IDENTIFICATION AND FUNCTIONAL CHARACTERISATION OF A CLASS ON NON-SELECTIVE CATION CHANNELS IN *SACCHAROMYCES CEREVISIAE*

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6. PQ loop repeat proteins in living systems.

6.1. Introduction

A large number of proteins from other living systems share sequence similarity with YDR352w and YOL092w (Figure 6-1). In general terms, the greater the similarity between two proteins, the higher the probability that they share common functions or properties. This premise drives the thought that proteins similar to the yeast PQ loop repeat proteins characterised are responsible for non-selective cation flux in other systems. By comparing protein sequences it is possible to infer which residues may be important for cation flux.

6.2. Results and Discussion

A difference in MA^+ toxicity phenotype was observed between the strains ∑1278b, 31019b and 26972c/2 (Figure 2-1). Analysis of the YDR352w, YOL092w and YBR147w genomic DNA sequences of each of these strains reveals differences predicted at the protein level (Figure 6-2). Of these changes, an alteration at Q30H in the 26972c2 sequence of YDR352w (Figure 6-2, A) is probably the most relevant. This mutation is within the very conserved first PQ loop repeat region. As it is so well conserved it is likely to perform an important role in the function of the cell. This mutation may result in the phenotypic differences observed between 31019b and 26972c2 in terms of MA^+ tolerance. Unfortunately there was no time to further validate this mutation and introduce similar substitutions in YDR352w and observe the effect on cation flux. Other mutations are also revealed through sequence alignment. YBR147w displays a higher degree of polymorphism between strains

compared to YDR352w and YOL092w (Figure 6-2, C). The introduction of a stop codon at 91Q in 31019b and frame shifts in the 31019b and ∑1278b sequences suggest YBR147w protein is of less functional importance than YDR352w and YOL092w and, since 31019b displays a viNSCC-like phenotype (Figure 2-1), YBR147w may not contribute to viNSCC mediated flux in yeast.

Knowing the molecular identity of these proteins allows for speculation of their function and regulation. Functional analysis of Stm1, a protein of the PQ loop repeat family found in *Schizosaccharomyces pombe* (Chung et al., 2001) has revealed conserved residues that may be involved with G protein interaction. Two residues on the Stm1 sequence are identified as potential interaction sites, I197 and K199. Comparison of the Stm1 sequence with the amino acid sequences of YDR352w and YOL092w show that the I197 equivalent is conserved in YOL092w (Figure 6-2, B) and the K199 equivalent is conserved in YDR352w (Figure 6-2, A).

Sequence alignments of proteins that share PQ loop repeat motifs may also reveal residues of particular interest (Figure 6-2). The loop regions that follow the PQ repeats that identify these proteins show a reasonable degree of conservation across phyla. The two putative G-coupled protein interaction residues described previously are found on the second loop motif (Figure 6-2). The PQ loop regions in these proteins show the greatest degree of conservation, in particular the P Q I motif found in the first PQ loop of YDR352w and in both domains of YOL092w and YBR147w. The second PQ loop of YDR352w has a conserved P Q L motif. For a more detailed analysis of structure-based function of these proteins, further work is required.

6.3. Materials and Methods

6.3.1. Interrogation of online databases for putative viNSCC sequences.

The protein sequence of YDR352w was retrieved from the Saccharomyces Genome Database (SGD) and the BLAST algorithm (Gish, 1996 - 2004) used to find similar sequences across all NCBI (http://www.ncbi.nlm.nih.gov/) databases available. Standard algorithm parameters were used. All hits were retrieved and downloaded in FASTA format.

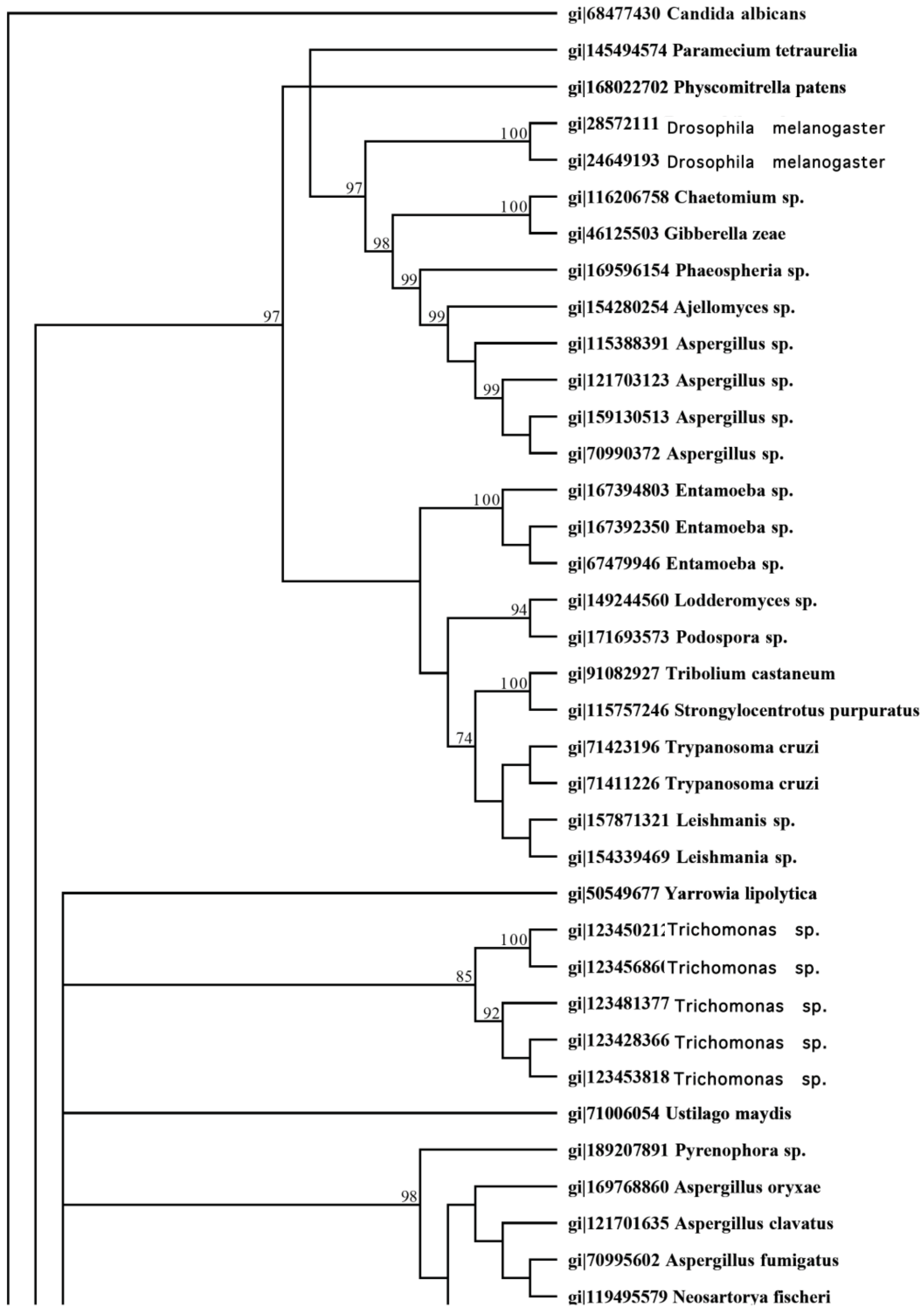
6.3.2. Sequence alignments and construction of phylogenetic trees

Protein sequences retrieved were opened in MacVector (Symantec) and compiled into a single protein alignment file. The ClustalW alignment algorithm (Chenna et al., 2003) was then used at standard settings with the exception of altering the matrix to BLOSUM and increasing the gap penalty for multiple alignments to 1. Once aligned, phylogenetic trees were constructed using the neighbour joining method, absolute difference and 1000 bootstrap replications.

6.3.3. Cloning of yeast PQ loop repeat proteins, sequencing, alignment and domain prediction

The genes YDR352w, YOL092w and YBR147w were sequenced from genomic DNA extracted from the strains 31019b, 26972c2, CY 152/162 and ∑1278b using flanking

primers (Figure 4-6) in conjunction with Big Dye v3.2 (Applied Biosystems). Resultant sequences were aligned with each other and those available on the Saccharomyces Genome Database (SGD) using MacVector (Accelrys). Prediction of trans-membrane domains, prediction of PQ loops and potential G protein interaction residues was done through the combination of protein alignments to *S. pombe* Stm1, and data generated from the software packages TMpred (Hofmann, 1993), DAS (Cserzo et al., 1997) and TMHMM (Sonnhammer, 1998).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gi|91082927 Tribolium castaneum
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gi|71423196 Trypanosoma cruzi
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gi|157871321 Leishmanis sp.
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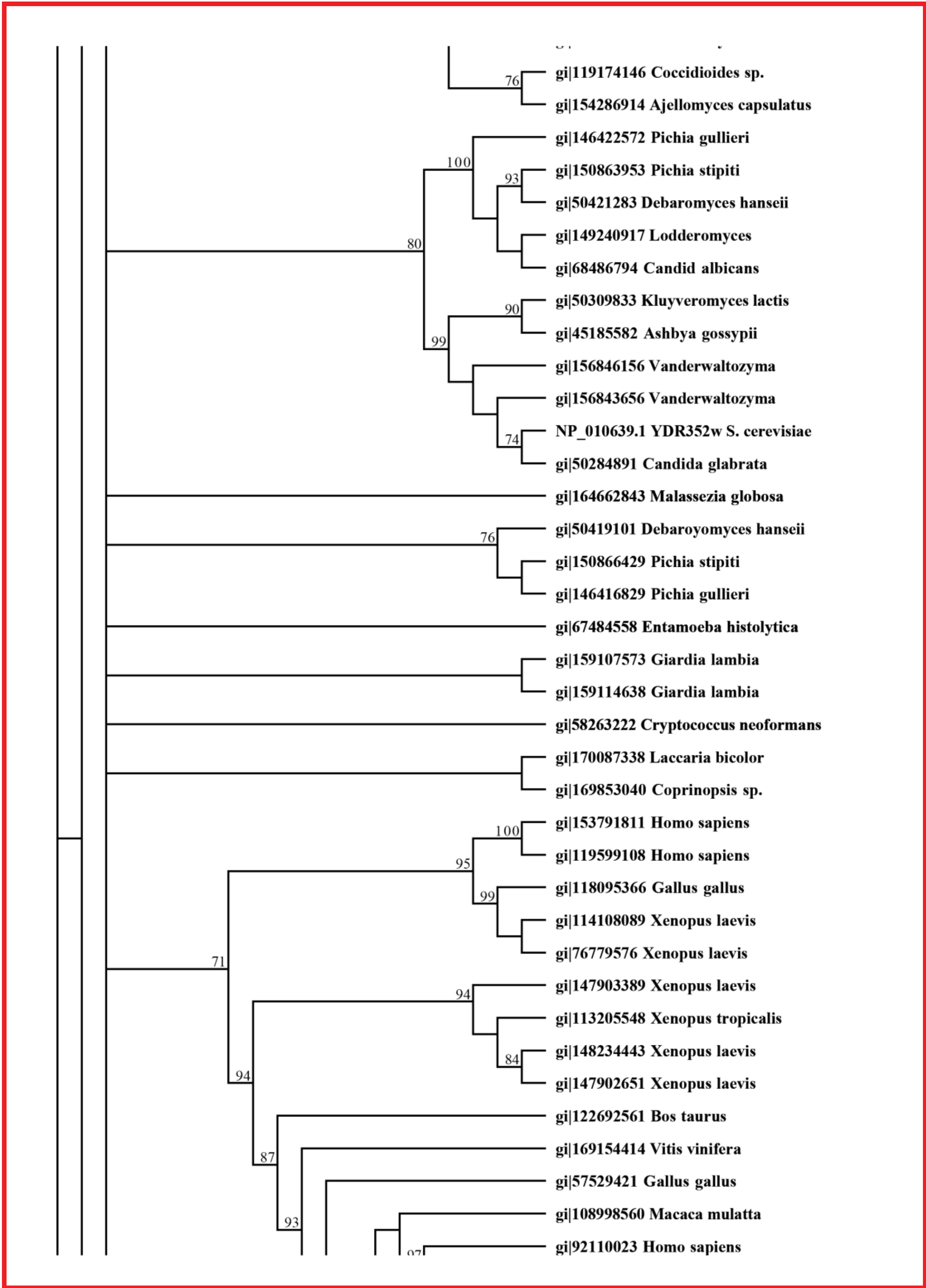

