The cloning and characterisation of a multidrug resistance associated protein (MRP) from wheat

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ABSTRACT

Multidrug resistance associated protein (MRP) is the prototype of an ATP Binding Cassette (ABC) transporter subfamily first identified in a drug resistant lung cancer cell line. In plants, MRP homologues mediate ATP-dependent transport of glutathione conjugates and are thought to play a role in the metabolism of herbicides and endogenously-generated toxicants, by sequestering conjugates in the vacuole. MRP homologues have been cloned from the model plant Arabidopsis but little is known about the MRP family in cereals. The aim of this thesis therefore, was to isolate MRP homologues from wheat.

Since MRP is induced in Arabidopsis by a range of xenobiotics, selective inducibility was employed as a tool to facilitate cloning of wheat MRP isoforms. The amino acid sequences of Arabidopsis MRPs were aligned and degenerate primers corresponding to conserved regions were designed. These primers were used in RT-PCR reactions containing total RNA from xenobiotic-treated wheat tissue as a template. A 791 bp fragment obtained was used to screen a cDNA library from xenobiotic-treated wheat shoots. A full-length cDNA containing an ORF of 4416 bp, encoding a protein with 60% amino acid identity to AtMRP3 was obtained and designated TaMRP2. Hydropathy analysis indicated a similar gross topology to other members of the MRP subfamily, with a hydrophobic N-terminal extension and two transmembrane domains separated by two nucleotide binding folds.

Northern analysis indicated that transcript levels of TaMRP2 were induced up to 3-fold by aminotriazole, menadione and phenobarbital. TaMRP2 was expressed in the Saccharomyces cerevisiae mutant, Δycf1, which lacks the tonoplast MRP homologue, YCF1, and was shown to restore resistance to cadmium. It was also shown that expression of TaMRP2 in Δycf1 alleviated the toxic effects of the glutathione S-transferase substrate 1-chloro-2,4-dinitrobenzene. These data suggest that TaMRP2 is a glutathione conjugate transporter which can also transport cadmium.
ACKNOWLEDGEMENTS

My first thank-you must go to Freddie. What can I say, you’ve taught me so much...what shops I should be buying my shoes from, if in doubt eat chocolate and a gin and tonic saves all! Seriously though, I wouldn’t have got through the past 3 years without your help and enthusiasm, you deserve this PhD nearly as much as I do.

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<th>Full Form</th>
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<tr>
<td>ATP</td>
<td>Adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>Base pairs</td>
</tr>
<tr>
<td>Bluo-gal</td>
<td>5-bromo-indolyl-ß-D-galactopyranoside</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BTP</td>
<td>Bis-Tris propane</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
</tr>
<tr>
<td>CDNB</td>
<td>1-chloro-2,4-dinitrobenzene</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic fibrosis transmembrane conductance regulator</td>
</tr>
<tr>
<td>cps</td>
<td>Counts per second</td>
</tr>
<tr>
<td>DES</td>
<td>Drosophila expression system</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNP-GS</td>
<td>S-(2,4-dinitrophenyl) glutathione</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced Chemiluminescence</td>
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<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
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<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic</td>
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<tr>
<td>EGTA</td>
<td>Ethyleneglycol-bis(ß-aminoethyl ether) N,N,N',N'-tetraacetic acid</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbant assay</td>
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<td>EST</td>
<td>Expressed sequence tag</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>g</td>
<td>Grams</td>
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<tr>
<td>GCG</td>
<td>Genetics Computer Group</td>
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<td>IAA</td>
<td>Isoamyl alcohol</td>
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<tr>
<td>IPTG</td>
<td>Isopropyl β-D-thiogalactopyranoside</td>
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<tr>
<td>IUB</td>
<td>International Union of Biochemists</td>
</tr>
<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
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<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistance</td>
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<tr>
<td>MES</td>
<td>2-(N-morpholino)-ethane sulphonic acid</td>
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<tr>
<td>MOPS</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<td>MRP</td>
<td>Multidrug resistance associated protein</td>
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<td>MSD</td>
<td>Membrane spanning domain</td>
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<td>NaOAC</td>
<td>Sodium acetate</td>
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<td>NaPi</td>
<td>Sodium phosphate</td>
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<td>nm</td>
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<td>Nucleotide binding fold</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Polymerase chain reaction</td>
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<td>pfu</td>
<td>Plaque forming units</td>
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<td>PIPES</td>
<td>Piperazine-N,N'-bis[2-ethanesulphonic acid]</td>
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<td>Polyvinyl pyrrolidone</td>
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<td>RNA</td>
<td>Ribonucleic acid</td>
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<td>RPM</td>
<td>Revolutions per minute</td>
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<td>Reverse transcriptase PCR</td>
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<td>S. cerevisiae</td>
<td><em>Saccharomyces cerevisiae</em></td>
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<td>SDS</td>
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<td>Serum free media</td>
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<td>w/v</td>
<td>Weight/volume</td>
</tr>
<tr>
<td>utr</td>
<td>Untranslated region</td>
</tr>
<tr>
<td>YAP1</td>
<td>Yeast activation protein 1</td>
</tr>
<tr>
<td>YCF1</td>
<td>Yeast cadmium factor 1</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION

1.1 ABC transporters; an overview
The ATP binding cassette (ABC) family is one of the largest protein families known and appears to be present in all organisms (Henikoff, et al., 1997). To date, several hundred clones have been identified, for example: 79 ABC protein genes have been identified in the complete genome of E. coli (Linton and Higgins, 1998), and completion of the Saccharomyces cerevisiae and Arabidopsis thaliana genomes has revealed 29 and 129 ABC proteins respectively (Decottignies and Goffeau, 1997; Sánchez-Fernández, et al., 2001). Most, but not all ABC proteins have a transport function. The first ABC transporters to be characterised were the periplasmic permeases of bacteria, which are unusual in that they require an additional periplasmic binding protein (Ames, 1986). Generally, however, ABC transporters do not require a periplasmic component.

A survey of various ABC transporters indicates that they are capable of transporting a wide range of substrates including: amino acids, sugars, inorganic ions, alkaloids, lipids, heavy metal chelates and peptides. Some, such as bacterial amino acid and sugar permeases are specific for single substrates, whereas others are capable of transporting a wide range of substrates, for example P-glycoprotein can transport diverse lipophilic compounds (Rea, et al., 1998).
Certain ABC transporters can act as uptake systems, whilst others export substrates from the cell.

Classification of the ABC superfamily is complex. Subfamilies clearly exist, however a systematic convention for their naming has yet to be adopted. From recent analyses it would appear that there are 7 mammalian ABC protein subfamilies (Dean, et al., 2001), 13 from Arabidopsis (Sánchez-Fernández, et al., 2001) and 6 in S. cerevisiae (Rogers, et al., 2001).

1.1.1 ABC transporter organisation: motifs, domains and modular construction

ABC transporters are composed of two major conserved domains: nucleotide binding folds (NBF), and transmembrane-bound regions, also known as multiple spanning domains (MSD). The NBFs are peripherally located at the cytoplasmic face of the membrane, binding ATP and coupling its hydrolysis to the transport process (Higgins, 1992). The NBF houses the highly conserved Walker A region, Walker B region and an ABC signature (or C motif), and whilst the Walker regions are common to other nucleotide-binding proteins the C motif is unique to the ABC transporters. Approximately 200 amino acids in length, the C motif typically shares 30-40 % identity between different subfamily members (Higgins, 1992).

The organisation of ABC protein domains varies between organisms, although a functional transporter is thought to consist of four domains (2 NBF and 2 MSD), and these may be encoded by single or separate genes (figure 1.1). Prokaryotic and organellar ABC protein domains are generally expressed as separate polypeptides, for example the oligopeptide permease of Salmonella typhimurium has 2 separate hydrophobic membrane-spanning domains (Opp B and Opp C), 2 separate peripheral membrane domains (Opp D and Opp F), and an additional domain that is not part of the core transmembrane translocation mechanism (Hiles, et al., 1987). The ABC protein domains of eukaryotic organisms are generally fused in various combinations to form larger multifunctional polypeptides, for example all four domains of P-glycoprotein are fused; this is also the case for the cystic fibrosis transmembrane conductance regulator.
(CFTR) gene product (Higgins, 1992). In so-called ‘half’ ABC transporters, two domains are encoded in a single gene, for example, TAP1 and TAP2 proteins are involved in antigen presentation (Spies, et al., 1990), and the white, brown and scarlet genes of Drosophila are involved in the transport of tryptophan and guanine (Ewart, et al., 1994). Each contains 1 NBF and 1 MSD and are thought to function as dimers.

In addition to the 4 core domains, some ABC transporters have additional domains that appear to have regulatory or other peripheral functions, for example the cystic fibrosis gene product CFTR (Riordan, et al., 1989) and the multidrug resistance associated protein gene product (Deeley and Cole, 1997) contain a regulatory or connector domain and MRPs have a unique hydrophobic N-terminal extension termed MSD₀.

1.2 ABC transporters and multidrug resistance

1.2.1 P-glycoprotein

The phenomenon of multidrug resistance has been characterised in tumour cells as the simultaneous acquisition of cross-resistance to a wide variety of dissimilar drugs. Multidrug resistance is therefore of great importance in the treatment of cancers since it can limit the efficacy of chemotherapy treatments. The initial approach to understanding the mechanism of multidrug resistance was to study lines of cultured cells selected for resistance to various anticancer drugs (Gottesman and Pastan, 1993). Studies on drug resistant mouse, Chinese hamster and human cells showed that resistance was due to the removal of the drug from the cell via an energy-dependent drug transport protein (Sugimoto and Tsuruo, 1991, Beck, et al., 1979; Shen, et al., 1986). Physiological and pharmacological studies of mutant cell lines indicated that the mechanism of resistance was due to a decrease in drug accumulation within the cell (Kessel, et al., 1968) by either increased efflux out of or decreased influx into the cell (Dano, 1973). Biochemical studies of multidrug-resistant cell lines indicated that alterations of specific glycosylated proteins were associated with resistance (Beck, et al., 1979; Shen, et al., 1986) and since these proteins were apparently unique to cells exhibiting altered drug permeability, they were designated P-glycoproteins (Juliano and Ling, 1976).
1.2.2 Multidrug resistance associated protein (MRP)

The overexpression of P-glycoprotein is responsible for numerous cases of multidrug resistance, however, drug resistance in certain human lung tumours is not correlated with P-glycoprotein overexpression, which indicates alternative mechanisms of drug resistance (Cole, et al., 1992). In 1992, Cole and co-workers cloned the first multidrug-resistance associated protein gene from human small cell lung cancer cells which was later shown to encode a plasma membrane drug efflux pump (Zaman, et al., 1994). This ATP-dependent export pump was shown to have a broad substrate specificity, being competent in the transport of glutathione, glucuronate and sulphate conjugates of drugs (Hollo, et al., 1996; Kool, et al., 1997; Leier, et al., 1994; Muller, et al., 1994; Zaman, et al., 1995); and also unaltered, lipophilic, cytotoxic drugs for example: anthracyclines, vincristine, and etoposide (Paul, et al., 1997).

1.2.3 Microbial drug transporters

Drug resistance is also well documented in microbes, but it is clear that ABC subfamilies distinct from P-glycoprotein and MRP are involved. Two yeast ABC transporters have been particularly well-studied: pleiotropic drug resistance 5 (PDR5) and SNQ2. In common with some other ABC transporters, PDR5 confers resistance to a number of drugs including cycloheximide and sulfometuron methyl (Balzi, et al., 1994; Leppert, et al., 1990; Meyers, et al., 1992) and SNQ2 confers resistance to 4-nitroquinoline-N-oxide (Servos, et al., 1993). Unlike P-glycoprotein, PDR5 and SNQ2 do not belong to the multidrug resistance (MDR) subfamily, but form a separate group the PDR subfamily according to the Saier classification (Saier, 2000). The classification of yeast ABC transporters varies between authors, however the International Union of the Biochemical and Molecular Biology Societies is at present considering endorsing the transporter classification system developed by Milton Saier. Studies on S. cerevisiae have shown that PDR5 encodes a 160-kDa protein, with a predicted duplicated six transmembrane-spanning domain, and a repeated putative ATP-binding domain homologous to the mammalian P-glycoprotein (MDR1; Balzi, et al., 1994). PDR5 and SNQ2 share similar domain organisations and a highly similar primary structure in the ABC regions. Distinctly they both display an
NBF1-MSD1-NBF2-MSD2 orientation, rather than an MSD1-NBF1-NBF2-
MSD2 orientation as displayed by other ABC transporters including: MDR,
STE6 and CFTR. Both PDR5 and SNQ2 have been shown to be targeted to the
plasma membrane (Bauer, et al., 1999). Other members of the PDR group
include PDR12, a weak acid transporter, and PDR10, PDR15 and YOR011, the
specific functions of which are undetermined however, given the amino acid
sequence homology between the later of these proteins and PDR5, it is possible
that they too may have an association with multidrug resistance (Rogers, et al.,
2001). Aside from one isolated report (Smart and Fleming, 1996), very little is
known about the functions of the PDR subfamily in plants.

Bacteria also exhibit the drug resistance phenotype which is significant in
antibiotic resistance (George, 1996; Ouellette and Kundig, 1997; van Veen and
Konings, 1998). One example of a bacterial ABC transporter involved in
multidrug transport that has been experimentally characterised is LmrA from
Lactococcus lactis (van Veen, et al., 1998). LmrA is a close relation of the
mammalian P-glycoprotein and can in fact substitute for P-glycoprotein when
transfected in human lung fibroblast cells to retain a similar drug specificity (van
Veen, et al., 1998). A significant difference between P-glycoprotein and LmrA is
that whereas P-glycoprotein is a full sized ABC transporter i.e. its four domains
are fused together as a single polypeptide, LmrA is a half sized transporter
consisting of one MSD and one NBF that function as a homodimer (van Veen,
et al., 2000). Similar to yeast ABC transporters, bacterial ABC transporters provide
excellent model systems for studying the function, structure and mechanisms of
ABC transporters belonging to higher eukaryotics.

1.3 P-glycoprotein
1.3.1 Multidrug resistance genes encode P-glycoproteins
Molecular studies in the 1980s demonstrated that P-glycoprotein is encoded by
the MDR gene, and belonged to the ABC protein family. In 1985, Riordan and
co-workers cloned a P-glycoprotein complementary DNA (cDNA) from a highly
multidrug-resistant cell line. The cDNA was subsequently used as a probe for the
analysis of hamster, mouse and human DNA, and it was shown that P-glycoprotein is conserved between different mammals. Members of this putative gene family were amplified in cells exhibiting the MDR phenotype. In 1986, Gros and co-workers isolated a member of this gene family from multidrug resistant hamster cell lines (LZ and C5), and showed that its overexpression in drug sensitive hamster cells conferred drug resistance (Ueda, et al., 1986). MDR genes were subsequently cloned in human (MDRI and MDR3; Bliek, et al., 1988; Chen, et al., 1986; Gros, et al., 1986b), mouse (mdr1, 2 and 3; Gros, et al., 1986a; Gros, et al., 1986b), rat (mdr 1, 2 and 3; Silverman, et al., 1991), and hamster (Ng, et al., 1989). However, not all of these genes conferred the multidrug resistance phenotype; transfection studies indicated that P-glycoproteins can be separated into two groups: those that confer the MDR phenotype and those that exhibit other phenotypes. The group of P-glycoproteins that can confer directly drug resistance to drug sensitive cells include human MDR1, and mouse mdr1 and mdr3 (Devault and Gros, 1990; Gros, et al., 1986a; Gros, et al., 1986b; Ueda, et al., 1987).

In addition to the multidrug resistance phenotype of P-glycoproteins, it seemed likely that they would have a physiological role in non-drug resistant cells, and this idea was supported by the expression patterns of P-glycoprotein in these cells. In 1993, Smit and co-workers created a mouse mdr2 mutant, which lacked phosphatidylcholine (the major phospholipid of bile), and it was later shown that mouse mdr2 was a phosphatidylcholine transporter (Ruetz and Gros, 1994a). Similarly, human MDR3 transports phosphatidylcholine and drug transport has yet to be demonstrated (Helvoort, et al., 1996). Interestingly the drug transporter human MDR1 can also transport a variety of short-chain lipids across the plasma membrane (Helvoort, et al., 1996). An additional function of P-glycoprotein is its channel activity: the overexpression of P-glycoprotein is associated with volume-activated channel activity and it seems likely that P-glycoprotein regulates this activity. The association of P-glycoprotein with channel activity is discussed in 1.3.6 (iv), and has been recently reviewed by Idriss and co-workers (2000).
1.3.2 P-glycoproteins are membrane ATPases

The ATPase activity of P-glycoprotein has been demonstrated in plasma membrane vesicles and reconstituted protein preparations from multidrug-resistant cells. The anti-cancer drug vinblastine was shown to be transported into plasma membrane vesicles prepared from multidrug-resistant cells, but not from drug-sensitive cells, and that the transport activities were dependent on a constant energy source (Horio, et al., 1988; Horio, et al., 1991; Lelong, et al., 1992). It is perhaps of interest that although ATP is the preferred energy source, GTP and ITP are hydrolysed and to a certain extent can substitute for ATP; non-hydrolysable analogues of ATP cannot (Lelong, et al., 1992). The first attempts to purify P-glycoprotein and demonstrate multidrug resistance were of limited success (Hamada and Tsuruo, 1988); ATPase activity was minimal and not drug-dependent. In this work, P-glycoproteins were isolated and assayed in the presence of the detergent CHAPS, and it was subsequently proposed that CHAPS inhibits drug-stimulated ATPase activity (Sarkadi, et al., 1992). However, Doige and co-workers (1993) suggest that this is unlikely, and that CHAPS results not in the loss of catalytic activity but in the partial uncoupling of the ATPase activity from drug binding. In addition, ATPases other than P-glycoprotein may have been present in the sample thus contributing to the drug-independent ATPase activity observed. Expression of P-glycoprotein in transfected SF9 insect cells (Sarkadi, et al., 1992), or MDR hamster cells (Alshawi, et al., 1994), has shown significant ATPase activity in multidrug-resistant cells. Activity was also demonstrated when partially-purified P-glycoprotein was reconstituted into proteoliposomes (Ambudkar, et al., 1992); for optimal ATPase activity to occur, the hydrophobic transmembrane domains require a lipid environment (Doige, et al., 1993). In summary, it is now established that P-glycoprotein binds ATP, and ATPase activity is essential for drug transport across the membrane.

1.3.3 Post translational modification

P-glycoprotein is post-translationally modified by glycosylation and phosphorylation. Glycosylation is not required for protein function, but appears to be involved in protein stability and routing (Schinkel, et al., 1993). Recent drug transport studies on wild type and mutated P-glycoproteins, showed that the phosphorylation of the linker region has no significant effect on the rates of drug
transport (Goodfellow, et al., 1996), but it does regulate channel activities associated with P-glycoproteins (Gill, et al., 1992) (see below).

1.3.4 Sequence analysis and membrane topology of P-glycoprotein
The ORF of the human MDR1 gene (Chen, et al., 1986) encodes a 1280 amino acid protein. Analysis of the DNA and predicted peptide sequences (Chen, et al., 1986; Gottesman and Pastan, 1993; Gros, et al., 1986b), and experimental data (Yoshimura, et al., 1989; Zhang and Ling, 1991) suggest that P-glycoprotein consists of two homologous halves, each containing 6 transmembrane domains and a large cytoplasmic loop containing ATP binding sites. The cytoplasmic region of the protein houses the Walker A and B, and the ABC signature motifs. Alternative topological models for P-glycoprotein have been suggested over the years, for example Zhang and Ling (1991), proposed that P-glycoprotein had 10 transmembrane spans based on the identification of a glycosylated region within the C-terminus of P-glycoprotein. However, photoaffinity labelling has shown that there was no apparent glycosylated region within the C-terminus of P-glycoprotein capable of binding drugs (Bruggemann, et al., 1992; Bruggemann, et al., 1989). The basic $6 + 6 \alpha$ helical model remains current and is supported by secondary structure predictions and experimental data including cysteine-scanning mutagenesis and epitope mapping (Georges, et al., 1993; Kast, et al., 1995; Loo and Clarke, 1995; Loo and Clarke, 1996). Alternative models are also supported by this data albeit to varying degrees; the majority of these models are adaptations of the $6 + 6 \alpha$ helical model, only differing in the number of transmembrane spans (for review see Jones and George, 1999). There is however an exception; an interesting, perhaps radical double-$\beta$-barrel model: which proposes that each half of P-glycoprotein contains 16 $\beta$ stands which form a barrel-like structure, and these provide P-glycoprotein with 2 membrane channels (Jones and George, 1998). The double-$\beta$-barrel model appears to accommodate most of the major experimental data available.

The first experimental insight into the three dimensional architecture of an ABC transporter was obtained by electron microscopy and image analysis of detergent-solubilised and lipid-reconstituted P-glycoprotein (Rosenberg, et al.,
1997). Consistent with biochemical data, the derived model was cylindrical in shape incorporating a large aqueous pore open at the extracellular face of the membrane and closed at the cytoplasmic face. It was speculated that the presence of two ‘thumbs’ represented two MSDs and that the lobes on each thumb reflect the predicted 12 transmembrane spans, each separated by extracellular loops and 2 NBFs. The data were however obtained in the absence of ATP and since P-glycoprotein undergoes conformational changes in the presence of ATP and substrate, an altered conformation of the MSDs is possible.

The recent crystallisation of MsbA, a half-sized transporter from E. coli, which is closely related to mammalian P-glycoprotein, has shown that this protein is organised as a homodimer and each subunit contains 6 α helical transmembrane spans and a NBF (Chang and Roth, 2001). Perhaps an unusual finding for a P-glycoprotein-like transporter was that MsbA contained an additional intracellular domain (ICD) that connected TM6 to the NBF; the significance of this region is undetermined, perhaps it is similar to the regulatory domain found in CFTR and MRP. In addition, the structure of MsbA did not appear to form a pore through the membrane but instead formed a ‘molecular machine’ that flips substrates from one side of the membrane to the other (1.3.6 (i)). Whether the structure of MsbA is representative of full-sized ABC transporters can only be determined by further experimentation.

1.3.5 Substrate specificity and binding

P-glycoprotein is capable of recognising a wide variety of substrates that include amphipathic drugs and short chain lipids (table 1.1). There are exceptions including human MDR3, and mouse mdr2, which are specific for phosphatidylcholine. Site-directed mutagenesis and protein mapping studies have indicated that drug binding sites occur within the transmembrane spans especially MSD 5, 6, 11 and 12 and it is possible that these interactions occur within the lipid bilayer (Sharom, et al., 1996). In addition, photoaffinity labelling of drugs has shown that it is possible that P-glycoprotein has two non-identical drug binding sites and this is perhaps significant since P-glycoproteins have a wide substrate specificity (Dey, et al., 1997; Gottesman and Pastan, 1993; Sharom, et al., 1996).
Table 1.1: Examples of P-glycoprotein substrates

<table>
<thead>
<tr>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Drug substrates</td>
</tr>
<tr>
<td>Anthracyclines: e.g. daunorubicin, doxorubicin</td>
</tr>
<tr>
<td>Anthracenes: e.g. bisantrene, mitoxantrone</td>
</tr>
<tr>
<td>Antibiotics: e.g. actinomycin D</td>
</tr>
<tr>
<td>Vinca alkaloids: e.g. vinblastine, vincristine</td>
</tr>
<tr>
<td>Epipodophyllotoxins: e.g. VP-16</td>
</tr>
<tr>
<td>Fluorophores: e.g. rhodamine 123</td>
</tr>
<tr>
<td>Others: e.g. taxol, topotecan</td>
</tr>
<tr>
<td>(B) Lipids</td>
</tr>
<tr>
<td>Phosphatidylcholine</td>
</tr>
<tr>
<td>C6-NBD-phosphatidylcholine</td>
</tr>
<tr>
<td>C6-NBD-phosphatidylethanolamine</td>
</tr>
<tr>
<td>C6-NBD sphingomyelin</td>
</tr>
<tr>
<td>C8-C8-glucosylceramide</td>
</tr>
<tr>
<td>(c) Antagonists</td>
</tr>
<tr>
<td>Calcium channel blockers: e.g. verapamil, azidopine</td>
</tr>
<tr>
<td>Calmodulin antagonists: e.g. Trifluoperazine, chlorpromazine</td>
</tr>
<tr>
<td>Antiarrhythmics: e.g. quinidine, amiodarone</td>
</tr>
<tr>
<td>Antihypertensives: e.g. reserpine, propranolol</td>
</tr>
<tr>
<td>Antibiotics: e.g. hydrophobic cephalosporins</td>
</tr>
<tr>
<td>Hormones: e.g. tirilazad, progesterone</td>
</tr>
<tr>
<td>Detergents: e.g. Tween-80</td>
</tr>
</tbody>
</table>

1.3.6 Models of action

(i) Protein pumps and ‘Flippase’

The most conventional model for P-glycoprotein substrate transport is a protein pump whereby the substrate is transported across the membrane energised by ATP hydrolysis. However, an alternative ‘flippase’ model was proposed by Higgins and Gottesman (1992). Rather than the substrate binding directly to P-glycoprotein, they suggested that it interacted with the lipid bilayer and gained access to the membrane bound P-glycoprotein in this way. The substrate is then ‘flipped’ from the inner to the outer bilayer, with ATP hydrolysis driving the associated conformational change in the protein (Higgins, 1994). Examples of this include human MDR1, mouse MDR3 and LmrA from *Lactococcus lactis* which transport short-chain lipids, phosphatidylcholine and phosphatidylethanolamine respectively across the plasma membrane via the flippase mechanism (Helvoort, *et al.*, 1996; Margolles, *et al.*, 1999; Smith, *et al.*, 1994). The flippase model goes some way to explaining the broad substrate specificity of P-glycoprotein: there is no precise structural requirement for substrates, other than that they are amphipathic. MsbA is a half-sized transporter from *E.coli* sharing greatest homology with human MDR1 (Chang and Roth, 2001). The recent crystallisation of MsbA supports the flippase model, showing that this transporter at least does not form a pore through the membrane but rather constitutes a ‘molecular machine’ which scans the lower bilayer for substrates, accepts them laterally and flips them to the outer membrane (Chang and Roth, 2001).

(ii) Tilting and rotational models

Despite the evidence to support the flippase model, especially the crystallisation of MsbA, inconsistencies in a variety of data imply that other models are equally viable. Low resolution analysis of the structures of P-glycoprotein and MRP1, although similar to that of MsbA, show that whereas the chamber of MsbA is closed on the extracellular face of the membrane, the chambers of P-glycoprotein and MRP1 appear to be open (Chang and Roth, 2001; Rosenberg, *et al.*, 1997; Rosenberg, *et al.*, 2001). Secondly, ABC transporters recognise both hydrophobic and hydrophilic substrates and the flippase model does not fully explain the transport of the hydrophilic substrates. Perhaps P-glycoprotein is
similar to the ABC transporter cystic fibrosis transmembrane conductance regulator and in addition to the flippase mechanism forms an aqueous channel through the membrane (1.3.6 (iv)) Two alternative models for the transport of substrates across the membrane that apply to the ABC transporter family in general include the tilting and rotation model (Higgins and Linton, 2001). Briefly, the tilting model proposes that the substrate enters the chamber formed by the transporter from the inner leaflet of the lipid bilayer and following a conformational change in the transporter, the chamber opens to the extracellular aqueous medium (closing to the intracellular lipid medium) and the substrate is released. A variation of this model suggests that the chamber does not open to the extracellular aqueous medium but instead the chamber re-orientates and the substrate is directed into the outer leaflet of the bilayer. The rotational model proposes that the chamber is continuously open to the extracellular medium and when the substrate binds to the transporter on the inner leaflet of the lipid bilayer, the transporter re-orientates releasing the substrate into the aqueous chamber, or the substrate partitions back into the outer leaflet of the bilayer. These models cannot explain the mechanisms of all ABC transporters, the tilting model for example was based on MsbA and does not reflect what is known about P-glycoprotein or human MRP1. Indeed, it is possible that specific members of the ABC transporter family may function by mechanisms specific to their subclass.

(iii) Two-cylinder model
The two-cylinder engine model or two-site model is based on the drug transport of the Lactococcus LmrA transporter (van Veen, et al., 2000). It was proposed that the transporter had two binding sites (van Veen, et al., 1998), accessible from both sides of the membrane: substrate binding results in a conformational change of the MSD which is coupled to ATP hydrolysis, releasing substrate on the other side of the membrane. A clear requirement of this mechanism is that both NBFs are required to hydrolyse ATP and this is achieved by an alternating catalytic cycle (Senior, et al., 1995).

(iv) Channels
In addition to a role in drug and lipid transport, P-glycoproteins have recently been associated with chloride channel activities. It has been demonstrated that
the expression of P-glycoprotein in cell lines that do not normally exhibit volume-regulated chloride channel activity, generated volume-regulated chloride currents (Valverde, et al., 1992), which suggests that P-glycoprotein is either a chloride channel or regulates other channel activities (Gill, et al., 1992). Subsequent studies have demonstrated the P-glycoprotein is indeed active as a channel regulator (Hardy, et al., 1995). Interestingly, the cystic fibrosis transmembrane conductance regulator (CFTR), another class of ABC protein is itself a cAMP-regulated chloride channel (Davis, et al., 1996).

1.3.7 P-glycoprotein homologues in plants
A recent inventory of Arabidopsis ABC proteins has revealed a total of 129 proteins which can be categorised into 12 subfamilies (table 1.2; Sánchez-Fernández, et al., 2001). Whilst the function of the majority of these proteins remains unknown, some like AtPGP1 and AtMRP1-3 can transport xenobiotics and toxic metabolites from the cytoplasm (see below; Rea, et al., 1998).

In 1992, Dudler and Hertig set out to isolate potential MDR-genes from Arabidopsis thaliana to determine their involvement, if any, in the cross-resistance to different herbicides. Many of the drugs transported by P-glycoproteins were derived from plants, so it seemed plausible that they may be the natural substrates of plant ABC transporters. Their work lead to the cloning of the first multidrug-resistance-like gene, Arabidopsis P-glycoprotein (AtPGP1). Since then, one additional Arabidopsis P-glycoprotein (AtPGP2; Dudler and Sidler, 1998) has been cloned; given that there are 22 MDR genes in Arabidopsis, understanding of this subfamily is incomplete. MDR homologues have also been cloned from potato (PMDRI; Wang, et al., 1996) and barley (HVMDR2; Davies, et al., 1997). Sequence comparisons of plant and mammalian MDR proteins indicate close sequence homology especially within the NBF. Since AtPGP1 shared the highest sequence similarity with mammalian P-glycoprotein it seemed likely that it may be involved in the removal of xenobiotics and xenobiotic metabolites from the cell. Studies with transgenic plants have shown that AtPGP1 is a plasma membrane protein and its overexpression confers increased cycloheximide resistance (Thomas, et al.,
In addition, analysis of transgenic plants showed that AtPGP1 is involved in light-dependent hypocotyl elongation (Sidler, et al., 1998).

The remaining part of this chapter will focus on the MRP subfamily, which is the subject of this thesis. Further information regarding plant ABC transporters is detailed in two comprehensive reviews; Rea (1998); Theodoulou (2000).

Table 1.2 Summary of Arabidopsis ABC protein subfamilies

<table>
<thead>
<tr>
<th>Subfamily</th>
<th>Number of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole molecules</td>
<td></td>
</tr>
<tr>
<td>Multidrug resistance/P-glycoprotein (MDR/P-gp)</td>
<td>22</td>
</tr>
<tr>
<td>Multidrug resistance associated protein (MRP)</td>
<td>15</td>
</tr>
<tr>
<td>Pleiotropic drug resistance (PDR)</td>
<td>13</td>
</tr>
<tr>
<td>ABC1 homologue (AOH)</td>
<td>1</td>
</tr>
<tr>
<td>Peroxisomal membrane protein (PMP)</td>
<td>1</td>
</tr>
<tr>
<td>Half Molecules</td>
<td></td>
</tr>
<tr>
<td>White-brown complex homologues (WBC)</td>
<td>29</td>
</tr>
<tr>
<td>ABC2 homologues (ATH)</td>
<td>16</td>
</tr>
<tr>
<td>ABC transporter of the mitochondria (ATM)</td>
<td>3</td>
</tr>
<tr>
<td>Transporter associated with antigen processing (TAP)</td>
<td>2</td>
</tr>
<tr>
<td>Peroxisomal membrane protein (PMP)</td>
<td>1</td>
</tr>
<tr>
<td>Soluble proteins</td>
<td></td>
</tr>
<tr>
<td>RNase L inhibitor (RIL)</td>
<td>2</td>
</tr>
<tr>
<td>General control non-repressible (GCN)</td>
<td>5</td>
</tr>
<tr>
<td>Structural maintenance of chromosomes (SMC)</td>
<td>4</td>
</tr>
<tr>
<td>Non-intrinsic ABC protein (NAP)*</td>
<td>15</td>
</tr>
</tbody>
</table>

* Heterogeneous group of soluble proteins, not classified as an Arabidopsis subfamily by Sánchez-Fernández and co-workers.

1.4 MRP-subclass ABC transporters

1.4.1 Human MRPs

P-glycoprotein overexpression is responsible for many cases of multidrug resistance, however, drug resistance is not always correlated with P-glycoprotein overexpression, indicating alternative mechanisms of drug resistance. The

P-glycoprotein and human MRP1 have distinct, but overlapping substrate specificities (figure 1.2). In the cases where both transporters accept the same substrate, P-glycoprotein transports the unmodified compound, whereas human MRP1 transports compounds which are conjugated to reduced glutathione (GSH), glucuronate or sulphate (Hollo, et al., 1996; Kool, et al., 1997; Leier, et al., 1994a; Muller, et al., 1994; Zaman, et al., 1995), or co-transport un conjugated drugs with GSH (Paul, et al., 1997). P-glycoprotein has a low affinity for these negatively charged compounds (Borst, et al., 2000).

