

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

**SCOTT ANTHONY WILLIAM CARTER**

School of Agriculture, Food and Wine

The University of Adelaide

September 2008

Thesis submitted for the degree of

Doctor of Philosophy

**Declaration:**

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

I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying, subject to the provisions of the Copyright Act 1968.

Signed: \_\_\_\_\_

Scott Carter

**Abstract:**

Na<sup>+</sup> accumulation is a significant component in salt toxicity in plants. Although many proteins that facilitate Na<sup>+</sup> flux have been identified in plants, investigations aimed at identifying the initial mechanism of Na<sup>+</sup> entry into plants have failed. The catalysis of rapid, high capacity flux of Na<sup>+</sup> across plant cell membranes, currently through unknown means, is of particular importance. Observations of Na<sup>+</sup> flux across cellular membranes, using techniques such as electrophysiology and radiotracer flux, suggests this is a protein-based catalysis (Demidchik, 2002a; Demidchik and Tester, 2002c) (Essah et al., 2003). Based on these data, a class of protein described as voltage insensitive non-selective cation channel(s) (viNSCCs) are considered a good candidate for this Na<sup>+</sup> flux. This is due in part to characteristics they possess. These characteristics include catalysis of high capacity / low affinity cation flux, being relatively non-selective to the point where Na<sup>+</sup>, K<sup>+</sup>, NH<sub>4</sub><sup>+</sup> and MA<sup>+</sup> flux with similar properties and this flux being sensitive to elevated Ca<sup>2+</sup> and changes in pH.

A screen using the toxic ammonium analogue methylammonium (MA<sup>+</sup>) was developed using *Saccharomyces cerevisiae* strains that have minimal high affinity ammonium uptake capability. An *in silico* screen was developed and a number of candidate genes were identified as being possible viNSCCs. Preliminary selection of these was then conducted using the developed *S. cerevisiae* screen. Two genes, belonging to the same protein family, were selected based upon these results.

Analysis of these proteins using radiotracer flux in *S. cerevisiae* and electrophysiological examination using *Xenopus laevis* oocytes revealed these

proteins catalyse the non-selective flux of mono-valent cations following unsaturable kinetics, indicative of a low affinity transport system. Further analysis revealed this cation flux is sensitive to external  $\text{Ca}^{2+}$ . These properties strongly indicate these proteins form voltage insensitive non-selective cation channels in their native system.

**Acknowledgements:**

First and foremost, my wife Lynda is the person to which I am most grateful. Through countless childish tantrums she has weathered me and for that I am eternally grateful. I never forget how happy I am since I met you, even though it may seem I do. I love you, sugar mamma. Next is the other female that keeps me in line, little Lucy. I hope you grow up to be the angel you are now, I can't imagine you any other way. I am so proud of everything you are and will be, no matter what. There is nothing that can cheer me up more than remembering that last hug. I have also to mention the next addition to our family, one who I won't meet for a number of months but to which I look forward to. I love you without ever meeting you and will do forever.

My parents have been a great support through my life. The bedrock of my life is the knowledge they are there for me whenever I may need them. For that I am always grateful. They provided me with the best upbringing I can imagine, I personally blame my father for my nerdiness. Without a thorough introduction to space, science and technology through a variety of models and books, I doubt I would have been nudged in this direction. My mum also played a great deal of a role, especially with reading. My brother and sisters I thank also. Although we held a great dislike for each other at times, I think we have grown to be the best friends. I miss you all when you are not nearby and I am happy when you are, you have all grown up to be adults any parent would be proud of. I would also like to thank my parents in law, without whom I would not have my beautiful wife nor the abundance of time to write while they took care of my daughter.

There is a saying that a man's friends are a measure of his character. That must be a lie because I don't have that good a character. If I don't start with you, please don't feel slighted; I have to start somewhere. Pat has been a great friend both at work and away. No matter how much of a nerd I am, I know Pat will first laugh at me, then join in. We have had some really stupid and some really stimulating conversations and I am thankful for all of them. Thanks especially for all of your help. Kate and Megs, I apologise for the torture of sharing a lab with us but, to quote a great person 'you love it'. Dan, Grant and Chris, you guys helped me through some tough times, thank you. Things would not be how they are now without you. Michael Frank and the gentlemen that frequent his house of disrepute, thanks for the many fun nights and I look forward to enjoying them without having to work on my thesis the next day.

