The role of aquaporins in the nitrogen induced hydraulic response of maize roots

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A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Agriculture, Food and Wine

The University of Adelaide



November 2017

Abstract

Water and nitrogen are essential for plant growth and development. In many plants, the soil nitrogen availability, often in the form of nitrate, influences the water uptake rate of the root system through changes to root hydraulic conductivity. This increase may be an evolutionary mechanism to increase nitrate transport to the roots via the flow of water through the soil in a process known as mass transport. A subset of the membrane intrinsic proteins, the aquaporins, are permeable to water and changes in their activity can lead to changes in hydraulic conductance and root water uptake. In maize, nitrate stimulates root water uptake through changes to root hydraulic conductivity in several inbred lines and varieties. However, the hydraulic response to nitrate of B73, the maize reference genome, is not known.

Maize aquaporin research is largely based on the set of aquaporins described by Chaumont et al. (2001) despite a reference maize genome being available since 2009. Here, a search of the maize (*Zea mays*) inbred line B73 genome revealed 43 aquaporins split among four subfamilies and included 14 ZmPIPs (Plasma membrane Intrinsic Proteins), 15 ZmTIPs (Tonoplast Intrinsic Proteins), 11 ZmNIPs (Nodulin-26 like Intrinsic Proteins), and 3 ZmSIPs (Small and Basic Intrinsic Proteins). This set of aquaporins shows diversity from sequenced clones in other studies, including nucleotide and amino acid changes within isoforms. The B73 genome does not contain ZmPIP2;7, but includes several isoforms not previously described, including ZmPIP2;9 and ZmPIP3;1. In addition, the upgraded APGv4 genome contains several differences to the previous genome version, AGPv3.

Aquaporin function has been often assessed through heterologous expression in *Xenopus laevis* oocytes. Many maize studies, along with plants in general, have examined the role and regulatory means of influencing water transport through the PIP subfamily of aquaporins. Here, cloning confirmed several sequence variations found in the B73 genome, and showed water conductance not only for ZmPIPs, but also high conductance through ZmTIP1;2b and ZmTIP2;1.

Originally, this research sought to characterise the hydraulic response to both nitrate starvation and resupply across maize inbred lines, including in B73. Conductance measurements made on individual maize root segments of B73 and F44 did not reveal a standard hydraulic response to nitrate across multiple growing conditions (hydroponics and aeroponics). Large variation in hydraulic conductance was observed that was examined as a function of changing light conditions, N nutrition, or switching from hydroponic to aeroponic growth. It was concluded that much of the variation occurred between individual root segments. This variation was observed in where along the root axis xylem vessels become conducting, seen as the proportion of root with high axial hydraulic conductance. Variations in root hydraulic conductivity was still present, and may be due to the activity of aquaporins.

The expression of the whole set of maize aquaporins was monitored at once under nitrate starvation along with genes for nitrogen uptake and assimilation using a real-time PCR gene expression system (OpenArray). This revealed the expression profile of root and shoot aquaporins, in the root, not previously studied in ZmTIPs, ZmNIPs or ZmSIPs. Under three day nitrogen starvation, both B73 and F44 had changes in gene expression, and each had root and shoot aquaporins significantly down regulated.

The aim of this research was to examine the mechanism used in maize to change root hydraulic conductance in response to nitrogen. The research presented in this thesis solidifies the genetic basis of water transport through aquaporins, provides evidence that some are water permeable, and that gene expression of aquaporins is responsive to nitrate conditions. This suggests the change in maize root hydraulics is in part mediated by the activity of aquaporins.

Declaration

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Jonathan Wignes November 2017

Acknowledgements

I would like first to thank my supervisor Prof. Steve Tyerman for your patience and generous support throughout my PhD. Thanks also to Associate Prof. Brent Kaiser, who convinced an Iowa boy to move around the world to study corn.

I have been helped and assisted by many in the laboratory. Thanks to Julie and Allen of the Kaiser lab for getting me up to speed and to the Adelaide Node of the Centre of Excellence in Plant Energy Biology for all the technical support and assistance. Special thank you to Dr. Rebecca Vandeleur for help with all things hydraulic, and a very special thank you to Wendy Sullivan for all the technical help around the laboratory.

I would like to gratefully acknowledge DuPont Pioneer and the Centre of Excellence in Plant Energy Biology for financially supporting my postgraduate studies.

Thank you is not enough for what my family, both blood and adopted, has done for me. To my parents, Grandparents, and in-laws – thank you for your support from afar. Thanks to Ben for being willing to listen.

I do not have the words to express what Molly and Judson mean to me and for what they have done to get me here especially at the end. Your love and support will always be cherished. Thank you for this adventure turned quest.

I will forever be indebted to my friends. To Rowan and Nyoli, without your support I would still be somewhere near Chapter 3. You not only pushed me to finish but pulled and prodded as well. Thank you.

To Kylee and Darren, who cared for Molly, Judson and I throughout. I hope to become a fraction of the friend you have been to us. I also want to thank Molly's friends, Clio, Michelle, Anna, Charlotte, and Diana who supported her as I finished.

Thank you Danny and Tim for listening to my struggles along the way. To Gav, Stef, Josh, and Anthony and for everyone at LifeChurch for loving and challenging me to be a better Christ follower.

And a final shout out to Nick, as per our agreement.

Table of contents

Abstract ..... **i**

Declaration ..... **v**

Acknowledgements ..... **vii**

Table of contents ..... **ix**

List of figures ..... **xv**

List of tables ..... **xix**

List of abbreviations ..... **xxi**

Chapter 1: General introduction **1**

1.1 Research context **1**

1.2 Forms of N and the water connection **3**

1.2.1 Soil N availability and mass flow 3

1.2.2 Transpiration and stomatal control 6

1.3 Linking N and water movement: basic root characteristics and environmental effects 7

1.3.1 Water flow through roots 7

1.3.2 Measurement of root hydraulic properties 8

1.3.3 Root components that influence water movement 10

1.3.4 Aquaporins 11

1.3.4.1 Aquaporin structure 12

1.3.4.2 Aquaporin selectivity 14

1.3.4.3 Aquaporins in plants 15

1.3.4.4 Plant aquaporin regulation 16

1.3.5 Diurnal variations 17

1.3.6 Temperature 18
1.3.7 Anoxia and hypoxia 19
1.3.8 Energy and nutrient signalling 21
1.3.9 Co-location of water and N uptake 22
1.4 N form and its concentration affect root hydraulic properties 25
1.4.1 Growth and development 25
1.4.2 Root hydraulics 27
1.4.3 NO_3^- versus NH_4^+ in the regulation of root hydraulics 29
1.4.4 Transcriptional responses of aquaporins in response to N 32
1.4.5 Post-translational regulation of aquaporins in response to N 33
1.5 Concluding remarks and general hypothesis 36
1.5.1 The aims and of this thesis: 37
1.5.2 Approach 37
1.6 Appendix 38
Chapter 2: An analysis of the aquaporins found in the maize inbred line B73 ..39
2.1 Introduction 39
2.2 Methods 43
2.3 Results 48
2.3.1 ZmPIPs 64
2.3.2 ZmTIPs 67
2.3.3 ZmNIPs and ZmSIPs 70
2.3.4 Aquaporin fragments 71
2.4 Discussion 78
2.5 Conclusion 80
2.6 Appendix 81
2.6.1 FASTA sequences used in this chapter 81
2.6.1.1 Protein Sequences 81

2.6.1.2 Predicted cDNA transcript Sequences 87

Chapter 3: Cloning of maize aquaporins and water channel characterisation in *Xenopus laevis* oocytes 111

3.1 Introduction 111

3.2 Methods 112

3.2.1 Cloning maize aquaporins 112

3.2.1.1 cDNA synthesis 112

3.2.1.2 Maize aquaporin cloning 114

3.2.1.3 cRNA synthesis of maize aquaporins for expression in *Xenopus* oocytes 122

3.2.1.4 ZmNRT1.1B cRNA 124

3.2.1.5 Expression in Xenopus oocytes 124

3.2.2 Water transport assay 125

3.3 Results 128

3.3.1 Cloning maize aquaporins 128

3.3.2 Water transport of maize aquaporins 135

3.3.3 Acid effects on maize aquaporin swelling rates 137

3.3.4 Nitrate and nitrate uptake effects on the water transport of ZmPIP2;6 139

3.4 Discussion 142

3.4.1 Maize aquaporin cloning 142

3.4.2 Water permeation 144

3.4.3 Errors in the calculation of water permeability 147

3.4.4 Future experiments 149

3.5 Conclusion 150

3.6 Appendix 151

3.6.1 Deriving Equation 1.2 151

3.6.2 Cloning alignments 151

3.6.3 Macros used for image processing and swelling assay 173

3.6.3.1 ImageJ macro for image processing 173

3.6.3.2 MATLAB macro for processing area files 176

Chapter 4: Maize root hydraulic responses to nitrogen 181

4.1 Introduction 181

4.2 Materials and methods 184

4.2.1 Plant materials and germination 184

4.2.2 Growth conditions 185

4.2.2.1 Low light growth room hydroponics 185

4.2.2.2 Medium light growth chamber hydroponics 187

4.2.2.3 High light glass house aeroponics 188

4.2.3 Plant measurements 192

4.2.3.1 Hydraulic measurements 192

4.2.3.2 Determination of open xylem 194

4.2.3.3 Root properties 194

4.3 Results 195

4.3.1 Hydraulic measurements in a low light growth room 196

4.3.1.1 Hydraulic conductance measurements measured with the Xyl'em Flow Meter 196

4.3.1.2 Hydraulic conductivity with HCFM in low light conditions in the growth room 200

4.3.2 Maize root hydraulics in medium light grown in hydroponics 209

4.3.2.1 Time after excision inhibits root hydraulic conductance 209

4.3.2.2 Open xylem vessels in hydroponically grown maize 212

4.3.2.3 Nitrate effects on root properties of maize plants grown in a medium light growth chamber 213

4.3.2.4 Physical properties of nodal roots 216

4.3.2.5 Hydraulic conductance in response to nitrogen starvation in medium light 218

4.3.2.6 Open xylem determination in maize hydroponics 220

4.4 Aeroponics experiments with HCFM 222

4.5 Discussion 227

4.5.1 Maize hydraulic responses to nitrogen 227

4.5.2 Tracking down the source of variance 228

4.5.3 The open xylem problem....230
4.5.4 Individual root contributions to total root conductivity....231
4.5.5 Data that would be useful for further interpretation....233
4.5.6 Conclusions....234

Chapter 5: Gene expression in response to nitrate starvation....235

5.1 Introduction....235

5.2 Materials and methods....237

5.2.1 Plant materials and tissue harvest....237
5.2.2 RNA extraction and processing....237
5.2.3 cDNA synthesis....239
5.2.4 qPCR on the OpenArray 12k Flex....239
5.2.4.1 Overview....239
5.2.4.2 OpenArray design....240
5.2.4.3 OpenArray loading....241
5.2.4.4 Instrument Run....242
5.2.4.5 TaqMan primer/probe quasi-validation....243
5.2.5 Analysis of qPCR data....244

5.3 Results....245

5.3.1 Isolating RNA and generating cDNA from root and shoot samples....245
5.3.2 Quality of reference genes....245
5.3.3 Relative expression of genes in tissue types....249
5.3.3.1 Maize aquaporin relative gene expression....249
5.3.3.2 Expression of genes for Nitrogen uptake, assimilation and regulation....253
5.3.4 Gene expression changes in response to nitrogen treatment....259
5.3.5 Validity of TaqMan probes....271

5.4 Discussion....273

5.4.1 C_{RT} and the validity of the results....273
5.4.2 Maize aquaporin gene expression....274

5.4.3 Further experiments 276
5.4.4 Conclusion 276
5.5 Appendix 277
5.5.1 Custom TaqMan probes with sequences available 278
5.5.2 Custom TaqMan primer and probes validated by Thermo Fisher Scientific (no sequences available) 280
Chapter 6: General discussion and conclusions 283
6.1 Maize aquaporin genes and gene expression 283
6.2 Maize aquaporin activity 288
6.3 Maize root hydraulic conductivity 289
6.4 Concluding remarks 292
Appendix 293
References 325

List of figures

Figure 1.1 Cramer model for nitrate control of root water flow 6

Figure 1.2 Composite transport model of water flow through roots 8

Figure 1.3 Aquaporin structure 13

Figure 1.4 ar/R selectivity filters 15

Figure 1.5 Regulation of root PIP aquaporins involves specific and probably parallel pathways of local and systemic signaling. 36

Figure 2.1 Phylogenetic tree of maize aquaporins 51

Figure 2.2 Monocot aquaporins 52

Figure 2.3 Mapping published aquaporins to those in the B73 genome 53

Figure 2.4 Aquaporin location on maize chromosomes 54

Figure 2.5 Maize aquaporin protein exon locations 57

Figure 2.6 Phylogenetic relationship of ZmPIP proteins 64

Figure 2.7 ZmPIP1;4 transcript alignments 65

Figure 2.8 ZmPIP1;7 cDNA and protein alignments with ZmPIP1;1 65

Figure 2.9 Protein and nucleotide alignments of ZmPIP2;6 and ZmPIP2;9 66

Figure 2.10 Phylogenetic relationship of the ZmTIP proteins 67

Figure 2.11 ZmTIP2;3 and ZmTIP5;1b genomic location 68

Figure 2.12 Phylogenetic relationship of the ZmNIP proteins 70

Figure 2.13 ZmSIP1;1 protein sequences from AGPv3 and AGPv4 71

Figure 2.14 Protein alignments of ZmSIP1;1 and fragments containing the ISY1-like domain 74

Figure 2.15 Protein alignments of ZmSIP1;1 and three fragments with predicted genes 75

Figure 2.16 Protein alignment of ZmTIP4;2b and three fragments 75

Figure 2.17 Protein alignment of ZmNIP2;1 and fragment ZmNIP2;5 76

Figure 2.18 Protein alignments of ZmPIP1;7 and four fragments. 77

Figure 2.19 Protein alignments of ZmTIP3;4 and three fragments 77

Figure 2.20 Protein alignment of ZmSIP1;1 and two fragments 77

Figure 2.21 Protein alignments of ZmNIP1;2 and fragments 78

Figure 3.1 cDNA tested for genomic DNA contamination 113

Figure 3.2 Location of primers used for amplication 118

Figure 3.3 pCR8/GW vector with insert 121

Figure 3.4 pGEMHE expression vector 122

Figure 3.5 PCR amplificaiton of maize aquaporins 130

Figure 3.6 Swelling assay in 1:5 dilution of Ringer's solution at pH 7.60 135

Figure 3.7 Acid effects on oocyte osmotic permeability 137

Figure 3.8 Acid sensitivity of maize aquaporins 139

Figure 3.9 Nitrate uptake on water transport in ZmPIP2;6 141

Figure 3.10 Nitrate and acid effect on oocytes expressing maize aquaporins 142

Figure 3.11 Alignments of the open reading frames (ORFs) of the sequenced ZmPIP1;2b clone, the genomic prediction (Zm00001d017256_T001.1) and published cDNA (AF131201.1) 152

Figure 3.12 Alignments of the ORFs of the sequenced ZmPIP1;4 clone, the genomic prediction (Zm00001d051403_T001.1) and published cDNAs (ZmPIP1;3 AF326487.1 and ZmPIP1;4 AF326488.1) 153

Figure 3.13 Alignments of the ORFs of the sequenced ZmPIP1;5 clone, the genomic prediction (Zm00001d051872_T001.1) and published cDNAs (ZmPIP1;5 AF326489.1 and ZmPIP1;5b AJ271796.1) 154

Figure 3.14 Alignments of the ORFs of the sequenced ZmPIP1;6b clone, the genomic predictions (AGPv4 Zm0001d020383_T001.1 and AGPv3 GRMZM2G136032_T01) and published cDNA (AF236490.1) 155

Figure 3.15 Alignments of the ORFs of the sequenced ZmPIP2;1 clone, the genomic predictions (Zm00001d019563_T006.1) and published cDNA (AF326491.1) 156

Figure 3.16 Alignments of the ORFs for the sequenced ZmPIP2;2 clone, the genomic prediction (Zm00001d005421_T001.1,) and cDNA (AF326492.1) 157

Figure 3.17 Alignments of the ORFs for the sequenced ZmPIP2;3 clone, the genomic prediction (Zm00001d051174_T002.1) and published cDNA (AF326493.1) 158

Figure 3.18 Alignments of the ORFs for the sequenced ZmPIP2;4 clone, the genomic prediction (Zm00001d017288_T001.1) and published cDNA (AF326494.1) 159

Figure 3.19 Alignments of the ORFs for the sequenced ZmPIP2;5 clone, the genomic predication (Zm00001d003006_T001.1) and published cDNA (AF130975.1) 160

Figure 3.20 Alignments of the ORFs for the sequenced ZmPIP2;6 clone, the genomic prediction (Zm00001d019565_T001.1) and cDNA (AF326495.1) 161

Figure 3.21 Alignments of the ORFs for the sequenced ZmTIP1;2b clone, the genomic prediction (Zm00001d011778_T001.1) and published cDNA (AF326500.1) 162

Figure 3.22 Alignments of the ORFs for the sequenced ZmTIP2;1 clone, the genomic prediction (Zm00001d051362_T001.1) and published cDNA (AF326501.1) 163

Figure 3.23 Alignments of the ORFs of the sequenced ZmTIP4;1b clone, the genomic prediction (Zm00001d037779_T001.1) and published cDNA (AF326505.1) 164

Figure 3.24 Alignments of the ORFs of the sequenced ZmTIP4;4b clone, the genomic prediction (Zm00001d039293_T001.1) and published cDNA (AF326508.1) 165

Figure 3.25 Alignments of the ORFs of the sequenced ZmNIP1;1 clone, the genomic prediction (Zm00001d016237_T001.1) and the published cDNA (AF326483.1) 166

Figure 3.26 Alignments of the ORFs of the sequenced ZmNIP2;1 clone, the genomic prediction (Zm00001d018037_T001.1) and published cDNA (AF326484.1) 167

Figure 3.27 Alignments of the ORFs of the sequenced ZmNIP2;2 clone, the genomic prediction (Zm00001d037228_T001.1) and published cDNA (AF326485.1) 168

Figure 3.28 Alignments of the ORFs of the sequenced ZmNIP3;1 clone, the genomic prediction (Zm00001d032461_T001.1) and published cDNA (AF326486.1) 169

Figure 3.29 Alignments of the ORFs of the sequenced ZmSIP1;1 clone, the genomic predictions (APGv4 Zm00001d048959_T002.1 and AGPv3 GRMZM2G113470_T01) and published cDNA (AF326497.1) 170

Figure 3.30 Alignments of the ORFs of the sequenced ZmSIP1;2 clone, the genomic prediction (Zm00001d010325_T001.1) and published cDNA (AF326498.1) 171

Figure 3.31 Alignments of the ORFs of the sequenced ZmSIP2;1 clone, the genomic prediction (Zm00001d028951_T001.1) and published cDNA (AF326499.1) 172

Figure 4.1 Maize hydroponic system 186

Figure 4.2 Light conditions in growth room 187

Figure 4.3 Light conditions in growth chamber 188

Figure 4.4 Aeroponic system 191

Figure 4.5 Experimental conditions summary 195

Figure 4.6 Maize seedling hydraulic responses to ammonium nitrate 198

Figure 4.7 Maize seedling hydraulic responses to ammonium nitrate 200

Figure 4.8 HCFM measurements of maize seedlings..... 201

Figure 4.9 Low light growth room water conductance measured with HCFM 203

Figure 4.10 Maize seedling hydraulic response to N resupply 204

Figure 4.11 Hydraulic response of hydroponic B73 seedlings in low light response to nitrate starvation over four days 207

Figure 4.12 Hydraulic response of hydroponic B73 seedlings in low light response to nitrate starvation over five days..... 208

Figure 4.13 Time inhibition of root hydraulic conductivity..... 212

Figure 4.14 Open xylem determination..... 213

Figure 4.15 Regression analysis of physical root properties in F44 215

Figure 4.16 Root properties..... 217

Figure 4.17 Lateral root emergence in B73..... 218

Figure 4.18 Hydraulic conductance measurements on maize plants in medium light . 219

Figure 4.19 Distance to open xylem from root tip 222

Figure 4.20 Open xylem determination in F44 225

Figure 4.21 Hydraulic conductance of maize roots grown in high light (glass house) in an aeroponics system 227

Figure 5.1 OpenArray plate..... 240

Figure 5.2 Example OpenArray amplification plot for one sample 243

Figure 5.3 Expression levels of the reference genes 247

Figure 5.4 Aquaporin expression in maize roots and shoots of B73..... 251

Figure 5.5 Maize aquaporins expressed in root and shoot tissue of F44..... 252

Figure 5.6 B73 root and shoot relative gene expression for N uptake genes 253

Figure 5.7 F44 root and shoot relative gene expression for N uptake genes 254

Figure 5.8 B73 root and shoot relative gene expression for N assimilation genes 255

Figure 5.9 F44 root and shoot relative gene expression for N assimilation genes..... 256

Figure 5.10 B73 relative root and shoot expression of N related regulatory genes 257

Figure 5.11 F44 relative expression of N related regulatory genes..... 258

Figure 5.12 Relative expression of reference genes..... 259

List of tables

Table 1.1 Nitrogen effect on root water transport 29

Table 1.2 Nitrogen form impact on root hydraulic properties 32

Table 2.1 Plant aquaporins revealed in genome searches 41

Table 2.2 Maize aquaporins used as query for TBLASTN genome search 46

Table 2.3 P. patens aquaporins used in TBLASTN genome search 47

Table 2.4 Full length aquaporins in the maize genome 55

Table 2.5 Mapping maize aquaporins between AGPv3 to AGPv4 56

Table 2.6 Maize genomic aquaporins compared to published sequences 58

Table 2.7 Maize aquaporin protein features 61

Table 2.8 Aquaporin fragments found in the maize B73 genome 63

Table 3.1 Primers used to test quality of cDNA 114

Table 3.2 Reaction conditions and PCR protocol for Taq DNA Polymerase 115

Table 3.3 Reaction conditions and PCR protocol for Platinum Taq DNA Polymerase High Fidelity 116

Table 3.4 Reaction conditions and PCR protocol for Q5 High Fidelity DNA Polymerase 116

Table 3.5 Primer sequences and PCR condidtions used to amplify maize aquaporin open reading frames 117

Table 3.6 Preliminary amplication of selected aquaporins from UTRs 118

Table 3.7 Cloning maize aquaporins 130

Table 4.1 Nutrient solutions for hydroponics and aeroponics 185

Table 5.1 C_{RT} values of the reference genes 248

Table 5.2 Aquaporin gene transcripts detected in each tissue type 250

Table 5.3 B73 root gene expression changes in response to nitrate starvation 260

Table 5.4 F44 root gene expression changes in response to nitrate starvation 263

Table 5.5 B73 shoot gene expression changes in response to nitrate starvation 266

Table 5.6 F44 shoot gene expression changes in response to nitrate starvation 269

Table 5.7 TaqMan probe quasi-validation 272

Table 5.8 TaqMan primer and probe sequences available 278

Table 5.9 Custom TaqMan primer and probe sets with target and context sequence .. 280

Table 6.1 Cloned aquaporins and gene expression on the OpenArray.....286
Table 6.2 Maize hydraulic response to nitrogen.....290

List of abbreviations

Abbreviation	Full Term
# or No.	Number
%	Percent
~	Approximately
±	Plus and minus
Δ	Delta, as prefix, used to denote change: initial conditions subtracted from final conditions
°C	Degree Celsius
µ	Micro (10^{-6})
µL	Micro-litre
µm	Micrometer
3’	Three prime, of nucleic acid sequence
5’	Five prime, of nucleic acid sequence
A	Area
AMF	Ammonium facilitator, gene family
AMT	Ammonium transporter, gene family
ar/R	Aromatic arginine selectivity filter
BLAST	Basic Local Alignment Search Tool
bp	Base pairs, of nucleic acid
c	Centi- (10^{-2})
Ca or Ca^{2+}	Calcium
cDNA	Complementary DNA
Cl or Cl^-	Chloride
C_t or C_{RT}	Cycle number, cycle at which qPCR signal hits a threshold value
C-terminal	Carboxyl terminal (protein)
C-terminus	Carboxyl terminus (protein)
CV	Coefficient of variance (as a %)
d	Day
Da	Dalton
DNA	Deoxyribonucleic acid
FW	Fresh weight
g	Gram

Abbreviation	Full Term
GIP	GlpF-like intrinsic protein
HATS	High Affinity Transport System, for nitrogen transport
HCFM	Hydraulic Conductance Flow Meter
HIP	Hybrid intrinsic protein
hr	Hour
iHATS	inducible High Affinity Transport System
k	Kilo (10^3)
kb	Kilo base pairs, nucleic acid
L	Litre
LATS	Low Affinity Transport System, for nitrogen transport
Lp_r	Root Hydraulic Conductivity
m	Milli (10^{-3}) or meter
M	Molar
M	Mega (10^6)
min	Minute
MIP	Major intrinsic protein
mol	Mole
n	Nano (10^{-9})
nL	Nano-litre
NO_3^-	Nitrate
NPA	Asparagin-proline-alanine motif found in aquaporins
NRT	Nitrate Transporter
N-terminal	Amine terminal (protein)
N-terminus	Amine terminus (protein)
OAc	Acetate ion
ORF	Open reading frame
Osm	osmolarity
P	Pressure
Pa	Pascal
PIP	Plasma membrane intrinsic protein
P_{os}	Osmotic Permeability Coefficient
RNA	Ribonucleic acid

Abbreviation	Full Term
RPM	Rotations per minute
s or sec	Second
SD	Standard deviation
SE or SEM	Standard error of the mean
SIP	Small, basic intrinsic protein
SO_4^{2--}	Sulfate
TIP	Tonoplast intrinsic protein
T_m	Primer melting temperature
UTR	Untranslated region
V	Volume
v/v	Volume per volume
v_w	Partial molar volume of water (18 mL·mol^{-1})
w/v	Weight per volume
w/w	Weight per weight
XIP	X-intrinic protein, family of plant aquaporin

Chapter 1: General introduction

1.1 Research context

In 2014, the global maize yield was estimated to be ~1.253 billion tonnes, and was with a gross production value of at least ~$246 billion (USD)[1]. Due to modelled population growth, the Food and Agriculture Organization of the United Nations forecast global production of maize, rice and wheat, will need to have a combined increase of around half a billion tonnes, from 2.5 to 3.0 billion above the (then) record harvest of 2014. Much of this increase will need to come at a time of increased sustainability efforts, and with pressure from predicted effects of climate change. These increases will have to occur with less water, fossil fuels, agrochemicals, and on aging soils that have been under intense production pressure. Additional challenges come from a changing climate, possibly including shifts in temperature (warmer earlier in the growing season, higher maximum range), drought, soil salinity increases, and emerging pest and diseases (FAO 2016). Developing sustainable agriculture through existing, and potentially engineered, genetic diversity will play a large part in overcoming these global issues.

Modern maize was domesticated from teosinte ~9000 years ago (Hastorf 2009; Piperno et al. 2009; Ranere et al. 2009). This has resulted in a species with genetic diversity, and varieties with advantages for specific environments (Matsuoka et al. 2002). Some of this diversity has been captured within inbred lines, and can be exploited in hybrid crosses between inbred lines (Duvick and Cassman 1999). Much of this artificial selection was done through trait breeding without the influence of modern genetics (Lee and Tracy 2009). Understanding genetic basis of the desired traits is complex, but will aid in the

[1] FAOSTAT (Food and Agriculture Organization of the United Nations, August 2017)

future design and prediction to tailor maize for specific conditions (Moose and Mumm 2008).

A set of traits that have large impacts on final yields centre on how a plant acquire and use nitrogen. Nitrogen (N) is essential for plant survival, being vital for cell chemistry and processes, and is the macronutrient taken up in the greatest quantity (80 % of total ion uptake) with the greatest energy requirement for transport and assimilation (Marschner 2012). While 78 % of the atmosphere is N_2 gas, it is unavailable for direct use by most plants except those that enter into symbiosis with N_2-fixing bacteria, where N_2 is converted to inorganic ammonium (NH_4^+). Once reduced, NH_4^+ can be assimilated into organic (amino acids, organic molecules) and inorganic (nitrate NO_3^-) forms by plants and soil microbes, respectively. These forms of N often present within the root rhizosphere have large effects on plant growth and development (Miller and Cramer 2004). Generally, low N stimulates increased biomass production in the roots, leading to increased root sizes (Chapin 1991). In maize, low N does affect, with wide diversity across varieties, the ability to retain biomass (Garnett et al. 2015). Additionally, low N can stimulate the nitrate (NO_3^-) uptake capacity, which may be related to short-term post-secondary modification of high affinity nitrate transporters (Garnett et al. 2013).