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gi|50309833 Kluyveromyces lactis
gi|45185582 Ashbya gossypii
gi|156846156 Vanderwaltozyma
gi|156843656 Vanderwaltozyma
NP_010639.1 YDR352w S. cerevisiae
gi|50284891 Candida glabrata
gi|164662843 Malassezia globosa
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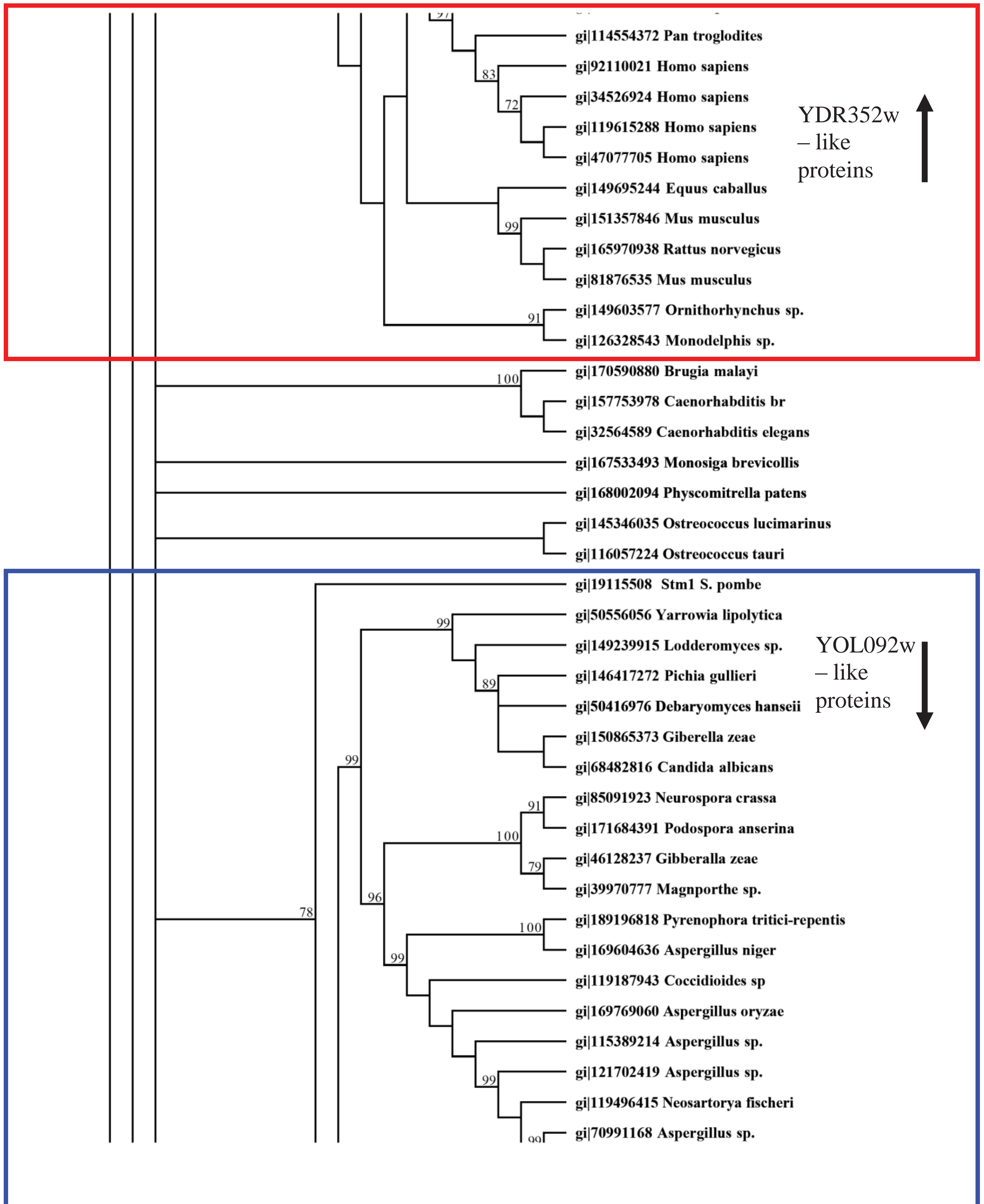

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YDR352w
– like
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gi|32564589 Caenorhabditis elegans
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gi|168002094 Physcomitrella patens
gi|145346035 Ostreococcus lucimarinus
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YOL092w
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gi|171684391 Podospora anserina
gi|46128237 Gibberalla zeae
gi|39970777 Magnporthe sp.
gi|189196818 Pyrenophora tritici-repentis
gi|169604636 Aspergillus niger
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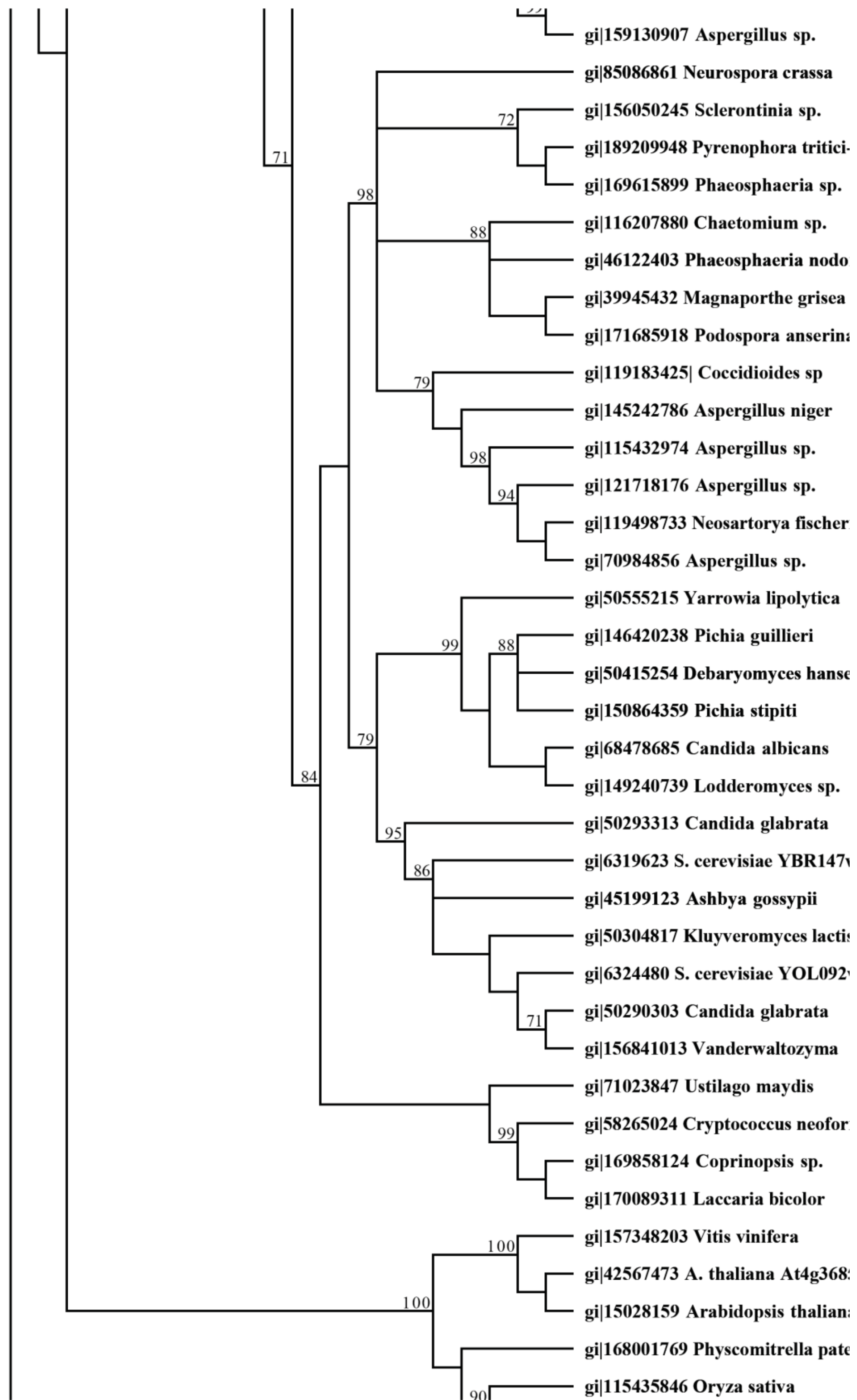
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gi|171685918 Podospora anserina
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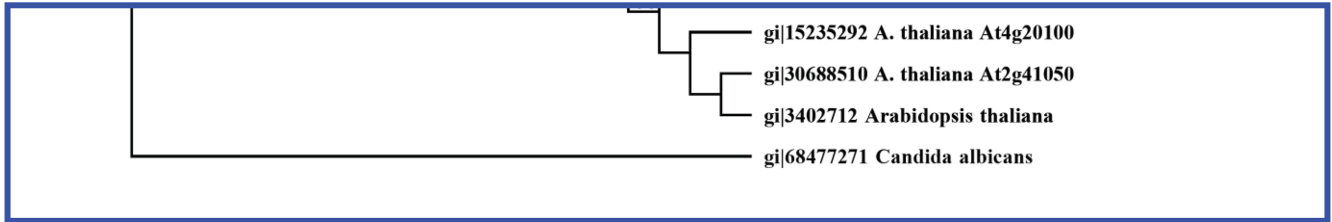
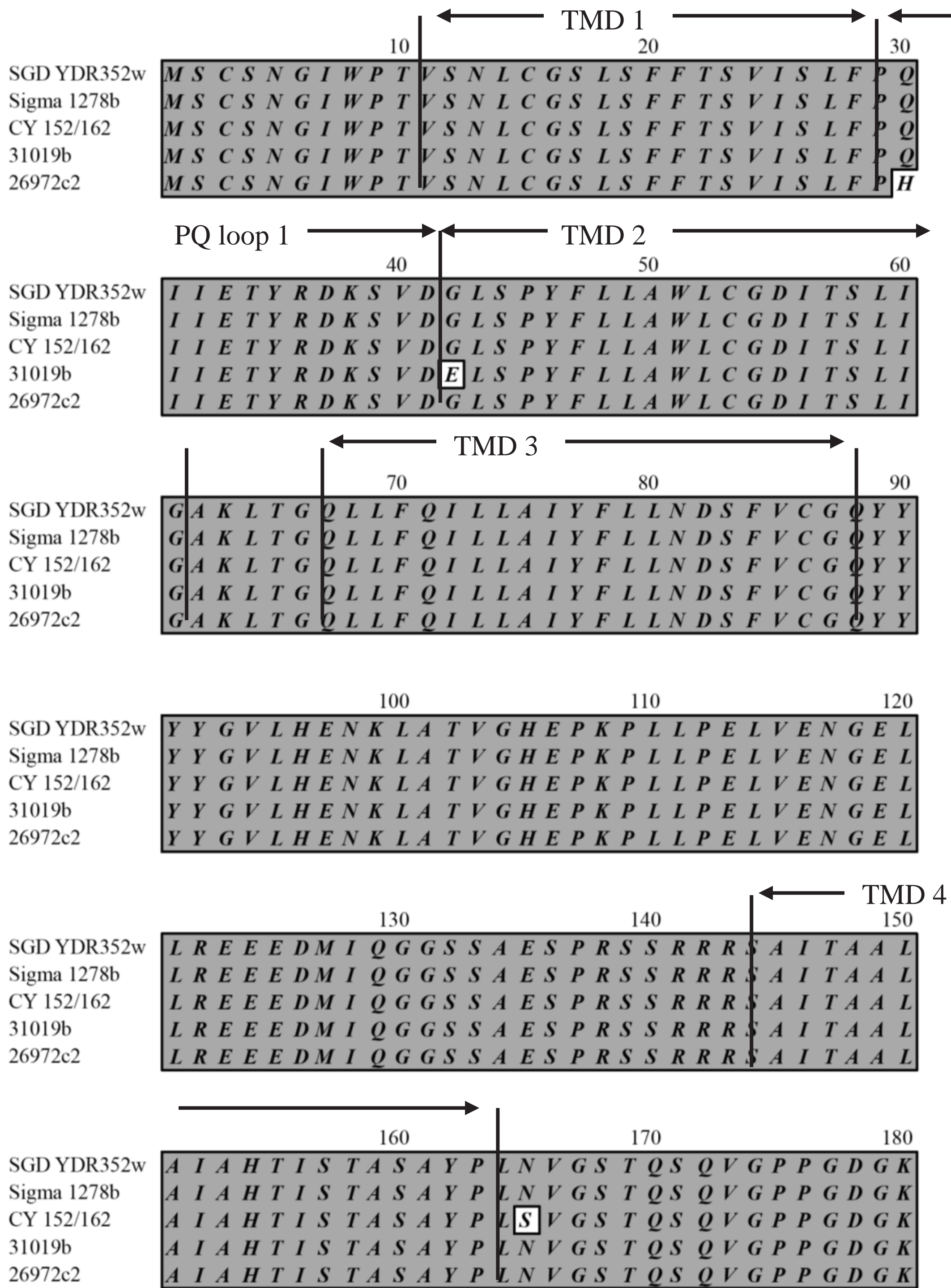
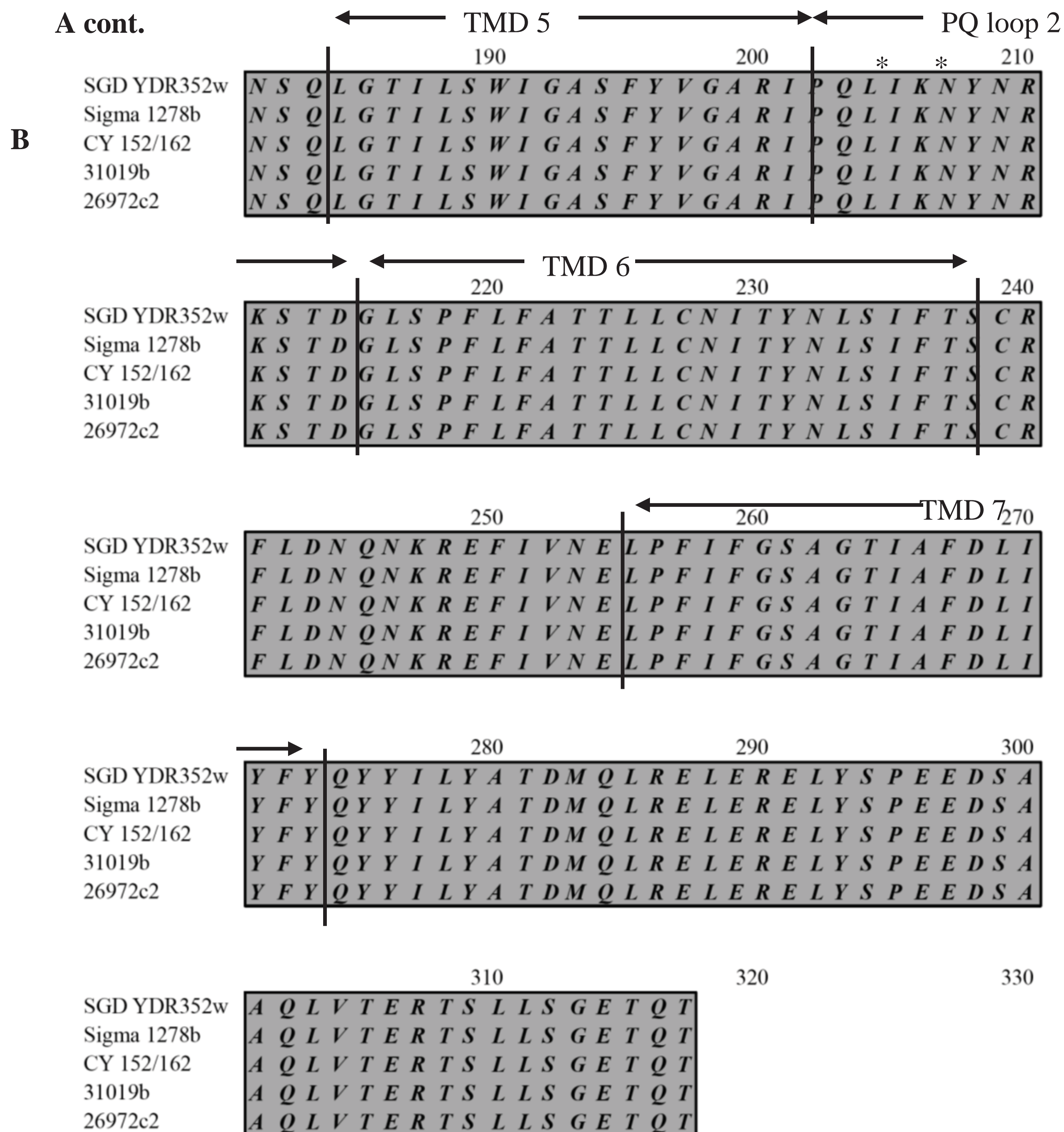