My supervisors Mark and Brent have been great to me. Mark as a mentor to show how big science works. It was certainly an eye opening experience, much different to how I thought it would be. Brent, thanks for all the time and for not getting too many grey hairs from my data. You have been a great teacher, you have basically taught me molecular biology from scratch and almost all my knowledge of how to do research. I look forward to keeping our friendship. Steve Tyerman has also been a great source of knowledge and suggestion, especially in electrophysiology but also for science in general. Thank you. I would also like to thank Sunita Ramesh for all the invaluable help she gave and continues to give. Without her expertise I would have probably floundered. Lastly I would like to thank Dale Sanders who, in a brief chat while visiting our campus, became the catalyst towards some of our discoveries.

<b>1. Introduction and review of literature .....</b>	<b>15</b>
<b>1.1. Introduction.....</b>	<b>16</b>
<b>1.2. Overcoming soil salinity .....</b>	<b>16</b>
<b>1.3. Plant based strategies for coping with salt stress .....</b>	<b>17</b>
1.3.1. Na <sup>+</sup> exclusion .....	17
1.3.2. Na <sup>+</sup> tolerance .....	18
1.3.2.1. Osmotic adjustment.....	18
1.3.2.2. Maintaining Na <sup>+</sup> / K <sup>+</sup> ratios.....	19
<b>1.4. Proteins that influence Na<sup>+</sup> flux in plant cells.....</b>	<b>19</b>
1.4.1. Na <sup>+</sup> ATPase pump .....	20
1.4.2. Na <sup>+</sup> / H <sup>+</sup> Exchanger NHX1.....	20
1.4.3. The low affinity cation transporter, LCT1 .....	21
1.4.4. The high affinity Na <sup>+</sup> / K <sup>+</sup> transporters, the HKT family .....	21
1.4.5. The Na <sup>+</sup> / K <sup>+</sup> co-transporter HAK1 .....	22
1.4.6. The SOS1 Na <sup>+</sup> / H <sup>+</sup> antiporter .....	22
1.4.7. The Non-Selective Cation Channels (NSCCs).....	22
<b>1.5. Candidate proteins that facilitate non-selective cation flux .....</b>	<b>23</b>
1.5.1. Glutamate activated NSCCs.....	23
1.5.2. Cyclic Nucleotide Gated Channels.....	24
1.5.3. Hyperpolarisation activated NSCCs.....	24
1.5.4. Depolarisation activated NSCCs.....	25
1.5.5. Ca <sup>2+</sup> activated NSCCs .....	25
1.5.6. Voltage insensitive NSCCs .....	25
<b>1.6. Enhancing Na<sup>+</sup> exclusion characteristics .....</b>	<b>27</b>
<b>1.7. Finding candidate NSCCs .....</b>	<b>27</b>
1.7.1. The strain 26972c as a NSC1 null mutant.....	28
<b>1.8. Conclusion .....</b>	<b>29</b>
<b>2. Development of a <i>Saccharomyces cerevisiae</i> based screen for NSCCs.....</b>	<b>38</b>
<b>2.1. Introduction.....</b>	<b>39</b>
2.1.1. A potential viNSCC null mutant in yeast.....	40
2.1.2. Development of a screen for viNSCCs .....	41
<b>2.2. Results .....</b>	<b>43</b>
2.2.1. Differences in MA <sup>+</sup> accumulation in $\Sigma$ 1278b, 26972c, 26972c2 and 31019b.....	43
2.2.2. Screening cDNA libraries for viNSCCs using the phenotypic differences between 31019b and 26972c2 .....	44
2.2.2.1. Positive screen using a <i>Glycine max</i> nodule cDNA library.....	44
2.2.2.2. Negative screen using a transposon insertion <i>Saccharomyces cerevisiae</i> cDNA library.....	45
<b>2.3. Discussion.....</b>	<b>47</b>
<b>2.4. Materials and Methods.....</b>	<b>52</b>
2.4.1. Yeast Phenotyping on Solid Media. Comparison of MA <sup>+</sup> toxicity phenotype between 31019b, 26972c, 26972c2 and $\Sigma$ 1278b.....	52
2.4.2. Phenotyping <i>Glycine max</i> cDNAs expressed in 26972c2 .....	52
2.4.3. Phenotyping transposon mutations of 31019b for improved MA <sup>+</sup> tolerance... ..	54
<b>3. An in silico screen for voltage insensitive non-selective cation channels (NSCCs) .....</b>	<b>74</b>
<b>3.1. Introduction.....</b>	<b>75</b>
<b>3.2. Results .....</b>	<b>77</b>
3.2.1. Screen of the <i>Saccharomyces cerevisiae</i> Genome Database (SGD) .....	77
3.2.2. Comparative screen of the <i>A. thaliana</i> database .....	77
<b>3.3. Discussion.....</b>	<b>78</b>
3.3.1. Interrogation of online databases .....	78

