient solution. SE(n=4) is shown as vertical bars. On D11, plant samples were taken before pH treatments were imposed.



**Fig. 3.6.** Effect of three levels of pH on Mn concentrations in the shoots of three barley genotypes grown in a chelate-buffered nutrient solution. SE(n=4) is shown as vertical bars. On D11, plant samples were taken before pH treatments were imposed.



**Fig. 3.7.** Effect of three levels of pH on Mn concentrations in the roots of three barley genotypes grown in a chelate-buffered nutrient solution. SE(n=4) is shown as vertical bars. On D11, plant samples were taken before pH treatments were imposed.

Fe concentrations in shoots of three genotypes were adequate at the three levels of pH throughout the experiment except that those treated at pH 6.00 were low on D19 (Table 3.2). Fe concentrations in roots of all three genotypes were low, which indicates that it is unlikely that Fe precipitates occurred in any pH treatments (Bell *et al.*, 1991a).

**Table 3.2. Effect of pH on the concentrations of Fe in three barley genotypes at three harvests.**

Genotype	plant part	Day 11	Day 15			Day 19		
		pH	pH			pH		
		6.00	6.00	6.85	7.70	6.00	6.85	7.70
<b>Iron (mg/kg DM)</b>								
Weeah	Roots	46 1	38 4	48 4	63 2	32 1	45 3	67 5
	Shoots	65 2	74 5	73 2	81 1	52 2	72 2	82 1
Schooner	Roots	47 1	42 9	47 2	65 8	28 1	46 3	52 2
	Shoots	61 0	59 3	73 3	82 6	44 3	68 2	73 3
Galleon	Roots	49 0	42 4	51 3	62 3	29 1	53 4	78 3
	Shoots	61 1	70 1	72 4	77 1	51 2	75 2	83 4

<sup>a</sup> Small number after means represents standard errors of means.

### 3.4 Discussion

Mn efficiency was expressed in soil under controlled conditions in terms of dry matter production and Mn concentration in shoots (Chapter 2). However, in the solution culture at pH 6.0, Mn efficiency was not expressed when Mn was supplied as Mn<sup>2+</sup> buffered by HEDTA, either in terms of dry matter production, Mn concentration of shoots or Mn accumulation rate. Although differences in Mn efficiency have been reported in solution culture, those results were either in dicotyledonous plants such as soybean (Ohki *et al.*, 1980), in single-salt solutions (Landi and Fagioli, 1983) or in nutrient solutions that may not have maintained Mn as the Mn<sup>2+</sup> form (Munns *et al.*, 1963; Brown and Jones, 1974). Nevertheless, by using the new chelate-buffering techniques in conjunction with a chemical speciation program (GEOCHEM-PC), complications such as co-precipitation or adsorption of Mn with other nutrients which might have confounded the interpretation of previous experiments, can presumably be

dismissed. Thus, the responses that we have measured can be attributed to concentrations of free  $Mn^{2+}$  in solution.

With increasing pH in the chelate-buffered nutrient solution, Mn accumulation in shoots was greatly increased in a manner not predictable from field experience. In contrast, results from conventional nutrient solutions showed a depression in Mn absorption when pH rose above 6.0 (Munns *et al.*, 1963), which may be due to Mn oxidation and precipitation (Munns *et al.*, 1963; Maas *et al.*, 1968), or may be related to co-precipitation or adsorption with other nutrients such as Fe (Bell *et al.*, 1991a). However, in the chelate-buffered nutrient solution used, the effect of Fe precipitation on Mn absorption through change of Mn concentration and Mn forms was less likely because no Fe was likely to be precipitated in the roots (Table 3.2). Any effect of Mn oxidation and precipitation, if they occurred, on Mn absorption would be insignificant. As the complicating factors were eliminated, the increase in Mn absorption with increasing pH in the chelate-buffered nutrient was probably related to a lack of  $H^+$  competition and/or to an increasing  $H^+$  efflux gradient in this experiment.

Higher Mn concentrations in roots were found with increasing pH in both absolute terms and relative to shoot concentrations even though high concentration of competing chelate was present in the nutrient solution. This suggests that the Mn in roots exists as immobile forms such as  $MnO_2$ , which could not be scavenged by the free chelate. The immobilisation of Mn as oxidised forms in rice root apoplasm was reported when the plants were grown in a nutrient solution with a high concentration of  $Mn^{2+}$  over a wide range of pH (Crowder and Coltman, 1993). However, Mn immobilisation in barley roots occurred only at the high pH in the chelate-buffered nutrient solution with a low concentration of Mn. Interestingly, the Mn concentrations in roots were different among the three genotypes. The Mn-efficient genotype, Weeah had a lower Mn concentration in roots than the Mn-inefficient, Galleon. This could, therefore, suggest that either less  $Mn^{2+}$  is immobilised from the nutrient solution to the



root apoplastic space of the Mn-efficient genotype, or more immobilised Mn in the apoplastic space can be remobilised by the Mn-efficient genotype via various processes (Welch, 1995). In either case, it would be expected that more  $Mn^{2+}$  in the nutrient solution is available to Weeah, but in fact it had little effect on the Mn concentrations of shoots in the Mn-efficient genotype. The explanation for this is that by calculation, the amount of immobilised Mn in roots of the Mn-inefficient genotypes at the high pH was not sufficient to significantly decrease  $Mn^{2+}$  activity in the nutrient solution, leading to decrease of Mn absorption by the Mn-inefficient genotype because of the chelate-buffering power. Alternatively, if any Mn were remobilised by the Mn-efficient genotype from apoplasm, it would be recaptured by free chelate in the solution so that  $Mn^{2+}$  activity in the nutrient solution would not be increased.

However, in soil, no large amount of excess Mn chelates is available in rhizosphere as there is in the stirred chelate-buffered nutrient solution. Therefore, any genotypic difference in Mn immobilisation or remobilisation would affect the amount of available Mn in rhizosphere, which would be expected to lead to a difference in uptake and ultimately to a difference in Mn concentration of shoots. This can explain the genotypic difference in Mn efficiency, but the actual process remains unknown. Further investigation was carried out in the next chapter.

## Chapter 4

### Assessment of $\text{Cu}^{2+}$ and $\text{Fe}^{3+}$ reduction by Mn-efficient and Mn-inefficient genotypes

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#### 4.1 Introduction

Higher Mn accumulation in shoots of Mn-efficient genotypes was observed in calcareous soils where the predominant forms of Mn are insoluble Mn oxides or carbonates (Reisenauer, 1988). Moreover, no genotypic difference in absorption of  $\text{Mn}^{2+}$  ions was found in the chelate-buffered nutrient solution over a range of Mn concentration and over a range of pH (Chapter 3). These results suggest that efficient Mn mobilisation is likely to be the mechanism of Mn efficiency. This suggestion was supported by genotypic differences in Mn concentration of roots occurring at high pH in the same chelate-buffered nutrient solution (Chapter 3).

Mobilisation of Mn oxides can be increased through a decrease in rhizosphere pH and by the release of reductants (Marschner, 1991). However, no clear differences in ability to lower rhizosphere pH or reduce Mn oxides were found among barley genotypes differing in Mn efficiency (Marcar, 1986; Harbard, 1992). Moreover, the reduction of  $\text{MnO}_2$  by reductants is pH-dependent (Bromfield, 1958b; Godo and Reisenauer, 1980), and thus it is unlikely to be effective at the pH levels found in such calcareous soil. However, Fe(III) reductases in plasma-membrane may be involved in Mn mobilisation by donating electrons either directly or indirectly to Mn(III, IV) as suggested by Welch (1995). Fe(III)-chelate reductase activities were high in Mn accumulator mutants of *Arabidopsis* (Delhaize *et al.*, 1993; Delhaize, 1995). In addition, Fe(III)-chelate reductases not only reduce Fe(III) to Fe(II), but also appeared to be able to reduce Cu(II) to Cu(I) (Welch *et al.*, 1993). The reduction of Cu(II) was reported in dicotyledons, but no work has been done in monocotyledons. Standard

reductases, which reduce ferric cyanide exist in both dicotyledons and monocotyledons (Bienfait, 1988). It is possible that Fe(III) reductases may contribute to Mn efficiency. The following experiments in this chapter were conducted to assess reduction of Cu<sup>2+</sup> and ferric cyanide by Mn-efficient and Mn-inefficient genotypes.

#### 4.2 Materials and methods

Three barley genotypes WA73S276 (Mn-efficient), Weeah (Mn-efficient) and Galleon (Mn-inefficient) were used in the experiments. Seeds had a low Mn content ( $\mu\text{g}$  Mn per seed): 0.19 for WA73S276, 0.12 for Weeah and 0.12 for Galleon. pH 6.0 was chosen for growing plants in the chelate-buffered nutrient solution to prevent the differential accumulation of MnO<sub>2</sub> in roots among the genotypes, which occurred at higher pH in Experiment 3B (Chapter 3). The experimental procedure and plant growth conditions were the same as in Experiment 3A. Two levels of Mn (0.004  $\mu\text{M}$  and 0.5  $\mu\text{M}$ ) were supplied in the nutrient solution. The activities of Mn expressed as negative logarithm of activity ( $\text{pMn}^{2+}$ ) was 10.2 and 8.1, respectively (Appendix B, Table B1 and B8, respectively).

The method for assessment of Cu<sup>2+</sup> reduction were similar to that described by Welch *et al.* (1993). The root system of intact plants was embedded in a gel containing 0.2 mM CaSO<sub>4</sub>, 0.75 (w/v) agarose (SeaPlaque, FMC corporation, USA), 5 mM MES-Tris (pH 6.0), 0.2 mM CuSO<sub>4</sub>, 0.4 mM Na<sub>2</sub>-2,9-dimethyl-4,7-diphenyl-1,10-phenanthrolinedisulfonic acid (BCDS) and 0.6 mM calcium citrate were added into gels. An orange colour of Cu(I)-BCDS complex around root tips indicates the active reduction of Cu(II) to Cu(I). For assessment of ferric reduction, 0.3 mM K<sub>3</sub>Fe(CN)<sub>6</sub>, 0.2 mM CaSO<sub>4</sub>, 0.75 (w/v) agarose, 5 mM MES-Tris (pH 6.8), and 0.6 mM calcium citrate were added into gels. The clearing of the light yellow colour of K<sub>3</sub>Fe(CN)<sub>6</sub> around roots indicates the reduction of Fe(III) to Fe(II). The concentration of K<sub>3</sub>Fe(CN)<sub>6</sub> and pH level used in this experiment were close to the optimum condition for the reductases (Moog and Brüggemann, 1994).

One plant from each genotype was taken from the chelate-buffered solution, and then the roots were put into 100 ml aerated solution for 30 min. The aerated solution contained 0.8 mM CaSO<sub>4</sub> and 5 mM MES-Tris buffer with the same pH as later embedding gel. Agarose was heated to dissolve in a solution of CaSO<sub>4</sub>, allowed to cool down to 50°C, and MES-Tris buffer and other reagents were added and mixed well. The liquefied agarose mixture was poured into plastic trays lined with plastic film. When the agarose mixture was cooled (35°C) to a viscous solution, the roots of three plants were submerged into it with the attached shoots in the air. Further cooling allowed the agarose to solidify around the roots, and then lids were placed on the trays, followed by wrapping the tray with aluminium foil to prevent light entry into the gels. The shoots were covered with a transparent plastic bag to reduce plant water loss by transpiration during colour development. The tray was put under light at 25°C. The reduction of Cu<sup>2+</sup> and Fe<sup>3+</sup> was repeated at least three times. One of them was presented in the results.

### **4.3 Results**

On day 16, symptoms of Mn deficiency had appeared in the youngest leaf of all three genotypes at 0.004 µM Mn supply, but the plants of all three genotypes were green and healthy at 0.5 µM Mn supply. The growth of seminal and lateral roots in all three genotypes was inhibited at 0.004 µM of Mn supply (Fig. 4.1).

For Cu<sup>2+</sup> reduction, no orange colour could be observed in roots of any genotypes within 1 hour, but the orange colour slowly developed with increasing time. After 6 hours, the orange colour was visible in the tips of seminal and lateral roots (Fig. 4.1), but no such colour was found in the elongation zone behind the root tips. The orange colour was weak in the root tips of plants grown at 0.004 µM of Mn supply, compared with those at 0.5 µM of Mn supply (Fig. 4.1). However, no genotypic differences in the reduction of Cu<sup>2+</sup> were found at either level of Mn supply (Fig. 4.1). Similar results were obtained when pH was increased to 6.85 (data not shown).

Reduction of ferric cyanide was observed in roots of all three genotypes in 26 hours (Fig. 4.2). Large clear zones were found around the roots of plants supplied with 0.5  $\mu\text{M}$  Mn, compared with those supplied with 0.004  $\mu\text{M}$  Mn. Clearing was greatest in the root zone with lateral roots, which appears associated with more lateral roots but no clear zone was found in root tips and the elongation zones. However, there were no genotypic differences in the reduction of ferric cyanide were found at either level of Mn supply (Fig. 4.2).



**Fig. 4.1.** Qualitative assessment of Cu(II) reduction by roots of barley seedlings of three genotypes grown at 0.004  $\mu\text{M}$  and 0.5  $\mu\text{M}$  of Mn.

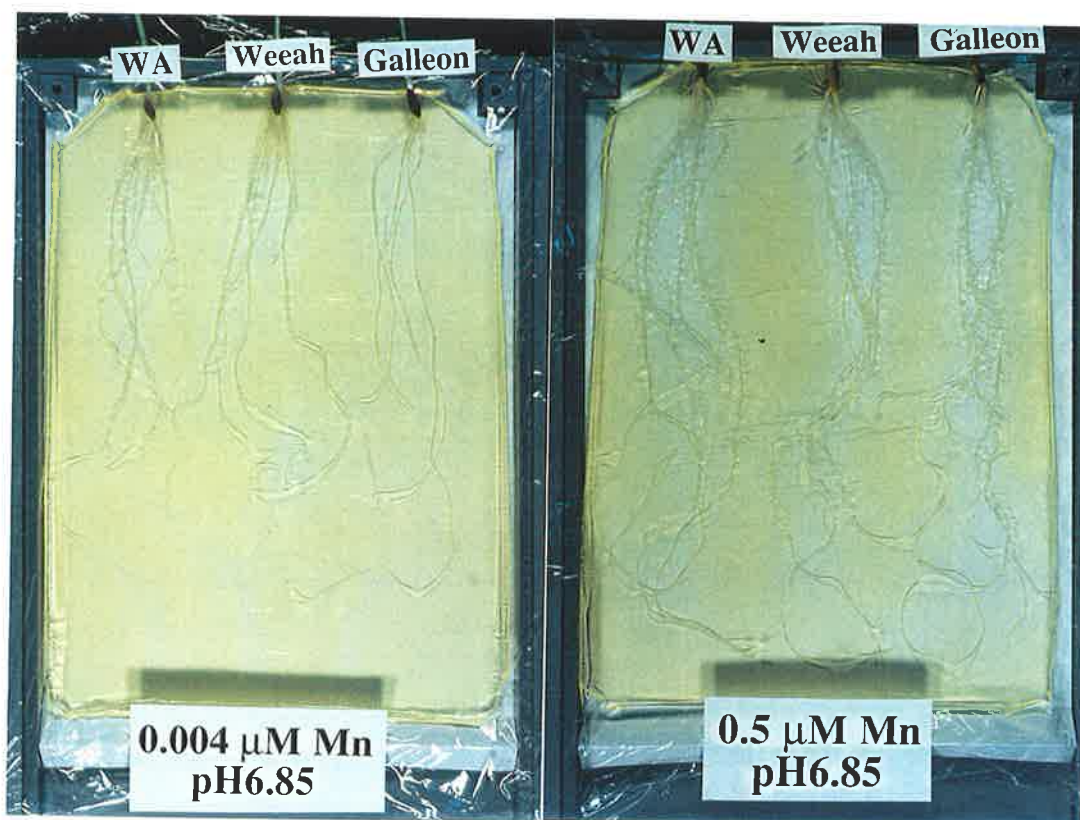


Fig. 4.2. Qualitative assessment of Fe(III) cyanide reduction by roots of barley seedlings of three genotypes grown at 0.004  $\mu\text{M}$  and 0.5  $\mu\text{M}$  of Mn.

#### 4.4 Discussion

Very weak reduction of Cu<sup>2+</sup> by barley roots was found using Cu(I) chelate BCDS, compared with that reported for dicotyledons such as pea (Welch *et al.*, 1993). The strong reduction of Cu<sup>2+</sup> in pea took place in half an hour, whereas only weak reduction in barley became visible after 6 hours. Mn deficiency did not enhance the reduction of Cu<sup>2+</sup> in any of the three genotypes differing in Mn efficiency, and no genotypic differences were observed among the three genotypes at either of the two levels of Mn supply. Thus, Cu<sup>2+</sup> reduction by barley is unlikely to be involved in Mn efficiency. Barley roots had a low activity in Cu<sup>2+</sup> reduction, which is similar to Fe(III)-chelate reduction (Bienfait, 1988). The Fe(III)-chelate reductases differ greatly in Strategy I and Strategy II plants (Bienfait, 1988) since they have different pathways for Fe acquisition. Strategy I plants such as pea have a high activity of Fe(III)-chelate

reductases commensurate to the requirement for reduction of Fe(III) to Fe(II), but Strategy II plants have a low activity consistent with the fact that Fe(III) reduction is not essential for Fe absorption in these plants. Therefore, Fe(III) reductases can contribute to Cu(II) reduction, and probably Mn(III, IV) reduction in dicotyledons (Delhaize, 1995), but this appears unlikely in monocotyledons such as barley.

The activities of ferric cyanide reductases exist in both Strategy I and Strategy II plants (Bienfait, 1988). The reduction of ferric cyanide in barley was not enhanced by Fe deficiency (Bienfait, 1988), nor by Mn deficiency in the present studies (Fig. 4.2). Inversely, an inhibition in reduction of ferric cyanide by Mn deficiency was observed in this experiment. This may be due to shortage of electron donors for the reduction because the inhibition of photosynthesis by Mn deficiency would result in fewer carbohydrates translocated from shoots to roots. No genotypic differences in reduction of ferric cyanide suggest that the ferric cyanide reductases may not contribute to Mn efficiency.

These results show that Fe(III)-chelate reductases and standard reductases are unlikely to be involved in Mn efficiency. However, other reductases, which may require specific substrates such as Mn(III) complexes formed with various chelates, may be involved in Mn mobilisation, and contribute to Mn efficiency. To clarify this issue, further study is needed.

## Chapter 5

### Differential expression of iron deficiency-induced genes in Mn-efficient and Mn-inefficient genotypes

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#### 5.1 Introduction

The mechanism of Mn efficiency is likely to be some aspect of enhanced Mn mobilisation from soils as discussed in Chapter 3. However, genotypic differences were not found in lowering rhizosphere pH, releasing reductants (Marcar, 1986; Harbard, 1992) or Fe(III) reductases (Chapter 4). Barley is classified as a Strategy II plant, possessing the pathway for Fe(III) acquisition through secretion of phytosiderophores and uptake of Fe(III) phytosiderophore complexes (Marschner *et al.*, 1986; Marschner *et al.*, 1989). Although phytosiderophores released under Fe deficiency can mobilise not only Fe, but also Zn, Cu and Mn from soil, Mn deficiency cannot induce the release of these natural ligands (Treeby *et al.*, 1989; Takagi *et al.*, 1988; Singh *et al.*, 1992). However, barley may have an analogous mechanism for Mn acquisition although no specific chelates have been found so far. On the other hand, Mn uptake was positively correlated both to Fe efficiency in oats (Mench and Fargues, 1994), and to Fe uptake by monocotyledonous plants in calcareous soil (Warden and Reisenauer, 1991b). Thus, it is still possible that Fe acquisition may have some connection with Mn efficiency.

It is difficult to study the effect of rhizosphere change enhanced by Fe acquisition on Mn mobilisation in soil because Mn mobilisation in soil is affected by many abiotic and biotic factors (Marschner, 1988). An alternative approach was taken to investigate the gene expression in response to low Mn availability in calcareous soils, where Fe availability is usually low. Only limited numbers of genes are available in higher plants, which are likely to be involved in Fe acquisition (Okumura *et al.*, 1992; 1994; Nakanishi, 1993). One of them, *Ids1*, which is induced specifically by Fe deficiency has a sequence related to metallothionein. The other two genes, *Ids2* and *Ids3*, share



high amino-acid homology, and both are expressed under Fe deficiency as well as Mn deficiency in solution culture (Nakanishi *et al.*, 1993; Okumura *et al.*, 1994). Therefore, in this chapter, the expression of *Ids1* and *Ids2* in Mn-efficient and Mn-inefficient genotypes was examined in a calcareous soil for any connection of Fe acquisition related gene expression with Mn efficiency.

## 5.2 Materials and methods

### 5.2.1 Plant growth

Two barley genotypes with a large difference in Mn efficiency, Mn-efficient Weeah and acutely Mn-inefficient WI2585 (Longnecker *et al.*, 1990) were grown in soil for analysis of gene expression. Galleon, a Mn-inefficient genotype (Longnecker *et al.*, 1990) was also included in part of this experiment. Seeds with low Mn content ( $\mu\text{g}$  per seed) were used in this experiment: 0.12 in Weeah, 0.23 in WI2585 and 0.12 in Galleon.

Wangary soil was used in this experiment. The source of soil and preparation were the same as described in Chapter 2. In this soil, Mn efficiency was consistently expressed in terms of plant growth and Mn concentration in shoots at levels of added Mn up to 30 mg/kg soil (Chapter 2). Thus, the soil was supplemented with two rates of Mn (15 and 100 mg Mn/kg dry soil). Pots (16 cm diameter x 15 cm) containing 2 kg dry soil were used in this experiment, which not only provided an adequate soil volume for plant growth, but also allowed sufficient plants to be grown for these studies. The conditions for plant growth were the same as described in Chapter 2. Surface sterilised barley seeds were germinated in petri dishes at 25°C overnight and sown at 20, 18, 14, and 12 seeds to a pot for harvest on day 14 (D14), D21, D28 and D37, respectively. On D7, seedlings were thinned to 17, 16, 12 and 10 seedlings per pot for harvest on D14, D21, D28 and D37, respectively. The genotypic difference in yields and Mn concentrations of shoots in the single pots used for this experiment was comparable to all replicated studies in Chapter 2.

At each harvest, plant roots were carefully washed free of soil with water, gently blotted in tissue, separated from shoots and weighed. Roots and a sub-sample of shoots were frozen in liquid nitrogen and stored at -80°C for RNA isolation. In order to freeze the plants as quickly as possible, time was not taken to weigh individual plants; accordingly, standard errors are not available for the means of Fig. 5.1.

### 5.2.2 Mn and Fe analysis

Sub-samples of five to seven shoots from each harvest were oven-dried (70°C), digested in 70% nitric acid and analysed by ICP spectrometry (Zarcinas *et al.*, 1987).