To date, 8 human MRP isoforms have been isolated and have been systematically termed MRP1-8. However, some of these MRPs are known by other names, related to their function (Bera, et al., 2001; Borst, et al., 1999; Borst, et al., 2000). Human MRP2 for example, was originally characterised as the canalicular multispecific organic anion transporter (cMOAT), a liver specific MRP-like glutathione conjugate pump (GS-X) isolated from TR[-hyperbilirubinemic mutant] rats (Oude Elferink, et al., 1991; Paulusma, et al., 1996). Sequence comparisons between MRP1 and MRP2/cMOAT have shown that they are 48 % identical (Borst, et al., 2000), and they also have similar substrate specificities, however their localisation and expression patterns are different (Konig, et al., 1999; Kool, et al., 1997; Paulusma, et al., 1996). Human MRP3, 4 and 5 have been termed MOAT-D, MOAT-B and MOAT-C respectively and like Human MRP1 can transport a wide range of substrates. Localisation and expression patterns vary suggesting that although these transporters are very similar, each MRP is likely to have a specific function. Comparatively less is known about human MRP6-8 but it seems likely that they too play a role in multidrug resistance.
1.4.2 MRP Structure and Topology

The basic structure of ABC transporters was outlined in an earlier section (1.1.1), however it is important to discuss the points that distinguish the MRP subgroup from other ABC transporters. Although the 190 kDa protein human MRPl and the 170 kDa P-glycoprotein share only 15 % sequence homology, the order of domains is conserved: MSD1-NBF1-MSD2-NBF2. Human MRPl has 5 additional transmembrane spans attached to the N-terminal (MSD₀), and an MRP-specific feature is the regulatory or R domain of 200 residues which is rich in charged amino acids.

Although the exact topology of MSD 1 and 2 remains uncertain, several predictions have been made. The first model was based on the work of Cole and co-workers (1992). Unlike the MDR/P-glycoprotein which was predicted to have 6 + 6 transmembrane helices (Gros, et al., 1986b), it proposed that human MRPl had 8 + 4 transmembrane helices and that the C- and N- termini were located within the cytosol. This initial prediction was based on sequence analysis and there is now a growing body of experimental data to challenge this model, although the cytosolic location of the C-terminus remains current. Monoclonal antibodies raised against epitopes within NBF1 and NBF2 of human MRPl bind to immunoblots or permeabilized cells but not to intact cells, suggesting that the NBFs and the C-terminus of human MRPl are cytosolic (Flens, et al., 1994; Hipfner, et al., 1996). The N-terminus of human MRP was shown by site-directed mutatagensis of its N-glycosylation site to be extracellular. The precise number of transmembrane spans within each MSD remains uncertain, current models predict a 5 + 6 + 6 topology and accordingly the recent two-dimensional crystallisation and single particle analysis of human MRPl has confirmed that there are indeed 5 transmembrane spans in MSD₀, and suggested that MSD1 and MSD2 each contained 6 transmembrane spans. At present there are no three-dimensional structures available for any MRPs however with the recent crystallisation of the bacterial ABC-half transporter (Chang and Roth, 2001), it appears only a matter of time until MRP is successfully crystallised.
1.4.3 Yeast MRPs

The yeast *Saccharomyces cerevisiae* has 6 MRP subfamily members (Decottignies and Goffeau, 1997; Taglicht and Michaelis, 1998) and of these the yeast cadmium factor 1 gene (*YCF1*) is the best characterised (Szczypka, *et al.*, 1994). As the name suggests, *YCF1* confers resistance to cadmium and studies with tonoplast vesicles have indicated that it encodes a vacuolar GS-X pump involved in the transport of organic glutathione conjugates, and bis(glutathionato)cadmium (CdGS₂; Li, *et al.*, 1997a; Li, *et al.*, 1996; Tommasini, *et al.*, 1996). The deletion of the *YCF1* gene from *S. cerevisiae* results in hypersensitivity to cadmium and prevents the transport of both Cd'GS₂ and DNP-GS. Transforming the same strain with human *MRPL* reinstates cadmium resistance and the transport of DNP-GS suggesting that the functions of *MRPL* and *YCF1* are closely related (Tommasini, *et al.*, 1996). Whereas *YCF1* is predominantly found on the vacuolar membrane, MRP is also located on other internal membranes, when expressed in yeast (Tommasini, *et al.*, 1996), however overexpression of membrane proteins can result in mis-localisation (d'Exaerde, *et al.*, 1996), and protein targeting in mammals and yeast is likely to differ.

More recent studies have shown that *YCF1* is also competent in the transport of other substrates including the glutathione conjugate leukotriene C4 (LTC₄) and metalloids: glutathione chelates (Falcon-Perez, *et al.*, 1999; Ghosh, *et al.*, 1999). Bilirubin is transported independently of glutathione providing the first evidence that *YCF1* can transport unconjugated substrates (Petrovic, *et al.*, 2000). In addition, there is evidence to suggest that *YCF1* transports GSH albeit at low affinity suggesting that *YCF1* might participate in glutathione efflux from the cytoplasm (Rebbeor, *et al.*, 1998). During nitrogen starvation in *S. cerevisiae*, GSH accumulates in the vacuole where it is further degraded, possibly providing the cell with amino acids required for growth; links between *YCF1* and vacuolar GSH transport under nitrogen starvation have yet to be established (Mehdi and Penninckx, 1997).

The remaining members of the yeast MRP subfamily are: BAT1, BPT1, YOR1, YKR and YHL035 (Decottignies and Goffeau, 1997; Taglicht and Michaelis,
1998). BAT1 (bile acid transporter) and BPT1 (bile pigment transporter) are both localised on the yeast vacuolar membrane where they transport taurocholate and bilirubin respectively (Ortiz, et al., 1997; Petrovic, et al., 2000). YOR1 (yeast oligomycin resistance) confers resistance to oligomycin and a range of organic anions however, unlike the other yeast MRPs characterised to date, it is located on the yeast plasma membrane (Cui, et al., 1996; Katzmann, et al., 1995). The location and function of YKR and YHL035 have yet to be determined. It should be noted that according to the Saier transporter classification system, YOR1 does not belong to the same family as MRP1, BAT1, BPT1, and YHL035 (YKR is not classified) however, previous classifications have always included YOR1 in the MRP-subfamily and it was therefore discussed here.

1.4.4 Xenobiotic detoxification and the vacuolar GS-conjugate pump
Detoxification of xenobiotics occurs in three main phases: phase I (conversion/transformation), phase II (conjugation) and phase III (deposition/compartmentalisation) (reviewed in Ishikawa, et al., 1997; Sandermann, 1992). During phase I, xenobiotics may be oxidised, hydrolysed or reduced to reveal or introduce a functional group, and these reactions are catalysed by cytochrome P450s, amidases, mixed function oxidases or esterases. However, some xenobiotics contain active functional groups and do not require phase I modification. During phase II, the reactive xenobiotic or activated derivative is covalently linked to an endogenous hydrophilic molecule such as glutathione (GSH), glucuronate or sulphate in animals, or GSH, malonate or glucose in plants. Conjugation is catalysed by the action of specific transferases, and the conjugated molecule is generally, but not always less toxic. Although all phase II reactions are important, glutathione conjugation has been best-studied in plants, since it is a predominant mechanism for herbicide metabolism. In the third phase of xenobiotic detoxification, the conjugates are transported from the cytosol, to the extracellular medium (mammals) or vacuole or apoplast (plants). It is possible that the conjugation partner added in phase II may serve as a recognition 'tag' for the transporter. There is a fourth phase in xenobiotic detoxification where the transported conjugates are inactivated by further degradation and substitution, for example, in plants, vacuolar carboxypeptidase catalyses the
removal of the glycine residue of glutathione conjugate complexes, but relatively little is known of this (Wolf, et al., 1996).

1.4.5 Transport of glutathione conjugates in plants
The detoxification of xenobiotics via conjugation to glutathione is one of the most studied mechanisms of detoxification. Glutathione acts as a nucleophile, binding to the electrophilic xenobiotics and modifying them so they become less- or even non-toxic. This process can occur spontaneously at pH values greater than 7, but in most cases the reaction is catalysed by glutathione-S-transferases (GST), located in the cytosol in plants. It is essential that once the GS-conjugates are formed they are removed from the cytosol as they can inhibit GST activity or even be modified by cytosolic enzymes to form toxic metabolites (Coleman, et al., 1997a).

In mammalian systems, glutathione conjugates are transported out of the cell into the extracellular medium, however in plants, glutathione conjugates are transported into the vacuole. Prior to the discovery of the GS-X pump, most solutes were thought to be transported into the vacuole by secondary active processes, energised by the vacuolar H⁺-ATPase (V-ATPase) and the H⁺-pyrophosphatase (V-PPase). Both V-ATPase and V-PPase catalyse the electrogenic translocation of H⁺ from the cytosol to the vacuole lumen to generate an inside-acid pH difference (ΔpH) and an inside-positive electrochemical potential difference (Rea and Poole, 1993), the energy stored in these gradients can be used to drive transport.

In 1993, Martinoia and co-workers demonstrated that transport of glutathione conjugates into barley mesophyll vacuoles was energised by MgATP and that free ATP or nonhydrolysable analogues could not be used as substitutes: for uptake to occur, the γ-phosphate of MgATP must be hydrolysed. Uptake was shown to be insensitive to V-ATPase inhibitors (bafilomycin and KNO₃), agents which dissipate the proton gradient (e.g. gramicidin-D) and to valinomycin which dissipates the inside positive membrane potential. GS-X transport was, however, inhibited by vanadate, an agent which has no effect on tonoplast H⁺
pumps but is known to be an inhibitor of ABC transporters and P-type ATPases. These findings were supported by Rea and co-workers, who studied GS-X uptake into tonoplast vesicles isolated from a range of plant species (Li et al. 1995a). Taken together, these data rule out secondary active transport of glutathione conjugates. Indeed, the pharmacological properties of vacuolar GS-X transport were very similar to those of the GS-X pump in the canalicular membrane of liver (Akerboom, et al., 1991, Ishikawa, 1992, Kobayashi, et al., 1990) and it was proposed that the transporter was an ABC protein.

### 1.4.6 Additional MgATP-dependent transporters in the vacuolar membrane

Isolated vacuoles have also been used to study the MgATP-dependent uptake of a range of other organic compounds, including: the *Brassica napus* nonfluorescent chlorophyll catabolite (*Bn*-NCC-1; Hinder, et al., 1996), the disulfonated 4-aminophthalimide, lucifer yellow CH (LYCH; Klein, et al., 1997) and the bile salt, taurocholate (Hortensteiner, et al., 1993). *Bn*-NCC-1 is transported at high affinity across the tonoplast of vacuoles isolated from barley mesophyll protoplasts and in addition to its requirement for an MgATP energy source, in common with the vacuolar GS-X pump, it is insensitive to agents that dissipate the inside-positive membrane potential, and is inhibited by vanadate. It has since been shown that *AtMRP2* encodes a GS-X pump competent in the high affinity transport of *Bn*-NCC-1 (Lu, et al., 1998).

The transport of LYCH into rye vacuoles is also energised by MgATP, and similar to transport of GS-X, LYCH transport is insensitive to inhibitors of the V-ATPase and V-PPase, and sensitive to vanadate. Whereas sulphated compounds strongly inhibited the uptake of LYCH, glutathione and glutathione conjugate had only a weak to moderate effect, and it was suggested that LYCH uptake is not mediated via the GS-X pump (Klein, et al., 1997). The recent kinetic data for *AtMRP2* has shown that the simultaneous transport of structurally dissimilar compounds is possible, therefore further experimental data are required to unequivocally prove the molecular identity of the LYCH transporter.
Taurocholate was shown to be taken up into barley vacuoles by an ATP-dependent process, but since plants do not contain cholate and its conjugates, the function of this transport activity is unknown (Hortensteiner, *et al.*, 1993). The identity of this transporter is different to that of the GS-X pump since taurocholate uptake was only slightly inhibited by oligomycin, a compound which strongly inhibits glutathione conjugate transport. The uptake of N-ethylmaleimide-glutathione (NEM-GS) and taurocholate was examined in the presence of several inhibitors and substrates. It was shown that there were differences between the compounds that caused inhibition suggesting that two different transport mechanisms were involved. These proteins were subsequently termed plant multispecific organic anion transporters (pMOATs; BlakeKalff and Coleman, 1996). The molecular identity of these transporter remains unknown. (Hinder, *et al.*, 1996 and Klein, *et al.*, 1997).

1.4.7 The MRP family in *Arabidopsis*: overview

At the beginning of this project 5 AtMRPs had been cloned, only 4 of which had been characterised (AtMRP1-4; Lu *et al.*, 1997; Lu *et al.*, 1998; Tommasini *et al.*, 1997; Tommasini *et al.*, 1998; Sánchez-Fernández *et al.*, 1998, Marin *et al.*, 1998). During the course of this study, AtMRP5 was characterised and an inventory of *Arabidopsis* ABC proteins has shown that there are 15 AtMRPs in total (see appendix I; Sánchez-Fernández, *et al.*, 2001).

*AtMRP1* and 2 have been isolated both as full-length cDNAs and genomic clones, and mapped to chromosome 1. Heterologous expression in yeast has shown that they encode MgATP-dependent GS-X pumps, which can transport xenobiotic GS-conjugates and endogenous substrates (Lu, *et al.*, 1998; Lu, *et al.*, 1997, Liu, *et al.*, 2001). Expression of *AtMRP1* and 2 transcripts revealed that the steady state levels of *AtMRP2* in root, leaf, stem and flower were considerably lower than those of *AtMRP1*. Whereas the expression of *AtMRP1* was similar in each of the tissues examined, the expression patterns of *AtMRP2* in these tissues varied. These findings remain to be explained. Recent studies have shown that AtMRP2 is localised within the *Arabidopsis* vacuolar membrane and also, when expressed in the ScYCF1 mutant cells AtMRP2 is targeted to the vacuolar membrane. This finding is significant, since it provides the first direct
experimental evidence that in plants a functional GS-X pump encoded by an MRP is located on the vacuolar membrane.

A full-length cDNA clone of \textit{AtMRP3} has been isolated and expressed in yeast (Tommasini, \textit{et al.}, 1998) and similar to \textit{AtMRP1} and 2 it encodes a GS-X pump (Tommasini, \textit{et al.}, 1993). The fourth member of this subfamily, \textit{AtMRP4} has been isolated as a genomic clone and shares most sequence similarity with \textit{AtMRP3} (Sánchez-Fernández, \textit{et al.}, 1998), indeed, phylogenetic analysis of the MRP and CFTR family members indicate that \textit{AtMRP3} and \textit{AtMRP4} belong to the same sub-division of this family. Martinoia and co-workers have shown that \textit{AtMRP4} encodes a GS-X pump (pers. comm). Recent studies on \textit{AtMRP5} have shown that, in common with \textit{AtMRP1-4}, it encodes a GS-X pump and in yeast exhibits transport activities typical for MRPs (Gaedeke, \textit{et al.}, 2001). In addition a mutant plant containing a T-DNA insertion in \textit{AtMRP5} was identified, and a phenotype determined (see section 1.4.9).

Functional comparisons between the \textit{AtMRPs} have indicated differences in their transport capabilities and substrate specificities. For example the transformation of the \textit{YCF1} mutant with \textit{AtMRP1-5} restores GS-conjugate transport, however \textit{AtMRP3-5} are capable of conferring cadmium resistance but \textit{AtMRP1} and 2 cannot (Gaedeke, \textit{et al.}, 2001; Lu, \textit{et al.}, 1998; Lu, \textit{et al.}, 1997; Martinoia, pers. comm.).

\textbf{1.4.8 Functional comparisons between the \textit{AtMRP1} and 2}

The transport activities of \textit{AtMRP1} and \textit{AtMRP2} have been studied extensively but comparable data from \textit{AtMRP3-5} is not available and therefore the main focus of this discussion will be \textit{AtMRP1} and 2. The affinities of \textit{AtMRP1} and 2 for glutathione conjugates are very similar (Km values between 60-75 \textmu M) however, the transport capacity of \textit{AtMRP2} greatly exceeds that of \textit{AtMRP1} and the affinity for oxidised glutathione (GSSG) differs (Lu, \textit{et al.}, 1998). It is interesting to note the substrate preference of the two \textit{AtMRPs}: \textit{AtMRP1}: cyanidin-3-glucoside-GS(C3G-GS) \textgreater metolachlor-GS \textgreater S-(2,4-dinitrophenyl) glutathione (DNP-GS) \textgreater GSSG. \textit{AtMRP2}: metolachlor-GS\textgreater C3G-GS\textgreater GSSG\textgreater DNP-GS. The substrates of \textit{AtMRP1} fall into the same order as the
substrates for mung bean vacuolar membrane vesicles (Li, et al., 1995a; Lu, et al., 1997); AtMRP2 is clearly very different. An additional substrate examined was the non-glutathionated chlorophyll catabolite of Brassica napus (Bn-NCC-1) a substrate for which AtMRP2 has a high affinity and transports at a high capacity, AtMRP1 transports Bn-NCC-1 at a low capacity.

Heterologously expressed AtMRP2 is also competent in the transport of E$_2$17βG and DNP-GS can stimulate its uptake (Liu, et al., 2001). The converse of this phenomenon is also true: E$_2$17βG can stimulate the AtMRP2-mediated uptake of DNP-GS. The effects of other GS-conjugates on AtMRP2-mediated uptake of E$_2$17βG were also examined but their effects were marginal and in some cases inhibitory. It was also shown that the uptake of E$_2$17βG and DNP-GS was simultaneous however counter-transport or trans-activation appeared unlikely because preloading experiments did not result in enhanced uptake, and the concentration of DNP-GS required for the half-maximal promotion of E$_2$17βG was 1-2 times lower than required for the half-maximal transport of DNP-GS alone. Furthermore, interactions between glutathione and E$_2$17βG were non-reciprocal; glutathione stimulated the transport E$_2$17βG but E$_2$17βG did not promote the transport of glutathione. Counter-transport and transactivation would only be possible if the interactions were reciprocal given that glutathione is a known co-transported species of MRP (Rappa, et al., 1997; Rebbeor, et al., 1998). It was concluded that different substrates are transported by distinct yet tightly coupled binding sites. The reciprocal activation of DNP-GS and E$_2$17βG transport is a phenomenon unique to AtMRP2 to date. Although AtMRP1, YCF1 and Human MRP3 can all catalyse the transport of E$_2$17βG and DNP-GS individually, they do not catalyse DNP-GS stimulated transport of E$_2$17βG (Liu, et al., 2001). These results further support the idea that individual MRPs have specific functions.

1.4.9 Functional capabilities of AtMRP5
AtMRP5, in common with AtMRP1-4, encodes a GS-X pump however it exhibited a low transport capacity for the GS-conjugates and glucuronides tested, suggesting that it may have other functions (Gaedeke, et al., 2001).
Arabidopsis plant with a T-DNA insertion in AtMRP5 (mrp5-1) was identified and the plant exhibited a decrease in root growth and an increase in lateral root formation. This phenotype is characteristic of an increase in the free auxin levels within a plant, and indeed the roots of mrp5-1 had increased auxin levels suggesting that AtMRP5 may function as an auxin conjugate transporter by directly transporting the conjugate or indirectly by altering ion uptake. Previous studies have indicated that a sulfonylurea receptor (SUR) and/or CFTR-like ABC protein which binds specific sulfonylureas may be involved in controlling stomatal opening/closing (Leonhardt, et al., 1999). AtMRP5 is sensitive to the sulfonylurea glibenclamide and mrp1-5 is insensitive, and since promoter-GUS studies have shown that AtMRP5 is expressed in the guard cells it would suggest that AtMRP5 may either control channel activity or act as a channel itself in stomata (Gaedeke, et al., 2001).

1.4.10 Mechanisms of transport

Section 1.3.6 details some of the mechanisms proposed for ABC transporter function; how these mechanisms relate to MRP is questionable. It would appear from the literature that the well-established flippase model is favoured for the transport of substrates via MRP; there is supporting experimental data but this model does not account for the pore identified in the structure of human MRP1 (Rosenberg, et al., 2001). Three additional models were described in section 1.3.6: the tilting model, the rotational model and the two-cylinder engine model, and of these only the two latter mechanisms are applicable to MRP. The tilting model is based on a transporter whose pore is closed to the extracellular medium; two-dimensional crystallisation and single-particle analysis of MRP indicated that the putative pore is open to the extracellular medium. The rotational and two-cylinder models are both equally applicable to MRP however, the latter of these models has greater experimental support. The transport mechanism proposed for AtMRP2 suggests that different substrates can be transported via semi-autonomous pathways (Liu, et al., 2001), an idea consistent with the two-cylinder model. At present however neither the rotational or the two-cylinder model can be ruled out.
Two additional MRP subfamily members operate as ion channels. The rabbit epithelial basolateral chloride conductance regulator (EBCR/MRP2), is 82% identical to human MRP2 and exhibits chloride channel conductance activities (Kuijck, et al., 1996), however, rabbit EBCR/MRP2 is not linked to glutathione transport or multidrug resistance. The second MRP subfamily member that exhibits channel activity is the cystic fibrosis transmembrane conductance regulator (CFTR; 1.3.6 (iv); Davis, et al., 1996), and more recently AtMRP5 has been shown to have a putative role in channel activity (Gaedeke, et al., 2001).

Several mechanisms of transport have been suggested however, it is not currently known which mechanism(s) applies to the MRP family.

1.4.11 Chemical induction of the AtMRPs

Several studies have shown that plant MRPs are differentially induced by xenobiotics (Sánchez-Fernández, et al., 1998; Tommasini, et al., 1998). Expression of AtMRPs was studied in Arabidopsis cell suspensions treated with sublethal doses of stress agents that were known to increase GSH levels (Sánchez-Fernández, et al., 1998). Aminotriazole, which leads to mild oxidative stress through the inhibition of catalases induced AtMRP3, and AtMRP4 was induced by menadione, a chemical which causes oxidative stress through the generation of superoxide (Sánchez-Fernández, et al., 1998). AtMRP1 was not significantly induced by any of the treatments and AtMRP2 was not examined. These findings suggest that AtMRP3 and AtMRP4, and even members of the AtMRP family not yet characterised, may play a role in defending cells against oxidative stress.

Further studies were carried out using herbicide safeners. Safeners are defined as chemicals that reduce the phytotoxicity of another chemical, for example herbicides, when used together (Cobb, 1992), and are employed to improve the tolerance of herbicides that show limited selectivity on crops, and improve those already available. Certain safeners have been shown to act by stimulating the metabolism of herbicides in crops, for example by increasing GST activity (Fuerst and Gronwald, 1986; Wiegand, et al., 1986). They may therefore also induce MRPs.
Biochemical studies have shown that the wheat safener, cloquintocet-mexyl increases the activity of the GS-X pump in barley (Gaillard, et al., 1994). Induction studies subsequently indicated that AtMRP3 was significantly (4-fold) induced by benoxacor and oxibetrinil, and was only moderately affected by clonquintocet, fenchlorozole, and flurazole. AtMRP1 and AtMRP4 were moderately affected by all of the safeners except oxabetrinil (Sánchez-Fernández, et al., 1998). It is apparent from the data outlined above, that AtMRP3 is the most inducible of the AtMRP isoforms characterised to date.

1.4.12 Transport of natural substrates; MRP and oxidative stress

The protein pumps encoded by MRP genes transport a wide range of substrates, yet these pumps did not evolve to transport metabolites of herbicides which have only been used systematically in the past century. It is likely that their primary function is to remove toxins, which have always been present in the environment or generated as a consequence of metabolism for example under oxidative stress. The main cause of oxidative damage in cells is the excess production of active oxygen species (AOS) which include superoxide (O$_{2}^•$), hydrogen peroxide (H$_2$O$_2$) and hydroxyl radicals (OH$_•$). Although H$_2$O$_2$ and O$_2^•$ can themselves be damaging, it is usually their conversion to OH$_•$ that then readily reacts with proteins, lipids, and DNA to cause severe cell damage (Inzé and Van Montagu, 1995). The cell has several defence mechanisms against AOS including the antioxidant glutathione that both binds to electrophilic products and reduces toxic compounds to less toxic metabolites, an action that is often catalysed by GSTs acting as peroxidases (Coleman, et al., 1997b). Oxidised glutathione (GSSG) is the product of antioxidant reactions. Under normal conditions GSH is regenerated by the action of glutathione reductase, however when under attack from AOS, the production of GSSG is accelerated (e.g. Akerboom, et al., 1982) and can be transported out of the cytoplasm by both plant and mammalian MRPs (Leier, et al., 1996; Lu, et al., 1998), therefore maintaining the redox status of the cell.
During oxidative stress, lipids can undergo peroxidation resulting in the formation of highly toxic fatty acid hydroperoxides which can be degraded to yield the corresponding hydroxide and GSSG. They can also be conjugated to glutathione (Vollenweider, 1999). It has recently been demonstrated that the glutathione conjugate derived from linoleic acid hydroperoxide is readily transported into isolated barley vacuoles (F Theodoulou and E Martinoia, unpublished results). Pharmacological characterisation suggests the GS-X pump as a likely candidate transporter. It seems likely that the transport of naturally occurring substrates including GSSG may be an ancestral function of the GS-X pump.

One additional observation concerns the involvement of GSTs and GS-X pumps in the formation and export of base propenals and hydroxyalkenals. Hydroxyalkenals are products of lipid peroxidation and in mammalian systems they have been shown to act as substrates for GSTs and the GS-X pump (Ishikawa, 1989; Ishikawa, et al., 1986; Ketterer, 1998). DNA peroxidation results in the formation of base-propenals that can be conjugated by GSTs to glutathione suggesting that they may serve as substrates for MRPs (Ketterer, 1998). These observations have yet to be made in plant systems, however Coleman and co-workers 1997a have shown that alkyl-s derivatives competitively inhibit the uptake of NEM-GS and taurocholate by vacuolar MRP-like transporters in barley.

1.4.13 Transport of natural substrates; MRP and secondary metabolites

Plants contain an array of naturally occurring compounds and there is evidence to suggest that MRPs can transport some of these compounds, such as anthocyanins and chlorophyll catabolites, out of the cytoplasm. In maize, the Bronze-2 (bz2) gene is involved in the last genetically defined step of the anthocyanin pathway. In wild-type plants, anthocyanins accumulate in the vacuole as purple or red derivatives. In Bronze-2 (bz2) mutant plants however, the anthocyanin precursor cyanidin-3-glucoside (C3G) accumulates in the cytosol where it is subjected to oxidation resulting in the generation of brown derivatives (Marrs, et al., 1995). It has been shown that bz2 encodes a type III GST, leading to the suggestion that C3G is conjugated to glutathione, prior to transport into the vacuole. Protoplasts
isolated from wild-type plants treated with vanadate resulted in the accumulation of C3G in the cytoplasm; this result suggested that glutathione conjugates of C3G (C3G-GS) were transported into the vacuole via a vanadate-inhibitable GS-X pump. In support of this, Li and co-workers (1995a,b) have shown that C3G-GS is transported by a MgATP-dependent mechanism into vacuolar membrane vesicles isolated from maize roots and mung bean hypocotyls, and more recently Lu and co-workers (1998) reported that C3G-GS transport is attributable to AtMRP1 and AtMRP2. However, the conjugate used in the transport experiments was not prepared using Bz2 and Walbot and co-workers (1998) have been unable to demonstrate conjugation of C3G by An9, the petunia analogue of Bz2 (Alfentio, et al., 1998). It appears that GSTs play an alternative role in anthocyanin sequestration: it has recently been shown that An9 acts as an anthocyanin binding protein, and might serve to deliver anthocyanin precursors to the vacuolar transporter (Mueller, et al., 2000).

One further endogenous substrate for MRP is the phytoalexin medicarpin. Phytoalexins play a key role in the defence of leguminous plants against pathogens, and the glutathione conjugate of medicarpin is readily taken up into the tonoplast vesicles isolated from etiolated hypocotyls of mung bean (Li, et al., 1997b). Similar to the transport of other GS-X conjugates, medicarpin-GS uptake is MgATP-dependent, vanadate sensitive and uncoupler insensitive. It was therefore suggested that GS-X pumps may play a role in the vacuolar storage of antimicrobial compounds in healthy plant cells surrounding the hypersensitive lesion (Li, et al., 1997b).

The transport of chlorophyll catabolites has been discussed previously (1.4.9). Briefly, Bn-NCC-1 is transported into barley vacuoles via an MgATP-dependent, vanadate-sensitive mechanism (Hinder, et al., 1996), and it has subsequently been shown that AtMRP2 is a competent Bn-NCC-1 transporter (Lu, et al., 1998). Interestingly, Bn-NCC-1 is a malonyl conjugate but it appears that malonylation is not essential for transport since Bn-NCC-2, a glucosylated derivative is transported at rates similar to those exhibited by Bn-NCC-1 (Hinder, et al., 1996).
1.5 Rationale and aims

The world's agrochemical market is growing at a rate of 6.6% per annum, herbicides in particular are valued at $15,050 million, however herbicide resistance is threatening this market. Furthermore, there is increasing political pressure to reduce the application of agrochemicals. An increased understanding in plant-herbicide interactions may help to overcome the problems encountered; studies have shown that the GS-X pumps are involved in the transport of herbicide metabolites and some of the genes which regulate their expression have been identified and termed MRP. Although MRPs have been characterised in Arabidopsis, with the exception of a partial MRP cDNA from wheat (Theodoulou, et al., 1998) they have yet to be characterised in an agronomically important species. The aim of this project was therefore to clone further members of the wheat MRP subfamily, examine their expression and characterise function.
Figure 1.1: Domain organisations of ABC proteins

(\text{MSD-NBF})_2: P-gp^1, ST6^2

\begin{center}
\includegraphics[width=0.5\textwidth]{domain1.png}
\end{center}

\begin{itemize}
\item MSD_2-(MSD-NBF)_2: MRP^4, YCF1^4, CFTR^5
\end{itemize}

\begin{center}
\includegraphics[width=0.5\textwidth]{domain2.png}
\end{center}

(\text{MSD-NBF}): ATMI^6, MDLI^7, TAPI^8

\begin{center}
\includegraphics[width=0.5\textwidth]{domain3.png}
\end{center}

(\text{NBF-NBF}): GNC20^9

\begin{center}
\includegraphics[width=0.5\textwidth]{domain4.png}
\end{center}

(\text{NBF-MSD})_2: PDR5^10, SNQ2^11

\begin{center}
\includegraphics[width=0.5\textwidth]{domain5.png}
\end{center}

(\text{NBF-MSD}): WBC^12, ADP1^13, ABC5^14

\begin{center}
\includegraphics[width=0.5\textwidth]{domain6.png}
\end{center}

\text{NBF only: NAP}^15

\begin{center}
\includegraphics[width=0.5\textwidth]{domain7.png}
\end{center}

The different domains are colour-coded: red, multiple spanning domain (MSD), blue MSD1-2, and white nucleotide binding fold 1-2 (NBF1-2). Examples for each organisation are given and references are given as superscripts above the gene names: 1, (Gottesman and Pastan 1993); 2, (Kuchler, et al., 1989); 3, (Cole, et al., 1992); 4, (Szczypka, et al., 1994); 5, (Davis, et al., 1996); 6, (Kispal, et al., 1999); 7, (Decottignies and Goffeau 1997); 8, TAPI (Reits, et al., 2000); 9, (Dealdana, et al., 1995); 10, (Balzi, et al., 1994); 11, (Servos, et al., 1993); 12, (Ewart, et al., 1994); 13, (Decottignies and Goffeau 1997); 14, (Berge, et al., 2000); 15, (Decottignies and Goffeau 1997). Figure not to scale. See text for further details.
Examples of substrates specific to P-glycoprotein or MRP, and those common to both. MRP commonly transports conjugated substrates, or co-transport substrates in the presence of reduced glutathione (indicated in red font) (After Litman, et al., 2001; König and et al., 1999 and references therein).
CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals
Chemicals were purchased from Sigma or Riedel-de Haën and were of analytical grade or higher. Radiochemicals were obtained from ICN and Amersham Pharmacia Biotech.

2.2 Oligonucleotide Primers
Oligonucleotide primers were synthesised by Cruachem and MWG Biotech.

2.3 Plant material

2.3.1 Growth of wheat in vermiculite
*Triticum aestivum* (cv Paragon, PBI) seeds were grown in moist vermiculite under controlled conditions (day: 20 °C, 16 h light, night: 16 °C) for a period of 8 d. Wheat leaves were harvested after 8 d of growth and stored under liquid nitrogen.

2.3.2 Hydroponic growth of wheat
A hydroponic environment was set up to grow wheat for membrane preparations. Prior to growth, wheat seeds (*Triticum aestivum* (cv Paragon, PBI)) were surface sterilised with 10 % (v/v) sodium hypochloride, rinsed well and imbibed in
distilled water with aeration for 3 h. Plastic containers (26 x 26 x 15 cm) [surface-sterilised with 10 % (v/v) sodium hypochlorite and rinsed well], were filled with growth medium (0.5 mM CaSO₄) to a depth of 9 cm, and covered with a mesh lid. A layer of sterile muslin was placed over the mesh lid and ca. 100 g of the prepared wheat seeds were placed on top. The seeds were covered with 2 layers of sterile muslin and a loose aluminium lid. Seeds were grown under controlled conditions (aerated growth media, 24 h darkness, 25 °C) for 7 d.