Nitrogen status of the plant influences the acquisition of another limiting resource: water. Plant water relations are heavily influenced by N availability on both a short (minutes to hours as in Gorska et al. (2008) and Górska et al. (2008)) and long term (weeks to months) basis through a variety of factors. Long term, N impacts root architecture, including dry weight, branching, and lengths (Gao et al. 2015). On a short term, N can influence water uptake through modification of the root hydraulic conductance, which may include the activity of the membrane water channels known as aquaporins. While cause and effect

are observed, the mechanism by which soil N availability acts to induce changes to plant water flow is unknown. Increasing understanding of these processes may aid in the quest to increase the sustainability of maize globally.

1.2 Forms of N and the water connection

The two major forms of available N in the soil are NO_3^- and NH_4^+, the availability of which depends on soil structure and composition, microbial activity, temperature and water (Warren 2009; Glass 2003). Under conditions of poor aeration, low temperatures and/or acidity, nitrifying bacteria become inactivated and the less mobile NH_4^+ increases relative to that of the more mobile NO_3^- (Marschner 2012; Laanbroek 1990). In contrast, in well-aerated pH-neutral soils, activity of nitrifying bacteria ensures NO_3^- is the predominant form of available N (Marschner 2012). Most plants have a preference for NO_3^- despite the overall energy costs of NO_3^- assimilation relative to that of NH_4^+ being higher (5–12 %) taking into account pH regulation (Raven 1985). The water costs per mole of N assimilated are also higher for NO_3^- by up to 12 % depending on where the NO_3^- is reduced and how pH is regulated (Raven 1985). There is a strong interaction between N uptake and water content of the soil, since this can have diverse effects on N availability through its effect on the degree of aeration and in the dissolution and transport of available N to the root surface (Moyano et al. 2013; Gonzalez-Dugo et al. 2010) (discussed below).

1.2.1 Soil N availability and mass flow

N availability has interacting effects on many physiological and developmental processes in plants including water relations, and there is cross talk in the regulation of N and water relations (Easlon and Bloom 2013). The link between N and water movement is also

important in biogeochemical models that influence predictions from global climate models (Šimůnek and Hopmans 2009; Seneviratne et al. 2010). Optimisation of water and NO_3^- uptake by root structure and architecture are also closely linked (Lynch 2013). In addition to supplying water for growth and nutrient movement in the xylem, water flow through plants has a role in nutrient acquisition and delivery from the soil.

Mass flow in the soil has been long observed (Barber 1962) and is the process by which water flow to the root brings soluble and mobile molecules to the root surface. In the absence of mass flow, or for nutrients that are poorly mobile in soil (e.g. inorganic phosphate), uptake of nutrients by the root cells reduces the surface concentration and creates a localised diffusion gradient to the root so that the rate of uptake may be limited by the rate of diffusion to the root surface (or hyphae of mycorrhizae) (Nye 1977). Mass flow of water during transpiration can convey (via convection) dissolved and mobile nutrients to the surface of the root (Cramer et al. 2008; Chapman et al. 2012) thus reducing or possibly reversing the concentration gradient and assisting the membrane transport systems by increasing the external concentration at the sites of uptake. The degree of "assistance" will depend on the flow rate to the root surface; concentration kinetics of the transport systems, i.e. high (HATS), low (LATS) or dual affinity; and the type of transport systems engaged (i.e. AMTs (Ammonium Transporters) and AMFs (Ammonium Facilitator) for NH_4^+ (Ludewig et al. 2007; Chiasson et al. 2014) or NRT2 (Nitrate Transporter) or NPF (Nitrate transporter/Peptide Transporter, previously known as NRT1) for NO_3^- (Tsay et al. 2007)), which in turn depends on the preference for different forms of N by the plant and the relative availability of these N forms in the soil solution. NH_4^+ and NO_3^- uptake kinetics are complicated since there are also inducible components, e.g. iHATS (inducible High Affinity Transport System). Most of the components recognised from physiological studies can be accounted for by specific

transporters in *Arabidopsis*, e.g. AtNRT2.1, AtNRT2.2, AtNRT2.4 and AtNRT2.5 as HATs NO_3^- transporters (Li et al. 2007; O'Brien et al.) and AtNRT1.1 and AtNRT1.2 as LATS NO_3^- transporters (Léran et al. 2014). The NRT2s interact with a nitrate assimilation- related protein (AtNAR2.1) to be active (Kotur et al. 2012). A whole plant model for the acquisition of NO_3^- by mass flow has been proposed (Cramer et al. 2009). By increasing total water flow through the plant when NO_3^- is sensed, more NO_3^- may be brought to the root surface. NO_3^- reduction to NH_4^+ is shifted from the root to the shoot as NO_3^- acquisition rises and closes stomata as it is reduced, providing a feedback mechanism to slow water uptake when NO_3^- levels are sufficient. NO_3^- at concentrations above 2 mM in the xylem reduces stomatal conductance in maize in an ABA-dependent manner (Wilkinson et al. 2007). The Cramer et al. (2009) model necessitates a root NO_3^- sensor and a hydraulic response triggered by NO_3^-, but not by NH_4^+.

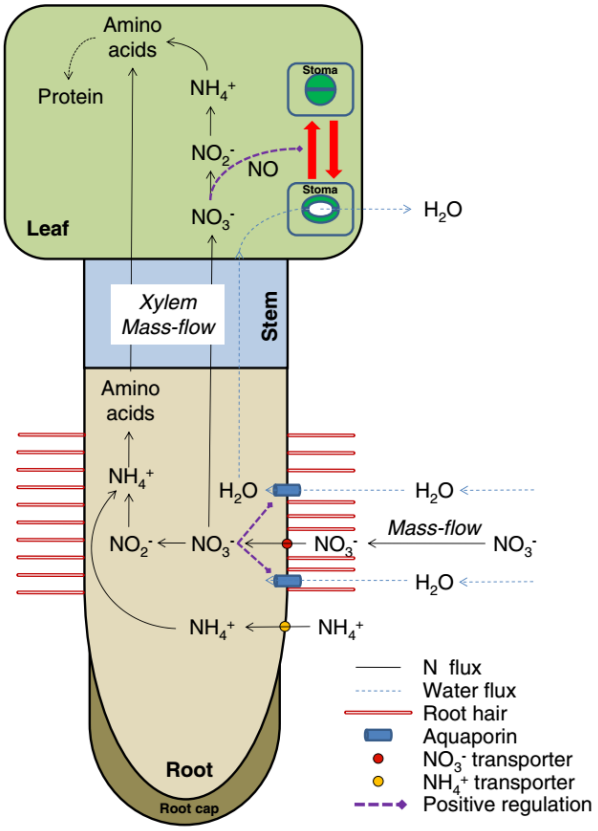


Figure 1.1 Cramer model for nitrate control of root water flow

The Cramer et al. (2009) model for nitrate sensitive water flux control by the root. Ammonium (NH_4^+) uptake and assimilation products do not affect water flux. Nitrate (NO_3^-) uptake and assimilation increase the flow of water via aquaporins. The flow of water brings additional nitrate to the surface for uptake. At some point, the nitrate uptake rate overtakes the roots nitrate reductase activy. Excess nitrate is transported to the shoot where NO production (biproduct of nitrate's reduction to nitrite) induce stomatal closure. Figure is from Cramer et al. (2009).

1.2.2 Transpiration and stomatal control

Transpiration is considered to be an essential driver of various forms of N uptake by roots by increasing both mass flow and diffusive fluxes of N to the root surface (Oyewole et al. 2014). Stomatal control of transpiration is regulated by N availability (Matimati et al. 2014; Cramer et al. 2008) and NO_3^- in the xylem in concert with ABA and xylem pH is a signal from roots that regulates stomatal conductance (Wilkinson et al. 2007). By inference from recent studies, stomatal regulation in response to NO_3^- is likely to involve

internal hydraulic conductivities in the leaf bundle sheath regulated by plasma membrane intrinsic proteins (PIP) aquaporins (Shatil-Cohen et al. 2011) as well as particular PIPs in the guard cell membrane (Grondin et al. 2015; Wang and Ruan 2016). In turn it is likely that transpiration itself regulates root hydraulic conductivity via root aquaporins (Vandeleur et al. 2014; Laur and Hacke 2013; Tardieu et al. 2015) thus providing a rather complex feedback regulation in concert with N availability. These factors add to the plethora of factors that must be integrated by the plant in regulating transpiration and nutrient uptake (Raven 2008).

1.3 Linking N and water movement: basic root characteristics and environmental effects

1.3.1 Water flow through roots

In most plants, water moves into the roots from the environment and flows through xylem vessels to the shoot. The composite model of water transport (**Figure 1.2**) has been proposed to explain how water moves through the root tissue to the xylem vessels (Steudle 2003, 1997; Steudle 2000). In this model water flows along two radial pathways across the root: an apoplastic pathway between cells, and a cell-cell pathway, across cells and plasmadesmata. The crossing of cell membranes allows plants to control water flow through the activity of aquaporins, and gives plants control over the symplastic pathway. Using apoplastc barriers, plants can nearly eliminate apoplastic flow and force the usage of the symplastic pathway, allowing the plant control over root water relations (Steudle and Peterson 1998).

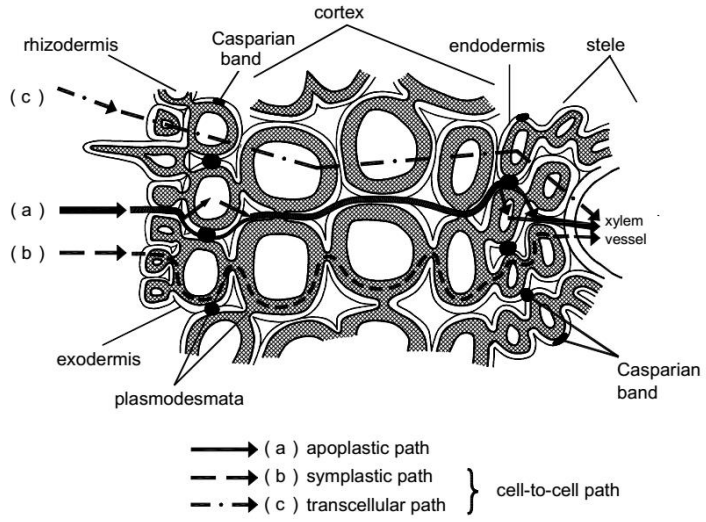


Figure 1.2 Composite transport model of water flow through roots
Water flows through two pathways from root surface to the xylem vessel. Between cells, known as the apoplastic path, or across cells and through plasmadesmata, the cell-cell path. Crossing the cell membranes incallows plants to control water flow through the activity of aquaporins. Apopastic barriers in the endodermis and exodermis can greatly reduce water flow through the apoplastic pathway. Figure from (Steudle 2000)

1.3.2 Measurement of root hydraulic properties

Root hydraulic properties can be measured using several methods, and these are important in the interpretation of the data obtained and the links with N transport. They apply to different root dimensions, tissue scales and rapidity of measurement at the level of single cells; root segments; whole roots in soil, intact or excised; and direction of flow. Each has advantages and disadvantages in interpretation of physiological significance. The water carrying capacity of the roots is generally measured as the hydraulic conductance variously normalised to account for root size (L_o) or hydraulic conductivity (L_p) in the strict sense when normalised to root surface area or root length. Here the term "root hydraulic conductivity" (Lpr) will be used rather loosely to indicate that conductance has been normalised to some component of root size.

Total flow of water through plants can be determined by observing the loss of water over time. This method is useful, since it avoids any wounding effects, but is unable to isolate root versus shoot responses or the effects of hydraulic conductance changes in different parts of the plant versus changes in water potential gradients unless these are measured (e.g. Franks et al. (2007)). Isolating the effect of the roots can be done by excising the shoot and observing only the root system. This opens the xylem to the atmosphere and may interfere with signals from the shoot (Vandeleur et al. 2014). Simple observations are done by determining the rate and volume of sap exuded over time at atmospheric or applied suction to the root system (e.g. Hachez et al. (2012)). Hydraulic conductivity and solute effects are determined from measurement of the osmotic concentration of the exuded sap and the osmolarity of the nutrient solution supplied. Hydraulic conductivity can also be determined by observing the rate of solution flow through the root at varying applied pressures to the whole root system forcing water to flow in the direction of the shoot (e.g. Gorska et al. (2008)). Alternatively pressure variations can be applied to the excised root system (high-pressure flowmeter, HPFM (Tyree et al. 1995) or single root segments (root pressure probe, e.g. Lee et al. (2004b)), and from relaxation kinetics or steady-state volume flow, the hydraulic conductance can be determined. These do not always give the same answer when compared and depending on driving force (osmotic or hydrostatic) (Bramley et al. 2007b). Some techniques can give conductances very rapidly after root system excision, for example, the transient method using the HPFM, and although water flow is in the reverse direction to normal transpirational flow, the values obtained correlate well with measurements of flow in the normal direction (Tsuda and Tyree 2000). While many have used excised root systems or roots for measurement of hydraulic properties, it has been reported that shoot excision itself causes a decrease in

hydraulic conductivity of grapevine, soybean and maize (Vandeleur et al. 2014). In soybean the reduction after excision is large and rapid (half-time ≈ 5 min).

Root hydraulics can also be observed on a cellular basis using the cell pressure probe (Tomos and Leigh 1999). The hydraulic conductivity of individual plant cells can be determined from the elasticity of the cell wall and the half-time of water flow from pressure pulse relaxations after the cell volume is rapidly changed (Hüsken et al. 1978). This is useful to attempt to reconstruct the components of radial flow across the root and to determine potential cells that could govern whole root hydraulic conductance (Bramley et al. 2009). It also allows a more direct link between root hydraulic conductance and the molecular components that determine cell membrane water permeability, i.e. the aquaporins.

1.3.3 Root components that influence water movement

Like N and other inorganic nutrients, water acquisition occurs via the root in nearly all plants. In the root, water is absorbed from the soil by flowing down its free energy gradient, the components of which in the plant can be modified through changing solute concentration and the rate of transpiration (Kramer and Boyer 1995). The free energy gradient (water potential gradient) for water to flow to roots through the soil, radially across roots, and then up to the shoot via the xylem, is generated by hydrostatic pressure and osmotic gradients, osmotic gradients only being effective when across a semipermeable membrane. Osmotic gradients may dominate when there is no transpiration, while pressure gradients established in the soil-root-xylem via the cohesion tension mechanism dominate during transpiration (Steudle 2001; Sperry et al. 2002). Given the relatively large energy requirements for the uptake of NO_3^- compared to other nutrients (Cannell and Thornley 2000; Poorter et al. 1991), it is not surprising that there

may have been selection pressure to evolve mechanisms to assist the process through coupling with the energetically downhill flow of water in the soil-plant-atmosphere continuum.

Plants can modify the rate that water flows down the water potential gradient via the hydraulic conductivity in the flow pathways. In roots, water can enter the root through the apoplast and a parallel cell-to-cell pathway to reach the xylem (Steudle and Peterson 1998). In many systems, barriers interrupt the apoplastic water flow, forcing water to use the cell-to-cell pathway (Steudle 2001). The *sgn3* mutant of *Arabidopsis* that has compromised development of the Casparian strip due to the lack of deposition of lignin has increased Lp_r but only shows signs of compromised nutrient transport for potassium (Pfister et al. 2014). Suberin is deposited later in development to surround the endodermal cells in *Arabidopsis*, and its deposition is dependent (reversibly) on nutrient and ionic stress (Barberon et al. 2016). This is mediated by ethylene (reduces suberisation) and ABA (increases suberisation), but of the nutrients examined by Barberon et al. (2016), NO_3^- or NH_4^+ were not included and should be examined in the future. Root ABA and ethylene are reported to be involved in N signalling (Kudoyarova et al. 2015; Ondzighi-Assoume et al. 2016; O'Brien et al. 2016), so it is possible that N nutrition could regulate suberisation of the endodermis and hence influence both water and N transport into the pericycle or the movement of a diffusible chemical signal from the pericycle out to the cortex and epidermis.

1.3.4 Aquaporins

The first aquaporin discovered was mammalian AQP1 isolated from red blood cells (Preston et al. 1992), but aquaporins were soon found in plants as well (Maurel et al. 1993). Membrane water permeability of roots is modulated by the activity of aquaporins,

certainly in the plasma membrane of certain cell layers, but probably also in the tonoplast, such that, depending on species, more than 50 % of water flow through roots may occur via aquaporins as determined by inhibitor effects (Chaumont and Tyerman 2014; Maurel et al. 2015). The plasma membrane intrinsic proteins (PIPs) will be largely discussed in this chapter related to root water movement, but tonoplast intrinsic proteins (TIPS) and NOD-26 like intrinsic proteins (NIPs) are also relevant to interactions with N transport.

1.3.4.1 Aquaporin structure

Known aquaporin structures all share common global structures and themes. Aquaporins have six transmembrane alpha helixes (H1-H6) with five connecting loops (Loop A-E) with both N and C termini located on the cytoplasmic side of the membrane. Loop B and Loop E are both partially submerged in the membrane and each contains a highly conserved NPA (asparagine-proline-alanine) motif that forms a key constriction region of the pore (**Figure 1.3**). The first high resolution x-ray crystal structrures were deduced for mammalian AQP1 (Murata et al. 2000; Sui et al. 2001) and bacterial GlpF (Fu et al. 2000). Since then, many crystal structures have become available, indluding in plants (Fotiadis et al. 2001; Törnroth-Horsefield et al. 2006; Frick et al. 2013a; Frick et al. 2013b; Kirscht et al. 2016). These studies all confirm the basic three dimensional structure and assembly into tetramers, shown in **Figure 1.3**.

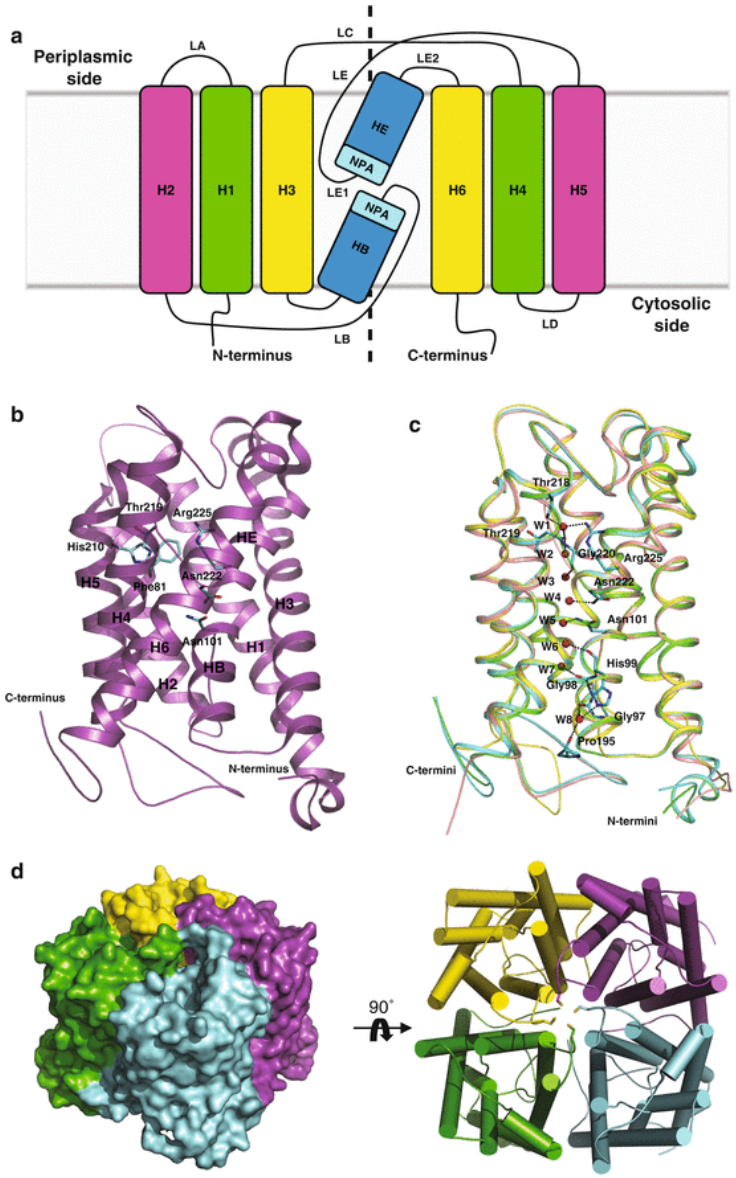


Figure 1.3 Aquaporin structure

The conserved structure of plant aquaporins. (A) Topology of aquaporin monomer in lipid bilayer. Six transmembrane alpha helicies and two alpha helix loops partially submerged in membrane, each with NPA (or derivative) domain central to forming the pore. (B) Model of spinich SoPIP2;1 in closed conformation. (C) Superposition of open and closed strucutres of SoPIP2;1 (pH 8 – closed, pH 6 – open). W1-W8 are water molecules in the pore. (D) Aquaporin tetramer. Four aquaporin monomers come to gether to form a tetramer. (E) Tetramer fro above, each monomer has its own solute conducting pore. Figure from Luang and Hrmova (2017)

1.3.4.2 Aquaporin selectivity

Aquaporins are highly selective and discriminate between molecules with similar properties and sizes. This is a result of slight structural differences in the pore, which can be modified by changing the amino acids that line the pore (Wallace and Roberts 2005). The most constrictive region of the pore has been called the aromatic/arginine (ar/R) selectivity filter (**Figure 1.4**). It is 8-9 Å away from the NPA motif conconsists of four residues: H2, H5, LE1 and LE2, found on helix 2, helix 5 and loop E, respectively (Murata et al. 2000; Sui et al. 2001). Plant aquaporin ar/R selectivity filters can be deduced from homology models (Bansal and Sankararamakrishnan 2007), with many variations been identified (Gupta et al. 2012).

While the ar/R region restrics the physical size of solute, the NPA motifs are essential in the aquaporins ability to transport water, but not protons. Molecular dynamics show the NPA motifs block protons by providing a hydrogen bond donor to ‘flip’ waters orientation in the pore which disrupts the single file chain of water molecules enough to prevent proton conductance (Tajkhorshid et al. 2002; Savage et al. 2010).

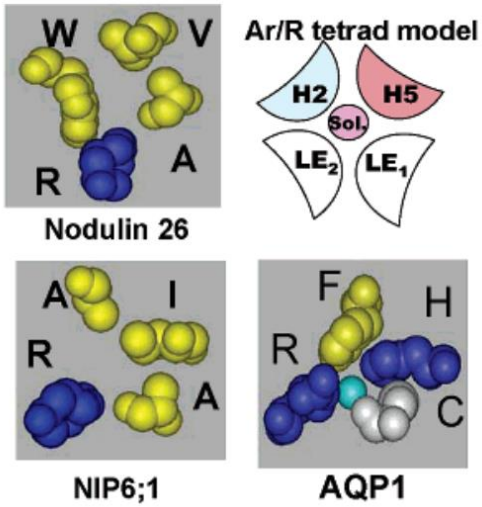


Figure 1.4 ar/R selectivity filters

The ar/R selectivitiy filters of soybean NOD26 , AtNIP6;1 and mammalian APQ1, demonstrating size exclusion possibilities. The slight increase in pore diameter in AtNIP6;1 is enough to allow both urea and formamide to conduct, whereas in NOD26, urea transport is blocked. Mutating A119, the H2 component of the selectivity filter of AtNIP6;1 to that of NOD26 eliminated urea transport, but maintained formamide transport. Figure from Wallace and Roberts (2005)

1.3.4.3 Aquaporins in plants

Based on sequence homology, plant aquaporins are divided into seven subgroups. The smallest two subclasses, the GIPs (GlpF-like intrinsic proteins) and HIPs (hybrid intrinsic protein) are rare and have been found only in moss (Danielson and Johanson 2008; Gustavsson et al. 2005). The XIPs (X intrinsic proteins) are a new major subfamily, but are not found in monocots or in A. thaliana, (Bienert et al. 2011; Danielson and Johanson 2008; Gupta and Sankararamakrishnan 2009). The remaining aquaporin groups are found in all plants: the PIPs (plasma membrane intrinsic proteins), the TIPs (tonoplast intrinsic proteins), the NIPs (NOD26-like intrinsic proteins) and the SIPs (small and basic intrinsic proteins) (Johanson et al. 2001). In general, PIPs are expressed in the plasma membrane and TIPs in the tonoplast membrane. However, each isoform's localization should be checked through experimentation as localization differences are possible (Barkla et al.

1999). The NIPs are highly homologous to NOD26, a highly expressed protein in the symbiosome membrane in soybean (Glycine max) (Dean et al. 1999; Rivers et al. 1997) and are expressed in a variety of membranes (Wallace et al. 2006). SIPs have been shown to be localized to the ER membrane, and are the least studied (Ishikawa et al. 2005).

1.3.4.4 Plant aquaporin regulation

The PIPs are regulated on many levels (transcription, protein amount, protein location and by gating (Boursiac et al. 2008; Chaumont and Tyerman 2014; Maurel et al. 2015)), and this can account for considerable variation in hydraulic conductivity, sometimes by an order of magnitude in roots depending on species (Vandeleur et al. 2014). In maize roots, aquaporin expression has been seen to change diurnally and circadially (Lopez et al. 2003; Caldeira et al. 2014), in different developmental stages (Hachez et al. 2006) and during water stress (Hachez et al. 2012). Aquaporin protein activity can be modified in several ways including protein-protein interactions (Fetter et al. 2004; Zelazny et al. 2007), pH gating (Hedfalk et al. 2006a; Törnroth-Horsefield et al. 2006; Nyblom et al. 2009), blocking with certain metals (Niemietz and Tyerman 2002; Verdoucq et al. 2008), ubiquitination (Lee et al. 2009), amidation (di Pietro et al. 2013), phosphorylation (Van Wilder et al. 2008; Grondin et al. 2015) and membrane trafficking (Chevalier et al. 2014; Chevalier and Chaumont 2015). Any one or a combination of these including different combinations of isoforms could be involved in regulating water flow through roots to influence N uptake. Apart from water, certain aquaporins also facilitate passive transport of small solutes, including N-containing molecules. Some members of the *Arabidopsis* TIP family have been shown to be permeable to urea in yeast complementation assays, and their expression can increase during N starvation (Liu et al. 2003b). Other *Arabidopsis* TIP members, maize NIP and TIP, and a cucumber NIP, have been found to

complement yeast for growth on urea (Klebl et al. 2003; Gu et al. 2012). ZmPIP1;5-b is induced with NO_3^- application and has been suggested to be water and urea permeable when expressed in *Xenopus* oocytes for 5 days (Gaspar et al. 2003). Wheat TaTIP2;1 and TaTIP2;2 have NH_4^+ permeability in yeast or *Xenopus* (Jahn et al. 2004; Holm et al. 2005; Bertl and Kaldenhoff 2007), and the AtTIP2;1 protein structure indicates a unique side pore through which protons can be extracted to deprotonate NH_4^+ and allow NH_3 to permeate (Kirscht et al. 2016). Currently no plant aquaporins have been found to be NO_3^- permeable, but a NO_3^- permeable mammalian aquaporin is known – AQP6 (Hazama et al. 2002; Liu et al. 2005; Yasui et al. 1999a; Yasui et al. 1999b).