### 5.2.3 RNA gel blot analysis

The materials and methods for RNA gel blot analysis are described in Section 8 of Appendix C. <sup>32</sup>P random labelled Ids1 probe was prepared by PCR (Section 4.8.2 of Appendix C) using a Ids1 cDNA clone as template (Okumura *et al.*, 1992). <sup>32</sup>P random labelled Ids2 probe was prepared using the NaeI fragment from the Ids2 genomic clone, which contains 781 base pairs of coding region (Okumura *et al.*, 1994). A radioactive probe prepared from the BamHI-EcoRI fragment (about 1.0 kb) of the 18S ribosomal DNA clone PHA1 (Jorgensen *et al.*, 1987) was also used to verify the equivalent loadings of total RNA on each lane of the same stripped membrane.

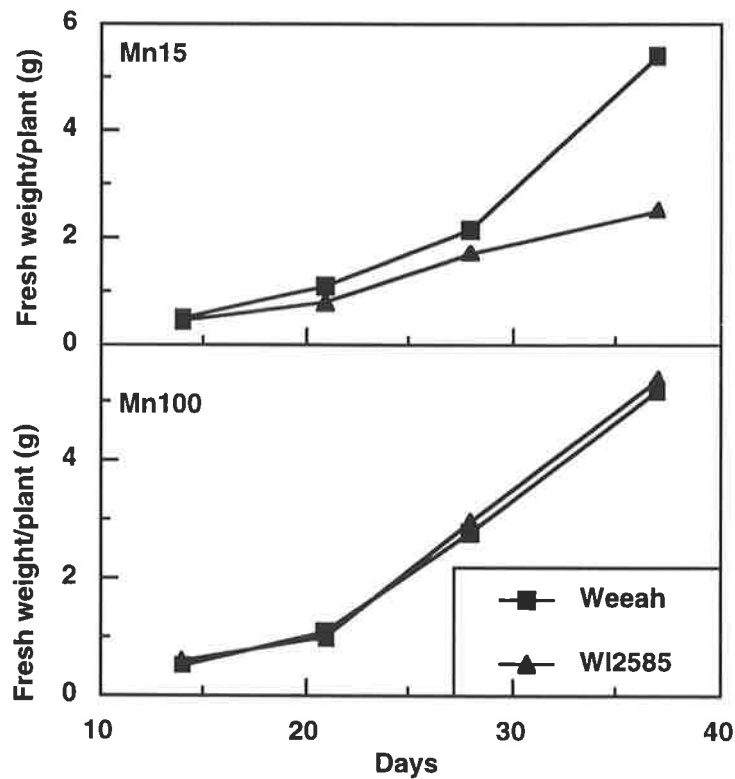
## 5.3 Results

### 5.3.1 Plant growth

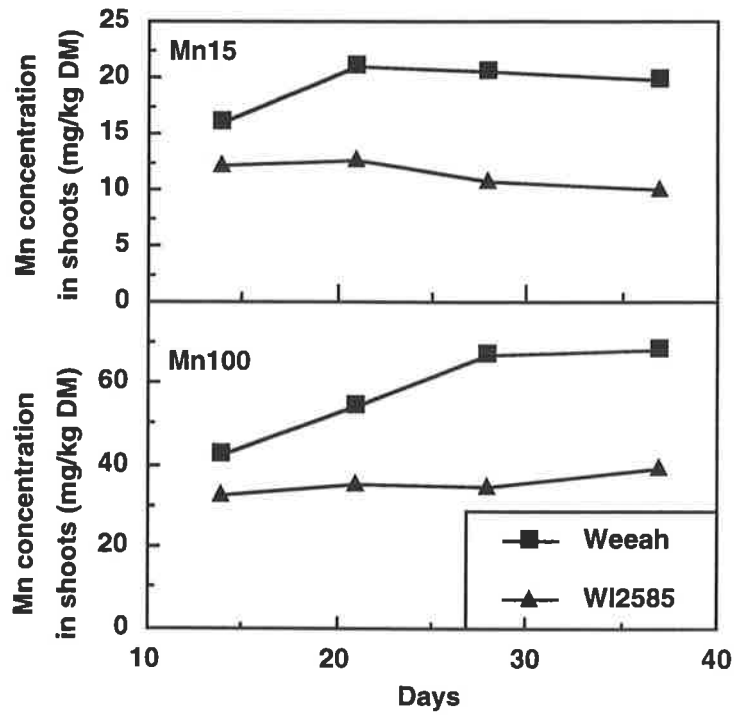
Plant growth of the two genotypes at two levels of Mn supply is shown in Fig. 5.1. At the low Mn level (15 mg Mn/kg soil), there was no clear difference in plant growth between the two genotypes during the first three weeks, but a marginal difference was observed on D28 and a large difference was found on D37. By this time, the plants of WI2585 (Mn-inefficient genotype) had a much lower fresh weight than those of Weeah (Mn-efficient genotype). There was no difference at any stage in plant growth between the two genotypes when grown at the high Mn level (100 mg Mn/kg soil).

**5.3.2 Mn and Fe accumulation**

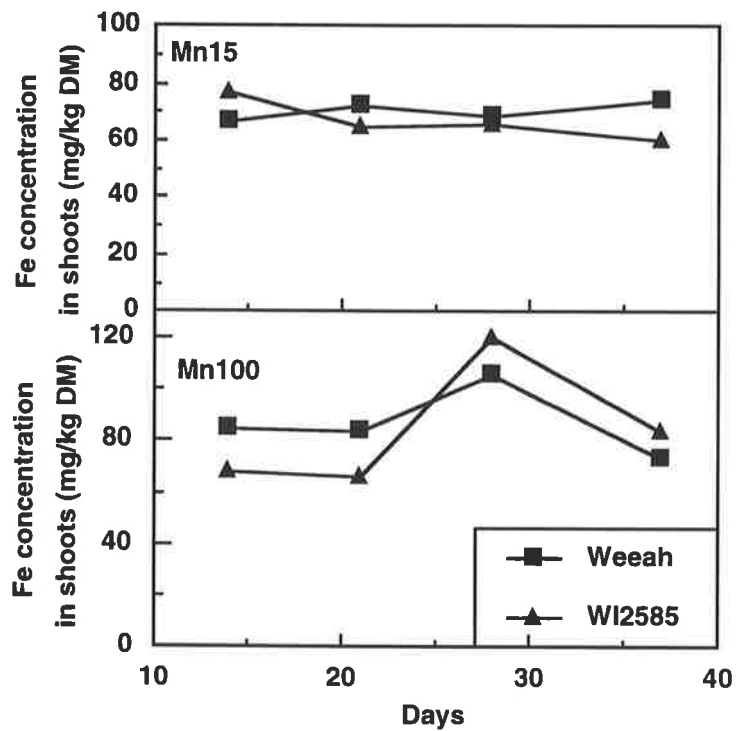
Mn and Fe concentrations in shoots of the two genotypes are shown in Fig. 5.2 and 5.3. At the low Mn level, Mn concentration in the shoots of Weeah increased from D14 to D21 and then remained constant above the critical level (Hannam *et al.*, 1987). However, Mn concentration in the shoots of WI2585 was low and close to the critical level in the period from D14 to D21, and then decreased to below the critical level at the later harvests (Fig. 5.2). A clear genotypic difference in Mn concentration of shoots was observed after D21. At the high Mn level, Mn concentrations of shoots in the two genotypes were well above the critical deficiency level, but the Mn-efficient genotype still had higher Mn concentration than the Mn-inefficient genotype (Fig. 5.2). Fe concentrations of shoots in the two genotypes were adequate at both levels of Mn, and unlike Mn concentration, no clear genotypic difference was found in Fe concentration of shoots (Fig. 5.3).



**Fig. 5.1.** Effect of two levels of Mn supply on the plant growth of two barley genotypes in a Mn-deficient soil under controlled conditions. Mn15 and Mn100 refer to 15 and 100 mg Mn/kg dry soil, respectively. Points are the average value of 17, 16, 12 and 10 plants harvested on D14, D21, D28 and D37, respectively.



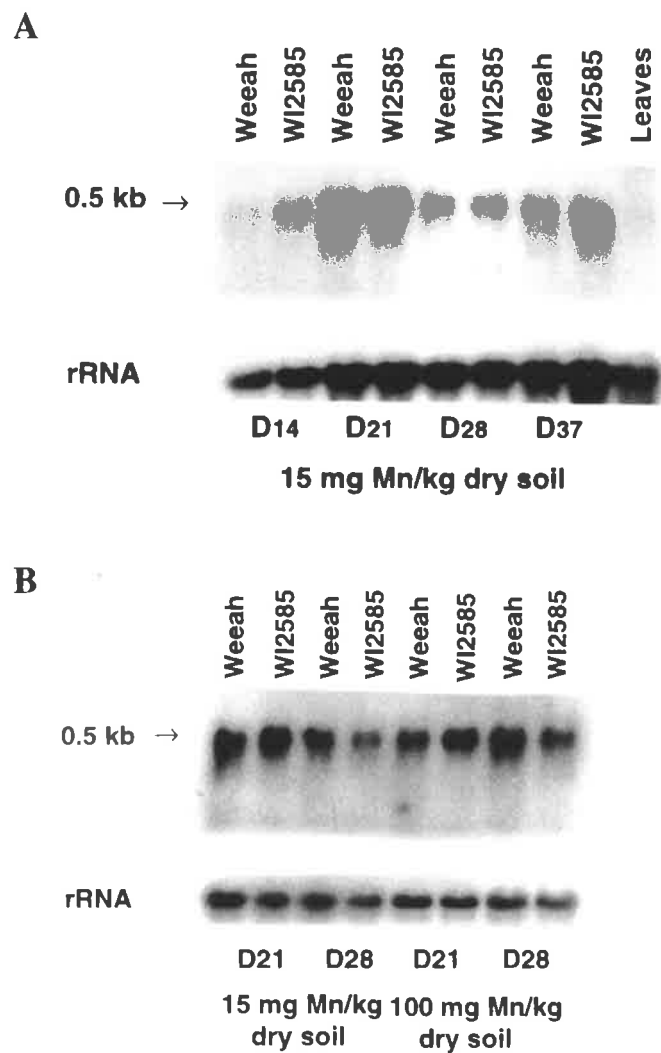
**Fig. 5.2.** Effect of two levels of Mn on Mn concentration of shoots in two barley genotypes. Mn15 and Mn100 refer to 15 and 100 mg Mn/kg dry soil, respectively.



**Fig. 5.3.** Fe concentration of shoots in two barley genotypes grown in a Mn-deficient soil supplied with two levels of Mn. Mn15 and Mn100 refer to 15 and 100 mg Mn/kg dry soil, respectively.

### 5.3.3 Expression of *Ids1*

RNA gel blot analysis showed that at the low Mn level, *Ids1* expression was low in the roots of the two genotypes on D14, but its expression was increased on D21, and then decreased afterwards (Fig. 5.4A). *Ids1* expression was not detectable in leaves (Fig. 5.4A). At the high Mn level, *Ids1* was expressed at a level similar to that at the low Mn level. No clear genotypic difference in the expression of *Ids1* was found at either level of Mn supply (Fig. 5.4B).



**Fig. 5.4.** *Ids1* RNA accumulation in two barley genotypes grown in a Mn-deficient soil. **A.** at the low level of Mn. **B.** at the low and high levels of Mn.

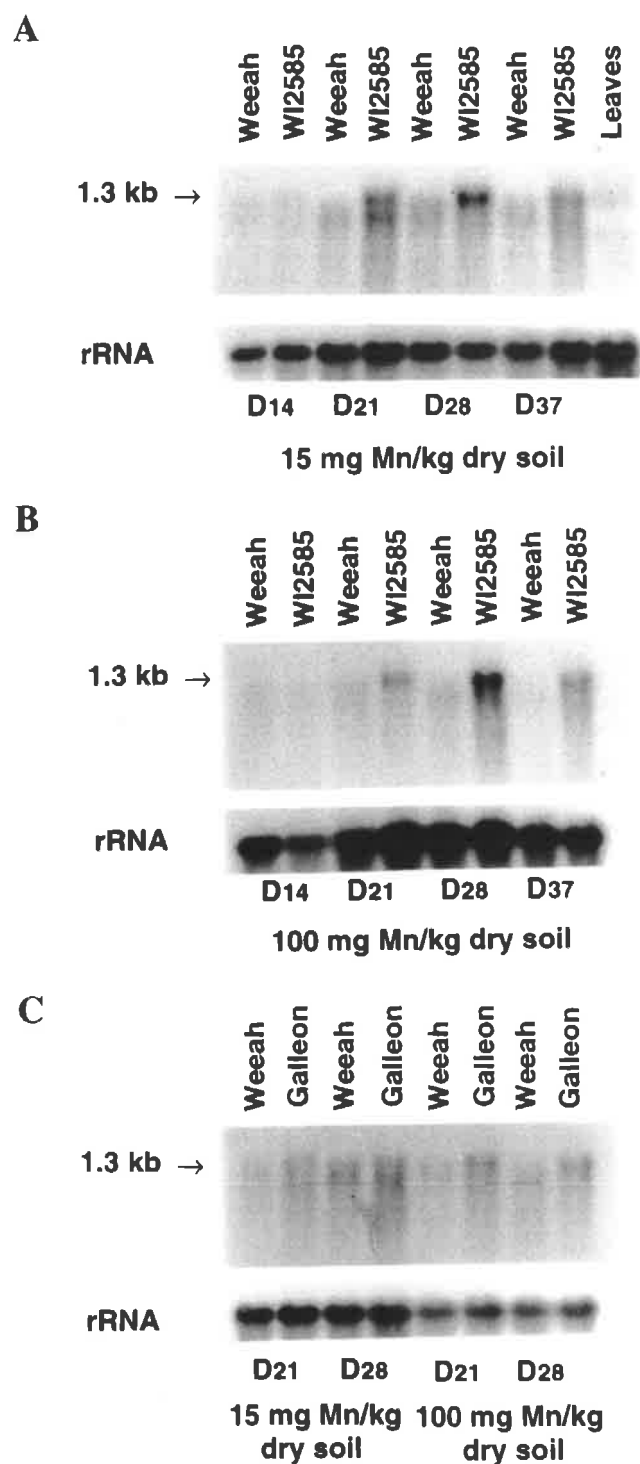
### 5.3.4 Expression of *Ids2*

At the low Mn level, *Ids2* had low expression in the two genotypes on D14, but its expression was increased in the Mn-inefficient genotype (WI2585) from D21 to D28, and then decreased on D37. The expression in the Mn-efficient genotype (Weeah) was low compared with that in WI2585, and no change was found in Weeah after D21. The expression of *Ids2* was not detectable in leaves (Fig. 5.5A). At the high Mn level, *Ids2* was also expressed, and WI2585 again had higher expression than Weeah (Fig. 5.5B) though Mn concentration in shoots was adequate (Fig. 5.2).

A similar pattern for the expression of *Ids2* was also found in another Mn-inefficient genotype, Galleon (Fig. 5.5C), which was grown under the same conditions as Weeah and WI2585. Mn concentration in the shoots of Galleon (mg/kg DM) was 16.3 on D21 and 18.2 on D28 at the low Mn level, and 45.3 on D21 and 52.0 on D28 at the high Mn level, which was between that of Weeah and WI2585. Galleon had higher expression of *Ids2* than Weeah, and Mn supply had no apparent effect on the expression of *Ids2* in Galleon (Fig. 5.5C).

### 5.4 Discussion

*Ids1* of barley is induced specifically by Fe deficiency in solution culture (Okumura *et al.*, 1992), and it is also expressed in the two barley genotypes Weeah and WI2585 when grown in a calcareous soil (Fig. 5.4). The expression of *Ids1* was not affected by Mn supply, and was similar in the two genotypes although they have different Mn efficiency. This suggests that *Ids1* responds to the low Fe availability in the calcareous soil used in this experiment to increase the Fe uptake, leading to adequate Fe in shoots. The expression of *Ids1* in roots may be important for Fe nutrition in a natural ecosystem. However, no difference in the expression of *Ids1* between the genotypes differing in Mn efficiency suggests that *Ids1* probably does not contribute to Mn efficiency.



**Fig. 5.5.** *Ids2* RNA accumulation in two barley genotypes grown in a Mn-deficient soil.

**A.** at the low level of Mn. **B.** at the high level of Mn. **C.** effect of the low and high levels of Mn on RNA accumulation of *Ids2* in Weeah and a second Mn-inefficient genotype, Galleon, at two harvests.

*Ids2* was also expressed in the two Australian barley genotypes when grown in a calcareous soil. A differential expression of *Ids2* was found between the genotypes which differ in Mn efficiency. The Mn-inefficient genotype had much higher induced expression than the Mn-efficient genotype. However, the expression of *Ids2* was not affected by Mn supply. As *Ids2* of barley is induced by Fe deficiency as well as Mn deficiency in solution culture (Okumura *et al.*, 1994), the expression of *Ids2* in the roots could be related to the plant response to low Fe availability or low Mn availability in the soil. Because Fe availability was low as indicated by the expression of *Ids1* at both low and high Mn treatments, and Mn supply did not affect the expression of *Ids2*, the expression of *Ids2* is possibly due to the plant response to the low available Fe rather than to the low available Mn. However, the higher expression of *Ids2* in the Mn-inefficient genotype did not correlate with a corresponding higher Fe uptake as indicated by the Fe concentration of shoots, but did have an inverse relation to Mn uptake. No corresponding higher Fe uptake may be due to other limiting factors involved in Fe uptake, such that enhanced expression of *Ids2* is not sufficient for higher Fe uptake. Analysis of additional genes involved in Fe acquisition, once these become identified, could clarify this point. The inverse relation of *Ids2* expression to Mn uptake suggests that *Ids2* is involved in Mn efficiency. However, the metabolic processes of the involvement are not known.

An interesting observation is the timing of change in expression of *Ids2*. Low expression of *Ids2* in the two barley genotypes was found in the first two weeks, which is presumably because until then seed Fe can supply the needs for seedling growth. A strong increase on D21, especially in the Mn-inefficient genotype possibly links to the switch in Fe supply from seed to soil, and an apparent decrease later may reflect a decrease in Fe deficiency stress resulting from Fe uptake from soil. A similar time course was found for the expression of *Ids1*. At a zero supply of Fe in solution culture, symptoms of Fe deficiency in barley plants appear after two weeks (Marschner *et al.*, 1987), which could reflect the exhaustion of Fe deposits in seed. These results suggest



that investigation of the expression of genes related to nutrient acquisition, especially micronutrients, should include consideration of the effect of seed nutrient content on the timing of the acquisition response.

## Chapter 6

### Isolation and characterisation of Mne-1 cDNA clone

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#### 6.1 Introduction

As the expression analysis of clones representing genes implicated in Fe acquisition did not show any positive correlation to Mn efficiency (Chapter 5), further attempts were made to isolate and characterise genes related to Mn efficiency. Under the conditions of low available soil Mn, Mn-efficient and Mn-inefficient plants could be expected to have differences in root mRNAs related to their genotypic differences in Mn efficiency. These differences in mRNAs may be detectable by differential screening whether the differences in mRNAs between the genotypes are constitutive or inducible by Mn conditions in soil. The approach of differential screening has been used to identify a number of genes which are involved in the root response to nutrient stresses (Okumura *et al.*, 1992; 1994; Nakanishi *et al.*, 1993; Dumont *et al.*, 1993; Snowden and Gardner, 1993; Winicov and Deutch, 1994; Ezaki *et al.*, 1995). However, no work has been done on the genes associated with differences in micronutrient efficiency traits. As Mn efficiency in barley is likely to be under simple genetic control (Longnecker *et al.*, 1990), theoretically it should be possible to identify the genes which are responsible for the Mn efficiency trait. However, no Mn-efficient isogenic lines or Mn-inefficient mutants were available when this project was initiated. Therefore, two genotypes greatly differing in Mn efficiency were used in the differential screening. The role of differentially expressed genes that were identified was assessed by investigating their expression in additional distinct Mn-efficient barley varieties.

## **6.2 Materials and methods**

### **6.2.1 Preparation of plant materials**

The growth conditions for Weeah and WI2585 and preparation of roots for RNA extraction were the same as described in Chapter 5. In order to verify the association of the identified mRNAs with Mn efficiency rather than other genetic differences, two other Mn-efficient genotypes with distinct pedigrees, WA73S276 and Amagi nijo, and the two tested genotypes, Weeah and WI2585, were grown under the same conditions as described in Chapter 5 with a low Mn supply (10 mg/kg soil) for 28 days. The Mn concentration in YEB (mg/kg DM) was  $7.0\pm 0.7$  for WA73S276,  $7.9\pm 0.9$  for Amagi nijo,  $9.2\pm 0.9$  for Weeah and  $3.6\pm 0.5$  for WI2585 (Margaret Pallotta, unpublished data).

### **6.2.2 Construction of cDNA library**

Poly(A)<sup>+</sup> RNA (approximately 1µg) was isolated from 28-day old Weeah roots grown at 15 mg Mn/kg soil with PolyAtract mRNA Isolation Systems IV (Promega). This poly(A)<sup>+</sup> RNA was used to synthesise cDNAs using a cDNA Synthesis Kit (Pharmacia), and the cDNA library was constructed in λgt10. The protocol was followed as described in Section 5 of Appendix C.

### **6.2.3 Differential screening and RNA gel blot analysis**

Differential screening and RNA gel blot analysis were performed as described in Section 6 and 8 of Appendix C.

### **6.2.4 DNA sequencing**

cDNA clones in λgt10 were subcloned into sequencing vectors (pTZ18U or pGEM5Zf) and sequenced from both directions using M13 primers, using Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc.), and a 373A DNA Sequencer, as described in Section 7 of Appendix C. The raw sequence data were analysed using SeqED software (Applied Biosystems Inc.). DNA sequences were compared to Genbank and EMBL data bases.

## 6.3 Results

### 6.3.1 Isolation of Mn efficiency related clones

At the low Mn level (15 mg Mn/kg soil), the time course of plant growth showed that there was no clear difference between the two genotypes differing in Mn efficiency during the first three weeks, but a marginal difference was observed on D28 and a large difference was found on D37 (see Fig. 5.1). Thus, the roots of 28-day old Weeah were chosen for the construction of the cDNA library. It was expected that the genes related to Mn efficiency (even if they were developmentally regulated) would be highly expressed by D28, but also stress genes would either be poorly expressed or expressed equally in both genotypes. This would maximise the chance of identifying Mn efficiency related clones upon a differential screening of this library.

Duplicate membranes, corresponding to around 30,000 plaques were differentially screened with <sup>32</sup>P-labelled cDNA probes derived from root mRNA of 28-day old Weeah or WI2585. Any clones that represented an mRNA of greater abundance in Weeah roots than WI2585 roots were isolated. One hundred and thirty-two putative differential clones were selected. Eighteen of those clones, having an insert size over 450 bp, were used for further analysis (Table 6.1). The relationships of the 18 clones were analysed with three of the clones (No 39, 107 and 139) by DNA gel blots. The results showed that several clones were related in sequence as indicated in Table 6.1.