Figure 6-1: Phylogenetic tree of proteins that show significant similarity to the sequence of YDR352w.

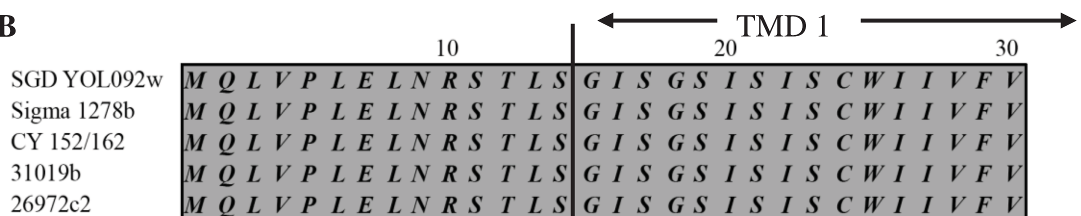
The protein sequence of YDR352w was used to BLAST NCBI databases and retrieve sequences that show similarity (See 6.3.1 and 6.3.2). The phylogenetic tree suggests YDR352w and YOL092w may be representative of two distinctions within this family as they form clear groupings.

A





* = Possible G protein binding residue(s) based on comparison to the *Schizosaccharomyces pombe* protein Stm1.


B
TMD 1
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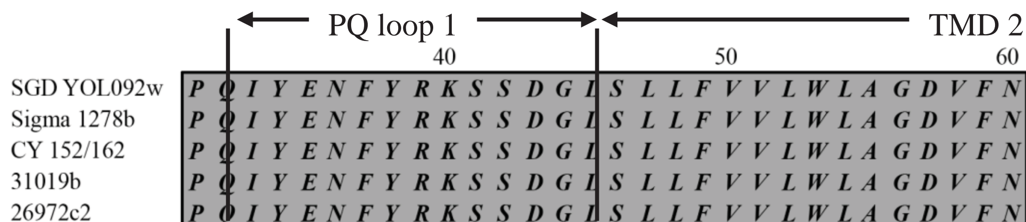
SGD YOL092w M Q L V P L E L N R S T L S G I S G S I S I S C W I I V F V
Sigma 1278b M Q L V P L E L N R S T L S G I S G S I S I S C W I I V F V
CY 152/162 M Q L V P L E L N R S T L S G I S G S I S I S C W I I V F V
31019b M Q L V P L E L N R S T L S G I S G S I S I S C W I I V F V
26972c2 M Q L V P L E L N R S T L S G I S G S I S I S C W I I V F V


PQ loop 1
TMD 2
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60
SGD YOL092w P Q I Y E N F Y R K S S D G I S L L F V V L W L A G D V F N
Sigma 1278b P Q I Y E N F Y R K S S D G I S L L F V V L W L A G D V F N
CY 152/162 P Q I Y E N F Y R K S S D G I S L L F V V L W L A G D V F N
31019b P Q I Y E N F Y R K S S D G I S L L F V V L W L A G D V F N
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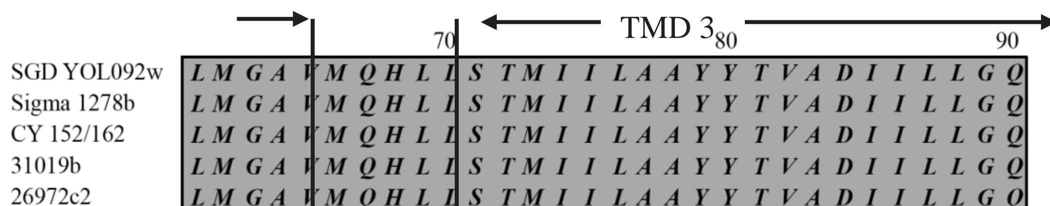
TMD 3
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SGD YOL092w L M G A V M Q H L L S T M I I L A A Y Y T V A D I I L L G Q
Sigma 1278b L M G A V M Q H L L S T M I I L A A Y Y T V A D I I L L G Q
CY 152/162 L M G A V M Q H L L S T M I I L A A Y Y T V A D I I L L G Q
31019b L M G A V M Q H L L S T M I I L A A Y Y T V A D I I L L G Q
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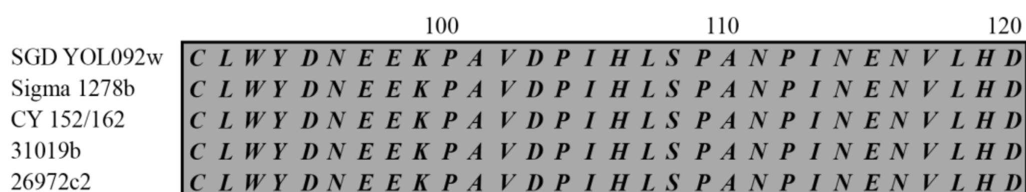
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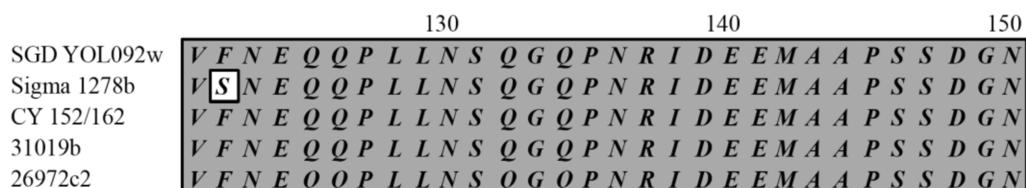
SGD YOL092w C L W Y D N E E K P A V D P I H L S P A N P I N E N V L H D
Sigma 1278b C L W Y D N E E K P A V D P I H L S P A N P I N E N V L H D
CY 152/162 C L W Y D N E E K P A V D P I H L S P A N P I N E N V L H D
31019b C L W Y D N E E K P A V D P I H L S P A N P I N E N V L H D
26972c2 C L W Y D N E E K P A V D P I H L S P A N P I N E N V L H D