2.3.3 Chemical treatment of wheat seedlings
Chemicals were applied by watering seedlings at 4 and 6 d with ca. 300 ml of one of the following: 2 mM aminotriazole dissolved in distilled water; 4 mM phenobarbital dissolved in distilled water or 0.1 M menadione dissolved in acetone and then diluted in distilled water to a final concentration of 100 μM.

2.4 E. coli strains
XL1-blue recA1, endA1, gyrA96, thi-1, hdR17, supE44, relA1, lac[F' proAB, lacrZAM15, Tn10(te')] (Stratagene).

DH5α F', φ 80dlacZ ΔM15, recA1, endA1, gyrA96, thi-1, hdR17(ri−, mri+), supE44, relA1, DeoR, Δ(lacZYA-argF) U169 (Obtained from Dr P. Madgwick, Rothamsted).

Y1090r araD139, hdR(ri−, mri+), mcrA−, rslL, supF, trpC22::Tn10, Δ lacU169, Δlon, (pMCp) (Clontech).

Y1090ZL ΔlacU169, proA+, hdR−, hdM+, Δlon, araD139, strA, supF, (trpC22::Tn10) (Life Technologies).

DH10B(ZIP) F mrcA Δ(mrr-hdRMS-mrcBC) φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697 araD139 galU galK nupG rpsL λxis6 ind P ZIP1 (pZIP1 = P1 ori-kanR-cre) (Life Technologies).

TOP10 F mrcA Δ(mrr-hdRMS-mrcBC) φ80dlacZΔM15 ΔlacX74 deoR recA1 araD139 Δ(ara,leu) 7697 galU galK rpsL (StrR) endA nupG ) (Invitrogen).
2.5 Maintenance and manipulation of bacterial strains

2.5.1 Bacterial growth media and antibiotics
Unless specified, *E. coli* were grown in Luria-Bertani (LB) broth (10 g/l Bacto-tryptone, 5 g/l yeast extract, 10 g/l NaCl, pH 7.4). Stock solutions of antibiotics were prepared according to table 2.1. All stock solutions were filter sterilised though a 22 μm filter prior to use, and added to media after autoclaving.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Working conc.</th>
<th>Stock conc.</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>50 μg/ml</td>
<td>50 mg/ml</td>
<td>H₂O, -20 °C</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>10 μg/ml</td>
<td>100 mg/ml</td>
<td>H₂O, -20 °C</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>10 μg/ml</td>
<td>5 mg/ml</td>
<td>Ethanol, -20 °C</td>
</tr>
</tbody>
</table>

2.5.2 Growth of bacterial cultures
Cultures were prepared by inoculating the appropriate medium containing antibiotics with a single colony, and incubated overnight at 37 °C at 250 rpm in an orbital shaker.

2.5.3 Maintenance and storage of bacterial cultures
For short term storage (1-2 month), bacterial cells were maintained on LB agar plates (LB broth set with 15 g/l Bactoagar) sealed with Parafilm and stored upside-down at 4 °C. For long term storage, bacterial cells were stored as glycerol stocks; 0.7 ml of an overnight culture mixed with 0.3 ml of 50 % (v/v) glycerol in sterile cryovials, frozen in liquid nitrogen and stored at -80 °C.

2.5.4 Transformation of *E. coli* using calcium chloride competent cells
Competent *E. coli* cells were prepared using a variation of a method from Dr P. Madgwick. Five ml of 2 x LB broth was inoculated with 5 individual colonies and grown overnight at 37 °C at 250 rpm in an orbital shaker. The overnight culture was used to inoculate 500 ml of 2 x LB broth and grown under the conditions above (2.5.2) to an OD₆₅₀nm of 0.2. The culture was chilled on ice for 10 min and the cells were pelleted by centrifugation at 4,500 g for 10 min at 4 °C. The pellet was resuspended in 250 ml of sterile TF buffer [50 mM CaCl₂, 15 %
(v/v) glycerol, 10 mM piperazine-N,N'-bis[2-ethanesulphonic acid](PIPES), pH 6.6] and incubated on ice for 20 min. The cells were pelleted by centrifugation as described previously, and resuspended in 25 ml of TF buffer. Competent cells were aliquoted into eppendorfs pre-chilled in liquid nitrogen, frozen in liquid nitrogen and stored at -80 °C.

To transform the *E. coli* competent cells, 2 µl of ligation mixture (2.6.6) or 10 to 50 µg of plasmid was added to 20-50 µl of competent cells and incubated on ice for 30 min in the presence of 19 mM β-mercaptoethanol. The cells were then heat shocked at 42 °C for 45 sec and cooled on ice for 2 min. One ml of pre-warmed LB was added to the cells and incubated for 1 h at 37 °C at 250 rpm in an orbital shaker. Cells were pelleted by centrifugation at 1,000 g for 3 min at room temperature, 800 µl of the LB was removed, and the cells were resuspended in the remaining liquid. Transformed cells were then selected by plating onto LB agar containing the appropriate antibiotics and incubated overnight at 37 °C.

### 2.6 Isolation, manipulation and modification of DNA

#### 2.6.1 Isolation of plasmid DNA

Bacterial plasmid DNA was isolated on a mini (10 ml LB culture) or midi (50 ml LB culture) scale, using a Qiagen Mini™ or Midi™ kit according to manufacturers’ instructions.

Alternatively, bacterial plasmid DNA was prepared by alkaline lysis as described by Sambrook *et al.*, (1989). Bacterial plasmid DNA was isolated from a 10 ml overnight culture. Cells were pelleted by centrifugation at 1,500 g for 10 min at room temperature, and resuspended in 0.3 ml of solution I [50 mM glucose, 25 mM Tris (hydroxymethyl) methylamine-hydrochloric acid (Tris-HCl) pH 8, 10 mM ethylenediaminetetraacetic (EDTA), 10 µg/ml RNase, filter sterilised though a 22 µm filter and stored at 4 °C], to which 0.3 ml of freshly prepared solution II [0.2 N NaOH, 1 % (w/v) SDS] was added. Tubes were inverted several times and incubated at room temperature for 5 min. 0.3 ml of solution III (60 ml 5 M potassium acetate, 11.5 ml glacial acetic acid, 28.5 ml sterile distilled water to
give 3 M potassium and 5 M acetate) was added, the tubes inverted several times and incubated on ice for 10 min. The bacterial lysate was centrifuged for 10 min at top speed in a microfuge, the supernatant removed into a fresh tube and centrifuged again to remove any remaining bacterial debris. The supernatant was removed to a fresh tube and precipitated with 0.65 ml of isopropanol at room temperature for 15 min. DNA was recovered by centrifugation at top speed in a microfuge for 15 min. The DNA pellet was washed with 70 % (v/v) ethanol, air-dried and resuspended in the desired volume of TE (10 mM Tris-HCl, pH 8, 1 mM EDTA) or sterile distilled water.

2.6.2 Restriction Digestion
Restriction digestion of DNA for subcloning or analysis was carried out using enzymes and buffers purchased from Promega, Boehringer Mannheim and NEB according to manufacturers’ instructions.

2.6.3 Phenol/chloroform extraction
Equal volumes of phenol:chloroform:isoamyl alcohol (IAA) (25:24:1) and DNA were vortexed thoroughly and centrifuged at 14,000 g for 10 min at room temperature. The aqueous phase was removed to a fresh tube, extracted with an equal volume of chloroform:IAA (24:1) and ethanol precipitated (2.6.4).

2.6.4 Ethanol precipitation of DNA
DNA was precipitated for a minimum of 30 min at -20 °C by adding 0.1 volume of 3 M sodium acetate (NaOAc) pH 5.8 and 2.5 volumes of 100 % ethanol (-20 °C). The DNA was pelleted by centrifugation at 14,000 g for 20 min at 4 °C. The supernatant was removed and the DNA was washed with 70 % (v/v) ethanol and centrifuged as described above for 10 min. The pellet was either air-dried or dried under vacuum and resuspended in an appropriate volume of TE or sterile distilled water.
2.6.5 Dephosphorylation of DNA
To prevent self-ligation of compatible ends of vector DNA during ligation, the 5' ends were dephosphorylated. Following digestion of ca. 5 μg of DNA with the appropriate restriction enzyme, the DNA was extracted with phenol:chloroform:IAA (2.6.3) and ethanol precipitated (2.6.4). The dried DNA pellet was resuspended in 50 μl of TE and quantified (2.7.3). The reaction was buffered with 1 x dephosphorylation buffer to which calf intestine alkaline phosphatase (Roche) was added at 1 unit per 1 pmol of 5' terminal phosphorylated DNA fragments (3' recessed, 5' recessed or blunt-ended), in a final volume of 100 μl. Reactions were incubated at 37 °C for 1 h then extracted with phenol:chloroform:IAA (2.6.3) and ethanol precipitated (2.6.4).

2.6.6 Ligation of DNA fragments
The ligation of DNA fragments was carried out according to manufacturers’ instructions (Promega). In a typical reaction, a 1:3 molar ratio of vector:insert DNA was used mixed with 1 x ligation buffer and 0.1-1 Weiss units of T4 DNA ligase in a total volume of 10 μl. Insert DNA was omitted from control reactions. Reactions were incubated overnight at 16 °C.

2.7 Analysis of DNA

2.7.1 Agarose gel electrophoresis
Agarose gel electrophoresis of DNA samples was carried out as follows: 1 x TAE (Tris-acetate: 0.04 M Tris, 20 mM glacial acetic acid, 0.001 M EDTA), agarose gels containing 1 μg/μl ethidium bromide were run in 1 x TAE buffer. The percentage of agarose was dependent on the expected size of the DNA. DNA samples mixed with 0.1 volumes of gel loading buffer [10 mM Tris-HCl, pH 7.5, 20 mM EDTA, 10 % (v/v) Ficoll (400, Pharmacia) 0.02 % (w/v) bromophenol blue, 0.02 % (w/v) xylene cyanol FF] were separated by electrophoresis at 100 V.

2.7.2 Purification of DNA from agarose gels
A small block of agarose containing the DNA of interest was excised from the gel with a scalpel blade and the DNA was purified using a Prep-A-Gene DNA
purification kit (BioRad) according to manufacturers’ instructions. DNA was eluted in 2 x pellet volume of elution buffer.

2.7.3 Determination of nucleic acid concentration
Nucleic acid concentrations were determined by UV spectroscopy (Carey 3, UV-visible) at 260 and 280 nm. An absorbance of 1 at 260 nm corresponds to approximately 50 µg/ml of double-stranded DNA and 40 µg/ml of single-stranded DNA/RNA. Alternatively, DNA concentration was determined using the DyNAQuant 2000 (Hoefer Pharmacia Biotech) fluorometer according to manufacturers’ instructions.

2.7.4 Sequencing
DNA was sequenced using a Dye Terminator Cycle Sequence, DNA sequencing kit (Perkin-Elmer) and an ABI 373 stretch automated DNA sequencer, according to manufacturers’ instructions. Alternatively, sequencing was performed by Genome Express S.A., Grenoble, France.

2.8 Extraction of RNA

2.8.1 Isolation of plant total RNA: Qiagen RNeasy plant mini kit
RNA was prepared from 8 d old wheat leaf tissue (2.3.1), using a RNeasy plant mini kit (Qiagen) according to manufacturers’ instructions. DNA was removed by digestion with RNase-free DNase: 100 µg of RNA was mixed with 40 mM Tris-HCl, pH 7.4, 6 mM MgCl₂, 2 mM CaCl₂, 40 U RNase block, 15 U RNase free DNase, 143 mM β-mercaptoethanol (final concentrations) and diethyl pyrocarbonate-treated (DEPC) water in a final volume of 100 µl, and incubated at 37 °C for 20 min. The RNA was then purified using RNeasy columns (Qiagen) according to manufacturers’ instructions.

2.8.2 Isolation of plant total RNA: Trizol™ method
RNA was prepared from 8 d old wheat tissue (2.3.1). Approximately 200 mg of tissue was ground in liquid nitrogen in a pre-chilled pestle and mortar. Trizol™ (Gibco) (1.5 ml) was added to the frozen tissue and allowed to melt, with occasional mixing. The tissue/Trizol™ mixture was pipetted into a 2 ml eppendorf tube and vortexed for 45 sec. The cell debris was pelleted by
centrifugation at 12,000 g for 10 min at 4 °C. Chloroform (400 µl) was added to the supernatant, vortexed for 15 sec and incubated at room temperature for 5 min. The aqueous phase was separated by centrifugation at 12,000 g, for 15 min at 4 °C. Isopropanol (0.6 vol) and DEPC-treated 3 M NaOAc (0.3 vol) were added to the aqueous phase and incubated at room temperature for 10 min. RNA was pelleted by centrifugation at 12,000 g for 10 min at 4 °C and washed twice in 70 % (v/v) ethanol. The pellet was air-dried for 10 min at room temperature and resuspended in 50 µl of DEPC-treated water. The integrity of the RNA was determined by gel electrophoresis on a 1.5 % (w/v) agarose/TAE gel (2.7.1).

2.8.3 Preparation of messenger RNA (mRNA)
Messenger RNA was prepared from total RNA (2.8.2) using a mRNA purification kit (Pharmacia Biotech) according to manufacturers’ instructions. The kit is based on the affinity purification of mRNA on oligo(dT)-cellulose spun columns.

2.9 Northern analysis
2.9.1 Electrophoresis and transfer
Samples for electrophoresis were prepared as follows: RNA in a volume of 20 µl was added to 4 µl of 10 x MEN [200 mM 3-(N-morpholino)-propane sulphonic acid (MOPS), 30 mM NaOAc, 10 mM EDTA, pH 7], 6 µl filtered formaldehyde [37 % (w/v)], and 20 µl formamide. The sample was incubated at 56 °C for 30 min and 5 µl of ethidium bromide buffer [100 µl 10 x DNA gel loading buffer: 50 % (v/v) glycerol, 1 mM EDTA 0.2 % (w/v) bromophenol blue, and 8 µl of ethidium bromide (10 mg/ml)] were added. The samples were then loaded into the wells of a 1.5 % (w/v) agarose gel made up with 1 x MEN and 16.7 % (v/v) formaldehyde. The gel tank was filled with 1 x MEN and covered with Clingfilm. The gel was electrophoresed at 40 V for 4 h. The RNA was transferred overnight to nylon membrane (Hybond NX, Amersham) by capillary action in 20 x SSC (173.3 g/l NaCl, 88.2 g/l trisodium citrate, pH 7). The membrane was air-dried and the RNA cross-linked by UV irradiation (Stratagene Stratalinker™ 1800, “autocrosslink” setting).
2.9.2 Hybridisation and autoradiography

The membrane was pre-hybridised for 4 h at 62 °C in 200 μl of hybridisation buffer [200 mM sodium phosphate (NaPi), pH 7.2, 6.6 % (w/v) SDS (sodium dodecyl sulphate), 1 mM EDTA, 1 % bovine serum albumin (BSA)]/cm² of membrane. The probe employed was a 791 bp fragment amplified by reverse transcriptase PCR (RT-PCR) from Triticum aestivum (TaMRP2) using primers 1b and 2b (2.11.3). The probe was labelled with α³²P-dCTP by random priming using Stratagene 'Prime it II' kit and subsequently purified using a Stratagene NucTrap column, according to manufacturers’ instructions. The probe was boiled for 5 min, cooled on ice, added to fresh hybridisation buffer, and hybridised overnight at 62 °C. After hybridisation, the probe was removed and the membrane was rinsed briefly with 2 x SSC, 0.1 % (w/v) SDS and then washed with 2 x SSC at 62 °C for 30 min. The stringency of the washes was increased systematically [1 x SSC, 0.1 % (w/v) SDS to 0.1 x SSC, 0.1 % (w/v) SDS] until 10 counts per second (cps) were recorded from the membrane using a hand held Geiger-Müller detector. The membrane was wrapped in Clingfilm and exposed to autoradiography film (X-omat, XB-200 Kodak) at −80 °C.

2.9.3 Membrane stripping

Membranes were stripped by immersion in boiling distilled water. Once the water had cooled, the membranes were removed from the water and wrapped in Clingfilm. To ensure that stripping was complete, membranes were exposed to an autoradiography film (X-omat, XB-200 Kodak) at −80 °C.

2.10 Library screening

2.10.1 cDNA libraries

Three libraries were available to the project:

(a) cDNA library constructed in λZipLox, Sal I, Not I sites (Life Technologies) using poly (A)⁺ RNA isolated from 7 d old etiolated wheat shoots (cv Darius). Plants were grown from seeds treated with the wheat herbicide safener cloquintocet-mexyl at 1 mg/g seed and watered with 4
mM phenobarbital. E. coli host strain: Y1090 ZL (50 μg/ml ampicillin). Provided by T. Thomas (Dept of Biology, Texas A&M University, USA).

(b) cDNA library constructed in λgt11. Eco RI site (Stratagene) using poly (A)⁺ RNA isolated from 13 d old wheat shoots (cv TAM 107). E. coli host strain: Y1090 r- (50 μg/ml ampicillin). Purchased from Clontech.

(c) cDNA library constructed in λZAP II. Eco RI site (Stratagene) using poly (A)⁺ RNA isolated from 10 d old hydroponically grown barley seedlings which had been induced with nitrate. E. coli host strain: XL1 blue (50 μg/ml tetracycline) (Davies et al. 1997).

2.10.2 Screening wheat cDNA library with a double stranded DNA probe; plating out phage and plaque lifts

Table 2.2: Volumes of agar, plating cells and agarose required for plating phage

<table>
<thead>
<tr>
<th>Size of Petri dish</th>
<th>Bottom agar (ml)</th>
<th>Plating cells (ml)</th>
<th>Top agarose (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9 cm Ø</td>
<td>30</td>
<td>0.1</td>
<td>3</td>
</tr>
<tr>
<td>15 cm Ø</td>
<td>80</td>
<td>0.3</td>
<td>7</td>
</tr>
<tr>
<td>20 cm²</td>
<td>200-250</td>
<td>3</td>
<td>50-60</td>
</tr>
</tbody>
</table>

To prepare plating cells a single colony of the desired strain was used to inoculate 50 ml of LB containing 0.2 % (w/v) filter-sterilised maltose and 10 mM MgSO₄, and incubated overnight at 37 °C, 250 rpm in an orbital shaker. Cells were pelleted by centrifugation at 1,000 g for 10 min at 4 °C, and resuspended in a half volume of sterile 10 mM MgSO₄. These phage-competent cells were stored at 4 °C for up to 7 d.

The library phage stock was diluted in SM buffer (5.8 g/l NaCl, 2.0 g/l MgSO₄, 50 mM Tris-HCl, pH 7.5, 0.01 % (w/v) gelatin) to give the desired titre of 10⁵ plaque forming units (pfu)/μl, of which 1 μl was added to 3 ml SM and 3 ml
plating cells, and incubated at 37 °C for 20 min. 50 ml of molten top agar (LB containing 0.7 % (w/v) agarose) at 47 °C was added to the phage and bacteria, inverted to mix, poured over the surface of a 20 cm² Petri dish of LB agar and allowed to set. Plates were incubated at 37 °C for at least 7 h, or until plaques were visible. Plates were kept at 4 °C for at least 1 h before performing the plaque lifts: a 20 cm square of nylon membrane, Hybond NX (Amersham/Pharmacia Biotech), was placed on the surface of the agar for 1 min. The position of the membrane was marked by piercing the membrane and agar with a sterile needle, and the position of the holes was then marked on the bottom of the Petri dish. The filter was peeled away and placed, phage side up, on a tray containing Whatman 3 MM paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 6 min. The membrane was then transferred on to Whatman 3 MM paper soaked in neutralising solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2) for 3 min, and then rinsed in 2 x SSC for 15 min. The membrane was air-dried and the DNA cross-linked by UV irradiation (Stratagene Stratalinker™ 1800, “autocrosslink” setting).

2.10.3 Hybridisation
Amersham prehybridisation tablets were dissolved according to manufacturers’ instructions, and the membrane was pre-hybridised at 42 °C for 1 h. The probe employed was a 330 bp fragment amplified by polymerase chain reaction (PCR) from an Arabidopsis EST clone (Tommasini et al. 1997), corresponding to bp 4222-4552 of AtMRP3 (accession number: U92650). The fragment was amplified with Pfu polymerase and the DNA was gel purified (2.7.2).

Primer sequences:
EST2/F1: 5'-d-GAATGGAGACAATTGGAGTATGGG -3'
EST2/R1: 5'-d-GGAACTAGATCTTGAGGTACTCAGC -3'

The probe was labelled with α-32P dCTP by random priming using a Stratagene ‘Prime it II’ kit, and subsequently purified using a Stratagene NucTrap column, according to manufacturers’ instructions. The probe was boiled for 5 min, chilled on ice for 2 min, added to the prehybridisation solution, and hybridised overnight.
at 42 °C. The membrane was rinsed briefly with 5 x SSC and then washed twice with 5 x SSC at 42 °C for 30 min. The membrane was wrapped in Clingfilm and exposed to autoradiography film (X-omat, XB-200 Kodak) at -80 °C.

2.10.4 Removal and storage of positive plaques
Positive plaques were excised from the agar using the wide end of a sterilised glass 1 ml pipette as a 'cutter'. The agar plug was then placed in a 1.5 ml microfuge tube to which 0.5 ml of SM buffer and 20 μl of chloroform was added. The contents were mixed thoroughly, and stored at 4 °C.

2.10.5 Secondary screening
Phage isolated from the primary screen (2.10.2) were diluted in SM buffer to give the desired titre of 1-200 pfu/μl. Phage were plated onto 9 cm Ø Petri dishes (table 2.2) and screened as described in section 2.10.2. Positive plaques from the secondary screen were excised as described in section 2.10.4.

2.10.6 Excision of DNA from λZipLox
The λZipLox system allows the recovery of cDNA in the autonomously-replicating plasmid pZL1 using a simple excision protocol. A 50 ml overnight culture of DH10B(ZIP) (10 μg/ml of kanamycin) was prepared. Positive plaques isolated from the λZipLox library screen (2.10.4) were vortexed vigorously, incubated for 5 min at room temperature and then incubated for an additional 5 minutes at room temperature with 100 μl of the DH10B(ZIP) culture. The phage/cell mix was plated onto a LB agar plate containing 10 mM MgSO₄, 100 μg/ml ampicillin and incubated overnight at 37 °C. Plasmid DNA was prepared from the isolated colonies using a Qiagen Mini-Prep™ kit according to manufacturers’ instructions. The presence and size of inserts was determined by a double restriction digestion using Sal I and Not I. Plasmids containing inserts were sequenced using T7 (5'-d-TAATACGACTCACTATAGGG-3') and SP6 (5'-d-TATTTAGGTGACACTATAG) primers (2.7.4).
2.10.7 Small scale purification of λ DNA
One hundred and fifty µl of the phage stock was mixed with 100 µl of competent cells (Y1090 r-) and 50 ml NZ medium [10 g/l casein enzymatic hydrolase (N-Z amine), 5 g/l NaCl, 2 g/l MgCl₂, pH 7.5], and incubated overnight at 37 °C, at 250 rpm. Cells were pelleted by centrifugation at 5,900 g for 10 min at 4 °C, and 45 ml of the supernatant was removed to a clean tube to which 5 ml of 20 % (w/v) polyethylene glycol (PEG; 8000), 2.5 M NaCl was added, mixed and incubated for 2 h on ice. This was then centrifuged at 12,100 g, 4°C for 10 min, the supernatant removed and the pellet resuspended in 500 µl of TE. The DNA was purified by phenol:chloroform:IAA extraction (2.6.3), and ethanol precipitation (2.6.4).

2.10.8 PCR amplification and analysis of λDNA
PCR was employed to amplify the λ DNA prepared by small-scale purification (2.10.7), for preliminary analysis. The PCR reaction mixture consisted of 1 µl λ DNA, 5 pmol each of λ forward and λ reverse primers (see below), 0.2 mM dNTPs, 1 x Pfu buffer (Stratagene), 0.625 U Pfu polymerase, and sterile distilled water to a final volume of 20 µl. The PCR cycle was carried out as follows: denaturation at 94 °C for 1 min, annealing at 50 °C for 1 min and extension at 72 °C for 4 min. A total of 30 cycles were performed. The final extension continued for 5 min at 72 °C. Fifteen µl of the PCR product was electrophorised in an 1.3 % (w/v) agarose/TAE gel to check for size and purity.

λ forward: 5’-d-CCATCGCTGGCGGA-3’
λ reverse: 5’-d-GACTCCTGGAGCCCG-3’

2.10.9 Large scale purification of λ DNA
For positives selected after PCR amplification of λ DNA (2.10.8), large-scale purification of λ DNA was carried out using the plate lysis method as described by Maughan (1997) with the following modifications: 20 large plates (15 cm Ø) of phage were prepared (table 2.2) and grown overnight at 37 °C to confluency. The top agarose was scraped off each plate using a sterile scalpel blade and pooled in a sterile 250 ml polypropylene centrifuge bottle. SM (10 ml) was added to each plate and the plates were incubated on a rotating platform for
approximately 10 min. The solution containing the remaining phage particles was recovered from each plate and added to the top agarose from the original scraping. The volume of the solution was made up to a total of 200 ml with SM and vortexed vigorously for several minutes to disperse the agarose. Chloroform (2.5 ml) was added and the suspension was shaken at 225 rpm for 15 min at 37 °C to facilitate lysis of any bacteria present. The suspension was then centrifuged at 4,000 g for 10 min at 4 °C. The supernatant was carefully recovered and filtered through sterile Miracloth (Calbiochem) to remove residual agarose. The phage DNA was then purified using a Qiagen Lambda Maxi™ kit according to manufacturers’ instructions.

2.10.10 Screening λgt11 library with polyclonal antiserum
The phage library was plated out (2.10.2) to give a titre of $10^5$ pfu/μl and incubated at 42 °C for 3-4 h until the plaques were just visible. A 20 cm square of nitrocellulose membrane, Hybond C Extra (Amersham/Pharmacia Biotech) was soaked in 10 mM filter-sterilised isopropyl β-D-thiogalactopyranoside (IPTG) and allowed to air dry. The membrane was then pre-wetted on a clean fresh agar plate and placed on to the library plate. The position of the membrane was marked by piercing the membrane and agar with a sterile needle, and the position of the holes was then marked on the bottom of the Petri dish. The library plate was inverted and incubated at 37 °C for 3.5 h. The plates were cooled at 4 °C for a minimum of 30 min before the membrane was removed and rinsed thoroughly in Tris-buffered saline (TBS: 20mM Tris-HCl, pH 7.6, 137 mM NaCl). The membrane was shaken overnight at 4 °C in blocking buffer [5 % (w/v) BSA in TBS], and rinsed 3 times in TBS the following day.

A C-terminal fragment of TaMRP1 was expressed in E. coli as a maltose-binding protein fusion, and used to raise a polyclonal antiserum (Theodoulou, et al., 1998). The antibody was diluted 1/1000 with TBS and 0.5 % (w/v) BSA, added to the membrane and incubated for 1 h at room temperature. The membrane was washed 3 times in TBS for 5 min and then incubated with the secondary antiserum, goat-anti-rabbit alkaline phosphatase conjugate (Sigma) diluted 1/3000 with TBS and 0.5 % (w/v) BSA, for 1 h at room temperature. The
washing steps were repeated and the membrane was then developed using alkaline phosphatase substrate tablets: BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate/Nitro blue tetrazolium), according to manufacturers’ instructions (Sigma). Positive plaques were removed and stored (2.10.4).

2.11 Amplification of MRP homologues by PCR

2.11.1 Design of degenerate primers
The design of the degenerate primers was based on (a) conserved motifs common to all plant MRPs, and (b) motifs specific to subgroups of plant MRPs. Peptide sequences of AtMRP 1-5 were obtained from public sequence databases (EMBL, Genbank and Swissprot). These sequences and the peptide sequence of TaMRP1 (Theodoulou, et al., 1998) were used as input to the Genetics Computer Group (GCG) LOCALPILEUP programme to produce multiple sequence alignments. Several rules for primer design were considered: the primers started at the 5' end where possible with a G or C; five exact matches were required at the 3' end; the primers were 18 nucleotides in length or longer; the minimum GC content was 40%; the primers were checked for secondary structure.

1a: 5'-d-GAGTGGYTXITGCTT YMGXIYTNG A Y ATG-3’
1b: 5'-d-GAGTGGYTXITGCTYTXIMGNATGG-3’
2a: 5'-d-CTXIGCXIGRNGCYTCRTC-3’
2b: 5'-d-GAXIGCXIGRNGCYTCRTC-3’

International Union of Biochemists (IUB) symbols are used to represent nucleotides and ambiguities.

2.11.2 PCR using degenerate primers
Degenerate primers (2.11.1) and vector primers (pUC/M13 forward: 5’-d-GTTTTCCCAGTCACGAC-3’ and SP6, see 2.10.6) were employed to amplify sequences from library (a) (2.10.1). Several sets of reaction conditions were tested. The optimal conditions were as follows: 0.5 µl cDNA library, 100 pmol of each primer, 0.2 mM dNTPs, 1 x PCR buffer, 2.5 U Taq polymerase and water to a final volume of 25 µl. The PCR cycle: denaturation 95 °C for 30 sec,
annealing at 55 °C for 30 sec and extension at 72 °C for 4 min. A total of 30 cycles were performed. The final extension continued for 5 min at 72 °C. The entire PCR reaction was electrophoresed in a 1.2 % (w/v) agarose/TAE gel. Amplified bands were excised from the gel and the DNA was gel purified (2.7.2), cloned into the vector PCR 2.1™ (Invitrogen) and transformed into TOP10 competent cells (Invitrogen) following manufacturers’ instructions. Plasmid DNA was prepared (2.6.1). The size of the DNA was checked by restriction digestion with Eco RI. Plasmids containing inserts were sequenced using T7 and reverse primers (2.7.4).

2.11.3 Reverse transcriptase-polymerase chain reaction (RT-PCR).

RT-PCR was carried out using the Access RT-PCR system (Promega), and degenerate primers (2.11.1). The kit was used according to manufacturers’ instructions, briefly, the reaction mix consisted of 1 μg RNA, 1 X AMV/Tfl reaction buffer, 1 μl dNTPs, MgSO₄ (1 mM), 5 U AMV reverse transcriptase, 5 U Tfl DNA polymerase, 50 pmol of each primer and sterile distilled water to a total volume of 50 μl. Several combinations of degenerate primers were employed; 1a and 2a, and 1b and 2b. The PCR reaction is described below.

First strand cDNA synthesis reverse transcription: 48 °C, 45 min.
AMV RT inactivation: 94 °C, 2 min.
RNA/cDNA/primer denaturation: 94 °C, 2 min.
Second strand cDNA synthesis and PCR amplification (40 cycles):

- Denaturation 94 °C, 30 sec.
- Annealing 60 °C, 1 min.
- Extension 68 °C, 2 min.

Final extension 68 °C, 7 min.

The PCR reaction (25 μl) was run out in an 1 % (w/v) agarose/TAE gel. The bands were excised, purified (2.7.2) and cloned into PCR 2.1™ according to manufacturers’ instructions (Invitrogen). Clones containing inserts were sequenced commercially (Genome Express SA, Grenoble, France).
2.12 Protein analysis

2.12.1 Quantitation of protein concentration
Protein concentration was determined using the Bio-Rad protein assay system according to manufacturers’ instructions, in a 96 well microtitre plate format. Sample buffer (80 µl) was pipetted into each well with the exception of the wells in row 1. Eighty µl of protein standard and sample was pipetted into triplicate wells in rows 1 and 2 and doubling dilutions were made. The final row contained buffer only (blank). Twenty µl of Bio-Rad protein assay dye reagent concentrate was added to each well, and the plate was gently shaken until an even distribution of colour. After 5 min the absorbance at 595 nm was determined in a plate reader (Dynatech MR500).

2.12.2 Electrophoresis of proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)
Polyacrylamide gel electrophoresis of proteins was carried out using a Bio-Rad PROTEAN minigel kit according to manufacturers’ instructions. SDS-PAGE gels consisted of a lower resolving gel [1.75 ml 30 % (v/v) bis-acrylamide (37.5:1 acrylamide:bis), 1.75 ml 1.5 M Tris-HCl pH 8.8, 10 µl ammonium persulphate (250 mg/ml), 30 µl 10 % (w/v) SDS, 10 µl NNN’N’-tetramethylethylenediamine (TEMED), 3.45 ml distilled water], which was overlaid with water-saturated butanol and allowed to polymerise for ca. 30 min at room temperature. Once set, the water-saturated butanol was poured off and the gel was rinsed with distilled water and dried with filter paper. The stacking gel [0.5 ml 30 % (v/v) bis-acrylamide (37.5:1 acrylamide:bis), 0.9 ml 0.5 M Tris-HCl pH 6.8, 5.1 µl ammonium persulphate (250 mg/ml), 36 µl 10 % (w/v) SDS, 5.1 µl TEMED, 2.17 ml distilled water] was poured on top of the resolving gel, a comb was inserted to allow the formation of wells, and the gel was allowed to set at room temperature.
Protein samples were prepared by mixing with an equal volume of 2 x SDS sample buffer [5 % (v/v) glycerol, 3 % (w/v) SDS, 63 mM Tris-HCl pH 6.8, 25 mg bromophenol blue, 5 % (v/v) β-mercaptoethanol (14.3 M), 2 mM phenylmethysulphonyl fluoride (PMSF) made up to a final volume of 50 ml with distilled water], and incubated at 42 °C for 20 min. Samples were loaded into the wells and electrophoresed for ca. 35 min at 200 V in gel running buffer [25 mM Tris-HCl pH 8.3, 250 mM glycine, 0.1 % (w/v) SDS]. To visualise the bands, gels were stained for 1 h in staining buffer [40 % (v/v) methanol, 20 % (v/v) acetic acid, 0.2 % (v/v) Coomassie Brilliant Blue R-250] and destained in 30 % (v/v) methanol, 10 % (v/v) acetic acid until bands became visible.