**Table 6.1.** Characterisation of eighteen clones

Clone No	cDNA size (bp) <sup>a</sup>	Size of mRNA (bp) <sup>b</sup>	Cross hybridisation <sup>c</sup>	Sequence information <sup>d</sup>
27	700			
30	500			
39	500			
55	450		39	
58	500			
64	450		139	
67	750			
70	450		39	
74	600			
87	1450			
107	578	1500	64	no homology to any known sequences
121	1450			
125	1400	1500		
130	500	1500	139	
139	492	500		strong homology to MPS-1
140	450		139	
142	450	1800	139	
144	650			

**a** cDNA size was estimated by EcoRI digestion of the clone in  $\lambda$ gt10 or the insert fragment amplified using PCR.

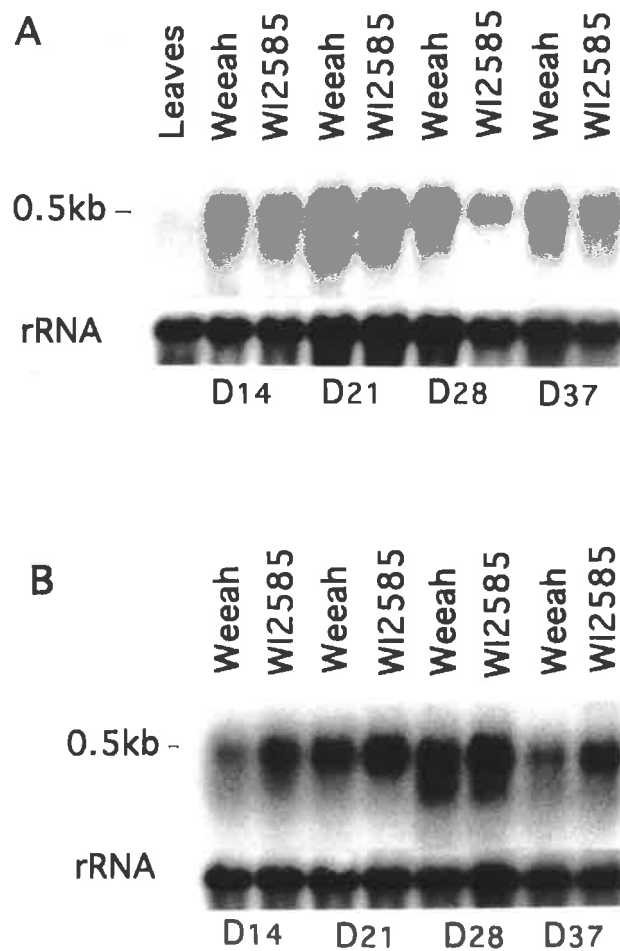
**b** mRNA size was estimated by RNA gel blot analysis.

**c** cross hybridisation was analysed by DNA gel blot analysis.

**d** computerised database research (GenBank and EMBL).

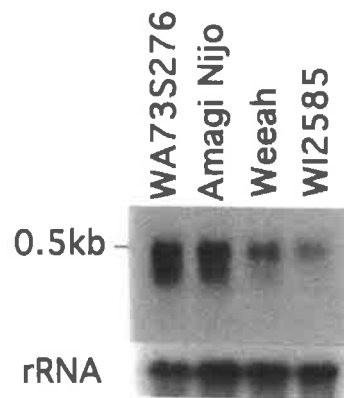
Two clones, No 107 and 125, that contained unique sequences and three clones, 130, 139 and 142, that represented cross hybridising sequences were further analysed by RNA gel blots. At the low Mn supply, no clear differences in the expression level of mRNA were found between Weeah and WI2585 in four of the five clones (No 107, 125, 130 and 142). However, the abundance of the mRNA represented by clone No 139 was different in the two genotypes as illustrated in Fig. 6.1A. No 139 mRNA was low in both genotypes on D14, but increased on D21 and slightly decreased afterwards.

The accumulation of No 139 mRNA was not different between the two genotypes on D14, but was higher in the Mn-efficient genotype Weeah on D21 than in the Mn-inefficient genotype WI2585, and this higher expression pattern was maintained for the rest of the experimental period. At the high Mn level, no clear difference in the expression of No 139 gene could be observed between the two genotypes (Fig. 6.1B), and No 139 mRNA was not detectably accumulated in leaves (Fig. 6.1A), suggesting a specificity in expression of the No 139 gene. The size of No 139 mRNA was 500 bp.



**Fig. 6.1.** Time course showing the levels of *Mne-1* mRNA in two barley genotypes grown in a Mn-deficient soil. **A** supplied with a low level of Mn (15 mg Mn/kg soil). **B** supplied with a high level of Mn (100 mg Mn/kg soil).

To confirm the results obtained for the two genotypes differing in Mn efficiency, four genotypes from diverse genetic backgrounds including the two test genotypes were analysed under low Mn conditions. RNA gel blot analysis indicated that the expression of No 139 gene was high in all three Mn-efficient genotypes, but low in the Mn-inefficient genotype (Fig. 6.2). Therefore, No 139 cDNA clone was designated *Mne-1*, representing the first gene found to be related to Mn efficiency.



**Fig. 6.2.** Levels of *Mne-1* mRNA in four barley genotypes grown in a Mn-deficient soil supplied with a low level of Mn (10 mg Mn/kg soil) and harvested on day 28.

### 6.3.2 Sequence of *Mne-1* cDNA and predicted properties of *Mne-1* protein

Sequence analysis of *Mne-1* cDNA was performed in order to determine a potential function for its predicted protein product. The sequence data (Fig. 6.3) showed that *Mne-1* cDNA was 492 nucleotides in length. It consisted of a 5'-untranslated sequence of 34 bp, an open reading frame of 297 bp starting at nucleotide 35 and ending at nucleotide 331, and a 3'-untranslated region of 158 bp from nucleotide 335 to 492. A potential polyadenylation signal (AATATA) was located in the 3'-untranslated region from nucleotide 480 to nucleotide 485. As the RNA gel blot analysis had revealed a 0.5-kb RNA, this sequence information indicated that the *Mne-1* clone was close to or full length. The open reading frame encoded a protein of 99 amino acids with an unmodified molecular size of 10.9 kDa.

GGCGCGGTTT	CCGAGCAAGC	CCTAGCCTCC	CACG	ATG	GTG	CTG	CAG	AAc	49						
			Met	Val	Leu	Gln	Asn		5						
GAC	ATC	GAC	CTG	CTG	AAc	CCG	CCG	GCG	GAG	CTC	GAG	AAG	CTC	AAG	94
Asp	Ile	Asp	Leu	Leu	Asp	Pro	Pro	Ala	Glu	Leu	Glu	Lys	Leu	Lys	20
CAC	AAG	AAG	AAG	CGC	CTC	GTC	CAG	TCC	CCC	AAc	TCC	TTC	TTC	ATG	139
His	Lys	Lys	Lys	Arg	Leu	Val	Gln	Ser	Pro	Asn	Ser	Phe	Phe	Met	35
GAC	GTC	AAG	TGC	CAG	GGC	TGC	TTC	AAc	ATC	ACT	ACT	GTG	TTC	AGC	184
Asp	Val	Lys	Cys	Gln	Gly	Cys	Phe	Asn	Ile	Thr	Thr	Val	Phe	Ser	50
CAT	TCC	CAG	ACT	GTT	GTG	GTG	TGC	CCA	GGC	TGC	CAG	ACG	GTG	CTC	229
His	Ser	Gln	Thr	Val	Val	Val	Cys	Pro	Gly	Cys	Gln	Thr	Val	Leu	65
TGC	CAG	CCA	ACG	GGT	GGC	AAG	GCC	AGG	CTA	ACC	GAG	GGT	TCT	CCT	274
Cys	Gln	Pro	Thr	Gly	Gly	Lys	Ala	Arg	Leu	Thr	Gln	Gly	Ser	Pro	80
TCC	GTC	GCA	AGG	GCG	ACT	AAA	CCT	GTG	GCC	AAc	TGG	AAA	AGC	AAG	319
Ser	Val	Ala	Arg	Ala	Thr	Lys	Pro	Val	Ala	Asn	Trp	Lys	Ser	Lys	95
CCG	TTA	TTG	AAc	TGA	GGTTTT	TTCAAGGGTG	TTGGTCTGAA	AATTTTGCAA	370						
Pro	Leu	Leu	Asn	***					99						
TGTCGGCAAA	GTGAACCCAT	CTTTGGTAAT	TTGATATCTA	GCTGAAGCAA	TATT	424									
ATGACACTGT	ACTATGTTCA	GTTTGGGTTG	TGAGATCAGA	CTAGTAGTAC	CTGG	478									
<u>CAATATATGT</u>	GGTA					492									

**Fig. 6.3.** Nucleotide sequence and deduced amino acid sequence of barley *Mne-1* cDNA clone.

The amino acid sequence is shown in the three-letter code. The numbers at the end of each line refer to the nucleotide position, and amino acid position, respectively. Asterisks mark the termination codon TGA.

A computerised database (Genbank and EMBL) search revealed that the *Mne-1* nucleotide sequence had high homology to two DNA sequences, a characterised human cDNA (*MPS-1*), and an uncharacterised rice cDNA (*RICC23*). *MPS-1* encodes a nuclear protein with a single zinc finger structure (Fernandez-Pol *et al.*, 1993). The



deduced amino acid sequence of the *Mne-1* protein has four cysteine residues in the sequence -Cys-X<sub>2</sub>-Cys-X<sub>15</sub>-Cys-X<sub>2</sub>-Cys- (where X<sub>2</sub> and X<sub>15</sub> refer to 2 and 15 amino acid residues, respectively), which is almost identical to that of *MPS-1* (Fig. 6.4). In addition, a second homology was a stretch of basic amino acid residues that was present in similar positions in the *Mne-1* protein sequence and in *MPS-1*, of which only one residue was different from *MPS-1*. Those basic residues may function as a nuclear localisation signal in *MPS-1* (Fernandez-Pol *et al.*, 1993; Garcia-Bustos *et al.*, 1991).

<i>Mne-1</i>	Met Val Leu Gln Asn Asp Ile Asp Leu Leu Asp Pro Pro Ala Glu	15
<i>MPS-1</i>	--- * * Pro Leu Ala Lys --- --- --- His --- Ser Pro ---	
<i>RICC23</i>	--- --- --- Ser --- --- --- --- --- --- --- Asn --- --- --- ---	
<i>Mne-1</i>	Leu Glu Lys <u>Leu Lys His Lys Lys Lys Arg</u> Leu Val Gln Ser Pro	30
<i>MPS-1</i>	Glu --- --- Arg --- --- --- --- --- --- --- --- --- ---	
<i>RICC23</i>	--- --- --- --- --- --- --- --- --- --- --- --- --- ---	
<i>Mne-1</i>	Asn Ser Phe Phe Met Asp Val Lys <u>Cys Gln Gly Cys</u> Phe Asn Ile	45
<i>MPS-1</i>	--- --- Tyr --- --- --- --- --- --- Pro --- --- Tyr Lys ---	
<i>RICC23</i>	--- --- --- --- --- --- --- --- --- --- --- --- --- Ser ---	
<i>Mne-1</i>	<u>Thr Thr Val Phe Ser His Ser Gln Thr Val Val Val</u> <u>Cys Pro Gly</u>	60
<i>MPS-1</i>	--- --- --- --- --- --- --- Ala --- --- --- Leu --- Val ---	
<i>RICC23</i>	--- --- --- --- --- --- --- --- --- --- --- --- --- ---	
<i>Mne-1</i>	<u>Cys</u> Gln Thr Val Leu Cys Gln Pro Thr Gly Gly Lys Ala Arg Leu	75
<i>MPS-1</i>	--- Ser --- --- --- --- --- --- --- --- --- --- --- ---	
<i>RICC23</i>	--- --- --- --- --- --- --- --- --- --- --- --- --- ---	
<i>Mne-1</i>	Thr Gln Gly Ser Pro Ser Val Ala Arg Ala Thr Lys Pro Val Ala	90
<i>MPS-1</i>	--- --- --- Cys Ser Phe Arg Arg Lys Gln His	
<i>RICC23</i>	--- Glu --- Cys Ser Phe Arg Arg Lys Gln His	
<i>Mne-1</i>	Asn Trp Lys Ser Lys Pro Leu Leu Asn	99

**Fig. 6.4.** Comparison of the deduced amino acid sequence of the barley *Mne-1* clone with *MPS-1* and *RICC23*. Only the amino acids that differ from *Mne-1* are given. The conserved zinc finger domains are boxed at the zinc binding regions and the connecting region is underlined. The putative nuclear localisation signal is indicated by a double bar.

## 6.4 Discussion

A gene (*Mne-1*) was identified by its differential expression in roots of Mn-efficient and Mn-inefficient genotypes grown at a low Mn supply. This gene was highly expressed in the Mn-efficient genotype but poorly in the Mn-inefficient genotype. The higher expression of *Mne-1* was also demonstrated in two other Mn-efficient genotypes with diverse genetic backgrounds. This suggests that the differential expression is related to Mn efficiency rather than an unrelated genotypic difference between Weeah and WI2585. In addition, the expression of *Mne-1* in the Mn-inefficient genotype was not increased with increasing Mn deficiency stress from D28 to D37, which indicates that *Mne-1* expression is not related to Mn deficiency stress.

When the plants of the two genotypes were grown at a high Mn supply, *Mne-1* was expressed in the two genotypes at an equal level, whereas the Mn concentrations in shoots, although well above the critical level, were still different between the two genotypes. These results not only indicate that the expression of *Mne-1* is important for plants grown in the calcareous soil, even when the Mn supply is high enough for plants to achieve adequate Mn in shoots, but also suggest that a translational or post translational regulation occurs so that the level of protein translated from *Mne-1* mRNA or the activity of protein may be different between the two genotypes, leading to the difference in Mn concentration in shoots. Alternatively, a second unidentified differentially active gene may also contribute to Mn uptake.

The deduced protein sequence reveals that *Mne-1* has a sequence characteristic of a zinc finger structure. Zinc finger proteins can form a finger like configuration by binding a zinc ion through co-ordination with four cysteine residues. This finger structure is able to bind to DNA. Such proteins are thus involved in DNA repair (Culp *et al.*, 1988; Tanaka *et al.*, 1990) and transcription regulation (Berg, 1990). Three classes of zinc-binding motif have been reported (Schwabe and Fairall, 1995). The

*Mne-1* protein has sequence similarity to the Class II zinc-binding motif, nuclear hormone receptors, found in mammals and insects. However, the nuclear hormone receptors in this class have two zinc fingers, both consisting of four cysteines (C-X<sub>2</sub>-C-X<sub>12-13</sub>-C-X<sub>2</sub>-C) (Evans, 1988), whereas *Mne-1* only has one zinc finger (C-X<sub>2</sub>-C-X<sub>15</sub>-C-X<sub>2</sub>-C). Two clones, No 130 and No 142 which cross hybridised to *Mne-1* but represented much larger mRNAs, may encode proteins with two zinc fingers.

Two plant DNA sequences encoding a protein having a single zinc finger of the four cysteine type have been registered with Genbank. One is the uncharacterised rice cDNA (RICC23) which has high protein sequence similarity with *Mne-1* (Fig. 6.4). The other is a barley cDNA, ES43 which is similar to the DNA binding domain of steroid hormone receptors (Speulman and Salamini, 1995), but ES43 shows similarity to *Mne-1* only in the zinc finger structure consisting of four cysteines (C-X<sub>2</sub>-C-X<sub>23</sub>-C-X<sub>2</sub>-C).

*Mne-1* protein, however, also has close sequence relationship to the characterised MPS-1 protein of humans (Fig. 6.4). MPS-1 protein can bind a zinc ion, bind to a cyclic AMP-responsive element, be phosphorylated and is localised in the nucleus (Fernandez-Pol *et al.*, 1994; Xynos *et al.*, 1994). This gene functions in the transcriptional regulation of genes containing a cyclic AMP-responsive element (Fernandez-Pol *et al.*, 1994), and appears to be involved in various signal transduction pathways in human cells (Fernandez-Pol *et al.*, 1993; Xynos *et al.*, 1994). The results of the sequence comparison strongly suggest that *Mne-1* functions as a transcriptional regulator in barley root cells. However, protein characterisation is deemed important before assigning this role to *Mne-1* (Chapter 7).

## Chapter 7

### Functional analysis of *Mne-1* gene

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#### 7.1 Introduction

*Mne-1* cDNA has a nucleotide sequence which predicts a protein containing a zinc finger motif of the four cysteine type (Chapter 6). A human gene, MPS-1, that has sequence similarity to *Mne-1*, functions in transcriptional regulation and possibly in signal transduction as well (Fernandez-Pol *et al.*, 1993; 1994; Xynos *et al.*, 1994). *Mne-1* may have a similar role, but this type of protein has not previously been functionally characterised in plants. Understanding of *Mne-1* function may help to reveal the role of *Mne-1* in Mn efficiency, and also provide information about how this type of protein operates in plant cells.

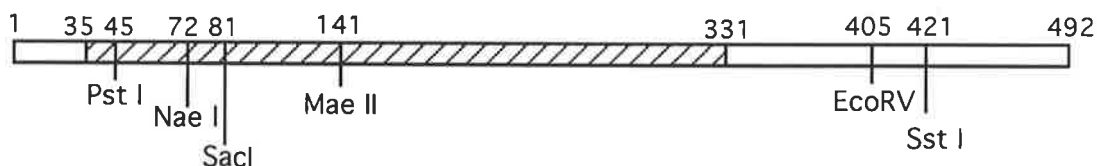
In order to study *Mne-1* function, the open reading frame of *Mne-1* was cloned into an *E. coli* expression vector and the recombinant *Mne-1* protein was isolated. Using the purified recombinant *Mne-1* protein, anti-*Mne-1* antibodies were raised, and then used to analyse the abundance of *Mne-1* protein in roots of barley genotypes differing in Mn efficiency. In addition, the renatured recombinant protein was analysed for metal content. The results provide a preliminary understanding of the possible role of *Mne-1* in Mn efficiency.

#### 7.2 Materials and methods

##### 7.2.1 Construction of expression vector

PQE-30 was chosen as the expression vector because a protein produced from this vector would contain a six-histidine tag in the amino terminal, that could be used for affinity purification. In order to prevent internal starts which result in expressed proteins without the six-histidine tag, the removal of the start codon (methionine) from the inserted DNA fragment is recommended by the manufacturer (QIAGEN). As

shown in Fig. 7.1, eleven nucleotides encoding for methionine and the next three amino acids were removed from the 5' end of the *Mne-1* coding region by *Pst*I digestion, which was the nearest restriction site in the 5' end. The desired 360-bp DNA fragment (287-bp coding region and 73-bp uncoding region) was cleaved from pGEM-5Zf(+)/*Mne-1* by *Pst*I-EcoRV digestion and purified. The 3' overhang generated by *Pst*I digestion was removed by T4 DNA polymerase to generate a blunt end for ligation with the *Sma*I-cut and dephosphorylated expression vector (PQE-30). The 360-bp fragment was ligated into the vector using T4 DNA ligase. The recombinant PQE-30/*Mne-1* plasmids derived from this ligation were transformed into *E. coli* M15 cells. The insert size and the orientation of the insert in PQE-30/*Mne-1* transformants were determined by restriction digestion. All DNA manipulation and analysis, and the transformation were performed using the methods described in Section 2, 3 and 4 of Appendix C.



**Fig. 7.1.** Restriction map of *Mne-1* cDNA

Numbers above the box designate the nucleotide pair located at the start of the sequence and each restriction site. The predicted coding region (▨) extends from base pair 35 to base pair 331 of the cDNA sequence. The non-coding region (□) is also indicated.

### 7.2.2 Expression and purification of *Mne-1* recombinant protein

Two cell lines obtained from the transformation, one carrying PQE-30/*Mne-1* with the right orientation of *Mne-1* insert, and another carrying PQE-30/*Mne-1* with the reverse orientation of the insert (as control), were selected for the expression of recombinant *Mne-1* protein. A small scale expression was carried out to investigate the

production of recombinant Mne-1 protein in *E. coli*. After the overexpression of the recombinant Mne-1 protein in *E. coli* was confirmed, a large scale expression and affinity purification were performed according to the manufacturer's instructions (QIAGEN). The details are described in Section 9.1 and 9.2 of Appendix C.

### **7.2.3 Production of anti-Mne-1 antiserum**

One rabbit was immunised by injection with a 600  $\mu$ l mixture of the affinity-purified recombinant Mne-1 protein (150  $\mu$ g) and Freund's complete adjuvant (1:1, v/v). After 3 weeks, the rabbit was given a booster injection with a 600  $\mu$ l mixture of the affinity-purified recombinant Mne-1 protein (150  $\mu$ g) and incomplete adjuvant (1:1, v/v). The rabbit was bled after 5 weeks. The blood was stored at 4°C overnight and the immune serum was obtained by centrifugation.

### **7.2.4 Extraction of soluble barley root protein**

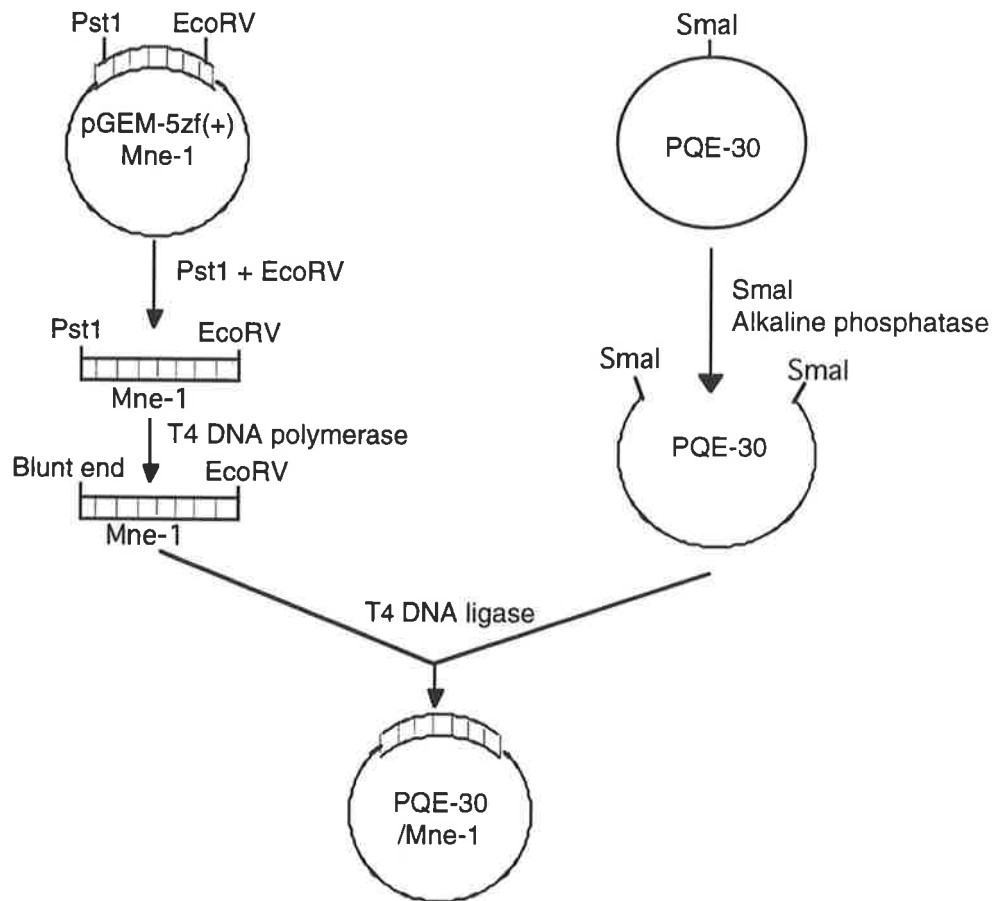
A small scale extraction was carried out. About 0.5 g of roots was ground to a fine powder in a prechilled mortar with liquid nitrogen. Then, 0.5 ml protein extraction buffer (Genenheimer, 1990) was added, which contained 50 mM Tris·HCl (pH 8.0), 5% glycerol, 100 mM KCl and 1 mM phenylmethylsulphonyl fluoride (PMSF). The slurry was ground rapidly with pestle for 1 min, followed by transferring the slurry to a 2-ml Eppendorf tube. The supernatant was obtained after the extract was spun at 4°C in a microfuge for 15 min and stored at -20°C. The concentration of total soluble protein was determined with BCA\* protein assay reagent (Section 9.3 of Appendix C).