130
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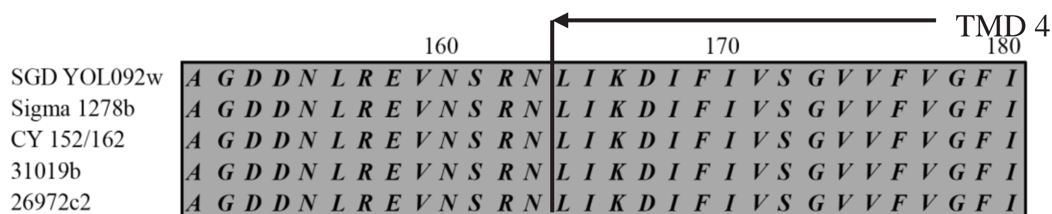
SGD YOL092w V F N E Q Q P L L N S Q G Q P N R I D E E M A A P S S D G N
Sigma 1278b V S N E Q Q P L L N S Q G Q P N R I D E E M A A P S S D G N
CY 152/162 V F N E Q Q P L L N S Q G Q P N R I D E E M A A P S S D G N
31019b V F N E Q Q P L L N S Q G Q P N R I D E E M A A P S S D G N
26972c2 V F N E Q Q P L L N S Q G Q P N R I D E E M A A P S S D G N


TMD 4
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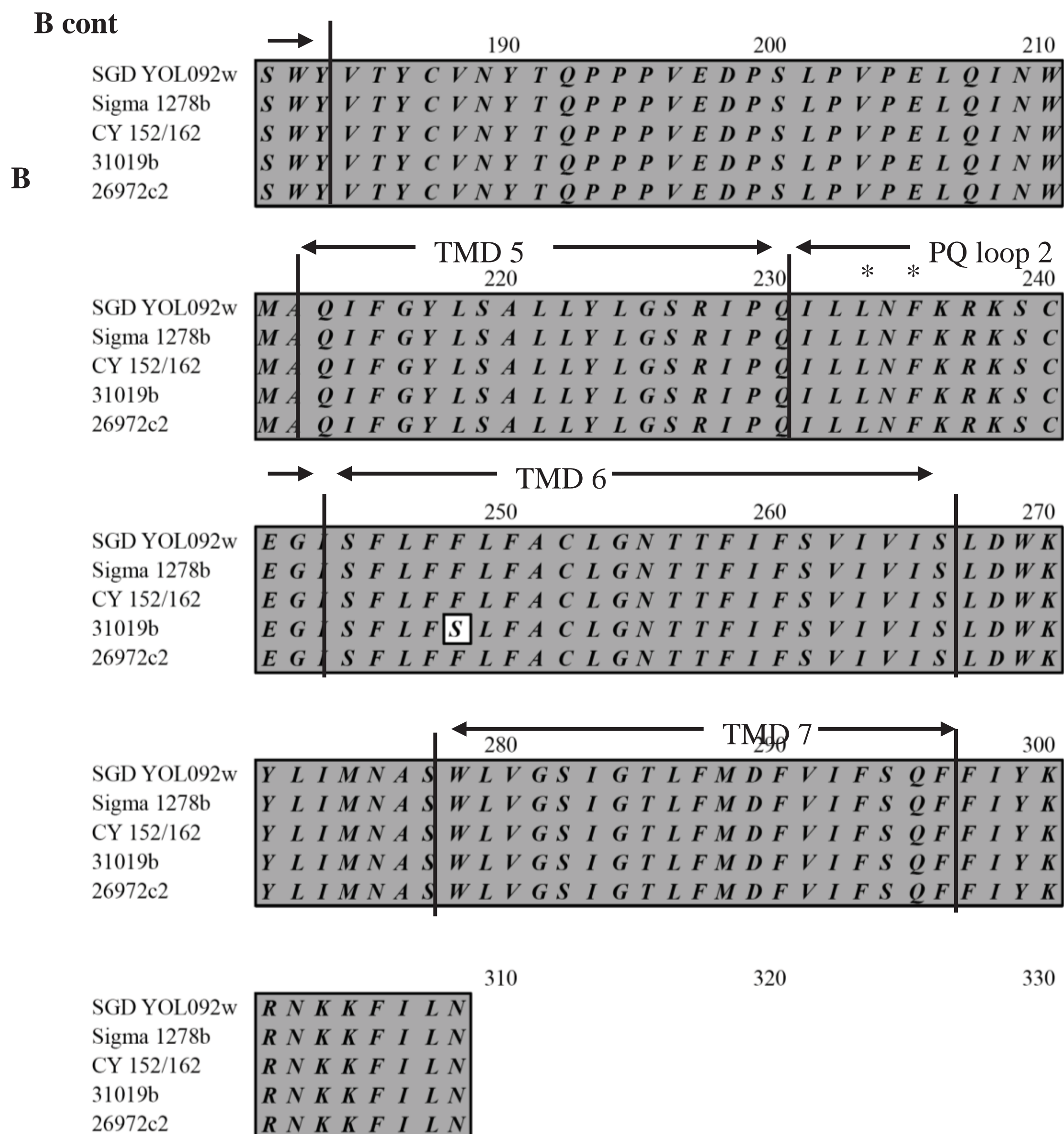
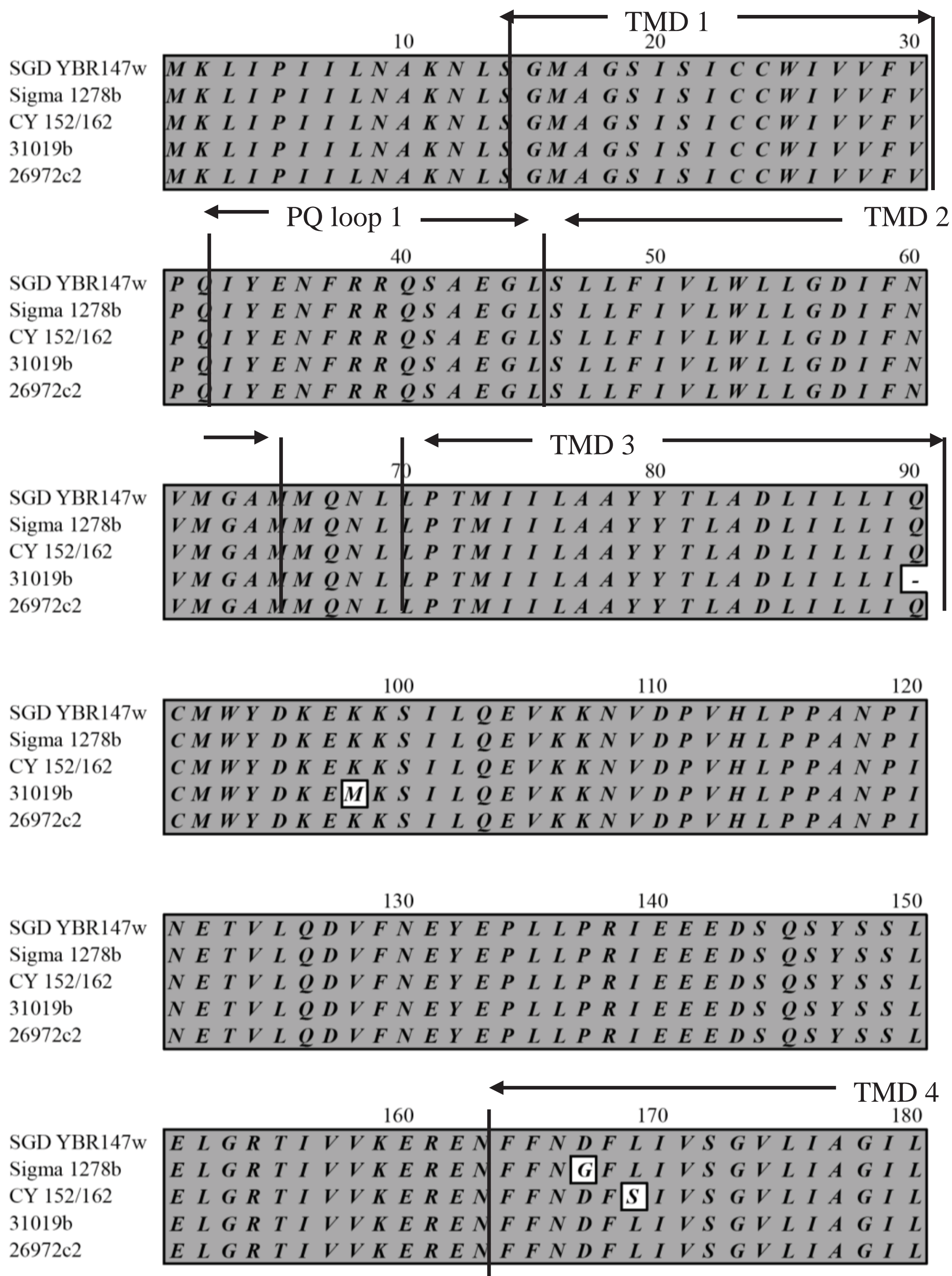
SGD YOL092w A G D D N L R E V N S R N L I K D I F I V S G V V F V G F I
Sigma 1278b A G D D N L R E V N S R N L I K D I F I V S G V V F V G F I
CY 152/162 A G D D N L R E V N S R N L I K D I F I V S G V V F V G F I
31019b A G D D N L R E V N S R N L I K D I F I V S G V V F V G F I
26972c2 A G D D N L R E V N S R N L I K D I F I V S G V V F V G F I



* = Possible G protein binding residue(s) based on comparison to the *Schizosaccharomyces pombe* protein Stm1.

C



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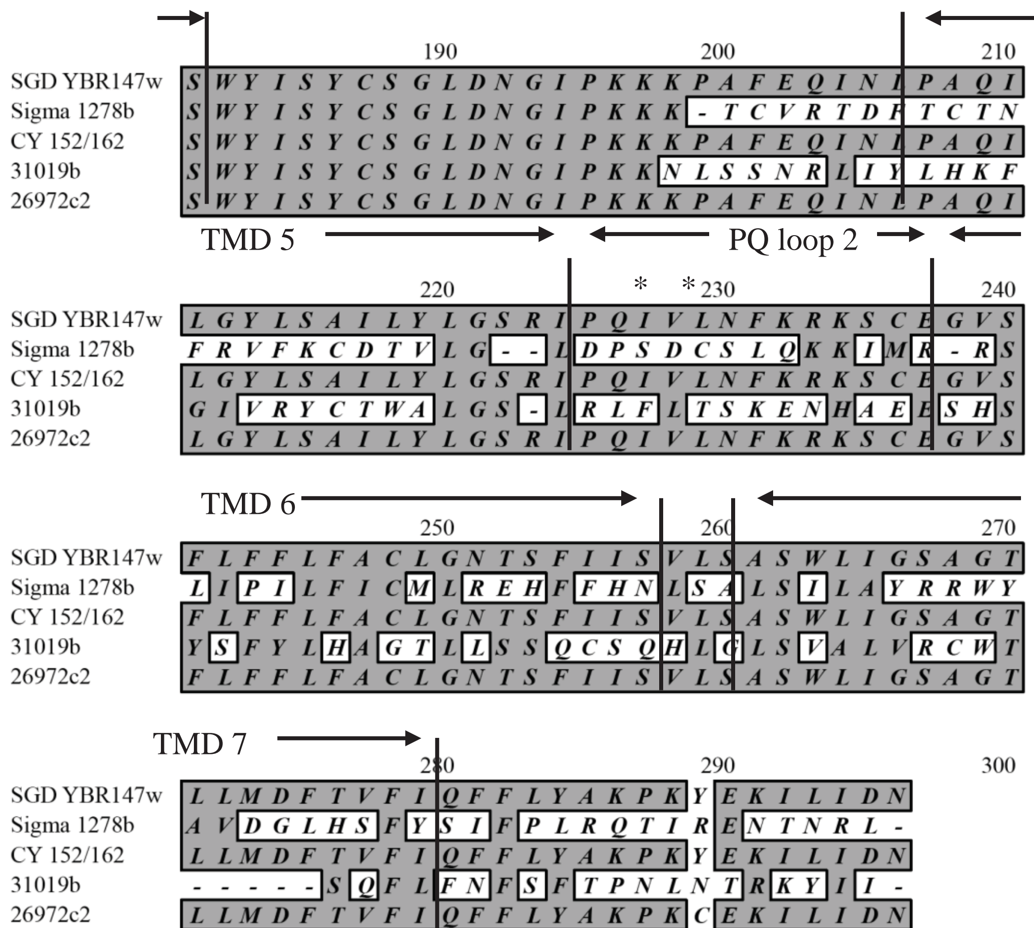


* = Possible G protein binding residue(s) based on comparison to the *Schizosaccharomyces pombe* protein Stm1.