### 2.12.3 Western blotting

Proteins were separated by SDS-PAGE (2.12.2) and ‘wet’ electro-transfer of proteins was performed using a Mini-PROTEAN II gel system (Bio-Rad) according to manufacturers’ instructions. Briefly, unstained gels were equilibrated in transfer buffer [24 mM Tris, 193 mM glycine, 20 % (v/v) methanol]. Proteins were then transferred to Hybond-C Extra (Amersham Pharmacia Biotech) in transfer buffer at 100 V for 1 h. Membranes were rinsed briefly in TBS (0.5 M Tris-HCl pH 7.6, 0.2 M NaCl), and blocked in 5 % (w/v) BSA/TBS for 1 h. The blocked membrane was rinsed briefly in TBS and incubated with primary antibody or pre-immune serum diluted 1:1000 in 1 % (w/v) BSA in TBS, for 1 h at room temperature or overnight at 4 °C. Membranes were washed 4 x 10 min in TBST [TBS, 0.1 % (v/v) Tween 20], and incubated with secondary antibody (goat anti-rabbit horseradish peroxidase conjugate, Sigma), diluted 1:50 000 in 1 % (w/v) BSA in TBS, for 1 h at room temperature. Membranes were washed 6 x 10 min in TBST. Membranes were developed using the Enhanced Chemiluminescence (ECL) kit (Amersham) according to manufacturers’ instructions. If required for stripping and reprobing, membranes were kept moist and stored at 4 °C.
2.13 Production and characterisation of polyclonal antisera

2.13.1 Expression of the TaMRP2 C-terminus in *E. coli*

In order to test whether TaMRP2 is recognised by anti-TaMRPl antiserum (Theodoulou, *et al.*, 1998), the 171 C-terminal amino acids of TaMRP2 were expressed as a GST fusion protein, using pGEX-4T-3 (Amersham Pharmacia). A 670 bp *Bgl II/Not I* fragment (corresponding to the C-terminus) excised from TaMRP2/pZL1 was cloned into the *Bam HI/Not I* of pGEX-4T-3. The resulting construct, TaMRP2/pGEX-4T-3, was transformed into XL1-blue (2.5.4). Overnight cultures of transformants were used to inoculate 20 ml of LB containing 10 μg/ml ampicillin. The cultures were grown to an OD$_{600}$ nm of 0.5 and 10 ml was removed to duplicate fresh tubes. One tube was inoculated with IPTG to a final concentration of 1 mM. The cultures were grown for a further 6 h and at hourly intervals from 3-6 h, 600 μl aliquots were removed, 100μl was used to determine the OD$_{600}$ nm, and the remaining 500 μl was pelleted in a bench top centrifuge at top speed, resuspended in distilled water (100 μl/ 1 OD unit measured at 600 nm), and stored at -20 °C until required. Pellets were lysed by boiling for 5 min in 1 x SDS-PAGE sample buffer and SDS-PAGE and Western analysis were performed as described in sections 2.12.2 and 2.12.3. Cells transformed with vector alone or with TaMRP1/pGEX-4T-3 (supplied by Dr. Theodoulou) were used as negative and positive controls, respectively.

2.13.2 Selection of peptide antigens

The protein sequences of AtMRP1-5, TaMRP1 and TaMRP2 were aligned using the LOCALPILEUP program (GCG). The alignment was examined manually to select regions specific to TaMRP1 and TaMRP2. Putative transmembrane domains were predicted [TopPredII program (http://bioweb.pasteru.fr/seqama/;interfaces/toppred.html)]. Hydropathy plots (http://bioinformatics.weizmann.ac.il/hydroph/) were performed according to Kyte and Doolittle (1982) with a window of 17 amino acids and these hydrophobic regions were not used for peptide design. Sequences of 20-25 amino acid residues, which were largely hydrophilic were selected, and this information submitted to Sigma-Genosys who advised on feasibility of peptide synthesis, and probable antigenicity. Two peptides were selected:
TaMRP1: CEGAAPVSDEKGETPAISRQPSRKG
TaMRP2: CGSLPSADKKDKQNVKQDDGHGQSG

These peptides were synthesised by Sigma-Genosys and conjugated to keyhole limpet hemocyanin (KLH).

2.13.3 Production of anti-peptide antisera
Lyophilised peptides were resuspended in phosphate buffered saline (PBS: 0.01 M KPi, 0.0027 M KCl, 0.137 M NaCl, pH 7.4) to a final concentration of 1 mg/ml. Antisera were raised using these antigens at the antibody facility at IACR-Rothamsted; licence number PPL 70/4356, under the Animals (Scientific Procedures) Act 1986. A rabbit was inoculated with up to 2 mg of the peptide at 3 weekly intervals. A pre-immune bleed was taken prior to any injections and a test bleed was taken 4 weeks after the first injection and the titre measured by ELISA. When the titre was sufficiently high (1:1000), bleeds were taken at 3 weekly intervals. The antisera was mixed with 50 % (v/v) glycerol to improve stability and stored at -20 °C.

2.13.4 Dot blotting
Dot blotting was used to determine the dilutions of the antiserum required for protein detection. Peptide (1 μg/μl) was serially diluted 10-fold in PBS and 1 μl of each dilution was spotted onto 6 replica strips of Hybond C extra (Amersham) which had been pre-wetted in transfer buffer (2.12.3). The membranes were left to dry, rinsed briefly in TBS (2.12.3) and blocked for 1 h in 5 % (w/v) BSA in TBS. The membranes were rinsed in TBS and then incubated for 1 h at room temperature with immune or preimmune serum at a range of dilutions in 1 % (w/v) BSA in TBS (10^2, 10^3, 10^4, 10^5, 10^6). The strips were then developed as described for Western blotting in section 2.12.3.

2.14 Plant membrane fractionation

2.14.1 Preparation of microsomes from wheat
Microsomes were prepared from 8-d-old xenobiotic-treated wheat shoots according to the method of Rea et al., (1992) by differential centrifugation. Briefly, wheat shoots grown in vermiculite (2.3.1) were cut into 1 cm segments.
Working at 4 °C, the tissue was homogenised with a pestle and mortar in 2 volumes of homogenisation buffer [1.1 M glycerol, 5 mM ethyleneglycol-bis(β-aminoethyl ether) N,N′,N′,N′-tetraacetic acid-Tris (EGTA-Tris), 1.5 % (w/v) polyvinyl pyrrolidone-40 (PVP), 1 % (w/v) ascorbic acid, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM Tris-HCl, pH 7.6]. The homogenate was filtered through 6 layers of muslin and centrifuged at 4,000 g for 10 min at 4 °C. The supernatant was decanted into fresh tubes and centrifuged at 38,000 g for 35 min at 4 °C. The resulting pellet was resuspended in 1 ml of suspension buffer [1.1 M glycerol, 1 mM EGTA-Tris, 5 mM Tris-2-(N-Morpholino)-ethane sulphonic acid (MES), pH 7.6] and in a dounce homogeniser, homogenised until no clumps were visible. Samples were stored in aliquots at -20 °C.

2.1.4.2 Preparation of plasma membranes from wheat
Plasma and intracellular membranes were prepared by aqueous two-phase partitioning according to the method of Olbe and Sommarin (1991 and 1997). Briefly, 100 g of hydroponically grown wheat roots and shoots (2.3.2), were cut into 1 cm segments. Working at 4 °C, the tissue was homogenised with a pestle and mortar in 2.5 volumes of homogenisation medium [0.25 M sucrose, 5 mM EDTA, 50 mM MOPS-Bis-Tris propane (MOPS-BTP), pH 7.5, 0.2 % (w/v) BSA, 0.2 % (w/v) casein, 2 mM DTT, 0.5 mM PMSF]. The homogenate was filtered though 4 layers of muslin and centrifuged at 10,000 g for 10 min at 4 °C, the pellet discarded and the supernatant centrifuged at 30,000 g for 30 min at 4 °C. The resulting microsomal preparation was resuspended in a small volume (ca. 100 μl) of resuspension medium [0.25 M sucrose, 5 mM KH₂PO₄, 1 mM dithiothreitol dithiothreitol (DTT), 4 mM KCl, 0.5 mM PMSF] and made up to a final volume of 6 ml. Four x 24 g phase systems [6.5 % (w/w) dextran, 6.5 % (w/w) PEG 3250, 0.25 M sucrose, 5 mM KH₂PO₄, 4 mM KCl to a final weight of 24 g] were prepared in 50 ml Falcon tubes by centrifugation of the phase mixed at 1,500 g for 5 min at 4 °C in a bench-top swinging bucket rotor. The microsomal preparation was partitioned in the 24 g phase system, at 1,500 g for 5 min at 4 °C in a bench-top swinging bucket rotor. The microsome-enriched upper phase was purified by counter-current distribution where the upper phase was continually
re-partitioned against fresh lower phase to yield 4 2-phase systems: 1 lower, 3 upper (Larsson, et al., 1994). Each phase was diluted in wash/resuspension medium [0.25 M sucrose, 10 mM EDTA, 10 mM N(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid-potassium hydroxide (HEPES-KOH), pH 7.5, 1 mM DTT] to give an appropriate volume (ca. 15 ml) for centrifugation at 100,000 g, for 1.5 h at 4 °C in a swinging bucket rotor (Beckman SW28). Membranes were resuspended in ca. 100 μl of wash/resuspension medium without EDTA and stored at -20 °C.

2.14.3 Wheat tonoplast preparation
Tonoplast vesicles were prepared according to the method of Allen et al., (1995). Briefly, 100 g of hydroponically grown wheat roots and shoots (2.3.2) were cut into 1 cm segments. Working at 4 °C, the tissue was homogenised with a pestle and mortar in 2.5 volumes of homogenisation medium [250 mM sorbitol, 3 mM EGTA, 25 mM HEPES-BTP, pH 7.4, 0.1 % (w/v) BSA, 1 mM DTT], filtered through 4 layers of muslin and centrifuged at 10,000 g for 10 min at 4 °C. The supernatant was decanted into swinging rotor bucket tubes (Beckman SW28.1) and centrifuged at 63,000 g for 30 min at 4 °C (Beckman SW28). Pellets were resuspended in 1 ml of resuspension medium (250 mM sorbitol, 2.5 mM HEPES-BTP, pH 7.4, 0.5 mM DTT) using a paint brush pre-wetted in the medium, pooled, and diluted to a final volume of 24 ml with resuspension medium. Aliquots of the suspension (4 ml) were layered onto 10 ml 6 % (w/v) dextran and centrifuged at 72,000 g for 2 h at 4 °C (Beckman SW28). Tonoplasts formed a layer on top of the dextran cushion, and were carefully removed with a glass Pasteur pipette. Tonoplasts were stored -20 °C.

2.15 Expression of TaMRP2 in Baculovirus

2.15.1 Construction of BacMRP-DB8/9
The pFastBac-TaMRP2 plasmid (BacMRP-DB8/9) was constructed in several steps to allow the removal of the 5' utr of TaMRP2.

1. A 4761 bp Eco RI-Not I fragment (nucleotides 101-4861) of TaMRP2 was sub-cloned into pFastBac (BacMRP)(Life Technologies).

2. A 1175 bp fragment corresponding to nucleotides 289-1450 of TaMRP2 was
amplified from BacMRP (from step 1) by PCR to introduce a \textit{Rsr} II restriction site and flanking region adjacent to the \textit{TaMRP2} ATG (13 bp) and maintain the internal \textit{Rsr}II site (nucleotide 1358) of \textit{TaMRP2}.

**PCR reaction mix:**
- 25ng DNA (template BacMRP, Step 1)
- 12.5 pmol (DB8 and DB9. See below)
- 0.2 mM dNTPs
- 1 X \textit{Pfu} Buffer (Promega)
- 0.75 U \textit{Pfu} polymerase
- 5 % (v/v) dimethylsulphoxide (DMSO)
- Sterile water to a final volume of 25 \mu l.

**PCR conditions:**
- 95 °C 1 min
- 30 cycles:
  - 95 °C 30 sec
  - 72 °C 30 sec
  - 72 °C 2 min
- Final extension:
  - 72 °C 10 min

**Primers**
- DB8 5'-d-CATTATCGGTCCGATGGCGGC-3'
- DB9 5'-d-CGATGTTGACCATCTCGCCG-3'

3. The resulting PCR product was gel-purified (2.7.2) and sub-cloned into pCR® Blunt (Invitrogen)(ZBDB8/9).

4. ZBDB8/9 (step 2) was digested with \textit{Rsr} II and the resulting 1074 bp fragment was cloned into BacMRP (step 1) digested with \textit{Rsr} II. The resulting clone was termed BacMRP-DB8/9

5. BacMRP-DB8/9 was end-sequenced (2.7.4) with DB9 to confirm that no errors had occurred during amplification and cloning.

### 2.15.2 Generation of recombinant Baculovirus

Recombinant Baculovirus was generated using the BAC-TO-BAC Baculovirus Expression System (Invitrogen) according to manufacturers’ instructions, with the following modifications. Plasmids BacMRP-DB8/9 and pFastBac (negative control) were transformed into DH10Bac, and the resulting bacmid DNAs were chosen at random, in place of IPTG/5-bromo-indolyl-\beta-D-galactopyranoside.
(Bluogal; Invitrogen) selection as recommended, and analysed by PCR (according to the BAC-TO-BAC manual) using DB29 (5'-CAGTCGAGCAGATTCTCG-3') and reverse primers.

Sf9 insect cells were cultured in 25 cm² Nunclon™A flasks (163371A) in serum-free Sf-900 II serum free media (SFM) media supplemented with 5 % (v/v) fetal calf serum (FCS) (Invitrogen). Cells were transfected with the bacmid DNAs using CELLFECTIN reagent, and virus was harvested after a 72 h incubation at 27 °C. The virus was amplified by infecting 10⁶ cells with 0.5 ml virus in a total volume of 2 ml in a 25 cm² flask.

2.15.3 Viral plaque assay
In a laminar flow hood, the wells of a 6 well plate (Nunclon™A multidishes (152795A). Life Technologies) were seeded with 1.1 x 10⁶ Sf9 cells/2 ml SF-900 II SFM media, supplemented with 5 % (v/v) FCS. A total of 2 plates were required for each virus or control requiring titration. The cells were left to attach for 1 h at room temperature or until a monolayer had formed. The viral stocks were serially diluted 10-fold, in 1 ml SF-900 II SFM media to give 10⁻³-10⁻⁷. The media from each well was removed using a sterile Pasteur pipette, and 200 µl of each dilution of virus was pipetted into duplicate wells, swirling gently to ensure virus covered the entire monolayer. The virus was left to fuse for 1 h, then the excess virus was removed with a 1 ml Gilson pipette tip. Each monolayer was then covered with 2 ml of equal volumes of molten sea-plaque agarose and SF-900 SFM II (1.3 x), by tilting the plate and allowing the mix to run over the monolayer. Once the agarose had set, it was overlaid with 1.5 ml of SF-900 II SFM media, containing 5 % (v/v) FCS, and incubated in a humid environment at 27 °C for 7 d. Cells were then stained with neutral red; the media was removed from each well and replaced with 2 ml of neutral red [0.33 % (v/v)] diluted 1:11 with PBS. Plates were incubated at 27 °C for 3 h, the stain removed and the plates wrapped in aluminium foil, inverted and incubated overnight at 27 °C. Plaques of 1-2 mm in diameter containing a few red (healthy) cells were visible. Infected cells are clear. Absence of cells indicated that the cells had been lysed by the virus.
2.15.4 Crude membrane preparation of Baculovirus infected Sf9 cells
Crude membrane extracts were prepared according to the method of Zimmermann et al., 1998. Flasks (85 cm²) of Sf9 cells (ca. 1 x 10⁶) infected with 500 µl of twice amplified virus were harvested by centrifugation at 500 g for 5 min at 4 °C. Pellets were washed in 1 ml of PBS, pooled (cells from 2 x 85 cm² flasks), resuspended in 1 ml of buffer A (10 mM NaPi, pH 8, 1 mM EDTA, 1 mM PMSF), and incubated on ice for 5 min. One hundred and thirty µl of 5 M NaCl was added to each sample which was then sonicated on ice for 30 sec at an amplitude of 5 microns (MSE Soniprep 150, Sanyo). Samples were diluted in ca. 11 ml of buffer A and centrifuged at 100,000 g for 30 min at 4 °C. Pellets were resuspended in 100 µl of buffer A and stored at -20 °C.

2.16 Expression of TaMRP2 in Saccharomyces cerevisiae

2.16.1 Cloning TaMRP2 into the yeast expression vector pYCT2/CT
The pYC2/CT-TaMRP2 plasmid (pYC2/TaMRP) was constructed in several steps to allow the removal of the 5' utr of TaMRP1.
1. A 4761 bp Eco RI-Not I fragment (nucleotides 101-4861) of TaMRP1 was sub-cloned into pYC2/CT (pYC2/TaMRP1) (Invitrogen).

2. A 1182 bp fragment corresponding to nucleotides 289-1450 was amplified from BacMRP-DB8/9 (2.15.1) by PCR to introduce a Eco RI restriction site and flanking region adjacent to the TaMRP1 ATG (10 bp) and maintain the internal RsrII restriction site (nucleotide 1358) of TaMRP2:

PCR reaction mix: 25ng DNA (template BacMRP-DB8/9 (2.15.1))
12.5 pmol (DB9 (2.15.1) and DB33. See below)
0.2 mM dNTPs
1 X Pfu Buffer (Promega)
0.75 U Pfu polmerase
5 % (v/v) DMSO
Sterile water to a final volume of 25 µl.
PCR conditions: 95 °C 1 min
30 cycles:
95 °C 30 sec
65.2 °C 30 sec
72 °C 2 min
Final extension: 72 °C 10 min
Primer: DB33 5'-d-CGAATTCATAATGGCGGCGACGGCGAGC-3'
The resulting PCR product was gel-purified (2.7.2) and sub-cloned into pCR®Blunt (Invitrogen)(ZBDB9/33).

4. ZBDB9/33 was digested with Rsr II and Eco RI and the purified 1060 bp fragment was cloned into pYC2/TaMRP2 (from step 1) digested with Rsr II and Eco RI.

5. pYC2/MRP was sequenced (2.7.4) to confirm that no errors had occurred during amplification and cloning.

2.16.2 Yeast strains
Yeast strains were purchased from EUROSCARF, Germany (http://www.rz.uni-frankfurt.de/FB/fb16/mikro/euroscarf/index.html). Accession numbers are indicated in brackets below the strain name.

Wild type         Matα; his3Δ1; leu2Δ0; ura3Δ0
(Y1000)

ΔYCF1         BY4742; Matα; his3Δ1; leu2Δ0; ura3Δ0; YDR135c::kanMX4
(Y146069)

ΔYAP1         BY4742; Matα; his3Δ1; leu2Δ0; ura3Δ0; YML007w::kanMX4
(Y10569)
2.16.3 Growth of yeast
Unless specified, yeast strains were grown in either rich media/YPD (10 g/l bactoyeast extract, 20 g/l bactopeptone, 20 g/l d-glucose), or minimal media/SD (1.7 g/l yeast nitrogen base, 5 g/l ammonium sulphate, 5 g/l d-glucose, 0.83 g/l amino acid supplements (as described on the Gietz yeast media web-page www.umanitoba.ca/faculties/medicine/biochem/gitez/media.html)). For yeast containing the plasmid pYC2/CT, uracil was omitted from the minimal media (SD-U), since the auxotrophic marker of the plasmid was uracil.

2.16.4 Maintenance and storage of yeast cultures
For short term storage, yeast cells were maintained on YPD agar plates (YPD set with 20 g/l Bactoagar) sealed with Parafilm and stored upside-down at 4 °C. For long term storage yeast cells were stored as glycerol stocks; yeast was scraped from one YPD agar plate, mixed with 1 ml of 15 % (v/v) glycerol in a sterile cryovial, frozen in liquid nitrogen and stored at -80 °C.

2.16.5 Determination of yeast cell count
A 100 µl aliquot of an overnight culture of yeast cells was diluted 1:10 in the appropriate growth medium, and ca. 50 µl was transferred to both chambers of a haemocytometer by touching the edge of the cover-slip with a pipette tip and allowing the chambers to fill by capillary action. The number of the cells in 5 diagonal squares of both central grids were counted. The central grid was 1 mm² equivalent to a volume of $10^{-4}$ ml. To calculate the number of cells per ml, the following calculation was used: cells counted x $5/2$ x dilution factor x $10^4$. 
2.16.6 Transformation of Saccharomyces cerevisiae
Yeast was transformed according to the Gitez ‘best’ method for lithium acetate/ss-DNA/PEG transformation (Agatep, et al., 1998). Approximately 1 µg of DNA in 2 µl of sterile distilled water was used for each transformation. Transformants were visible after 2-3 d of growth at 28 °C.

Transformants were either used directly from the initial plate for further study or selected on CdCl₂. For selection on CdCl₂, it was necessary to use galactose in place of glucose to induce expression of the GAL promoter. Cells were washed off the plate in 1-2 ml of yeast nitrogen base (1.7 g/l)/ammonium sulphate (5 g/l) using a spreader. The transformants were vortexed vigorously and 100 µl was plated onto a minimal media agar plate containing 20 g/l galactose instead of glucose (SG-U), containing 100 µM CdCl₂. Transformants were incubated at 28 °C for 2-3 d.

2.16.7 Drop test
A 10 ml overnight culture of yeast cells grown in SG-U was diluted in SG-U to give 3 x 10⁴ cells/6 µl. Ten-fold and 3-fold dilutions were made to give 3 x 10⁴, 3 x 10³, 1 x 10³, 3 x 10², 1 x 10² and 3 x 10 per 6 µl in SG-U. In a flow bench, 6 µl of each dilution of yeast was spotted in a horizontal line, onto a SG-U agar plate containing the test compound (SG-U as an untreated control). If comparisons between a number of cultures were required, they were spotted onto the same plate. Plates were incubated at 28 °C for 2-3 d.

2.16.8 Growth assay
A 10 ml overnight culture of yeast cells grown in SG-U was diluted to an OD₆₅₀nm of 0.1 in 10 ml SG-U, before inoculation with the test compound. Cultures were incubated at 28 °C, at 225 rpm in an orbital shaker, and at given time periods the OD₆₅₀nm was recorded and plotted on a graph against time. When the yeast had reached lag phase the experiment was terminated.
2.16.9 Challenge assay
A 10 ml overnight culture of yeast cells grown in SG-U was diluted to $2 \times 10^7$ cells/3 ml in SD-U for each test compound at each required concentration. After the addition of the cells at time 0, the test compound was added and cultures were incubated at 28 °C, 225 rpm in an orbital shaker. After 1 h, cells were serially diluted 10-fold ($10^2$, $10^3$, $10^4$, $10^5$) in SD-U and plated in triplicate on SD-U agar plates. After incubation at 28 °C for 2-3 d, the cells were counted, and compared on a percentage basis to the untreated control.
CHAPTER 3

MOLECULAR CLONING OF WHEAT MRP2 (TaMRP2)

3.1 Introduction

The objective of this project was to clone and characterise wheat MRP isoforms and investigate their role in xenobiotic metabolism. This chapter describes the cloning of a full length MRP from wheat.

Several different strategies are available for the detection of cDNA clones (reviewed in; Sambrook, et al., 1989), and the choice of method is dependent upon the nature of the gene and the information and tools available. The resources available at the outset of this project were: 5 Arabidopsis cDNA sequences encoding MRP homologues; AtMRP1 (AF00814), AtMRP2 (AF020288), AtMRP3 (U92650), AtMRP4 (AF234509), AtMRP5 (Y11250) (Lu, et al., 1998; Lu, et al., 1997; Marin, et al., 1998; Sánchez-Fernández, et al., 1998; Tommasini, et al., 1998; Tommasini, et al., 1997; Weigmann, et al., 1998. (pers. comm.), a partial wheat cDNA (TaMRP1 which encodes the 766 C-terminal amino acids; Theodoulou, et al., 1998), and a polyclonal antiserum raised to the C-terminus of TaMRP1 (Theodoulou, et al., 1998). Since the specific aim of the project was to isolate MRP isoforms involved in xenobiotic metabolism, a decision was made to concentrate on chemically inducible isoforms in the first phase of the project. Of the Arabidopsis isoforms, AtMRP3
exhibited the greatest transcriptional response to xenobiotics, such as herbicide safeners (Tommasini, et al., 1997). Whilst safener inducibility does not guarantee that herbicides act as substrates, inducibility by foreign chemicals might suggest a role for MRP transport activities in xenobiotic detoxification. Additionally, previous preliminary work indicated that screening a wheat cDNA library with AtMRP3-derived probes would be successful (Theodoulou, pers. comm.), therefore the project was initiated with this approach.

3.2 Library screening with a double stranded DNA probe

Three wheat cDNA libraries (2.10.1) were screened using an heterologous DNA probe corresponding to AtMRP3. Although the full-length AtMRP3 clone was not available to the project, an 660 bp EST (EST2: accession number: T22571; Tommasini, et al., 1997) representing this cDNA was obtained from the Ohio Arabidopsis stock centre. The EST contained 417 bp of coding sequence including the Walker B motif and other conserved sequences, and 243 bp of untranslated region. Only the coding sequence was required as a probe and this fragment (330 bp) was therefore amplified by PCR. This probe was believed to have sufficient sequence identity to wheat MRP isoforms to permit library screening. TaMRP1 and its closest Arabidopsis homologue, AtMRP4, are 69 % identical at the nucleotide level; it was therefore assumed that AtMRP3 and its closest wheat homologue might share similar levels of homology. Hybridisation conditions were chosen based on the estimated likely percentage identity between AtMRP3 and its putative wheat homologue, therefore the libraries were initially screened at a low/medium stringency (42 °C, 5 x SSC). Four hundred thousand plaques were screened from each library. Duplicate filter lifts were made in an attempt to eliminate any false positives. A number of plaques from all 3 of the libraries were isolated (table 3.1) however, none of these clones proved positive in secondary screens and this approach was therefore discontinued.
Table 3.1: Screening cereal cDNA libraries with an AtMRP specific probe

<table>
<thead>
<tr>
<th>cDNA Library</th>
<th>Number of positive plaques from primary screen</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Etiolated, safener treated wheat shoots</td>
<td>1</td>
</tr>
<tr>
<td>(b) Light grown wheat shoots</td>
<td>13</td>
</tr>
<tr>
<td>(c) Nitrate-induced barley roots</td>
<td>10</td>
</tr>
</tbody>
</table>

3.3 Library screening with a polyclonal antibody

An alternative cloning strategy was library screening with a polyclonal antibody. An anti-TaMRP1 polyclonal antiserum was available to the project: a C-terminal fragment of TaMRP1 was expressed in *E. coli* as a maltose-binding protein fusion, and used to raise a polyclonal antiserum in rabbit (Theodoulou, et al., 1998). From amino acid sequence comparisons of AtMRP3 and AtMRP4 with TaMRP1 (figure 3.1 and table 3.2), it is possible that this antiserum will recognise not only TaMRP1 and AtMRP4, but also AtMRP3. Therefore it could be used to isolate wheat homologues of AtMRP3. Since Western blot analysis of green leaves showed that the antigen is abundant in this tissue even in the absence of chemical inducing treatments (as discussed in chapter 4), the antiserum was used to screen a cDNA library constructed from RNA isolated from 13-day old light-grown wheat shoots (see 2.10.1, library (b)). Four hundred thousand plaques were screened from library (b). Duplicate lifts were made in an attempt to eliminate any false positives. Twenty-five clones were obtained from the primary screen, 23 clones from the secondary screen and 21 clones were taken through to the tertiary screen. λ DNA was prepared from the positive clones (2.10.7), amplified by PCR and cloned into the vector PCR Blunt™ (Invitrogen). The clones were then sequenced using the ABI 373 (M13 and reverse sequencing primers) and analysed using the Genetics Computer Group (GCG) (University of Wisconsin, USA) molecular biology analysis program FASTA (Pearson, et al., 1997). The results of the sequence analysis are summarised in Table 3.3. Eleven of the plaques from the tertiary screen did not contain inserts and were eliminated from further analysis. The remaining 10 plaques were sequenced either directly from the PCR product or after sub-cloning into PCR 2.1™. No MRP isoforms were isolated.
Table 3.2: Amino acid comparisons of TaMRP1 with *Arabidopsis MRP* isoforms

<table>
<thead>
<tr>
<th><em>Arabidopsis</em> isoform</th>
<th>Over 2307 amino acids (Entire clone)</th>
<th>Over region used for antibody production</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtMRP3</td>
<td>48</td>
<td>58</td>
</tr>
<tr>
<td>AtMRP4</td>
<td>72</td>
<td>84</td>
</tr>
</tbody>
</table>

Table 3.3: Homology matching of wheat cDNAs

<table>
<thead>
<tr>
<th>Clone</th>
<th>Length of Sequence (nucleotides)</th>
<th>Closest homologue</th>
<th>Percentage identity</th>
<th>Number of nucleotides sharing homology</th>
<th>Accession number of closest homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>496</td>
<td>Ribulose bisphosphate carboxylase from wheat</td>
<td>97 %</td>
<td>455</td>
<td>D00206</td>
</tr>
<tr>
<td>2</td>
<td>367</td>
<td><em>E. coli</em> hypothetical protein</td>
<td>100 %</td>
<td>361</td>
<td>AE000451</td>
</tr>
<tr>
<td>3</td>
<td>275</td>
<td>Chloroplast DNA from wheat</td>
<td>98 %</td>
<td>245</td>
<td>AB042240</td>
</tr>
<tr>
<td>4</td>
<td>233</td>
<td><em>E. coli</em> hypothetical protein</td>
<td>100 %</td>
<td>226</td>
<td>AE000334</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>Sequence too short to analyse</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>Sequence too short to analyse</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>Sequence too short to analyse</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>322</td>
<td>CTD-binding protein</td>
<td>64 %</td>
<td>148</td>
<td>AC004216</td>
</tr>
<tr>
<td>9</td>
<td>258</td>
<td>Barley vacuolar H* pyrophosphatase gene</td>
<td>98 %</td>
<td>240</td>
<td>AB042240</td>
</tr>
<tr>
<td>10</td>
<td>318</td>
<td>Wheat MAP kinase</td>
<td>94 %</td>
<td>305</td>
<td>AF079318</td>
</tr>
</tbody>
</table>

Nucleotide sequences were analysed using the GCG FASTA program.
3.4 Amplification of MRP homologues by PCR using degenerate primers

PCR is a sensitive method for the amplification of DNA fragments. It was hoped that this method may circumvent the problems of low abundance of MRP, by permitting PCR screening of library sub-pools (Israel, 1993), a strategy which has proved effective for cytochrome P450s (Clark, et al., 1997). Several plant MRP sequences were available in public databases (EMBL, Genbank and Swissprot and were used together with the sequence of TaMRPl (Theodoulou, et al., 1998) as input to the GCG LOCALPILEUP programme, to produce multiple sequence alignments (figure 3.2). Primers were designed: (a) to conserved motifs common to all plant MRPs, and (b) to motifs specific to subgroups of plant MRPs (figure 3.3). The primers were subjected to FASTA searches of Genbank to determine whether they contained regions of homology to sequences other than MRP. No such matches were found. The MRP-specific primers (1a and 2a, 1b and 2b) were used in combination with vector primers (SP6 and M13 forward) to permit nested PCR on a safener-treated wheat cDNA library (2.10.1 a). Figure 3.4 summarises the PCR strategy. Some optimisation of PCR was required; optimal conditions are described in section 2.11.2. Bands of approximate expected size were obtained (figure 3.5). These bands were then excised, gel-purified, cloned into PCR 2.1™ (Invitrogen) and sequenced. FASTA and BLAST results indicated that the clones were all lambda DNA (data not shown) although these homologies were not picked up in the original FASTA search when designing primers. At this stage redesign of primers was one appropriate strategy, but an RT-PCR approach employing the original degenerate primers was used instead, with the advantage that different inducing treatments could be tested.

3.5 RT-PCR

Due to the low abundance of MRP, methods for increasing the number of transcripts were required. RT-PCR is a quick and sensitive method for amplifying a specific target RNA, and was used in combination with the degenerate primers to attempt to isolate MRP isoforms. It will also circumvent amplification of lambda DNA since no vector sequences are present. Previous studies have shown that it is possible to induce the expression of MRP isoforms
with several compounds: these include aminotriazole and menadione which induce *AtMRP3* and *AtMRP4* respectively (Sánchez-Fernández, *et al.*, 1998), and phenobarbital which is a known inducer of wheat *MRP1* (Theodoulou, *et al.*, 1998) and of xenobiotic detoxifying enzymes in plants and mammals (Nebert and Gonzalez, 1987; Pickett and Lu, 1989). RNA from wheat treated with a variety of xenobiotics (100 μM menadione, 4 mM phenobarbital, 2 mM aminotriazole) was therefore used for RT-PCR (2.1.1.3). RNA was also isolated from control plants treated with solvents only. Various combinations of degenerate primers were employed (1a/2a and 1b/2b; figures 3.3 and 3.4). Where cDNA from aminotriazole and menadione-treated plants was used as a template, bands of ca. 800bp were obtained with primers 1a/2a and 1b/2b (figure 3.6). These DNA fragments were cloned into PCR 2.1™ (Invitrogen), and sequenced in both directions. BLAST results indicated that the closest match to these 791 bp clones was *AtMRP3* (accession no.: O24510), with 78 % identity and 69 % similarity at the amino acid level (table 3.4). The predicted amino acid sequences of the two clones obtained with primers 1b and 2b were 99 % identical, and also 98 % identical to the clone obtained with the primers 1a and 2a, it was therefore assumed that all of the clones obtained derived from the same isoform of wheat MRP. The clones were also compared to *TaMRP1*, and were found to be 53 % identical, 65 % similar at the amino acid level. The partial clone was designated partial-*TaMRP2*.