### **7.2.5 Protein gel blot analysis**

The materials and methods for protein gel blot analysis were described in Section 9.4-9.6 of Appendix C.

### 7.2.6 Metal content analysis

The insoluble fraction of *E. coli* crude lysate was used to purify sufficient amount of recombinant Mne-1 protein for metal content analysis. After the cells were lysed by sonication, the insoluble fraction was obtained by centrifugation at 10K, 4°C in JA 20 for 15 min. The pellets were washed twice with Sonication buffer (Appendix D) and once with Sonication buffer containing 1M urea in which the solubility of recombinant Mne-1 protein was low as observed by SDS -PAGE gel. Then the pellet was resuspended with Buffer B which contained 8 M urea, to dissolve the recombinant protein. After centrifugation, the recombinant protein at approximately 80% purity (estimated by SDS-PAGE) was obtained in the supernatant. In order to allow the denatured recombinant protein in buffer B to refold, the supernatant was diluted to 1M urea with 10 mM Tris·HCl, pH 8.0, 0.2 M NaCl, 5% (v/v) glycerol buffer (TNG) containing 1 mM DTT and 10 µM ZnSO<sub>4</sub> or 10 µM MnSO<sub>4</sub>. The diluted protein solution in 2-ml tubes was mixed slowly in a rotary mixer at 4°C for 60 min, and then spun at 12K, 4°C for 5 min. The supernatant was transferred to dialysis tubing (molecular cut-off 8 kDa), and dialysed in 1-litre dialysis solution (10 mM Tris·HCl, pH 8.0, 0.2 M NaCl buffer containing 1M urea and 1mM EDTA for 24 hours with three changes. In the dialysis solution, the addition of 1 M urea could help decrease the recombinant protein precipitation during the dialysis and thus a sufficient amount of the recombinant Mne-1 protein could be obtained for analysis. The low concentration of urea (1M) appeared not to interfere with the protein binding to zinc (Von Arnim and Deng, 1993). The addition of 1 mM EDTA in the dialysis solution was to remove any zinc ions not tightly bound to the protein (Von Arnim and Deng, 1993). After the dialysis was completed, the dialysed protein solution was transferred to a 2-ml Eppendorf tube and spun at 12K, 4°C for 5 min to remove any precipitate formed during the dialysis. Finally, the concentration of the renatured recombinant protein which had high purity (no observable contaminating protein upon SDS-PAGE) was measured, and the metal concentration was determined by ICP.



**Fig. 7.2.** Construction of recombinant expression vector PQE30/Mne-1

### 7.3 Results

#### 7.3.1 Cloning of *Mne-1* coding sequence into an *E. coli* expression vector

An *E. coli* plasmid expression vector, PQE-30/Mne-1 was constructed using PQE-30 and a 360-bp fragment of *Mne-1* from pGEM-5Zf(+)/Mne-1 as described in Materials and methods and Fig. 7.2. Both orientations of the insert in the plasmids were expected as the insert was blunt ended. After the PQE-30/Mne-1 constructs were transformed into M15 cells, clones containing recombinant plasmid with the right sized insert were determined by restriction digestion (Fig. 7.3). Double digestion with BamHI and HindIII showed that two transformed M15 cell lines were carrying a plasmid with the desired sized insert, around 400 bp (Fig. 7.3, Lane 1 and 2, white



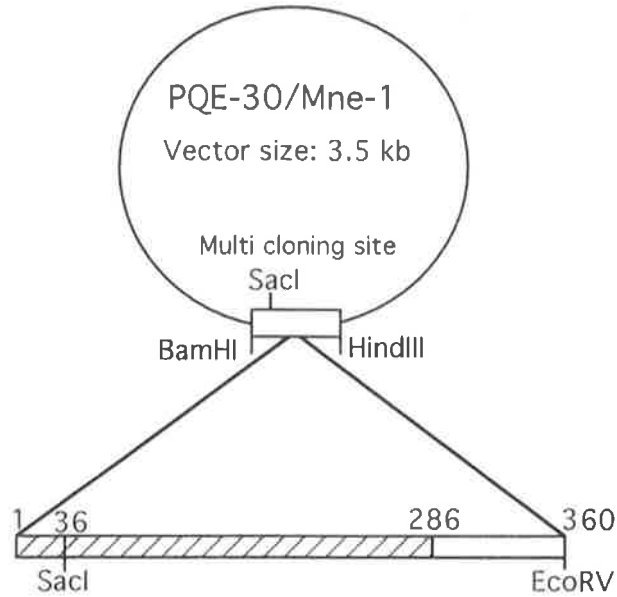
arrow). An additional digestion with *SacI* indicated that the *Mne-1* inserts carried by the two cell lines were in opposite orientations (Fig. 7.3, Lane 3 and 4). *SacI* digestion of the reverse orientation could release a 341-bp fragment, which appeared in the digest run in Lane 3 (Fig. 7.3), but the right orientation was expected to release a 45-bp fragment, which was too small to be observed in the digest run in Lane 4 (Fig. 7.3). From this evidence, the clone run in Lane 3 was designated the backward orientation and the clone run in Lane 4 was the forward orientation.

### 7.3.2 Expression and purification of *Mne-1* recombinant protein

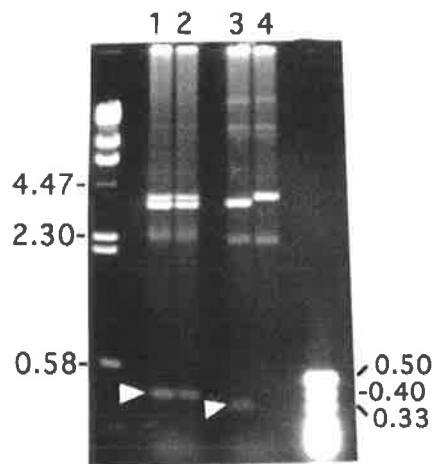
The PQE-30/*Mne-1* constructs allow the expression of *Mne-1* in *E. coli* under the control of the T5 RNA polymerase promoter after addition of IPTG. Both soluble and insoluble proteins, before and after IPTG treatment of clone cultures, were analysed (Fig. 7.4). In the soluble protein fraction, no visible overexpressed protein band from the IPTG-induced cell line carrying the PQE-30/*Mne-1* construct with the right orientation was observed in the expected size range of the recombinant *Mne-1* protein (calculated size, MW 12.5 kDa). However, in the insoluble protein, an IPTG-induced overexpressed protein band close to 14.3 kDa was observed, but only in the cell line carrying PQE-30/*Mne-1* construct with the right orientation (Fig. 7.4). These results indicated that the overexpressed protein band was the recombinant *Mne-1* protein, and that the recombinant protein was insoluble in *E. coli*.

The recombinant protein denatured by 8 M urea in Buffer B (Appendix D) was affinity-purified under denaturing conditions using the recombinant protein property, a six-histidine tag (QIAGEN), as shown in Fig. 7.5. From this result, it was concluded that the 14.3 kDa overexpressed protein was the recombinant *Mne-1* protein.

**A**

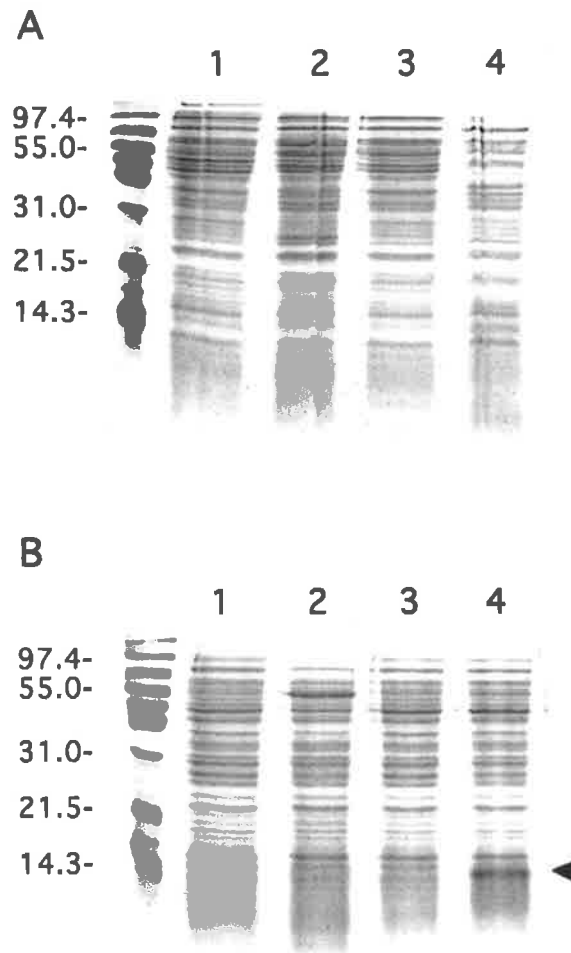


**B**



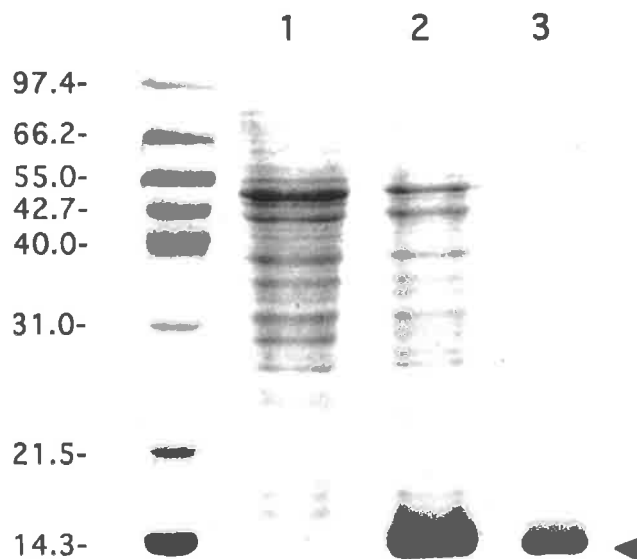
**Fig. 7.3.** Restriction analysis of the recombinant PQE30/Mne-1

**A.** restriction map of PQE-30/Mne-1. Numbers represent the nucleotide pair of the sequence indicated. and represent the coding region and the non-coding region, respectively. **B.** restriction analysis of the recombinant PQE30/Mne-1. Two transformed cell lines carrying PQE30/Mne-1 were used in the analysis. Double digestion with BamHI and HindIII (Lane 1 and 2). SacI digestion (Lane 3 and 4). The small (faint) restriction fragments are indicated by white arrows.



**Fig. 7.4.** Expression analysis of the recombinant Mne-1 protein

**A.** the soluble protein from *E. coli*. **B.** the insoluble protein from *E. coli*. Lane 1 and 3 were the control cell line carrying PQE30/Mne-1 with the reverse orientation of the insert. Lane 2 and 4 were the cell line carrying PQE30/Mne-1 with the right orientation of the insert. Lane 1 and 2 were before IPTG induction, and Lane 3 and 4 were after the induction. The overexpressed recombinant Mne-1 protein is indicated by the black arrow. The protein was separated by SDS-PAGE and stained with Coomassie blue.



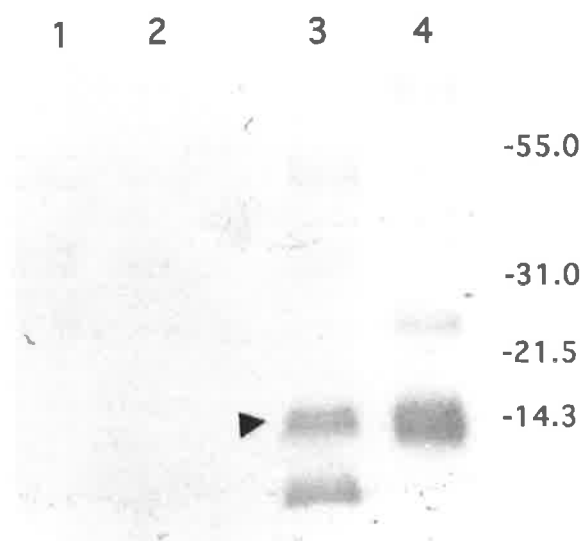
**Fig. 7.5.** Affinity purification of the recombinant Mne-1 protein

**Lane 1.** the insoluble protein from the control cell line carrying PQE30/Mne-1 with reverse orientation of the insert after ITPG induction. **Lane 2.** the insoluble protein from the cell line carrying PQE30/Mne-1 with right orientation of the insert after ITPG induction. **Lane 3.** affinity-purified Mne-1 recombinant protein. The recombinant Mne-1 protein is indicated by the black arrow. The protein was separated by SDS-PAGE and stained with Coomassie blue.

### 7.3.3 Immunoassay of recombinant Mne-1 protein and barley root protein

Affinity-purified recombinant Mne-1 protein was used to generate an antiserum for further functional analysis of Mne-1 in barley roots. The specificity of the anti-Mne-1 immune serum was determined by protein gel blot analysis. As shown in Fig. 7.6, no immunoreactive protein was detected in either soluble or insoluble protein fraction extracted from the control cell line carrying PQE-30/Mne-1 with the reverse orientation of the insert, but cross-reacting protein could be detected in both soluble and insoluble protein fractions. In addition to the expected size band, another smaller

immunoreactive protein band was also detected, but only in the soluble protein extract (Fig. 7.6, Lane 3). The identity of this second band is unclear, but it may result from specific processing of the full-length protein. Nevertheless, these results indicate that anti-*Mne-1* antibodies in the immune serum were specific for the recombinant *Mne-1* protein as no cross-reacting protein was found in the extracts from the cell line with the reverse orientation clone.



**Fig. 7.6.** Specificity analysis of the anti-*Mne-1* antibodies against the recombinant *Mne-1* protein

The soluble and insoluble protein from the control cell line (Lane 1 and 2), and from the cell line carrying PQE-30/*Mne-1* with the right orientation of the insert after IPTG induction (lane 3 and 4). The recombinant *Mne-1* protein is indicated by the black arrow.

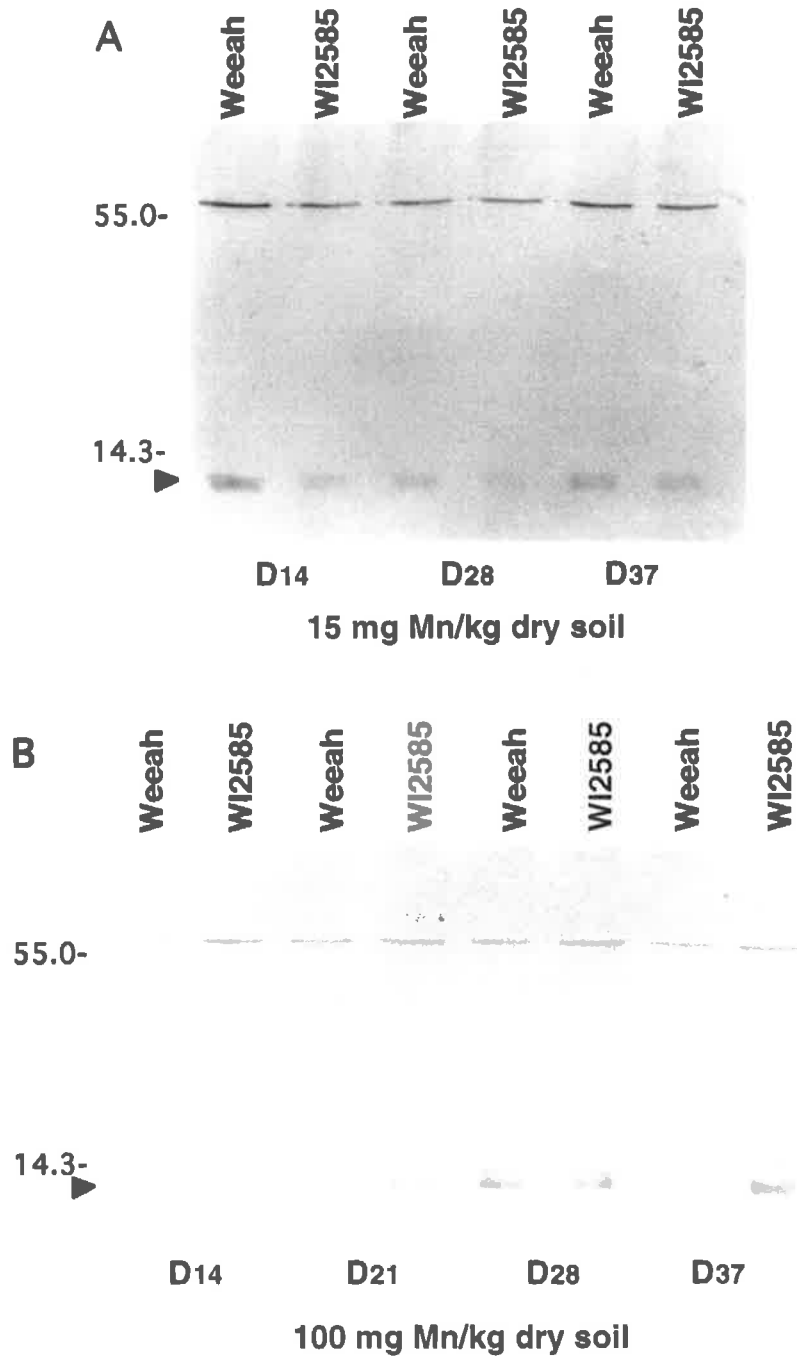
The soluble barley root protein was analysed with the preimmune serum and immune serum to determine the reactivity of anti-*Mne-1* antibodies with the native *Mne-1* protein. The preimmune serum did not immunoreact with any soluble barley root protein, but the immune serum detected two barley protein species (Fig. 7.7). The large protein species was about 60 kDa. The small protein species was around 11 kDa, which was close to the calculated size of *Mne-1* protein (MW 10.9 kDa).



**Fig. 7.7.** Specificity analysis of the anti-Mne-1 antibodies using barley root protein

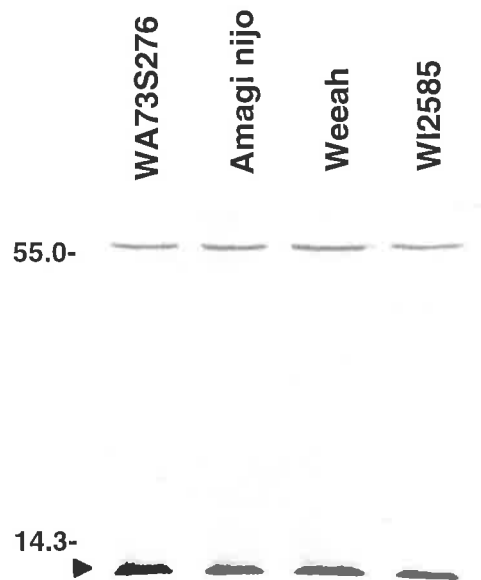
The soluble root protein extracted from Weeah was separated by SDS-PAGE, transferred to cellulose membrane, and incubated with the preimmune serum (Lane 1) or with the immune serum (Lane 2). The anti-Mne-1 antibodies were detected by goat anti-rabbit IgG alkaline phosphatase conjugate, as described in materials and methods. The small protein is indicated by the black arrow.

The immune serum was also used to analyse the soluble root protein of the two barley genotypes differing in Mn efficiency (Fig. 7.8). No clear genotypic difference in the accumulation of the large protein species was found in the plants grown at the low Mn supply from D14 to D37 (Fig. 7.8A). This indicated that the large protein was a Mne-1 related protein, which may share cross-reacting protein epitope(s) with Mne-1 protein. However, the expression level of the small protein species was different in the two genotypes, being higher in the Mn-efficient genotype Weeah than in the Mn-inefficient genotype WI2585 from D14 to D37 (Fig. 7.8A). Moreover, the higher expression of the small protein species at the low Mn supply was also demonstrated in two other Mn-efficient genotypes with diverse genetic backgrounds compared to that of Weeah (Fig. 7.9). At the high Mn supply, no genotypic difference in the expression of the small protein species was found (Fig. 7.8B).



**Fig. 7.8.** Immunoblot analysis of the expressed Mne-1 protein in the roots of two barley genotypes.

The soluble root protein was extracted from Weeah and WI2585 grown at low Mn supply (**A**) and at high Mn supply (**B**). The small protein is indicated by the black arrow.



**Fig. 7.9.** Immunoblot analysis of the expressed Mne-1 protein in the roots of four barley genotypes.

The soluble root protein was extracted from four genotypes grown at low Mn supply (10 mg/kg soil). The small protein is indicated by the black arrow.

#### 7.3.4 Metal content analysis of recombinant Mne-1 protein

According to the sequence data comparisons, the deduced Mne-1 protein sequence contains a zinc finger motif. As Mn ions have similar chemical properties to those of zinc ions, the metal content of renatured recombinant Mne-1 protein was analysed (Table 7.1). The *in vitro* renatured recombinant Mne-1 protein contained zinc ions under both sets of renaturation conditions. The ratio of zinc to protein when zinc salt was added was below the expected value of one, which may be due to some of the protein not refolding properly in the conditions used in this study. However, in neither set of renaturation conditions was Mn found associated with the protein. This evidence strongly supports the designation of Mne-1 as a zinc finger protein.



**Table 7.1. Metal content of recombinant Mne-1 protein**

Treatment	Protein concentration ( $\mu\text{M}$ )	Zn concentration ( $\mu\text{M}$ )	Mn concentration ( $\mu\text{M}$ )	Zn/protein ratio
10 $\mu\text{M}$ $\text{Zn}^{2+}$	5.68 $\pm$ 0.44	3.19 $\pm$ 0.34	0	0.56 $\pm$ 0.03
10 $\mu\text{M}$ $\text{Mn}^{2+}$	5.87 $\pm$ 0.32	1.70 $\pm$ 0.14	0	0.29 $\pm$ 0.03

The data are the average of three replicates  $\pm$ SE.