1.3.5 Diurnal variations

Root hydraulic conductivity can change diurnally with higher conductivity observed in the daytime, which has been correlated with expression patterns of PIP aquaporins (Henzler et al. 1999). This has been shown for a number of species (Vandeleur et al. 2009; Almeida-Rodriguez et al. 2011; Hachez et al. 2008) and in some cases related to circadian oscillation in aquaporin activity or expression (Caldeira et al. 2014; Takase et al. 2011; Lopez et al. 2003) and additionally via a transpiration signal from the shoots to the root (Sakurai-Ishikawa et al. 2011; Almeida-Rodriguez et al. 2011; Vandeleur et al. 2014). Though contrary to this, transition from shade to light for several tropical tree species over a long period caused a reduction in Lp_r despite increased transpirational demand (Shimizu et al. 2005). This was correlated with a reduced inhibition by mercury indicating that root aquaporins had a diminished role after the transition to light and further growth. It is not surprising that NO_3^- or NH_4^+ transport by roots also varies diurnally with higher rates observed in the day or light period (e.g. in soybean (Delhon et al. 1995) and citrus (Camañes et al. 2007)). In citrus the expression of a high-affinity NH_4^+ transporter in the

root is regulated by sugar, independently of a circadian control (Camañes et al. 2007). In *Arabidopsis* both NH_4^+ and NO_3^- uptake by roots appears to be regulated via sugar from photosynthesis. In addition to the NH_4^+ transporters (*AMT1.1, 1.2, 1.3*), *NRT1.1* (a NO_3^- transceptor, now called NPF6.3 (O'Brien et al. 2016)) and the high-affinity NO_3^- transporter and transceptor AtNRT2.1 (see below) are both sugar (sucrose and glucose) inducible, and deletion of AtNRT2.1 results in the loss of the regulation of root NO_3^- uptake by light and sugar (Lejay et al. 2003). In rice NRT transcripts are also upregulated by light and sugar (Feng et al. 2011). Aquaporins can be upregulated by a protein kinase sugar sensor in seedlings (Wu et al. 2013) (see below) that could lead to entrainment of root water movement to N transport and shoot photosynthesis in the light. Recently a shoot-to-root mobile bZIP transcription factor activated by light (HY5) that interacts with sugar signalling in *Arabidopsis* increased expression of *AtNRT2.1* in roots (Chen et al. 2016). It is not known if this changes expression of any aquaporins.

1.3.6 Temperature

Root hydraulic conductivity is also sensitive to temperature. Cucumber roots experience a decline in both root pressure and hydraulic conductivity in response to temperature reductions below 25 °C (Lee et al. 2004a; Lee et al. 2004b). In temperature-resistant rice (Ahamed et al. 2012) and maize (Aroca et al. 2005), aquaporin activity is linked with maintenance of hydraulic conductivity and recovery. In cucumber roots exposed to lowered temperature, there was a larger reduction in the osmotic component of water flow compared with the hydrostatic component. This was associated with an effect on root aquaporins (specific type not identified), while the reduction in root pressure was associated with reduced activity of the plasma membrane proton pump (Lee et al. 2004b). The kinetics of NH_4^+ uptake across the plasma membrane of rice roots also differs with

temperature, where the high-affinity transport system (HATS) is more strongly reduced by low temperature than that of the low affinity transport system (LATS) (Wang et al. 1993). The relative uptake of NH_4^+ and NO_3^- also changes at low temperature due to different temperature effects on NO_3^- and NH_4^+ transport in ryegrass (Clarkson and Warner 1979). The interaction between soil temperature, water movement and N transport is poorly understood (Pregitzer et al. 2000; Kreuzwieser and Gessler 2010), and this is important in the context of global warming and predictions of biogeochemical fluxes (Davidson et al. 2006).

1.3.7 Anoxia and hypoxia

Anoxia (lack of environmental oxygen) or hypoxia (insufficient environmental oxygen) in the soil as a result of soil water logging also influences water flux through roots, leading to an immediate decrease in water flux and Lp_r (Vandeleur et al. 2005), in a species-dependent way (Bramley and Tyerman 2010). This again can have ramifications for predictions of fluxes at ecosystem scales (Shaw et al. 2013).

Anoxia reduced the hydraulic conductivity of *Arabidopsis* roots, which coincided with cytosolic acidification (Tournaire-Roux et al. 2003). This acidification caused protonation of a histidine on loop D of PIP proteins, which led to a conformational change that reduced water movement. Anoxia greatly reduced the water flow of NO_3^-fed tomato, cucumber (Gorska et al. 2008), maize (Górska et al. 2008) and sunflower (Gloser et al. 2007) indicating that aquaporins are important in hydraulic responses to N in some species. Increase in reactive oxygen species under hypoxia (van Dongen and Licausi 2015; Shabala et al. 2014) may also inhibit aquaporin activity (Wudick et al. 2015). Several aquaporin transcripts are increased under anoxia/hypoxia (Bramley et al. 2007a) that may be linked to tolerance mechanisms related to gas transport (CO_2 or O_2) or

signalling (Bramley and Tyerman 2010) or release of a fermentation product (Choi and Robers 2007). Despite being passive transporters, it is interesting to note how dependent aquaporin function is on cell energy status as indicated by one of the best inhibitors of aquaporins, azide (Postaire et al. 2010; Grondin et al. 2016), which inhibits mitochondrial respiration (Hodges and Elzam 1967). Since the uptake of NH_4^+ and NO_3^- are highly energy-dependent processes requiring a proton electrochemical gradient established by the plasma membrane H^+-ATPase to drive their influx, it is not surprising that anoxia and soil water logging have profound effects on N uptake, e.g. wheat (Herzog et al. 2016; Robertson et al. 2009), probably due to collapse of pH gradients (Felle 2005). There are, however, differences between species in N uptake under flooding and in the amount of NH_4^+ versus NO_3^- taken up (Kreuzwieser et al. 2002) partly related to NO_3^- becoming less available relative to NH_4^+ in water logged soils due to decreased nitrification (Laanbroek 1990). An acidification of root cell cytoplasm, that can result from anoxia (Felle 2005), could increase NO_3^- efflux via the AtNAXT NO_3^- transporter (Segonzac et al. 2007) exacerbating the reduced availability in the soil. NO_3^- is particularly interesting in relation to the integration of water and N fluxes because of its stimulatory effect on root water movement (examined below) (Gloser et al. 2007) via aquaporins (Górska et al. 2008) and because of the ameliorative effects of NO_3^- and nitrite on cytosolic pH regulation under anoxia (Libourel et al. 2006). Recently it was shown that the *Arabidopsis* NRT1.1 is required for acid tolerance, and when knocked out, the roots were unable to increase the pH of the external medium when acidic. This was linked to NO_3^- uptake rather than NO_3^- sensing (Fang et al. 2016).

1.3.8 Energy and nutrient signalling

Energy and nutrient sensing in plant cells is also an area of potential overlap between NO_3^- transport and assimilation, and aquaporin activity, especially since PIP aquaporins seem to be energy-dependent. The TOR (Target of Rapamycin) and SnRK1 (Sucrose non-fermenting-1 (SNF1)-related kinase 1) signaling pathways interact to regulate metabolism and development in response to energy supply and nutrition (Robaglia et al. 2012). SnRK1 is a central energy sensor important for metabolic homeostasis in plants (Baena-Gonzalez et al. 2007; Baena-González and Sheen 2008) and is partly controlled via trehalose-6-phosphate sensing among other signals (Emanuelle et al. 2016; Jossier et al. 2009). It is activated under energy deficient conditions, e.g. by hypoxia and carbon starvation (Cho et al. 2016; O'Hara et al. 2013) and inhibits NO_3^- reductase activity via phosphorylation (Sugden et al. 1999). Out of the 1021 gene transcripts reported to be up-regulated by SnRK1.1 in *Arabidopsis* only 16 are channels or transporters and three of these are aquaporins (AtTIP2;1, AtTIP1;3 and AtPIP1;3) (Baena-Gonzalez et al. 2007). AtTIP2;1 is of special interest because of its ability to facilitate the diffusion of NH_4^+ directly or indirectly as NH_3 (Holm et al. 2005; Kirscht et al. 2016) and its correlation to stomatal conductance (Pou et al. 2013). The TOR protein kinase on the other hand is a regulatory hub involved in nutrient sensing (Dobrenel et al. 2016). The TOR pathway is proposed to increase N assimilation under energy and nutrient replete conditions (Dobrenel et al. 2016; Lillo et al. 2014). Protein phosphatase 2A is a component of the TOR pathway via the regulatory subunit (Tap46). When Tap46 is overexpressed in *Arabidopsis* this results in growth stimulation, increased NO_3^- reductase activity and induction of transcription of genes involved in N transport including AtNRT1.1 (AtNPF6.3) and AtNRT2.1 (Ahn et al. 2015). Both are components of the NO_3^- transceptor signalling cascade, which regulates the primary nitrate response in plant roots

(O'Brien et al. 2016) with AtNRT2.1 proposed to have a role in influencing root aquaporin activity in response to NO_3^- (see below) (Li et al. 2016).

Sugar, and hence energy supply, also regulates aquaporins in *Arabidopsis* via a sucrose-activated receptor kinase in whole seedling plasma membrane (SIRK1), able to phosphorylate aquaporins and thereby positively affecting their activity (Wu et al. 2013). Several PIP aquaporins were shown to interact with SIRK1 including those that have been shown to be associated with Lpr (e.g. AtPIP2;1) (Wudick et al. 2015; Sutka et al. 2011). Sugar signaling in plants is linked to C:N balance, and root/shoot coordination with cross-talk with hormone signaling (Wang and Ruan 2016). Altogether these data suggest that aquaporins could be added as an important component of nutrient signaling pathways, but location in roots needs to be determined for SIRK1.

1.3.9 Co-location of water and N uptake

For mass flow to have an effect on NO_3^- transport to the root, it would be expected that the location of NO_3^- influx along the axis of the root or in different root classes should correspond to the location of the maximum volume-flux density of water inflow (Górska et al. 2008). It is not a trivial exercise to determine where along the axis of roots or the type of roots where water inflow is maximal. Work with lupin and wheat has indicated contrasting locations for water flow based on the scaling of root hydraulic conductance with root length (Bramley et al. 2009). Wheat tends to have a greater concentration of water flow near the root tip and is more dependent on aquaporins than lupins (Bramley et al. 2009). Using neutron radiography to image deuterated water movement in and through roots in soil during transpiration with a model to separate convection from diffusion, Zarebanadkouki et al. (2014) concluded for lupin roots that radial flow into roots was maximal 12–16 cm from the root tip of lateral roots. For maize roots, lateral roots show

a greater uptake of water relative to primary and seminal roots as determined by the same technique (Ahmed et al. 2016). It was concluded that lateral roots were the primary sites of water absorption and that primary and seminal roots functioned to axially transport water to the shoot. Since whole maize roots have been shown to have a positive response of water movement to NO_3^- (Górska et al. 2008), it would be expected from the results of Ahmed et al. (2016) that NO_3^- uptake would occur mainly through lateral roots. NO_3^- uptake kinetics measured for different root classes at two plant ages and in response to NO_3^- deprivation showed large differences, with lateral roots giving the highest affinity (lowest Michaelis-Menten constant, K_m) and highest maximum rate of uptake, which depended on plant age (York et al. 2016). Interestingly linear uptake kinetics indicating LATS transport of NO_3^- were not observed for laterals but occurred in all other root classes. Modelling of plant growth based on these kinetics and taking into account mass flow to the roots indicated that the maximum rate of influx was important in determining plant growth rather than the concentration kinetics (York et al. 2016).

Ion sensitive electrodes have been used to scan NH_4^+ and NO_3^- fluxes at finer scale along roots. In young maize roots, NO_3^- influx reached a maximum at 3.5– 8.5 mm from the root tip and then remained more or less constant with distance to 21–61 mm (limit of measurements) (Taylor and Bloom 1998). NH_4^+ influx was high at the root tip and increased with distance but was about 1/3 that of NO_3^- It was concluded that NH_4^+ was most actively taken up at the root tip reflecting the energy advantage over NO_3^-. In fine wheat roots, highest net fluxes of NH_4^+ and NO_3^- occurred at 20–25 mm from the root tip (Zhong et al. 2014), which would correspond to maximum hydraulic conductivity measured by Bramley et al. (2009). In rice, maximum net influx of NH_4^+ and NO_3^- occurred at 10–20 mm from the apex and then declined (Colmer and Bloom 1998). This was different from maize since maize did not show a decline with distance. It was

concluded that sclerenchymatous fibres on the outer side of the cortex of older parts of the rice root could impede influx. However, later measurements of water flow across this region indicated that it was largely determined by apoplastic flow (Ranathunge et al. 2004). Examination of NO_3^- uptake kinetics, expression of *ZmNRT2.1* and plasma membrane H^+-ATPase activity along maize primary roots using a compartmental system showed that the root tip (0–4 cm) had higher capacity to take up NO_3^- due to higher maximum influx and more rapid induction of a high-affinity transport system (Sorgonà et al. 2011). This did not correlate with the expression of *ZmNRT2.1*, which was highest at 4–6 cm from the root tip. To date there are no studies that have attempted to define water and NO_3^- or NH_4^+ fluxes simultaneously with root position or root type, and this data is required to better understand the link between water and N uptake. In *Arabidopsis* specific NRT2-HATS transporters are associated with root development, plant age and specific cell types (Kiba and Krapp 2016). AtNRT2.4 and AtNRT2.5 are expressed in a polarised manner in epidermal cells (facing the external medium) with a greater level of expression of AtNRT2.4 in young plants and AtNRT2.5 in mature plants (Lezhneva et al. 2014; Kiba et al. 2012). *AtNRT2.4* is down-regulated with high NO_3^- and is only increased to levels equivalent to *AtNRT2.1* without NO_3^- in young plants (Kiba et al. 2012). In mature plants *AtNRT2.1* has higher expression than both *AtNRT2.4* and *AtNRT2.5* after 1 day of N starvation but then declines with *AtNRT2.5* and *AtNRT2.4* expression increasing to dominate by 10 days (Lezhneva et al. 2014). Many experiments investigating the link between root water transport and NO_3^- transport initially starve plants of N or have them grown in low NO_3^- for various periods, then resupply with NO_3^- to examine the response in water transport. In this case HATS NRT2s would be induced and may account for a portion of the NO_3^- uptake when NO_3^- is introduced to the roots. However, the concentrations of NO_3^- used are generally well above the HATS concentration range and

would require NRT1-LATs transporters or anion channels to carry the load of NO_3^- uptake. In roots of *Arabidopsis*, these would be AtNRT1.1, AtNRT1.2 (AtNPF4.6) and AtNAXT1 (AtNPF2.7) (O'Brien et al. 2016) or possibly an anion channel (initially before cytoplasmic NO_3^- increases) (Skerrett and Tyerman 1994). The location of the inducible LATS transporter *AtNRT1.1* mRNA is in the epidermis near the root tip and in the cortex or endodermis in more mature regions (Huang et al. 1996). Likewise the constitutively expressed *AtNRT1:2* (*AtNP4.6*) is located in root hairs and epidermis in mature regions of roots. The location of AtNRT2.1 as a NO_3^- sensor that regulates PIP activity in *Arabidopsis* (Li et al. 2016) makes sense, since mass flow of water to the root would be expected to build NO_3^- concentration in the apoplast of cortex cells due to endodermal barriers blocking apoplastic movement. However, being a high-affinity transporter with saturation occurring around 0.5 mM NO_3^-, one would expect that mass flow under conditions of NO_3^- resupply (often several millimolar NO_3^-) would require a LATS transport or dual-affinity transporter to do most of the influx, i.e. AtNRT1.1 and AtNRT1.2. The locations of these transporters (discussed above) would match with our present view of high water transport intensity, particularly in the endodermis and epidermis.

1.4 N form and its concentration affect root hydraulic properties

1.4.1 Growth and development

Over the long term, N affects both root growth and development (Wilson 1988; Robinson 1994; Lopez-Bucio et al. 2003; Guo et al. 2007c; Desnos 2008). In *Arabidopsis* local patches of NO_3^- promote lateral root outgrowth via NO_3^-- inducible MADS-box gene (ANR1) and coordinated by a systemic signal dependent on the plant's N status (Zhang and Forde 2000). N responses on root growth can affect different properties in different

species. Górska et al. (2010) showed that cucumber, tomato, maize, *Arabidopsis* and *Festuca arundinacea* have increased relative root growth on 2 mM NO_3^- compared to 0.2 mM, whereas *Populus trichocarpa* and *Nephrolepis exaltata* do not have a relative root growth response. Tomato, maize, *Arabidopsis* and *Festuca arundinacea* also have increased root/shoot ratios on low NO_3^- nutrition, whereas cucumber, *Populus trichocarpa* and *Nephrolepis exaltata* do not.

The form of N available to roots impacts growth and development as well. French bean grown in NO_3^- as sole N source had larger roots and shoots than when grown on NH_4^+ (Guo et al. 2002). These results were repeated with split root experiments where one half of the root was grown on NO_3^- and the other on NH_4^+ (Guo et al. 2007b). This demonstrated a repression of growth of roots grown in NH_4^+. In experiments where half the roots were grown on N (NH_4^+ or NO_3^-) and the other half starved of N, either form resulted in increased growth. Still NO_3^--grown roots had increased growth. In tomato, roots grown in NH_4^+ had stagnated growth over 3 weeks compared to plants grown in NO_3^- (Pill and Lambeth 1977). Stimulation of lateral root growth in response to $NO_3^-$is regulated by multiple pathways related to initiation of the lateral root, early development and elongation of lateral root primordia (Forde 2014). There is an interaction between auxin and NO_3^- signalling in this process. Aquaporins also play a role in lateral root outgrowth. Auxin-mediated repression of AtPIP2;1 accounts for reduced polarised water flow into overlying tissue for optimum extension (Péret et al. 2012), while the tonoplast located aquaporins AtTIP1;1, AtTIP1;2 and AtTIP2;1 are also involved in the development of lateral root primordia in a complex manner, each isoform having different spatio-temporal expression patterns (Reinhardt et al. 2016). It is yet unknown if these aquaporins also play similar roles in lateral root outgrowth under NO_3^- stimulation. AtTIP2;1 is particularly interesting given that it complemented the triple *tip1;1/1;2/2;1*

knockouts (Reinhardt et al. 2016) and that its expression can be linked to N and energy signalling (Baena-Gonzalez et al. 2007). However, neither *AtTIP2;1* nor *AtPIP2;1* comes up as regulated genes in local NO_3^-stimulation in wild-type *Arabidopsis* or the NO_3^- signalling mutant of NRG2 (Xu et al. 2016), while *AtTIP1;1, 2;2* and *2;3* are subject to changes in expression under local NO_3^- supply (Li et al. 2014; Ruffel et al. 2011). Maize cultivars show large differences in the response to NO_3^- of lateral root outgrowth both in terms of homogeneous and locally high NO_3^- (Yu et al. 2016). In this study pericycle cell transcriptomes were examined for all root classes and compared between locally high NO_3^- and uniform low NO_3^-. The transcript responses were root-class dependent with brace roots showing the greatest response of some 2,740 NO_3^--regulated genes with unique cell-cycle control genes being strongly represented. Brace roots would be worthy of further physiological studies to examine the water: NO_3^- link.

1.4.2 Root hydraulics

Root hydraulic properties are sensitive to N form and concentration and are dependent on species and length of treatment. For many species, an increase in water flow and hydraulic conductivity are observed (**Table 1.1**). For example, in wheat, plants grown on NO_3^- (0.5–16 mM), de-topped root exudation rates are increased in a dose-dependent manner with increasing NO_3^-(Barthes et al. 1996a). When starved of NO_3^- for 5 days, wheat plants showed a decrease in hydraulic conductivity and water flow (Carvajal et al. 1996a). Resupplying NO_3^- to N-starved wheat caused quick increase in flow and hydraulic conductivity, resulting in a return to control values by 24 h (Carvajal et al. 1996a). A rapid stimulation of Lp_r by NO_3^- is not always observed (Górska et al. 2010), for example, in the tree *Eucalyptus grandis*, no root response was observed, though in this species there was a longer-term increase in whole plant hydraulic conductance when gown on high N

(Graciano et al. 2016). It may be that the root cellular concentration of NO_3^- plays an important role in these responses. Tomato and cucumber both increased water flow through roots in response to NO_3^- (Gorska et al. 2008). This was observed at the cellular level as well using a cell pressure probe to measure the half-time of water flow to applied pressure relaxations. The half-time fell when roots were exposed to NO_3^-, indicating water flowed with smaller resistance in these cells. In the presence of tungstate, a NO_3^- reductase inhibitor, tomato root responses at the cellular and whole root levels were unaffected, but the response of cucumber roots and cells was eliminated. It was shown that tomato roots maintained NO_3^- uptake, but tungstate treatment inhibited uptake of NO_3^- by cucumber roots. Using the cell pressure probe, injecting NO_3^- directly into cucumber root cells treated with NO_3^- reductase recovered the cellular response. In maize, a delay is observed between NO_3^- application and increase in hydraulic flow (Górska et al. 2008). Tungstate application to maize roots resulted in a decreased NO_3^- uptake rate over 4 h and also eliminates the hydraulic response to NO_3^-. Tungstate application greatly inhibited *PIP* expression not seen in starved plants, indicating tungstate may disrupt many cellular pathways (Górska et al. 2008). In this case, diurnal patterns in maize root sap flow and osmolality (Lopez et al. 2003) or de-topping effects may have confounded the hydraulic response to increased cellular NO_3^- levels.

Observations across several species show that a species' NO_3^- uptake rate is linearly correlated with the magnitude of the hydraulic response (Górska et al. 2010). In these experiments, species with high NO_3^- uptake rates had large increases in water flow after NO_3^- application, while species with low NO_3^- uptake rates lacked a hydraulic response to NO_3^- (Górska et al. 2010). Cellular NO_3^- levels may have been important in the hydraulic response to NO_3^- in these plants. The link between NO_3^- uptake rate and hydraulic response also supports the mass flow hypothesis for the basic hydraulic

response described in Cramer et al. (2009). Larger NO_3^- uptake rates are quicker to deplete the root zone of NO_3^-, and mass flow could be a mechanism to quickly resupply NO_3^- pools near the root surface when NO_3^- is present (Górska et al. 2008).

Table 1.1 Nitrogen effect on root water transport

Plant species response to nitrogen of any form (NO_3^-, NH_4^+, mixture, other)

Species	+N led to increased conductance (Lp_r) and/or increased exudation rate (J_v)	+ N had no effect	+ N led to decreased conductance, and/or decreased exudation rate
Tomato	Minshall (1964) Chapin et al. (1988) Lee et al. (2002) Gorska et al. (2008) Górska et al. (2010)		
Barley	Chapin et al. (1988) Skobeleva et al. (2010) Ktitorova and Skobeleva (2008) Ruggiero and Angelino (2007)		Chapin et al. (1988)
Cotton	Radin and Matthews (1989)		
Maize	Barthes et al. (1995) Barthes et al. (1996a) Hoarau et al. (1996) Schraut et al. (2005) Górska et al. (2008) Górska et al. (2010)		
Wheat	Barthes et al. (1996b) Carvajal et al. (1996a) Carvajal et al. (1996a) Ruggiero and Angelino (2007)		
Lotus japonicas	Clarkson et al. (2000)		
Clover	Belastegui-Macadam et al. (2007)		
Ryegrass	Belastegui-Macadam et al. (2007)		
Sunflower	Radin and Boyer (1982) Gloser et al. (2007)		
Cucumber	Gorska et al. (2008) Górska et al. (2010)		
Arabidopsis	Górska et al. (2010) di Pietro et al. (2013)		
Populus trichocarpa		Górska et al. (2010)	
Festuca arundinacea	Górska et al. (2010)		
Boston Fern		Górska et al. (2010)	
Figleaf gourd	Rhee et al. (2011)		
Pistacia lentiscus	Trubat et al. (2006) Trubat et al. (2012)		
Aspen	Siemens and Zwiazek (2013)		
Phaseolus vulgaris	Guo et al. (2002) Guo et al. (2007b)		
Rice	Yang et al. (2012) Ishikawa-Sakurai et al. (2014)		
Oak	Steudle and Meshcheryakov (1996)		Steudle and Meshcheryakov (1996)

1.4.3 NO_3^- versus NH_4^+ in the regulation of root hydraulics

The form of N supplied affects root hydraulic responses and is species dependent (**Table 1.1**). In French bean, roots exposed to NO_3^- (whole root or in split root) have increased

water uptake compared to roots exposed to NH_4^+. Water uptake in root systems split between NO_3^- and zero N has more total uptake from the NO_3^- fed roots, but when normalised by root volume, starved roots absorb more water per root volume. Under NH_4^+ or starvation, roots absorb more water and more water per unit root volume on the starvation side. Root volume and dry mass are larger when in NO_3^- or NH_4^+ compared to starvation and was more pronounced during the day than at night (Guo et al. 2002; Guo et al. 2007a; Guo et al. 2007b; Guo et al. 2007c).

Ammonium nutrition has mixed results in other species. Like French bean, maize NO_3^- resupply led to an increase in water flow through the root in N starved plants, whereas urea and NH_4^+ had slightly negative effects on water flow (Górska et al. 2008). In tomato, application of either NO_3^- , NH_4^+ or $NH_4^+ + NO_3^-$ increased the total exudate from de-topped plants, with NH_4^+ having a slightly larger effect than NO_3^- (Minshall 1964). Clover plants grown in 5 mM NO_3^- had a slight increase in Lpr compared to 0.5 mM, but no increase was seen with NH_4^+. Contrasting to this, ryegrass had a 300 % increase to either 5 mM NO_3^- or NH_4^+ compared to 0.5 mM (Belastegui-Macadam et al. 2007).

In rice, NO_3^- and NH_4^+ have mixed effects on plant hydraulics. In cultivar "Shanyou63" hybrid *indica*, root dry weights between NO_3^- and NH_4^+ fed plants are not different (Li et al. 2009; Yang et al. 2012), whereas root fresh weight is significantly greater for NO_3^- grown plants (Gao et al. 2010). When normalised to fresh weight, the water uptake was found to be significantly greater for NH_4^+ grown plants in two out of three of the studies (Li et al. 2009; Yang et al. 2012) and that total root uptake of water (not normalised) was greatest for $NH_4^+ + NO_3^-$ fed plants, intermediate for NO_3^- and least with NH_4^+ (Gao et al. 2010). All three of these studies show that for NO_3^- fed plants, xylem water flow and total water uptake (normalised or not) decrease by greater amounts during water stress

(PEG). These studies used the same rice cultivar and were treated with different N forms for up to 2 weeks potentially masking the hydraulic effects behind growth changes. In mixed N (NH_4^+ NO_3^-) treatment, lowering N from 20 to 0.2 ppm led to a decrease in xylem sap flow and hydraulic conductivity over 3 days (Ishikawa-Sakurai et al. 2014). Upon resupply, xylem sap flow rate and hydraulic conductivity were raised. Interestingly, the initial response for starvation or resupply was opposite from the final response. The first 6 h of starvation saw an increase in the hydraulic conductivity and xylem flow rate in N-starved plants. At 24 h, rates began to fall compared to plants in full N treatment. Between 0 and 6 h, N-fed plants showed a slight increase in xylem sap flow but a decrease in Lpr. By the next light period, a large increase in both root xylem sap flow and Lpr were observed. This highlights that the magnitude of hydraulic response to N is time dependent and suggests that the direction of change may be time dependent as well probably linked to circadian and diurnal regulation. Further study on rice comparing two cultivars that have different water relations responses to N supply during growth showed a strong increase in Lpr with increasing N supply (as equal NH_4^+ and NO_3^-) with larger responses observed for Yangdao6 than Shanyou63 (Ren et al. 2015). This difference was reflected in the response of leaf water potential, which became more negative in Shanyou63 and was constant in Yangdao6 with increasing N. It was concluded that the increase in Lpr was due to a combination of reduced aerenchyma and lignin as well as increased PIP aquaporin expression in roots (see below). The greater response of Yangdao6 was linked to aquaporin expression and more reduced aerenchyma (Ren et al. 2015).