3.3.2.	Preliminary analysis of viNSCC candidates .....	79
<b>3.4.</b>	<b>Materials and Methods</b> .....	<b>83</b>
3.4.1.	In silico screen of the <u>Saccharomyces Genome Database (SGD)</u> and <u>The Arabidopsis Genome Resource (TIGR)</u> for potential viNSCCs.....	83
<b>4.</b>	<b>Identification of a protein family with voltage-dependent non-selective cation channel activity</b> .....	<b>87</b>
<b>4.1.</b>	<b>Introduction</b> .....	<b>88</b>
4.1.1.	PQ loop repeat proteins as putative viNSCCs in <i>Saccharomyces cerevisiae</i> ...	88
<b>4.2.</b>	<b>Results</b> .....	<b>90</b>
4.2.1.	MA <sup>+</sup> toxicity based screen for putative viNSCC proteins.....	90
4.2.2.	MA <sup>+</sup> flux into <i>Saccharomyces cerevisiae</i> over expressing YDR352w or YOL092w.....	91
4.2.3.	Na <sup>+</sup> flux into <i>Saccharomyces cerevisiae</i> over expressing YDR352w or YOL092w.....	92
<b>4.3.</b>	<b>Discussion</b> .....	<b>93</b>
<b>4.4.</b>	<b>Materials and Methods</b> .....	<b>96</b>
4.4.1.	Heterologous expression of PQ loop repeat proteins in a <i>S. cerevisiae</i> expression system.....	96
4.4.1.1.	Growth phenotypes on solid media of <i>S. cerevisiae</i> strain 31019b over-expressing candidate genes.....	96
4.4.1.1.1.	Cloning of YDR352w and YOL092w into the yeast expression vector pYES3-Dest	97
4.4.1.2.	<sup>14</sup> C Labelled MA flux and <sup>22</sup> Na labelled Na <sup>+</sup> flux analysis.....	97
<b>5.</b>	<b>Identification of a protein family with voltage dependent non-selective cation channel activity</b> .....	<b>110</b>
<b>5.1.</b>	<b>Introduction</b> .....	<b>111</b>
5.1.1.	viNSCCs and cation flux in plants .....	111
5.1.1.1.	viNSCCs and their role in Na <sup>+</sup> and NH <sub>4</sub> <sup>+</sup> uptake in plants.....	111
5.1.1.2.	PQ loop repeat proteins as candidate viNSCCs .....	112
<b>5.2.</b>	<b>Results</b> .....	<b>114</b>
5.2.1.	Characterisation of fluxes mediated by PQ Loop proteins YDR352w and YOL092w expressed in <i>Xenopus laevis</i> oocytes.....	114
5.2.2.	Relationship between K <sup>+</sup> and Na <sup>+</sup> flux through YDR352w and YOL092w .....	117
5.2.3.	Investigation of potential anionic flux through YDR352w and YOL092w .....	117
5.2.4.	Non-selectivity of YDR352w and YOL092w to mono-valent cations .....	118
5.2.5.	Influence of Ca <sup>2+</sup> on Na <sup>+</sup> flux catalysed by YDR352w and YOL092w .....	118
<b>5.3.</b>	<b>Discussion</b> .....	<b>119</b>
5.3.1.	YDR352w and YOL092w elicit responses concurrent with recorded viNSCCs	119
5.3.2.	Differences in the physiology of YDR352w and YOL092w .....	119
5.3.3.	Time dependence of currents elicited by YDR352w and YOL092w.....	121
5.3.4.	Potential Ca <sup>2+</sup> flux through viNSCCs.....	121
5.3.5.	The role of PQ loop repeat proteins in biological systems.....	122
<b>5.4.</b>	<b>Materials and Methods</b> .....	<b>123</b>
5.4.1.	Heterologous expression of PQ loop repeat proteins in the <i>Xenopus laevis</i> oocyte expression system .....	123
5.4.1.1.	Synthesis of cRNA and injection into <i>X. laevis</i> oocytes .....	123
5.4.1.2.	Electrophysiology of <i>S. cerevisiae</i> PQ loop repeat proteins expressed in <i>Xenopus laevis</i> oocytes.....	124
<b>6.</b>	<b>PQ loop repeat proteins in living systems</b> .....	<b>147</b>
<b>6.1.</b>	<b>Introduction</b> .....	<b>148</b>
<b>6.2.</b>	<b>Results and Discussion</b> .....	<b>148</b>