Figure 6-2: ClustalW alignments of PQ loop repeat proteins sequenced from various yeast strains.

Genomic DNA sequences from 31019b, 26972c2, CY 152/162 and ∑1278b were sequenced and aligned with sequences available on the SGD, from the strain S288 (6.3.3). Alignments show sequence specific polymorphisms and putative domains of importance (6.3.3).

7. General Discussion

7.1. S. cerevisiae PQ loop repeat proteins as viNSCCs

The *S. cerevisiae* PQ loop repeat proteins YDR352w and YOL092w have been shown to catalyse a viNSCC current in *Xenopus oocytes* (see Chapter 5). Data obtained shows they both catalyse the flux of numerous cations, including K^+, Na^+ and NH_4^+ across biological membranes. They have also been shown to be sensitive to external Ca^{2+} concentration, cation flux through these proteins decreases with increased external Ca^{2+} concentration. These data indicate these members of the PQ loop repeat protein family induce cation flux that is physiologically similar to other available viNSCC data (Tyerman et al., 1995; Bihler, 1998; Davenport, 1998; Davenport and Tester, 2000; Bihler, 2002; Demidchik, 2002a).

7.2. viNSCCs as ubiquitous cation flux facilitators

Evidence of viNSCC activity can be found across many phyla (Hagiwara et al., 1992; Siemen, 1993; Bihler, 1998; Davenport, 1998; Bihler, 2002; Demidchik, 2002a). Common physiology observed suggests a fundamental functionality is associated with the activity of these channels. As an extension of common function, the conservation of key residues in the amino acid sequences of the associated proteins is likely to maintain structure and function.

7.3. PQ loop repeat proteins as cation channels

Over-expression of YDR352w and YOL092w in the strain 31019b resulted in increased accumulation of MA^+ and Na^+ according to radiotracer studies (Chapter 4).

To complement these data, expression was induced in oocytes of *Xenopus laevis* and current, due to the flux of various cations, measured (Chapter 5).

Properties that suggest these proteins are viNSCCs were gathered using both the yeast and *Xenopus* systems. When over-expressed in yeast, low affinity cation flux was observed (Figures 4-5, 4-6 and 4-7). Sensitivity of cation flux to Ca^{2+} and pH was also strongly suggested when these proteins were over-expressed (Figure 4-1 B and C). When expressed in the *Xenopus* system, evidence of channel like cation flux for numerous cations (Figures 5-1 to 5-10) was observed. Sensitivity of this flux to increasing Ca^{2+} concentrations was also observed in voltage clamped *Xenopus* oocytes (Figures 5-9 and 5-10).

These data strongly suggest a viNSCC current in being induced, resulting in an increase of non-selective cation flux across these biological membranes. It does not, however, show the flux is occurring directly through these proteins. It is important to consider other possible causes of these phenotypes, especially since the expression patterns of these proteins in the systems will probably alter the '*status quo*' of the systems used. It is possible the phenotypes observed are the result of an artefact from the expression of these proteins. Over expression of native yeast proteins could have a variety of effects, which may induce native viNSCCs and the use of voltage clamped *Xenopus* oocytes is far from a 'natural' system. Although inferences made from the observation of these data need to be approached cautiously, there is, however, evidence that supports the idea that ion flux occurs through these proteins.

The induction of this current through the expression of these proteins in both yeast and in *Xenopus* oocytes supports the hypothesis that these proteins directly catalyse the cation flux rather than induce native systems. If expression of YDR352w and YOL092w induced a native viNSCC, it is unlikely that it would do so in both yeast and *Xenopus* oocytes owing to the significant divergence in their cell biochemistries. Secondly, differences in currents catalysed by the expression of YDR352w and YOL092w in *Xenopus* oocytes suggest they catalyse cation flux themselves (Figures 5-5, 5-6, 5-9 and 5-10). If they both catalysed the production of native viNSCCs, it is very unlikely they would be able to influence their physiology differently in such a manner.

In *Schizosaccharomyces pombe* the PQ loop repeat homolog, STM1, is believed to interact with the heterotrimeric G protein subunit GPA2 and is termed a G protein coupled receptor (GPCR) (Chung et al., 2001). The interaction is predicted to be protein – protein and putatively involves amino acids in the PQ loop region (Figure 6-2). Interestingly, other GPCRs have also been associated with cation flux in plants (Wang et al., 2001). AtGPA1 is a heterotrimeric G protein subunit in *Arabidopsis thaliana* which is associated with guard cell signalling through association with an unidentified GPCR. Null mutants of GPA1 show defects in ABA response and associated K^+ flux profiles in guard cells. AtGPA1 shares 33% identity with the *S. pombe* GPA2, shown to interact with a PQ loop repeat protein. The association of G proteins, cation currents and potential interactions with PQ loop repeat proteins seem tantalisingly possible, although further work is required to demonstrate this.

Mammalian systems are well studied in terms of G protein complexes and their interaction with GPCRs. Roughly 40 - 45% of drugs available target GPCRs and

subsequently signalling pathways (Filmore, 2004; Bjarnadottir et al., 2006). They are involved in numerous signalling pathways that involve cation flux, including olfactory responses, glutamate responses and sight signal transduction (Bjarnadottir et al., 2006). Several proteins that share similar protein sequences to YDR352w and YOL092w have been identified in the human genome. These six or seven trans-membrane domain proteins may play a role in direct ion flux facilitation for one or more of these processes.

7.4. Further Investigation

Whilst the data gathered to this point strongly suggests the PQ loop repeat proteins YDR352w and YOL092w act as viNSCCs, further investigation is required to fully characterise them.

Localisation of these proteins to specific membrane(s) will provide considerable information as to the function of these proteins. Large-scale protein screens have not revealed a specific membrane these proteins may be active in (Huh, 2003). Definitive intercellular localisation is required for these genes.

A more comprehensive assay of the electrophysiological properties of these proteins is also required. This includes measurement of flux capacity for as many cations as possible to determine the true non-selective nature of this channel. Measurement of cation flux, pH influence and the exploration of channel blockers are also required, especially due to the structural information such an experiment will reveal.

Expression of truncated and mutated versions of these proteins is required to gain further structural information. This will also confirm the flux measured is due to ions travelling through these proteins and not due to an induced secondary reaction.

The most exciting future work is, however, the analysis of related proteins found in other systems. The identification of plant viNSCCs has huge potential in terms of modulating salt stress in crops.

8. Appendix

8.1. Optimisation of conditions for the analysis of PQ loop repeat proteins in Xenopus laevis oocytes.

Xenopus laevis oocytes are useful for exploring the electrophysiology of membrane bound proteins. We synthesised cRNA of YDR352w and used it to optimise conditions for cation flux analysis. Initial experiments used choline-Cl as the predominant cation in the bath solution. This is often used to allow good current flow without being transported itself due to its size. $Choline^{+}$ was initially used in the bathing solution for the characterisation of PQ loop repeat proteins.

8.1.1. Materials and Methods

A minimal bath solution was used for these optimisation experiments, 200 mM mannitol buffered to pH 7.0 with a trace (approximately 2 mM) amount of Tris-Cl. Further cations were added as required. Synthesis and injection of cRNA were as 5.4.1.1. Electrophysiology was carried out as in 5.4.1.2 with the exception that in these experiments a trace amount of Tris-Cl was used as a buffer whereas in the experiments in Chapter 5 used 5mM MES / Tris at pH 7.0.

8.1.2. Results

These experiments revealed a strong induction of current in oocytes injected with YDR352w cDNA when voltage was clamped at hyperpolarising potentials (Figure 8-1A, Figure 8-2, A and B). This is indicative of the native Xenopus Ca^{2+} activated Cl^{-}

channel (Weber, 1999). The induction of this current suggests that either the expressed protein is inducing the native Ca^{2+} activated Cl^- channel or that YDR352w in facilitating the flux of Ca^{2+} into the oocyte and driving this Ca^{2+} activated Cl^- channel current.

With 1 mM $CaCl_2$ and 2 mM $MgCl_2$ in this basic buffer (Figure 8-1B; Figure 8-2 C and D), currents resembling the Ca^{2+} activated Cl^- channel were present, although much reduced when compared to the choline-Cl based buffered traces.

Replacing Ca^{2+} with Ba^{2+} further reduced the difference between the YDR352w injected oocytes and the water injected oocytes (Figure 8-1 C; Figure 8-2 E and F). Removal of all divalent cations, leaving only 5 mM MES/Tris, effectively abolished any inward positive / outward negative ion flux at negative potentials (Figure 8-1 D; Figure 8-2 G and H).

8.1.3. Discussion

The data gathered from this buffer optimisation suggests that $choline^+$ is carried by YDR352w (see Chapter 5) and that Ca^{2+} influx is also increased into the oocyte. The induction of the Ca^{2+} activated Cl^- current indicates that either the higher external Cl^- concentration was inducing the Ca^{2+} activated Cl^- channel or Ca^{2+} was being carried through YDR352w, which in turn activates the Cl^- current.

8.2. Investigation of the NH_4^+ flux properties of AtAMT 1;4 and AtAMT 1;5

8.2.1. Introduction

Much research has been carried out in the characterisation of proteins of the MEP/AMT/Rh class. Heterologous expression in yeast mutants has played a major role in this characterisation, from initial identification of the *S. cerevisiae* MEPs (Marini et al., 1997) and the first AtAMT (Gazzarrini et al., 1999) to the subsequent characterisation of remaining *Arabidopsis* AMTs and the properties of the human RhAG and RhGK proteins. Recently, advances have been made using other systems such as *Xenopus laevis* oocyte expression (Ludewig et al., 2003)(Wood, 2006) and the construction of an *Arabidopsis* effectively devoid of functional AMTs for the characterisation of NH_4^+ flux (Yuan et al., 2007; Yuan et al., 2007). A potential flaw exists in data collected in such a manner as NH_4^+ efflux is difficult to measure and is often ignored. Using heterologous expression in the *S. cerevisiae* strain 31019b (Marini et al., 1997) the catalysis of NH_4^+ influx and efflux due to the high affinity NH_4^+ transporters AtAMT1;1, AtAMT1;2, AtAMT1;4 and AtAMT1;5 were investigated.

8.3. Results

8.3.1. MA^+ influx and efflux through AtAMTs expressed in Saccharomyces cerevisiae

The NH_4^+ analogue, methylammonium (MA^+) was labelled with ^{14}C MA^+ and the influence of AtAMTs on the influx and efflux of MA^+ measured in a *S. cerevisiae* expression system. Overall, all AtAMTs investigated showed a net influx of MA^+ into yeast cells (Figure 8-3). This is consistent with published data (Gazzarrini et al., 1999; Shelden, 2001) (Loque and von Wiren, 2004; Yuan et al., 2007). To investigate the hypothesis that NH_4^+/MA^+ efflux was catalysed through AMTs (Britto, 2006) (Loque and von Wiren, 2004), a series of experiments were designed to further exploit the *S. cerevisiae* expression system (8.5.2 and 8.5.3).

The efflux of MA from all samples tested followed a biphasic pattern. Initial efflux, from 0 to 5 minutes, was characterised by a rapid release of MA^+ from the cells to the efflux buffer. The rate of efflux reduced from 5 to 30 minutes.