Table 1.2 Nitrogen form impact on root hydraulic properties

N form has variable impacts, with many plants preferring either nitrate or ammonium. In several plants, ammonium inhibited root hydraulic properties

Species	NO_3^- treated plants had increased conductance (Lp_r) and/or increased exudation rate (J_v) compared to NH_4^+	No difference in root hydraulics between NO_3^- or NH_4^+	NO_3^- treated plants had decreased conductance (Lp_r) and/or decreased exudation rate (J_v) compared to NH_4^+	NH_4^+ inhibited conductance and or exudation
Conifers		Gebauer and BassiriRad (2011)	Gebauer and BassiriRad (2011)	
Aspen	Siemens and Zwiazek (2013)	Siemens and Zwiazek (2013)		
Barley	Skobeleva et al. (2011)			
Clover			Belastegui-Macadam et al. (2007)	
Ryegrass		Belastegui-Macadam et al. (2007)		
Phaseolus vulgaris	Guo et al. (2002) Guo et al. (2007a) Guo et al. (2007b) Guo et al. (2007c) Schulze-Till et al. (2009)			
Muskmelon	Adler et al. (1996)			
Rice		Yang et al. (2012) Gao et al. (2010)	Li et al. (2009) Gao et al. (2010)	
Sunflower				Ragab (1980)
Tomato	Pill and Lambeth (1977)			Pill and Lambeth (1977)

1.4.4 Transcriptional responses of aquaporins in response to N

Aquaporins undoubtedly play a role in the N responses as indicated above. Transcriptional responses of aquaporins to changes in external N status, if they occur, tend to take days to become evident. Caution must be used, as transcription and protein amount are not always correlated (Hachez et al. 2012). In tomato, increased hydraulic conductivity to N is seen quickly (Górska et al. 2010; Gorska et al. 2008) , but aquaporin gene responses were not seen before 48 h of treatment (Wang et al. 2001). Exposing *Arabidopsis* to NO_3^- from NH_4^+ growth for 20 min only repressed *AtNIP2;1* expression but left all other aquaporin levels the same (Wang et al. 2003). In *Arabidopsis* resupply of NO_3^- to N-stressed plants strongly induced a *TIP* member, and several others weakly (Scheible et al. 2004).

In maize, NO_3^- addition was not reported to change *PIP* gene expression within 4 h, while tungstate treatment greatly inhibited the expression of most *PIP* genes (Górska et al. 2008). However eight hours after NO_3^-addition, *ZmPIP1-5b* transcript level was strongly induced (Gaspar et al. 2003). These studies utilised different maize varieties (a hybrid "Hybrid corn – white sweet" and a F7F2 respectively). It remains to be seen how the PIP expressin profiles of the B73 inbred line, with a published genome, responds to varying N forms and amounts. Additionally, the transcriptional behaviour of the other aquaporin families (TIPs, NIPs and SIPs) is unknown.

In French bean, NO_3^--induced *PIP1* expression occurred around 4 days after addition, but no PIP1 gene induction was seen with NH_4^+(Guo et al. 2007a). In rice, switching from 10 ppm NH_4^+ + NO_3^- to 0.5 ppm led to a slight repression of *OsPIP1;1*, *OsPIP2;3*, *OsTIP1;1* and *OsTIP2;2* expression. Major reduction in expression was seen for *OsPIP2;4* and *OsPIP2;5* with induction for *OsTIP2;1* and *OsPIP2;6*. During resupply, *OsPIP2;4*, *OsPIP2;5* and *OsTIP2;2* gene transcription was largely induced. Several other genes were slightly induced. This correlated with decreased hydraulic conductivity during starvation and increased during resupply (Ishikawa-Sakurai et al. 2014).

1.4.5 Post-translational regulation of aquaporins in response to N

Aquaporin blockers result in reduced hydraulic conductivity under N treatments of wheat (Carvajal et al. 1996b), barley (Ruggiero and Angelino 2007) and figleaf gourd (Rhee et al. 2011). An investigation of the link between Lpr and aquaporin protein abundance and post-translational responses in *Arabidopsis* revealed that AtPIP2 protein amount correlated with Lpr responses to various treatments (di Pietro et al. 2013). A higher correlation was observed with the PIP phosphorylated peptides. In contrast PIP1 and TIP abundance did not correlate with Lpr. N starvation tended to have the largest impact on

PIP protein abundance. They also observed differences in deamination in PIP2 protein fragments, suggesting a role in postsecondary modification of aquaporins during the response. Largely, however, these pathways, including pH-mediated gating, localization and phosphorylation, have remained unexplored in N regulation of plant hydraulics (di Pietro et al. 2013). Other connections in this regard between NO_3^- signalling and factors that regulate aquaporins include elevation of ABA in certain cells (Ondzighi-Assoume et al. 2016) and transient increased cytosolic Ca^{2+} concentration (Riveras et al. 2015) in response to NO_3^-. The Ca^{2+} response was abolished in mutants of NRT1.1 (NPF6.3). Both cytosolic Ca^{2+} and ABA are known to regulate aquaporins (Wan et al. 2004; Alleva et al. 2006). Global protein phosphorylation patterns have been examined in N-starved *Arabidopsis* seedlings when resupplied with either NO_3^- or NH_4^+ compared with potassium chloride after 30 min (Engelsberger and Schulze 2012). Distinct phosphorylation patterns were observed for proteins that have signalling functions and transporters/channels. Phosphopeptides from aquaporins were identified, and these were rapidly dephosphorylated in response to KCl and NH_4Cl, but KNO_3 produced a weaker effect, and there was a subsequent increase in phosphorylation over time.

Recently a significant advance was made in understanding the regulation by NO_3^- of Lpr and aquaporins in *Arabidopsis* (Li et al. 2016). Examination of the Lpr of NO_3^- transporter mutants including the transceptors NRT1.1 (NPF6.3) (dual HATS LATS) and NRT2.1 (iHATS) showed that only the NRT2.1 knockout had reduced Lpr. Though Lpr was lowered in the NRT2.1 mutant, it still responded to reduced NO_3^- supply. Overall there was a better correlation between Lpr and shoot NO_3^- concentration than root NO_3^- across all mutants and WT. Although NRT1.1 mutant was not considered further in the work, it did appear that the correlation between Lpr and shoot NO_3^- was depressed in this mutant compared to WT. Transcripts of both PIP1;1, PIP1;2, PIP2;1 and PIP2;3

correlated with changes in Lpr for NRT2.1 mutants under different NO_3^- concentrations, and both PIP1 and PIP2 protein abundance were also correlated with Lpr. In WT, however, the regulation appeared to be mostly at the post-translational level. The conclusion from this study was that root aquaporins were regulated by a shoot-to-root signal communicating shoot NO_3^- status as well as the function of NRT2.1 (**Figure 1.5**). It would be worthwhile examining a split root protocol to distinguish between local and systemic signals to test this model, especially since NO_3^- initially upregulates the level of the NRT2.1 transcript after starvation and split root experiments indicate that a shoot-to-root signal is involved in transmitting shoot N-demand to changes in NRT2.1 expression (Kudoyarova et al. 2015).

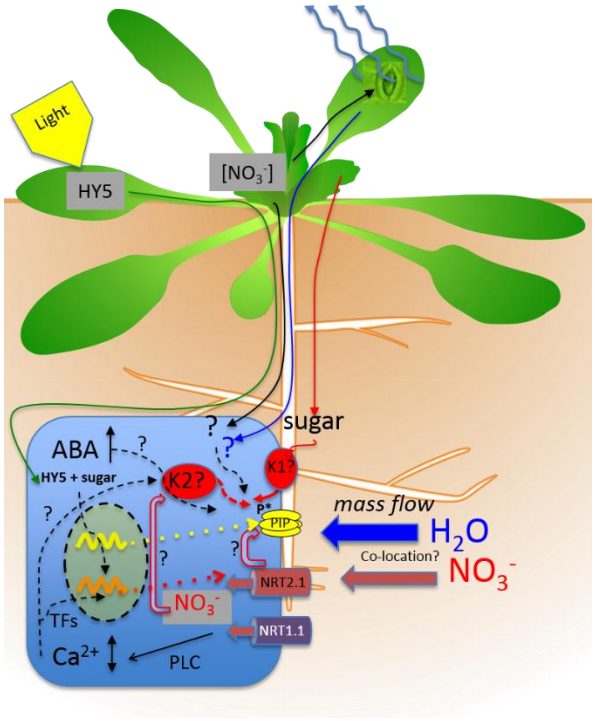


Figure 1.5 Regulation of root PIP aquaporins involves specific and probably parallel pathways of local and systemic signaling.

Local: The presence of NRT2.1 is required for maintaining a high Lpr in Arabidopsis (Li et al. 2016) and while the signaling cascade to PIP aquaporins is unknown, it is likely to be via phosphorylation by an unidentified kinase(s) (denoted K2) (Engelsberger and Schulze 2012). NRT1.1 is also required for nitrate signaling and regulation of expression of NRT2.1 (Bouguyon et al. 2015) (short-term up-regulation and long-term downregulation) via a cytosolic Ca2+ signal involving phospholipase C (PLC) and various transcription factors are activated (TFs) (O'Brien et al. 2016; Riveras et al. 2015). Although Ca2+ is known to directly regulate PIP aquaporins via gating (Verdoucq et al. 2008), the presence of NRT1.1 was not required for Lpr responses to NO_3^- in Arabidopsis (Li et al. 2016). Abscisic acid (ABA) is also elevated in certain cells (endodermis) of the root tip of Arabidopsis in response to NO_3^- (Ondzighi-Assoume et al. 2016). ABA has been shown to have a positive effect on root cell water permeability by an unknown but rapid mechanism (Wan et al. 2004; Olaetxea et al. 2015). Transcription and translation to increase protein abundance are indicated as dotted arrows to PIPs and NRTs. Systemic: Shoot NO_3^-positively correlates with Lpr in Arabidopsis and unknown shoot to root signal was suggested (black arrow) (Li et al. 2016). Sugar has been shown to activate a kinase (K1? = SIRK1), which phosphorylates PIP aquaporins to activate them in Arabidopsis seedlings (Wu et al. 2013) and it is assumed that this occurs in roots in the diagram (red arrow, K1?). Sugar signals also regulate root nitrate uptake and NRT2.1 (Wang and Ruan 2016). A shoot-to-root mobile bZIP transcription factor activated by light (HY5) that interacts with sugar signaling in Arabidopsis increases expression of AtNRT2.1 in roots (green arrow) (Chen et al. 2016). NO_3^- is also a signal to control stomatal aperture (Wilkinson et al. 2007), which will impact on transpiration and thence mass flow to the root. Also an unidentified shoot-to-root signal (blue arrow) conveying transpiration or leaf water potential regulates Lpr in rice (Sakurai-Ishikawa et al. 2011) and probably other plants (Vandeleur et al. 2014) this could be ABA in wheat (Kudoyarova et al. 2011). Not shown in the diagram is the probable signaling involving NH_4^+, the role of certain aquaporins in lateral root initiation in response to NO_3^- and outgrowth that is important for NO_3^- foraging in soil, nor the relative expression patterns of NO_3^- transporters in the root that may influence the efficiency of coupling of water flow with PIP activated increase in Lpr. Other hormones involved in N signaling are also not included and some of these e.g. auxin have also been demonstrated to have effects on aquaporin regulation (Péret et al. 2012). Figure from Tyerman et al. (2017)

1.5 Concluding remarks and general hypothesis

Variation exists between maize varieties on a wide variety of factors. Maize plants are documented to have a hydraulic response to nitrogen treatments, and hydraulic responses are likely to be the result of changing aquaporin activity. Studies have examined hydraulic responses to resupply, however the effects of other N treatments are unclear. Additionally, knowledge of aquaporin expression and N treatment are limited to a subset of the ZmPIPs, and to within 4h of N resupply. The water transport of maize aquaporins is largely understood in the ZmPIPs, however most ZmTIP and ZmNIP water transport is theoretical based on ar/R filter predictions.

1.5.1 The aims and of this thesis:

1. Deduce the genetic basis of water transport by mining the maize genome for aquaporins
2. Clone and characterise maize aquaporins response to water, acid, and nitrate
3. Measure hydraulic response of maize in response to N to test the hypothysis that different maize lines have variable hydraulic responses to nitrate from changes in hydraulic conductivity.
4. Measure gene expression of the complete set of aquaporins, as well as genes involved in nitrate uptake and regulation.

1.5.2 Approach

These hypotheses were tested by monitoring the root hydraulic conductivity maize inbred lines B73 and F44 in hydroponics and aeroponics during starvation (3-5 days) and resupply (4hr-3 days) experiments. B73 was selected as its genome has been sequenced, and F44 as it was shown to be genetically distinct from B73 (Liu et al. 2003a) and could offer insight to the effect across maize lines. To link root hydraulics to aquaporin activity, decreases in hydraulic conductivity were monitored in a single root over time post-excision. Vandeleur et al. (2014) demonstrated that hydraulic conductance decreases over time due to aquaporin activity. To examine aquaporin effects, a complete set of aquaporins in maize inbred B73 was deduced by screening the genome form maize aquaporins. Select aquaporins open reading frames were cloned, sequenced, and expressed heterologously in *Xenopus laevis* oocytes. Oocytes were exposed to hypotonic solutions to determine the water permeability of select aquaporin isoforms. Finally, the expression levels of all the aquaporins, along with genes involved in N transport and assimilation, were monitored though the use of a high throughput qPCR assay,

(OpenArray (Thermo Fisher Scientific, Waltham, MA USA)) during nitrogen starvation conditions.

1.6 Appendix

This chapter was modified from a published book chapter in Tyerman et al. (2017). It is reproduced in original form in the Appendix at the end of this thesis.

Chapter 2: An analysis of the aquaporins found in the maize inbred line B73

2.1 Introduction

Research on maize aquaporins has been primarily based on the set of aquaporins described by Chaumont et al. (2001). The identified 33 full-length cDNA sequences obtained from expressed sequence tag libraries (EST) were constructed from multiple tissue sources from multiple maize lines. These sequences split into the four characteristic plant major intrinsic protein (MIP) subfamilies: plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), Nodulin-26 like intrinsic proteins (NIPs) and small and basic intrinsic proteins (SIPs), just as those found in *Arabidopsis* (Johanson et al. 2001). While the set covered the four subfamilies, the authors conceded it was possible additional maize aquaporin sequences existed.

The authors were correct, as additional maize aquaporin sequences have been since discovered. Gaspar et al. (2003) identified ZmPIP1;5b in the maize inbred line F7F3. The transcript shared 95.2% nucleotide identity with the previously identified ZmPIP1;5, differing by just two base pairs in the coding region (both silent mutations). While the 5' and 3' UTRs had significant sequences differences (82.7% and 87.9% identity, respectively), without promotor information for either gene it is unknown whether they are distinct genes or different alleles resulting from genetic diversity in maize. Gu et al. (2012) identified and cloned ZmNIP2;4, showing it had urea transport abilities when expressed in yeast. A second NIP, ZmNIP5;1 was listed in a transcriptomics study by Yue et al. (2012), but no source was given nor any gene or protein characteristics. Finally, ZmPIP2;8 was identified by Lawrence et al. (2013) in the inbred line B73. Its expression

was modulated by the presence of southern corn rootworm infection. Expressed in *Xenopus* oocytes, it was shown to be a pH sensitive water channel. The first plant genome to be sequenced was *Arabidopsis* (Arabidopsis Genome Initiative 2000). Johanson et al. (2001) used a TBLASTN search on the *Arabidopsis* genome to uncover the complete set of aquaporins it contained. Since then many plant genomes have been sequenced, and some have been searched for the presence of aquaporins (**Table 2.1**). The maize genome for inbred line B73 has also been published (Jiao et al. 2017; Schnable et al. 2009), and a scan for aquaporins has also been published based on the AGPv3 genome (Deshmukh et al. 2015), revealing several aquaporins not included in Chaumont et al. (2001). The results listed here partially unknowingly overlapped with Deshmukh et al. (2015), with initial analysis of the AGPv3 genome presented in this chapter taking place in June 2014, prior to their publication. Additionally, Deshmukh et al. (2015) was part of a broad study which searched the genomes of 25 plants, including moss, monocots and dicots, and was focused on the structural determinants of silicon transport in the NIP subfamily. While it did contain lists of the NPA motifs, ar/R sequences and transmembrane locations, it did not perform a deeper analysis of the aquaporins found in the maize genome. This chapter goes further and analyses the aquaporin set in the AGPv4 genome as well.

The aim of this chapter is to search the maize genome of inbred line B73 for aquaporins, analyse the set produced and to test the hypothesis that the aquaporin set described by Chaumont et al. (2001) is not equivalent to the set found in the B73 genome.

Table 2.1 Plant aquaporins revealed in genome searches

The plant genomes that have been searched for aquaporins reveal four major subfamilies (PIPs, TIPs, NIPs and SIP) shared by all plants and moss. A fifth major subfamily (XIPs – X intrinsic proteins) are absent in monocots. The moss *P. patens* had two additional aquaporin subfamilies, labelled GIPs (GlpF-like intrinsic proteins) and HIPs (hybrid intrinsic protein). Each subfamily can be further subdivided into subclass

Plant			**Major Intrinsic Proteins**							**Reference**
Common Name	**Species**	**Type**	**Total**	**PIPs**	**TIPs**	**NIPs**	**SIPs**	**XIPs**	**Other**	
Arabidopsis	*Arabidopsis thaliana*	Dicot	35	13	10	9	3	-	-	Johanson et al. (2001)
			35	13	10	9	3	-	-	Quigley et al. (2001)
			35	13	10	9	3	-	-	Deshmukh et al. (2015)
			35	13	10	9	3	-	-	Diehn et al. (2015)
Rice	*Oryza sativa*	Monocot	33	11	10	10	2	-	-	Sakurai et al. (2005)
			39	13	11	13	2	-	-	Bansal and Sankararamakrishnan (2007)
			34	11	10	11	2	-	-	Deshmukh et al. (2015)
Moss	*Physcomitrella patens*	Moss	23	8	4	5	2	2	1 GIP 1 HIP	Danielson and Johanson (2008)
			23	9	4	5	2	2	-	Deshmukh et al. (2015)
Western poplar	*Populus trichocarpa*	Dicot	55	15	17	11	6	6	-	Gupta and Sankararamakrishnan (2009)
			58	15	18	11	7	7	-	Deshmukh et al. (2015)
Upland cotton	*Gossypium hirsutum*	Dicot	71	28	23	12	7	1	-	Park et al. (2010)
Tomato	*Solanum lycopersicum*	Dicot	47	14	11	12	4	6	-	Reuscher et al. (2013)
			44	14	10	11	3	6	-	Deshmukh et al. (2015)
Potato	*Solanum tuberosum*	Dicot	41	15	11	10	3	8	-	Venkatesh et al. (2013)
			44	15	11	11	2	5	-	Deshmukh et al. (2015)
Soybean	*Glycine max*	Dicot	66	22	23	13	6	2	-	Zhang et al. (2013)
			72	22	23	17	8	2	-	Deshmukh et al. (2013)
Chinese cabbage	*Brassica rapa*	Dicot	53	20	14	13	6	-	-	Tao et al. (2014)
			59	22	16	15	6	-	-	Deshmukh et al. (2015)
			60	23	16	15	6	-	-	Diehn et al. (2015)
Common bean	*Phaseolus vulgaris*	Dicot	41	12	13	10	4	2	-	Ariani and Gepts (2015)

(continued)

Plant			Major Intrinsic Proteins							Reference
Common Name	**Species**	**Type**	**Total**	**PIPs**	**TIPs**	**NIPs**	**SIPs**	**XIPs**	**Other**	
Spikemoss	*Selaginella moellendorffii*	Spike-moss	19	3	2	8	1	3	2 HIPs	Anderberg et al. (2012)
			19	3	3	8	1	3	1 HIP	Deshmukh et al. (2015)
Norway Spruce	*Picea abies*	Gymno-sperm	39	18	6	13	2	-	-	
Maize	*Zea mays*	Monocot	43	10	13	13	7	-	-	
Oil Palm	*Elaeis guineensis*	Monocot	30	9	10	9	2	-	-	
Lyrate rockcress	*Arabidopsis lyrata*	Dicot	39	14	12	10	3	-	-	
Papaya	*Carica papaya*	Dicot	28	10	7	7	2	2	-	
Mandarin	*Citrus clementina*	Dicot	37	14	10	9	3	1	-	
Grapevine	*Vitis vinifera*	Dicot	30	9	9	9	1	2	-	
Pigeon pea	*Cajanus cajan*	Dicot	40	12	13	10	4	1	-	
Strawberry	*Fragaria vesca*	Dicot	39	10	9	14	4	2	-	
Peach	*Prunus persica*	Dicot	29	7	8	9	3	2	-	
Cabbage	*Brassica oleracea*	Dicot	67	25	19	17	6	-	-	Diehn et al. (2015)
Barley	*Hordeum vulgare*	Monocot	40	19	11	8	2	-	-	Hove et al. (2015)
Banana	*Musa acuminate*	Monocot	47	18	17	9	3	-	-	Hu et al. (2015)
			51	21	18	9	9	-	-	Deshmukh et al. (2015)
Sweet orange	*Citrus sinensis*	Dicot	34	8	11	9	3	3	-	de Paula Santos Martins et al. (2015)
			34	11	9	8	3	3	-	Deshmukh et al. (2015)
Castor bean	*Ricinus communis*	Dicot	37	10	9	8	4	6	-	Zou et al. (2015b)
			36	10	9	8	4	5	-	Deshmukh et al. (2015)
Rubber tree	*Hevea brasiliensis Muell. Arg.*	Dicot	51	15	17	9	4	6	-	Zou et al. (2015a)
Switchgrass	*Panicum virgatum*	Monocot	68	21	20	23	4	-	-	Azad et al. (2016)
Foxtail millet	*Setaria italica*	Monocot	42	12	15	12	3	-	-	Azad et al. (2016)
			50	16	16	15	3	-	-	Deshmukh et al. (2015)
			41	12	14	12	3	-	-	McGaughey et al. (2016)
Sorghum	*Sorghum bicolor*	Monocot	38	13	11	11	3	-	-	Azad et al. (2016)
			40	14	13	10	3	-	-	Deshmukh et al. (2015)
Purple false brome	*Brachypodium distachyon*	Monocot	28	10	10	7	1	-	-	Azad et al. (2016)
			32	11	10	9	2	-	-	Deshmukh et al. (2015)
Moso bamboo	*Phyllostachys edulis*	Monocot	26	10	6	8	2	-	-	Sun et al. (2016)
Physic nut	*Jatropha curcas*	Dicot	32	9	9	8	4	2	-	Zou et al. (2016)
Chickpea	*Cicer arietinum*	Dicot	40	9	12	16	3	-	-	Deokar and Tar'an (2016)

2.2 Methods

Thirty seven known aquaporins from maize (**Table 2.2**) were selected as query for a TBLASTN search against the maize genome of inbred line B73. To search for more divergent sequences, the 23 aquaporins from the moss *Physcomitrella patens* (Danielson and Johanson 2008) were also selected (**Table 2.3**) for an independent search of the maize genome. The moss genome encodes aquaporins in the PIPs, TIPs, NIPs and SIPs, as well as XIPs (not found in monocots), and seemingly moss specific GIPs and HIPs.

A TBLASTN search was performed at ensemble.gramene.org/Zea_mays on the genome assembly AGPv3 with search parameters set to search for distant homologies. This resulted in 101,210 total hits for the maize query and 67,069 for *P. patens*. However, many of these hits were duplicates resulting from the similarity of query sequences (two or more query hitting the same genomic location), multi exon genes (one query hitting each exon as an individual result), and from E-values below 10 (E-values are a statistic of the number of times a fragment would be randomly found in the genome).

The results were consolidated and screened for hits with TBLASTN scores above 99, at least 30% identity and lengths of at least 70 bp (base pairs). Results were sorted based on chromosome and position. Due to the similarity of the aquaporin subfamily, only regions with hits from multiple queries were considered. Regions with predicted genes were analysed. Those regions that displayed aquaporin features (an NPA domain) were kept. This list was then analysed and used as query for an additional TBLASTN search.

A second independent search was performed on the maize genome (AGPv3) searching for predicted genes that encoded proteins with an aquaporin domain (InterPro domains IPR023271, IPR000425, or IPR022357).

Some of the genes identified had several splice variants listed. The splice variants were compared to known cDNA sequences looking for a close match. If an obvious match was not available, the variants were analysed for known aquaporin features: six transmembrane helices and two NPA domains, and a preferred length between 250 and 320 amino acids.

In August 2016 (published in May 2017 as Jiao et al. (2017)), the maize genome underwent a major update from AGPv3 to AGPv4. A second TBLASTN search was performed at http://ensembl.gramene.org/Zea_mays/Tools/Blast using the known possible aquaporins and fragments from the previous genome searches. TBLASTN settings were set to look for distant homologies, and screened with an E-value of less than 10. This resulted in 4,986 total hits to the genome, counting all hits from all maize sequence queries. Three queries used were aquaporin fragments with regions that did not align to known aquaporins. Locations with only TBLASTN hits to these non-aquaporin regions were removed (about 500). Hits that were to known aquaporins were noted and removed. Finally, the resulting regions were individually examined, looking at hits in the genomic region, the alignment with the query, looking for genes or fragments with aquaporin features, namely alignment near an NPA domain. Comparisons were made between genes present in both genome versions (AGPv3 and AGPv4). If differences were present, greater weighting was given to the most complete (six TM domains, two NPA domains) and on alignment to known cDNAs.

Phylogenetic analysis was performed on the set of resulting aquaporins. Unless noted otherwise, deduced amino acid sequences were aligned using CLUSTAL Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). Phylogenetic trees were constructed using MEGA 7, utilizing the Neighbour-Joining method to infer evolutionary relationships and

estimating genetic distances with the p-distance method (assuming a gamma distribution of variation among sites). All alignments and trees were presented using Geneious version 8.1 by Biomatters, available from http://www.geneious.com (Kearse et al. 2012).

Protein transmembrane domains were calculated using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), SOSUI (version 1.10) (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuiG/sosuigsubmit.html), TMHMM (version 2.0) (http://www.cbs.dtu.dk/services/TMHMM/) and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The Phyre2 predictions were taken from the homology models it produced, modelled off known aquaporin crystal structures (Kelley et al. 2015). From the Phyre2 models and CLUSTAL Omega alignments, the ar/R filter and NPA domains were obtained for each sequence.

Table 2.2 Maize aquaporins used as query for TBLASTN genome search

37 full length maize protein sequences derived from the cDNA (NCBI number) or transcript ((*) Gramene ID from AGPv3). The coding region of each transcript was translated and used to search the maize genome for aquaporin sequences. The 37 sequences listed above encoded 35 unique proteins (ZmPIP1;3 and ZmPIP1;4 encode the same protein, as do ZmPIP1;5 and ZmPIP1;5b).

No.	**Query Name**	**Genebank accession number or Gramene ID***	**Reference**
1	ZmNIP1;1	AF326483	Chaumont et al. (2001)
2	ZmNIP2;1	AF326384	
3	ZmNIP2;2	AF326485	
4	ZmNIP2;3	AF342810	
5	ZmNIP3;1	AF326486	
6	ZmPIP1;1	X82633	
7	ZmPIP1;2	AF131201	
8	ZmPIP1;3	AF326487	
9	ZmPIP1;4	AF326488	
10	ZmPIP1;5	AF326489	
11	ZmPIP1;6	AF326490	
12	ZmPIP2;1	AF326491	
13	ZmPIP2;2	AF326492	
14	ZmPIP2;3	AF326493	
15	ZmPIP2;4	AF326494	
16	ZmPIP2;5	AF130975	
17	ZmPIP2;6	AF326495	
18	ZmPIP2;7	AF326496	
19	ZmSIP1;1	AF326497	
20	ZmSIP1;2	AF326498	
21	ZmSIP2;1	AF326499	
22	ZmTIP1;1	AF037061	
23	ZmTIP1;2	AF326500	
24	ZmTIP2;1	AF326501	
25	ZmTIP2;2	AF326502	
26	ZmTIP2;3	AF326503	
27	ZmTIP3;1	AF326504	
28	ZmTIP3;2	AF342809	
29	ZmTIP4;1	AF326505	
30	ZmTIP4;2	AF326506	
31	ZmTIP4;3	AF326507	
32	ZmTIP4;4	AF326508	
33	ZmTIP5;1	AF326509	
34	ZmPIP1;5b	AJ271796	Gaspar et al. (2003)
35	ZmNIP2;4	GRMZM2G082184_T01*	Gu et al. (2012)
36	ZmNIP5;1	GRMZM2G000471_T01*	Yue et al. (2012)
37	ZmPIP2;8	GRMZM2G432926_T01*	Lawrence et al. (2013)

Table 2.3 P. patens aquaporins used in TBLASTN genome search

23 full length aquaporin sequences from the moss *P. patens* and the protein ID from PpDB at the Joint Genome Institute (http://www.jhi.doe.gov). The original search was performed on the maize genome version AGPv3. The moss aquaporins were selected in order to give greater diversity in the TBLASTN search. This query list did not reveal any additional maize sequences that were not found using the maize queries.