<b>6.3.</b>	<b>Materials and Methods</b> .....	<b>150</b>
6.3.1.	Interrogation of online databases for putative viNSCC sequences. ....	150
6.3.2.	Sequence alignments and construction of phylogenetic trees .....	150
6.3.3.	Cloning of yeast PQ loop repeat proteins, sequencing, alignment and domain prediction.....	150
<b>7.</b>	<b>General Discussion</b> .....	<b>163</b>
7.1.	<i>S. cerevisiae</i> PQ loop repeat proteins as viNSCCs .....	164
7.2.	viNSCCs as ubiquitous cation flux facilitators .....	164
7.3.	PQ loop repeat proteins as cation channels .....	164
7.4.	Further Investigation.....	167
<b>8.</b>	<b>Appendix</b> .....	<b>169</b>
8.1.	<b>Optimisation of conditions for the analysis of PQ loop repeat proteins in <i>Xenopus laevis</i> oocytes</b> .....	<b>170</b>
8.1.1.	Materials and Methods .....	170
8.1.2.	Results .....	170
8.1.3.	Discussion .....	171
8.2.	<b>Investigation of the NH<sub>4</sub><sup>+</sup> flux properties of AtAMT 1;4 and AtAMT 1;5....</b>	<b>172</b>
8.2.1.	Introduction .....	172
8.3.	<b>Results</b> .....	<b>173</b>
8.3.1.	MA <sup>+</sup> influx and efflux through AtAMTs expressed in <i>Saccharomyces cerevisiae</i> .....	173
8.4.	<b>Discussion</b> .....	<b>175</b>
8.4.1.	Putative AtAMT mediated MA <sup>+</sup> efflux from yeast.....	175
8.4.2.	Concentration gradient effects.....	175
8.4.3.	Membrane depolarisation effects .....	176
8.4.4.	MA <sup>+</sup> / NH <sub>4</sub> <sup>+</sup> efflux.....	176
8.4.5.	AtAMTs as effluxers of MA <sup>+</sup> / NH <sub>4</sub> <sup>+</sup> .....	177
8.5.	<b>Materials and Methods</b> .....	<b>179</b>
8.5.1.	Transformation of yeast expression vectors containing <i>Arabidopsis</i> AMTs into yeast.....	179
8.5.2.	<sup>14</sup> C labelled methylammonium influx into <i>S. cerevisiae</i> strain 31019b expressing <i>Arabidopsis</i> AMTs. ....	180
8.5.3.	<sup>14</sup> C labelled MA <sup>+</sup> efflux from <i>S. cerevisiae</i> strain 31019b expressing <i>Arabidopsis</i> AMTs. ....	180
<b>9.</b>	<b>References</b> .....	<b>190</b>