#### 7.4 Discussion

The overexpression of the recombinant Mne-1 protein in *E. coli* was achieved, but most of the recombinant Mne-1 protein produced was insoluble in *E. coli*. The reason for this may be that the expression of the eukaryotic gene in a prokaryotic system generates some complicating artefacts such as incorrect post-translational modification. Affinity-purified recombinant Mne-1 protein was used to generate anti-Mne-1 antiserum and this antiserum was highly specific for the recombinant Mne-1 protein. Thus, the antiserum was used to analyse the level of Mne-1 protein in barley roots. The results showed that the small protein detected by the polyclonal antibodies was in the size range of the calculated value of the Mne-1 protein (10.9 kDa). Moreover, the accumulation patterns of the small protein resembled those of Mne-1 mRNA in the different barley genotypes tested (Chapter 6 and Fig. 7.8 and 7.9), and these patterns corresponded to the Mn efficiency ranking of the barley genotypes. These results indicate that the small protein is the native Mne-1 protein, and that *Mne-1* functions in root cells through its protein product.

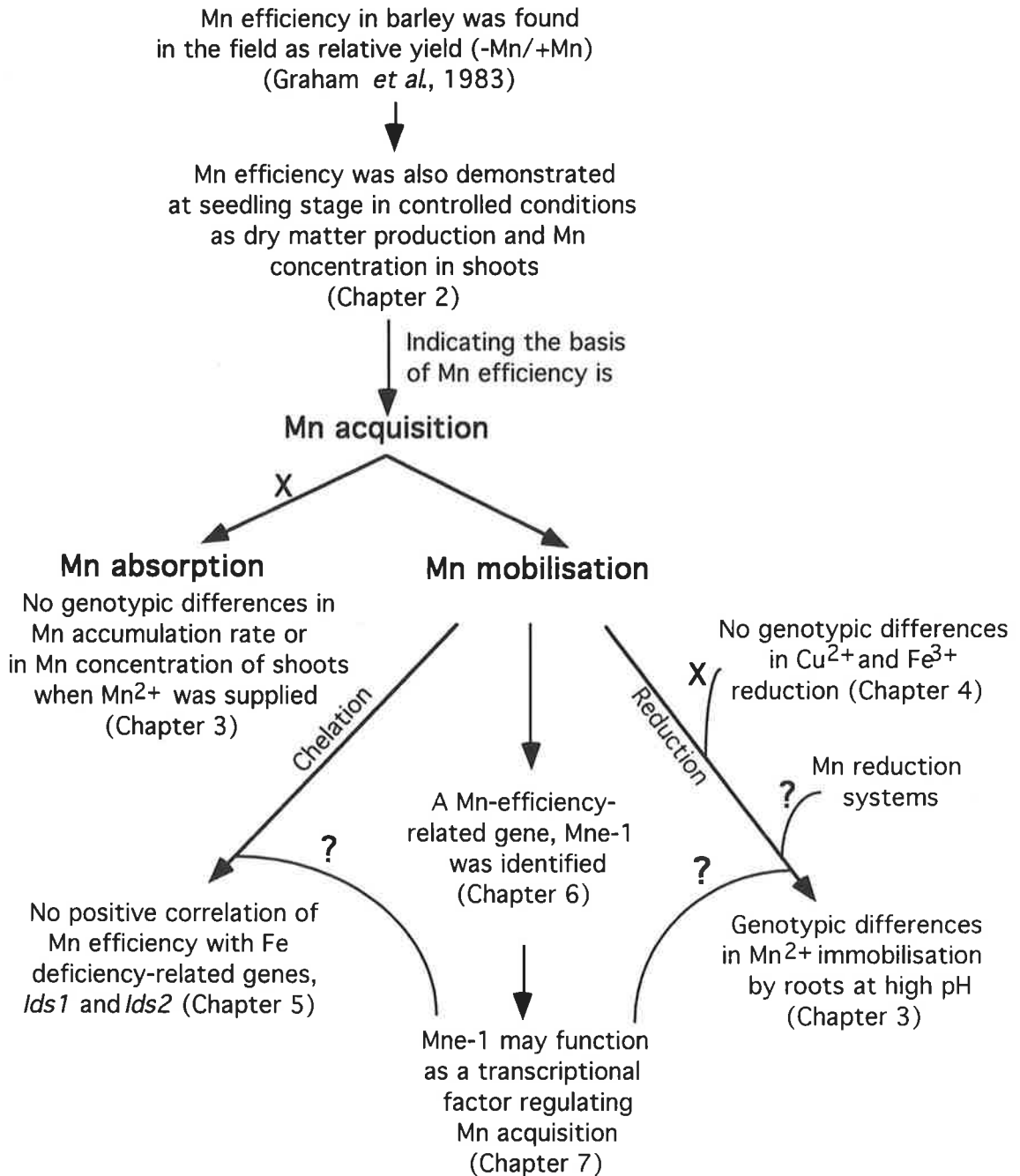
Another large protein species (~60 kDa) was also detected in barley roots, suggesting that it may have a similar protein epitope to Mne-1 protein. However, the level of this large protein did not vary in the different samples (Fig. 7.8 and 7.9). Therefore, there is no evidence for any role of this cross-reacting protein in Mn efficiency.

Sequence data base comparisons reveal that the deduced *Mne-1* protein sequence contains a zinc finger motif which may be able to bind Zn ions and possibly other ions as well (Fernandez-Pol *et al.*, 1994; Giedroc *et al.*, 1986; Li *et al.*, 1991). As Mn ions have similar chemical properties to those of Zn, and the higher expression of this gene is correlated with Mn efficiency under Mn-deficient soil conditions, *Mne-1* protein may be able to bind Zn or Mn ions, leading to two distinct functions. Thus, both Zn and Mn contents of the renatured recombinant protein were analysed. The results showed that the recombinant *Mne-1* protein contained Zn ions *in vitro*, but no Mn ions. This suggests that *Mne-1* is probably not involved in Mn efficiency through its protein product directly binding with Mn ions, but that *Mne-1* may function through the transcriptional regulation of genes related to Mn acquisition.

*Mne-1* protein was accumulated at an equal level in both Mn-efficient and Mn-inefficient genotypes at high Mn supply. This indicates that the accumulation of *Mne-1* protein is needed for plants grown in the calcareous soil supplied even with a level of Mn high enough for plants to achieve adequate Mn in shoots, and suggests that regulatory activities of *Mne-1* protein may be different between Mn-efficient and Mn-inefficient genotypes, or that different activities of genes regulated by *Mne-1* result in differences in Mn acquisition between Mn-efficient and Mn-inefficient genotypes. Further studies are needed to clarify these possibilities.

## Chapter 8

### General discussion



**Fig. 8.1.** Diagram showing approaches and conclusions of the investigation into the mechanism of Mn efficiency in barley.

Barley growth and yield are restricted in a soil which is limiting in available Mn, but Mn fertilisers can only relieve Mn deficiency temporarily (Reuter *et al.*, 1973a, b). The effective and economic solution for Mn deficiency in problem soils is to breed and grow Mn-efficient genotypes (Graham, 1984; 1988). Thus, a clear understanding of the mechanism of Mn efficiency is required for more effective genetic manipulation of Mn efficiency. The experiments conducted in this thesis aimed to identify such mechanism(s). The approaches and conclusions of this research are summarised in Fig. 8.1.

Genotypic variation in Mn efficiency was found in the field in terms of relative grain yield (-Mn/+Mn) as well as dry matter production and Mn accumulation (Graham *et al.*, 1983). For screening Mn efficiency, a faster laboratory procedure was needed. It was therefore necessary to demonstrate that Mn efficiency is expressed in terms of dry matter production and Mn accumulation at the seedling stage under laboratory conditions. Previous work showed that Mn efficiency was correlated to symptoms of Mn deficiency under controlled conditions (Longnecker *et al.*, 1990), but the association of the symptoms with the mechanism of Mn efficiency was not known. Moreover, under those conditions, genotype ranking was not consistent over time when assessed by symptom scores (Webb *et al.*, 1993a). The inconsistency was shown to be due to the change of Mn availability in the soil (Webb *et al.*, 1993a). At that time, it had not been realised that an especially low Mn supply, which was essential to induce Mn deficiency symptoms, limited the ability of Mn-efficient genotypes from fully expressing Mn efficiency. A small increase in Mn supply could allow Mn efficiency to be fully expressed, but restriction to plant growth by pot size was observed. Thus, the effect of pot sizes on the expression of Mn efficiency was studied. The results showed that pot size was a major factor in limiting the expression of Mn efficiency in Mn-efficient genotypes. By using a larger size of pot and measuring Mn concentration in YEB or shoots, genotype ranking in Mn efficiency (Chapter 2) was consistent with that established in the field studies (Longnecker *et al.*, 1990). The significance of the

results from Chapter 2 is that Mn efficiency is demonstrated under controlled conditions in terms of dry matter production and Mn accumulation. Thus, breeding materials can be screened in controlled conditions, and plant materials may be produced by the same procedure for molecular studies as carried out in this thesis. The newly developed screening procedure also can facilitate genetic studies of Mn efficiency and development of molecular markers for rapid screening. In addition, the soil-based pot experiments in controlled conditions further confirm that the basis of Mn efficiency is high Mn accumulation.

The high Mn accumulation in Mn-efficient genotypes is due to the high Mn acquisition from soil rather than more efficient translocation of absorbed Mn from roots to shoots (Chapter 2). Two possible mechanisms may be involved in the high Mn accumulation in shoots of the Mn-efficient genotype. One is a high absorption rate, by which plants can absorb more available Mn from soil solution, such as  $Mn^{2+}$  ions. The other is a high Mn mobilisation, by which plants can increase Mn absorption through an increase in Mn availability. The first proposed mechanism, genotypic differences in the absorption rate, was investigated in a chelate-buffered nutrient solution (Chapter 3), because in soil, Mn absorption can not be easily separated from Mn mobilisation. No genotypic differences in  $Mn^{2+}$  absorption could be shown over a range of Mn supply and over a range of pH. Moreover, genotypic differences in Mn concentration of roots occurred when the plants were grown in the chelate-buffered nutrient solution at high pH (Chapter 3) though the actual processes of mobilisation were not known. Thus, it is concluded that the high accumulation of Mn in shoots of the Mn-efficient genotype grown in the soil is not likely to be related to a high absorption rate of  $Mn^{2+}$  from soil solution by the Mn-efficient plants, but is likely to be related to a high Mn mobilisation from soil.

Several processes such as a decrease of rhizosphere pH, reduction of  $MnO_2$  and Mn chelation can increase Mn release from immobile forms in the soil at the root

surface, which may lead to the expression of genotypic differences in Mn concentration of roots at high pH. However, no clear genotypic difference was found in the change of rhizosphere pH (Marcar, 1986; Harbard, 1992). Little is known about genotypic variation in reduction of  $\text{MnO}_2$ , especially by reductases. Standard oxidation-reduction potentials ( $E_0$ ) of some metabolites, metals and metal complexes are shown in Table 8.1.  $\text{Cu}^{2+}$  reductase system is poised at a value lower than that of  $\text{Cu}^{2+}/\text{Cu}^+$  (+0.17 V), and standard reductase system is at a value lower than that of Fe(III)-cyanide/Fe(II)-cyanide (+0.36 V). Both systems may be able to reduce Mn(III, IV). Because Fe(III)-chelate reductases appeared to be involved in Mn mobilisation by Strategy I plants, and they are easily studied, the reduction of  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  were investigated (Chapter 4). The activities of Fe(III)-chelate reductases in  $\text{Cu}^{2+}$  reduction and the activities of standard reductases in  $\text{Fe}^{3+}$  cyanide reduction were not clearly different among genotypes differing in Mn efficiency. It is concluded that barley, a Strategy II plant for Fe acquisition, may be different in Mn mobilisation from Strategy I plants. It is speculated that barley may have a specific Mn reduction pathway, or produce some ligands to chelate Mn, leading to an increase in Mn mobility and consequently also in absorption (Warden and Reisenauer, 1991b). However, no specific reduction systems or specific chelates have been found so far.

**Table 8.1. Standard oxidation-reduction potentials ( $E_0$ ) of some metabolites, metals and metal complexes**

Reduction reaction couple	$E_0$ at pH 7.0 in volts
Mn(III)/Mn(II)	+1.49
$\text{Fe}^{3+}/\text{Fe}^{2+}$	+0.77
Fe(III)-cyanide/Fe(II)-cyanide	+0.36
$\text{Cu}^{2+}/\text{Cu}^+$	+0.17
Fe(III)-EDTA/Fe(II)-EDTA	+0.13
NADP <sup>+</sup> /NADPH	-0.32
NAD <sup>+</sup> /NADH	-0.32

Values from Welch (1995).

Phytosiderophores, natural Fe(III) ligands, not only mobilise Fe(III), but also Mn and other micronutrients (Takagi *et al.*, 1988; Treeby *et al.*, 1989; Singh *et al.*, 1992). Moreover, Mn uptake was positively correlated both to Fe efficiency in oats (Mench and Fargues, 1994), and to Fe uptake by grass species in calcareous soil (Warden and Reisenauer, 1991b). It is difficult to study the effect of rhizosphere change enhanced by Fe acquisition on Mn mobilisation in soil, because Mn mobilisation is affected by many abiotic and biotic factors (Marschner, 1988). Thus, the expression of two barley genes, *Ids1* and *Ids2*, which are induced by Fe deficiency and are likely to be involved in Fe acquisition (Okumura *et al.*, 1992; 1994), was investigated to determine their connection with Mn efficiency under soil conditions (Chapter 5). *Ids1* and *Ids2* were induced in the calcareous soil where the expression of Mn efficiency occurred among barley genotypes. Moreover, adequate Fe in shoots was observed in the plants grown under that condition. This indicates that *Ids1* and *Ids2* are involved in Fe acquisition. However, the expression of *Ids1* and *Ids2* did not show positive connection with Mn efficiency. Therefore, Fe acquisition as represented by the expression of *Ids1* and *Ids2*, probably does not increase Mn acquisition. Further work is needed for a clear understanding of the involvement of Fe acquisition in Mn efficiency, and this may proceed in the future as other Fe acquisition related genes become available.

Isolation of genes related to Mn efficiency was further explored as a means of better understanding the mechanism of Mn efficiency. *Mne-1* cDNA was identified by differential screening of a Mn-efficient root cDNA population, that was prepared using growth conditions to specifically ensure that Mn efficiency genes were being expressed. *Mne-1* was more abundant at both mRNA and protein levels in the Mn-efficient plant than in the Mn-inefficient plant under low Mn conditions (Chapter 6 and 7). The greater abundance of mRNA and protein of *Mne-1* was correlated with greater Mn efficiency. *Mne-1* shows high protein sequence similarity to a human zinc finger protein, MPS-1 (Fernandez-Pol *et al.*, 1994; Xynos *et al.*, 1994) and binds zinc ions *in vitro* (Chapter 6, and 7), which suggests that *Mne-1* may function as a transcriptional

factor that regulates genes responsible for Mn mobilisation. To confirm the transcriptional function of *Mne-1*, the localisation of Mne-1 protein in cells and the responsive element to which Mne-1 binds need to be determined. The role of *Mne-1* in Mn mobilisation may be able to be determined by gene expression disruption in transgenic plants, and analysis of the genes that *Mne-1* putatively regulates.

Two pathways, reduction of Mn oxides and/or Mn chelation are most likely to lead to enhanced Mn efficiency. The reduction of Mn oxides is necessary for increase of their solubility and mobility in the rhizosphere because Mn oxides are immobile. Chelation can stabilise  $Mn^{2+}$  ions to prevent their reoxidation in the rhizosphere, especially in calcareous soils with high pH, and the chelation can favour further Mn reduction owing to the decreased concentrations of  $Mn^{2+}$  ions. Therefore, the reduction of Mn oxides and/or  $Mn^{2+}$  chelation can result in more Mn available for plant uptake. *Mne-1* may regulate genes responsible for such processes.

Some features of Mn efficiency were observed in this thesis. Any rhizosphere change enhanced by the Mn-efficient plants to increase Mn mobilisation, is limited to a narrow spatial zone, because mixed root systems of Mn-efficient and Mn-inefficient genotypes does not confer any cross-advantage to the Mn-inefficient plants (Chapter 2). Thus, this shows that any Mn mobilisation enhanced by reduction and/or chelation would be expected both in a specific root zone, and in a limited, inner zone of the rhizosphere. Moreover, the expression of Mn efficiency may be constitutive in nature or conditionally inducible. The constitutive system was demonstrated as a higher Mn acquisition by Mn-efficient genotypes over a range of Mn supply in the calcareous soils from plants subject to Mn deficiency to plants in which the Mn concentration was well above the critical level (Chapter 2). The conditionally inducible system was implied by the fact that a higher Mn acquisition by Mn-efficient genotypes did not appear in chelate-buffered nutrient solutions containing only  $Mn^{2+}$  ions, but occurred in calcareous soils where various forms of Mn oxides exist besides  $Mn^{2+}$  ions. It seems



that the Mn forms may be an important factor in the expression of Mn efficiency. Further investigation into the effect of various forms of Mn oxides on Mn efficiency may be able to identify their roles in Mn efficiency. Identification of such factor which controls the expression of Mn efficiency will be an important step forward in the investigation of the biochemical processes in roots that are responsible for Mn efficiency.

## Appendix A

### Analysis of variance tables

Analysis of variance was conducted by SuperANOVA™. Homogeneity of variance was examined by a plot of the residual values versus the fitted values of the dependent variable.

**Table A1. Analysis of variance table for shoot dry matter from Experiment 2A (Fig. 2.1a).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	4	.093	.023	277.892	.0001
Genotype	1	3.213E-4	3.213E-4	3.821	.0624
Replication	3	9.122E-5	3.041E-5	.362	.7813
Mn supply * Genotype	4	.001	2.921E-4	3.474	.0225
Residual	24	.002	8.410E-5		

Dependent: Shoot DM

NOTE: 3 rows have been excluded from calculations because of missing values.

**Table A2. Analysis of variance table for Mn concentration in shoots from Experiment 2A (Fig. 2.1b).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	4	12704.709	3176.177	766.630	.0001
Genotype	1	1.446	1.446	.349	.5603
Replication	3	35.354	11.785	2.844	.0589
Mn supply * Genotype	4	27.177	6.794	1.640	.1968
Residual	24	99.433	4.143		

Dependent: Mn conc in shoots

NOTE: 3 rows have been excluded from calculations because of missing values.

**Table A3. Analysis of variance table for root/shoot ratio from Experiment 2A (Fig. 2.1c).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	4	1.015	.254	72.706	.0001
Genotype	1	.018	.018	5.073	.0337
Replication	3	.017	.006	1.598	.2160
Mn supply * Genotype	4	.049	.012	3.513	.0215
Residual	24	.084	.003		

Dependent: R/S ratio

NOTE: 3 rows have been excluded from calculations because of missing values.

**Table A4. Analysis of variance table for shoot dry matter from Experiment 2A (Fig. 2.1d).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	6	.156	.026	234.535	.0001
Genotype	1	.015	.015	132.071	.0001
Replication	2	1.903E-4	9.514E-5	.856	.4363
Mn supply * Genotype	6	.007	.001	10.246	.0001
Residual	26	.003	1.111E-4		

Dependent: Shoot DM

**Table A5. Analysis of variance table for Mn concentration in shoots from Experiment 2A (Fig. 2.1e).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	6	5453.286	908.881	686.005	.0001
Genotype	1	307.802	307.802	232.323	.0001
Replication	2	6.973	3.486	2.631	.0910
Mn supply * Genotype	6	149.743	24.957	18.837	.0001
Residual	26	34.447	1.325		

Dependent: Mn conc in shoots

**Table A6. Analysis of variance table for root/shoot ratio from Experiment 2A (Fig. 2.1f).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	6	.110	.018	26.480	.0001
Genotype	1	.009	.009	13.610	.0010
Replication	2	1.476E-4	7.381E-5	.106	.8995
Mn supply * Genotype	6	.009	.001	2.096	.0883
Residual	26	.018	.001		

Dependent: R/S ratio

**Table A7. Analysis of variance table for Mn concentration in roots from Experiment 2A (Fig. 2.3).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn supply	6	14.178	2.363	109.782	.0001
Genotype	1	.002	.002	.106	.7470
Replication	2	.190	.095	4.405	.0225
Mn supply * Genotype	6	.160	.027	1.242	.3179
Residual	26	.560	.022		

Dependent: Mn conc in roots (LN transformed data).