No.	Query Name	Protein ID in PpDB	Reference
1	PpGIP1;1	171260	Danielson and Johanson (2008)
2	PpHIP1;1	91611	
3	PpNIP3;1	94322	
4	PpNIP5;1	115513	
5	PpNIP5;2	186237	
6	PpNIP5;3	179749	
7	PpNIP6;1	16763	
8	PpPIP1;1	62169	
9	PpPIP1;2	166091	
10	PpPIP1;3	171662	
11	PpPIP2;1	202226	
12	PpPIP2;2	209703	
13	PpPIP2;3	196472	
14	PpPIP2;4	135286	
15	PpPIP3;1	68172	
16	PpSIP1;1	112053	
17	PpSIP1;2	200882	
18	PpTIP6;1	73809	
19	PpTIP6;2	191107	
20	PpTIP6;3	214518	
21	PpTIP6;4	219971	
22	PpXIP1;1	71087	
23	PpXIP1;2	71489	

2.3 Results

The TBLASTN search of the maize inbred line B73 AGPv4 genome revealed 43 full length aquaporin sequences (**Figure 2.1** and **Table 2.4**), and 21 possible fragments (**Table 2.8**). Full length sequences were defined as having six transmembrane domains and an NPA domain in Loop B and in Loop E. Searching with either subset of query sequences (from moss or from maize) resulted in identical lists of aquaporins. Researching the maize genome using the 43 full length aquaporins did not yield any additional sequences. The B73 AGPv3 genome yielded only 40 full length aquaporin sequences (ZmPIP3;1, ZmTIP1;1, ZmTIP5;1b were not annotated in the AGPv3 genome). Searching the AGPv3 genome for proteins with aquaporin domains also resulted in the same 40 aquaporins. Searching the AGPv4 genome for specific protein domains was not available at the time of analysis. The 43 full-length aquaporin sequences in the AGPv4 genome split into the expected four major subfamilies and included 14 ZmPIPs, 15 ZmTIPs, 11 ZmNIPs, and 3 ZmSIPs. At least one aquaporin was found on each of the maize chromosomes (**Figure 2.4**).

The naming of the aquaporins in the maize genome was carefully considered. The *Arabidopsis* aquaporin set (Johanson et al. 2001) established a nomenclature system based on phylogenetic relationships of the aquaporins. While many of the *Arabidopsis* aquaporins had been known before, Johanson et al. (2001) assigned all the aquaporins a unique name under their system. Chaumont et al. (2001) used this system to name the maize aquaporins, however several inconsistencies are present in dealing with cDNA clones with identical protein sequences. In the original set of 33, ZmPIP1;3 and ZmPIP1;4 encode identical proteins, and each cDNA, which were ~95% identical, were assigned sequential numerical names. When Gaspar et al. (2003) identified another ZmPIP1;5

clone with identical protein sequence, they chose to append the letter b to the end (to get ZmPIP1;5b) to distinguish the cDNAs. The nomenclature described below sought to preserve names of previously established maize aquaporins, conform to the conventional plant nomenclature established with the *Arabidopsis* aquaporins, but also highlight differences in similar protein sequences. While Deshmukh et al. (2015) has published a list of maize aquaporins found in the maize inbred line B73 genome (AGPv3), their nomenclature was not used here as it was not consistent with the previously established nomenclature (**Table 2.5**)

The nomenclature selected here assigned a unique identifying number to each aquaporin in the maize genome. Assignment was first given to proteins that had 100% identity (length and amino acid sequence) to those described before. If an aquaporin gene in the genome was close (98% or more identical) but not the same as one previously known, and that name was still available, it was given that name appended with the letter b. This allowed for their relatedness but also demonstrated sequences that were not quite identical. If a name was not available or if they were less than 98% to the closest known aquaporin, they were assigned the next available number in the subgroup they are found via phylogenetic analysis. For phylogenetic analysis (based on protein sequences), comparisons were made between the assumed tree of all the available sets of aquaporins from the monocots listed in **Table 2.1**.

Overall, 31 of the 33 aquaporins described by Chaumont et al. (2001) were found in the B73 genome (**Table 2.6**). 22 had identical protein sequences, while 9 had at least 1 amino acid difference to their predicted genome gene. Two genes were not mapped to the genomes, but only ZmPIP2;7 did not have any near matches. While ZmPIP1;1 was not in the genome as well, it seemed to be closely related to a gene named ZmPIP1;7 (see

below). The genome includes 11 additional aquaporins that were not included in the set of 33: ZmPIP2;8 (Lawrence et al. 2013), ZmPIP2;9, ZmPIP3;1, ZmTIP2;4, ZmTIP3;3, ZmTIP3;4, ZmNIP4;1, ZmNIP5;1 (Yue et al. 2012), and ZmNIP5;2.

Additionally, 39 of the 43 described by Deshmukh et al. (2015) were found in the genome (**Table 2.5**). That study used the AGPv3 genome, but several differences were present beyond minor updates to sequences and the additions of ZmPIP3;1, ZmTIP1;1 and ZmTIP5;1b. First, ZmNIP1;1 was not present in their study, despite having a transcript in the AGPv3 genome. Additionally, the authors included four ZmSIP fragments that did not have six transmembrane domains and two NPA domains (**Table 2.8**).

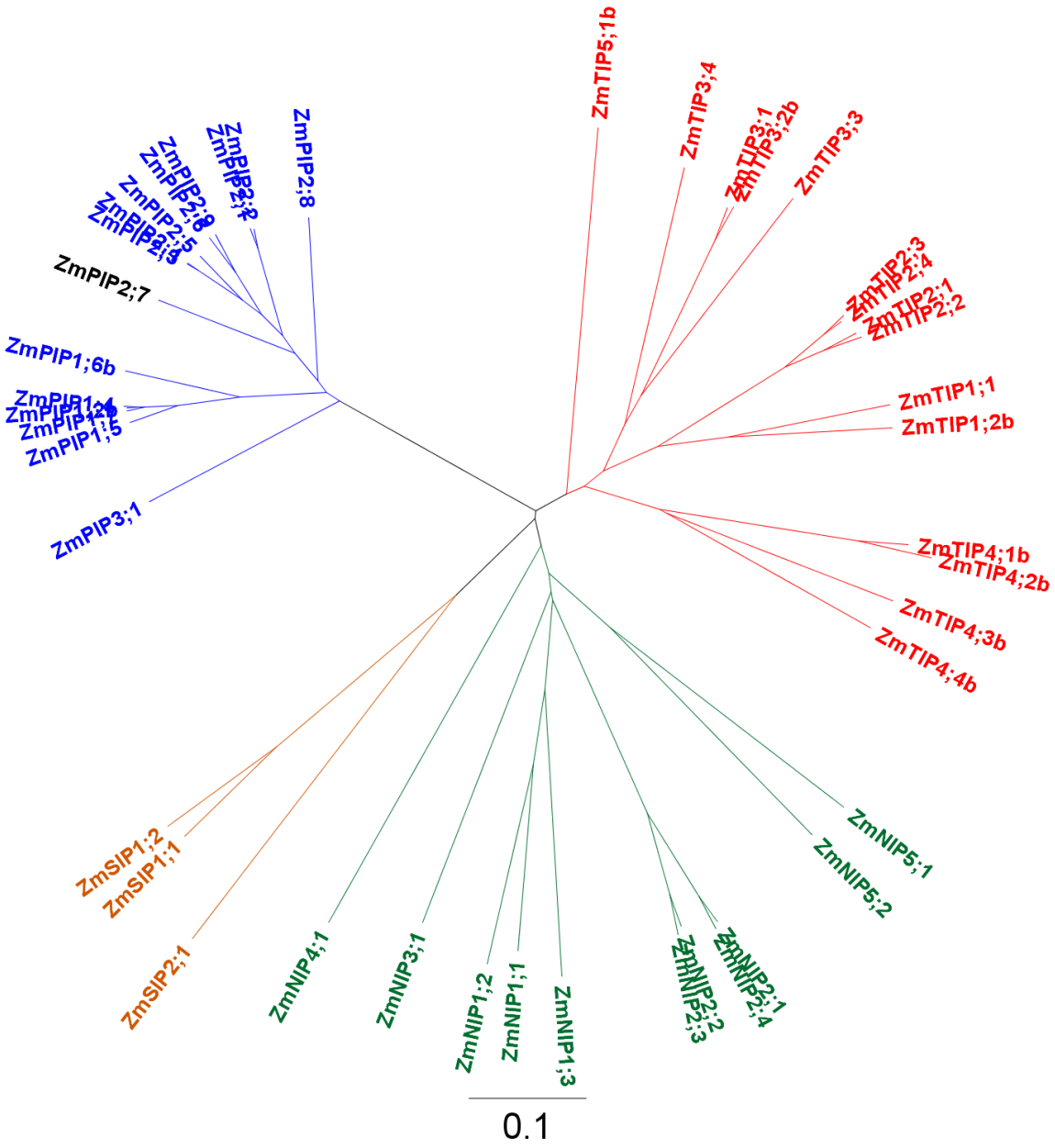


Figure 2.1 Phylogenetic tree of maize aquaporins

A phylogenetic analysis of maize aquaporins is presented, drawn to scale, with branch lengths representing the number of bamino acid differences per site. Deduced aquaporin sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The evolutionary history was inferred using the Neighbour-Joining method, estimating the genetic distance using the p-distance method and assuming a gamma distribution (shape parameter =1) of variation among sites. Ambiguous positions were removed between each sequence pair during analysis. This analysis includes the 43 full length aquaporins found in the B73 genome and ZmPIP2;7 from Chaumont et al. (2001). Scale represents number of amino acid differences per site. The tree was constructed using MEGA7 and aesthetically edited in Geneious version 8.1. Blue = PIPs, Red = TIPs, Green = NIPs, Tan = SIPs

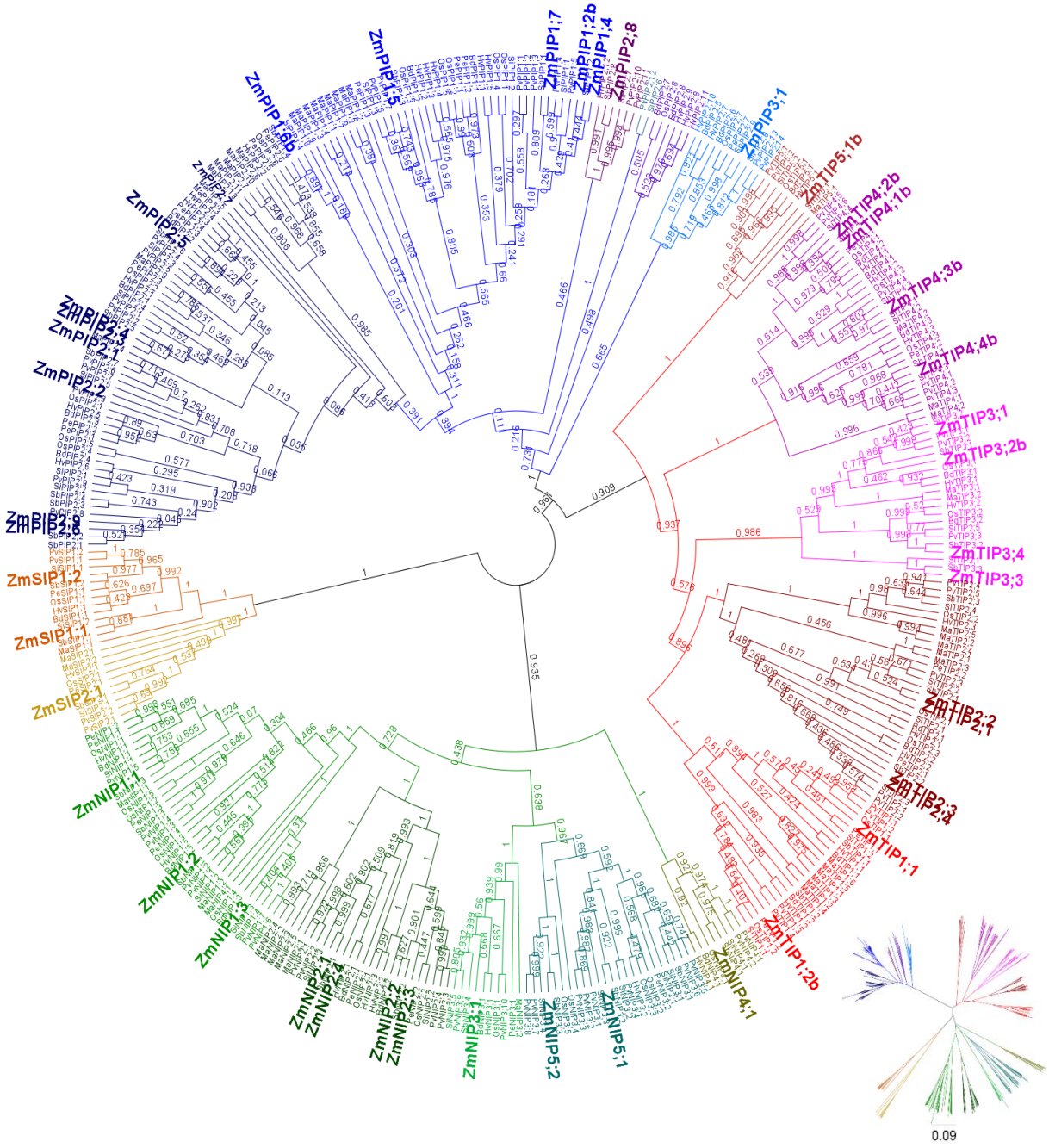


Figure 2.2 Monocot aquaporins

A phylogenetic analysis of aquaporins from complete genome sets of the monocots listed in **Table 2.1** and those in the maize B73 genome. Deduced aquaporin protein sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/). The evolutionary history was inferred using the Neighbour-Joining method, estimating the genetic distance using the p-distance method and assuming a gamma distribution of variation (shape parameter = 1) among sites. The analysis included 370 aquaporin sequences. The tree was constructed using MEGA7 and aesthetically edited using Geneious version 8.1. Maize aquaporins are highlighted and each subfamily was colour coded (PIPs = Blue, TIPs = Red, NIPs = Green, SIPs = Orange), with each shading to distinguish subgroups. Numbers represent the bootstrap consensus after 1000 resampling's of the original tree. The inset at the bottom right is the equivalent tree with branch lengths reflecting site substitutions per site. The major subdivisions are present and are generally supported with bootstrap support.

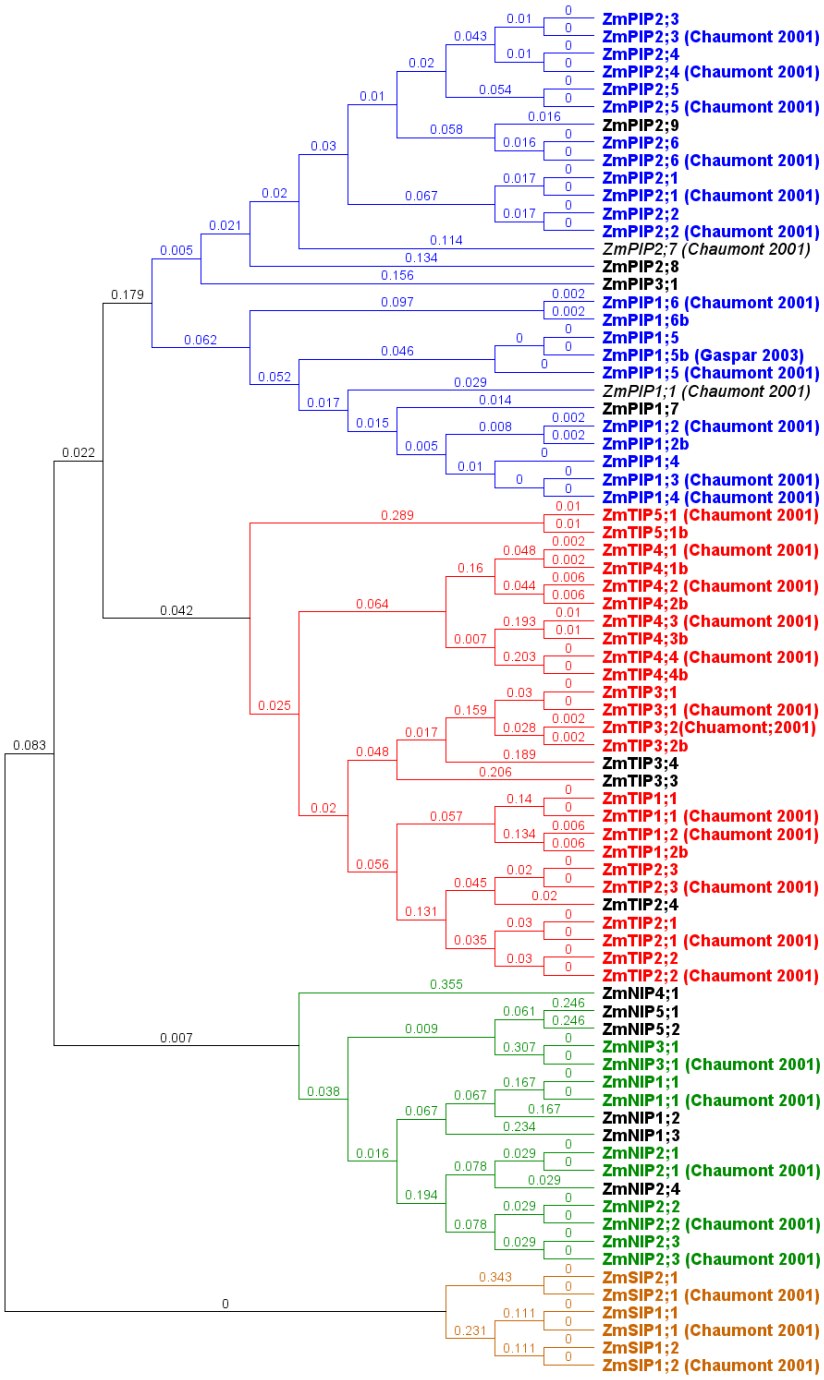


Figure 2.3 Mapping published aquaporins to those in the B73 genome

The aquaporins in the maize genome were aligned with those from Chaumont et al. (2001). Sequences were aligned with CLUSTAL Omega. As only mapping was needed, a simple UPGMA tree was constructed using the p-distance method. Breach labels represent amino acid changes per site. If proteins were at least 98% similar, the numerical nomenclature was unchanged, but a letter was added to denote differences to the known gene. For ZmTIP4;4, the protein predicted in the genome exactly matched the published, but had an N-terminal extension. Details of the protein differences can be found in Table 2.6. Only ZmPIP2;7 and ZmPIP1;1 were not found in the genome (italic). Names in black represent sequences not found by Chaumont et al. (2001).

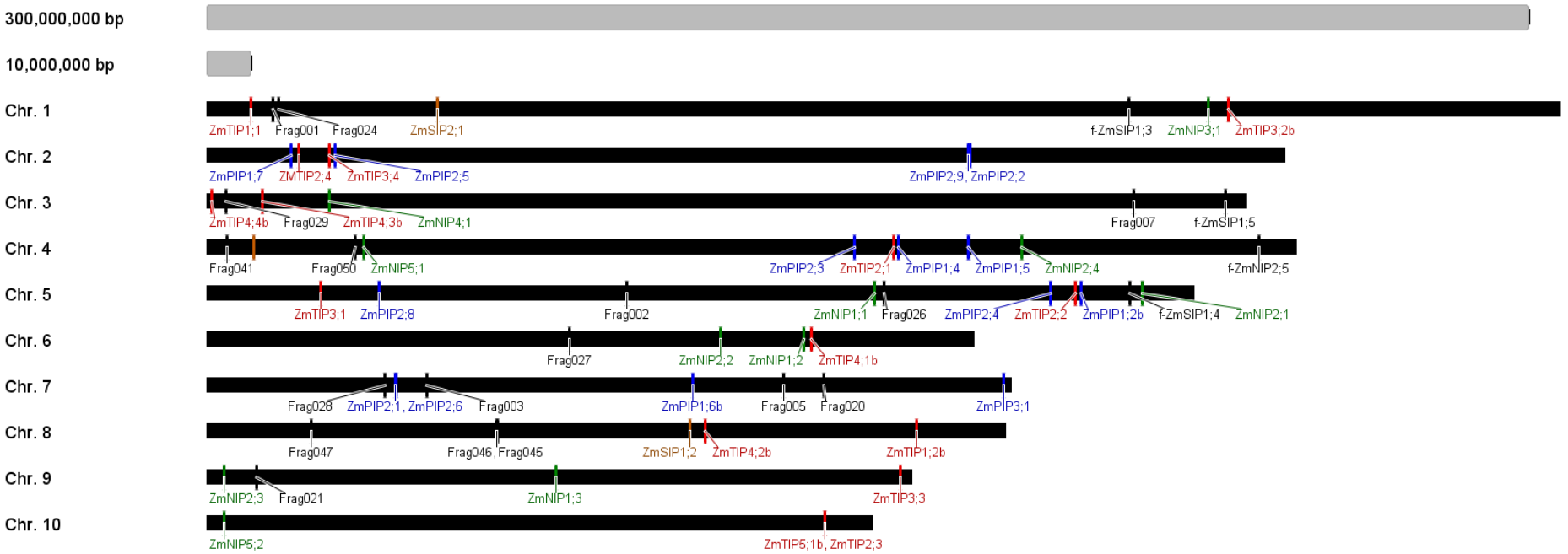


Figure 2.4 Aquaporin location on maize chromosomes

The relative positions of the maize aquaporins (full length and fragments) on their respective chromosomes. ZmTIP2;3 and ZmTIP5;1b are the closest genes to one another, separated by just 162 bp from the end of ZmTIP2;3 and the start of ZmTIP1;5b on chromosome 10. The next closest pair of genes are ZmPIP2;9 and ZmPIP2;2 on chromosome 2, separated by around 250 kb. Blue = ZmPIPs, Red = ZmTIPs, Green = ZmNIPs, Tan = ZmSIPs, Black = Fragments

Table 2.4 Full length aquaporins in the maize genome

The B73 genome contains 43 aquaporin genes with full-length aquaporin sequences. The initial search was performed on genome version AGPv3. In August 2016, an early release of AGPv4 was released. There were differences between the versions (see **Table 2.5**). AGPv4 newly revealed ZmPIP3;1, ZmTIP1;1 and ZmTIP5;1b, however, the predicted genes for ZmPIP1;6b, ZmNIP2;4 and ZmSIP1;1 were truncated versions. For these three, the AGPv3 predictions were used with genome coordinates converted to AGPv4. Chr = chromosome number. The number of splice variants was determined by Gramene in the laboratory of Doreen Ware UCSHL/USDA) using an algorithm (MAKER-P) trained on Iso-Seq (long read RNA-Seq), known cDNAs and other RNA-Seq sequences.

#	Gene Name	ID	Amino Acids	Splice Variants	Exons	Chr.	Start	End	Orientation
ZmPIPs									
1 (1)	ZmPIP1;2b	Zm00001d017526_T001.1	289	5	4	5	198,707,054	198,710,453	forward
2 (2)	ZmPIP1;4	Zm00001d051403_T001.1	292	3	4	4	157,200,400	157,203,987	forward
3 (3)	ZmPIP1;5	Zm00001d051872_T001.1	288	1	2	4	172,964,877	172,966,249	reverse
4 (4)	ZmPIP1;6b	GRMZM2G136032_T01 (AGPv3)	296	1	2	7	110,507,409	110,508,756	forward
5 (5)	ZmPIP1;7	Zm00001d002690_T001.1	288	7	4	2	19,342,470	19,344,477	reverse
6 (6)	ZmPIP2;1	Zm00001d019563_T006.1	290	18	3	7	42,951,729	42,954,764	reverse
7 (7)	ZmPIP2;2	Zm00001d005421_T001.1	292	19	5	2	173,378,742	173,382,478	forward
8 (8)	ZmPIP2;3	Zm00001d051174_T002.1	289	3	4	4	147,173,978	147,176,860	forward
9 (9)	ZmPIP2;4	Zm00001d017288_T001.1	288	2	4	5	191,817,119	191,819,914	forward
10 (10)	ZmPIP2;5	Zm00001d003006_T001.1	285	1	3	2	29,275,928	29,278,172	reverse
11 (11)	ZmPIP2;6	Zm00001d019565_T001.1	288	3	3	7	43,071,903	43,073,370	reverse
12 (12)	ZmPIP2;8	Zm00001d014285_T001.1	283	1	1	5	39,448,633	39,449,484	reverse
13 (13)	ZmPIP2;9	Zm00001d005410_T001.1	286	1	3	2	173,113,641	173,114,720	forward
14 (15)	ZmPIP3;1	Zm00001d022608_T001.1	288	1	4	7	181,006,347	181,007,450	reverse
ZmTIPs									
15 (1)	ZmTIP1;1	Zm00001d027652_T001.1	250	1	2	1	10,150,836	10,152,891	reverse
16 (2)	ZmTIP1;2b	Zm00001d011778_T001.1	254	1	2	8	161,412,063	161,413,479	reverse
17 (3)	ZmTIP2;1	Zm00001d051362_T001.1	249	1	2	4	155,984,860	155,986,408	reverse
18 (4)	ZmTIP2;2	Zm00001d017485_T002.1	250	4	2	5	197,280,413	197,281,892	reverse
19 (5)	ZmTIP2;3	Zm00001d026177_T001.1	248	1	3	10	140,377,176	140,378,531	forward
20 (6)	ZmTIP2;4	Zm00001d002738_T001.1	248	1	3	2	21,104,664	21,105,814	reverse
21 (7)	ZmTIP3;1	Zm00001d013956_T003.1	262	4	2	5	26,132,033	26,133,292	forward
22 (8)	ZmTIP3;2b	Zm00001d032610_T003.1	266	6	2	1	232,015,987	232,017,309	reverse
23 (9)	ZmTIP3;3	Zm00001d048520_T001.1	267	17	3	9	157,714,272	157,715,705	reverse
24 (10)	ZmTIP3;4	Zm00001d002952_T001.1	265	1	3	2	27,986,031	27,988,042	reverse
25 (11)	ZmTIP4;1b	Zm00001d037779_T001.1	255	1	3	6	137,396,807	137,399,127	forward
26 (12)	ZmTIP4;2b	Zm00001d010410_T001.1	311	1	3	8	113,392,026	113,394,414	reverse
27 (13)	ZmTIP4;3b	Zm00001d039713_T001.1	249	1	2	3	12,824,022	12,826,924	forward
28 (14)	ZmTIP4;4b	Zm00001d039293_T001.1	287	1	2	3	1,167,256	1,168,292	reverse
29 (15)	ZmTIP5;1b	Zm00001d026178_T001.1	260	2	3	10	140,378,693	140,379,967	forward
ZmNIPs									
30 (1)	ZmNIP1;1	Zm00001d016237_T001.1	282	1	4	5	151,835,826	151,838,198	forward
31 (2)	ZmNIP1;2	Zm00001d037733_T001.1	284	1	4	6	135,558,617	135,560,426	forward
32 (3)	ZmNIP1;3	Zm00001d046303_T001.1	317	1	6	9	79,505,477	79,507,136	forward
33 (4)	ZmNIP2;1	Zm00001d018037_T001.1	295	1	5	5	212,580,241	212,583,888	forward
34 (5)	ZmNIP2;2	Zm00001d037228_T001.1	294	2	5	6	116,839,545	116,843,339	reverse
35 (6)	ZmNIP2;3	Zm00001d044826_T001.1	301	4	5	9	4,090,119	4,093,538	forward
36 (7)	ZmNIP2;4	GRMZM2G082184_T01 (AGPv3)	303	1	5	4	185,174,497	185,177,596	reverse
37 (8)	ZmNIP3;1	Zm00001d032461_T001.1	302	2	4	1	227,449,316	227,453,079	forward
38 (9)	ZmNIP4;1	Zm00001d040108_T001.1	299	1	5	3	27,850,931	27,852,740	forward
39 (10)	ZmNIP5;1	Zm00001d049578_T001.1	296	1	4	4	35,941,537	35,942,756	forward
40 (11)	ZmNIP5;2	Zm00001d024634_T001.1	279	1	4	10	81,353,456	81,354,480	forward
ZmSIPs									
41 (1)	ZmSIP1;1	GRMZM2G113470_T01 (AGPv3)	245	2	3	4	10,754,425	10,760,360	reverse
42 (2)	ZmSIP1;2	Zm00001d010325_T001.1	243	1	3	8	109,745,230	109,750,097	reverse
43 (3)	ZmSIP2;1	Zm00001d028951_T001.1	249	1	3	1	52,645,733	52,648,243	reverse

Table 2.5 Mapping maize aquaporins between AGPv3 to AGPv4

AGPv4 introduced a new nomenclature for the maize genome. The AGPv4 genome contained three additional aquaporin genes not found in the AGPv3 genome: ZmPIP3;1, ZmTIP1;1, and ZmTIP5;1b. Five genes had different amino acid sequences, due to insertions or deletions around exon boarders. This included ZmPIP1;6b, ZmNIP2;4 and ZmSIP1;1. For two others, ZmNIP5;1 and ZmNIP5;2, there is no experimental evidence to suggest one version over another, so the AGPv4 was chosen. The genes with differences in amino acids between versions are highlighted in grey (names in yellow).