**Table 3.4: Homologies of partial *TaMRP2* cDNA clones from RT-PCR**

<table>
<thead>
<tr>
<th>cDNA template (RNA from treated wheat leaves)</th>
<th>PCR Primers</th>
<th>Best score from BLAST (accession no.)</th>
<th>Percentage amino acid identity/similarity to:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Menadione</td>
<td>1b + 2b</td>
<td>O24510</td>
<td><em>AtMRP3</em> (O24510) 69/78</td>
</tr>
<tr>
<td>Aminotriazole</td>
<td>1a + 2a</td>
<td>O24510</td>
<td><em>TaMRP1</em> 70/79</td>
</tr>
<tr>
<td>Aminotriazole</td>
<td>1b + 2b</td>
<td>O24510</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1b + 2b</td>
<td>O24510</td>
<td></td>
</tr>
</tbody>
</table>

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3.6 Library screening using the PCR-fragment TaMRP2 as a probe

Since the TaMRP2 PCR fragment was 791 bp in size, and based on the size of AtMRP3 (Tommasini, et al., 1998) represented only ca. 17% of the full-length transcript, two cDNA libraries (2.10.1, a and b) were screened in order to obtain a longer clone(s). Four hundred thousand plaques were screened from each library. The use of a homologous probe (791 bp fragment of TaMRP2; section 3.5) permitted high stringency screening of the libraries (65 °C, 0.1 x SSC).

Putative positive clones from both libraries were obtained and excised and taken through to a secondary screen. End-sequencing verified that all clones were MRP, and BLAST analysis indicated that they all shared closest homology with AtMRP3 (table 3.5). The clones were also compared to each other, and were found to originate from the same wheat MRP isoform. The sequence data for the longest clone (TTii) was incomplete because the clones were end-sequenced. The 3' end of this clone was 68% identical to AtMRP3, and although the 5' end of this clone was not homologous to any sequence data in the public databases, Arabidopsis MRPs share very little sequence homology at the 5' end, so this result was not unexpected. The clone was then fully sequenced to publication quality by gene walking (Genome Express, Grenoble). The 5 Kb clone was confirmed as the full-length MRP2 from wheat.

<table>
<thead>
<tr>
<th>Library</th>
<th>Name allocated to clone</th>
<th>Length (bp)</th>
<th>Closest homologue</th>
<th>% amino acid identity to closest homologue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Etiolated wheat shoot</td>
<td>Ttii</td>
<td>512</td>
<td>AtMRP3</td>
<td>70</td>
</tr>
<tr>
<td>(2.10.1a)</td>
<td>Ttiii</td>
<td>Ca. 5000*</td>
<td>AtMRP3</td>
<td>68 (at the 3' end)</td>
</tr>
<tr>
<td></td>
<td>Ttiv</td>
<td>1225</td>
<td>AtMRP3</td>
<td>67</td>
</tr>
<tr>
<td>Wheat shoot (2.10.1b)</td>
<td>JA2ii</td>
<td>376</td>
<td>AtMRP3</td>
<td>74</td>
</tr>
<tr>
<td></td>
<td>JA2iv</td>
<td>392</td>
<td>AtMRP3</td>
<td>62</td>
</tr>
</tbody>
</table>

Sequencing primers: clones from the etiolated wheat shoot library (2.10.1a) were sequenced with SP6 and T7. Clones from the wheat shoot library (2.10.1b) were sequenced directly with λ forward and λ reverse primers. * approximation of size by restriction enzyme analysis.
3.7 Analysis of TaMRP2 sequence and computational analysis of topology

The largest clone isolated from the safener-treated library was subjected to further sequence analysis. After the removal of the pZL1 vector sequence, TaMRP2 was 4837 bp in length, with an open reading frame of 4416 bp. The sequence at the 3' end included a TGA stop codon, 130 bp of 3' untranslated region, and a poly A+ tail. The 5' end of the sequence had an ATG start site (289 bp from the beginning of the sequence), an in-frame stop codon in the putative 5' untranslated region, and contained the most influential nucleotides of the Kozak sequence (a G at position -3 and a G at position +4; Kozak, 1996). The full length sequence of TaMRP2 is located in the appendix II, and all of the features mentioned here have been outlined. BLAST results indicated that the closest homologue of TaMRP2 was AtMRP3, as expected, sharing 60% identity and 68% similarity at the amino acid level. Further sequence analysis indicated that TaMRP2 possessed the ABC signature motifs (SGGQKQRVQIA and WSVGQRQQLVC) and the Walker A (GTVGS and IYLFD) and Walker B (GRTGS and ILVLD) motifs (figure 3.7; Higgins and Gottesman, 1992).

For an initial assessment of the overall topology of TaMRP2, a hydropathy plot was performed according to Kyte and Doolittle (1982), with a window of 17 amino acids. For comparative analysis, hydropathy plots of AtMRP3 (O24510) and human MRP1 (HuMRP1; P33527) were determined (figure 3.8). Hydropathy plots indicate that the topologies of the three MRPs shown are fairly similar, although given the quality of the graphs, it was difficult to determine if a peaked area contained 1 individual peak or 2 separate peaks. For a more detailed assessment of the topology of TaMRP2, the putative positions of transmembrane domains (TMD) were computed using the programs, DAS (http://www.sbc.su.se/~miklos/DAS/), TMpred (http://www.ch.embnet.org/cgibin/TMPRED_form.html), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM) used previously by Martinoia, et al., (2000) to predict the TMDs of AtMRP3. For comparison, the positions of putative TMDs for HuMRP1 and AtMRP3 were computed using the same programs, and these data are summarised in table 3.6. The data is based on the established model that MRP houses 3 membrane spanning domains (MSD) (Hipfner, et al., 1997), and is presented as the number of TMDs in each MSD. The topologies shown in table
3.6 are based on comparisons between the numerical and graphical data determined, and the published data available (Hipfner, et al., 1997). Predictions for HuMRP1 using all 3 programs were in agreement with each other and published data (Hipfner, et al., 1997), indicating the presence of 5+6+4 TMDs. The published model for AtMRP3 best reflects the result from the DAS program, with a topology of 5+6+4 (Martinoia, et al., 2000), although TMPred and TMHMM both predicted topologies of 5+5+4. The predicted topology of TaMRP2 based on computational data is either 5+5+4 (DAS and TMPred) or 5+3+6 (TMHMM), and in combination with the published data for HuMRP1 (Hipfner, et al., 1997), was used to generate the models in figure 3.9. Model (a) is based on the 5+5+4 topology predicted by the DAS and TMPred programs; the numbers on the model delimiting the starts of the TMDs of the MSD0, MSD1, nucleotide binding fold 1 (NBF1), MSD2 and NBF1 were deduced from the DAS program which fitted the published model for HuMRP1 more closely (TMDs predicted by DAS and TMPred only differed by a few amino acids, data not shown). Model (b) is based on the 5+3+6 topology predicted by the TMHMM program. Both proposed models suggest that the N-terminus of TaMRP2 is cytosolic.

Finally, two additional protein analysis prediction programs were used to detect putative chloroplast transit peptides, and putative N-glycosylation sites. The amino acid sequence of TaMRP2 was used as input into the ChloroP 1.1 Prediction Server (http://www.cbs.dtu.dk/services/ChloroP/), and resultant data indicated that TaMRP2 did not have a chloroplast transit peptide. The amino acid sequence of TaMRP2 was analysed using the PROSITE program (http://ca.expasy.org/cgi-bin/scanprosite) that indicated the presence of three N-glycosylation sites at amino acids 397-400 (NSSW), 725-728 (NLSG) and 1364-1367 (NWSV).
Table 3.6: Putative number of TMDs of HuMRP1, AtMRP3 and TaMRP2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Program</th>
<th>DAS</th>
<th>TMPred</th>
<th>TMHMM</th>
</tr>
</thead>
<tbody>
<tr>
<td>HuMRP1</td>
<td>DAS</td>
<td>5+6+4</td>
<td>5+6+4</td>
<td>5+6+4</td>
</tr>
<tr>
<td>AtMRP3</td>
<td>DAS</td>
<td>5+6+4</td>
<td>5+5+4</td>
<td>5+5+4</td>
</tr>
<tr>
<td>TaMRP2</td>
<td>DAS</td>
<td>5+5+4</td>
<td>5+5+4</td>
<td>5+3+6</td>
</tr>
</tbody>
</table>

The amino acid sequences of HuMRP1 (P33527), AtMRP3 (O24510) and TaMRP2 were used as input for 3 membrane prediction programs: DAS (http://www.sbc.su.se/~miklos/DAS/), TMPred (http://www.ch.embnet.org/cgi-bin/TMPRED_form.html), and TMHMM (http://www.cbs.dtu.dk/services/TMHMM). Data represents the predicted number of TMDs in MSD0, MSD1 and MSD2, respectively based on the computational analysis and the published data for HuMRP1 (Hipfner, et al., 1997).

3.8 Phylogenetic analysis of TaMRP2

The amino acid sequences of the AtMRP1-15, TaMRP2, YCF1, HuMRP1, RatMOAT, HuCFTR, AtPGP and HuMDR were aligned using the ClustalX program (version 1.8) (Thompson, et al., 1997). Interestingly, AtMRP11 and AtMRP15 lacked the hydrophobic MSD0 region. Likewise, HuMDR, AtPGP and HuCFTR also lacked the MSD0 although these topologies had been reported previously (reviewed by; Varadi et al., 1998). Regions within the alignment containing 4 or less sequences and poor alignments were ‘masked’ from the final alignment. The optimal alignment was used as input to the PHYLogeny Inference Package (PHYLIP) version 3.5., PROTDIST (PAM matrices; Dayhoff, 1978) was used to calculate distances between proteins, and FITCH to estimate the phylogenies from the distance matrix data. The resulting phylogram is depicted in figure 3.10. The confidence level of each hypothetical node was assessed by the analysis of 100 computer-generated trees (bootstrap replicates), and was above the accepted 70 % cut-off (Dr. F. Wright Pers. comm.), indicating statistical significance. The MRP, MDR and CFTR sub-families formed distinct groups, with the MRPs closer to the CFTR sub-family than the MDR sub-family. The MRP sub-family itself forms 2 distinct groups, one containing TaMRP2 and AtMRPs 3-10, 14 and 15. AtMRP1, 2, 12 and 13 form a second group that
includes the yeast MRP orthologue, YCF1, HuMRP1 and the MRP sub-family member RatMOAT. AtMRP11 lies on its own between the 2 MRP groups. TaMRP2 is more closely related to AtMRP3, than to other members of the Arabidopsis MRP family, with only 2 hypothetical ancestors separating them (indicated by branches on the tree).
The *Arabidopsis* MRP (AtMRP) sequences were obtained from public databases: EMBL, Genbank and Swissprot [AtMRP3 (U92650) and AtMRP4 (AJ002584)] and were used together with the sequence of TaMRP1 (Theodoulou, 1998 #240) as input to the GCG LOCALPILEUP programme to produce multiple sequence alignments. The GeneDoc programme was used to highlight conserved regions. The antiserum was raised to a fusion of maltose-binding protein and amino acids 543-768 of TaMRP1.
The *Arabidopsis* MRP (AtMRP) sequences were obtained from public databases: EMBL, Genbank and Swissprot [AtMRP1 (AF008124), AtMRP2 (Af020288), AtMRP3 (U92650), AtMRP4 (AJ002584), AtMRP5 (Y11250)], and were used together with the sequence of TaMRP1 (Theodoulou et al., 1998) as input to the GCG PILEUP programme to produce multiple sequence alignments. The GeneDoc programme was used to highlight conserved regions. Boxed areas indicate where degenerate primers were designed (figure 3.3), and arrows indicate the direction of the primers.
Figure 3.3: Design of degenerate primers based on Arabidopsis MRP and wheat MRPl sequences

IUB symbols are used to represent nucleotides and ambiguities. Single letter amino acid symbols are used. The nucleotide symbol for guanine is in lower case for clarity.

I = inosine

**Primer la** (most similar to AtMRP3)

amino acid seq. and back translation:

E W L C F R L D M

gAA Tgg CTN TgT TTT AgA TTA gAT ATg

g T A C C C g g C

choice of bases:

5'-gAg Tgg YTI TgC TTY MgI YTN gAY Atg-3

**Primer lb** (most similar to AtMRP5)

amino acid seq. and back translation:

E W L C L R M E

gAA Tgg CTN TgT CTN AgA ATg g..

g TTA C T A g

g CgN

choice of bases:

5'-gAg Tgg YTI TgC YTI MgN ATg g-3'

**Primer 2a** (common to all plant MRPs)

amino acid seq. and back translation:

D E A T A S V D

gAT gAA gCN ACN gCN TCN gTN GAT

choice of bases:

gAY gAR gCN ACI gci Ag

reverse complement:

5'-CTI gCI gTN gCY TCR TC-3'

**Primer 2b** (common to all plant MRPs)

amino acid seq. and back translation:

D E A T A S V D

gAT gAA gCN ACN gCN TCN gTN GAT

choice of bases:

gAY gAR gCN ACI gCI TC

reverse complement:

5'-GAI gCI gTN gCY TCR TC-3'
Figure 3.4: PCR strategy for amplification of MRP from directional cDNA libraries

Schematic diagram showing a generalised clone from the cDNA library. The hatched area represents vector DNA. Figure not to scale. Possible combinations of vector and degenerate primers are indicated below.

Combinations of vector/degenerate primers:

\[
\begin{align*}
&1a + SP6 & & 1a + 2a \\
&1a + For & & 1a + 2b & & \text{Expected insert} \\
&1b + SP6 & & 1b + 2a & & \text{Expected insert} \\
&1b + For & & 1b + 2b \\
\end{align*}
\]

1a/1b - Motifs specific to subgroups of plant MRP\(\text{s}\)

2a/2b - Conserved motifs common to all plant MRP\(\text{s}\)
Figure 3.5: Electrophoretic analysis of PCR products amplified from a directional wheat cDNA library

PCР of a safener-treated wheat shoot cDNA library (2.10.1a) using degenerate and vector primers. DNA was separated by electrophoresis on a 1 % agarose/TAE gel. 1 Kb and 123 bp markers were used to determine the size of the bands, sizes are indicated at the left hand side of the gel image. Bands excised and cloned are marked with an arrow.

Primer combinations:
1. 1a + SP6  7. 1b + 2b  13. positive control
2. 1a + For   8. 1b + 2a
3. 1b + SP6  9. 1a + 2a
4. 1b + For  10. 1b + 2b
5. 1a + 2a   11. 1a + SP6
6. 1a + 2b  12. 1b + For
1-8: 1μl of library (a), and 12.5 pmol of each primer
9-12: negative controls without DNA, 12.5 pmol of each primer
13: positive control: 25 ng TaMRPl DNA + TaMRPl specific primers (12.5pmol).

PCR conditions:
Denaturation 95 °C for 30 sec, annealing at 45 °C for 30 sec and extension at 72 °C for 4 min. A total of 30 cycles were performed. The final extension continued at 72 °C for 10 min.

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Figure 3.6: Electrophoretic analysis of RT-PCR products

RT-PCR using RNA from treated wheat shoots and degenerate primers. DNA was separated by electrophoresis in a 1% agarose/TAE gel. 1 Kb and 123 bp markers were used to determine the size of the bands, sizes are indicated at the left hand side of the gel image. Bands excised and cloned are marked with an arrow.

Wheat treatments and primer combinations:
1. Control (1a + 2a)  
2. Control (1b + 2b)  
3. Phenobarbital (1a + 2a)  
4. Phenobarbital (1b + 2b)  
5. Menadione (1a + 2a)  
6. Menadione (1b + 2b)  
7. Aminotrizole (1a + 2a)  
8. Aminotrizole (1b + 2b)  
9. positive control*  
10. negative control*

50 ng of template and 12.5 pmol of each primer was used in each reaction.
*Control primers, RNA, and water were provided in the kit for positive and negative control reactions.

PCR conditions:
First strand cDNA synthesis: reverse transcription, 45 min at 48°C, AMV RT inactivation and RNA/cDNA/primer denaturation for 2 minutes at 94°C.
Second strand cDNA synthesis and PCR amplification: denaturation for 30 sec at 94°C, annealing for 1 min at 60°C, extension for 2 min at 68°C. A total of 40 cycles were performed. The final extension continued for 7 min at 68°C.
Figure 3.7: Amino acid sequence of TaMRP2

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1  MAATASERSL PCLFLHGASA GAHLILALAV AGSRLFPRRG KDTAVAVGGF
51  RCYAVAAACAT WALTAAFLLLL AAYSYCLGAG AGWPLDAVVE QADAARARAVA
101  WLLLAAYLQF EFRREERFPA PLRLWALFL LLSVVAVAVH AATSLLGDPV
151  PARSWALDAV SVLAAVVLVL AGLFAGKSELA GGSAESEPLL DGASESDSAD
201  ASAFAGAGLQ GVLAFSWMGP LLAVGHKKAL GLEDVPEDDP GDVSAGLLPS
251  FKNLLETLSG DQFPCQVTAF FKLAQKVLRT FRWHYAVT ALYLVYNVATY
301  VGYLIDSLV QYLGQDDERH ARKGQLLLLQ LAIFAPFPGF PRLPSSSSDA
351  QGIRARSLA VLYQYKGGLA GSSRRAHSGG AGMWNVGVV DADVGNSSSW
401  YIHDMLVLPL QYGRMAMFLY STGRLASLAA LGATAAVMLV NVPKVQVEK
451  LQQNLMRSDK VRMKATSEIRL RNMRIKQGQ WEMKFLSKII ALRKTETWML
501  KKYLTBSTII TPIFWSAPTF IAVTVFGACW LGMIPLHSGK VLALATLRLR
551  LQESIYNLDP RSNAIQTQKV SLDRIASPLC LEFVPTAVQ RLPQSSDVA
601  IEVSNQGFSW DASPEMPTKL DLSIFARQGM RVACCTVGSG CKSSLSSCIL
651  GEPKLQGSGV KTCTGVTAYVS QSAWIKGQK QENILFGKQM DSEKDYVRLE
701  LCSLKDLES FPSGQTVTQL GREGNQLGGQ KQRVQAIRAL YQQADTVLYLD
751  DPFSAVDAHT GSHIPECQG LALAQTLYVL VHNLQLPLLA ADLILVKEGD
801  VIAQISGRYND ILSSGEEFMQ LVGAHQQDALA AIDAIQVPNG ASEAFSSSSDA
851  ASLSSLPSA DKKLQMNQKQ DSGHQQSAPQ VQEEERERGR VGFVYMKYL
901  TLAYGALVP FVLLQALMFLVE VHLSASYNWN AWAPASQDVE EPPSMYLYLI
951  YYVVALAGLS SVCTFVRALF LVPAAYKTAT LFPKMNHSV LPRMSFSSDS
1001  TPSGRILNRA STDQULVTS IANRMGSAIF AFIQLEGTVI VMSQVAVQVF
1051  VVFIPVIAIC LWQRYIITDE ARELQRMVGI CKAPIQHFP EFIGSTIIR
1101  SFGKEQQLSL TNPQLNMDAY RPKFYNAGAM EWLFRMDML SSLTPAISLI
1151  PLINDPTGI DPNAGLQVT YGLNINLNSV TLVSTMNLG KXISSERIL
1201  QYLTSPPAEAP LSMSDEGLIA NPWQIGEINL HNLHVYAPQ LFPVNLGTIV
1251  TFGGMRKGTI GSRTRGSGKST LIQALFRIMD PTHQQITVGD VDICTGILHD
1301  LSLRISIPQ DPTMDFGTVR HNLDPGLETY DNYQWALDH CQGDEVKRR
1351  ELKDLTPVVE NGEMWSVQLQ QRQVLCGRLML RRRKLTVLIE ATASVADTATD
1401  NLIQKTLQOH QSGATVITI HRITSVLHSD IVLDDNGMA VEHQTARLL
1451  EDKSSLFSKSL VAETYTMSTR T* 
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The Walker A and B motifs (sequences GTVGS and IYLFD and GRTGS and ILVLD respectively) and the ABC signature motifs (sequences SGGGKQRQIA and WSVGQRQQLVC) of the deduced amino acid sequence of TaMRP2 are indicated by the turquoise and yellow shaded boxes. Putative transmembrane spans were identified using a transmembrane prediction program: DAS (http://www.sbc.su.se/~miklos/DAS/) and are outlined in bold. The positions of MSD1 and the R domain (sub-class specific features of MRPs), are indicated by underlining and italic letters respectively, and are based on the model of HuMRP1 (Hipfner, et al., 1997).
Hydropathy plots were performed according to Kyte and Doolittle (1982) with a window of 17 amino acids (http://bioinformatics.weizmann.ac.il/hydrop/). The GenBank/EMBL accession numbers for the proteins used in this comparison are the following: AtMRP3 (U92650); HuMRP1 (L05628). Positions of the multiple spanning domains (MSDs), the nucleotide binding folds (NBFs), and the regulatory (R) domain were based on the model of HuMRP1 (Hipfner, et al., 1997), and the hydropathy plots.
The topological models of TaMRP2 are based on the proposed models of AtMRP3 (Martinoia et al., 2000) and HuMRP1 (Hipfner et al., 1997). The predicted membrane spans of TaMRP2 were predicted using (a) the transmembrane prediction programs DAS (http://www.sbc.su.se/~miklos/DAS/) and TMPred http://www.ch.embnet.org/cgi-bin/TMPRED_form.html, or (b) the TMHMM prediction program (http://www.cbs.dtu.dk/services/TMHMM). The numbers delimit the starts of the membrane-spanning section of the membrane spanning domain 1 (MSD1), MSD2, nucleotide binding fold 1 (NBF1), MSD3 and NBF2. The Walker A and B, the ABC signature motifs, and the regulatory domain are denoted A, B, C and R respectively. Please refer to text for discussion regarding cytosolic locations of the NBFs, and vacuolar localisation of TaMRP2. Model not drawn to scale.
Protein sequence alignments were determined using CLUSTAL X, 1.8 (Thompson et al., 1997). Regions containing 4 or less sequences, and poor homology were masked from the final alignment. Phylogenetic analysis was carried out using the PHYLogeny Inference Package (PHYLIP), version 3.5. PROTDIST (PAM matrices) was used to calculate the distance between the proteins, and FITCH to estimate the phylogenies from the distance matrix data. Confidence levels of the hypothetical nodes were assessed by the analysis of 100 computer-generated trees (bootstrap replicates), and were above the established 70% cut-off. The Genbank/EMBL accession numbers for the proteins used in this comparison are as follows: AtMRP1 (AF008124); AtMRP2 (AF020288); AtMRP3 (U92650); AtMRP4 (AJ002584); AtMRP5 (Y11250); AtMRP6 (Q9LU34); AtMRP7 (Q9LK62); AtMRP8 (Q9LK63); AtMRP9 (Q9MIC7); AtMRP10 (QJLZJ5); AtMRP11 (Q9SKX0); AtMRP12 (Q9C8H1); AtMRP13 (Q9C8H0); AtMRP14 (Q9LYS2); AtMRP15 (AL358732); HuMRP1 (P33527); YCF1 (L35237); RatMOAT (L49379); HuCFTR (M28668); HuMDR (M14758); AtPgp (X61370). TaMRP2 is highlighted in red.
3.9 Discussion

In summary, four approaches to clone *MRP* homologues from wheat were adopted: library screening with a double-stranded DNA probe derived from an *AtMRP3* EST; library screening with anti-TaMRP1 polyclonal antiserum; amplification of *MRP* homologues by PCR using degenerate primers and RT-PCR employing degenerate primers. Of these, RT-PCR was successful. Partial wheat cDNA clones were isolated by RT-PCR performed with degenerate primers (1a/2a and 1b/2b) and RNA extracted from wheat treated with menadione and aminotriazole. All clones were shown to be partial *MRP* homologues from wheat. One of these clones (aminotriazole treated wheat, primers 1a/2a) was used as a probe to screen a safener-treated etiolated wheat shoot cDNA library, from which 5 longer clones were isolated, one of which proved to be a full length MRP homologue from wheat, and was designated wheat *MRP2 (TaMRP2)*.

The approach adopted at the outset of the project was to screen 3 cDNA libraries with *Arabidopsis EST2* (Tommasini, *et al.*, 1997), a DNA sequence which was most similar to *AtMRP3*. It seemed likely that this approach may be successful, since positive plaques were obtained from a preliminary screen of one of the libraries (Theodoulou, pers. comm.). The approach however, was unsuccessful. There are several possibilities why no true positives were obtained: *AtMRP3* homologues may not be present in the library or may be present at a low abundance, and therefore insufficient pfu were screened. Alternatively, there may be insufficient homology between the *Arabidopsis EST2* and wheat *MRP* for positives to be obtained given the hybridisation conditions employed. Since carrying out the library screening, an alternative method led to the cloning of *TaMRP2*. It was therefore possible to compare the *Arabidopsis EST2* sequence with the *TaMRP2* sequence to determine how similar they were, and if indeed, *EST2* should have hybridised with wheat *MRP*. At the amino acid level, the two sequences were 69 % identical, which suggests that *EST2* should have recognised at least wheat *MRP2* if conditions were optimal. It is therefore likely that the conditions used for the primary screen were not optimal, as there is a very fine line between the conditions being too stringent and not stringent enough. It is
possible that if the conditions were too stringent, the probe would simply not have annealed with wheat MRPs, due to insufficient matching. Alternatively, if the stringency was too low the probe may have bound non-specifically, and given the low abundance of MRP, the probe may have preferentially annealed to other more abundant genes. As a general rule, well-matched hybrids should be hybridised at 65-68 °C (5-25 °C below $T_m$), and washed at similar temperatures, in low salt concentrations (0.1 x SSC). Poorly-matched hybrids should be hybridised at low temperatures (35-42 °C), and washed at intermediate temperatures (40-60 °C) in high salt concentrations (2-6 x SSC). For a detailed review on hybridisation conditions see Anderson and Young (1985). For this experiment it was assumed that given the probe was heterologous, although corresponding to a fairly conserved area, a low/medium stringency would be sufficient. To determine if the conditions used were too stringent or not stringent enough, a series of calculations and further experimentation would be necessary, although since alternative methods of isolating MRP cDNAs from wheat were available, this approach was not continued.

The second approach was to employ the TaMRP1 antiserum as a probe for library screening. Immunoscreening is one of the many accepted methods for isolating cDNAs (Young and Davis, 1983), and since the polyclonal antiserum was available for use, it was appropriate and also convenient. It was possible that the antiserum may have recognised TaMRP1, possibly isolating longer clones, or it may have recognised another wheat MRP isoform novel to the project. The clones isolated however, were not MRP isoforms. There are several possibilities why no true positives were obtained: firstly, MRP homologues may not be present in the library or be present at a low abundance and insufficient pfu were screened to detect low abundance clones. Secondly, the λ library was non-directional and in theory, only one in six inserts would be in the correct reading frame, significantly decreasing the probability of isolating a clone in-frame. Finally, membrane proteins are commonly toxic when expressed in E. coli (Serrano and Villalba, 1995), and phage containing inserts encoding partial or full-length MRP homologues may have proved toxic to the cell when their
expression was induced by IPTG. It may be due to one if not all of these reasons why TaMRP1 was not re-isolated, nor were other MRP isoforms.

The third approach was to employ degenerate primers to amplify fragments from a cDNA library. Peptide sequences of AtMRP 1-5 genes were obtained from public sequence databases (EMBL, Genbank and Swissprot), and these were used in conjunction with the sequence of TaMRP1 for degenerate primer design. Since PCR is more sensitive than the two previous approaches adopted, and degenerate primers employed were based on 6 sequences, it seemed likely that this method may be successful. Reviewing these methods with hindsight it would have seemed more appropriate to start the project using the most sensitive method available, due to the fact that MRP genes are large and non-abundant. RT-PCR, the most sensitive of the four approaches taken was tried last and gave rise to a novel, partial cDNA clone encoding a MRP. This result was significant for two reasons, the first and perhaps most obvious reason was that a second MRP isoform had been isolated in wheat, and secondly, this isoform was most similar to AtMRP3, the most xenobiotic-inducible Arabidopsis isoform cloned to date (Sánchez-Fernández, et al., 1998; Tommasini, et al., 1998). This information together with the data from the RT-PCR (MRP was isolated from menadione and aminotriazole-induced tissue) suggested that TaMRP2 was inducible.

Five MRP clones were isolated from a cDNA wheat shoot library, and a safener-treated wheat cDNA library, using the TaMRP2 PCR fragment as a probe. Sequence analysis indicated that all five clones isolated corresponded to the same TaMRP2 isoform, and one was a full length cDNA. The 5’ untranslated region of TaMRP2 contained a number of sequence characteristics expected at the start of a coding region, and sequence comparisons identified AtMRP3 as the closest homologue of TaMRP2. Further sequence analysis of TaMRP2 outlined the presence of Walker A and Walker B motifs and the ABC signature motif, which are consistent with other members of the MRP-subfamily and also the ABC transporter super-family.
The topologies of MRPs were assigned by computational analysis, using algorithms to predict where the TMDs are located. However, these 'models' can only be substantiated once biochemical data is available. Unlike other members of the ABC superfamily that have 2 MSDs, the majority of MRPs have an additional hydrophobic N-terminus (MDS0). The best studied topological model of MRP is that of HuMRP1, where the computer-generated model is supported by a growing body of biochemical data, although its precise topology still requires further experimental evidence. The topology of HuMRP1 is discussed in detail in chapter 1. Briefly, monoclonal antibodies that recognise epitopes within NBF1 (Flens, et al., 1994; Hipfner, et al., 1996) and NBF2 (Flens, et al., 1994) of HuMRP1 bind only to immunoblots or permeabilized cells, but not intact cells. These results provide the experimental evidence required to support the likely cytosolic location of the NBFs. Site-directed mutagenesis of N-glycosylation sites prove that the N-terminus of HuMRP1 is extracytosolic (Hipfner, et al., 1997). The precise number of TMDS within each MSD remains uncertain, although based on computer models and with biochemical data providing evidence for the location of specific regions of MRP, it is generally assumed that there are 5+4/6+4/6 TMDs. Recently, two-dimensional crystallisation and single-particle analysis (Rosenberg, et al., 2001) has shown that there are indeed 5 TMDs in MSD0, and the data also suggested that core structure of HuMRP contains 6 TMDs within both MSD1 and MSD2. A topological model for AtMRP3 has been proposed (Martinoia, et al., 2000), although it was derived solely from computational analysis and the analysis of HuMRP1 model (Hipfner, et al., 1997), and there is no biochemical data to support this model. One of the most obvious differences between HuMRP1 and AtMRP3 is their sub-cellular localisation. Whilst HuMRP is predominantly located in the plasma membrane (Hipfner, et al., 1994), it is assumed that AtMRP3 is vacuolar (Tommasini, et al., 1998). The working hypothesis for this thesis is that TaMRP2 is vacuolar (the ChloroP 1.1 Prediction Server indicated the absence of a transit peptide which suggests that TaMRP2 is not located within the chloroplast). With all of this information in mind, three transmembrane prediction programs (DAS, TMpred, TMHMM) previously employed to predict the TMDs of AtMRP3 (Martinoia, et al., 2000), were used to predict the TMDs for TaMRP2.
analysis, HuMRPl and AtMRP3 were also analysed. The number of TMDs predicted for HuMRPl by all 3 programs were in agreement with experimental data (Hipfner, *et al.*, 1997), HuMRPl had 5+6+4 TMDs. TMDs predicted for AtMRP3 and TaMRP2 varied between the 3 programs, although it was apparent that the predictions from the DAS program for AtMRP3 closely fitted the published model for AtMRP3 of 5+6+4 TMDs (Martinoia, *et al.*, 2000). Despite all 3 computer programs being referred to in the proposed model of AtMRP3, the models predicted by TMPred and TMHMM of 5+5+4 TMDs were rejected, with no given explanation. It must therefore be assumed that these 2 models were disregarded because they were not in agreement with the experimental data published for HuMRPl (Hipfner, *et al.*, 1997). The TMD predictions for TaMRP2 were 5+5+4 (DAS and TMPred) and 5+3+4 (TMHMM), and given these predictions and the experimental data for HuMRPl, two topological models for TaMRP2 were proposed. Both models suggest that the NBFs are cytosolic, and given the experimental data for HuMRPl, and the sub-cellular localisation of ATP pools, both plasma membrane and vacuolar MRPs would be predicted to have their NBFs in the cytosol. Assuming the location of the NBFs to be correct, the N-terminus of both of these models is located in the cytosol, and this prediction does not agree with current experimental data for HuMRPl. The PROSITE prediction program was used to indicate the location of putative N-glycosylation sites; a N-glycosylation site on the N-terminus would have suggested that the N-terminus was extracytosolic, since the process of N-glycosylation in mammalian cells occurs within the lumenal side of the endoplasmic reticulum (Kaplan, *et al.*, 1987) however, no such sites were found, in fact the three sites predicted were all cytosolic, hence inaccessible for N-glycosylation *in vivo*. The unequivocal assignment of the TMDs, and the position of the N-terminus will only be possible with further experimentation which is beyond the scope of this thesis.

Phylogenetic analysis (figure 3.10) suggests that the ABC transporters selected for inclusion in the phylogram form three distinct groups: MRP-like sequences form two groups designated a (AtMRP3-10, 14, 15, TaMRP2) and b (AtMRP1, 2, 11-13, YCF1, HuMRPl, RatMOAT, HuCFTR) for the purpose of this
discussion. MDR-like sequences form a separate group. Interestingly, the MRP groups do not appear to have a common ancestor that is distinct from MDR. It would have been interesting to include TaMRP1 on the phylogram, however this has not been done since the sequence of TaMRP1 is incomplete. Only the 3' half of the TaMRP1 cDNA is available, and it is within this area that the MRPs and other members of the ABC superfamily share the greatest homology. This might bias the resultant phylogram, possibly resulting in an inaccurate representation of the MRP/ABC superfamily ancestry.