In cells expressing AtAMT1;1, initial MA^+ accumulation was 7.32 μmol MA^+ / mg protein which fell to 1.41 μmol / mg protein within 5 minutes of efflux commencing in the NH_4^+ / MA^+ buffer (Figure 8-4). AtAMT1;2 ranged from 4.12 μmol MA^+ / mg protein to 1.28 μmol MA^+ / mg of protein , AtAMT1;4 from 3.08μmol MA^+ / mg protein to 0.92 μmol MA^+ / mg protein and AtAMT1;5 from 576 nmol MA^+ / mg protein to 317 nmol MA^+ / mg protein in this same buffer (Figure 8-4).

With the exception of AtAMT1;5 expressing cells, which is much lower overall, all other cells expressing AtAMTs had ^{14}C labelled MA^+ efflux curtailed when resuspended in a buffer consisting of 20 mM K_2HPO_4 / KH_2PO_4 only (Figure 8-4).

To investigate if membrane potential influenced observed MA^+ efflux, the protocol was modified to introduce high concentrations of K^+ in the efflux buffer. Flux of K^+ through native yeast proteins would certainly depolarise the membrane potential and recreate in some way conditions akin to the large-scale movement of MA^+ through over-expressed AMTs. Depolarisation due to the addition of excess K^+ did not induce MA^+ efflux, unlike buffers containing MA^+ / NH_4^+ (Figure 8-5).

8.4. Discussion

8.4.1. Putative AtAMT mediated MA^+ efflux from yeast

AMT mediated efflux of NH_4^+ has been suggested as a possible mechanism of observed NH_4^+ efflux phenotypes in plants (Loque and von Wiren, 2004). These data suggest that this may be the case, although further work is required to decisively answer this.

The observed efflux is due either to the expressed AtAMTs facilitating MA^+ efflux from the cells or through the activity of native yeast protein(s) (Figure 8-4). The influx of positively charged MA^+ into the yeast cells will in part act to depolarise the plasma membrane (Borst-Pauwels, 1992; Wood, 2006) and thus reduce the energy requirements of MA^+ efflux. Any passive diffusion through a native channel-like protein would increase with lowered membrane potential. Addition of 200 mM K^+, depolarising membrane potential did not increase MA^+ efflux. It is therefore unlikely that MA^+ efflux is catalysed through native yeast channels using membrane potential as a driving force. Concentration gradients may also influence MA^+ flux through channel-like proteins, as ion flux will occur from high to low concentration. This is particularly relevant in the event of membrane depolarisation occurring.

8.4.2. Concentration gradient effects

These data do not support MA^+ flux down a concentration gradient as being the causative effect of observed efflux. Greater ^{14}C labelled MA^+ efflux is measured from cells resuspended in buffer containing MA^+ and NH_4^+ than without, therefore the

conditions with a reduced concentration gradient actually encourage MA^+ efflux. Internal cellular MA^+ concentration was also not a factor impacting observed efflux. Empty vector transformed cells loaded with MA^+ at 50 mM concentration, to a point where their internal MA^+ concentration is comparable to AMT expressing cells, did not exhibit the efflux response observed in AMT expressing yeast (Figure 8-4).

8.4.3. Membrane depolarisation effects

The rapid flux of NH_4^+ or MA^+ could lead to a depolarisation of membranes that would increase the probability of cations leaving the cell through passive transport (Wood, 2006). The addition of 200 mM KCl will result in strong membrane depolarisation (Borst-Pauwels, 1992; Maresova, 2006). Therefore, if efflux of MA^+ is favoured by increasing depolarisation of cellular membranes, an increase of MA^+ efflux should occur under these conditions. Our data showed very little difference in the amount of MA^+ effluxed with the addition of 200 mM K^+ to the efflux buffer, indicating that changes in yeast membrane potential has little influence on the rate of MA^+ efflux in AtAMT expressing cells (Figure 8-5).

8.4.4. MA^+ / NH_4^+ efflux

NH_4^+ efflux has been long observed in plant and yeast systems (Epstein, 1962; Wang et al., 1993, 1993; Ninnemann, 1994; Marini et al., 1997; Palková, 1997; Marini, 2000). Increased NH_4^+ efflux from plant roots as a response to high external NH_4^+ concentration has been reported (Kronzucker, 2001) and theorised this to be an attempt by the plant to control cellular NH_4^+ concentrations. A marked reduction in

the efflux of NH_4^+ on the basis of the concentration external to the plant was observed, with lower concentrations inducing less efflux (Kronzucker, 2001).

8.4.5. AtAMTs as effluxers of MA^+ / NH_4^+

AtAMTs have been classified as NH_4^+ uniporters, a characteristic supported by recent *Xenopus laevis* oocyte expression analyses of AtAMT 1;1 (Wood, 2006) and AtAMT 1;2 (Neuhauser et al., 2007), which show no evidence of NH_4^+ efflux at depolarised membrane potentials. Likewise, expression of LeAMT1;2 in *X. laevis* oocytes strongly suggested a net inward flow of positive charge (Mayer, 2006). In this same study, however, the human Rhesus-like protein RhCG was shown to facilitate the electroneutral influx of MA^+ into *X. laevis* oocytes. When expressed in the ammonium transport deficient strain 31019b, RhAG did not confer a growth rescue phenotype on media containing low NH_4^+ nor did it result in toxicity when grown on media containing 125 mM MA. This phenotype is also observed when 31019b is transformed with the closely related RhAG protein (Marini et al., 2000), which was shown to contribute to NH_4^+ efflux from yeast cells.

Import / export are considered to be linked to the substrate binding site of the pore within the Mep / AMT / Rh proteins. The crystallisation of the *E. coli* AMTB protein strongly suggests that the unprotonated species, NH_3, is transported rather than the NH_4^+ that is generally accepted to be transported through plant AMTs. Mayer et. al, (2006) suggest the electro-neutral accumulation of MA^+ in oocytes through RhCG occurs either through the passage of NH_3 through the protein or the outward movement of a cation, probably H^+, to compensate for the inward flux of NH_4^+. These would both explain the accumulation of MA^+ and the alkalinisation of the oocytes

cytosol observed. It is conceivable that in the case of Rh proteins, the maintenance of electro-neutral transport is achieved through the import of MA^+ / NH_4^+ coupled with the export of another cation. In conditions of high cellular NH_4^+ / MA^+ concentrations, the exported cation could quite possibly be MA^+ / NH_4^+. This would be an elegant and simplistic mechanism for the maintenance of NH_4^+ concentrations within a cell type.

Each AMT has a different affinity to NH_4^+ / MA^+ which in part limits the rate of NH_4^+ uptake. If this was coupled to an efflux capability, presumably also with differing substrate affinities, it could lead to an effective and simplistic manner with which cellular NH_4^+ could be controlled.

This analysis of MA^+ efflux and its relationship to AtAMTs in 31019b has resulted in data remarkably similar to that gathered during investigations of NH_4^+ efflux from plants (Kronzucker, 2001). When empty vector transformed 31019b, loaded with a comparable amount of MA^+, is subjected to varying external concentrations of MA^+ and NH_4^+, this response is absent. The presence of expressed AtAMTs undoubtedly contributes to this phenotype although further work is required to complete this line of research.

8.5. Materials and Methods

8.5.1. Transformation of yeast expression vectors containing *Arabidopsis* AMTs into yeast.

Clones of the *Arabidopsis thaliana* Col 0 AMTs in the yeast expression vector pYES3 (Smith et al., 1995) were obtained from Dr. Brent Kaiser (AtAMT1;1), Dr. Megan Shelden (AtAMT1;2) and Dr. Sunita Ramesh (AtAMT1;4 and AtAMT1;5). Yeast strain 31019b (*mep1*Δ, *mep2*Δ, *mep 3*Δ, *URA3*) was grown in liquid YPAD (yeast extract 1% (w/v), peptone 2% (w/v), D-glucose 2% (w/v) and adenine sulfate 0.004% (w/v) pH 6.5) at 28°C with constant shaking at 200 rpm to late-log phase. An aliquot of culture was used to inoculate 100ml of YPAD to OD_{600} = 0.1. Cells were grown at 28°C with constant shaking at 200 rpm to an OD_{600} = 0.4 – 0.6. Cells were harvested by centrifugation at 4000 x *g* for 4 minutes and washed twice with sterile MilliQ H_2O. Yeast were transformed using the lithium acetate / polyethylene glycol method (Gietz, 1995). 100µL aliquots of transformed cells were plated on to YNB glucose solid media (yeast nutrient base (BD biosciences, San Jose, USA) 0.67% (w/v), D-glucose 2% (w/v) pH 6.5) and incubated at 28°C for 2 days. Individual colonies were selected for their ability to overcome the URA3 deficiency in yeast.

8.5.2. ^{14}C labelled methylammonium influx into *S. cerevisiae* strain 31019b expressing *Arabidopsis* AMTs.

Cells were initially grown to late log phase in liquid YNB supplemented with 2% D-glucose (w/v). Cells were harvested by centrifugation and washed in milliQ H_2O and then used to inoculate Grensons liquid media (pH 6.5) containing 0.1% L-proline and 2% D-galactose to a final OD_{600} = 0.1. Cells were incubated overnight at 28°C with constant shaking (200 rpm) and harvested once they reached an OD_{600} of 0.4 – 0.7. Cells were washed twice in MilliQ H_2O and then re-suspended in 20mM K_2HPO_4 / KH_2PO_4 buffer pH 6.5 containing 2% (w/v) D-galactose to give a final OD_{600} between 4-6. A 2x reaction buffer containing a defined concentration of ^{14}C (Amersham) labelled MA^+ and 20mM K_2HPO_4 / KH_2PO_4 buffer (pH 6.5) was added to an equal volume of resuspended cells at T = 0 and were shaken continuously in a 2 ml Eppendorf tube. At the specified time, samples were removed, passed through a 0.45⌠M nitrocellulose filter (Whatman) and washed with 10 ml of ice-cold 20 mM K_2HPO_4 / KH_2PO_4 buffer to cease MA^+ flux. Membranes were collected, placed in a 7 ml scintillation vial (Sarstedt) and 4 ml of aqueous scintillation fluid added (Perkin Elmer). Samples were counted in a liquid scintillation counter (Packard) and counts (CPM) converted into the equivalent amount of MA^+ and samples were normalised against total yeast protein in a typical reaction volume (Peterson, 1977).

8.5.3. ^{14}C labelled MA^+ efflux from *S. cerevisiae* strain 31019b expressing *Arabidopsis* AMTs.

Cells were initially grown to late log phase in liquid YNB supplemented with 2% D-glucose (w/v). Collected and washed cells were transferred to Grensons liquid media (pH 6.5) containing 0.1% L-proline and 2% D-galactose to a final OD_{600} = 0.1. Cells

were grown to an OD_{600} = 0.4 – 0.7 and then resuspended in 20mM K_2HPO_4 / KH_2PO_4 reaction buffer (pH 6.5) containing 2% (w/v) D-galactose to give a final OD_{600} of 4-6. Cells were then mixed with ^{14}C-MA^+ reaction buffer containing either 0.5 mM MA^+ (AMT containing cells) or 50 mM MA^+ (pYES3 controls) for 30 minutes. Cells were harvested by centrifugation at 13000 x *g* and the ^{14}C labelled supernatant aspirated. Resuspension of the cells was in a 20mM K_2HPO_4 / KH_2PO_4 based buffer at pH 6.5 with either no addition, 0.5mM MACl or 0.5mM MACl and 5mM NH_4Cl. In depolarisation experiments, resuspension was carried out in buffers as above but with the addition of 200 mM KCl. Samples were withdrawn at the specified time and filtered through a 0.45μM syringe filter (Bio Lab, Adelaide, Australia) and both the filter and the filtrate collected and counted separately.