#	Gene Name	AGPv4			AGPv3			
		ID	Amino Acids	Base Pairs	Deshmukh 2015 ID	ID	Amino Acids	Base Pairs
ZmPIPs								
1 (1)	ZmPIP1;2b	Zm00001d017526_T001.1	289	1,420	ZmPIP1;1	AC209208.3_FGT002	289	870
2 (2)	ZmPIP1;4	Zm00001d051403_T001.1	292	1,484	ZmPIP1;2	GRMZM2G392975_T01	292	1,674
3 (3)	ZmPIP1;5	Zm00001d051872_T001.1	288	1,280	ZmPIP1;4	GRMZM2G081843_T01	288	1,408
4 (4)	ZmPIP1;6b	Zm00001d020383_T001.1	192	1,201	ZmPIP1;5	GRMZM2G136032_T01	296	1,552
5 (5)	ZmPIP1;7	Zm00001d002690_T001.1	288	1,389	ZmPIP1;3	GRMZM2G174807_T01	288	1,388
6 (6)	ZmPIP2;1	Zm00001d019563_T006.1	290	1,186	ZmPIP2;1	GRMZM2G014914_T01	290	1,957
7 (7)	ZmPIP2;2	Zm00001d005421_T001.1	292	1,635	ZmPIP2;2	GRMZM2G092125_T01	292	1,470
8 (8)	ZmPIP2;3	Zm00001d051174_T002.1	289	1,495	ZmPIP2;4	GRMZM2G081192_T01	289	1,661
9 (9)	ZmPIP2;4	Zm00001d017288_T001.1	288	1,281	ZmPIP2;5	GRMZM2G154628_T01	288	1,501
10 (10)	ZmPIP2;5	Zm00001d003006_T001.1	285	1,219	ZmPIP2;6	GRMZM2G178693_T01	285	1,238
11 (11)	ZmPIP2;6	Zm00001d019565_T001.1	288	1,265	ZmPIP2;8	GRMZM2G047368_T02	288	1,166
12 (12)	ZmPIP2;8	Zm00001d014285_T001.1	283	852	ZmPIP2;3	GRMZM2G432926_T01	283	1,213
13 (13)	ZmPIP2;9	Zm00001d005410_T001.1	286	861	ZmPIP2;7	GRMZM2G018649_T01	286	1,205
14 (15)	ZmPIP3;1	Zm00001d022608_T001.1	288	867	-	-	-	-
ZmTIPs								
15 (1)	ZmTIP1;1	Zm00001d027652_T001.1	250	1,146	-	-	-	-
16 (2)	ZmTIP1;2b	Zm00001d011778_T001.1	254	1,340	ZmTIP1;1	GRMZM2G168439_T01	254	1,126
17 (3)	ZmTIP2;1	Zm00001d051362_T001.1	249	1,386	ZmTIP2;1	GRMZM2G027098_T01	249	1,283
18 (4)	ZmTIP2;2	Zm00001d017485_T002.1	250	1,366	ZmTIP2;2	GRMZM2G056908_T01	250	1,091
19 (5)	ZmTIP2;3	Zm00001d026177_T001.1	248	1,079	ZmTIP2;4	GRMZM2G125023_T01	248	2,505
20 (6)	ZmTIP2;4	Zm00001d002738_T001.1	248	946	ZmTIP2;3	GRMZM2G121275_T01	248	1,116
21 (7)	ZmTIP3;1	Zm00001d013956_T003.1	262	1,145	ZmTIP3;2	GRMZM2G305446_T01	262	1,227
22 (8)	ZmTIP3;2b	Zm00001d032610_T003.1	266	1,195	ZmTIP3;1	GRMZM2G103983_T01	266	1,161
23 (9)	ZmTIP3;3	Zm00001d048520_T001.1	267	1,202	ZmTIP3;3	GRMZM2G037327_T01	267	1,139
24 (10)	ZmTIP3;4	Zm00001d002952_T001.1	265	1,223	ZmTIP3;4	GRMZM2G039845_T01	265	1,177
25 (11)	ZmTIP4;1b	Zm00001d037779_T001.1	255	1,309	ZmTIP4;2	GRMZM2G103945_T02	255	1,304
26 (12)	ZmTIP4;2b	Zm00001d010410_T001.1	311	1,309	ZmTIP4;1	GRMZM2G108273_T01	311	1,333
27 (13)	ZmTIP4;3b	Zm00001d039713_T001.1	249	1,187	ZmTIP4;3	GRMZM2G146627_T01	249	1,153
28 (14)	ZmTIP4;4b	Zm00001d039293_T001.1	287	973	ZmTIP4;3	GRMZM2G093090_T01	287	1,058
29 (15)	ZmTIP5;1b	Zm00001d026178_T001.1	260	1,037	-	-	-	-
ZmNIPs								
30 (1)	ZmNIP1;1	Zm00001d016237_T001.1	282	1,369	-	GRMZM2G041980_T02	282	1,363
31 (2)	ZmNIP1;2	Zm00001d037733_T001.1	284	1,288	ZmNIP1;1	GRMZM2G126582_T01	284	1,289
32 (3)	ZmNIP1;3	Zm00001d046303_T001.1	317	954	ZmNIP1;2	AC234180.1_FGT004	317	954
33 (4)	ZmNIP2;1	Zm00001d018037_T001.1	295	1,432	ZmNIP2;2	GRMZM2G028325_T01	295	1,641
34 (5)	ZmNIP2;2	Zm00001d037228_T001.1	294	1,407	ZmNIP2;3	GRMZM2G137108_T01	294	1,760
35 (6)	ZmNIP2;3	Zm00001d044826_T001.1	301	1,237	ZmNIP2;4	GRMZM2G081239_T01	301	1,507
36 (7)	ZmNIP2;4	Zm00001d052261_T001.1	211	2,461	ZmNIP2;1	GRMZM2G082184_T01	303	1,558
37 (8)	ZmNIP3;1	Zm00001d032461_T001.1	302	1,920	ZmNIP3;3	GRMZM2G176209_T01	302	2,100
38 (9)	ZmNIP4;1	Zm00001d040108_T001.1	299	1,250	ZmNIP4;1	GRMZM2G103214_T01	299	1,250
39 (10)	ZmNIP5;1	Zm00001d049578_T001.1	296	891	ZmNIP3;2	GRMZM2G000471_T01	312	1,167
40 (11)	ZmNIP5;2	Zm00001d024634_T001.1	279	840	ZmNIP3;1	GRMZM2G358161_T01	297	894
ZmSIPs								
41 (1)	ZmSIP1;1	Zm00001d048959_T002.1	235	1,015	ZmSIP1;4	GRMZM2G113470_T01	245	1,276
42 (2)	ZmSIP1;2	Zm00001d010325_T001.1	243	1,196	ZmSIP1;5	GRMZM2G060922_T01	243	1,201
43 (3)	ZmSIP2;1	Zm00001d028951_T001.1	249	1,184	ZmSIP2;1	GRMZM2G175038_T01	249	1,249

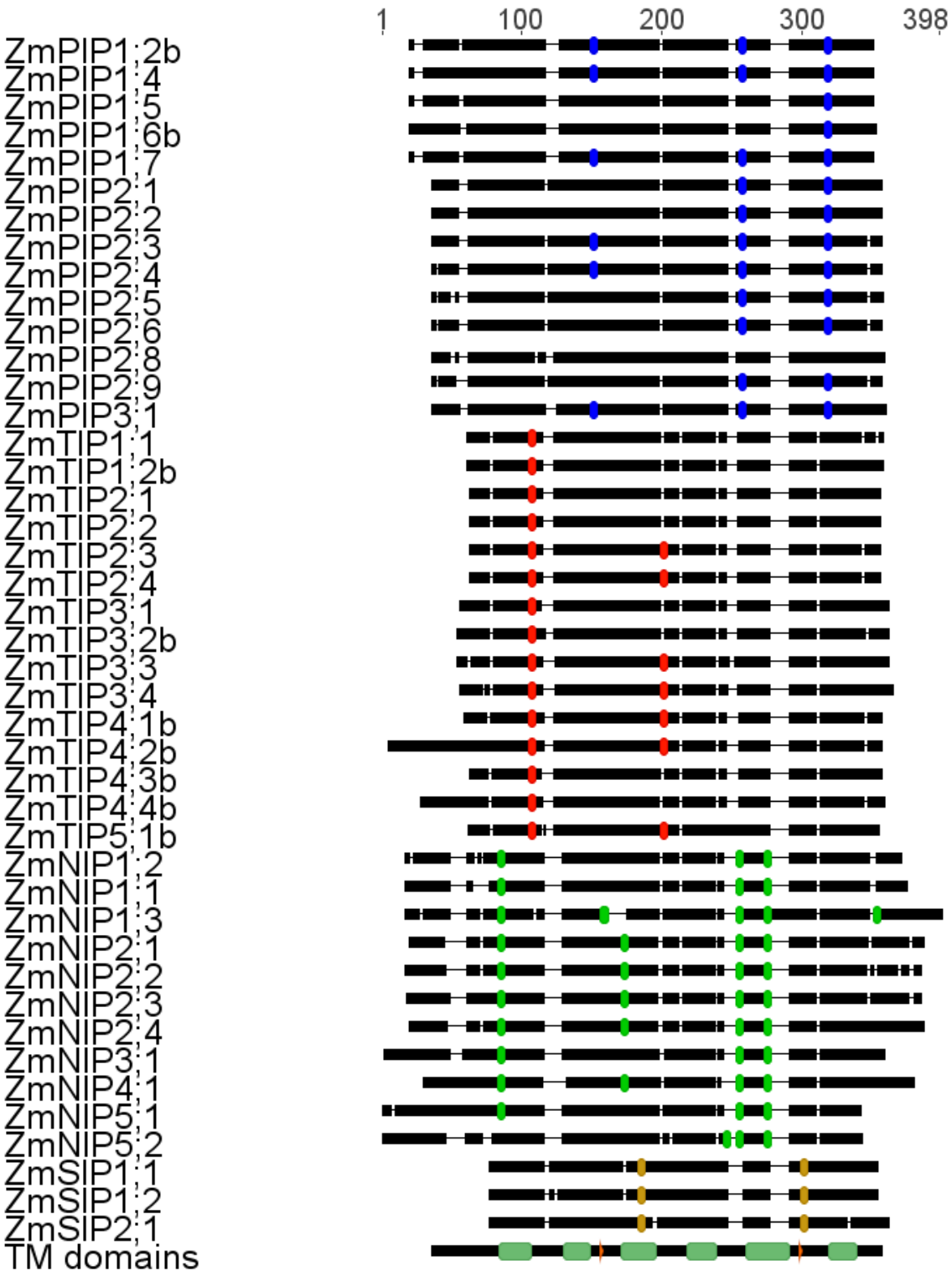


Figure 2.5 Maize aquaporin protein exon locations

Locations of introns in the protein sequences aligned with CLUSTAL omega. Coloured bars represent the intron location in the protein sequence (Blue = PIP, Red = TIP, Green = NIP, Yellow = SIP). For reference, the approximate location of the six trans- membrane domains (green rectangles) and two NPA domains (Orange triangles) are shown at the bottom. Only ZmPIP2;8 did not have any introns.

Table 2.6 Maize genomic aquaporins compared to published sequences

Of the 31 unique aquaporin protein sequences in Chaumont et al. (2001), only one did not match a genomic sequence (ZmPIP2;7). An arbitrary threshold of 98% protein identity was selected to match names. At this cut off, only one did not match (ZmPIP1;1 and ZmPIP1;7 at 96.2%). Two others matched within the cut off (*), but had N-terminal extensions: ZmTIP4;2b and ZmTIP4;4b. Amino acid changes are from those previously published to the genome.

Genome				Published cDNAs				Base pair % identity	# of base pair differences in open reading frame	Amino acid % identity	# amino acid changes	Amino acid changes (from cDNA to genome)	Location in protein
Gene Name	ID	Base Pairs	Amino Acid	Gene name	NCBI ID	Base pairs	Amino Acids						
ZmPIPs													
ZmPIP1;2b	Zm00001d017526_T001.1	1,420	289	ZmPIP1;2	AF131201.1	1,280	289	99.4	2	99.7	1	V147I	TM 3
ZmPIP1;4	Zm00001d051403_T001.1	1,484	292	ZmPIP1;3	AF326487.1	1,296	292	95.0	6	100	-	-	-
				ZmPIP1;4	AF326488.1	1,153	292	100	-	100	-	-	-
ZmPIP1;5	Zm00001d051872_T001.1	1,280	288	ZmPIP1;5	AF326489.1	1,338	288	100	-	100	-	-	-
				ZmPIP1;5b	AJ271796.1	1,364	288	95.1	2	100	-	-	-
ZmPIP1;6b	GRMZM2G136032_T01 (AGPv3)	1,552	296	ZmPIP1;6	AF326490.1	1,325	296	99.2	3	99.7	1	E12A	N-terminus
ZmPIP1;7	Zm00001d002690_T001.1	1,389	288	ZmPIP1;1	X82633.1	1,169	287	97.9	24	96.2	11	H19Q A20P	N-terminus
												P55A	TM 1
												L101F	TM 2
												113G (ins.)	Loop B
												R149A	TM 3
												R166G R167A	Loop C
												R202N	Loop D
												M229I G230P	TM 5
ZmPIP2;1	Zm00001d019563_T006.1	1,186	290	ZmPIP2;1	AF326491.1	1,186	290	99.8	1	100	-	-	-
ZmPIP2;2	Zm00001d005421_T001.1	1,635	292	ZmPIP2;2	AF326492.1	1,268	292	96.3	-	100	-	-	-
ZmPIP2;3	Zm00001d051174_T002.1	1,495	289	ZmPIP2;3	AF326493.1	1,156	289	100	-	100	-	-	-
ZmPIP2;4	Zm00001d017288_T001.1	1,281	288	ZmPIP2;4	AF326494.1	1,171	288	100	-	100	-	-	-
ZmPIP2;5	Zm00001d003006_T001.1	1,219	285	ZmPIP2;5	AF130975.1	1,207	285	99.3	1	100	-	-	-
ZmPIP2;6	Zm00001d019565_T001.1	1,265	288	ZmPIP2;6	AF326495.1	1,257	288	100	-	100	-	-	-
-	-	-	-	ZmPIP2;7	AF326496.1	929	287	-	-	-	-	-	-
ZmPIP2;8	Zm00001d014285_T001.1	852	283	-	-	-	-	-	-	-	-	-	-
ZmPIP2;9	Zm00001d005410_T001.1	861	286	-	-	-	-	-	-	-	-	-	-
ZmPIP3;1	Zm00001d022608_T001.1	867	288	-	-	-	-	-	-	-	-	-	-

(*continued*)

Genome				Published cDNAs				Base pair % identity	# of base pair differences in open reading frame	Amino acid % identity	# amino acid changes	Amino acid changes (from cDNA to genome)	Location in protein
Gene Name	ID	Base Pairs	Amino Acid	Gene name	NCBI ID	Base pairs	Amino Acids						
ZmTIPs													
ZmTIP1;1	Zm00001d027652_T001.1	1,146	250	ZmTIP1;1	AF037061.1	1,097	250	97.7	5	100	-	-	-
ZmTIP1;2b	Zm00001d011778_T001.1	1,340	254	ZmTIP1;2	AF326500.1	1,021	254	98.9	8	98.8	3	T30M A53T L130I	TM 1 Loop A Loop C
ZmTIP2;1	Zm00001d051362_T001.1	1,386	249	ZmTIP2;1	AF326501.1	1,110	249	100	-	100	-	-	-
ZmTIP2;2	Zm00001d017485_T002.1	1,366	250	ZmTIP2;2	AF326502.1	1,073	250	100	-	100	-	-	-
ZmTIP2;3	Zm00001d026177_T001.1	1,079	248	ZmTIP2;3	AF326503.1	1,042	248	100	-	100	-	-	-
ZmTIP2;4	Zm00001d002738_T001.1	946	248	-	-	-	-	-	-	-	-	-	-
ZmTIP3;1	Zm00001d013956_T003.1	1,145	262	ZmTIP3;1	AF326504.1	989	262	100	-	100	-	-	-
ZmTIP3;2b	Zm00001d032610_T003.1	1,195	266	ZmTIP3;2	AF342809.1	1,156	266	98.2	3	99.6	1	F164L	TM 4
ZmTIP3;3	Zm00001d048520_T001.1	1,202	267	-	-	-	-	-	-	-	-	-	-
ZmTIP3;4	Zm00001d002952_T001.1	1,223	265	-	-	-	-	-	-	-	-	-	-
ZmTIP4;1b	Zm00001d037779_T001.1	1,309	255	ZmTIP4;1	AF326505.1	1,125	255	99.0	3	99.6	1	P18L	N-terminus
ZmTIP4;2b	Zm00001d010410_T001.1	1,309	311	ZmTIP4;2	AF326506.1	1,255	257	97.7	8*	98.8*	54+3	MSHSPLPPPV QNISRFSESF SLQGTETTG AFTPPAFPSP PGTTGLLAII RPS(0) D69H R200S N300Y	N-terminal extension N-terminus Loop C C-terminus
ZmTIP4;3b	Zm00001d039713_T001.1	1,187	249	ZmTIP4;3	AF326507.1	1,045	249	89.6	11	98.0	5	A110G P138A V208I M235I D242A	TM 3 Loop C Loop E C-terminus
ZmTIP4;4b	Zm00001d039293_T001.1	973	287	ZmTIP4;4	AF326508.1	955	252	100	-*	100*	35	MYPSPPPHLF YGVSVSRQH SPRSNRHFFN PLAKRA(0)	N-terminal extension
ZmTIP5;1b	Zm00001d026178_T001.1	1,037	260	ZmTIP5;1	AF326509.1	973	260	95.6	15	98.1	5	T47A G56W S102R M104I F124L	Loop A TM 3

(*continued*)

Genome				Published cDNAs				Base pair % identity	# of base pair differences in open reading frame	Amino acid % identity	# amino acid changes	Amino acid changes (from cDNA to genome)	Location in protein
Gene Name	ID	Base Pairs	Amino Acid	Gene name	NCBI ID	Base pairs	Amino Acids						
ZmNIPs													
ZmNIP1;1	Zm00001d016237_T001.1	1,369	282	ZmNIP1;1	AF326483.1	1,145	282	100	-	100	-	-	-
ZmNIP1;2	Zm00001d037733_T001.1	1,288	284	-	-	-	-	-	-	-	-	-	-
ZmNIP1;3	Zm00001d046303_T001.1	954	317	-	-	-	-	-	-	-	-	-	-
ZmNIP2;1	Zm00001d018037_T001.1	1,432	295	ZmNIP2;1	AF326484.1	1,293	295	100	-	100	-	-	-
ZmNIP2;2	Zm00001d037228_T001.1	1,407	294	ZmNIP2;2	AF326485.1	1,387	294	100	-	100	-	-	-
ZmNIP2;3	Zm00001d044826_T001.1	1,237	301	ZmNIP2;3	AF342810.1	1,300	301	98.2	-	100	-	-	-
ZmNIP2;4	GRMZM2G082184_T01 (AGPv3)	1,558	303	-	-	-	-	-	-	-	-	-	-
ZmNIP3;1	Zm00001d032461_T001.1	1,920	302	ZmNIP3;1	AF326486.1	1,409	302	100	-	100	-	-	-
ZmNIP4;1	Zm00001d040108_T001.1	1,250	299	-	-	-	-	-	-	-	-	-	-
ZmNIP5;1	Zm00001d049578_T001.1	891	296	-	-	-	-	-	-	-	-	-	-
ZmNIP5;2	Zm00001d024634_T001.1	840	279	-	-	-	-	-	-	-	-	-	-
ZmSIPs													
ZmSIP1;1	GRMZM2G113470_T01 (AGPv3)	1,276	245	ZmSIP1;1	AF326497.1	1,084	245	100	-	100	-	-	-
ZmSIP1;2	Zm00001d010325_T001.1	1,196	243	ZmSIP1;2	AF326498.1	936	243	100	-	100	-	-	-
ZmSIP2;1	Zm00001d028951_T001.1	1,184	249	ZmSIP2;1	AF326499.1	1,186	249	95.6	3	100	-	-	-

Table 2.7 Maize aquaporin protein features

The aquaporins found in the maize genome were investigated for major motifs: transmembrane domains, NPA sequences, and ar/R filter sequences. Transmembrane domains were predicted by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html), Sosui v1.0 (http://harrier.nagahama-i-bio.ac.jp/sosui/sosuiG/sosuigsubmit.html), TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/), and Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index). The ar/R filter sequences were determined from alignments and referencing to the maize ar/R filters from Bansal and Sankararamakrishnan (2007). Yellow boxes highlight NPA domains with derivative sequences.

Gene name	ID	Amino acids	Transmembrane domain predictions				NPA Domains		ar/R filter				Aquaporin InterPro Domains		
			TMpred	SOSUI	TMHMM	Phyre2	Loop B	Loop E	H2	H5	LE1	LE2	IPR000425	IPR023271	IPR022357
ZmPIPs															
ZmPIP1;2b	Zm00001d017526_T001.1	289	6	6	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP1;4	Zm00001d051403_T001.1	292	6	6	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP1;5	Zm00001d051872_T001.1	288	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP1;6b	GRMZM2G136032_T01 (AGPv3)	296	6	6	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP1;7	Zm00001d002690_T001.1	288	6	6	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;1	Zm00001d019563_T006.1	290	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;2	Zm00001d005421_T001.1	292	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;3	Zm00001d051174_T002.1	289	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;4	Zm00001d017288_T001.1	288	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;5	Zm00001d003006_T001.1	285	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;6	Zm00001d019565_T001.1	288	6	5	7	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;8	Zm00001d014285_T001.1	283	6	6	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP2;9	Zm00001d005410_T001.1	286	6	5	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes
ZmPIP3;1	Zm00001d022608_T001.1	288	6	6	6	6	NPA	NPA	F	H	T	R	Yes	Yes	Yes

(*continued*)

Gene name	ID	Amino acids	Transmembrane domain predictions				NPA Domains		ar/R filter				Aquaporin InterPro Domains		
			TMpred	SOSUI	TMHMM	Phyre2	Loop B	Loop E	H2	H5	LE1	LE2	IPR000425	IPR023271	IPR022357
ZmTIPs															
ZmTIP1;1	Zm00001d027652_T001.1	250	6	6	6	6	NPA	NPA	H	I	A	V	Yes	Yes	Yes
ZmTIP1;2b	Zm00001d011778_T001.1	254	6	6	7	6	NPA	NPA	H	I	A	V	Yes	Yes	Yes
ZmTIP2;1	Zm00001d051362_T001.1	249	6	6	6	6	NPA	NPA	H	I	G	R	Yes	Yes	Yes
ZmTIP2;2	Zm00001d017485_T002.1	250	6	6	6	6	NPA	NPA	H	I	G	R	Yes	Yes	Yes
ZmTIP2;3	Zm00001d026177_T001.1	248	6	6	6	6	NPA	NPA	H	I	G	R	Yes	Yes	Yes
ZmTIP2;4	Zm00001d002738_T001.1	248	6	6	6	6	NPA	NPA	H	I	G	R	Yes	Yes	Yes
ZmTIP3;1	Zm00001d013956_T003.1	262	6	6	6	6	NPA	NPA	H	V	A	R	Yes	Yes	Yes
ZmTIP3;2b	Zm00001d032610_T003.1	266	6	6	6	6	NPA	NPA	H	V	A	R	Yes	Yes	Yes
ZmTIP3;3	Zm00001d048520_T001.1	267	6	6	5	6	NPA	NPA	H	V	A	R	Yes	Yes	Yes
ZmTIP3;4	Zm00001d002952_T001.1	265	6	0	6	6	NPA	NPA	H	I	A	R	Yes	Yes	Yes
ZmTIP4;1b	Zm00001d037779_T001.1	255	6	6	6	6	NPA	NPA	H	S	A	R	Yes	Yes	Yes
ZmTIP4;2b	Zm00001d010410_T001.1	311	6	6	6	6	NPA	NPA	H	S	A	R	Yes	Yes	Yes
ZmTIP4;3b	Zm00001d039713_T001.1	249	6	6	6	6	NPA	NPA	Q	S	A	R	Yes	Yes	Yes
ZmTIP4;4b	Zm00001d039293_T001.1	287	6	6	6	6	NPA	NPA	H	V	A	R	Yes	Yes	Yes
ZmTIP5;1b	Zm00001d026178_T001.1	260	6	6	6	6	NPA	NPA	Q	V	A	R	Yes	Yes	Yes
ZmNIPs															
ZmNIP1;1	Zm00001d016237_T001.1	282	6	6	6	6	NPA	NPA	W	V	A	R	Yes	Yes	Yes
ZmNIP1;2	Zm00001d037733_T001.1	284	6	6	5	6	NPA	NPA	W	V	A	R	Yes	Yes	Yes
ZmNIP1;3	Zm00001d046303_T001.1	317	6	6	6	6	NPA	NPA	W	I or A	A	R	Yes	Yes	-
ZmNIP2;1	Zm00001d018037_T001.1	295	6	6	6	6	NPA	NPA	G	S	G	R	Yes	Yes	Yes
ZmNIP2;2	Zm00001d037228_T001.1	294	6	6	6	6	NPA	NPA	G	S	G	R	Yes	Yes	Yes
ZmNIP2;3	Zm00001d044826_T001.1	301	7	7	6	6	NPA	NPA	G	S	G	R	Yes	Yes	Yes
ZmNIP2;4	GRMZM2G082184_T01 (AGPv3)	303	6	6	6	6	NPA	NPA	G	S	G	R	Yes	Yes	Yes
ZmNIP3;1	Zm00001d032461_T001.1	302	6	6	6	6	NPS	NPV	A	I	G	R	Yes	Yes	Yes
ZmNIP4;1	Zm00001d040108_T001.1	299	6	6	6	6	NPA	NPI	C	G	G	R	Yes	Yes	Yes
ZmNIP5;1	Zm00001d049578_T001.1	296	6	6	6	6	NPA	NPA	G	A	A	R	Yes	Yes	Yes
ZmNIP5;2	Zm00001d024634_T001.1	279	6	6	6	6	NPA	NPA	A	A	P	R	Yes	Yes	Yes
ZmSIPs															
ZmSIP1;1	GRMZM2G113470_T01 (AGPv3)	245	6	6	5	6	NPT	NPA	L	I	P	N	Yes	Yes	-
ZmSIP1;2	Zm00001d010325_T001.1	243	7	5	6	6	NPT	NPA	L	V	P	N	Yes	Yes	-
ZmSIP2;1	Zm00001d028951_T001.1	249	6	4	6	6	NPL	NPA	S	H	G	S	Yes	Yes	-

Table 2.8 Aquaporin fragments found in the maize B73 genome

There were several locations in the B73 that contained sequences with similarity to aquaporins, but did not have full length aquaporins. The TBLASTN results were examined for alignment to aquaporins. The genomic region was examined near the alignment and the predicted genes for alignments to known maize aquaporins. Included in this list were the regions that shared some overlap with at least one NPA domain. The AGPv3 and AGPv4 genes were both included for f-ZmSIP1;3, f-ZmSIP1;4, and f-ZmSIP1;5. Each pair of versions shared the same alignment to ZmSIP1;1 with each other, but differed in N and C domains, resulting in different % identities with ZmSIP1;1 (†). Frag020 and Frag007 (*) had extensions to the gene predicted. Four genes from Deshmukh et al. (2015) did not have the requisite 6 transmembrane domains and two NPA motifs to be labelled full length aquaporin sequences. These genes aligned well to the AGPv3 annotation, except ZmSIP1;6 where an AGPv3 annotation was unavailable. The ZmSIP1;6 aligned to Frag007, but the AGPv4 gene was identical, but shortened (857 amino acids to 338 amino acids).