Ancestrally, the closest MRP to TaMRP2 is AtMRP3 as expected, given that it has previously been shown that TaMRP2 is most homologous to AtMRP3. The simplest hypothesis from the phylogram is that AtMRP3 and TaMRP2 evolved from common ancestors which suggests, but does not prove that TaMRP2 might have similar functions to those exhibited by AtMRP3 [a glutathione S-conjugate (GS-X) pump]. AtMRP1 and AtMRP2 diverged from the same hypothetical node, and despite having different substrate specificities, both are GS-X pumps. As previously mentioned, the MRP-like proteins analysed form two groups: one consisting of plant MRPs, the other of mammalian, yeast and plant MRPs. This grouping suggests that all plant MRPs have evolved from a common ancestor that functioned as a GS-X pump, and diverged later. The demonstration of the chlorophyll catabolite Bn-NCC-1 transport by AtMRP2 and 3 (Lu, et al., 1998; Tommasini, et al., 1998) indicates that this indeed might be the case. Additionally, the cystic fibrosis transmembrane conductance regulator (CFTR) functions as a channel and mediates channel-channel interactions (reviewed by Davis et al., 1996)), and a recent report indicates that AtMRP5 has channel-like activities (Gaedeke, et al., 2001).

However, caution must be taken when analysing phylogenograms, and making assumptions regarding homology and functional correlations based on two proteins sharing a common ancestor. The phylogram is merely hypothetical; the programs used to make the analyses, especially pairwise protein comparisons, are based on matrix tables that ‘score’ the likelihood of one amino acid changing to another. These programs do not take into consideration that if a gene has not
evolved by duplication (although unlikely), two identical proteins may have different functions. Furthermore, studies on transporters from other protein families have shown that single amino acid substitutions can result in dramatic changes in substrate specificity, for example, if the highly conserved tryptophan at position 1246 in HuMRP1 is substituted with cysteine, the transport of 17β-estradiol 17-(β-D-glucuronide)(E217βG) is eliminated (Ito Ki, et al., 2001). Thus, proteins with a close evolutionary relationship and high overall amino acid identity can have quite different functions.

Now that TaMRP2 has been cloned, it is necessary to address its location, expression and function, which are discussed in chapter 4 and 5 respectively.
CHAPTER 4

EXPRESSION OF TaMRP2

4.1 Introduction

The aims of this chapter were to examine the chemical induction of TaMRP2 and its subcellular localisation. Since TaMRP1 and TaMRP2 were both cloned on the basis of their selective induction by chemicals (see chapter 3 and Theodoulou et al., 1998), it was necessary to test whether the transcripts and protein were indeed induced. Moreover, as discussed in Chapter 1, the Arabidopsis MRPs, most notably AtMRP3, are increased by chemical induction (Sánchez-Fernández, et al., 1998; Tommasini, et al., 1997). It was therefore likely that TaMRP2, which shared the greatest sequence similarity with AtMRP3, may also be inducible by a wider range of compounds. The expression of TaMRP2 was therefore examined on two levels; RNA and protein, by Northern and Western analysis, respectively. The induction of TaMRP1 was also examined for comparison.

The production of anti-MRP antibodies offers a route not only to examine induction of proteins but also to localising the proteins in the plant. Glutathione S-conjugate transport activity has been demonstrated in isolated vacuoles and tonoplast vesicles from plants (Li, et al., 1995a; Martinoia, et al., 1993), and AtMRP2 was recently located in the tonoplast (Liu, et al., 2001). It therefore seems probable that TaMRP2 might be vacuolar. The anti-TaMRP1 antiserum
that recognises a band of ca. 170 kDa in treated wheat shoot vacuolar membrane fractions may also recognise TaMRP2 (Theodoulou et al., 1998; see Chapter 3), however to distinguish between the two wheat MRP isoforms in planta, specific antibodies were raised.

4.2 Northern analysis: the effect of chemical treatment on RNA abundance
Northern analysis was employed to determine if the expression of TaMRP2 was constitutive, or if transcript abundance could be increased by chemical induction. Total RNA was extracted from wheat shoots treated with aminotriazole (2 mM), menadione (100 μM) and phenobarbital (4 mM), untreated tissue was used as a control. High stringency Northern blots (0.1 x SSC, 65 °C) were performed employing the partial TaMRP2 clone amplified by RT-PCR as a probe (section 3.5). Due to the low abundance of MRP however, blots were not of publication quality (data not shown). This problem was circumvented by using messenger RNA (mRNA) prepared from pooled samples of total RNA: a band of ca. 5.3 Kb, was detected in untreated tissue; aminotriazole and phenobarbital slightly increased expression, but the greatest effect was observed in menadione treated tissue (figure 4.1a). The Northern blot was also probed with TaMRP1 (obtained from Dr F. Theodoulou, IACR-Rothamsted) to determine if inducibility patterns of the two wheat MRP isoforms were similar (figure 4.1b). TaMRP1 was detected in all tissues, although the band in the untreated lane was barely detectable. Both menadione and aminotriazole increased expression although the greatest effect was observed in phenobarbital treated tissue. A constitutively expressed clone, wheat actin, was used to confirm equal loading on the blot (figure 4.1c).

TaMRP1 was isolated as a safener-induced gene and Northern analysis confirmed that it was safener inducible (4.2a). It was therefore interesting to test whether TaMRP2 was also safener inducible. Chemical induction by herbicide safeners was examined by probing a pre-existing blot with TaMRP2 (obtained from Dr F. Theodoulou). The blot was of mRNA extracted from etiolated wheat seedlings treated with phenobarbital, the safener cloquintocet-mexyl and phenobarbital, the safener fenchlorazole-ethyl and an untreated control. The blot
was previously probed with *TaMRP1* (figure 4.2a), and whilst expression was observed in all tissue samples, herbicide safeners induced expression most significantly in cloquintocet-mexyl and phenobarbital treated tissue. The blot probed with *TaMRP2* (figure 4.2b) showed similar expression patterns, although levels of expression appeared lower compared to those exhibited by *TaMRP1*.

### 4.3 Detection of TaMRP2 protein

#### 4.3.1 Assessment of anti-TaMRP1 antiserum for detection of TaMRP2

Antibodies that recognise TaMRP2 were required to monitor protein expression in plants and in heterologous systems, and to determine the subcellular location of TaMRP2 in plant cells. As discussed in Chapter 3, an anti-TaMRP1 antiserum was available to the project, and from amino acid comparisons, it seemed likely that the antiserum may recognise TaMRP2. To test the antiserum, the 171 C-terminal amino acids of TaMRP2 were expressed as a GST fusion protein in *E. coli*. A 670 bp PCR fragment encoding amino acids 1302-1472 of TaMRP2 containing a stop codon was sub-cloned into the GST fusion vector pGEX-4T-3 (Amersham Pharmacia; pGEX/MRP2). This vector contains an inducible *tac* promoter and a GST from *Schistosoma japonicum*, and a cDNA cloned in frame with the GST can be induced by isopropyl β-D-thiogalactopyranoside (IPTG). *E. coli* strain XL1 Blue was transformed with the construct pGEX/MRP2. Cells were grown to an OD$_{600nm}$ of 0.5 and treated with 1 mM (final concentration) IPTG to induce expression. Samples were collected 0, 3 and 6 hours after induction with IPTG, samples from uninduced cells and cells transformed with empty vector were collected as negative controls. A construct containing the 110 C-terminal amino acids of TaMRP1 fused to GST (pGEX/MRP1; Theodoulou, unpublished) was used as a positive control. To determine if the protein of interest was induced over time and in the presence of IPTG, identical amounts of the crude lysates were separated in 12.5 % (w/v) SDS-polyacrylamide gels and visualised by staining with Coomassie Blue. The results indicated that 3 and 6 hours after induction (figure 4.3), bands of approximately 25 kDa (pGEX-4T-3), 38 kDa (pGEX/MRP1) and 45 kDa (pGEX/MRP2) were present at higher levels than those exhibited at 0 hours (data not shown). There were no differences between samples in the presence or absence of IPTG (data not shown). To test the anti-TaMRP1 antiserum, crude lysates taken at 0 and 3 hours after IPTG
treatment were separated in a 12.5 % (w/v) SDS-polyacrylamide gel, and transferred onto nitrocellulose membrane (Hybond C Extra, Amersham Pharmacia). The pre-immune serum did not recognise any bands (data not shown). The anti-TaMRPI antiserum recognised bands of approximately 25 kDa (pGEX-4T-3), 38 kDa (pGEX/MRPI) and 45 kDa (pGEX/MRP2) (figure 4.4). The expression of pGEX/MRPI and pGEX/MRP2 was induced in the presence of IPTG. The antiserum also recognised additional bands to those expected, and it is likely that they were a result of non-specific binding or proteolysis (figure 4.4).

4.3.2 Western analysis of wheat microsomes using anti-TaMRPI antiserum
Since transcript levels of TaMRP2 can be chemically induced, and the anti-TaMRPI antiserum recognises TaMRP2, chemical induction of wheat MRP protein was examined by Western blotting. Wheat microsomes were prepared from wheat shoots treated with aminotriazole (2 mM), menadione (100 μM) and phenobarbital (4 mM), and untreated tissue was used as a control. Microsomes (10 μg) were separated in a 7.5 % (w/v) SDS-polyacrylamide gel, and blotted onto a nitrocellulose membrane (Hybond C Extra, Amersham Pharmacia). The TaMRPI antiserum recognised multiple bands (figure 4.5) however, comparison with the pre-immune serum indicated that the anti-TaMRPI antiserum recognised a novel band of approximately 170 kDa present in the phenobarbital-treated sample.

4.4 Selection of peptide antigens for specific antibody production
Antibodies that distinguished between TaMRPI and TaMRP2 were required, therefore specific peptides were selected for antibody production. Multiple sequence alignments of TaMRPI, TaMRP2 and AtMRPs 1-5 were produced in GCG using the LOCALPILEUP program and were examined for regions specific to TaMRPI and TaMRP2 (figure 4.6). It was important that the peptides were designed to a region that was not located within the membrane, therefore the TopPredII program (http://bioweb.pasteru.fr/seqama:/interfaces/toppred.html) was employed to predict transmembrane domains (TMDs) of TaMRPI and TaMRP2, and these were used in conjunction with a hydropathy plot of TaMRP2 (see Chapter 3). The data considered for peptide design is summarised in figure 4.7: a GAP (GCG) alignment of TaMRPI and TaMRP2 indicating; the region
where similarities between the AtMRPs and TaMRPs were low (see also figure 4.6), putative TMDs, and the region to which the anti-TaMRP1 antiserum was raised. Sequences of 20-25 amino acid residues, which were largely hydrophilic were selected, and this information was submitted to Sigma-Genosys who advised on feasibility of peptide synthesis, and probable antigenicity. Two peptides were selected:

TaMRP1/pep: CEGAAPVSDEKGETPAISRQPSRKG
TaMRP2/pep: CGSLPSADKKDKQNVKQDDGHGQSG

These peptides were synthesised by Sigma-Genosys and conjugated to keyhole limpet haemocyanin (KLH). Antisera were raised using the two peptides at the antibody facility at IACR-Rothamsted, and the pre-immune sera and antisera from the second bleeds were assessed by dot blotting and Western analysis. Analysis of the dot blots (data not shown) indicated that the pre-immune serum for TaMRP1/pep and TaMRP2/pep did not recognise the respective peptides. The anti-TaMRP1/pep antiserum detected 100 mg of the corresponding peptide at a dilution of 1 in 1,000, although the signal was very weak. The anti-TaMRP2/pep antiserum detected 1 µg of the corresponding peptide at a dilution of 1 in 1,000, once again the signal was weak.

To determine whether the sera recognised TaMRP1 and TaMRP2 in planta, Western blot analysis of wheat shoot microsomes and tonoplast vesicles was performed and comparisons between the anti-TaMRP1/pep and TaMRP2/pep antisera, and the original anti-TaMRP1 antiserum were made. Fifteen µg of wheat shoot tonoplast vesicles were separated in a 7.5 % (w/v) SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (Hybond C Extra, Amersham Pharmacia). The anti-TaMRP1 antiserum recognised a band of ca. 170 kDa (figure 4.8), whereas the anti-TaMRP1/pep and TaMRP2/pep antisera did not recognise bands of the approximate expected size.
Figure 4.1: Northern analysis of *TaMRP1* and *TaMRP2*: effect of chemical treatments

Light grown wheat seedlings were treated on days 4 and 6 by watering with: 2 mM aminotriazole (A), 100 μM menadione (M), 4 mM phenobarbital (P). Untreated plants (U) as a control. Seedlings were harvested on day 8. Messenger RNA was separated by electrophoresis, and transferred to nitrocellulose membrane (Hybond NX, Amersham Pharmacia). (a) Filter probed with *TaMRP2*. (b) Blot from (a) stripped and reprobed with *TaMRP1*. (c) Constitutively expressed wheat *actin* was used to confirm equal loading on the blot (a). Blots were screened at high stringency: 0.1 x SSC; 65 °C.
Dark grown wheat seedlings were treated with phenobarbital (P), cloquintocet-mexyl and phenobarbital (C/P), or fenchlorazole-ethyl (F). Cloquintocet-mexyl was applied as a seed drench at 0.1 % seed weight. Fenchlorazole-ethyl was applied as a seed drench at 10 mg/ml. Phenobarbital (4 mM) was watered on to germinated seedlings. Messenger RNA was separated by electrophoresis, and transferred to nitrocellulose membrane (Hybond NX, Amersham Pharmacia). (a) Filter probed with TaMRP1 (Image provided by F. Theodoulou). (b) Blot (a) stripped and reprobed with TaMRP2.
TaMRP2 171 C-terminal amino acids and TaMRP1 110 C-terminal amino acids were expressed in *E.coli* as a GST fusion protein from plasmid pGEX-4T-3 (Amersham Pharmacia). Cells were grown to an OD$_{600}$nm of 0.5 and samples were collected at 3 h and 6 h after induction with IPTG. Samples from cells transformed with empty vector were collected as controls. Identical amounts of crude bacterial lysate were subjected to SDS-PAGE on a 12.5 % (w/v) gel, and visualised by Commassie Blue staining. Samples 1, 2 and 3 refer to pGEX-4T-3, pGEX/MRP1 and pGEX/MRP2 respectively.
Figure 4.4: Western analysis of GST-fusion proteins: assessment of the anti-TaMRP1 antiserum for the detection of TaMRP2

TaMRP2 171 C-terminal amino acids and TaMRP1 110 C-terminal amino acids were expressed in E.coli as a GST fusion protein from plasmid pGEX-4T-3 (Amersham Pharmacia). Cells were grown to an OD$_{600nm}$ of 0.5 and samples were collected at 0 and 3 h after induction with IPTG. Samples from uninduced cells and cells transformed with empty vector were collected as negative controls. Identical amounts of crude bacterial lysate were subjected to SDS-PAGE on a 12.5 % (w/v) gel, transferred onto nitrocellulose membrane (Hybond C Extra, Amersham Pharmacia) and probed with the anti-TaMRP1 antiserum. The pre-immune serum did not react to produce bands. Samples 1, 2 and 3 refer to pGEX-4T-3, pGEX/MRP1 and pGEX/MRP2 respectively. Additional bands are likely to be a result of non-specific binding.
Wheat microsomes were prepared from wheat shoots treated by watering on days 4 + 6 with: 2 mM aminotriazole (A), 100 μM menadione (M), 4 mM phenobarbital (P) and an untreated control (U). Seedlings were harvested on day 8. 10 μg of microsomes were subjected to SDS-PAGE on 12.5 % (w/v) gels, blotted onto nitrocellulose membranes (Hybond C Extra, Amersham Pharmacia) and probed with the anti-TaMRP1 antiserum or pre-immune serum.
The \textit{Arabidopsis} MRP (AtMRP) sequences were obtained from public databases: EMBL, Genebank and Swissport (AtMRP1 [AF008124], AtMRP2 [AF020288], AtMRP3 [U92650], AtMRP4 [AJ002584], AtMRP5 [Y11250]), and were used together with the sequences of TaMRP1 [Theodoulou, 1998 #240] and TaMRP2 as input to the GCG PILEUP program to produce multiple sequence alignments. The GeneDoc program was used to highlight conserved regions. The area considered for peptide design is highlighted in red.
Figure 4.7: Peptide design

GAP alignment of TaMRP1 and TaMRP2. The area marked in blue indicates where sequence similarities between the wheat and *Arabidopsis* are low. Putative transmembrane domains were identified using the TopPred II program (Claros and von Heijne, Karolinska Institute, Stockholm, Sweden), and are indicated by bold face letters. The TaMRP1 MBP fusion protein antiserum (Theodoulou *et al.*, 1998) was raised to the area highlighted in yellow. Specific AtMRP1 and AtMRP2 peptides are highlighted in turquoise and named TaMRP1/pep and TaMRP2/pep respectively.
Figure 4.8: Western analysis of wheat shoot tonoplast vesicles: comparison of anti-TaMRP1, MRP1/pep and MRP2/pep antiserum

Wheat shoot tonoplast vesicles (15 μg) were separated in a 7.5 % (w/v) SDS denaturing gel, transferred onto nitrocellulose membrane (Hybond C Extra) and probed with anti-TaMRP1 (Theodoulou et al., 1998), MRPl/pep and MRP2/pep antisera. The pre-immune sera did not react to produce bands (data not shown).
4.5 Discussion

This chapter examined the expression of TaMRP2 from 2 perspectives; RNA expression and protein expression. Initially, the effect of chemicals on transcript abundance was examined, two groups of chemicals were employed; those known to induce stress, and herbicide safeners, which were originally used to help clone both TaMRP1 and TaMRP2. The first group comprised: aminotriazole, menadione and phenobarbital. Aminotriazole and menadione cause oxidative stress, through the inhibition of catalase and the generation of superoxide respectively, and phenobarbital is a barbiturate known to up-regulate genes involved in oxidative metabolism (Reichhart, et al., 1980). Northern analysis of mRNA indicated that all three chemicals induced the transcript levels of both TaMRP1 and TaMRP2 compared to an untreated control, however the two TaMRPs were differentially regulated. Whereas phenobarbital was the strongest inducer of TaMRP1, menadione was clearly the strongest inducer of TaMRP2 compared to the untreated control. The differential expression of TaMRP1 and TaMRP2 is not unexpected, the AtMRPs are differentially expressed upon treatment with different chemicals (Sánchez-Fernández, et al., 1998; Tommasini, et al., 1997). Interestingly, the two TaMRPs do not exhibit the same patterns of induction as their closest orthologues in Arabidopsis. Whereas menadione was the strongest inducer of TaMRP2, in Arabidopsis menadione did not significantly induce AtMRP3, the closest orthologue of TaMRP2, although it did induce AtMRP4 the AtMRP most similar to TaMRP1 (Sánchez-Fernández, et al., 1998). The induction of TaMRP1 and TaMRP2 by the three compounds tested indicates that they may both play a role in oxidative stress tolerance, and there are several lines of evidence for this in the literature (see section 1.4.12). Glutathione-S-conjugate (GS-X) pumps can transport oxidised glutathione (GSSG), a product of antioxidant actions (Lu, et al., 1998; Tommasini, et al., 1993). Products derived from the action of active oxygen species (AOS) can be transported by GS-X pumps (Ishikawa, 1992; Konig, et al., 1999), and also, compounds known to react to form AOS can up-regulate the expression of these GS-X pumps. Moreover phenobarbital can also up-regulate plant and animal glutathione-S-transferases (GSTs) which precede MRP in the detoxification pathway (Ogawa, et al., 2000; Pickett and Lu, 1989; Schrenk, et al., 2001; Theodoulou,
unpublished observations). Finally, the YAP1 gene encoding a yeast transcription factor regulates a network of genes known to be involved in defence against oxidative stress, including the MRP orthologue YCF1 and GSH1 which encodes the first enzyme in glutathione synthesis (DeRisi, et al., 1997; Wemmie, et al., 1994; Wu and Moyrowley, 1994).

Considering what is already known about MRP and oxidative stress tolerance, it seems likely TaMRP1 and 2 may play a role in oxidative stress tolerance, however further experimental data is required to determine if they exhibit any of the characteristics exhibited by MRP and MRP-like genes, and other genes known to be involved in oxidative stress tolerance.

The effect of herbicide safeners on the expression of TaMRP2 was examined for several reasons: firstly, safener inducibility was employed to facilitate the cloning of TaMRP1; secondly, herbicide safeners have been shown to induce AtMRP1, 3 and 4 (Sánchez-Fernández, et al., 1998), and finally experiments conducted in our laboratory indicated that TaMRP1 transcripts were induced by safeners. The blot used to obtain this result was available to re-probe with TaMRP2. Two safeners: cloquintocet-mexyl and fenchlorazole-ethyl induced the transcriptional levels of the TaMRP isoforms compared to an untreated control. However, the effects of cloquintocet-mexyl were examined in the presence of phenobarbital, and although the mix had an additive effect on the induction of TaMRP1 and 2, compared to treatment with phenobarbital alone, and it is likely that cloquintocet-mexyl does induce the two TaMRPs, Northern analysis of RNA from wheat treated only with cloquintocet-mexyl is required.

Herbicide safeners are used to protect selectively monocotyledonous crops against certain herbicides, without affecting the ability of the herbicide to control weeds. Safeners predominantly function by inducing herbicide metabolism within the crop for example, through the transcriptional regulation of P450 mono-oxygenases and GSTs (Davies and Caseley, 1999). The safeners examined here, fenchlorazole-ethyl and cloquintocet-mexyl have been shown to induce the metabolism of their respective herbicides (Kreuz, et al., 1991; Romano, et al., 1993). In wheat, fenchlorazole-ethyl induces the level of GSH (Tal, et al., 1995),
and the activity of GSTs (Cummins, et al., 1997). May et al., (1998a) have examined the effect of fenchlorazole acid on glutathione metabolism in Arabidopsis suspension cultures and found that GST activity and levels of GSH were induced. GSH is synthesised in two ATP-dependent steps, catalysed by γ-glutamylcysteinylglycine (γECS) and glutathione synthetase (GSHS). Whilst the activity of γECS was induced by fenchlorazole acid, transcription of the γECS gene was not induced, which suggested that γECS is post-transcriptionally regulated. In contrast, the activity of the GSHS, and the transcription levels of the corresponding gene, were not induced by fenchlorazole acid. This is consistent with the established hypothesis that γECS is the rate-limiting step in GSH biosynthesis.

Fenchlorazole also induces the transcriptional response of AtMRP1, 3 and 4 in the same cell cultures (Sánchez-Fernández, et al., 1998). Similarly, the safener cloquintocet-mexyl induces GST activity in wheat (Riechers, et al., 1997), and the activity of the vacuolar GS-X pump in isolated barley vesicles, where induction is due to the increased expression of the transporter (Gaillard, et al., 1994). Thus the regulation of TaMRP1 and 2 by herbicide safeners is consistent with the up-regulation of herbicide metabolism by safeners, providing further evidence that TaMRP1 and 2 may play a role in xenobiotic detoxification.

The next question addressed was whether an existing antibody (anti-TaMRP1, Theodoulou, et al., 1998) would recognise TaMRP2 permitting its use, for example, in tracking recombinant TaMRP2 in heterologous systems. Specific antibodies were also needed to distinguish between TaMRP1 and 2 in planta to monitor protein expression and investigate the subcellular localisation. Theoretical considerations suggest that an anti-TaMRP1 antiserum would recognise not only TaMRP1 but also TaMRP2 (see Chapter 3), and Western analysis of TaMRP2 C-terminus expressed in pGEX-4T-3 provided direct proof of this. The antiserum had been used previously to demonstrate that immunoreactive protein is inducible by cloquintocet-mexyl, phenobarbital and by a combination of these two compounds in wheat microsomes (Theodoulou et al., 1998). These results are consistent with the Northern data presented in this chapter. The anti-TaMRP1 antiserum was used in this study to examine the
effects of aminotriazole, menadione and phenobarbital on protein expression. Western analysis showed that although phenobarbital induced the expression of a band of ca. 170 kDa, aminotriazole and menadione did not cause appreciable induction. This was surprising, given the effects of these compounds on transcription.

The recognition of TaMRP2 protein by the TaMRP1 antiserum also has implications for the immunolocalisation of both proteins: previous studies using the anti-TaMRP1 antiserum in Western blots of phenobarbital-treated and untreated wheat membrane fractions, revealed bands of ca. 170 kDa present in microsomes, intracellular membranes and tonoplast vesicles, however, the band was not present in the plasma membrane (Theodoulou, unpublished data). This result is consistent with the vacuolar localisation of AtMRP2 and plant GS-X pump activity (Li, et al., 1995a; Liu, et al., 2001; Martinoia, et al., 1993), and also with MRP-like transport of glutathionated and nonglutathionated compounds into plant vacuoles (Coleman, et al., 1997b; Swanson, et al., 1998; Wolf, et al., 1996). Our data suggests, but does not prove that TaMRP2 is also located at the tonoplast. Indeed, since the anti-TaMRP1 antiserum recognises both TaMRP1 and TaMRP2, it is possible that it will also recognise other wheat MRP isoforms, especially since the serum was raised to the C-terminus of TaMRP1, a region that is conserved at the amino acid level. Hence, from present data, it can only be concluded that MRP isoforms from wheat are present in microsomes, intracellular membranes and tonoplast vesicles, and to distinguish between the wheat MRP isoforms, specific antibodies would be required.

Whilst the TaMRP1 antiserum may prove useful to identify TaMRP2 in heterologous systems, it was concluded that specific antibodies for TaMRP1 and 2 were required. To distinguish between the TaMRP isoforms at protein level, TaMRP1- and 2-specific peptides were designed and used for antibody production. Dot blots and Western analysis were used to analyse the two antisera, however the results indicated that neither of the antisera recognised bands of the approximate expected size. There are several possible reasons why the antisera were unsuccessful: firstly, due to personnel changes at the antibody unit, a suboptimal schedule of immunisation and serum collection was carried out. This
may have resulted in a very low titre of specific antibodies. The animals have subsequently received booster immunisation and at the time of writing, the results of this are awaited. Additionally, it is possible that the peptides selected for antibody production had low antigenicity despite following accepted guidelines for design; the reasons why some peptides make good antibodies and others do not remain unknown and commonly, several peptides must be tested before a good antiserum is obtained (Dr Gordon-Weeks, pers. comm.). To do this was beyond the financial and time constraints of this project.

In summary, this chapter has shown that expression of TaMRP1 and TaMRP2 is inducible, and it was subsequently concluded that the two TaMRPs may play a role in oxidative stress tolerance and xenobiotic detoxification. To determine if this is indeed the case, the functional expression of TaMRP2 was examined, and these data are discussed in chapter 5.
CHAPTER 5

FUNCTIONAL EXPRESSION OF TaMRP2

5.1 Introduction

MRPs in mammals, plants and yeast can function as glutathione S-conjugate pumps capable of transporting a wide range of electrophilic substrates. In addition, MRPs can also transport non-glutathionated substrates for example, AtMRP2 can transport the malonylated Brassica napus nonfluorescent chlorophyll catabolite 1 (Bn-NCC-1), and the glucuronide 17-β-estradiol 17 (β-D-glucuronide) (E217βG). Similarly, human MRPl (HuMRPl) which can also transport glucuronate conjugates, transports sulphate conjugates, which have not yet been identified as substrates for plant MRPs. In light of these findings it appears possible that the wheat MRP isoform identified in this study may also function as a GS-X pump/transporter. Data from the TaMRP2 expression studies supports this hypothesis, however to test if TaMRP2 was indeed a GS-X pump, it was expressed in an heterologous system.

The expression of membrane proteins in heterologous systems is challenging. In order to function, many membrane proteins undergo complex post-translational modifications and conformational folding prior to membrane insertion, it is therefore necessary that the heterologous host has the appropriate suite of enzymatic and translational machinery (Grisshammer and Tate, 1995). Whereas most soluble proteins can be expressed in E.coli, heterologous membrane
proteins are often toxic to bacteria, possibly due to the depolarisation of the bacterial membrane caused by incorrect assembly of the membrane protein (Eraso and Serrano, 1990), and the overloading of the membrane with the expressed heterologous protein. Therefore, eukaryotic systems are generally used for expression of membrane proteins, these include: yeasts, Baculovirus-mediated and non-viral expression in insect cells, mammalian systems and Xenopus oocytes.

To maximise the chances for the successful expression of TaMRP2, the following criteria were considered: (1) Which system(s) will allow the expression of GC-rich membrane proteins? (2) Will TaMRP2 be targeted to the appropriate membrane? And (3) can the function of TaMRP2 be easily be assayed? In addition to these considerations, systems previously used for the successful expression of ABC transporters, in particular MRPs, were reviewed and these ranged from human or animal multidrug resistant cell lines, to yeast, and Baculovirus. Taking the above points into consideration, and the necessity to choose affordable systems, Baculovirus and a Saccharomyces cerevisiae mutant in which the vacuolar MRP, YCF1 is deleted (Δycf1), where selected for the functional expression of TaMRP2.

5.2 Insect expression systems

The Baculovirus expression system is widely used for the expression of heterologous genes in insect cells and has been used successfully for a range of plant membrane proteins including the inwardly rectifying potassium channel from potato (SKT1; Zimmermann, et al., 1998), and an outward rectifying potassium channel from Arabidopsis (Czempinski, et al., 1997). The Baculovirus system is advantageous because post-translational modifications within infected cells, including phosphorylation, glycosylation and peptide cleavage, are similar to those of higher eukaryotes (Grisshammer and Tate, 1995). A strong polyhedrin promoter of the Autographa californica nuclear polyhedrosis virus (AcNPV) controls transcription of the gene of interest, and during late stages of infection the recombinant protein is expressed, often at high levels (Luckow, et al., 1993). One further advantage is that the Baculovirus host, Spodoptera frugiperda has a
relatively high GC content (50.1 \%; see table 5.1 and section 5.3) which may aid the successful expression of the GC-rich wheat MRP (55.7 \%). In addition, the Baculovirus system has been used not only for the expression of HuMRP1 but also for the ScYCF1 vacuolar membrane transporter which was targeted to the plasma membrane (Ren, et al., 2000). Moreover Liu and co-workers recently published experiments detailing Baculovirus-mediated expression of AtMRP2 for characterisation of anti-peptide antibodies (Liu, et al., 2001).

The construction of recombinant Baculovirus occurs in two stages due to the large size (138 Kb) of the viral genome (Grisshammer and Tate, 1995). Initially the gene of interest is inserted into the donor plasmid downstream from the polyhedrin promoter, and flanked by Baculovirus DNA. The recombinant plasmid is then introduced into the insect cell DNA. Using traditional transfection and plaque assay methods, this process can take up to 6 weeks (Luckow, et al., 1993), however technologies have been developed to speed up these processes, and many commercial kits are now available that claim the construction of recombinant Baculovirus within a week.

Figure 5.1 outlines the commercially-available BAC-TO-BAC expression system (Invitrogen). The gene of interest is cloned into a pFastBac donor plasmid downstream from the polyhedrin promoter. The donor plasmid contains the bacterial transposon Tn7 which allows transposition of the recombinant plasmid into the linearised Baculovirus DNA (contained within the DH10BAC competent cells). A helper plasmid within the DH10BAC cells aids transposition by providing the necessary proteins and also confers tetracycline resistance for selection. The Tn7 attachment site is inserted into the N-terminus of the LacZα gene of the bacmid DNA and allows blue/white selection of recombinant bacmids in the presence of 5-bromo-indoly-β-D-galactopyranoside (Bluo-gal) and isopropyl β-D-thiogalactopyranoside (IPTG). Recombinant bacmid DNA is then purified and used to transfect Spodoptera frugiperda(Sf9) insect cells. The recombinant Baculovirus particles generated are then used to infect Sf9 cells for expression of the protein.
An alternative system, which also employs insect cells is the *Drosophila* expression system (DES™; Invitrogen). As the name of the system suggests, this method utilises a cell line derived from *Drosophila melanogaster* Schneider 2 (S2) cells, and a choice of expression vectors depending upon user requirements. There are several advantages for using this expression system over the BAC-TO-BAC system: firstly, whereas the BAC-TO-BAC system is invasive to the cell causing cell lysis, DES does not, and it is therefore possible to generate stable lines expressing heterologous proteins. Secondly, vectors for constitutive and inducible expression are commercially available. Finally, a further advantage of DES is that *Drosophila*, in common with *Spodoptera*, has a high GC content compared to other common hosts (54 %). Despite obvious advantages, this system is relatively new and has not yet been used for the expression of MRPs.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Percentage GC content</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>51.3</td>
</tr>
<tr>
<td><em>Saccharomyces cerevisiae</em></td>
<td>39.7</td>
</tr>
<tr>
<td><em>Schizosaccharomyces pombe</em></td>
<td>39.8</td>
</tr>
<tr>
<td><em>Pichia pastoris</em></td>
<td>43.2</td>
</tr>
<tr>
<td><em>Hansenula polymorpha</em></td>
<td>49.5</td>
</tr>
<tr>
<td><em>Xenopus laevis</em></td>
<td>47.7</td>
</tr>
<tr>
<td><em>Spodoptera frugiperda (Baculovirus host)</em></td>
<td>50.1</td>
</tr>
<tr>
<td><em>Drosophila melanogaster</em></td>
<td>54</td>
</tr>
<tr>
<td>Human</td>
<td>52.5</td>
</tr>
<tr>
<td><em>Arabidopsis</em></td>
<td>44.3</td>
</tr>
<tr>
<td>Tobacco</td>
<td>43.9</td>
</tr>
<tr>
<td><em>Wheat</em></td>
<td>55.7</td>
</tr>
<tr>
<td>Maize</td>
<td>55.7</td>
</tr>
<tr>
<td>Rice</td>
<td>56.3</td>
</tr>
</tbody>
</table>

Table 5.1: Codon usage of experimental host organisms and gene sources

The percentage GC contents presented in the table were obtained from the Kazusa codon usage data base (http://www.kazusa.or.jp/codon/). Common hosts are given at the top of the table working down, and sources for gene expression start at the bottom of the table and work up. The organisms linked to this project are in bold text.
5.3 *Saccharomyces cerevisiae* as a heterologous expression system

As a unicellular eukaryotic organism, yeast provides a useful tool for studying the expression of heterologous plant proteins. The most widely used yeast is *S. cerevisiae* because in addition to its ease of use, rapid growth, and low maintenance costs, its well-characterised genetics allow the generation of null mutants. The yeast genome sequence was completed in 1996 (Mewes, *et al*., 1997) and a total of 6 MRPs have been identified (Decottignies and Goffeau, 1997; Rea, 1999). Of these, the yeast cadmium factor 1 (*YCF1*), isolated by its ability to confer cadmium resistance, is best characterised (Li, *et al*., 1996; Szczypka, *et al*., 1994; 1.3.1). Since the yeast cadmium factor 1 mutant (*Aycf1*) is hypersensitive to cadmium, lacks a vacuolar GS-X transporter and determination of its phenotype is relatively simple and rapid, it has proved useful in the study of MRP function. Human MRPI and AtMRP3 can complement *Aycf1* to alleviate cadmium sensitivity and restore the transport of S-(2,4-dinitrophenyl)glutathione (DNP-GS) in recombinant vesicles (Lu, *et al*., 1998; Tommasini, *et al*., 1996; Tommasini, *et al*., 1998). In addition, complementation with AtMRP1, 2 and 5, although unable to confer cadmium tolerance, can restore DNP-GS transport (Gaedeke, *et al*., 2001; Lu, *et al*., 1998; Lu, *et al*., 1997). The *Aycf1* mutant therefore provides a useful tool to ascertain the phenotype(s) of putative MRPs.