**Table A8. Analysis of variance table for plant dry matter from D37 of Experiment 3A (Fig. 3.1).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
Mn	1	2.767	2.767	7.11E2	.0001
Genotype	2	.069	.034	8.812	.0029
Replication	3	.008	.003	.716	.5576
Mn * Genotype	2	.042	.021	5.343	.0177
Residual	15	.058	.004		

Dependent: Dry matter

**Table A9. Analysis of variance table for plant dry matter from D15 of Experiment 3B (Fig. 3.5).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
pH	2	6.119E-5	3.060E-5	3.541	.0449
Genotype	2	3.321E-4	1.661E-4	19.216	.0001
Replication	3	1.646E-5	5.488E-6	.635	.5997
pH * Genotype	4	1.128E-4	2.821E-5	3.264	.0285
Residual	24	2.074E-4	8.642E-6		

Dependent: Dry matter

**Table A10. Analysis of variance table for plant dry matter from D19 of Experiment 3B (Fig. 3.5).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
pH	2	4.601E-4	2.300E-4	6.330	.0062
Genotype	2	.001	.001	16.203	.0001
Replication	3	4.116E-4	1.372E-4	3.776	.0237
pH * Genotype	4	1.428E-5	3.569E-6	.098	.9820
Residual	24	.001	3.634E-5		

Dependent: Dry matter

**Table A11. Analysis of variance table for Mn concentration in shoots from D15 of Experiment 3B (Fig. 3.6).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
pH	2	308.751	154.375	1.49E2	.0001
Genotype	2	33.441	16.720	16.186	.0001
Replication	3	1.205	.402	.389	.7620
pH * Genotype	4	13.261	3.315	3.209	.0303
Residual	24	24.792	1.033		

Dependent: Mn concentration\*

**Table A12. Analysis of variance table for Mn concentration in shoots from D19 of Experiment 3B (Fig. 3.6).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
pH	2	1337.236	668.618	1.86E2	.0001
Genotype	2	75.511	37.755	10.521	.0005
Replication	3	41.041	13.680	3.812	.0229
pH * Genotype	4	29.981	7.495	2.089	.1137
Residual	24	86.124	3.588		

Dependent: Mn conc in shoots•

**Table A13. Analysis of variance table for Mn concentration in roots from D15 of Experiment 3B (Fig. 3.7).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
pH	2	1940.167	970.083	2.34E2	.0001
Genotype	2	101.627	50.813	12.250	.0002
Replication	3	33.903	11.301	2.724	.0665
pH * Genotype	4	62.077	15.519	3.741	.0167
Residual	24	99.557	4.148		

Dependent: Mn conc in roots

**Table A14. Analysis of variance table for Mn concentration in roots from D19 of Experiment 3B (Fig. 3.7).**

Source	df	Sum of Squares	Mean Square	F-Value	P-Value
pH	2	5898.352	2949.176	3.14E2	.0001
Genotype	2	297.542	148.771	15.847	.0001
Replication	3	30.863	10.288	1.096	.3700
pH * Genotype	4	179.107	44.777	4.770	.0056
Residual	24	225.304	9.388		

Dependent: Mn conc in roots

**Appendix B**  
**GEOCHEM-PC calculation for free metals and ligands in chelate-  
buffered nutrient solutions**

**Table B1. GEOCHEM-PC calculation of free metals and ligands for the treatment of  
pMn<sup>2+</sup> 10.2, Experiment 3A.**

Ionic Strength = 7.523E-03 (computed)  
Fixed pH = 6.000  
pCO<sub>2</sub> = 3.090E-04

	Tot. Conc	-log Tot. Conc	Free Activity	-log Free Activity	Free Conc	-log Free Conc	Remainder
Ca	1.000E-03	3.000	6.495E-04	3.187	9.361E-04	3.029	-1.098E-13
Mg	2.500E-04	3.602	1.665E-04	3.779	2.399E-04	3.620	3.839E-12
K	3.767E-03	2.424	3.434E-03	2.464	3.762E-03	2.425	3.697E-11
Fe+3	2.000E-05	4.699	4.268E-18	17.370	9.711E-18	17.013	6.440E-11
Mn+2	3.999E-09	8.398	6.689E-11	10.175	9.640E-11	10.016	6.305E-15
Cu+2	1.000E-06	6.000	1.714E-14	13.766	2.471E-14	13.607	-9.744E-14
Zn	1.000E-05	5.000	1.082E-10	9.966	1.560E-10	9.807	-9.460E-12
Ni	1.000E-07	7.000	3.416E-15	14.466	4.923E-15	14.308	9.101E-14
CO <sub>3</sub>	1.000E-04	4.000	2.197E-10	9.658	3.166E-10	9.499	0.000E+00
SO <sub>4</sub>	2.612E-04	3.583	1.606E-04	3.794	2.315E-04	3.635	-5.951E-12
Cl	5.000E-05	4.301	4.560E-05	4.341	4.996E-05	4.301	6.699E-13
NH <sub>3</sub>	2.000E-04	3.699	1.827E-07	6.738	1.823E-07	6.739	-5.821E-11
PO <sub>4</sub>	2.000E-04	3.699	4.581E-12	11.339	1.042E-11	10.982	1.620E-10
B(OH) <sub>4</sub>	1.250E-05	4.903	7.894E-09	8.103	8.649E-09	8.063	-9.095E-12
MoO <sub>4</sub>	1.000E-07	7.000	6.837E-08	7.165	9.853E-08	7.006	4.441E-16
NO <sub>3</sub>	3.565E-03	2.448	3.253E-03	2.488	3.565E-03	2.448	-7.271E-11
HEDTA	5.610E-05	4.251	1.071E-11	10.970	2.438E-11	10.613	5.229E-1
MES	5.000E-03	2.301	1.383E-03	2.859	1.515E-03	2.820	-2.328E-10

The solution contains 6.335E-03 equivalents per liter of cationic species, -5.865E-03 eq/L of anionic species, and thus has a computed net charge of 4.694E-04 eq/L. This represents an error equal to 7.41 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is 4.107E-06 eq per L.

The total dissolved inorganic carbon (Ct) remaining in this solution is 1.591E-05 mol per L.

**Table B2. GEOCHEM-PC calculation of free metals and ligands for the treatment of  
pMn<sup>2+</sup> 9.2, Experiment 3A.**

Ionic Strength = 7.523E-03 (computed)  
Fixed pH = 6.000  
pCO<sub>2</sub> = 3.090E-04

	Tot. Conc	-log Tot. Conc	Free Activity	-log Free Activity	Free Conc	-log Free Conc	Remainder
Ca	1.000E-03	3.000	6.495E-04	3.187	9.361E-04	3.029	1.446E-11
Mg	2.500E-04	3.602	1.665E-04	3.779	2.399E-04	3.620	7.082E-13
K	3.767E-03	2.424	3.434E-03	2.464	3.762E-03	2.425	2.378E-11
Fe+3	2.000E-05	4.699	4.273E-18	17.369	9.724E-18	17.012	-5.082E-12
Mn+2	3.999E-08	7.398	6.698E-10	9.174	9.653E-10	9.015	3.445E-15
Cu+2	1.000E-06	6.000	1.717E-14	13.765	2.474E-14	13.607	2.136E-12
Zn	1.000E-05	5.000	1.084E-10	9.965	1.562E-10	9.806	1.328E-11
Ni	1.000E-07	7.000	3.421E-15	14.466	4.930E-15	14.307	7.468E-14
CO3	1.000E-04	4.000	2.198E-10	9.658	3.167E-10	9.499	0.000E+00
SO4	2.612E-04	3.583	1.606E-04	3.794	2.315E-04	3.635	2.682E-13
Cl	5.000E-05	4.301	4.560E-05	4.341	4.996E-05	4.301	8.996E-13
NH3	2.000E-04	3.699	1.827E-07	6.738	1.824E-07	6.739	-2.910E-11
PO4	2.000E-04	3.699	4.582E-12	11.339	1.043E-11	10.982	-2.949E-11
B(OH)4	1.250E-05	4.903	7.895E-09	8.103	8.650E-09	8.063	-8.185E-12
MoO4	1.000E-07	7.000	6.837E-08	7.165	9.853E-08	7.006	2.887E-15
NO3	3.565E-03	2.448	3.253E-03	2.488	3.565E-03	2.448	-7.272E-11
HEDTA	5.610E-05	4.251	1.070E-11	10.971	2.434E-11	10.614	5.083E-11
MES	5.000E-03	2.301	1.383E-03	2.859	1.515E-03	2.820	-1.164E-09

The solution contains 6.335E-03 equivalents per liter of cationic species, -5.865E-03 eq/L of anionic species, and thus has a computed net charge of 4.693E-04 eq/L. This represents an error equal to 7.41 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is 4.108E-06 eq per L.

The total dissolved inorganic carbon (Ct) remaining in this solution is 1.591E-05 mol per L.

**Table B3. GEOCHEM-PC calculation of free metals and ligands for the treatment of pMn<sup>2+</sup> 8.2, Experiment 3A.**

Ionic Strength = 7.524E-03 (computed)

Fixed pH = 6.000

pCO<sub>2</sub> = 3.090E-04

	Tot. Conc	-log Tot. Conc	Free Activity	-log Free Activity	Free Conc	-log Free Conc	Remainder
Ca	1.000E-03	3.000	6.495E-04	3.187	9.360E-04	3.029	-3.285E-11
Mg	2.500E-04	3.602	1.665E-04	3.779	2.399E-04	3.620	-9.775E-12
K	3.767E-03	2.424	3.434E-03	2.464	3.762E-03	2.425	-9.818E-11
Fe+3	2.000E-05	4.699	4.266E-18	17.370	9.709E-18	17.013	-2.696E-11
Mn+2	3.999E-07	6.398	6.688E-09	8.175	9.638E-09	8.016	3.687E-13
Cu+2	1.000E-06	6.000	1.714E-14	13.766	2.470E-14	13.607	-1.380E-13
Zn	1.000E-05	5.000	1.082E-10	9.966	1.559E-10	9.807	-9.460E-12
Ni	1.000E-07	7.000	3.416E-15	14.467	4.922E-15	14.308	-1.458E-13
CO3	1.000E-04	4.000	2.197E-10	9.658	3.167E-10	9.499	0.000E+00
SO4	2.618E-04	3.582	1.610E-04	3.793	2.320E-04	3.634	-2.915E-12
Cl	5.000E-05	4.301	4.560E-05	4.341	4.996E-05	4.301	1.407E-12
NH3	2.000E-04	3.699	1.827E-07	6.738	1.824E-07	6.739	-2.910E-11
PO4	2.000E-04	3.699	4.581E-12	11.339	1.043E-11	10.982	-2.622E-10
B(OH)4	1.250E-05	4.903	7.895E-09	8.103	8.650E-09	8.063	5.457E-12
MoO4	1.000E-07	7.000	6.837E-08	7.165	9.853E-08	7.006	-2.554E-15
NO3	3.565E-03	2.448	3.253E-03	2.488	3.565E-03	2.448	-7.284E-11
HEDTA	5.649E-05	4.248	1.071E-11	10.970	2.438E-11	10.613	-3.447E-11
MES	5.000E-03	2.301	1.383E-03	2.859	1.515E-03	2.820	-1.397E-09

The solution contains  $6.335\text{E-}03$  equivalents per liter of cationic species,  $-5.867\text{E-}03$  eq/L of anionic species, and thus has a computed net charge of  $4.677\text{E-}04$  eq/L. This represents an error equal to 7.38 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is  $4.108\text{E-}06$  eq per L.

The total dissolved inorganic carbon (Ct) remaining in this solution is  $1.591\text{E-}05$  mol per L.

**Table B4. GEOCHEM-PC calculation of free metals and ligands for the treatment of pH 6.00, Experiment 3B.**

Ionic Strength =  $4.052\text{E-}03$  (computed)

Fixed pH = 6.000

pCO<sub>2</sub> =  $3.090\text{E-}04$

	Tot. Conc	-log Tot. Conc	Free Activity	-log Free Activity	Free Conc	-log Free Conc	Remainder
Ca	$2.500\text{E-}04$	3.602	$1.661\text{E-}04$	3.780	$2.190\text{E-}04$	3.660	$-5.869\text{E-}12$
Mg	$2.500\text{E-}04$	3.602	$1.810\text{E-}04$	3.742	$2.386\text{E-}04$	3.622	$-4.800\text{E-}12$
K	$1.807\text{E-}03$	2.743	$1.684\text{E-}03$	2.774	$1.804\text{E-}03$	2.744	$-5.317\text{E-}11$
Fe+3	$2.500\text{E-}06$	5.602	$1.534\text{E-}19$	18.814	$2.857\text{E-}19$	18.544	$-4.899\text{E-}12$
Mn+2	$2.148\text{E-}07$	6.668	$1.056\text{E-}09$	8.976	$1.392\text{E-}09$	8.856	$4.678\text{E-}15$
Cu+2	$1.000\text{E-}06$	6.000	$4.948\text{E-}15$	14.306	$6.522\text{E-}15$	14.186	$1.277\text{E-}12$
Zn	$1.000\text{E-}05$	5.000	$3.123\text{E-}11$	10.505	$4.117\text{E-}11$	10.385	$8.854\text{E-}12$
Ni	$1.000\text{E-}07$	7.000	$9.859\text{E-}16$	15.006	$1.300\text{E-}15$	14.886	$3.869\text{E-}14$
CO <sub>3</sub>	$1.000\text{E-}04$	4.000	$2.188\text{E-}10$	9.660	$2.884\text{E-}10$	9.540	$0.000\text{E+}00$
SO <sub>4</sub>	$2.636\text{E-}04$	3.579	$1.891\text{E-}04$	3.723	$2.492\text{E-}04$	3.603	$-8.287\text{E-}12$
Cl	$5.000\text{E-}05$	4.301	$4.664\text{E-}05$	4.331	$4.998\text{E-}05$	4.301	$1.497\text{E-}12$
NH <sub>3</sub>	$1.000\text{E-}04$	4.000	$9.333\text{E-}08$	7.030	$9.324\text{E-}08$	7.030	$-3.638\text{E-}11$
PO <sub>4</sub>	$1.000\text{E-}04$	4.000	$2.397\text{E-}12$	11.620	$4.463\text{E-}12$	11.350	$-6.633\text{E-}11$
B(OH) <sub>4</sub>	$1.250\text{E-}05$	4.903	$7.883\text{E-}09$	8.103	$8.447\text{E-}09$	8.073	$8.185\text{E-}12$
MoO <sub>4</sub>	$1.000\text{E-}07$	7.000	$7.467\text{E-}08$	7.127	$9.843\text{E-}08$	7.007	$2.109\text{E-}15$
NO <sub>3</sub>	$2.061\text{E-}03$	2.686	$1.923\text{E-}03$	2.716	$2.061\text{E-}03$	2.686	$-1.835\text{E-}11$
Tris	$1.387\text{E-}03$	2.858	$1.387\text{E-}03$	2.858	$1.386\text{E-}03$	2.858	$-4.294\text{E-}11$
HEDTA	$3.882\text{E-}05$	4.411	$3.762\text{E-}11$	10.425	$7.005\text{E-}11$	10.155	$4.997\text{E-}12$
MES	$3.614\text{E-}03$	2.442	$1.007\text{E-}03$	2.997	$1.079\text{E-}03$	2.967	$-2.328\text{E-}10$

The solution contains  $2.828\text{E-}03$  equivalents per liter of cationic species,  $-3.842\text{E-}03$  eq/L of anionic species, and thus has a computed net charge of  $-1.013\text{E-}03$  eq/L. This represents an error equal to 35.83 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is  $3.975\text{E-}06$  eq per L.

The total dissolved inorganic carbon (Ct) remaining in this solution is  $1.575\text{E-}05$  mol per L.

**Table B5. GEOCHEM-PC calculation of free metals and ligands for the treatment of pH 6.85, Experiment 3 B.**

Ionic Strength =  $4.559\text{E-}03$  (computed)

Fixed pH = 6.850

pCO<sub>2</sub> =  $3.090\text{E-}04$



	-log Tot.		Free		-log Free		-log Free Conc Remainder
	Tot. Conc	Conc	Activity	Activity	Free Conc	Conc	
Ca	2.500E-04	3.602	1.592E-04	3.798	2.131E-04	3.671	1.365E-11
Mg	2.500E-04	3.602	1.737E-04	3.760	2.326E-04	3.633	-4.148E-12
K	1.726E-03	2.763	1.602E-03	2.795	1.723E-03	2.764	-5.770E-11
Fe+3	2.000E-05	4.699	1.541E-19	18.812	2.969E-19	18.527	2.190E-11
Mn+2	2.500E-07	6.602	1.076E-09	8.968	1.441E-09	8.841	4.769E-13
Cu+2	1.151E-06	5.939	4.984E-15	14.302	6.671E-15	14.176	-4.184E-13
Zn	1.151E-05	4.939	3.145E-11	10.502	4.209E-11	10.376	-9.712E-12
Ni	1.000E-07	7.000	8.639E-16	15.064	1.156E-15	14.937	4.912E-14
CO3	1.000E-04	4.000	1.097E-08	7.960	1.468E-08	7.833	0.000E+00
SO4	2.812E-04	3.551	1.992E-04	3.701	2.666E-04	3.574	1.120E-11
Cl	5.000E-05	4.301	4.647E-05	4.333	4.998E-05	4.301	-1.563E-12
NH3	1.000E-04	4.000	6.546E-07	6.184	6.539E-07	6.185	-7.276E-12
PO4	1.000E-04	4.000	7.933E-11	10.101	1.529E-10	9.816	-1.712E-10
B(OH)4	1.250E-05	4.903	5.516E-08	7.258	5.933E-08	7.227	5.457E-12
MoO4	1.000E-07	7.000	7.456E-08	7.128	9.980E-08	7.001	-1.638E-15
NO3	2.061E-03	2.686	1.916E-03	2.718	2.061E-03	2.686	9.781E-11
Tris	2.188E-03	2.660	2.189E-03	2.660	2.186E-03	2.660	6.540E-11
HEDTA	5.808E-05	4.236	4.284E-11	10.368	8.256E-11	10.083	3.501E-11
MES	2.710E-03	2.567	1.884E-03	2.725	2.027E-03	2.693	-5.821E-11

The solution contains 2.727E-03 equivalents per liter of cationic species, -4.895E-03 eq/L of anionic species, and thus has a computed net charge of -2.168E-03 eq/L. This represents an error equal to 79.49 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is 3.547E-05 eq per L.

The total dissolved inorganic carbon (Ct) remaining in this solution is 4.624E-05 mol per L.

**Table B6. GEOCHEM-PC calculation of free metals and ligands for the treatment of pH 7.70, Experiment 3B.**

Ionic Strength = 4.714E-03 (computed)

Fixed pH = 7.700

	-log Tot.		Free		-log Free		-log Free Conc Remainder
	Tot. Conc	Conc	Activity	Activity	Free Conc	Conc	
Ca	2.500E-04	3.602	1.561E-04	3.807	2.099E-04	3.678	6.614E-12
Mg	2.500E-04	3.602	1.697E-04	3.770	2.282E-04	3.642	2.125E-12
K	1.726E-03	2.763	1.600E-03	2.796	1.723E-03	2.764	1.204E-11
Fe+3	2.000E-05	4.699	1.994E-20	19.700	3.883E-20	19.411	-5.164E-11
Mn+2	2.600E-07	6.585	1.087E-09	8.964	1.462E-09	8.835	-3.508E-14
Cu+2	1.199E-06	5.921	5.046E-15	14.297	6.785E-15	14.168	1.908E-12
Zn	1.199E-05	4.921	3.184E-11	10.497	4.281E-11	10.368	-1.048E-11
Ni	1.000E-07	7.000	8.394E-16	15.076	1.129E-15	14.947	-3.873E-15
CO3	1.000E-04	4.000	2.078E-07	6.682	2.795E-07	6.554	-3.593E-11
SO4	2.812E-04	3.551	1.986E-04	3.702	2.670E-04	3.573	-1.017E-11
Cl	5.000E-05	4.301	4.641E-05	4.333	4.998E-05	4.301	-8.515E-13
NH3	1.000E-04	4.000	4.452E-06	5.351	4.447E-06	5.352	0.000E+00
PO4	1.000E-04	4.000	1.149E-09	8.939	2.238E-09	8.650	-1.835E-11
B(OH)4	1.250E-05	4.903	3.787E-07	6.422	4.078E-07	6.390	9.095E-13
MoO4	1.000E-07	7.000	7.437E-08	7.129	9.999E-08	7.000	8.639E-16
NO3	2.061E-03	2.686	1.914E-03	2.718	2.061E-03	2.686	6.937E-11
Tris	2.685E-03	2.571	2.686E-03	2.571	2.684E-03	2.571	9.615E-12
HEDTA	5.861E-05	4.232	4.448E-11	10.352	8.658E-11	10.063	-6.031E-11
MES	2.317E-03	2.635	2.046E-03	2.689	2.204E-03	2.657	2.183E-11

The solution contains 2.709E-03 equivalents per liter of cationic species, -5.164E-03 eq/L of anionic species, and thus has a computed net charge of -2.455E-03 eq/L. This represents an error equal to 90.63 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is 9.684E-05 eq per L.

NOTE: All of the atmospheric CO<sub>2</sub> has been absorbed by the solution, indicating inadequate total carbonate in the system. The results are thus inaccurate, and the case should be rerun with a higher input total CO<sub>3</sub>.

**Table B7. GEOCHEM-PC calculation of free metals and ligands for the pretreatment of pH experiment, Experiment 3B.**

Ionic Strength = 4.161E-03 (computed)

Fixed pH = 6.000

pCO<sub>2</sub> = 3.090E-04

	Tot. Conc	-log Tot. Conc	Free Activity	-log Free Activity	Free Conc	-log Free Conc	Remainder
Ca	2.500E-04	3.602	1.648E-04	3.783	2.179E-04	3.662	3.600E-11
Mg	2.500E-04	3.602	1.797E-04	3.745	2.377E-04	3.624	-1.672E-13
K	1.807E-03	2.743	1.682E-03	2.774	1.804E-03	2.744	-5.787E-11
Fe+3	5.000E-05	4.301	3.109E-18	17.507	5.832E-18	17.234	8.520E-11
Mn+2	2.148E-07	6.668	1.049E-09	8.979	1.388E-09	8.858	6.270E-14
Cu+2	1.000E-06	6.000	4.915E-15	14.308	6.501E-15	14.187	2.056E-12
Zn	1.000E-05	5.000	3.103E-11	10.508	4.104E-11	10.387	9.760E-12
Ni	1.000E-07	7.000	9.794E-16	15.009	1.295E-15	14.888	1.335E-13
CO <sub>3</sub>	1.000E-04	4.000	2.177E-10	9.662	2.879E-10	9.541	0.000E+00
SO <sub>4</sub>	3.112E-04	3.507	2.224E-04	3.653	2.942E-04	3.531	5.835E-12
Cl	5.000E-05	4.301	4.660E-05	4.332	4.998E-05	4.301	1.002E-12
NH <sub>3</sub>	1.000E-04	4.000	9.325E-08	7.030	9.316E-08	7.031	-7.276E-12
PO <sub>4</sub>	1.000E-04	4.000	2.376E-12	11.624	4.458E-12	11.351	1.603E-11
B(OH) <sub>4</sub>	1.250E-05	4.903	7.870E-09	8.104	8.440E-09	8.074	-1.273E-11
MoO <sub>4</sub>	1.000E-07	7.000	7.441E-08	7.128	9.843E-08	7.007	-1.110E-15
NO <sub>3</sub>	2.061E-03	2.686	1.921E-03	2.716	2.061E-03	2.686	-2.239E-11
Tris	1.387E-03	2.858	1.387E-03	2.858	1.386E-03	2.858	1.364E-11
HEDTA	8.630E-05	4.064	3.746E-11	10.426	7.028E-11	10.153	1.389E-10
MES	3.614E-03	2.442	1.006E-03	2.998	1.078E-03	2.967	0.000E+00

The solution contains 2.824E-03 equivalents per liter of cationic species, -3.978E-03 eq/L of anionic species, and thus has a computed net charge of -1.154E-03 eq/L. This represents an error equal to 40.88 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is 3.970E-06 eq per L.

The total dissolved inorganic carbon (C<sub>t</sub>) remaining in this solution is 1.575E-05 mol per L.