Name	**Deshmukh 2015 Gene**	**Gene (AGPv4 unless noted)**	**Chr.**	**Start**	**End**	**AA**	**Aquaporin InterPro Domains**			**Most Similar ZmMIP**		
							IPR000425	**IPR023271**	**IPR022357**	**Gene**	**% Identity**	**Alignment Figure**
f-ZmSIP1;3	ZmSIP1;3	GRMZM2G373447_T01.1 (AGPv3) Zm00001d032015__T001.1 (AGPv4)	1	209,513,196	209,514,778	507 250	Yes	Yes	-	ZmSIP1;1	42.0(†) 73.8(†)	**Figure 2.14** **Figure 2.15**
f-ZmSIP1;4	ZmSIP1;2	GRMZM2G494600_T01.1 (AGPv3) Zm00001d017903_T001.1 (AGPv4)	5	209,829,962	209,834,459	487 423	Yes	Yes	-	ZmSIP1;1	55.1(†) 53.8(†)	**Figure 2.14**
f-ZmSIP1;5	ZmSIP1;1*	GRMZM2G065958_T01.1 (AGPv3) Zm00001d044543_T001.1 (AGPv4)	3	231,358,323	231,359,375	262 253	Yes	Yes	-	ZmSIP1;1	72.4(†) 75.8(†)	**Figure 2.14**
f-ZmNIP2;5	-	Zm00001d053690_T001.1	4	239,074,645	239,075,707	258	Yes	Yes	Yes	ZmNIP2;1	41.6	**Figure 2.17**
Frag001	-	Zm00001d027849_T001.1	1	15,126,907	15,130,354	384	-	yes	-	ZmSIP1;1	48.1	**Figure 2.15**
Frag002	-	Zm00001d015518_T001.1	5	95,499,574	95,499,894	106	yes	yes	-	ZmTIP4;2b	85.3	**Figure 2.16**
Frag003	-	Zm00001d019676_T001.1	7	50,195,791	50,196,111	106	yes	yes	-	ZmTIP4;2b	84.4	**Figure 2.16**
Frag005	-	Zm00001d020747_T001.1	7	131,061,325	131,061,969	107	yes	yes	-	ZmTIP4;2b	82.2	**Figure 2.16**
Frag007	ZmSIP1;6	Zm00001d043804_T001.1*	3	210,575,986	210,592,288	338	-	-	-	ZmSIP1;1	48.7	**Figure 2.15**
Frag020	-	Zm00001d021008_T001.1*	7	140,187,961	140,188,269	161	yes	-	-	ZmSIP1;1	86.7	**Figure 2.15**
Frag021	-	no gene	9	11,410,626	11,418,868	227	yes	yes	-	ZmSIP1;1	80.3	**Figure 2.20**
Frag024	-	no gene	1	16,607,420	16,615,524	58	yes	yes	yes	ZmTIP3;4	79.3	**Figure 2.19**
Frag026	-	no gene	5	153,889,248	153,897,388	47	yes	yes	-	ZmPIP1;7	74.5	**Figure 2.18**
Frag027	-	no gene	6	86,432,239	82,440,331	31	yes	yes	yes	ZmTIP3;4	96.8	**Figure 2.19**
Frag028	-	no gene	7	40,694,006	40,694,188	61	yes	yes	-	ZmTIP3;4	88.5	**Figure 2.19**
Frag029	-	no gene	3	4,456,430	4,456,671	73	yes	yes	-	ZmNIP1;2	56.4	**Figure 2.21**
Frag041	-	no gene	4	5,661,403	4,661,468	22	-	-	-	ZmPIP1;7	77.3	**Figure 2.18**
Frag045	-	no gene	8	66,123,416	66,131,517	41	-	-	-	ZmPIP1;7	58.5	**Figure 2.18**
Frag046	-	no gene	8	66,095,830	66,103,910	27	-	-	-	ZmPIP1;7	63.0	**Figure 2.18**
Frag047	-	no gene	8	23,846,677	23,854,778	108	yes	yes	-	ZmNIP1;2	47.7	**Figure 2.21**
Frag050	-	no gene	4	33,915,627	33,923,755	43	-	-	-	ZmSIP1;1	74.4	**Figure 2.20**

2.3.1 ZmPIPs

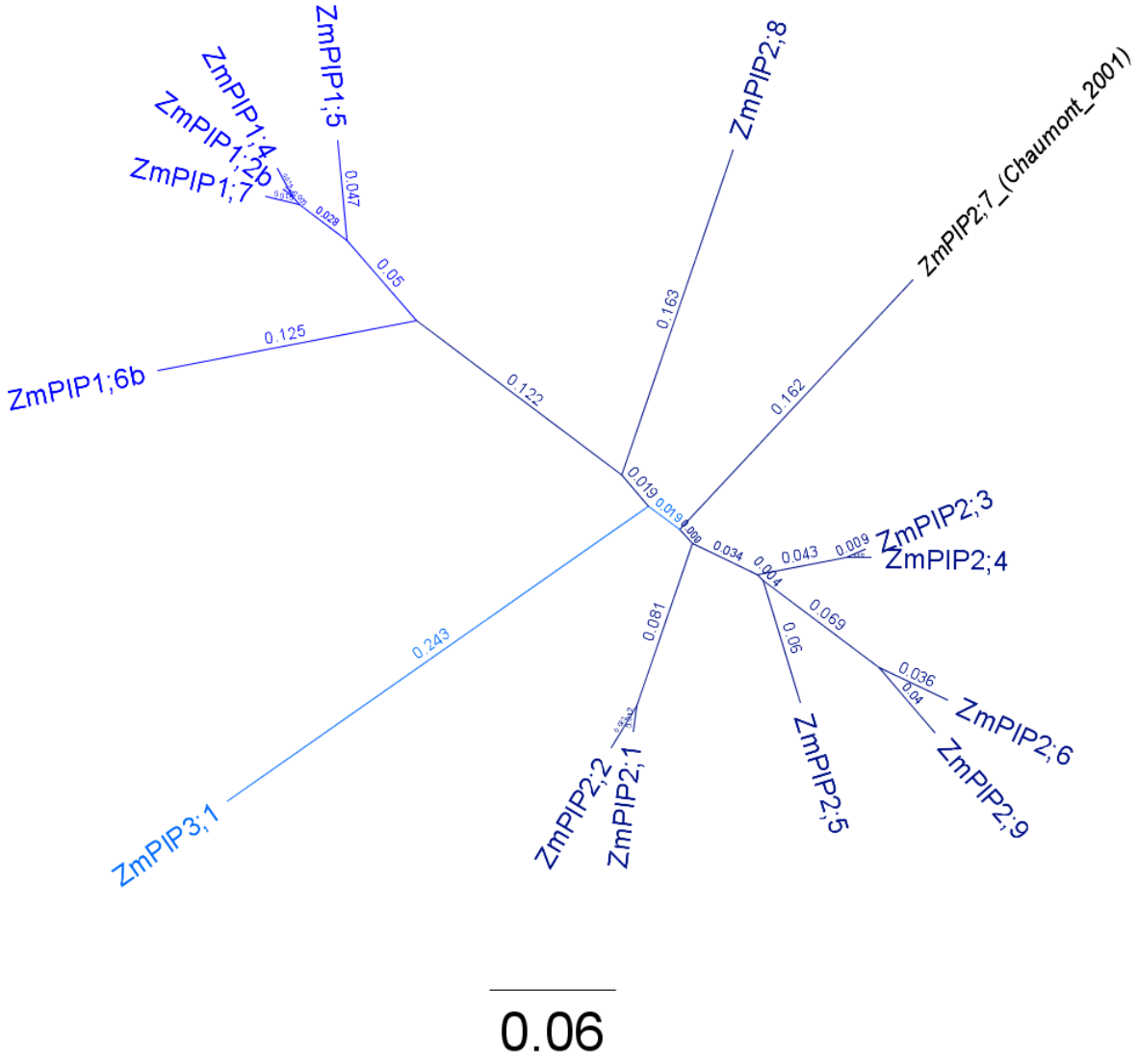


Figure 2.6 Phylogenetic relationship of ZmPIP proteins

The ZmPIP subset from the phylogenetic analysis of maize aquaporins from **Figure 2.1**. ZmPIP2;7 was included although it does not appear in the genome. Branch labels and scale represent number of changes per site.

There were five ZmPIP1s, eight ZmPIP2s, and one ZmPIP3 in the B73 genome. Two ZmPIP1s, ZmPIP1;2b and ZmPIP1;6b, had different amino acid sequences from those described previously (**Table 2.6**). Only one ZmPIP1;3/ZmPIP1;4 clone was found. Based

on nucleotide sequence alignments, ZmPIP1;4 was chosen as the name (**Figure 2.7**). ZmPIP1;7 was found, but may be related to ZmPIP1;1, which was absent. While their amino acid sequences share 96.2% identity and their cDNAs are 97.8% identical, they differ by 13 amino acids, including an insertion in ZmPIP1;7 (**Figure 2.8**). Based on the degree of difference in their amino acid sequences, a new name was chosen.

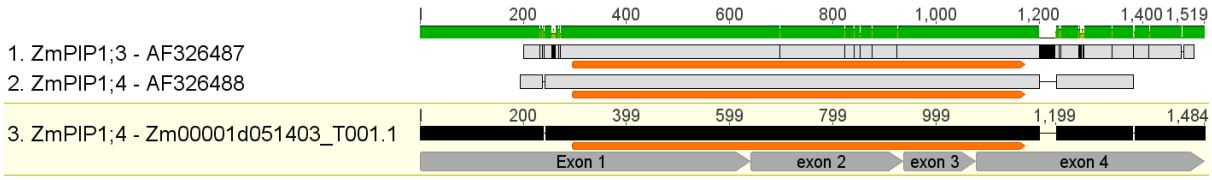


Figure 2.7 ZmPIP1;4 transcript alignments

Nucleotide alignments of the predicted cDNA sequence from the B73 genome and the ZmPIP1;3 and ZmPIP1;4 cDNA sequences described by Chaumont et al. (2001). The protein sequences are identical, but only one copy was found in the B73 genome, which is nearly identical to ZmPIP1;4, just 5' and 3' UTR extensions. The coding sequence is shown as an orange bar in each gene. Black bars denote differences from the genomic sequence.

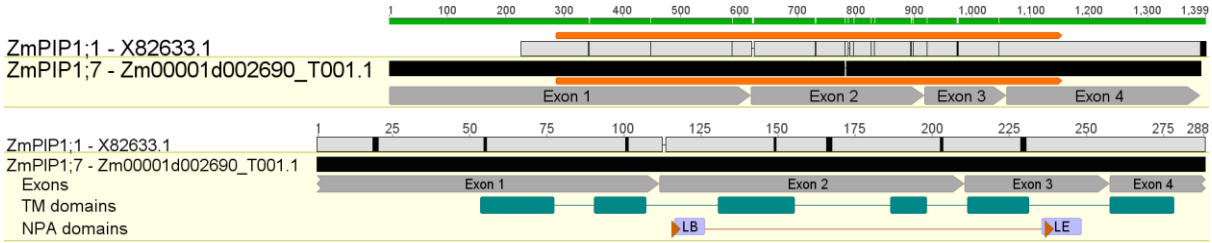


Figure 2.8 ZmPIP1;7 cDNA and protein alignments with ZmPIP1;1

cDNA (top) and protein (bottom) alignments of ZmPIP1;1 from Chaumont et al. (2001) and ZmPIP1;7 in the B73 genome. The genes are very similar, having 97.9% identity between the cDNA sequences and 96.2% in the amino acid sequences. Together, the coding region (orange) had 24 base pair differences resulting in 10 changed amino acids and 1 insertion in the protein sequence.

The rest of the ZmPIPs are identical to those previously described except for the absence of ZmPIP2;7 and the addition of ZmPIP2;8 (Lawrence et al. 2013), ZmPIP2;9 and ZmPIP3;1. ZmPIP2;9 is very similar to ZmPIP2;6, differing by nine amino acids (**Figure 2.9**). ZmPIP3;1 was not predicted nor present in the AGPv3 genome.

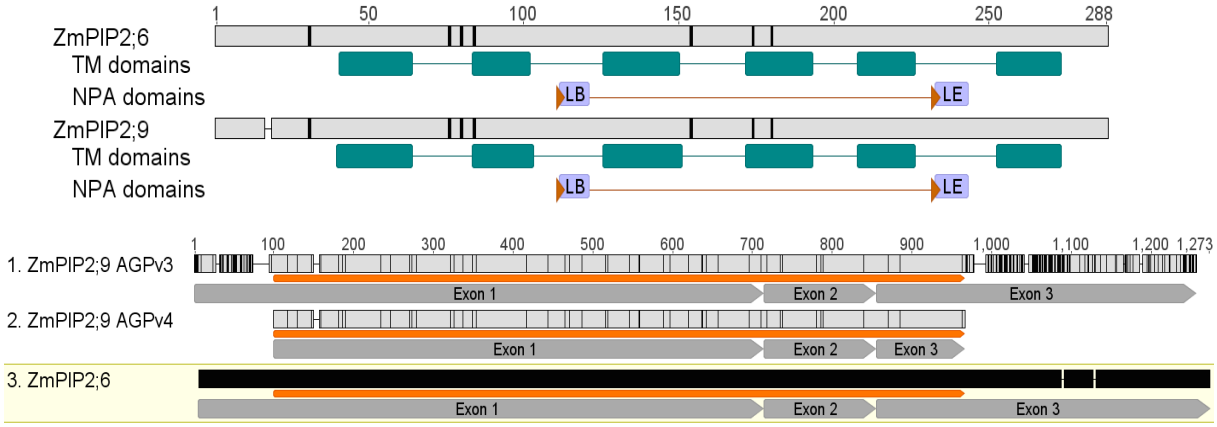


Figure 2.9 Protein and nucleotide alignments of ZmPIP2;6 and ZmPIP2;9
ZmPIP2;9 has not been previously described and is most similar to ZmPIP2;6, sharing 96.9% pairwise identity to their protein sequences (top). Their cDNAs (bottom) are 94.1% identical, however in the AGPv4 gene prediction, no 5' or 3' UTRs are predicted for ZmPIP2;9. In the AGPv3 genome annotation, UTRs are predicted, and differ significantly from the 5' and 3' UTRs of ZmPIP2;6

Based on phylogenetic analysis with monocot aquaporins, ZmPIP3;1 was made into a new subgroup (**Figure 2.2**). No other maize aquaporins belong to this clade, but other members are represented in barley (HvPIP2;7 and HvPIP2;10), brachypodium (BdPIP2;5 and BdPIP2;6), rice (OsPIP2;7), moso bamboo (PePIP2;7), sorghum (SbPIP2;9), setaria (SiPIP2;8) and switchgrass (PvPIP2;13 and PvPIP2;14). ZmPIP2;8 could be considered separate from the ZmPIP2s, however bootstrap support for this branch was not significant (21% vs 100% for the ZmPIP3s - **Figure 2.2**), so the name ZmPIP2;8 was retained to remain consistent with Lawrence et al. (2013).

While ZmPIP3;1 is a separate clade phylogenetically, it shares many features of the ZmPIPs. It has both NPA features and its ar/R filter (FHTR) is identical with the ZmPIP1s and ZmPIP2s. ZmPIP3;1 contains the LxxxA motif on helix 3, a highly conserved motif in plant PIP2 proteins and is necessary (but not sufficient) for plasma membrane localization (Chevalier et al. 2014).

2.3.2 ZmTIPs

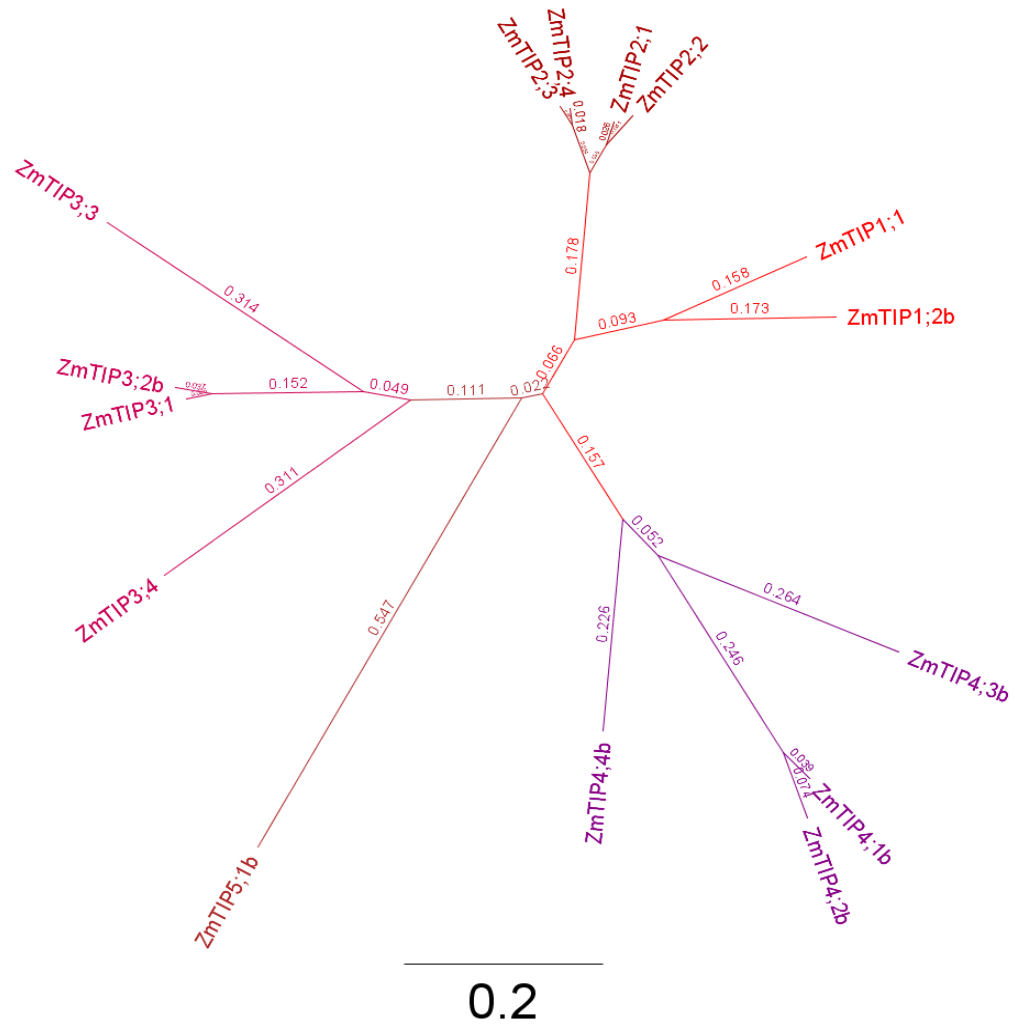


Figure 2.10 Phylogenetic relationship of the ZmTIP proteins

The ZmTIP subset from the phylogenetic analysis of maize aquaporins from **Figure 2.1**. Branch labels and scale represent number of amino acid changes per site.

The B73 genome revealed two ZmTIP1s, four ZmTIP2s, four ZmTIP3s, four ZmTIP4s, and one ZmTIP5. Three ZmTIPs (ZmTIP2;4, ZmTIP3;3 and ZmTIP3;4) had not been previously described. Interestingly, the AGPv3 genome did not have genes for ZmTIP1;1 or ZmTIP5;1b present. The AGPv3 genome had a partial ZmTIP1;1 gene, but due to missing about half of the nucleotides in exon 2, a gene was not predicted. In the updated AGpv4 genome, exon 2 was restored and ZmTIP1;1 was predicted, with identical protein sequence to the deduced amino acid sequence from the cDNA described before (Chaumont et al. 2001). Interestingly, ZmTIP5;1b is only 162 bp downstream of ZmTIP2;3 on chromosome 10. In AGPv3, these two genes were combined into one cDNA with just the ZmTIP2;3 coding sequence predicted (**Figure 2.11**).

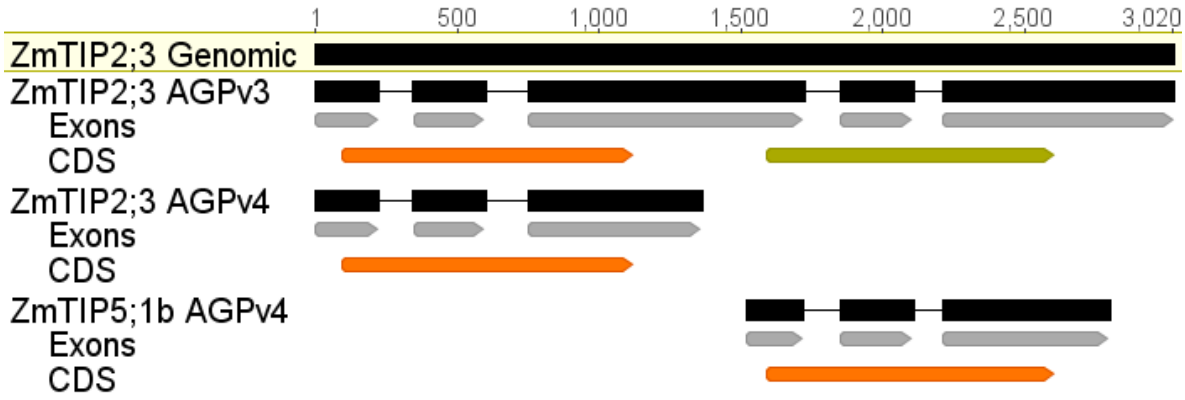


Figure 2.11 ZmTIP2;3 and ZmTIP5;1b genomic location
ZmTIP2;3 and ZmTIP5;1b are located very close together on chromosome 10. In AGPv3, just one gene was predicted that covered both regions. Only the ZmTIP2;3 part was predicted to encode a protein, although a fully functional second coding sequences was present on the cDNA. In AGPv4, these genes were split, with 162 genomic base pairs separating them. CDS = coding sequences (Orange = predicted, yellow = present but not predicted).

Seven ZmTIPs amino acid sequences were different than previously described, including all the ZmTIP4s. Two (ZmTIP4;2b and ZmTIP4;4b) had earlier start codons, extending the N terminus. ZmTIP4;4b was identical to the ZmTIP4;4 described by Chaumont et al. (2001) except for this extension.

Bansal and Sankararamakrishnan (2007) calculated the ar/R filter for many ZmTIPs and found several filters not found in *Arabidopsis* (Wallace and Roberts 2004). Alignments with these sequences show the ar/R in ZmTIP2;4 (HIGR) matches the other ZmTIP2s. ZmTIP3;2b and ZmTIP3;3 have identical ar/R to ZmTIP3;1, and ZmTIP3;4 forms an ar/R filter (HIAR) similar to the other ZmTIP3s, and is seen in 37 other plant aquaporins (Gupta et al. 2012).

2.3.3 ZmNIPs and ZmSIPs

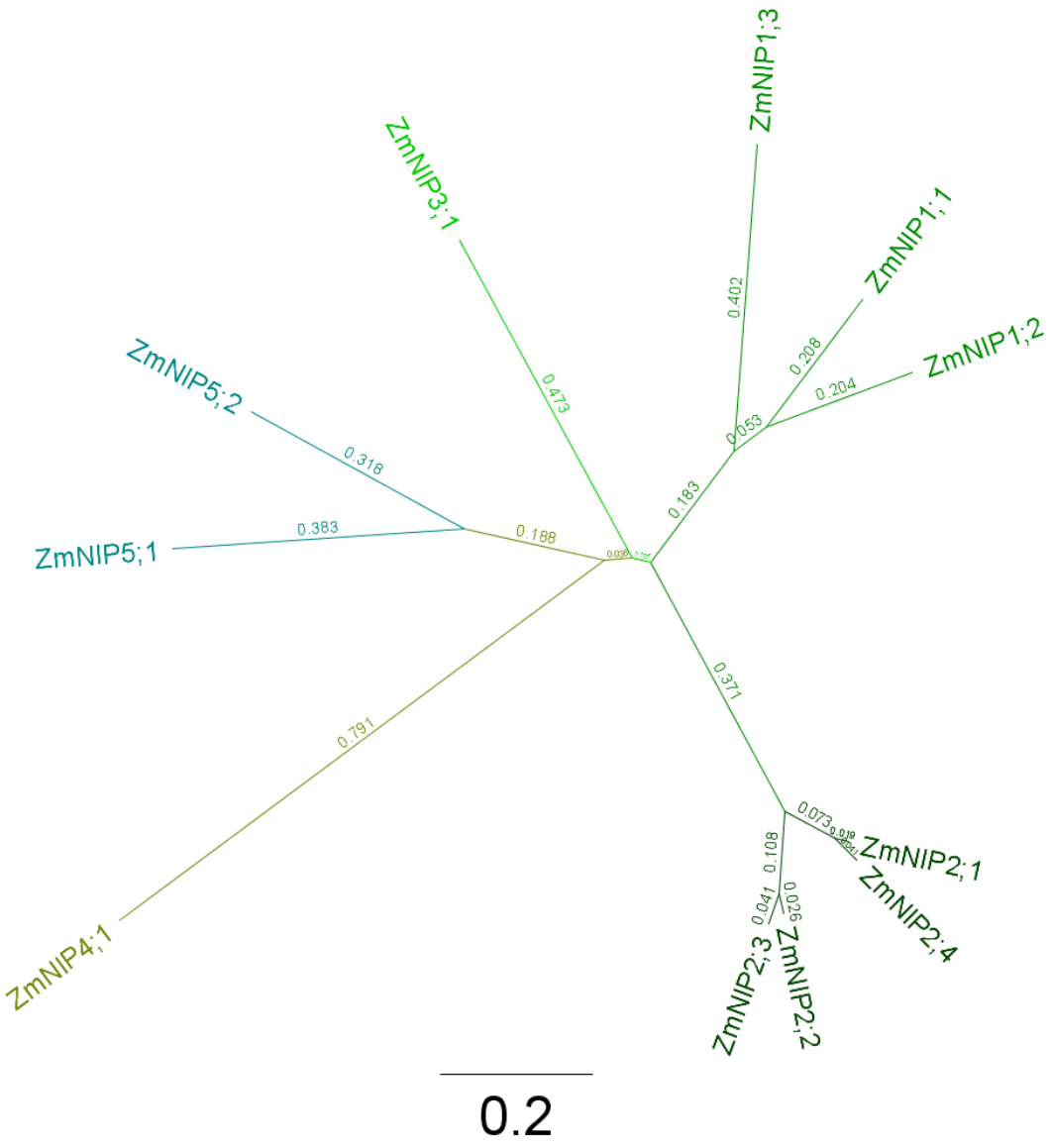


Figure 2.12 Phylogenetic relationship of the ZmNIP proteins
The ZmNIP subset from the phylogenetic analysis of maize aquaporins from **Figure 2.1**. Branch labels and scale represent number of changes per site.

The ZmNIPs consisted of three ZmNIP1s, four ZmNIP2s, one ZmNIP3, one ZmNIP4 and two ZmNIP5s. Chaumont et al. (2001) found only 5 ZmNIPs, less than half that are present in the AGPv4 maize genome. The six new ZmNIPs are ZmNIP1;2, ZmNIP1;3, ZmNIP2;4, ZmNIP4;1, ZmNIP5;1 and ZmNIP5;2. As with the ZmPIPs, the phylogenetic

analysis with the complete genome sets of aquaporins from monocots (**Figure 2.2**) suggests a new clade exists, the NIP5s, currently contained within the NIP3 subgroup. ZmNIP2;1 has the same ar/R as ZmNIP1;1, and ZmNIP3;1 (W(I/A)AR) shares is seen in 4 (WIAR) or 6 (WAAR) other plant aquaporins (Gupta et al. 2012). The ZmNIP2s all share the same ar/R filter (GSGR). The filter for ZmNIP4;1 (CGGR) and ZmNIP5;2 (AAPR) are seen in three other plant aquaporins each, while the filter for ZmNIP5;1 (GAAR) has not been seen in other plant aquaporins (Gupta et al. 2012).

The ZmSIPs found in the genome were the same as those previously described in Chaumont et al. (2001). A potential difference occurred in ZmSIP1;1. The AGPv3 notation was identical to ZmSIP1;1 described before, however, in AGPv4, 10 amino acids are truncated from the end of exon 1, predicted to be most of the third transmembrane domain (**Figure 2.13**). As AGPv3 contained the cDNA previously found, its sequence was used in the analysis performed in this report.