To aid successful expression of heterologous proteins in yeast, many commercial vectors are available (for review see; Trueman, 1995). The main components of these vectors are: selectable markers, a constitutive or inducible promoter, and an origin of replication that regulates copy number (see table 5.2). Vector choice is an important factor in the regulation of expression levels of potentially toxic proteins (Romanos, *et al*., 1992).
Table 5.2: Promoters and origins of replication commonly used in yeast expression vectors

<table>
<thead>
<tr>
<th>Vector component</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Promoter</strong></td>
<td></td>
</tr>
<tr>
<td>Phosphoglycerate kinase (PGK)</td>
<td>Very strong/inducible</td>
</tr>
<tr>
<td>Galactokinase (GAL1)</td>
<td>Strong/inducible</td>
</tr>
<tr>
<td>Alcohol dehydrogenase II (ADH2)</td>
<td>Medium/inducible</td>
</tr>
<tr>
<td><strong>Origin of replication</strong></td>
<td></td>
</tr>
<tr>
<td>2µ</td>
<td>Multiple copies/cell (−40)</td>
</tr>
<tr>
<td>Cen/Ars</td>
<td>1-2 copies/cell</td>
</tr>
</tbody>
</table>

Despite the obvious utility of yeast as an expression host for plant membrane proteins, it was important to consider its genetic make-up. Highly expressed genes have a strong codon bias; *S. cerevisiae*, for example has a codon bias for 26 of the 61 possible triplets (Serrano and Villalba, 1995). The basis of codon-bias is related to availability of transfer RNAs (tRNAs) within the cell, and the nucleotide frequency at position three which is affected by the GC content of the gene. Table 5.1 shows the GC content of a variety of host species and potential sources for gene expression. Wheat has a high GC content (55.7 %) compared to *S. cerevisiae* (39.7 %); indeed, a detailed examination of TaMRP2 indicated that 14 % of its codons are unfavourable for yeast and this may affect expression. Recoding the gene of interest is one way of overcoming the problem of unfavourable codons. The literature contains many examples showing that modifying the codon usage of a foreign gene optimises its expression in heterologous systems, for example: the expression of the green fluorescent protein (GFP) from *Aquorea victoria* is enhanced in tobacco and *Chlamydomonas* when codon usage is modified (Fuhrmann, *et al.*, 1999; Rouwendal, *et al.*, 1997), and in *S. cerevisiae*, the expression of the mouse immunoglobulin kappa chain protein is increased at least 50-fold when yeast-preferred codons were used. Recently Batard and co-workers (2000) successfully expressed two wheat cytochrome P450s in *S. cerevisiae* and tobacco by re-engineering their 5' ends employing a PCR ‘megaprimer’, but this process is time consuming and expensive.
5.4 Expression of TaMRP2 in the Baculovirus expression system

The Baculovirus system has been used for the expression of many proteins including that of the yeast YCFL vacuolar membrane transporter, which was targeted to the plasma membrane (Ren, et al., 2000). It was hoped that since ScYCF1 and HuMRP1 had been successfully expressed in this system, it might also be possible to express and characterise TaMRP2. Since the Baculovirus system and the insect cell lines were available at IACR-Rothamsted, it was the first system employed for the expression of TaMRP2.

The pFastBac-TaMRP2 plasmid was constructed in several steps to allow the removal of the 5' utr of TaMRP2, and ensure that the TaMRP2 ATG start site was as close to the polyhedrin promoter of pFastBac (Invitrogen) as possible. The cloning strategy adopted is outlined in figure 5.2. Briefly, (1) a 4761 bp Eco RI-Not I fragment of TaMRP2 (nucleotide 101-4861) was cloned into pFastBac (BacMRP). (2) Two PCR primers were designed to introduce a Rsr II site and a 6 bp flanking region before the TaMRP1 ATG (DBS), incorporating the unique internal Rsr II site (nucleotide 1358) of TaMRP2 (DB9). Primer sequences are shown in red font in figure 5.2. The two primers were then employed to amplify a 1175 bp fragment corresponding to nucleotides 289-1450 of TaMRP2 and an additional 13 bp upstream of the ATG engineered by DB8. (3) The resulting PCR product was gel-purified and cloned into pCR® Blunt (Invitrogen; ZBDB8/9). (4) ZBDB8/9 was digested with Rsr II and the resulting 1074 bp fragment was cloned into BacMRP (from step 1) digested with Rsr II, and termed BacMRP-DB8/9. Sequencing indicated that the BacMRP-DB8/9 was amplified and constructed without error.

Recombinant Baculovirus encoding TaMRP2 was generated in several steps. The initial step involved the transposition of the BacMRP-DB8/9 construct into DB10Bac cells. Empty vector (pFastBac) was used as a control. Colonies containing recombinant bacmid were initially selected on IPTG and Bluo-gal, however no recombinant bacmids were isolated. It is possible that TaMRP2 is toxic to E. coli for reasons outlined in 5.1, therefore the transposition was repeated, omitting IPTG and Bluo-gal from the media; colonies were screened by
PCR using a *TaMRP2* specific primer (DB29 5'-CACTGCAGCAGCATTTCTCG-3'), and a M13/pUC reverse primer that annealed to the bacmid DNA. Figure 5.3 outlines the transposition region of the bacmid DNA, and expected sizes of PCR products. PCR products were separated in 1.5% agarose gels, an example of which is shown in figure 5.4. PCR amplification of a bacmid transposed with BacMRP-DB8/9 with one *TaMRP2* specific primer (DB29) and one universal primer (reverse), produced a product of approximately 1000 bp (lane 1). If the bacmid is not transposed, no product is produced (lane 2). To ensure that empty vector was transposed into the bacmid DNA, PCR with M13/pUC forward and reverse primers was carried out and produced a band of approximately 2300 bp as expected (lane 3).

Recombinant bacmids containing *TaMRP2* and empty vector were used to transfect Sf9 insect cells, cultured in 25 cm² Nunclon™ flasks (Invitrogen), using CELLFECTIN reagent. After 3 days, the virus was harvested and amplified twice before using 500 µl to infect duplicate flasks of ca. 1 x 10⁶ Sf9 cells for protein expression. Viral amplification and volumes of virus used were based on Liggins, 2001, since attempts to determine and optimise the multiplicity of infection (MOI) were unsuccessful. Forty-eight hours after infection, cells were harvested in phosphate buffered saline (PBS), duplicates pooled, and crude membrane preparations were made. The membrane preparations (0.5 µg) were separated by SDS-PAGE in a 7.5% gel, blotted onto nitrocellulose membrane (Hybond C Extra, Amersham Pharmacia) and probed with anti-*TaMRP1* antiserum (figure 5.5). The anti-*TaMRP1* antiserum failed to recognise a band predicted to be 170 kDa if *TaMRP2* was expressed in Sf9 cells. A positive control (affinity-purified MBP-*TaMRP1* fusion protein; 55 kDa) was employed to determine the viability of the antiserum and whether the technique was working.

At this stage of the project there were several options regarding continuation: optimisation of the Baculovirus system, or expression in an alternative system, such as *Drosophila S2* cells or yeast. A significant amount of time had already
been spent on trying to optimise the Baculovirus expression system to no avail, therefore the expression of TaMRP2 in Δycf1 was examined.

5.5 Expression of TaMRP2 in the *Saccharomyces cerevisiae* mutant ycf1

In view of the considerations outlined in section 5.3, it was decided to attempt expression of TaMRP2 at low copy number in the Δycf1 mutant, with the aim of obtaining functional protein without saturating the translational apparatus.

5.5.1 Construction of pYC2/MRP and transformation into Δycf1

The expression vector selected for the expression of TaMRP2 in Δycf1 was pYC2/CT (Invitrogen). It contained an inducible galactose promoter, a *URA3* auxotrophic marker, and a CEN6/ARSH2 origin of replication. The pYC2/CT-TaMRP2 plasmid was constructed in several steps to allow the removal of the 5' utr and engineer a yeast Kozak sequence to ensure initiation of translation. The first cloning strategy adopted was a direct PCR approach; a specific *TaMRP1* primer was designed to engineer the correct yeast Kozak sequence, and an *Eco RI* restriction site upstream of the Kozak sequence (primer DB33, see box 1). DB33 and a M13/pUC forward primer were employed to amplify a product of 4678 bp from *TaMRP2* in pZL1 (Life Technologies). The PCR product was digested with *Eco RI-Not I* and cloned into pYC2/CT also digested with *Eco RI-Not I*. Sequence analysis of the construct revealed that the DB33 primer had annealed to the *Eco RI* site within the 5' utr of *TaMRP2*, hence part of the 5' utr was still present and the Kozak sequence had not been modified and was therefore not optimal for yeast. The second cloning strategy is similar to that described in section 5.4 (figure 5.6): (1) a 4761 bp *Eco RI - Not I* fragment of *TaMRP2* (nucleotide 101-4861) was cloned into pYC2/CT (pYC2/TaMRP2). (2) PCR primers DB9 and DB33 (see section 5.2 and box 1) were employed to amplify a 1182 bp fragment corresponding to nucleotides 289-1450 of *TaMRP2* and an additional 10 bp upstream of the ATG engineered by DB33. The template used for the PCR was the Bac/MRP construct from 5.4. (3) The PCR product was gel-purified and cloned into pCR®Blunt (ZBDB9/33). (4) ZBDB9/33 digested with *Eco RI-Rsr II* was cloned into pYC2/TaMRP2 digested with *Eco RI-Rsr II*, and
the resulting plasmid was designated pYC2/MRP. Sequencing was used to confirm there were no errors made during PCR and construction of the plasmid.

### Box 1: Primer DB33

\[
5' - \text{CGAATTCATAATGGCGGCGACGGCGAGC} - 3' \\
\]

- **Eco RI**
- **Additional nucleotides to aid Eco RI digestion**
- **Nucleotides contained in yeast Kozak sequence**

The mutant Δycfl and the isogenic parent strain were obtained from EUROSCARF, Germany (accession numbers: Y146069 and Y10000). The pYC2/MRP plasmid (1 µg) was transformed into Δycfl using the Gietz ‘best’ lithium acetate method (Agatep, et al., 1998) and plated onto selective medium (SD-U; synthetic minimal glucose medium without uracil). The resulting transformants were termed Δycfl-pYC2/MRP. For comparative analysis and a control, the isogenic wild type strain and Δycfl were transformed with pYC2/CT (termed WT-pYC2/CT and Δycfl-pYC2/CT respectively). Since individual yeast transformants are known to exhibit different phenotypes (Dr. M Klein, pers. comm.), a pre-selection process was devised. Primary transformants were washed from the agar plates in 1 ml of medium containing yeast nitrogen base (0.17 %) and ammonium sulphate (0.5 %); 200 µl aliquots were then plated onto selective minimal media containing 25, 50, 75, 100 and 125 µM CdCl₂. This represents a range from subtoxic to toxic levels of cadmium. To promote expression of the gene, glucose was replaced by galactose to induce the galactose promoter (SG-U). Plates were incubated for 2-3 days at 28 °C. To obtain comparable results, WT-pYC2/CT and Δycfl-pYC2/CT transformsants were also selected in this way. In general, after pre-section on cadmium the number of transformants on the WT-pYC2/CT and Δycfl-pYC2/MRP plates were higher than those present on the Δycfl-pYC2/CT plates.
5.5.2 The effect of cadmium on growth of TaMRP2 transformants

The simplest screen for TaMRP2 expression was to use drop tests to determine if TaMRP2 was capable of restoring cadmium tolerance compared to ΔycfI-pYC2/CT. Therefore, duplicate independent transformants of WT-pYC2/CT, ΔycfI-pYC2/CT and ΔycfI-pYC2/MRP from the 100 µM (sub-toxic level) selection plates were subjected to drop test analysis on plates containing varying amounts of CdCl₂ (figure 5.7): overnight cultures of yeast cells grown in SG-U were serially diluted (3 x 10⁴, 3 x 10³, 1 x 10³, 3 x 10², 1 x 10², 10), and 6 µl of each dilution was pipetted onto a SG-U agar plate containing CdCl₂. Figure 5.7 indicates that on the control plates (no CdCl₂), there was no difference in growth between WT-pYC2/CT, ΔycfI-pYC2/CT and ΔycfI-pYC2/MRP, or their duplicates. As the CdCl₂ concentration increased, the growth of ΔycfI-pYC2/CT was inhibited compared to both WT-pYC2/CT and ΔycfI-pYC2/MRP. There was little difference between the growth of WT-pYC2/CT and ΔycfI-pYC2/MRP. At 125 µM CdCl₂, the growth of WT-pYC2/CT and ΔycfI-pYC2/MRP was slightly inhibited compared to the untreated control. Expression of TaMRP2 in wild-type yeast did not confer additional resistance to cadmium (data not shown).

An alternative, more quantitative method for examining the effect of CdCl₂ on yeast growth was to determine the survival rates of yeast after treatment with lethal doses of CdCl₂, a so-called ‘challenge assay’. Primary transformants were washed from the agar plates (no pre-selection) in 1 ml of yeast nitrogen base (0.17 %) and ammonium sulphate (0.5 %), and a 10 µl aliquot of the ‘wash’ was used to inoculate 10 ml of SG-U and grown overnight at 28 °C. Approximately 2 x 10⁷ cells from the overnight culture were used to inoculate fresh media (SG-U) containing varying concentrations of CdCl₂, and incubated for 1 hour at 28 °C. Cells were then serially diluted 10-fold (10⁻², 10⁻³, 10⁻⁴ and 10⁻⁵) and plated in triplicate onto SD-U agar and incubated at 28 °C for 3 days. This method was used to compare the survival of WT-pYC2/CT, ΔycfI-pYC2/CT and ΔycfI-pYC2/MRP (figure 5.8); results are plotted as percentage survival compared to an untreated control. Standard error is represented by error bars in figure 5.8. A general trend that can be observed for all three classes of transformant is the
decrease in survival rate with increasing cadmium concentrations. The survival rates of WT-pYC2/CT and \( \Delta ycf1 \)-pYC2/MRP are however higher than \( \Delta ycf1 \)-pYC2/CT for example at 200 \( \mu \)M, the survival rate of WT-pYC2/CT and \( \Delta ycf1 \)-pYC2/MRP remain above 50 % whereas the survival rate of \( \Delta ycf1 \)-pYC2/CT fell below 40 %.

5.5.3 Effects of CDNB on growth of TaMRP2 transformants

1-chloro-2,4-dinitrobenzene (CDNB) is a model GST substrate which can also spontaneously conjugate to GSH to form DNP-GS (figure 5.9). The effects of CDNB on the growth of \( \Delta ycf1 \) (DTY167) and wild type (DTY165) have been previously demonstrated by Li et al., 1996, showing that the addition of 40 \( \mu \)M CDNB to rich medium (YPD), resulted in a slower, more linear, growth rate for both strains, and the growth rate of \( \Delta ycf1 \) was severely retarded compared to wild type.

In this study, synthetic complete medium lacking uracil was used to maintain selection of the pYC2/CT and TaMRP2 plasmids. Preliminary experiments have indicated that yeast grown in minimal medium is more sensitive to xenobiotics than when grown in rich medium (data not shown). Initially, the drop test method used to examine the effects of cadmium was performed using CDNB at 5, 10, 15 and 20 \( \mu \)M. The growth of WT-pYC2/CT, \( \Delta ycf1 \)-pYC2/CT and \( \Delta ycf1 \)-pYC2/MRP transformants were inhibited by 20 \( \mu \)M CDNB but there were no visible differences between the three classes of transformants (data not shown). The effect of CDNB on yeast survival was also examined using the challenge assay. The three concentrations of CDNB examined (30, 60 and 90 \( \mu \)M) inhibited the survival of WT-pYC2/CT, \( \Delta ycf1 \)-pYC2/CT and \( \Delta ycf1 \)-pYC2/MRP compared to untreated controls, yet there were no apparent trends in the data i.e. an increase in CDNB concentration did not necessarily correlate to a decrease or plateau in survival as expected, and the most successful class of transformant at each given concentration varied (data not shown).
The toxicity of CDNB to Δycfl was originally demonstrated using a more sensitive liquid culture assay (Li, et al., 1996), and this technique was therefore used to assess the effects of TaMRP2 on CDNB toxicity to Δycfl. The effects of CDNB on yeast growth (WT-pYC2/CT, Δycfl-pYC2/CT and Δycfl-pYC2/MRP) were examined over a time course of 28 hours: transformants from the selection plates were incubated overnight at 28 °C in SG-U. The overnight cultures were diluted in fresh medium (SG-U) to an OD$_{600mm}$ of 0.1, treated with 5, 10 and 15 µM of CDNB and incubated at 28 °C. At given time intervals the OD$_{600mm}$ of each culture was recorded, and is represented graphically (figure 5.10). All three classes of transformant grew at approximately the same rate in the absence of CDNB and in 5 µM CDNB. At 10 µM CDNB the differences between the growth rates of WT-pYC2/CT, Δycfl-pYC2/CT and Δycfl-pYC2/MRP are clearly visible; the growth rate decreased in all cases, but most notably Δycfl-pYC2/CT, the growth of which was almost linear in 10 µM CDNB. The growth rate of Δycfl-pYC2/MRP was inhibited compared to WT-pYC2/CT, however its growth rate is less affected by CDNB than Δycfl-pYC2/CT. Fifteen µM of CDNB retarded all yeast growth, and the differences between WT-pYC2/CT, Δycfl-pYC2/CT and Δycfl-pYC2/MRP were less evident.
The gene of interest is cloned into a pFastBac donor plasmid, the recombinant plasmid is transformed into DH10Bac competent cells which contain the bacmid with a mini-attTn1 target site and helper plasmid. The mini-Tn7 element on the pFastBac donor plasmid can transpose to the mini-attTn7 target on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the lacZa gene. High molecular weight mini-prep DNA is prepared from selected E. coli colonies containing recombinant bacmid, and the DNA is then used to transfect insect cells (figure reproduced with permission from the Baculovirus manual, Invitrogen).
Figure 5.2: Strategy for the subcloning of TaMRP2 in pFastBac1 (Invitrogen), to allow the removal of the 5' utr of TaMRP2.

1. A 4761 bp Eco RI-Not I fragment (nt 101-4861) of TaMRP2 was sub-cloned into pFastBac (BacMRP)

2. A 1175 bp fragment was amplified by PCR using Pfu to introduce a Rsr II restriction site and flanking region adjacent to the TaMRP2 ATG and maintain the internal Rsr II site of TaMRP2.

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3. The PCR fragment from (2) was cloned into pCR®-Blunt (ZBDB8/9)

4. Pooled ZBDB8/9 were digested with Rsrl II. The resulting gel-purified fragment was cloned into Bac/MRP digested with Rsrl II.
Figure 5.3: Schematic diagram of the bacmid transposition region indicating the size of PCR products expected

Transposition region of bacmid DNA

Sample | PCR product
--- | ---
Bacmid DNA | ~300 bp \((128 + 145)\)
Bacmid DNA transposed with pFastBac, employing forward and reverse primers | ~2300 bp \((1522 + 6 + 48 + 458 + 128 + 145)\)
Bacmid DNA transposed with Bac/MRP-DB33/9, Employing DB26 and reverse primers | ~1000 bp \((357 + 48 + 458 + 145)\)
Figure 5.4: Electrophoretic analysis of PCR products amplified from putative recombinant bacmids

PCR analysis of transposition of BacMRP-DB8/9 and pFastBac into DH10Bac cells. DNA was separated on by electrophoresis on a 1.5 % agarose/TAE gel. A 1Kb marker was used to determine the size of the DNA.

1. Bacmid DNA transposed with Bac/MRP-DB8/9; 1 µl plasmid preparation, DB29 and M13/pUC reverse primers (12.5 pmol)
2. Unsuccessful transposition of 1 (see above)
3. Bacmid DNA transposed with pFastBac; 1 µl plasmid preparation, M13/pUC forward and reverse primers (12.5 pmol)
+ve. Positive control; 25 ng TaMRP1 DNA + specific forward and reverse primers (12.5 pmol)

PCR conditions:
Initial denaturation at 94 °C for 2min. Denaturation at 95 °C for 45 sec, annealing at 55 °C for 45 sec and extension at 72 °C for 2 min. A total of 30 cycles were performed. The final extension continued at 72 °C for 10 min.
Crude membrane preparations (0.5 μg) of Sf9 insect cells transfected with BacMRP-DB8/9 or empty vector (pFastBac) were subjected to SDS-PAGE on a 7.5 % (w/v) gel, transferred onto nitrocellulose membrane (Hybond C Extra, Amersham Pharmacia) and probed with the anti-TaMRP1 antiserum. To determine the viability of the antiserum, a positive control (gift from Dr Theodoulou) known to react with a band of ca. 55 kDa was employed. The pre-immune serum did not react to produce bands.
Figure 5.6: Strategy for the subcloning of TaMRP2 in pYC2/CT (Invitrogen) for the removal of the 5' utr of TaMRP2, and introduction of the yeast Kozak sequence

1. A 4761 bp Eco RI-Not I fragment (nt 101-4861) of TaMRP2 was sub-cloned into pYC2/CT (pYC2/TaMRP2)

   ![Diagram of TaMRP2/ZL and pFastBac1 vectors showing subcloning strategy]

2. 1182 bp fragment was amplified by PCR using Pfu to introduce a Eco RI restriction site, nucleotides of the yeast Kozak sequence and an additional nucleotide for digestion adjacent to the TaMRP2 ATG, and maintain the internal Rsrl site of TaMRP2. Bac/MRP (5.4) was employed as a template.

   ![Primers DB33 and DB9 for PCR amplification]

   - Primer DB33:
     - Eco RI site: *CGAATTCTATGGCGGCGACGGCGAGC* 3'
     - Additional nucleotide to aid Eco RI digestion: *CGCAGATGGTCAACATCG*

   - Primer DB9:
     - Nucleotides contained in the yeast Kozak sequence: TACCGCCGCTGCCCCTCG
     - Additional nucleotide: GCGCTCTACCAGTTGTAGC

Continued over page...
3. The PCR fragment from (2) was cloned into pCR®-Blunt (ZBDB9/33)

4. Pooled ZBDB9/33 were digested with Eco RI and Rsr II. The resulting gel-purified fragment was cloned into Bac/MRP digested with Eco RI and Rsr II.
Wild type and Δycf1 mutant strains of *Saccharomyces cerevisiae* were transformed with the yeast expression vector pYC2/CT. Δycf1 was also transformed with pYC2/CT containing TaMRP2 (pYC2/MRP). Overnight cultures of the yeast cells grown in minimal selective media (SG-U) were serially diluted, and 6 μl of each dilution was pipetted in duplicate (vertically) onto a minimal selective agar plate (SD-U) containing CdCl₂. CdCl₂ was omitted from the control plate. The results are representative of three experiments.
Wild type and Δycfl mutant strains of *Saccharomyces cerevisiae* were transformed with the yeast expression vector pYC2/CT. Δycfl was also transformed with pYC2/CT containing TaMRP2 (pYC2/MRP). Overnight cultures of the yeast cells grown at 28 °C in minimal selective media were diluted to an OD<sub>600</sub>nm of 1 and treated with 100, 200, 400 μM CdCl₂ for one hour. Cells were diluted and plated in triplicate onto minimal selective agar plates to monitor cell viability. Percentage survival (average of four replicates) is expressed relative to an untreated control culture (100 %). Standard error was calculated (standard deviation / √n, where n=3), these values are represented by bars on the graph. The experiment was repeated to yield similar results, data not shown.
In plants and mammals the conjugation of CDNB to GSH is catalysed by glutathione S-transferases (GSTs), but can also occur spontaneously at pH values above 7.
Wild type and Δycf1 mutant strains of *Saccharomyces cerevisiae* were transformed with the yeast expression vector pYC2/CT. Δycf1 was also transformed with pYC2/CT containing *TaMRP2* (pYC2/MRP). Overnight cultures of the yeast cells grown at 28 °C in minimal selective media were diluted to an OD$_{600}$nm of 0.1 in minimal media containing CDNB, and grown at 28°C. OD$_{600}$nm was measured at the times indicated. CDNB was omitted from the control. The experiment was repeated to yield similar results, data not shown.
5.6 Discussion

5.6.1 Insect expression systems

This chapter describes the expression of TaMRP2 in the Baculovirus expression system (Invitrogen) and the ycf1 mutant of *S. cerevisiae*. Initially, the Baculovirus expression system was examined since it has a codon usage compatible with wheat genes and has been employed for the successful expression of the yeast YCF1 vacuolar membrane transporter, human MRPs (Ren, *et al.*, 2000; Sun, *et al.*, 1996; Van Aubel, *et al.*, 1999), and several plant genes including the inwardly rectifying potassium channel from potato (SKT1; Zimmermann, *et al.*, 1998). However, expression of TaMRP2 in this system was unsuccessful.

Prior to sub-cloning *TaMRP2* into pFastBac, Invitrogen was contacted for advice on constructing a plasmid containing a GC rich plant membrane gene. Removal of the 5' utr was considered essential since this region can be important in defining the level of transcription due to the presence of secondary structure in the mRNA (Grisshammer and Tate, 1995). The Kozak consensus sequence, although generally considered essential for initiation of translation (Kozak, 1996), is not apparently utilised in insect cells and therefore it was decided that optimisation was unnecessary (Damodarasamy, *et al.*, 2000; Tien, *et al.*, 1994). The guidelines from Invitrogen were followed and the 5' utr of *TaMRP2* was removed and the Kozak sequence left unaltered. It is possible that not altering the Kozak sequence may have effected the expression of TaMRP2 in Sf9 cells. The constructs employed for the expression of both *S. cerevisiae* YCF1 and HuMRP1 (Batard, *et al.*, 2000; Gao, *et al.*, 1996) included the Kozak sequence (GCGGCCATG). To prove if the expression of TaMRP2 was unsuccessful for this reason, a plasmid containing *TaMRP2* with the correctly engineered Kozak sequence would have to be generated and expressed. An alternative explanation for why expression of TaMRP2 failed may be related to the multiplicity of infection (MOI) at which the recombinant virus was used to infect Sf9 cells. Determination of the MOI failed, therefore the volumes and number of amplifications of virus were based on the work of Liggins, (2001), who had encountered similar problems when trying to determine the MOI using the BAC-
TO-BAC expression system. Although Zimmerman, and co-workers (1998) successfully expressed SKT1 without determination of the MOI, and in fact employed the same viral amplification and infection regime as described in this thesis, both Ren, and co-workers (2000), and Van Aubel, and co-wokers (1999), used a MOI of 1-5 for the expression of *S. cerevisiae* YCF1 and human MRPs in *Spodoptera frugiperda*. Until the MOI for recombinant TaMRP2 can be determined and optimised, its effect on TaMRP2 expression in Sf9 cells remains uncertain.

At this stage of the project a decision was made to discontinue the Baculovirus approach in favour of two alternatives; expression in the *S. cerevisiae* Δycf1 or the *Drosophila* expression system (DES). Prior to employing the DES system for the expression of TaMRP2, it was necessary to obtain approval from the safety committee since DES is classified as containment level 2. This allowed ample opportunity to examine expression of TaMRP2 in *S. cerevisiae* Δycf1.

5.6.2 Expression in yeast

Expression of *AtMRP3* and *HuMRP1* cDNAs in the *S. cerevisiae* ycf1Δ can restore or partially restore phenotypes exhibited by the YCF1 gene, cadmium tolerance and GS-X transport activity (Tommasini, *et al.*, 1996; Tommasini, *et al.*, 1998). Since the closest orthologue of TaMRP2, AtMRP3 could complement Δycf1, alleviating cadmium sensitivity and restoring transport of the model substrate DNP-GS, it seemed possible that TaMRP2 might complement the cadmium sensitive phenotype of Δycf1. As discussed in the introduction to this chapter, expression of TaMRP2 in *S. cerevisiae* may not be feasible given the high GC content of the *TaMRP1* cDNA compared to *S. cerevisiae*, and the use of unfavourable yeast codons. One method that may circumvent these problems is to minimise the expression of TaMRP2, and there are a few commercially available vectors designed specifically for this purpose. The vector utilised here (pYC2/CT, Invitrogen) was selected because expression of TaMRP2 could be controlled by utilising the inducible promoter (*GAL1*), and a CEN6/ARSH4 origin of replication that gives rise to 1-2 copies per cell. To ensure initiation of translation, it was essential that the pYC2/MRP plasmid contained the correct
Kozak sequence. The 5' utr of TaMRP2 was removed and a PCR-based cloning strategy was devised to amplify the full coding sequence of TaMRP2 with optimal flanking sequences. This initial cloning strategy was unsuccessful because the forward primer (DB33), that contained an Eco RI restriction site designed to anneal upstream of the TaMRP2 ATG preferentially annealed to the Eco RI site within the 5' utr of TaMRP2, not removing the 5' utr as originally intended. Theoretically, DB33 should not have annealed to the Eco RI site within the 5' utr of TaMRP2, however primers often undergo non-specific binding to redundant sequences. An alternative approach, analogous to the method used to construct the Baculovirus construct, was therefore adopted. Rather than amplify the whole TaMRP2 sequence, a fragment encoding the 285-1450 5' bp of TaMRP2 was amplified, utilising the BacMRP-DB8/9 construct as a template (where the 5' utr of TaMRP2 had been removed). Once this initial hurdle was overcome, further construction of the pYC2/MRP plasmid via subcloning the PCR fragment to reconstruct a full-length expression cassette was successful, and the pYC2/MRP plasmid was transformed into Δycf1 following the Gietz 'best method' (www.umanitoba.ca/faculties/medicine/biochem/gitez/media.html). For comparative analysis, pYC2/CT was transformed into both wild type and Δycf1 cells.

5.6.3 Cadmium tolerance in transformed yeast
The first putative TaMRP2 substrate examined was cadmium. The cadmium drop test and the challenge assay demonstrated that pYC2/MRP complements Δycf1 to restore cadmium tolerance, to a level similar to WT-pYC2/CT. The cadmium drop test examined individual transformants (WT-pYC2/CT, Δycf1-pYC2/CT, Δycf1-pYC2/MRP) that had been pre-selected by their ability to grow on sub-toxic levels of CdCl₂ (100 μM). This selection process was devised to enhance the probability of recovering transformants with optimal expression levels since individual yeast transformants can exhibit dramatic variability in protein expression (Dr M Klein, pers. comm.; Gaedeke, et al., 2001). Pre-selected transformants were more tolerant to cadmium compared to those that were not pre-selected (data not shown). Distinct differences between WT-pYC2/CT, Δ
ycfl-pYC2/CT and Δycfl-pYC2/MRP were apparent i.e. Δycfl-pYC2/CT was more sensitive to cadmium compared to WT-pYC2/CT and Δycfl-pYC2/MRP, suggesting that TaMRP2 conferred cadmium tolerance.

The drop test is a measure of the ability of cells to grow in the presence of a toxic agent. An alternative approach to examine the effects of TaMRP2 on cadmium toxicity to Δycfl, was to determine the survival rate of transformants employing the quantitative challenge assay. Additionally, a mixed population of transformants was employed for this experiment: individual transformants exhibit varying phenotypes, so by testing a random mixed population of transformants, it was likely that a proportion of these would exhibit optimal expression levels. Furthermore, this method does not impose a pre-selection of individual transformants. Results from this assay indicated that at each cadmium concentration tested, pYC2/MRP was able to complement Δycfl to restore cadmium tolerance to levels similar or higher than those exhibited by WT-pYC2/CT.