**Table B8. GEOCHEM-PC calculation of free metals and ligands for the treatment of 500 nM of Mn<sup>2+</sup> ions, Chapter 4.**

Ionic Strength = 7.524E-03 (computed)

Fixed pH = 6.000

pCO<sub>2</sub> = 3.090E-04

	Tot. Conc	-log Tot. Conc	Free Activity	-log Free Activity	Free Conc	-log Free Conc	Remainder
Ca	1.000E-03	3.000	6.495E-04	3.187	9.361E-04	3.029	5.539E-11
Mg	2.500E-04	3.602	1.665E-04	3.779	2.399E-04	3.620	2.962E-12
K	3.767E-03	2.424	3.434E-03	2.464	3.762E-03	2.425	9.505E-11
Fe+3	2.000E-05	4.699	4.283E-18	17.368	9.748E-18	17.011	4.045E-11
Mn+2	5.000E-07	6.301	8.394E-09	8.076	1.210E-08	7.917	9.806E-13
Cu+2	1.000E-06	6.000	1.721E-14	13.764	2.480E-14	13.605	2.136E-12
Zn	1.000E-05	5.000	1.086E-10	9.964	1.566E-10	9.805	1.329E-11
Ni	1.000E-07	7.000	3.429E-15	14.465	4.942E-15	14.306	2.953E-13
CO <sub>3</sub>	1.000E-04	4.000	2.197E-10	9.658	3.167E-10	9.499	0.000E+00
SO <sub>4</sub>	2.618E-04	3.582	1.610E-04	3.793	2.320E-04	3.634	8.223E-12
Cl	5.000E-05	4.301	4.560E-05	4.341	4.996E-05	4.301	-1.580E-12
NH <sub>3</sub>	2.000E-04	3.699	1.827E-07	6.738	1.824E-07	6.739	-2.910E-11
PO <sub>4</sub>	2.000E-04	3.699	4.581E-12	11.339	1.043E-11	10.982	-3.819E-13
B(OH) <sub>4</sub>	1.250E-05	4.903	7.895E-09	8.103	8.650E-09	8.063	-8.185E-12
MoO <sub>4</sub>	1.000E-07	7.000	6.837E-08	7.165	9.853E-08	7.006	2.887E-15
NO <sub>3</sub>	3.565E-03	2.448	3.253E-03	2.488	3.565E-03	2.448	-7.281E-11
HEDTA	5.649E-05	4.248	1.067E-11	10.972	2.428E-11	10.615	1.025E-10
MES	5.000E-03	2.301	1.383E-03	2.859	1.515E-03	2.820	-1.164E-09

The solution contains 6.335E-03 equivalents per liter of cationic species, -5.867E-03 eq/L of anionic species, and thus has a computed net charge of 4.679E-04 eq/L. This represents an error equal to 7.39 percent of the total charge of cationic species in solution.

The computed alkalinity for this solution is 4.108E-06 eq per L.

The total dissolved inorganic carbon (Ct) remaining in this solution is 1.591E-05 mol per L.

## Appendix C

### Materials and methods for molecular biology

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#### 1 Materials

##### 1.1 Enzymes

###### 1.1.1 Restriction Enzymes

Most restriction enzymes were bought from Boehringer Mannheim, Promega and Pharmacia.

###### 1.1.2 Other Enzymes

Calf intestinal alkaline phosphatase (CIP):	Promega
DNase I:	Boehringer Mannheim
Klenow DNA polymerase:	Pharmacia
Proteinase K:	Boehringer Mannheim
RNase A:	Boehringer Mannheim
Superscript MMLV reverse transcriptase (RNaseH <sup>-</sup> ):	Gibco-BRL
T4 DNA ligase:	Boehringer Mannheim, Promega
T4 DNA polymerase:	New England Biolabs
Taq DNA polymerase:	Promega

##### 1.2 Other proteins

RNasin:	Promega
Bovine serum albumin (BSA):	Sigma

##### 1.3 Antisera

Goat anti-rabbit-IgG-alkaline phosphatase conjugate: Sigma

##### 1.4 Blotting Membranes

Hybond-N <sup>+</sup> (DNA and RNA):	Amersham
Nitrocellulose (protein):	Bio-Rad

##### 1.5 Plasmids for probes

PHA1: A pea genomic DNA clone containing 18S ribosome DNA (Jorgensen *et al.*, 1987).

Ids1: A 0.5 kb cDNA insert in pBluescript(II) SK(+) (Okumura *et al.*, 1992).

Ids2: 4.5 kb genomic DNA insert in pBluescript(II) SK(-) (Okumura *et al.*, 1994).

### 1.6 Primers for PCR and labelling

$\lambda$ gt10 forward primer (Promega): 5'-d(CTTTTGAGCAAGTTCAGCCTGGT  
TAAG)-3'

$\lambda$ gt10 reverse primer (Promega): 5'-d(GAGGTGGCTTATGAGTATTTCTT  
CCAGGGTA)-3'

pUC/M13 forward primer (Promega): 5'-d(TTTCCCAGTCACGAC)-3'

pUC/M13 reverse primer (Promega): 5'-d(CAGGAAACAGCTATGAC)-3'

Random primer: 9mer

### 1.7 Vectors

#### 1.7.1 DNA cloning vectors

pGEM5Zf(+) (T-vector): Promega

pTZ18U: Promega

#### 1.7.2 Protein expression vectors

PQE30: QIAGEN

### 1.8 Bacterial strains

*E. coli* C600 hfl: F<sup>-</sup>, thi-1, thr-1, leuB6, lacY1, tonA21, supE44,  
hflA150 [chr:: Tn10].

*E. coli* DH5 $\alpha$ : F<sup>-</sup>,  $\phi$ 80dlacZ $\Delta$ M15, endA1, recA1, hsdR17(rk<sup>-</sup>.mk<sup>+</sup>),  
supE44, thi-1. gyrA96, relA1,  $\Delta$ (lacZYA-argF), U169,  $\lambda^-$ .

*E. coli* M15 (pREP4): Nal<sup>s</sup> str<sup>s</sup> rif<sup>s</sup>, lac<sup>-</sup> ara<sup>-</sup> gal<sup>-</sup> mtl<sup>-</sup> F<sup>-</sup> recA<sup>+</sup> uvr<sup>+</sup>.

### 1.9 Nucleotides and radio nucleotides

Deoxynucleotide triphosphates (dNTP): Promega

Dideoxynucleotide triphosphates (ddNTP): Applied Biosystems Inc.

$\alpha$ -<sup>32</sup>P-dCTP (10 mCi/ml, 3000 Ci/mM): Amersham

Labelling mix: Amersham

## 2 Transformation of *E. coli*

Competent cells were prepared according to the method described in Sambrook *et al.* (1989). A single *E. coli* colony was inoculated into 2 ml SOB medium with or without appropriate antibiotics and grown in a 37°C shaker overnight. 0.5 ml overnight culture was added to 50 ml SOB medium supplemented with 0.5 ml 1M MgSO<sub>4</sub>, 0.5 ml 1M MgCl<sub>2</sub> and with or without appropriate antibiotics, followed by incubation at 37°C with vigorous shaking for approximately 2.5 hours. When the OD<sub>600nm</sub> reached about 0.5, the cell suspension was chilled on iced water for 10 min, and 25 ml of the cell suspension was transferred to a sterile centrifuge tube. The cells were pelleted at 2.5K rpm, 4°C in JA20 (Model J2-21M, Beckman) for 12 min and resuspended with 8.5 ml TFB buffer. The cell suspension was left on ice for 10-15 min and centrifuged at 2.5K rpm, 4°C in JA20 for 10 min. The cell pellet was resuspended again with 2 ml TFB buffer, followed by adding 70 µl redistilled DMSO, mixing gently and leaving on ice for 5 min. Then, the cell suspension was mixed with 157.5 µl 1M DTT and left on ice for 10 min. After that, 75 µl DMSO was added, mixed gently and left on ice for 5 min. Until then, the cells were ready for transformation. The typical transformation efficiency was 10<sup>6</sup>-10<sup>7</sup> (colony forming unit/µg DNA).

100 µl of fresh competent cells was usually mixed with 5 µl ligation mixture or 5 µl plasmid DNA (0.2 ng/µl) and incubated on ice for 30 min. The cell suspension was heated at 42°C for 2 min, chilled on ice for 3 min, and then 400 µl SOC (1 ml SOB medium containing 7 µl 50% glucose) was added, followed by incubation in a 37°C shaker for 60 min. The cell suspension was then plated onto selection LB plates and incubated at 37°C overnight. Desired single colonies were selected for restriction analysis of plasmid DNA.

## 3 DNA isolation

### 3.1 Mini-prep of plasmid DNA

A single colony was inoculated into 1.5 ml LB medium or Terrific broth with

appropriate antibiotics and grown in a 37°C shaker overnight. An alkaline lysis method described by Sambrook *et al.* (1989) was used in mini-scale plasmid isolation.

### 3.2 Large scale isolation of plasmid

The alkaline lysis and equilibrium centrifugation in CsCl gradients (Sambrook *et al.*, 1989) were used to the large scale isolation of plasmid DNA.

### 3.3 Small scale isolation of $\lambda$ DNA

The methods are described in the  $\lambda$ gt10 cDNA cloning manual of Amersham. DEAE-cellulose is used to adsorb chromosomal DNA, RNA and protein in *E. coli* lysate. The resultant  $\lambda$ DNA is easily digestible and free of RNA and chromosomal DNA. Briefly, 100  $\mu$ l of prepared *E. coli* host (C600*hfl*) was mixed with 50  $\mu$ l of phage buffer containing phage particles diffused from a single plaque core or diluted phage lysate at 37°C for 30 min, and transferred to 5 ml LB supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub>. The cells were grown at a 37°C shaker until they were completely lysed. The cellular debris was removed by centrifugation at 3K rpm, 4°C in JA20.1 (Model J2-21M, Beckman) for 10 min. The supernatant was transferred to a new tube. Then, DNase I and RNase A (1  $\mu$ g/ml) were added to the supernatant and incubated at 37°C for 30 min, followed by addition of an equal volume of 20% PEG 6000 (w/v) and 2M NaCl in SM buffer to precipitate phage particles in ice water for one hour or overnight. After that, the phage particles were pelleted by centrifugation at 9K rpm in JA20.1 for 10 min. The pellet was resuspended with 750  $\mu$ l LB, and then 750  $\mu$ l of DEAE-cellulose DE52 LB suspension was added and mixed. The supernatant was obtained by spinning at 12K rpm, RT in microfuge for 5 min. The phage DNA was released by addition of 13  $\mu$ l proteinase K (0.1 mg/ml) and 32  $\mu$ l 10% SDS at RT for 5 min. Then, 130  $\mu$ l of 3M potassium acetate was added and incubated at 88°C for 20 min, followed by chilling on ice to precipitate SDS and protein. After centrifugation, the supernatant was obtained. The DNA was precipitated with iso-propanol, and the pellet was washed with 70% ethanol. Finally, the pellet was resuspended in 20  $\mu$ l TE.

### **3.4 Plant DNA isolation**

One fresh, young barley leaf (10-15 cm long) was folded and put into a 2-ml Eppendorf tube. The tube was frozen in liquid nitrogen, and then the leaf was crushed into powder with a small rod. 600  $\mu$ l of DNA extraction buffer was added and mixed well. An equal volume of Tris·HCl saturated phenol was added to the tube and mixed gently on a rotatory mixer at 4°C for 30 min. The supernatant was obtained by centrifugation at 12K rpm, 4°C in microfuge for 15 min, and re-extracted with phenol/chloroform/iso-amyl alcohol (25:24:1). The DNA was precipitated with ethanol, dried briefly in vacuum and dissolved in 50  $\mu$ l R40 buffer.

## **4 Analysis and manipulation of DNA**

### **4.1 Restriction digestion of DNA**

DNA (usually 0.5-5  $\mu$ g) was digested with restriction endonucleases (3-4 units/ $\mu$ g DNA) in restriction buffer recommended by the manufacturer at 37°C except for SmaI at 25 °C for 1-2 hours.

### **4.2 Separation and analysis of restriction fragments**

Agarose gel electrophoresis was used to separate and analyse DNA fragments. Digested DNA with DNA sample loading buffer and appropriate DNA size markers were loaded into 0.8-1.5% (w/v) agarose gels, and run in TAE buffer at 20-100V as required. After electrophoresis, the gels were stained in H<sub>2</sub>O containing 1 $\mu$ g/ml ethidium bromide for 10-15 min. DNA bands were visualised with UV light and photographed.

### **4.3 Purification of DNA fragments from agarose gel**

A small piece of gel containing the desired size of fragments was excised from agarose gel with a surgical blade under UV light, and stored in an Eppendorf tube. GeneClean Kit (Bio 101, Bresatec) was used to purify DNA fragments from agarose gel according to the manufacturer's instructions. Briefly, the piece of excised gel was



dissolved with 2.5X 6M NaI (w/v) at 50°C. Appropriate volume of glass milk (usually 5-10 µl) was added, mixed and then placed on ice for 5 min, allowing DNA to bind to glass particles. The particles were pelleted by brief centrifugation and washed three times with New Wash Solution. The particle-bound DNA was redissolved with TE or H<sub>2</sub>O at 50°C for 3 min, and spun briefly to pellet the particles. The clear DNA solution was carefully transferred to a new tube and stored at -20°C.

#### **4.4 Precipitation of DNA**

One tenth volume of 3M sodium acetate pH 4.8 and 2.5 volumes of pure ethanol or 1 volume of iso-propanol were added into DNA solution and mixed. The mixture was incubated at -20°C for 30 min to overnight to precipitate DNA. DNA was recovered by centrifugation at 12K rpm, 4°C in microfuge for 15 min. DNA pellet was washed with cold 70% (v/v) ethanol, dried in vacuum for 1-2 min and resuspended in an appropriate volume of TE or H<sub>2</sub>O.

#### **4.5 Quantification of DNA by spectroscopy**

DNA samples were diluted to 1 ml with water and scanned against a water blank between 200-300 nm wavelength. DNA concentration was calculated using 1 unit of  $A_{260} = 50 \mu\text{g/ml DNA}$ .

#### **4.6 Dephosphorylation of plasmid vector**

Plasmid DNA (usually 10 µg) was completely digested with the appropriate restriction enzyme. The digestion solution was extracted with phenol/chloroform and then precipitated with ethanol. The DNA was resuspended in 42 µl 10 mM Tris·HCl (pH 8.0), and then 5µl 10X CIP buffer (0.5 M Tris·HCl, pH 9.0, 10 mM MgCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub> and 10 mM spermidine) and 3µl (1U/µl) of CIP were added to dephosphorylate the DNA at 37°C for 30 min. For blunt ends or protruding 3' ends, additional 3µl CIP was added and incubated at 37°C for another 30 min. The reaction was stopped by adding 300 µl stop buffer (10 mM Tris·HCl, pH 7.5, 1 mM EDTA, pH 7.5, 200 mM

NaCl, 0.5% SDS). The reaction solution was extracted with equal volume of phenol/chloroform, and the DNA was precipitated with ethanol. The DNA was electrophoresed on 1% agarose gel. The linear DNA band was cut and purified with GeneClean (Bio 101). The DNA was resuspended in an appropriate volume of TE.

#### **4.7 Ligation of insert to cloning vector**

Ligation reaction (usually in a volume of 10  $\mu$ l) was performed using about 100 ng of vector DNA, insert DNA at a molar ratio of 1:3-4, ligation buffer (10X ligation buffer, 300 mM Tris·HCl, pH 7.8, 100 mM MgCl<sub>2</sub>, 100 mM DTT and 5 mM ATP) and 1 unit of T4 DNA ligase at 12°C overnight.

#### **4.8 Synthesis of insert by PCR**

##### **4.8.1 Amplification of $\lambda$ gt10 insert by PCR**

Amplification of  $\lambda$ gt10 insert by PCR was performed in a 0.5-ml Eppendorf tube with 50  $\mu$ l reaction solution containing Taq buffer (10X 500 mM Tris-HCl, pH 8.0, 100 mM NaCl, 0.1 mM EDTA, 5 mM DTT, 10% Triton X-100), approximately 0.2  $\mu$ g  $\lambda$ DNA or diluted phage lysate, 0.5-1.0  $\mu$ g of  $\lambda$ gt10 forward and reverse primers, 200  $\mu$ M dNTP, 2.5 mM MgCl<sub>2</sub>, 0.5 unit of Taq DNA polymerase. The tubes were placed in a Hybaid IHB 2024 thermal cycler and run through Program U55. After the reaction was completed, 10  $\mu$ l of the reaction solution was used in agarose gel electrophoresis to check the size of insert, or the reaction solution was used to isolate the insert as required for making DNA probe.

##### U55 program

1. 4 min, 94°C (Denaturing)
2. 1 min, 94°C
3. 2 min, 55°C (annealing)
4. 2 min, 72°C (extension)
5. Go to step 2, Cycle 35 times

6. 10 min, 72°C

7. 5 min, 25°C

8. end

#### **4.8.2 Amplification of Ids1 cDNA by PCR**

Ids1 cDNA insert in pBluescript(II) SK(+) plasmid (Okumura *et al.*, 1992) was amplified by PCR, using approximately 0.1 µg plasmid DNA and 0.3 µg M13 forward and reverse primers. Other PCR conditions were the same as the amplification of λgt10 insert (Section 4.8.1).

### **4.9 Preparation of DNA probe for hybridisation**

#### **4.9.1 Labelling DNA probe with random or specific primer**

Template DNA (approximately 100 ng) and a random primer or a specific primer (300 ng) in a volume of 7.5 µl were boiled for 3 min and chilled on ice water for 3 min. Then 12.5 µl labelling mix, 3 µl α-<sup>32</sup>P-dCTP (10 µCi/µl) and 1 µl Klenow enzyme (1 U/µl) were added and mixed. Then, the labelling reaction mixture was incubated at 37°C for 60 min.

#### **4.9.2 Separation of unincorporated radionucleotide from DNA probe**

A mini column was prepared using a pasteur pipette packed with Sephadex G100 and equilibrated with TE. The labelling reaction mixture was loaded to the mini column and eluted with TE. The radioactivity of eluent was monitored with a Geiger counter, and the first peak of eluent (about 0.4 ml) was collected. The eluent was boiled for 5 min to denature the DNA and cooled in ice water for 3 min. Then, it was ready for use in hybridisation.

### **4.10 DNA gel blot analysis**

#### **4.10.1 Transfer of DNA to membrane**

DNA transfer to membrane was carried out as described by Sambrook *et al.*

(1989). Before transfer, gels were soaked in denaturing solution for 20 min, in neutralising solution for 20 min and in 20X SSC for 20 min, respectively. Capillary transfer of DNA from gels to Hybond-N<sup>+</sup> membrane was carried out with 20X SSC for at least 6 hours, followed by briefly rinsing in 5X SSC and blotting dry with Whatman 3MM paper. Then, the membrane was dried in air or in vacuum at 80°C for 60 min, and DNA on the membrane was fixed under UV light (15 Watt, 254 nm, 24 cm above the membrane) for 5 min.

#### **4.10.2 Hybridisation**

The membrane was rolled, and placed in a hybridisation bottle, followed by adding a prehybridisation solution (1.5X HSB, 0.2X Denhardt's III, 2% SDS, 500µl/ml denatured salmon sperm DNA). The bottle was installed in a hybridisation oven and revolved at 65°C for 4-6 hours. After that, the prehybridisation solution was drained, and a hybridisation solution was added, which contained 1.5X HSB, 0.2X Denhardt's III, 2% SDS, 5% Dextran sulphate, 250µg/ml boil-denatured salmon sperm DNA and a boil-denatured probe. The hybridisation was carried out at 65°C overnight.

After hybridisation, the membrane was washed sequentially with 2X SSC + 0.1% SDS at 65°C for 15 min, 1X SSC + 0.1% SDS for 15 min, 0.5X SSC + 0.1% SDS at 65°C for 15 min, 0.2X SSC + 0.1% SDS for 15 min until no background signals could be detected. Then, the membrane was blotted dry, wrapped with a plastic bag and exposed to X-ray films in a cassette at -80°C for 3-10 days depending on the intensity of radioactivity on the membrane. The films were developed in Curix 60 Developer (AGFA).

#### **5 Construction of cDNA Library from roots of barley**

A cDNA library was constructed using cDNA synthesis kit from Pharmacia. The manufacturer's instructions were followed.

### 5.1 First strand cDNA synthesis

First-strand cDNA was synthesised by moloney murine leukemia virus (MMLV) reverse transcriptase in presence of dNTP and DTT, using poly(A)<sup>+</sup> RNA as template and oligo (dT)<sub>12-18</sub> as primer. Briefly, approximately 1 µg of poly(A)<sup>+</sup> mRNA in 20 µl H<sub>2</sub>O was denatured at 65°C for 10 min and chilled on ice. Then, 1µl DTT and the heat-denatured RNA were added into First-Strand Reaction Mix, and the reaction was incubated at 37°C for 1 hour.

### 5.2 Second strand synthesis

Second-strand synthesis involves that RNaseH nicks the RNA strand of the RNA:cDNA duplex and DNA polymerase I uses these nicks to replace RNA with DNA by nick translation. After the second-stand synthesis was completed, klenow fragment was added to ensure the double-strand cDNA with blunt ends. The actual processes were carried out as follows. The first-strand reaction solution (Section 5.1) was transferred to Second-Strand Reaction Mix, mixed gently, and incubated at 12°C for 1 hour and then 22°C for 1 hour. After that, 1 µl of klenow fragment was added and incubated at 37°C for 30 min. To stop the reaction, the reaction solution was heated at 65°C for 10 min, and 100 µl phenol/chloroform was added at RT to remove protein. After phenol/chloroform extraction, the upper aqueous layer was loaded to the centre of bed of a prepared S-300 spun column. The column was spun in a swinging bucket rotor at 400g for 2 min. The effluent was collected in a clean Eppendorf tube.

### 5.3 Ligation of EcoRI/NotI adaptors to cDNA and phosphorylation of the ligated adaptors

The effluent from Section 5.2 was mixed with 2µl EcoRI/NotI adaptors, 1µl ATP solution and 3µl T4 DNA ligase, and then incubated at 12°C overnight. The reaction was heated at 65°C for 10 min and chilled on ice. After that, 10 µl of ATP solution and 1µl T4 polynucleotide kinase were added, incubated at 37°C for 30 min. To stop the reaction, the reaction solution was heated at 65°C for 10 min and chilled on ice,

followed by adding 100  $\mu$ l phenol/chloroform at RT to remove protein. Then, the upper aqueous layer was loaded to another prepared S-300 spun column to remove free adaptors.

#### **5.4 Ligation of cDNA into $\lambda$ gt10**

Firstly, cDNA and  $\lambda$ gt10 arms were precipitated with ethanol. 15  $\mu$ l of the effluent from Section 5.3 was mixed with 15  $\mu$ l of STE buffer (TE buffer containing 150 mM NaCl) and 2  $\mu$ l of  $\lambda$ gt10 arms (0.5  $\mu$ g/ $\mu$ l, Promega), followed by adding 1  $\mu$ l of 3M sodium acetate and 60  $\mu$ l of cold ethanol to precipitate DNA at -80°C for 15 min. The DNA was pelleted by centrifugation and dried briefly in vacuum. The ligation was carried out in the same conditions as described in the ligation of adaptors to cDNA (Section 5.3).

#### **5.5 Packaging of ligated $\lambda$ gt10 DNA**

5  $\mu$ l of ligation mixture was added to 50  $\mu$ l of Packaging Extract (Promega), mixed and incubated at 22°C for 2 hours. The packaged phage solution (55  $\mu$ l) was diluted to 500  $\mu$ l with phage buffer and 25  $\mu$ l chloroform was added to prevent growth of contaminating bacteria.