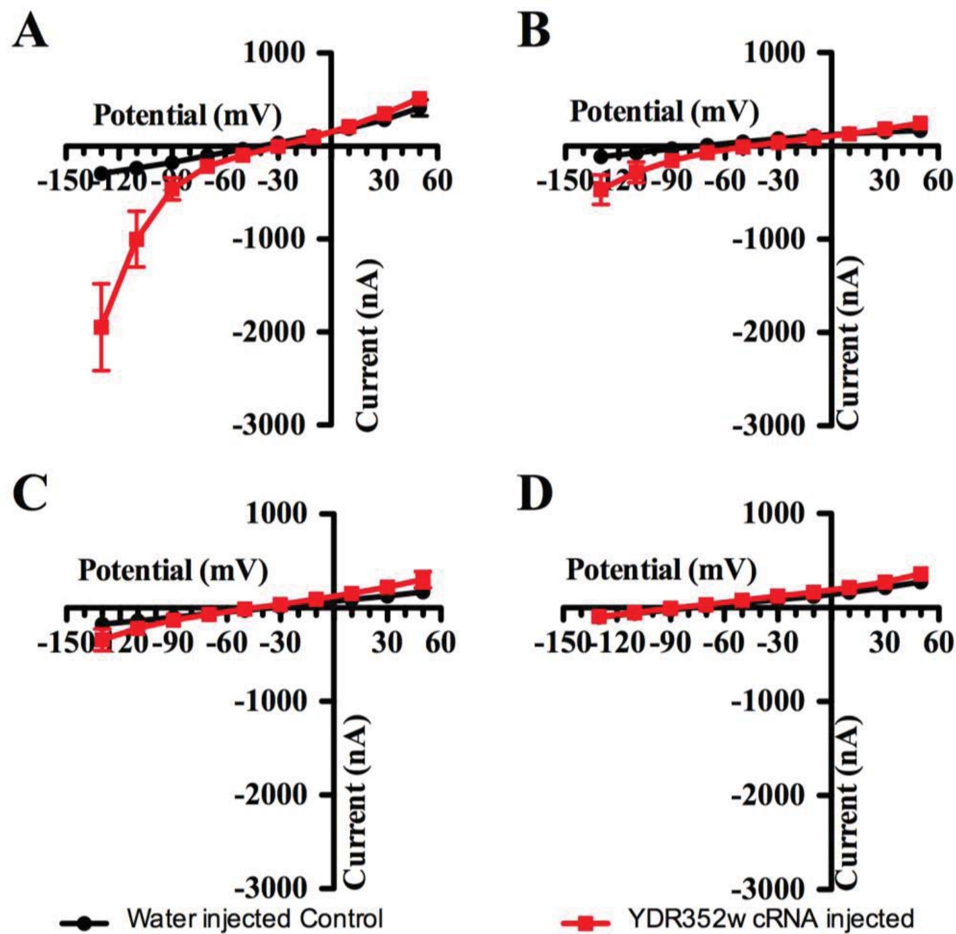


Figure 8-1: Optimisation of bath solutions to analyse cation flux in *Xenopus laevis* oocytes expressing PQ loop repeat proteins

Xenopus laevis oocytes were injected with either YDR352w cRNA or nuclease free H_2O and incubated as described in the methods of Chapter 6. Oocytes were exposed to the standard voltage protocol (Chapter 5 Figure 12) in various bath solutions. (A) 100 mM Choline Cl, 2mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM MES/Tris pH 6.5; (B) 200 mM Mannitol, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.0 Tris; (C) 200 mM Mannitol, 2 mM $MgCl_2$, 2 mM $BaCl_2$, pH 7.0 Tris; (D) 200 mM Mannitol, 5mM MES/Tris pH 7.0. Buffer D showed no evidence of the Ca^{2+} activated Cl^- channel and was used as a base for further experiments. Data presented is the mean ± SE ($n \geq 4$ oocytes).

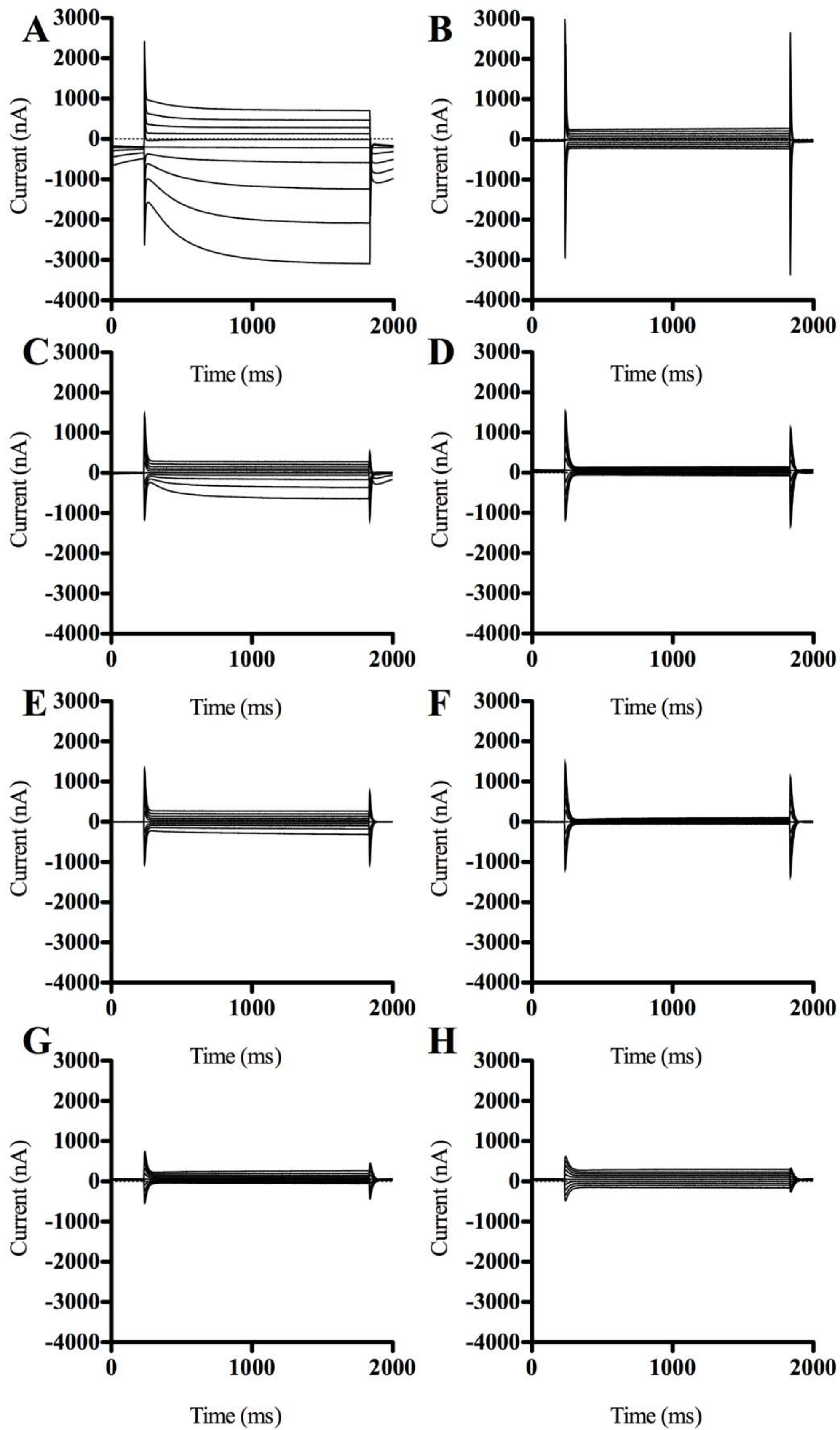

A
B
C
D
E
F
G
H
Current (nA)
Time (ms)
3000
2000
1000
0
-1000
-2000
-3000
-4000
0
1000
2000

Figure 8-2: Representative traces produced through the optimisation of solutions.

Representative traces of traces generated as part of collecting data for figure 1 A. Oocytes injected with cRNA of YDR352w are in panels A, C, E & G and H_2O injected oocytes in panels B, D, F and H. Bathing solutions were: 100 mM Choline Cl, 2mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM MES/Tris pH 6.5 (A and B); 200 mM Mannitol, 2 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.0 Tris (C and D); 200 mM Mannitol, 2 mM $MgCl_2$, 2 mM $BaCl_2$, pH 7.0 Tris (E and F); 200 mM Mannitol, 5mM MES/Tris pH 7.0 (G and H).