## Table of Figures

Figure 1-1: Inhibition of $\text{Rb}^+$ uptake by HKT1 in the presence of increasing concentrations of $\text{Na}^+$ and $\text{K}^+$ .....	31
Figure 1-2: The effect of $\text{Ca}^{2+}$ on NSC1 activity .....	32
Figure 1-3: The flux of $\text{NH}_4^+$ through NSC1 .....	33
Figure 1-4: The effect of pH on NSC1 .....	34
Figure 1-5: Comparison of 26972c and CY 152/162 .....	35
Figure 1-6: Comparison of currents through the viNSCC of wheat protoplasts induced by the flux of $\text{Na}^+$ or $\text{MA}^+$ .....	36
Figure 1-7: Rapid accumulation of $\text{Na}^+$ into <i>Arabidopsis thaliana</i> roots .....	37
Figure 2-1: The influence of media pH and $\text{Ca}^{2+}$ concentration on $\text{MA}^+$ toxicity phenotypes of different <i>S. cerevisiae</i> strains .....	61
Figure 2-2: Representative plates of <i>S. cerevisiae</i> strain 26972c2 transformed with a <i>Glycine max</i> cDNA library and arrayed using the BioRad Colony Picking and Array System. ....	63
Figure 2-3: Confirmatory screening of increased $\text{MA}^+$ toxicity phenotypes under 0.2 mM $\text{Ca}^{2+}$ conditions of <i>S. cerevisiae</i> strain 26972c2 transformed with a <i>Glycine max</i> cDNA library .....	64
Figure 2-4: BLAST alignments of <i>Glycine max</i> cDNAs shown to impart $\text{MA}^+$ toxicity phenotypes when over-expressed in the <i>S. cerevisiae</i> strain 26972c2 .....	70
Figure 2-5: Improved $\text{MA}^+$ resistance in <i>S. cerevisiae</i> strain 31019b mutated with an mTn transposon library .....	71
Figure 2-6: BLAST alignments of <i>S. cerevisiae</i> genes shown to improved $\text{MA}^+$ tolerance in 31019b through their disruption by mTn insertion events. ....	73

Figure 3-1: Hydrophilicity plots of candidate viNSCCs .....	86
Figure 4-1, A: Variation in MA <sup>+</sup> sensitivity in <i>S. cerevisiae</i> strain 31019b transformed with the empty pYES3 vector .....	99
Figure 4-1, B: MA <sup>+</sup> induced toxicity and uptake in <i>S. cerevisiae</i> strain 31019b transformed with the YDR352w in the galactose inducible vector BG1805.....	100
Figure 4-1, C: MA <sup>+</sup> toxicity and uptake in <i>S. cerevisiae</i> strain 31019b transformed with YOL092w in the galactose inducible vector BG1805.....	101
Figure 4-1, D: MA <sup>+</sup> toxicity and uptake in <i>S. cerevisiae</i> strain 31019b transformed with YFL034w in the galactose inducible vector BG1805.....	102
Figure 4-2: Vector map of BG1805 .....	103
Figure 4-3: Primers used in the amplification of Gateway (Invitrogen) adapted primers for full-length clones of YDR352w and YOL092w from yeast genomic DNA.....	104
Figure 4-4: The yeast expression vector pYES3-DEST .....	105
Figure 4-5: Time dependent uptake of <sup>14</sup> C-MA by <i>S. cerevisiae</i> strain 31019b expressing YDR352w, YOL092w or the empty vector pYES3 control.....	106
Figure 4-6: Concentration-dependent <sup>14</sup> C-MA uptake by YDR352w and YOL092w. ....	108
Figure 4-7: Time dependent uptake of <sup>22</sup> Na <sup>+</sup> by <i>S. cerevisiae</i> strain 31019b expressing YDR352w, YOL092w or the empty vector pYES3 control.....	109
Figure 5-1 : Comparison of the current / voltage relationships of <i>Xenopus laevis</i> oocytes expressing <i>S. cerevisiae</i> PQ loop repeat proteins as a function of external Na <sup>+</sup> concentration.....	125