#### **5.6 Titration of packaged phage**

A single colony *E. coli* C600hfl was inoculated into 2 ml LB with tetracycline and grown overnight in a 37°C shaker. 0.5 ml overnight culture was inoculated into 50 ml LB supplemented with 0.2% maltose and 10 mM MgSO<sub>4</sub> and incubated with vigorous shaking at 37°C for 2-3 hours until the OD<sub>600nm</sub> reached about 0.5. The cells were chilled on ice. A dilution series from 5 to 500 fold of packaged phage were prepared using phage buffer. 100  $\mu$ l of the diluted phage was mixed with 100  $\mu$ l of the C600hfl prepared cells, and incubated at 37°C for 30 min. 200  $\mu$ l of the cell suspension was added into 12 ml top agarose supplemented with 0.2 % maltose and 10 mM MgSO<sub>4</sub> (42°C), mixed gently and then plated on a plate (150 mm in diameter). The plate was

incubated at 37°C overnight, and then the number of plaque forming unit (pfu) was counted. After the titration, the library was plated on the plate (150 mm in diameter) at a density of 1,000 plaque forming unit per plate.

## **6 Differential screening bacteriophage lambda library**

### **6.1 Transfer of phage DNA to membrane**

Two plaque lifts were made for each phage plate, using Hybond-N<sup>+</sup> circle membrane (137 mm in diameter). The membrane was marked with three dots on the edge, carefully laid over the plate, and then the outside wall of the plate was marked corresponding to the three dots with Texta. The first lift was left on top of the plate for 30 seconds and the second lift for 60 seconds. The membrane was carefully lifted off the plate, laid on a sheet of Whatman 3MM paper saturated with denaturing solution with DNA side up for 3 min, and then transferred to a neutralising solution for 3 min, followed by rinsing the membranes in 5X SSC for a few seconds, blotting excess 5X SSC on the membranes with Whatman 3MM paper and drying at 80°C for 1 hour. After that, the membrane was fixed under UV light (see Section 4.10.1) for 5 min.

### **6.2 Hybridisation of plaque lifts**

The two plaque lifts from each plate were rolled, and placed in a different hybridisation bottle, prehybridised in a solution containing 1.5X HSB, 0.2X Denhardt's III, 2% SDS, 500µl/ml boil-denatured salmon sperm DNA at 65°C for 4-6 hours. Then, each copy of plaque lifts was hybridised in a solution containing 1.5X HSB, 0.2X Denhardt's III, 2% SDS, 5% Dextran sulphate, 250µg/ml denatured salmon sperm DNA and a boil-denatured cDNA probe as required at 65°C overnight. After hybridisation, the membranes were treated as the same as that described in Section 4.10.2.

$\alpha$ -<sup>32</sup>P dCTP cDNA probes were synthesised by MMLV reverse transcriptase, using total RNA as template and oligo (dT)<sub>15</sub> as primer. Briefly, approximately 15 µg of total RNA and 1µg of oligo(dT)<sub>15</sub> in a volume of 7.0 µl H<sub>2</sub>O were heated to 72°C

for 3 min and cooled to RT. Then, 4 $\mu$ l of 5X reverse transcriptase buffer (250 mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM MgCl<sub>2</sub>), 2 $\mu$ l 0.1 M DTT, 1 $\mu$ l dNTP mix (10 mM dATP, dGTP and dTTP, and 2 mM dCTP), 0.5  $\mu$ l (40U/ $\mu$ l) RNasin, 1.5  $\mu$ l (200U/ $\mu$ l) SuperScript RNaseH<sup>-</sup> reverse transcriptase (Gibco-BRL), and 4  $\mu$ l  $\alpha$ -<sup>32</sup>P dCTP were added, mixed gently and incubated at 37°C for 60 min. The labelled cDNA was separated from unincorporated radio active dCTP using a mini column (Section 4.9.2).

### 6.3 Selection of putative clones

A single plaque was isolated from the plate using a sterile pasteur pipette according to the different intensity in two plaque lifts.

### 7 DNA sequencing

A Taq DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems Inc.) was used in dideoxy-mediated sequencing reactions. Four separate reactions, A, C, G, T, were performed in four 0.5-ml Eppendorf tubes. In A or C reaction, 1 $\mu$ l of DNA (about 0.25 $\mu$ g/ $\mu$ l) and 4 $\mu$ l correspondent mix containing four deoxynucleotides, an appropriate primer and a chain-terminating dideoxy nucleotide (ddATP or ddCTP) were mixed. In G and T reaction, 2 $\mu$ l of DNA and 8 $\mu$ l of correspondent mix containing four deoxynucleotides, an appropriate primer and a chain-terminating dideoxy nucleotide (ddGTP or ddTTP). Then, 20  $\mu$ l mineral oil was layered over the aqueous reaction solution. The tubes were placed in a thermal cycler (Hybaid IHB 2024) and run through Program SEQ55.

#### SEQ55.

1. 5 min, 94°C (Denaturing)
2. 30 sec, 94°C
3. 30 sec, 55°C (annealing)
4. 1 min, 72°C (synthesis)



5. Go to step 2, Cycle 14 times
6. 30 sec, 94 °C
7. 1 min, 72°C
- 8 Go to step 6, Cycle 14 times
9. 30 min 4°C.

After the reactions were completed, all four reaction solutions were carefully transferred to a clean 1.5 ml Eppendorf tube containing 100µl ethanol and 2µl 3M sodium acetate (pH 5.5), and mixed thoroughly. The tube was placed on ice for 15 min to precipitate the DNA. The DNA was pelleted at 12K rpm, 4°C for 15 min, washed with 70% ethanol, and then dried briefly in vacuum. Then, the DNA was separated electrophoretically and analysed using 373A DNA sequencer (Applied Biosystems Inc.).

## **8 Isolation and analysis of RNA**

### **8.1 Total RNA isolation**

The method of Cox and Goldberg (1988) was used in extraction of total RNA with minor modification. Briefly, 1-2 g of roots or leaves was ground to a fine powder with liquid nitrogen in a mortar. 7.5 ml of RNA extraction buffer was added and mixed thoroughly. The icy slurry was transferred to a pre-cooled 30-ml Corex tube and centrifuged at 10K rpm, 4°C in JA20 centrifuge (Model J2-21M, Beckman) for 10 min. 7.5 ml supernatant was transferred to a flask containing 8g CsCl. Then, 0.5 ml of 30% sarkosyl was added and mixed until CsCl was dissolved. The supernatant was carefully layered onto the top of 2.5 ml CsCl cushion (0.96 g CsCl/ml H<sub>2</sub>O) in an ultracentrifuge tube, and centrifuged in a Ti 65 rotor (Model L8-70, Beckman) at 38K rpm, 4°C for 16 hours. After DNA and protein in the supernatant were removed with sterile cotton buds and sterile pasteur pipettes, RNA pellet was resuspended in 500 µl of 1% sarkosyl in TE. The RNA solution was extracted with phenol/chloroform, and then precipitated with one tenth volume of 5 M NaCl and 2.5 volume of cold ethanol at -20°C overnight.

The RNA was recovered by centrifugation at 12K rpm, 4°C in microfuge for 15 min, washed with 70% ethanol, and dried briefly in vacuum. The pellet was resuspended in an appropriate volume of H<sub>2</sub>O, and stored at -80°C.

A<sub>260</sub> of RNA samples was determined using spectrophotometer, and RNA concentration was calculated using 1 unit of A<sub>260</sub>=40 µl/ml RNA. The quality of RNA was assessed by RNA electrophoresis to view the integrity of ribosomal RNA bands with UV light.

### **8.2 Isolation of poly(A)<sup>+</sup> mRNA**

A poly(A)<sup>+</sup> mRNA isolation kit (PolyTract mRNA Isolation System IV, Promega) was used for isolation of poly(A)<sup>+</sup> mRNA. Total root RNA (300 µg) in a volume of 500 µl was denatured at 65°C for 10 min. Then 3 µl of biotinylated-Oligo(dT) probe and 13 µl of 20X SSC were added into the total RNA solution and incubated at RT for 30 min to anneal. After that, the total solution was transferred to a tube containing 100 µl of washed streptavidin-paramagnetic particles (SA-PMPs), mixed and incubated at RT for 10 min. SA-PMPs were captured using a magnetic stand and carefully remove supernatant. The particles were washed four times with 0.1X SSC. To elute mRNA, the particles were resuspended in 100 µl RNase-free H<sub>2</sub>O and captured magnetically. The aqueous phase was transferred to a new tube. The elution step was repeated once more with 150 µl RNase-free H<sub>2</sub>O. The eluted mRNA solutions were combined (250µl), and precipitated with ethanol. The RNA pellet was resuspended in 20 µl RNase-free H<sub>2</sub>O. A parallel sample of poly(A)<sup>+</sup> mRNA was used to determine the concentration of poly(A)<sup>+</sup> mRNA by spectrophotometer.

### **8.3 RNA electrophoresis**

Agarose gels (1.2% Seakem GTG agarose) containing MOPS buffer and 3% formaldehyde were prepared. 2.5 µl of total RNA (approximately 10 µg) was mixed with 2.0 µl of 10X MOPS, 10 µl deionised formamide, and 3.5 µl formaldehyde, heated

at 65°C for 10 min, and chilled on ice. 2 µl of 10X loading buffer was added to each sample. The RNA samples were loaded into gels and run in MOPS buffer at 60 V for 2-3 hours. Then, the gels were stained in 1 µg/ml ethidium bromide for 15 min, followed by destaining in water for 30 min to view ribosome bands with UV light, or used for RNA gel blot analysis.

## **8.4 RNA gel blot analysis**

### **8.4.1 RNA transfer to membrane**

After electrophoresis, gels were washed with water, and soaked in high efficiency transfer solution (Biotechx) for 15 min. Capillary transfer of RNA from gels to Hybond-N<sup>+</sup> membrane was carried out as same as DNA transfer (Section 4.10.1) using the high efficiency transfer solution overnight, followed by briefly rinsing in H<sub>2</sub>O and blotting dry with Whatman 3MM paper. The membranes were dried under vacuum at 80°C for 1 hour. Ribosomal RNA bands in the membranes were viewed under UV light to check quality of RNA and relative loading of total RNA in each lane.

### **8.4.2 Hybridisation**

The membrane was prehybridised in a solution containing 45% deionised formamide, 5X SSPE, 7.5X Denhardt's reagent, 0.5% SDS and 500 µg/ml denatured salmon sperm DNA at 42°C overnight, and then hybridised in a solution containing 45% deionised formamide, 5X SSPE, 7.5X Denhardt's reagent, 0.5% SDS, 2.5% dextran sulphate, 250 µg/ml denatured salmon sperm DNA and a denatured <sup>32</sup>P-labelled probe at 42°C for 48 hours. The probe was synthesised by random labelling cDNA inserts amplified by PCR. The membrane was washed sequentially in 2X SSC + 0.1% SDS at 65°C for 15 min, in 1X SSC + 0.1% SDS at 65°C for 15 min, 0.5X SSC + 0.1% SDS at 65°C for 15 min, and 0.2X SSC + 0.1% SDS at 65°C for 15 min until no background signals could be detected. Then, the membrane was blotted dry, wrapped with a polyethylene bag and exposed to X-ray film in a cassette at -80°C. The film was developed in Curix 60 developer (AGFA).

The equal loading of total RNA was also tested by hybridising the stripped membranes (see Section 8.4.3) with a radioactive 18S ribosomal DNA probe prepared from BarnHI-EcoRI fragment of PHA1 clone (Jorgensen *et al.*, 1987).

#### **8.4.3 Stripping membranes for reuse**

Used membranes were put into a container containing boiling stripping solution (2 mM EDTA, pH 8.0, 0.1% SDS). The container was put at RT to cool down, and then incubated at 65°C for 30 min, followed by pouring off the solution, washing with 5X SSC, blotting excess 5X SSC, sealing in plastic bags and storing at 4°C.

### **9 Protein gel blot analysis**

#### **9.1 Extraction of *E. coli* protein**

Small scale of extraction was carried out to check expression of recombinant protein according to the manufacturer's instructions (QIAGEN). 2 ml cell culture was pelleted by centrifugation at 12k rpm, RT in microfuge for 20 seconds. The pellet was frozen at -20°C and resuspended in 300µl Sonication buffer or Buffer B. The cells were lysed by sonication at 50 W for 3 min with intermittently cooling on ice. The supernatant (soluble protein in Sonication buffer and insoluble protein in Buffer B) was obtained by centrifuging the crude lysate at 12k rpm, 4°C in microfuge for 15 min.

Large scale of extraction was performed to purify the recombinant protein from the insoluble fraction according to the manufacturer's instructions (QIAGEN). 500 ml of cell culture was harvested by centrifugation at 4000 rpm, 4°C in JA14 for 12 min. The cell pellet was resuspended in 1:5 (w/v) Sonication buffer, and frozen at -20°C. The frozen cell suspension was thawed on ice and lysed by sonication at 50 W for 3 min with intermittently cooling on ice. The soluble protein was removed by centrifugation at 10K rpm, 4°C in JA20 for 15 min. The pellet was resuspended in Buffer B (pH 8.0), and then the supernatant containing insoluble fraction was obtained by centrifugation at 10K rpm, 4°C in JA20 for 10 min.

## 9.2 Affinity purification of recombinant Mne-1 protein

10 ml supernatant containing the insoluble fraction was mixed with 0.5 ml of Ni<sup>2+</sup>-nitrilotriacetic acid (Ni-NTA) resin, and stirred at room temperature for 45 min, allowing recombinant Mne-1 protein with a six-histidine tag to bind to the resin. Then, the resin was loaded into a 1-cm diameter column, and washed with Buffer B (pH 8.0) and Buffer C (same as Buffer B except for pH 6.3), respectively to remove unbound protein until A<sub>280</sub> of the effluent was < 0.01. The recombinant protein was eluted with Buffer C containing 250 mM imidazole.

## 9.3 Quantification of protein

Protein concentration of samples from *E. coli* was determined by BCA\* protein assay reagent (PIERCE), and BSA was used as standard. Briefly, 0.5 ml of Coomassie protein assay reagent was added into 0.5 ml of diluted protein solution, and mixed briefly by vortex. Then, absorbance at 595 nm was measured within one hour. Using the standard curve, the sample concentration was determined.

## 9.4 Protein gel electrophoresis

The protein gel electrophoresis was performed using the method described by Bollag and Edelstein (1991). Briefly, each protein sample (approximately 20 µg) was mixed with 5X protein loading buffer, heated at 94°C for 7 min to denature protein, and then chilled on ice. After that, the samples were spun shortly, loaded on 12.5% SDS-polyacrylamide gels and run at 200V.

## 9.5 Transfer of protein to nitrocellulose membrane

After gel electrophoresis, gels were soaked in transfer solution, and then placed into a transfer cassette (Bio-Rad). The protein in the gel was transferred to 0.45 µm nitrocellulose membranes (Bio-Rad) at 100V with cooling in a Bio-Rad electroblot apparatus for one hour.

## 9.6 Protein immunoassay

Protein immunoassay was carried out according to the procedure described by Bollag and Edelstein (1991). After transfer, the membrane was blocked with 5% non-fat dry milk powder in phosphate buffered saline (PBS) for one hour, and rinsed with PBS twice. Then the membranes were incubated in 1:500 dilution of primary antibody with PBS containing 1% (w/v) BSA at RT overnight. After that, the membrane was washed three times with TPBS (PBS containing 0.05% Tween 20) and twice with PBS, respectively. For detection of the primary antibody-antigen reaction, the membrane was incubated in 1:5000 dilution of goat anti-rabbit IgG (H+L) alkaline phosphatase conjugate (EIA grade, Bio-Rad) with PBS buffer containing 1% (w/v) BSA at RT for 3 hours, followed by washing the membranes three times with TPBS, twice with PBS and three times with Tris buffered saline (TBS), respectively. For colour development, the membrane was immersed in alkaline phosphatase buffer for 5 min, followed by pouring off the alkaline phosphatase buffer. The membrane was incubated at RT in 10 ml alkaline phosphatase buffer containing 66  $\mu$ l of 50 mg/ml p-nitro blue tetrazolium chloride in 70% dimethylformamide (NBT solution) and 33  $\mu$ l of 50 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100% dimethylformamide (BCIP solution) until dark purple bands appeared. Then, the incubation was stopped by rinsing the membrane with TBS containing 20 mM EDTA.

## Appendix D

### Buffers, stock solutions and media

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#### 1 Buffers and stock solutions

**Acrylamide stock solution:** 30% (w/v) acrylamide, 0.8% bis-acrylamide.

**Alkaline phosphatase buffer:** 0.1 M Tris·HCl (pH 9.5), 0.1 M NaCl, 5 mM MgCl<sub>2</sub>.

**Coomassie gel stain solution** (per litre): 1 g Coomassie blue R-250, 450 ml methanol, 450 ml H<sub>2</sub>O, 100 ml glacial acetic acid.

**Coomassie gel destain solution** (per litre): 100 ml methanol, 100 ml glacial acetic acid, 800 ml H<sub>2</sub>O.

**DEAE-cellulose DE52 LB suspension:** 100 g Whatman DE52 was suspended in 500 ml 0.05 M HCl. Then, 2 M NaOH was slowly added until pH 7.5. The resin was washed several times with LB until pH reached equilibrium. Finally, the resin was made to about 75% (v/v) with LB.

**Denhardt's III:** 2% gelatin, 2% Ficoll, 2% polyvinyl pyrrolidone, 10% SDS, 5% tetrasodium pyrophosphate.

**Denaturing solution:** 1.5 M NaCl, 0.5 M NaOH.

**DNA extraction buffer:** 1% sarkosyl, 0.1 M Tris·HCl (pH 8.5), 0.1 M NaCl, 0.01 M Na<sub>2</sub>EDTA.

**10X DNA sample loading buffer:** 100 mM Tris·HCl, 200 mM Na<sub>2</sub>EDTA, 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% Ficoll type 4000.

**5X HSB:** 3 M NaCl, 0.1 M PIPES, 25 mM Na<sub>2</sub>EDTA, pH 6.8.

**5X MMLV reverse transcriptase buffer:** 250 mM Tris·HCl (pH 8.3), 375 mM KCl, 15 mM MgCl<sub>2</sub>, 50 mM DTT (DTT was stored separately as a 100 mM stock).

**10X Calf intestinal alkaline phosphatase buffer:** 500 mM Tris·HCl, 10 mM MgCl<sub>2</sub>, 1 mM ZnCl<sub>2</sub>, 10 mM spermidine, pH 9.0.

**10X DNase buffer:** 20 mM Tris·HCl, 5 mM MgCl<sub>2</sub>, 10 mM DTT, pH 7.6.

**10X MOPS Buffer:** 500 mM MOPS (pH 7.0), 10 mM Na<sub>2</sub>EDTA.

**Neutralising solution:** 1.5 M NaCl, 0.5 M Tris·HCl, 0.01 M Na<sub>2</sub>EDTA, pH 7.0.

**10X PCR buffer:** 500 mM KCl, 200 mM Tris-HCl, 25 mM MgCl<sub>2</sub>, 1 mg/ml BSA, pH 8.4.

**10X PBS** (phosphate buffered saline, pH 7.5): 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>.

**Phage precipitating buffer:** 20% PEG, 2 M NaCl, 5 mM Tris·HCl (pH 7.5), 0.01% gelatine, 10 mM MgSO<sub>4</sub>.

**Phenol/chloroform/iso-amyl alcohol (25:24:1):** redistilled phenol was saturated with 0.5 M Tris·HCl (pH 8.0) and then mixed with chloroform and iso-amyl alcohol.

**Protein gel electrophoresis buffer:** 25 mM Tris, 192 mM glycine, 0.1 % SDS, pH around 8.3.

**5X Protein sample buffer:** 60 mM Tris·HCl (pH 6.8), 25% glycerol, 2% SDS, 14.4 mM 2-mercaptoethanol, 0.1% bromophenol blue.

**Protein transfer buffer:** 25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.05 % SDS, pH 8.1-8.3.

**R40:** 40 µg DNase-free RNase A/ml 1X TE buffer.

**RNA extraction buffer:** 0.2 M Tris, 0.1 M KCl, 0.025 M EGTA, 0.035 M MgCl<sub>2</sub>, pH adjusted to 9.0 with KOH, 1% Triton X-100, 0.5% sodium deoxycholate, 1 mM spermidine, 5 mM β-mercaptoethanol, 0.5 M sucrose (Cox and Goldberg, 1988).

**10X RNA loading buffer** (500 µl): 250 µl 50% glycerol, 1 mM EDTA, 10 mg bromophenol blue, 249 µl DEP'd H<sub>2</sub>O.

**4X Separating gel buffer:** 1.5 M Tris·HCl (pH 8.8), 0.4% SDS.

**SM buffer:** 0.1 M NaCl, 10 mM MgSO<sub>4</sub>, 5 mM Tris·HCl pH 7.5, 0.01% gelatine.

**Sonication buffer:** 50 mM Na-phosphate pH 7.8, 300 mM NaCl (pH adjusted with NaOH).

**20X SSPE:** 3.6 M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 0.02M Na<sub>2</sub>EDTA, pH 7.4.

**20X SSC:** 3 M NaCl, 0.3 M Na<sub>3</sub>citrate.

**4X Stacking gel buffer:** 0.5 M Tris·HCl (pH 6.8), 0.4% SDS.



**10X TAE buffer:** 400 mM Tris-HCl, 10 mM Na<sub>2</sub>EDTA, pH 8.0 with glacial acetic acid.

**10X TBE buffer:** 1 M Tris base, 10 mM Na<sub>2</sub>EDTA, 0.863 M boric acid (pH 8.3).

**10X TBS (Tris buffered saline):** 0.15 M NaCl, 10 mM Tris-HCl (pH 7.5).

**1X TE buffer:** 10 mM Tris-HCl (pH 8.0), 1 mM Na<sub>2</sub>EDTA.

**TFB buffer:** 10 mM MES (pH 6.3 with KOH), 45 mM MnCl<sub>2</sub>, 100 mM RbCl, 10 mM CaCl<sub>2</sub>, 3 mM hexamine cobalt chloride, sterile filter.

## 2 Media

**LB medium (per litre):** 10 g Bacto-Tryptone, 5 g yeast extract, 10 g NaCl, pH 7.0.

**LB agar:** 15 g agar per litre LB medium.

**SOB medium (per litre):** 20 g Bacto-Tryptone, 5 g yeast extract, 0.6 g NaCl, 0.19 g KCl, 10 mM MgSO<sub>4</sub>, 10 mM MgCl<sub>2</sub>.

**Terrific Broth (per litre):** 12 g Bacto-Tryptone, 24 g yeast extract and 4 ml glycerol were made to 900 ml with H<sub>2</sub>O, autoclaved and then 100 ml of sterile 10X phosphate buffer (0.17 M KH<sub>2</sub>PO<sub>4</sub> and 0.72 M K<sub>2</sub>HPO<sub>4</sub>) was added.

**Top agarose (per litre):** 10 g Bacto-Tryptone, 5 g NaCl, 2.5 g MgSO<sub>4</sub>·7H<sub>2</sub>O.

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