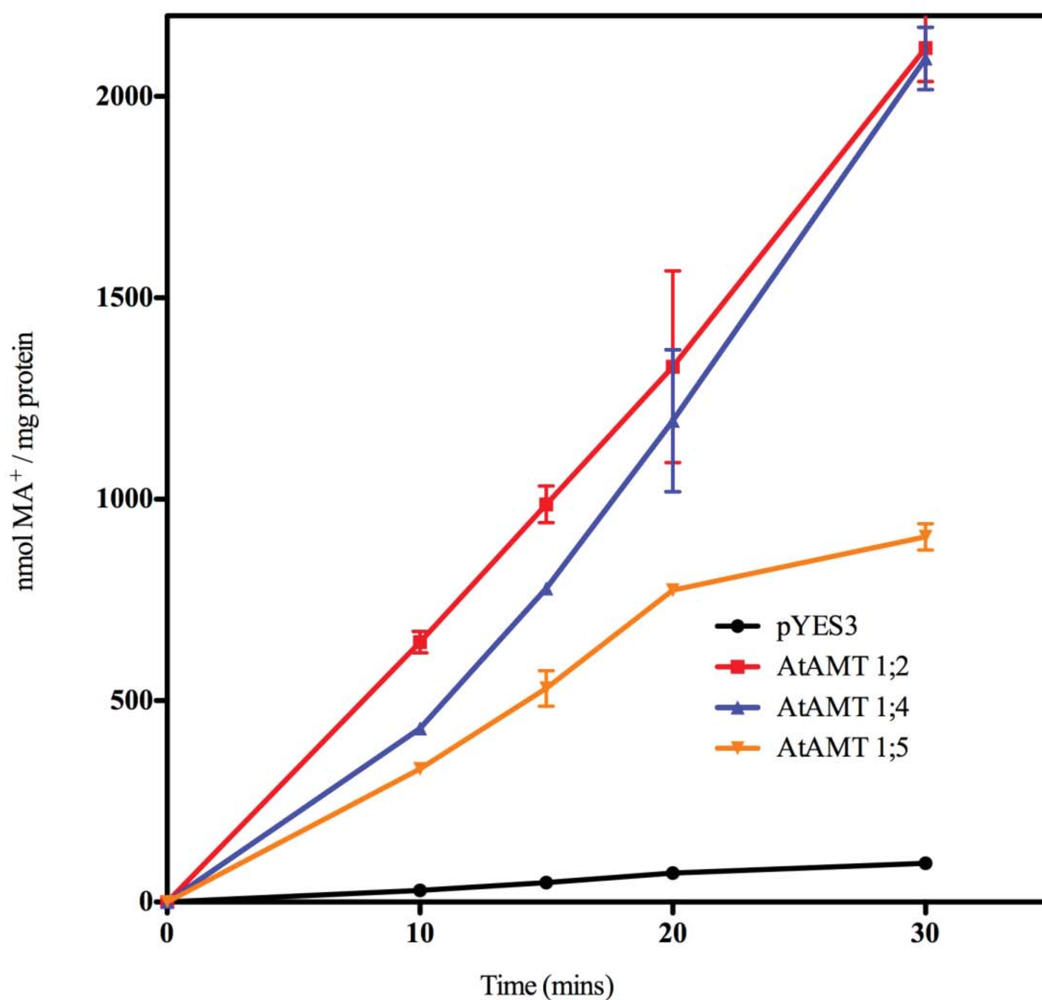
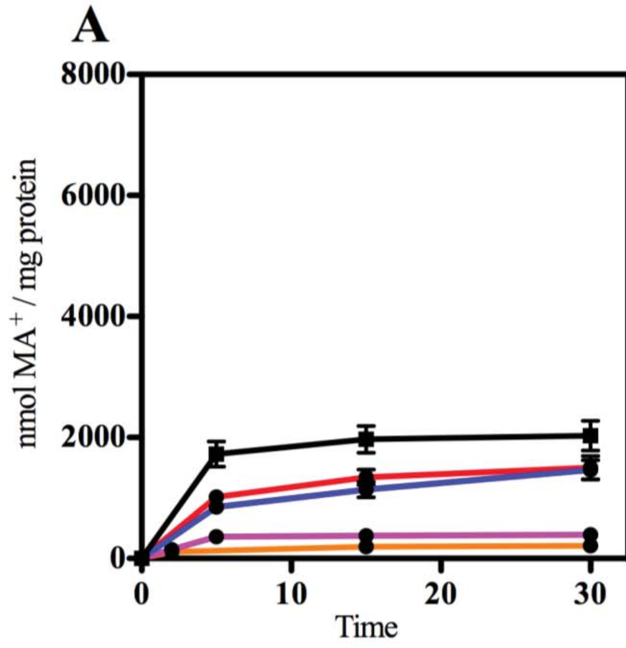
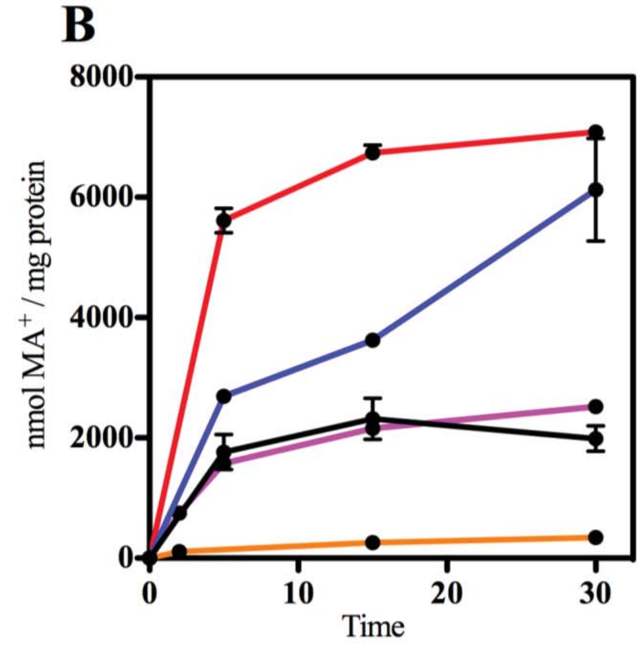
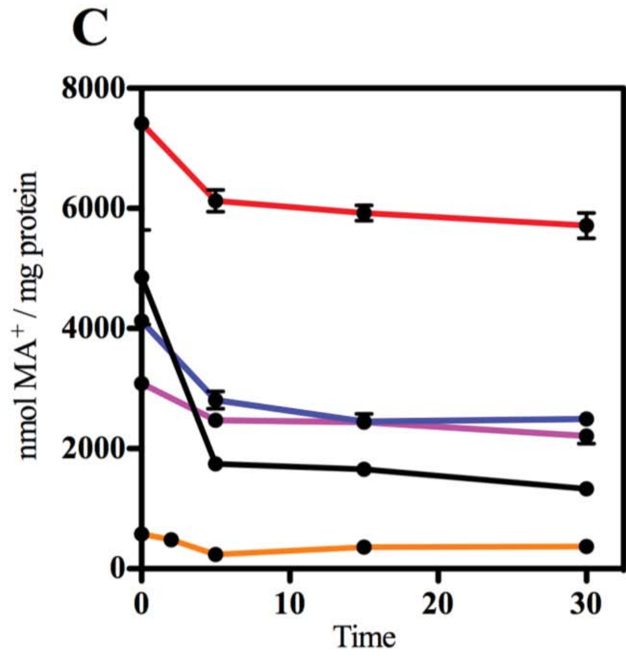


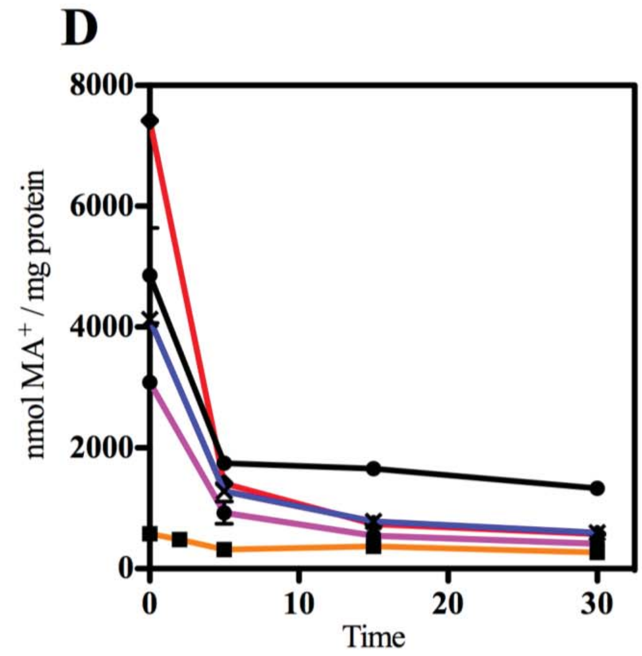
Figure 8-3: Accumulation of ^{14}C labelled MA^+ into yeast cells expressing Arabidopsis AMTs

S. cerevisiae strain 31019b was transformed with AtAMTs and net influx of MA^+ measured (section 8.5.2). Data expressed relative to total protein. Data is mean ± SE (n = 4).


A
8000
6000
4000
2000
0
0
10
20
30
Time
nmol MA+ / mg protein


B
8000
6000
4000
2000
0
0
10
20
30
Time
nmol MA+ / mg protein


C
8000
6000
4000
2000
0
0
10
20
30
Time
nmol MA+ / mg protein


D
8000
6000
4000
2000
0
0
10
20
30
Time
nmol MA+ / mg protein

pYES3
AtAMT 1;1
AtAMT 1;2
AtAMT1;4
AtAMT1;5

Figure 8-4: The influence of expressing AtAMTs and external MA^+ / NH_4^+ concentration on ^{14}C labelled MA^+ efflux from yeast cells.

S. cerevisiae strain 31019b was loaded with ^{14}C labelled MA^+ (section 8.5.3) and placed into an efflux buffer containing either 20 mM K_2HPO_4 / KH_2PO_4 (A and C) or 20 mM K_2HPO_4 / KH_2PO_4 + 0.5 mM MA^+ + 5 mM NH_4^+ (B and D). Efflux of ^{14}C labelled MA^+ was measured both by measuring efflux into the surrounding buffer (A and B) and depletion from loaded cells (C and D).

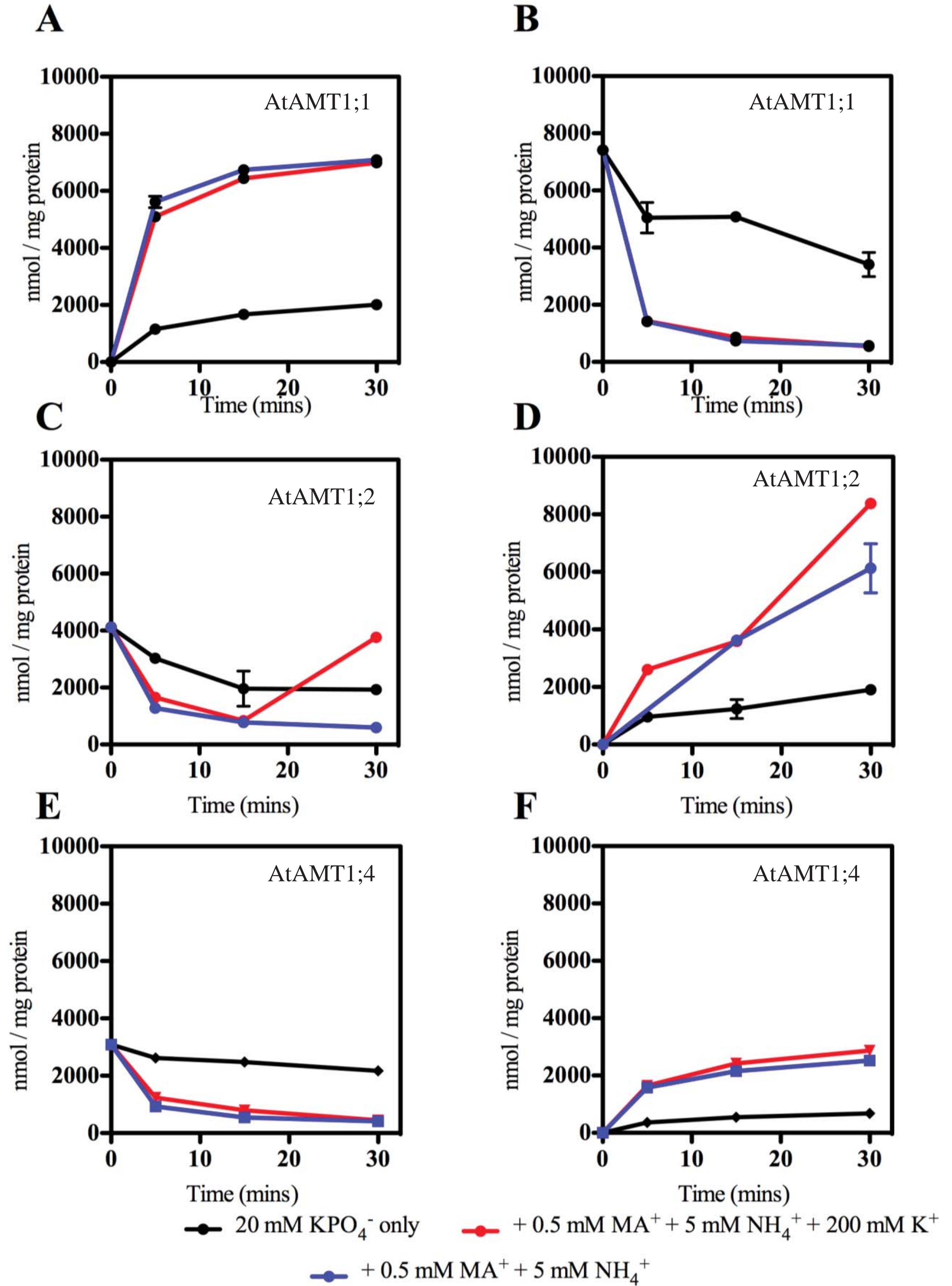

A
B
C
D
E
F
AtAMT1;1
AtAMT1;1
AtAMT1;2
AtAMT1;2
AtAMT1;4
AtAMT1;4
nmol / mg protein
Time (mins)
20 mM KPO4- only
+ 0.5 mM MA+ + 5 mM NH4+ + 200 mM K+
+ 0.5 mM MA+ + 5 mM NH4+

Figure 8-5: Effect of membrane depolarisation on MA^+ efflux from *S. cerevisiae* expressing Arabidopsis AMTs.

S. cerevisiae strain 31019b was loaded with ^{14}C labelled MA^+ (section 8.5.3) and placed into an efflux buffer containing either 20 mM K_2HPO_4 / KH_2PO_4, 20 mM K_2HPO_4 / KH_2PO_4 + 200 mM KCl or 20 mM K_2HPO_4 / KH_2PO_4 + 0.5 mM MA^+ + 5 mM NH_4^+ + KCl. Efflux of ^{14}C labelled MA^+ was measured for cells expressing AtAMT1;1 (A and B), AtAMT1;2 (C and D) and AtAMT1;4 (E and F). MA^+ efflux was measured both as decreasing content in the cells (A, C and E) and as increasing concentrations in the buffer (B, D and F).

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