Figure 5-2: Comparison of currents induced in <i>Xenopus laevis</i> oocytes expressing YDR352w or YOL092w with those injected with H <sub>2</sub> O over various Na <sup>+</sup> concentrations. ....	128
Figure 5-3: The effect of the K <sup>+</sup> channel blocker TEA <sup>+</sup> on Na <sup>+</sup> flux through YDR352w and YOL092w. ....	130
Figure 5-4: The effect of TEA <sup>+</sup> on Na <sup>+</sup> induced currents in <i>Xenopus laevis</i> oocytes expressing YDR352w or YOL092w with those injected with H <sub>2</sub> O. ....	132
Figure 5-5: The influence of anions on Na <sup>+</sup> based current in <i>Xenopus laevis</i> oocytes injected with cRNA from YDR352w or YOL092w. ....	134
Figure 5-6: Representative currents elicited from <i>Xenopus laevis</i> oocytes injected with H <sub>2</sub> O or cRNA of YDR352w or YOL092w in the presence of 100 mM Cl <sup>-</sup> or 50 mM SO <sub>4</sub> <sup>2-</sup> . ....	136
Figure 5-7: Catalysis of cation flux as a function of YDR352w and YOL092w expression in <i>Xenopus laevis</i> oocytes. ....	138
Figure 5-8: Representative currents elicited from <i>Xenopus laevis</i> oocytes injected with H <sub>2</sub> O or cRNA of YDR352w or YOL092w in the presence of varied cations. ...	140
Figure 5-9: Influence of bathing solution Ca <sup>2+</sup> concentrations on the conductance of Na <sup>+</sup> elicited current in <i>Xenopus laevis</i> oocytes injected with H <sub>2</sub> O, YDR352w cRNA or YOL092w cRNA. ....	142
Figure 5-10: Representative currents elicited from <i>Xenopus laevis</i> oocytes injected with H <sub>2</sub> O or cRNA of YDR352w or YOL092w in the presence of 100 mM NaCl and varied Ca <sup>2+</sup> concentrations. ....	144
Figure 5-11: Vector map of pGEMHE-DEST .....	145
Figure 5-12: Voltage protocol employed for all voltage clamp data presented in this thesis. ....	146

Figure 6-1: Phylogenetic tree of proteins that show significant similarity to the sequence of YDR352w. ....	156
Figure 6-2: ClustalW alignments of PQ loop repeat proteins sequenced from various yeast strains. ....	162
Figure 8-1: Optimisation of bath solutions to analyse cation flux in <i>Xenopus laevis</i> oocytes expressing PQ loop repeat proteins .....	182
Figure 8-2: Representative traces produced through the optimisation of solutions...	184
Figure 8-3: Accumulation of <sup>14</sup> C labelled MA <sup>+</sup> into yeast cells expressing Arabidopsis AMTs .....	185
Figure 8-4: The influence of expressing AtAMTs and external MA <sup>+</sup> / NH <sub>4</sub> <sup>+</sup> concentration on <sup>14</sup> C labelled MA <sup>+</sup> efflux from yeast cells. ....	187
Figure 8-5: Effect of membrane depolarisation on MA <sup>+</sup> efflux from <i>S. cerevisiae</i> expressing Arabidopsis AMTs.....	189

List of Abbreviations used:

AMT:	ammonium / methylammonium transporter
cAMP:	cyclic adenosine monophosphate
cDNA:	complimentary deoxyribonucleic acid
cGMP:	cyclic guanosine monophosphate
CNGC:	cyclic nucleotide gated channel
cRNA:	complimentary ribonucleic acid
GLR:	glutamate-like receptor
GPCR:	G protein coupled receptor
HKT:	A transporter family originally thought to catalyse H <sup>+</sup> and K <sup>+</sup> flux
iPCR:	inverse polymerase chain reaction
MA:	methylammonium
MEP:	A transporter family originally known as methylammonium permeases
MES:	2-( <i>N</i> -morpholino)ethanesulfonic acid
NSCC:	non-selective cation channel
ORF:	open reading frame
PCR:	polymerase chain reaction
TMD:	trans-membrane domain
Tris:	tris(hydroxymethyl)aminomethane
viNSCC:	voltage insensitive non-selective cation channel
WU-BLAST:	basic local alignment search tool maintained by Washington University