It is possible that enhanced cadmium tolerance of pre-selected transformants may be due to an MRP-independent mechanism, such as increased production of glutathione. However, use of transformants not pre-selected on cadmium i.e. those used in the challenge assay, also indicated that TaMRP2 confers cadmium tolerance to Δycfl, strongly suggesting that the transporter was responsible for the observed phenotype. Ideally, the cadmium drop test should have been repeated using transformants that were not pre-selected on cadmium, however given the time constraints and unforeseen technical problems, this was not feasible.

The question that must now be addressed is what role does TaMRP2 play in cadmium tolerance? Transport studies using S. cerevisiae tonoplast vesicles from wild type and Δycfl strains have shown that ScYCFl mediates DNP-GS and Cd'GS2 transport into the vacuole (Li, et al., 1997a). Interestingly, Tommasini and co-workers (1996) showed that HuMRPl could restore cadmium tolerance and the ability of DNP-GS transport when expressed in ycf1Δ, however it
appeared unable to mediate GSH-promoted cadmium transport, and neither cadmium nor GSH could inhibit the uptake of DNP-GS. Li and co-workers (1997a) suggested that the likely reason for this inability to measure GSH-promoted cadmium uptake was due to a technical error, and it is therefore possible, though not yet proven, that HuMRP1 could transport CdGS₂. Additionally, AtMRP3 can also restore cadmium tolerance and DNP-GS transport when expressed in ycfIΔ, although whether it can transport CdGS₂ has yet to be examined. AtMRP1, 2 and 5 can only complement ΔycfI to restore the transport of organic GS-conjugates and non-glutathionated substrates; cadmium tolerance was not restored and AtMRP1 and 2 (AtMRP5 has yet to be examined) were both incapable of transporting CdGS₂ (Gaedeke, et al., 2001; Liu, et al., 2001; Lu, et al., 1998; Lu, et al., 1997). These results suggest that, in common with other transport activities (Ishikawa, et al., 2000; Rea, 1999), cadmium detoxification is limited to specific MRP isoforms: ScYCFl, HuMRP1, AtMRP3 and AtMRP4 (E. Martinoia, pers. comm.). Although ScYCFl confers cadmium tolerance by transporting CdGS₂, it is possible that other MRPs confer cadmium tolerance via alternative or additional mechanisms. One suggestion proposed by Rea (1998) is that HuMRP1 requires a coreactant(s) in addition to or instead of GSH, that is present in intact yeast cells yet absent in in vitro assays for the transport of CdGS₂, however ScYCFl does not require this coreactant. The identity of the TaMRP2 transport substrate(s) remains to be demonstrated experimentally. Other mechanisms for cadmium detoxification have been shown in animals, yeast and plants, and these may not be related to MRP. In plants, GSH-derived peptides known as phytochelatins form complexes with heavy metals such as cadmium, aiding its detoxification (Rauser, 1990). In oat roots, phytochelatins and Cd-phytochelatin complexes are transported into the vacuole by an ATP-dependent, vanadate-sensitive mechanism, which might represent an ABC transporter (Salt and Rauser, 1995). Saccharomyces cerevisiae does not synthesise phytochelatins, but in the fission yeast, Schizosaccharomyces pombe, Cd-phytochelatin complexes are transported into the vacuole by Heavy Metal Tolerance 1 (HMT1), an ABC transporter which does not belong to the MRP subclass, (Ortiz, et al., 1995). There are no strict HMT homologues in the
Arabidopsis genome (Sánchez-Fernández, et al., 2001) and the plant phytochelatin transporter has yet to be identified at the molecular level.

An alternative, perhaps additional, hypothesis is that MRPs transport a product of cadmium toxicity, for example a metabolite generated as a result of oxidative stress, and as discussed in the introduction, there is a growing body of evidence to support this idea. Under oxidative stress, the ratio of reduced to oxidised glutathione (GSH: GSSG) falls. Since this can lead to changes in gene expression and elevated GSSG can cause protein thiolation, GSSG must be removed. This usually achieved by the action of NADPH-dependent glutathione reductase; however, if GSH:GSSG is particularly low and reducing power is limiting, other mechanisms may be required. The affinity of plant MRPs ranges from 73 to 400 μM, indicating that at least some isoforms are competent to remove GSSG from the cytosol (Foyer, Theodoulou and Delrot, 2001). Another consequence of oxidative stress is the peroxidation of lipids, resulting in the formation of fatty acid hydroperoxides. Since these products are toxic, and in the case of mammals, can act as intracellular signals, their levels must be tightly regulated. In plants, lipid hydroperoxides are degraded by glutathione peroxidases, to yield the corresponding hydroxide and GSSG. Certain GSTs also possess this catalytic activity (Bartling, et al., 1993; Cummins, et al., 1997), but other GSTs can catalyse bona fide conjugation of GSH and lipid hydroperoxides (Vollenweider, 1999). Recent work has shown that the glutathione conjugate of linoleic acid hydroperoxide is readily transported into isolated barley vacuoles, and pharmacological characterisation suggests that MRP is a likely candidate transporter (F Theodoulou and E Martinoia, unpublished results). Similarly, in mammals, arachidonic acid is metabolised in several steps to yield the glutathione conjugate, leukotriene C₄ (LTC₄), which is transported with high affinity by human MRP (Leier, et al., 1996) and also, interestingly by YCF1 (Falcon-Perez, et al., 1999).

One mechanism by which cadmium exerts toxicity is via lipid peroxidation. However, the membranes of S. cerevisiae contain relatively little polyunsaturated fatty acids, unless they are supplied in the growth medium (Avery, et al., 1996;
Howlett and Avery, 1997), therefore under standard growth conditions, yeast is not highly susceptible to cadmium-induced lipid peroxidation. For this reason, it would appear that MRP-mediated cadmium tolerance of *S. cerevisiae* is unlikely to be a direct result of the transport of a metabolite generated under oxidative stress. In summary then, the cadmium-resistant phenotype of the *ycfl* mutant expressing TaMRP2 is likely to be due to TaMRP2-mediated transport of a cadmium species. This does not however, rule out lipid hydroperoxide conjugates as substrates of plant MRPs. This possibility could be tested directly using membrane vesicles from yeast expressing TaMRP2 and other MRPs.

5.6.4 Resistance of transformed yeast to CDNB

The effects of CDNB on yeast growth were examined using three different techniques: growth on agar containing CDNB (drop test), survival rates after treatment with CDNB (challenge assay), and growth in liquid cultures containing CDNB. Data from the drop test indicated that there were no differences between the growth of WT-pYC2/CT, Δycfl-pYC2/CT and Δycfl-pYC2/MRP, at any given concentration of CDNB, however, above 20 µM CDNB, growth of all classes of transformant was inhibited compared to an untreated control. Data from the challenge assay were erratic and no clear trends were observed. The final experiment examined the effect of CDNB on the growth of WT-pYC2/CT, Δycfl-pYC2/CT, Δycfl-pYC2/MRP in liquid culture. In the absence of CDNB, all transformants grew at the same rates, however, the addition 10 µM CDNB to the media inhibited the growth rate of Δycfl-pYC2/CT compared to WT-pYC2/CT and Δycfl-pYC2/MRP. Thus in this assay, TaMRP2 can complement Δycfl to restore tolerance to CDNB.

The precise mechanism of CDNB toxicity is unclear, however, a key step in its detoxification is its conjugation to glutathione (GSH) to form DNP-GS, which can be transported out of the cytoplasm by the action of MRPs, including: HuMRP1, AtMRP1-3, and ScYCFl. In plants and mammals the conjugation of CDNB to GSH is catalysed by glutathione S-transferases (GSTs), but can also occur spontaneously at pH values above 7. In *S. cerevisiae* it was thought that
conjugation is spontaneous since no GST had been identified in this organism. Recently however, three genes encoding GSTs were identified and characterised (Choi, et al., 1998; Kim, et al., 2001). GSTs Gtt1 and Gtt2 exhibited activity with CDNB as a substrate, but a double mutant strain (gtt1Δ gtt2Δ), did not show increased sensitivity to CDNB compared to wild type. This suggests that, whilst spontaneous conjugation to GSH may be sufficient to permit MRP-mediated detoxification, other mechanisms for the detoxification of CDNB might exist. An example of this is ROD1, (Resistance to O-Dinitrobenzene), which has only been characterised genetically to date (Wu, et al., 1996).

The existence of alternative mechanisms for CDNB detoxification may provide an explanation for the inability of drop test and challenge assays to distinguish between wild type and Δycf1 yeast in the presence of CDNB. Other laboratories have obtained similar results using these tests (E. Martinoia, pers. comm.). However, the CDNB-sensitive phenotype of Δycf1 was readily apparent in liquid culture (fig. 5.10 and Li et al 1996). This may reflect the differences between the toxicity tests employed. Cell division is particularly sensitive to xenobiotics, especially electrophiles such as CDNB which can react with nucleic acids; indeed, a common response to chemical stress is to arrest the cell cycle in the G1 phase, preventing replication of damaged DNA (Reichheld, et al., 1999). Cells in aerated liquid culture divide more rapidly than those grown on agar plates and would be expected to be more sensitive to chemical insult. In the light of this, the CDNB challenge assay could be repeated employing higher concentrations of CDNB. Interestingly, a cadmium-sensitive phenotype was detected in both drop tests and challenge assays, and this may be because cadmium primarily exerts its toxic effects on processes other than cell division.

Although it is likely that TaMRP2 can alleviate the sensitivity of CDNB by transporting it out of the cytoplasm, as is the case for its closest orthologue, AtMRP3 (Tommasini, et al., 1998), and the other MRPs described above, further experiments are required. Resistance to both cadmium and CDNB could, theoretically be achieved by efflux at the plasma membrane or sequestration in the vacuole. Cadmium measurements of transformed yeast would shed light on
this, but a more direct approach would be to determine the location of TaMRP2 in yeast using an antibody or GFP fusion. Then, membrane vesicles could be prepared from recombinant yeasts and used for transport assays, to identify the transported species (Li, et al., 1996).

Whilst expression of TaMRP2 in the Δycf1 mutant was able to partially or fully restore wild type levels of tolerance to cadmium and CDNB, expression of TaMRP2 did not increase further the tolerance of wild type cells to cadmium or CDNB. This may be because a CEN vector was used and expression levels were low. The CEN origin of replication results in 1-2 copies/cell whereas vectors containing the 2µ origin typically yield 40 copies per cell. Falcon-Perez and co-workers (1999) showed that expression of ScYCF1 in the 2µ vector increases the cadmium resistance of Δycf1 compared to Δycf1 containing YCF1 cloned into a CEN vector. Similar trends were observed for the uptake of the glutathione conjugate, LTC₄. If TaMRP2 was cloned into a high copy vector perhaps tolerance towards cadmium or CDNB may have increased, however tRNA availability may have limited translation. Also, overexpression of membrane proteins can result in mis-localisation (d’Exaerde, et al., 1996), which may subsequently lead to loss of function.

5.6.5 Summary
The implications of the role of TaMRP2 in xenobiotic detoxification and oxidative stress tolerance have already been discussed. Based on the ability of TaMRP2 to confer cadmium and CDNB tolerance, and what is known about other MRPs, it is likely that TaMRP2 may recognise additional substrates and hence exhibit other functional capabilities. The mechanism of TaMRP2 transport has yet to be elucidated, however based on the data presented in this thesis, and the role of other MRPs, it can be speculated that TaMRP2 is a GS-X pump. The ability of TaMRP2 to confer cadmium tolerance is interesting; perhaps it has the capacity to transport other heavy metals. ScYCF1 for example, confers resistance to arsenical compounds by transporting As.GS₃ into the vacuole (Ghosh, et al., 1999). To test if this were the case for TaMRP2, one or all of the methods described in this chapter for testing CDNB and cadmium could be used to test...
arsenite and arsenate. On a note of caution, *S. cerevisiae* has 2 independent mechanisms for the detoxification of arsenite, YCF1 and arsenic compounds resistance (ARC3; Ghosh, *et al.*, 1999), and this should be taken into consideration when interpreting data.

In summary, this chapter has described the functional characterisation of TaMRP2 in the *S. cerevisiae* mutant *ycf1*. TaMRP2 has been shown to play a role in the detoxification of both cadmium and CDNB. This data supports and extends the findings presented in chapters 3 and 4, suggesting that TaMRP2 may play a role in xenobiotic detoxification and oxidative stress, and it is likely to achieve this by transport out of the cytoplasm.
CHAPTER 6

DISCUSSION

6.1 Overview
The first MRP cDNA (human MRPl) was cloned from a drug-selected lung cancer cell line resistant to multiple chemotherapeutic agents (Cole, et al., 1992), and further members of this ABC transporter subfamily have since been cloned from all major eukaryotic taxa. As GS-X pumps, they play an important role in protecting the cell against electrophilic compounds, and in addition transport a range of non-glutathionated substrates including glucuronide conjugates and non-conjugated substrates. Human MRPs are well characterised because of their importance in multidrug resistance, and evidence suggests that MRPs play a similar role in plants (for review see Rea, 1998) in the metabolism of xenobiotics. Apart from the cloning of MRPs in the model plant species Arabidopsis, and the partial cDNA clone from wheat (TaMRPl), comparatively little is known about MRPs in plants, especially in agronomically important species. This thesis, with aims to further the understanding of MRPs in a crop species, described the cloning and characterisation of a MRP isoform from wheat (TaMRP2), providing evidence that MRP in wheat may play a role in xenobiotic detoxification and additionally in oxidative stress tolerance.
6.2 Implications of cloning and the sequence analysis of TaMRP2

Sequence comparisons of TaMRP2 with sequences in public databases indicated that AtMRP3, the most inducible AtMRP isoform (Sánchez-Fernández, et al., 1998; Tommasini, et al., 1997), was the closest orthologue of TaMRP2. Detailed sequence analysis of TaMRP2 indicated the presence of sequence motifs common not only to the ABC transporter family as a whole, but also to the MRP-subfamily and phylogenetic analysis confirmed that TaMRP2 was a likely member of the MRP subfamily. The phylogram suggested that TaMRP2 and AtMRP3 evolved from a common ancestor, and it was therefore possible that they might be functionally related. Functional characterisation of TaMRP2 in the *S. cerevisiae ycf1* mutant provided evidence to support this hypothesis: TaMRP2, like AtMRP3 confers resistance to both cadmium and CDNB in yeast. To date however, there is no evidence to suggest that MRPs confer resistance to either cadmium or CDNB *in planta*.

The topological models assigned to TaMRP2 were different from the current model of human MRP1. Although structural divergence within the MRP subfamily does exist, for example human MRP5 lacks the N-terminal extension common to other MRPs, the predicted gross topology of all full-sized ABC transporters, including human MRP5, MSD1 and MSD2 are predicted to contain 6 + 6 transmembrane spanning domains (Borst, et al., 1999). It is of course possible that plant MRPs have a topology that is different from human MRPs, and given the potential mechanistic differences between human MRP1 and AtMRP2 (Liu, et al., 2001), it is a possibility that should not be ruled out, even though the published predicted model of AtMRP2 is the same as human MRP1. It remains likely however that *in silico* analysis of TaMRP2 may have been misleading, but without experimental evidence e.g. localisation of the nucleotide binding folds (NFBs), the precise topology of TaMRP2, and other plant MRPs remains unknown.
6.3 Localisation of TaMRP2
Sequence comparisons between TaMRP2 and other MRP subfamily members suggested that TaMRP2 may function as a GS-X pump, and therefore play a role in xenobiotic detoxification. Expression and functional data were required to determine if this was the case, thus the location, chemical inducibility and functional capabilities of TaMRP2 were examined. Plant MRP and MRP-like transport activity involved in xenobiotic detoxification have been localised to the vacuolar membrane (Martinoia, et al., 1993). The anti-TaMRP1 antibody employed in localisation studies was not specific for a single wheat MRP isoform, and although it recognised a TaMRP2 fusion expressed in E.coli and a band of the approximate expected size in wheat tonoplast vesicles and microsomes, the presence of TaMRP2 on the vacuolar membrane could not be confirmed unequivocally. To address this, a specific TaMRP2 peptide antibody was raised but the antisera available did not recognise the TaMRP2 peptide. New antibodies or alternative approaches for the localisation of TaMRP2 in planta are therefore required. Although it is likely that a TaMRP2-specific peptide antibody will only recognise the MRP2 isoform in wheat, it is possible that it may recognise other wheat MRP isoforms, given that the identity and number of other wheat MRPs remain unknown. Two experiments that could test the specificity of such an antibody include: (1) Comprehensive Western analysis of wheat MRP-fusion proteins in E. coli, however, since other putative wheat MRPs remain elusive, this experiment is not currently possible. (2) Construct an antisense mutation to reduce expression of TaMRP2 in wheat, however this method is difficult because of the hexaploid nature of wheat (Triticum aestivum); an alternative approach may be to examine a diploid wheat such as Triticum sp. A specific TaMRP2 antibody could also be employed to determine the localisation of TaMRP2 in yeast.

Alternative approaches to determine the subcellular localisation of TaMRP2 in plants include epitope-tagging approaches [(e.g. c-myc), which were successfully employed to determine the location of AtPGP1 (Dudler and Sidler, 1998)], and fusions with reporter proteins such as green fluorescent protein (GFP), which
have been used to determine the location of a novel pyrophosphatase (H+-PPase; ACP2) from *Arabidopsis* (Mitsuda, *et al.*, 2001).

### 6.4 Chemical induction of TaMRP2

The chemicals employed to determine the inducibility of *TaMRP2* were either stress inducers e.g. aminotriazole that leads to mild oxidative stress through the inhibition of catalases, or herbicide safeners, chemicals that are linked to increased levels of GSH and/or GST activities, both of which play important roles in xenobiotic detoxification, and oxidative stress tolerance. It therefore seems likely that since these chemicals also induce *TaMRP2*, it too may play a role in the detoxification of xenobiotics and oxidative stress tolerance. To examine the induction of *TaMRP2* further, it would be interesting to test a broader range of chemicals, for example cadmium and CDNB. If cadmium and CDNB did increase the transcript abundance of *TaMRP2*, it would suggest that the tolerance exhibited by *TaMRP2* in response to these chemicals is at least in part due to its expression. Interestingly, the transcript levels of *AtMRP3* are induced by CDNB (Tommasini, *et al.*, 1997), but not by cadmium (Sánchez-Fernández, *et al.*, 1998), and whereas *AtMRP3* is known to transport CDNB, the mechanism of cadmium tolerance remains unknown but it remains likely that cadmium is transported.

Other chemicals that could be tested include: herbicides used to control weeds in wheat, metals and metalloids e.g. aluminium, copper and arsenite, and products of oxidative stress i.e. lipid hydroperoxides. In addition to examining the induction of *TaMRP2* by different chemicals, it may prove interesting to study the effects of one chemical over a time course.
6.5 Heterologous expression

TaMRP2 was expressed successfully at low copy number in the ycf1 mutant of *S. cerevisiae*, restoring both cadmium tolerance and CDNB tolerance to levels similar to those exhibited by the wild type strain. Although the use of yeast mutants is a common tool employed for examining protein function, the expression of GC-rich genes encoding membrane proteins is not easy and therefore the expression of TaMRP2 in yeast was significant, paving the way for studying the expression of other wheat MRP genes. An important point that arose from this work was the utility of expression systems available for the study of wheat membrane proteins. *S. cerevisiae* was chosen for its relative ease of use, low cost, availability of mutants and successful application for the expression of human and *Arabidopsis* MRPs, however this is not to say alternative expression systems are less useful. Yeasts including: *Schizosaccharomyces pombe*, *Hansenula polymorpha* and *Pichia pastoris* have all been used for the expression of heterologous proteins, and although they are not as well characterised as *S. cerevisiae*, they do have certain advantages. For example, the translational-initiation mechanism of *Schizosaccharomyces pombe* is more similar to that of higher eukaryotes than *S. cerevisiae* (for review see; Giga-Hama and Kumagai, 1999), and the methylotrophic yeasts *Hansenula polymorpha* and *Pichia pastoris* show high expression levels of heterologous proteins in comparison to *S. cerevisiae* (Gellissen and Hollenberg, 1997; Sudbery, 1996). Two features of *Hansenula polymorpha* that are particularly attractive for the specific expression of TaMRP2 are its high GC content (49.5 % compared to 39.7 % GC content of *S. cerevisiae*), and the formation of peroxisomes which can occupy up to 80 % of the total cell volume. The latter contain relatively few homologous membrane proteins and heterologous membrane proteins can be targeted to these organelles (van Dijk, *et al.*, 2000). An alternative expression outlined in the introduction of Chapter 5 was the relatively new *Drosophila* expression system (DES; Invitrogen). Two obvious advantages of this system were the high GC content of *Drosophila melanogaster* host and the ability to generate stable lines expressing heterologous protein. One final expression system that merits discussion is Baculovirus. This system was adopted for the expression of TaMRP2, and
although it was unsuccessful, given more time and optimisation, this system may provide a useful tool for studying the expression of TaMRP2. To best characterise TaMRP2, it may be necessary to utilise several expression systems.

6.6 Functional capabilities of TaMRP2

6.6.1 TaMRP2 and cadmium
In chapter 5, it was demonstrated that TaMRP2 is capable of conferring tolerance to both cadmium and CDNB in intact yeast cells. Cadmium tolerance is a phenotype limited to ScYCF1, human MRP1 and AtMRP3, although to date only membrane vesicles containing ScYCF1 have been shown to transport Cd'GS$_2$ complexes. The species transported by human MRP1, AtMRP3 and TaMRP2 are unknown. Vesicle studies are required to answer this question, however based on the current literature, several suggestions can be proposed, and perhaps the most obvious of these is that the aforementioned MRPs may transport Cd'GS$_2$ complexes. Evidence that should not be overlooked is the inability of AtMRP1 and 2 to transport Cd'GS$_2$ complexes unless transport of these complexes requires a co-reactant that is not available in vitro, and not required by ScYCF1. As discussed in detail in Chapter 5, it is possible that MRPs may confer cadmium tolerance through the transport of products of cadmium toxicity e.g. GSSG or lipid metabolites. In support of this, human MRP1, AtMRP1 and 2 can transport GSSG (Jedlitschky, et al., 1996; Lu, et al., 1998; Lu, et al., 1997), and there is also evidence to suggest that MRP is the likely candidate for the transport of a glutathione conjugate of linoleic acid hydroperoxide into barley vacuoles. However, the direct detoxification of cadmium by MRPs in yeast via Cd'GS, transport seems more probable.

It should be noted that _S. cerevisiae_ has other potential mechanisms for dealing with cadmium toxicity in addition to ScYCF1, and these may or may not be related to MRPs: the heavy metal carrier proteins metallothioneins can neutralise the toxic effects of free cadmium ions by binding with them (for reviews see: Klaassen, _et al._, 1999; Robinson, _et al._, 1993; Viarengo, _et al._, 2000), and
interestingly, expression of *Arabidopsis* metallothioneins in Δycf1 restores cadmium tolerance (unpublished data quoted by; Tommasini, *et al*., 1996). In *S. cerevisiae*, the cadmium-resistant gene (*CAD2*), a mutant of a copper-transporting P-type ATPase is thought to control the intracellular level of cadmium through an enhanced cadmium efflux system (Shiraishi, *et al*., 2000).

In yeast, YCF1 plays an essential role in the detoxification of cadmium, but the importance of MRPs for cadmium detoxification in plants remains uncertain. Plants have several possible mechanisms for protection against metals and these include: reduced transport across the cell membrane, metal binding to the cell wall, active efflux, compartmentalisation, and chelation to metallothioneins and phytochelatins. The latter of these mechanisms is thought to be the most prevalent mechanism for protection against cadmium (Prasad, 1995; Robinson, *et al*., 1993). It would therefore be interesting to examine the role of MRPs in cadmium detoxification *in planta*, however this is not an easy goal to achieve. Initial experiments would include examining the effect of altering TaMRP2 function. In *Arabidopsis* the altered function of an MRP could be examined by antisense and insertional mutagenesis i.e. T-DNA and transposons. Indeed, T-DNA insertion lines are freely available for *Arabidopsis* (Krysan, *et al*., 1999). The advantage of using the *Arabidopsis* mutants is that due to the completion of its genome sequence, the entire family of MRPs have been identified. This may prove important: it is possible that the mutation of one AtMRP may result in a phenotypic response in the presence of cadmium, however given that there are 15 AtMRPs in total, it is also possible that they may be able to compensate for the defective MRP due to functional redundancy. Due to the hexaploid nature of wheat, the examination of a specific gene function in wheat is complex: T-DNA insertions are not currently a option due to problems with transformation, and although transposon tagged lines are being generated at the Institute of Arable Crops Research, knockouts are not guaranteed. An alternative approach would be to examine gene function in diploid monocotyledonous species; T-DNA insertion lines have been generated in rice (Jeon, *et al*., 2000) and transposon tagged maize and rice are also available (Swarbreck pers. comm.; Izawa, *et al*., 1997).
6.6.2 TaMRP2 and oxidative stress
A number of observations can be made from this work to link TaMRP2 and oxidative stress tolerance: (1) sequence comparisons and functional data indicate that TaMRP2 may be a GS-X pump, and human MRPl and AtMRPl and 2 that encode these pumps are capable of transporting GSSG, a product of antioxidant actions (Jedlitschky, et al., 1996; Lu, et al., 1998; Lu, et al., 1997). (2) Chemicals shown to induce TaMRP2 are known to cause oxidative stress and in addition these chemicals can also induce proteins (e.g. GST) that play an important role in the defence against oxidative stress. (3) Heavy metals such as cadmium can cause oxidative stress directly through lipid peroxidation or indirectly by depleting free-radical scavengers such as GSH. Since TaMRP2 confers cadmium resistance in yeast, an indirect link between TaMRP2 and oxidative stress can be made.

Initial experiments, which may help to elucidate the potential role of TaMRP2 in oxidative stress tolerance in yeast, include transport assays to determine if TaMRP2 can transport products of antioxidant action, for example GSSG. An extension of this experiment would be to determine the intracellular ratio of GSH/GSSG in recombinant yeast cells in the presence of stress-inducing chemicals. One possible consequence of oxidative stress is lipid peroxidation, and there is a growing body of evidence to suggest that human MRPl, ScYCFl and an MRP-like transporter in plants can transport the products of this action (Falcon-Perez, et al., 1999; Leier, et al., 1994a; F. Theodoulou and E. Martinoia, unpublished results). Vesicles prepared from recombinant yeast cells could be employed to determine if TaMRP2 could transport substrates such as LTC\textsubscript{4} and glutathione conjugate derivatives of other oxidised lipids. One final chemical that merits discussion is menadione. Menadione causes oxidative stress through the generation of superoxide, and work in this thesis revealed that menadione is a
potent inducer of TaMRP2 transcripts. In yeast menadione can be conjugated to glutathione (Zadzinski, et al., 1998), and it would therefore be of interest to determine if TaMRP2 could transport the menadione-GS conjugate. It should be noted that the menadione-GS conjugate is still capable of redox cycling and therefore must be removed from the cytosol for full detoxification.

In addition to examining putative substrates that may link TaMRP2 to oxidative stress tolerance, alternative factors could be analysed. The regulation of oxidative stress has received considerable attention, and there is now compelling evidence to suggest that transcription factors play an important role in the regulation of specific cellular responses to oxidative stress (e.g. Jaiswal, 1994; MoradasFerreira, et al., 1996). In both mammals and yeast transcription factors have been identified (activator protein-1; yeast, yAP1; humans, AP-1), and evidently control the transcription of human MRPl and ScYCF1 in response to oxidative stress. AP-1-type or other transcription factors regulating MRPs in plants have yet to be identified (1.4.12), however Rea and co-workers (1998) have reported that the promoter of AtMRP2 contains a bZIP recognition sequence (found in AP-1), and the promoter of AtMRP1 contains putative antioxidant response elements (Rea, et al., 1998). In order to determine if the promoter region of TaMRP2 contains similar antioxidant responsive elements (AREs) it would be necessary to isolate a genomic clone. The promoter region of this clone could then be analysed for the presence of consensus binding sites for known transcription factors using computational programs like the Transcription Factor Database (http://transfac.gbf.de/), that includes programs MatInspector V2.2 and PatSearch V1.1. The usefulness of this approach however is questionable because the consensus sequences of yAP1 and human AP-1 binding sites (and possibly those of TaMRP2, if present) are different. Additionally, Arabidopsis for example has over 1500 transcription factors, and 81 of these belong to the same family as AP-1 (Riechmann, et al., 2000); this makes the identification of a specific transcription factor very difficult.
6.6.3 TaMRP2 and herbicides
The initial aim of this thesis was to determine if wheat MRP isoforms played a role in detoxification of xenobiotics such as herbicides. Work outlined in this thesis details how herbicide safeners can induce TaMRP2 transcripts, and although this suggests that TaMRP2 may participate in the detoxification of herbicides further experimental evidence is required. It is possible that TaMRP2 may operate in the detoxification of electrophilic herbicides such as atrazine, metolachlor and fenoxaprop-ethyl which are known to be conjugated to glutathione (for review see Cole and Edwards, 2000). Once again, a yeast vesicle system could be used to examine the TaMRP2-transport of a range of herbicides, however on a whole cell level yeast does not provide an effective system for the study of herbicides since yeast cells are lacking many herbicide target sites present in plants. Transgenics may provide an alternative way to examine the phenotypic effects of xenobiotics on TaMRP2 in planta, however as described above, this is a long-term approach, which is technically challenging.

6.7 Future perspectives for TaMRP2
The association between TaMRP2 and xenobiotic metabolism could be of interest commercially in the future. Herbicide resistance in weeds is an ever-increasing problem (see www.weedscience.com), but one that can be improved by having a complete understanding of the metabolic pathways that control herbicide selectivity. Isolation and characterisation of individual genes such as TaMRP2 involved in the metabolic pathways of herbicide detoxification could potentially be exploited to help overcome resistance in weeds. For example, the over expression of TaMRP2 by genetic modification of a crop may enhance the metabolism of a specific herbicide, a principle that has previously been demonstrated in transgenic wheat, where the ectopic expression of a maize GST increased the resistance of the plant to alachlor (Milligan, et al., 2001). The potential downside of this strategy is that since herbicides are detoxified by a multi-step pathway, and TaMRP2 is one step in that pathway; the expression of TaMRP2 alone may not be able to achieve effective herbicide tolerance. It is not
known whether transport is rate-limiting in the pathway, wild-type plants may have sufficient transport capacity for rapid xenobiotic detoxification and other factors such as GSH levels or GST activity may be limiting.

TaMRP2 confers tolerance to cadmium and possibly other heavy metals or metalloids such as arsenite and antimony not yet examined. Heavy metal pollution in the soil, can result in accumulation of heavy metals in the grain of wheat resulting in toxicity to the consumer. Accumulation of cadmium in the grain might be prevented by overexpression of TaMRP2 in the root. It should not be forgotten however that plants have more than one mechanism for responding to heavy metals, e.g. metallothioneins and phytochelatins, and the overexpression of TaMRP2 alone may not be sufficient to enhance heavy metal detoxification.

As further mechanisms of TaMRP2 are elucidated, its practical implications are likely to increase.
### APPENDIX I

#### Inventory of Arabidopsis MRPs

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein identification number</th>
<th>Accession number</th>
<th>Amino acids</th>
<th>Functional information</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtMRP1</td>
<td>AAG28284</td>
<td>Q9C8G9</td>
<td>1622</td>
<td>GS-X pump</td>
<td>Lu et al., (1997); Lu et al., (1998).</td>
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Summary of the data currently available for the Arabidopsis MRPs. Protein identification numbers are as designated by Sánchez-Fernández and co-workers (2001). To date only five of the AtMRPs have been cloned (AtMRP1-5), functional data is not available for AtMRPs 6-15 (shaded area). EST data has not been included for reasons of space: for further details please refer to Sánchez-Fernández et al., (2001) or The Institute for Genomic Research (TIGR) database (http://www.tigr.org/tdb/tgi.shtml).
APPENDIX II

Nucleotide sequence of the full length TaMRP2

The flanking nucleotides of pZL1 (Life Technologies) are highlighted in red, the nucleotide and amino acid sequences of TaMRP2 are in black text. The ATG initiation site is underlined and nucleotides important for initiation of translation (Kozak sequence) are highlighted in bold. The ABC signature motif and Walker A and B motifs are double underlined. Primers employed for PCR are marked with arrows.

```
Rsr II   Eco RI   Sal I   pZL polylinker
   |       |       |            1
AGGTACGGTGTCGGAATTCCCGGGTCGACCCACGCGTCCGATTCAACCCAAGTCAAGAAT
                        ATTCACCCCAAGTCAGAAAT
                        TCAAGTTGGGTTCAGTTCTTA

21
AGGTACGGTGTCGGAATTCCCGGGTCGACCCACGCGTCCGATTCAACCCAAGTCAAGAAT
               +------------------
               80
ACTGTTTCTGAAACGGCGGTGATTGTGATGACCCGGCTACCGGTAGCTACCGGAGGTAC

81
GTCGTAATCCGATCTATTCGGGCTCTTCTGCACTAGGCAAGGCTGGGCTTGGGCGG

141
GGAGAGGACGGCGCTTCGAGGCAAGGCGGCGCAGGCGCTTCGCCGCGGCG

201
CTCCTTCCTGGGCGCAACCGTATGGGCGTTCGCTCTGGGCGGCGTACG

261
GCTCGCGTGCACGGCGCGGCGCCGTCGCTACCGCCGCTGCCGCTCGCTCGCTAGTGACG

321
AGCCGAGCGCTTCGAGGCAAGGCGGCGCAGGCGCTTCGCCGCGGCG

381
TGCGAGCGCTTCGAGGCAAGGCGGCGCAGGCGCTTCGCCGCGGCG
```

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