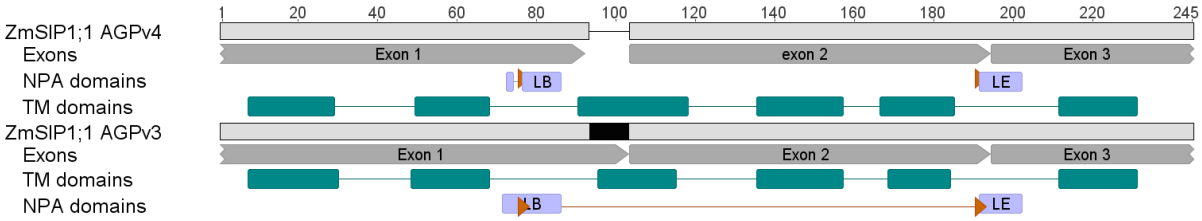


Figure 2.13 ZmSIP1;1 protein sequences from AGPv3 and AGPv4
The ZmSIP1;1 protein sequences differ by 10 amino acids, located at the end of exon 1. While this is part of the third TM domain, Phyre 2 homology modelling still predicts six transmembrane domains for each ZmSIP1;1. As AGPv3 has the 10 amino acids, its sequence was used in this thesis.

2.3.4 Aquaporin fragments

The B73 maize genome contained 21 fragments with aquaporin features. The genomic location of these fragments was analysed and no other fragments with aquaporin features

were found within 5 kb upstream or downstream of their location, indicating there was not a full length aquaporin gene at these loci (assuming there were no sequencing errors masking complete genes). Of the 21 fragments found, ten had genes predicted by the genome annotation: six similar to ZmSIP1;1, three similar to ZmTIP4;2b, and one similar to ZmNIP2;1. The other 11 fragments did not have genes predicted by the genome annotation. Of these, four were similar to ZmPIP1;7, three with ZmTIP3;4, two with ZmSIP1;1 and two with ZmNIP1;2.

Of the six fragments with genes resembling ZmSIP1;1, three fragments, had substantial similarity, labelled ZmSIP1;3, ZmSIP1;4 and ZmSIP1;5. The included close similarity over TM2, TM3, TM4 and TM5 included both NPA domains. These three proteins all had extensions on their N-terminal that have a domain associated with ISY1, a protein involved in pre-mRNA splicing in yeast (Dix et al. 1999; Villa and Guthrie 2005). The maize genome versions AGPv3 and AGPv4 differ in the exon structure predicted. This is due to the differences in the prediction algorithms used, as the nucleotides for the exons of both proteins are present. Both versions for the three genes are shown in **Figure 2.14**. Interestingly, both versions of ZmSIP1;4 had five TMs predicted. In AGPv3, this included an N-terminal TM and TM2-TM5 while the AGPv4 version had TM2-TM5 and a C-terminal TM predicted. Combined into one gene (as the material is present in the genome), six transmembrane domains could be present.

The three other fragments resembling ZMSIP1;1 all aligned with the first half of the protein, including TM1, TM2, Loop B and the first NPA motif, and TM3. Frag020 had loop C alignment as well, and with extensions added to the predicted genes, Frag001 and Frag007 did as well (**Figure 2.15**). Frag001 and Frag007 had C-terminal extensions that included putative protein kinase domains based on InterPro scans.

Of the four fragments associated with genes, three (Frag002, Frag003 and Frag005) had very high similarity to the start of exon 3 of ZmTIP4;2b (**Figure 2.16**). These fragments were 106-107 amino acids in length and covered from loop C to the start of TM6 (including loop E with the second NPA motif). The final fragment associated with a gene aligned well with exon 2 of ZmNIP2;1 (**Figure 2.17**). This includes TM1, TM2 and loop B with the first NPA motif. A thirst TM domain is predicted by Phyre2, but does not align with aquaporins. Both the N and C-terminals of the gene do not align with aquaporins.

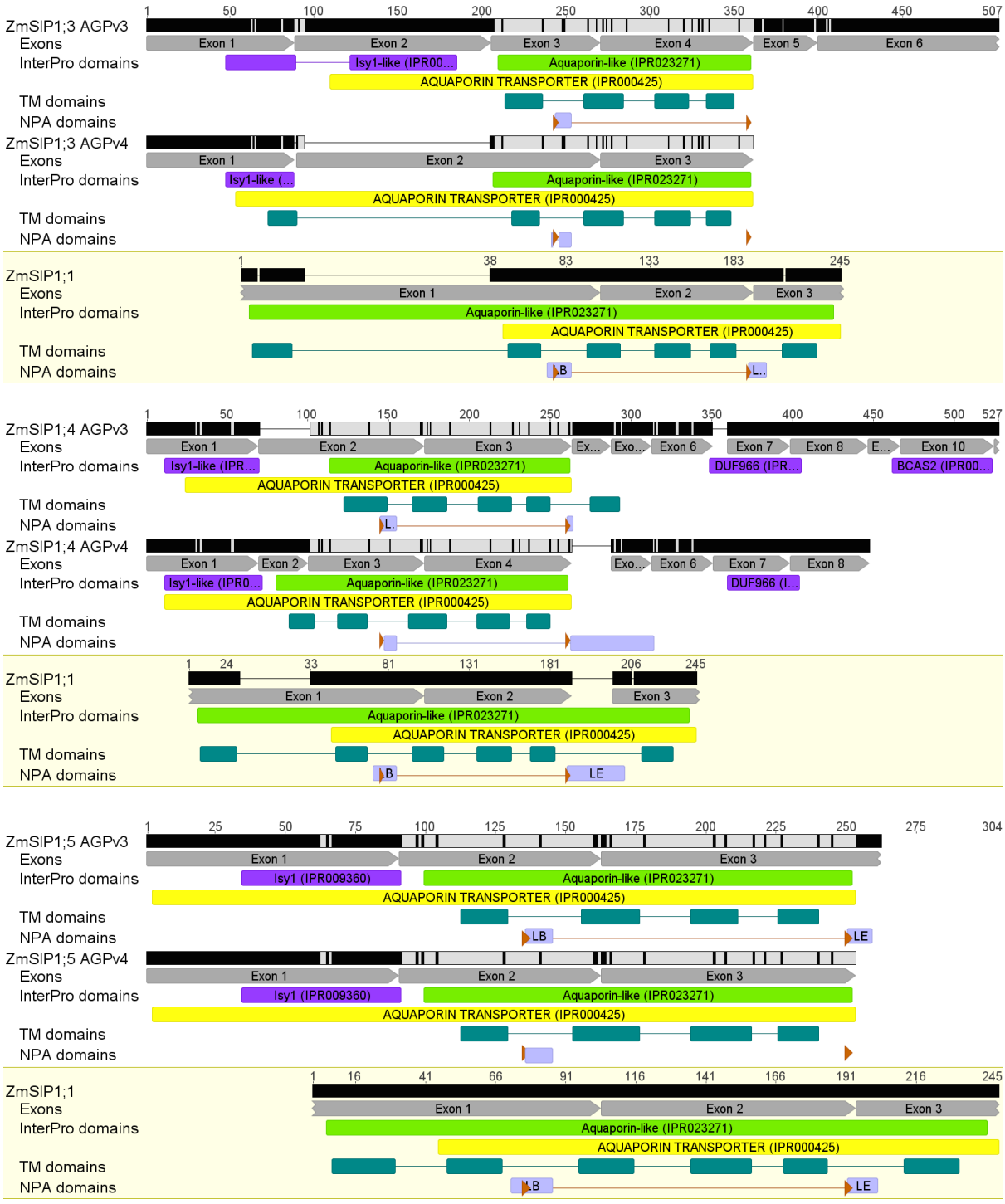


Figure 2.14 Protein alignments of ZmSIP1;1 and fragments containing the ISY1-like domain

ZmSIP1;1 with the fragments ZmSIP1;3, ZmSIP1;4 and ZmSIP1;5. Documented were the annotations from both AGPv3 and AGPv4. These fragments contain both NPA domains, and at least four TM helices that align well to ZmSIP1;1. The N-terminus has an ISY1-like domain. Highlighted are the major domains from InterPro, the predicted exon structure from the genome annotation, the TM domains from Phrye2 and the NPA domains.

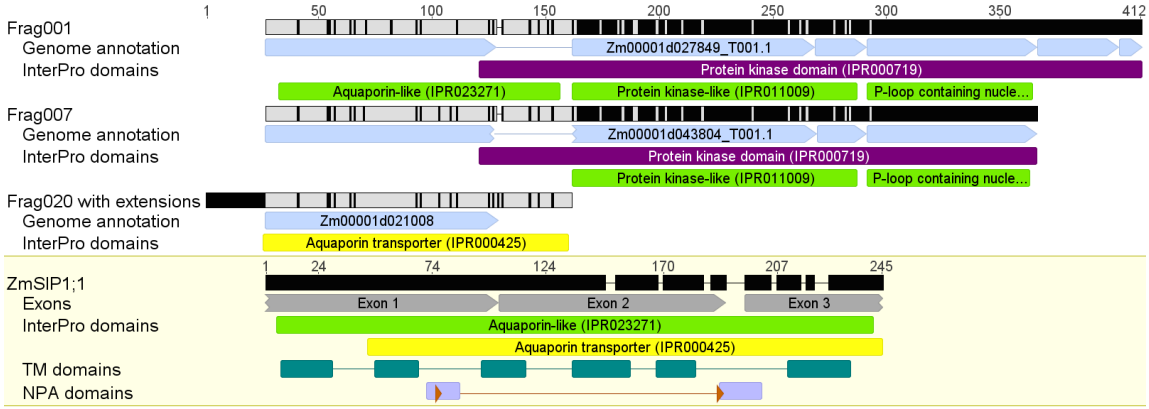


Figure 2.15 Protein alignments of ZmSIP1;1 and three fragments with predicted genes
Frag001, Frag007, and Frag020 had extensions from their predicted genes (light blue). These three aligned well to the first half of ZmSIP1;1. Frag001 and Frag007 had an additional protein kinase domain at the C-terminus (InterPro domains IPR000719, IPR011009, and IRP027417).

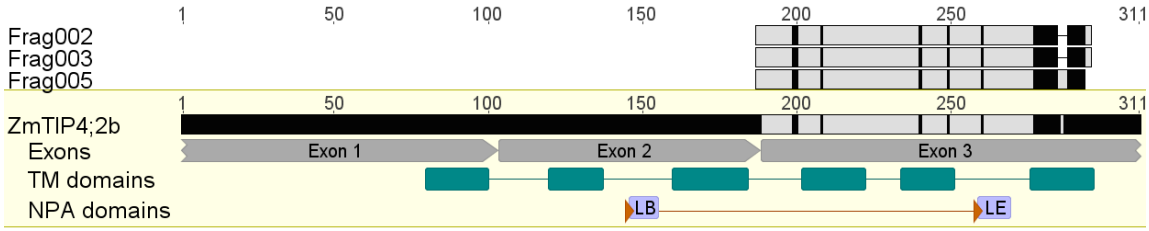


Figure 2.16 Protein alignment of ZmTIP4;2b and three fragments
Frag002, Frag003 and Frag005 are very similar and very similar to the first part of exon 3 in ZmTIP4;2b. These were predicted to be genes in the genome, but did not encode the first or final parts of the protein within 5000 bp upstream or downstream of the respective genes.

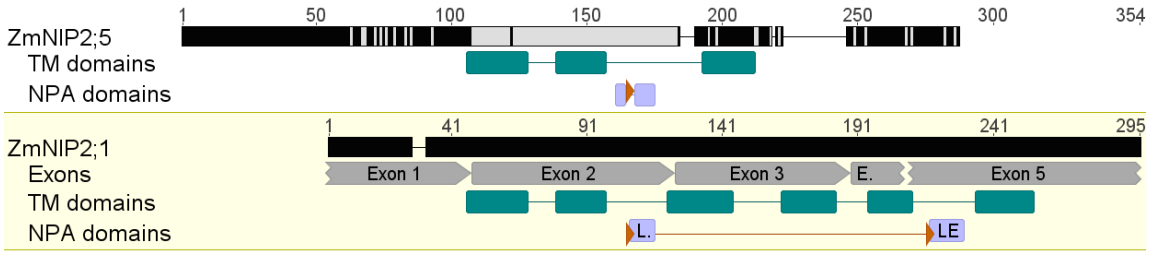


Figure 2.17 Protein alignment of ZmNIP2;1 and fragment ZmNIP2;5
ZmNIP2;5 is a gene with an aquaporin fragment similar to ZMNIP2;1. It has N and C terminus extensions that do not align to aquaporins.

The other 11 fragments were not associated with genes. Four fragments, Frag026, Frag045, and Frag046, covered parts of the third exon of ZmPIP1;7, a region with TM5 and the second NPA motif (**Figure 2.18**). Three fragments (Frag024, Frag027 and Frag028) resembled part of exon 2 of ZmTIP3;4. This region included part of TM2, the first NPA motif and TM3 (**Figure 2.19**). Two fragments covered parts of ZmSIP1;1 (**Figure 2.20**). Frag021 covered exon 1 and exon 2, including the N-terminal to loop E, including the second NPA motif. This fragment however contained multiple stop codons, preventing it from being an active gene. Frag050 also aligned with ZmSIP1;1, including TM5 and the second NPA motif. The remaining two fragments aligned with ZmNIP1;2 (**Figure 2.21**). Frag029 covered exon 4, including the second NPA motif and TM6. Frag047 covered parts of exon 2 and exon 4, including parts of TM2, TM3, TM4 and TM6, but due to gaps, neither NPA motif was covered.

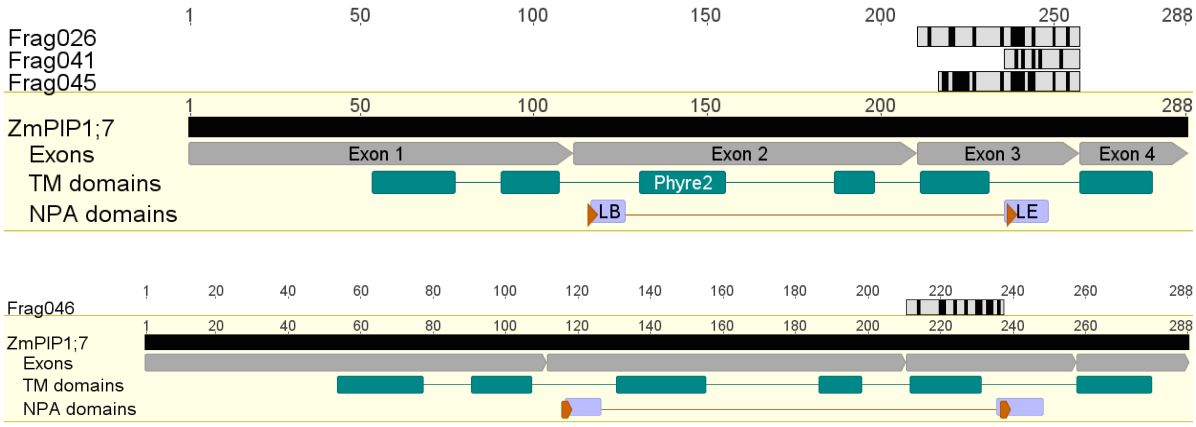


Figure 2.18 Protein alignments of ZmPIP1;7 and four fragments.
Each of these four fragments aligns to part of exon 3 of ZmPIP1;7. These fragments did not have a predicted gene associated with them, and no additional aquaporin alignments were within 5,000 bp upstream or downstream of the fragment location.

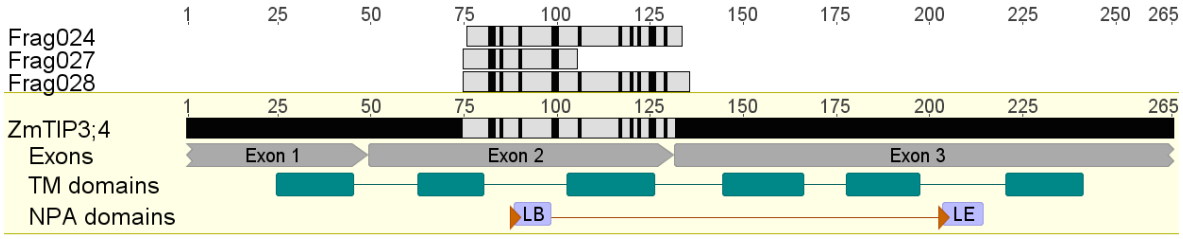


Figure 2.19 Protein alignments of ZmTIP3;4 and three fragments
These fragments share similarity to exon 2 of ZmTIP3;4.

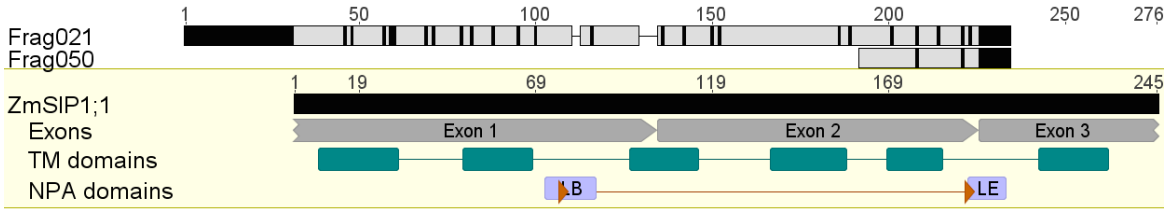


Figure 2.20 Protein alignment of ZmSIP1;1 and two fragments
Frag021 has three stop codons.

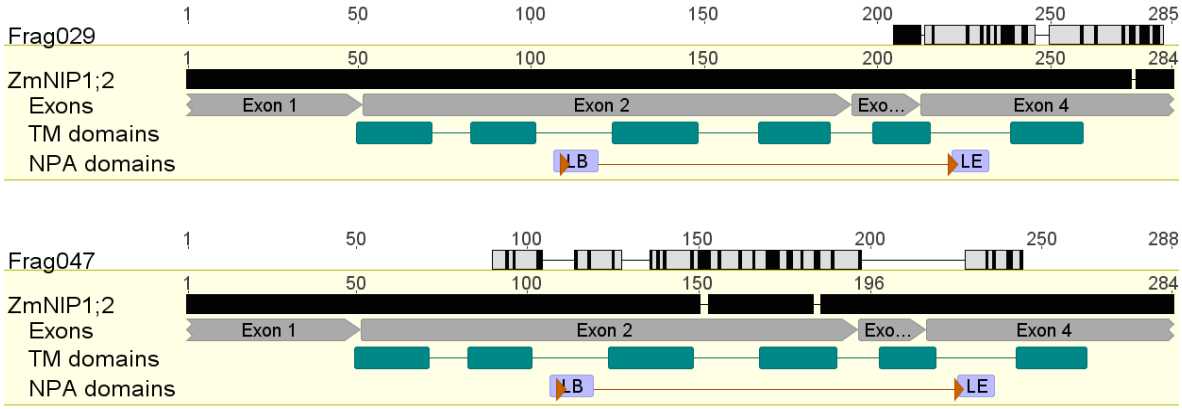


Figure 2.21 Protein alignments of ZmNIP1;2 and fragments
Frag029 and Frag047 did not have genes associated with them. Frag029 is similar to exon 4 of ZmNIP1;2. Frag047 is similar to around 150 amino acids, but has significant gaps, including both NPA domains.

2.4 Discussion

Mining the maize genome for aquaporins revealed nine aquaporins that had not been described or documented before. This was not entirely unexpected. Most maize aquaporins were described by Chaumont et al. (2001) by screening EST libraries. The authors were unable to clone full length aquaporin genes of several partial genes (one ZmPIP, two ZmTIPs, and one ZmNIP), attributing it to potential cloning problems (such as being toxic to *Escherichia coli*). In addition, ZmNIP ESTs were rare, just 38 of 1319 of the aquaporin ESTs collected. In comparison to *Arabidopsis*, Johanson et al. (2001) suggested more maize aquaporins could be found after the genome was published.

Interestingly, it was found that ten of the aquaporin sequences in the B73 genome had different sequences to those in Chaumont et al. (2001). The source of the cDNA libraries used was not explicitly stated. These differences could be because the B73 inbred line was related but distinct from the varieties used to construct the cDNA libraries used to screen the ESTs. With multiple variations of the same gene (ZmPIP1;3/ZmPIP1;4 and

ZmPIP1;5/ZmPIP1;b) known before this search, there seem to be some genetic variation for individual genes. In rice, genomes are available for two varieties. These have been examined for the aquaporin sets, but they were not found to be 100% identical (Bansal and Sankararamakrishnan 2007; Sakurai et al. 2005). Maize is known to have high genetic diversity among its various lines and may also have differences in aquaporins, both in sequence and amount.

Chaumont et al. (2001) described a ZmSIP cDNA with "exonic sequences of an unrelated gene". It did not appear to be a cloning artefact, as it was independently found in several libraries from the variety 'Black Mexican Sweet'. This cDNA had several stop codons and was not included in their analysis. The B73 genome also had several aquaporin fragments with predicted genes that had non-aquaporin domains based on InterPro scans, but did not have multiple stop codons. It remains to be seen if these are active genes in B73 that may have been lost in other maize varieties, or if they are the result of some sort of sequencing error. While RNA-Seq data sets are available, reconstructing transcripts from short reads is difficult with current technology (Conesa et al. 2016).

While the maize genome was found to have 43 aquaporin sequences, the expression levels for most are unknown, especially under nitrogen stress. A comparative RNA-Seq analysis picked up decreased expression for ZmPIP2;8 (GRMZM2G432926) and ZmTIP1;2b (GRMZM2G168439) from maize transcriptomes under reduced N (4 mM nitrate to 0.04 mM nitrate) at 12-96 hours after treatment (He et al. 2016), however RNA-Seq can be limiting, especially when in families of similar sequences (Conesa et al. 2016), as is seen with the ZmPIPs (Chaumont et al. 2001). Baseline B73 ZmPIP (not including ZmPIP2;8, ZmPIP2;9 or ZmPIP3;1) gene expression has been established for roots and leaves (Hachez et al. 2008; Hachez et al. 2006), and Górska et al. (2008) examined root

expression of the same set of ZmPIPs under nitrate stress in a hybrid variety "Hybrid corn-white sweet". While they were unable to correlate changes in ZmPIP expression with changes in water conductivity through maize roots under changing nitrogen conditions, the variety of maize used was unusual and the content of the nutrient solution used had potential differences under nitrate supply and nitrate starvation. In addition, very little is known about other maize subfamily gene expression under any conditions.

2.5 Conclusion

There were 43 full-length aquaporin genes found in the maize B73 genome. These differed, both in number and in sequence to those previously described by other studies. The next steps of this study are to examine the actual composition of B73 aquaporins through cloning and sequencing, look at the basis of water transport through these aquaporins, to create gene expression probes specific to each aquaporin sequences and monitor expression of the aquaporin gene family in maize under nitrogen stress.

2.6 Appendix

2.6.1 FASTA sequences used in this chapter

The protein and nucleotide sequences used are provided in FASTA format for independent use. AGPv4 sequences were provided, except where open reading frame was in conflict with AGPv3, where both were provided.

2.6.1.1 Protein Sequences

>ZmNIP1;1 Zm00001d016237_T001.1
MAGGGDHSQTNGGHVDQRALEEGRKEEFADQGCAAMVVSVPFIQKIIAEIFGTYFLMFAGCGA
VTINASKNGQITFPGVAIVWGLAVMVMVYAVGHISGAHFNPAVTLAFATSGRFPWRQLPAYVL
AQMLGATLASGTLRLMFGGRHEHFPGTLPTGSEVQSLVIEIITTFYLMFVISGVATDNRAIGELAG
LAVGATILLNVLIAGPVSGASMNPARSVGPALVSGEYTSIWVYVVGPVVGAVAGAWAYNLIRFT
NKPLREITKSTSFLKSTSRMNSAASA

>ZmNIP1;2 Zm00001d037733_T001.1
MAGAEVANGVHDGALDLEEGRGGGVDGAGCESSEQDGAGRRPMFSVPFVQKILAEALGTYFLI
FAGCAAVAVNLRTGGTVTFPGICAVWGLAVMVMVYSVGHISGAHLNPAVSLAFATCGRFPWR
QVPAYAAAQVTGATAASLTLRLLFGSAREHFFGTVPAGSDAQSLVVEFIISFNLMFVVSGVATDN
RAIGELAGLAVGATVLLNVLFAGPISGASMNPARTLGPALVVGRYAGIWVYFAGPICGTVAGAW
AYNLIRFTDKPLREITQTSSFLRSVRRSSS

>ZmNIP1;3 Zm00001d046303_T001.1
MARREDDSYTNASVFETSVEDGRKDKSESYAVDEPPQPVDDALCGMSTSVSFIQQLIAEFLATFF
LIFAGCGVIAVNDKNGMATFPGIAVVWGMVVMAMIYAVGHVSGAHINPAVPAYMLVQTVAAT
MASLVLRLMFGRQHELASVTVPAPGGSIFQSLVLEFIITFYLMFVVMAVATDDRAVGQMAGLAV
GGTIMLNALFAGSEVSRFFRSIAMGRPVSGASMNPARSIGPALVSNKFRALWVYIFGPFAGAAAG
AWAYNLIRHTDKTLAEEHEPNVWENLKETNVAKMLDVYGIIFLEHEPNVWKKLNETDVINP

>ZmNIP2;1 Zm00001d018037_T001.1
MSTNSRSNSRANFNNEIHDIGTAQNSSMPPTYYDRSLADIFPPHLLKKVVSEVVSTFLLVFVTCGA
AGIYGSDKDRISQLGQSVAGGLIVTVMIYAVGHISGAHMNPAVTLAFAVFRHFPWIQVPFYWAA
QFTGSICASFVLKAVLHPIAVLGTTTPTGPHWHSLVIEIIVTFNMMFVTLAVATDTRAVGELAGLA
VGSAVCITSIFAGAVSGGSMNPARTLGPALASNLYTGLWIYFLGPVLGTLSGAWTYTYIRFEEAP
SHKDMSQKLSSFKLRRLQSQSVAVDDDELDHIQV

>ZmNIP2;2 Zm00001d037228_T001.1
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VTCGAASIYGEDNRRISQLGQSVAGGLIVTVMIYATGHISGAHMNPAVTLSFACFRHFPWIQVPF
YWAAQFTGAMCAAFVLKAVLHPIAVIGTTTPSGPHWHALLIEIVVTFNMMFVTCAVATDSRAV
GELAGLAVGSAVCITSIFAGPVSGGSMNPARTLAPAVASNVFTGLWIYFLGPVIGTLSGAWVYTY
IRFEEAPAAKDTQRLSSFKLRRMQSQLAADEFDTV

>ZmNIP2;3_ Zm00001d044826_T001.1
MAASTTSRTNSRVNYSNEIHDLSTVQGGSAAAAAAALFYPDSKSIADIFPPHLGKKVISEVVATFL
LVFVTCGAASIYGEDNARISQLGQSVAGGLIVTVMIYATGHISGAHMNPAVTLSFACFRHFPWIQ
VPFYWAAQFTGAMCAAFVLKAVLQPIAVIGTTTPSGPHWHALAIEIVVTFNMMFVTCAVATDSR
AVGELAGLAVGSAVCITSIFAGPVSGGSMNPARTLAPAVASNVFTGLWIYFLGPVVGTLSGAWV
YTYIRFEEAPAAAKPDTQRLSSFKLRRMQSQSALAADEFDTV

>ZmNIP2;4 Zm00001d052261_T001.1
MSTNSRANSRANFNNEIHDIGTAVHNSSSLPPAYYDRSLADMFPPHLLKKVVSEVVSTFLLVFVT
CGAAGIYGSDKDRISQLGQSVAGGLIVTVMIYAVGHISGAHMNPAVTLAFAVFRHFPWIQVPFY
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Chapter 3: Cloning of maize aquaporins and water channel characterisation in *Xenopus laevis* oocytes

3.1 Introduction

In Chapter 2, the genome of maize inbred line B73 revealed 43 aquaporins, and showed potential variation not only with known maize aquaporins (Chaumont et al. 2001), but also between genome versions (Deshmukh et al. 2015). The aromatic/Arginine (ar/R) sequence of each was obtained. The ar/R sequence has been proposed to be a major determinate of water and solute permeability though the pore of each aquaporin monomer (Wallace and Roberts 2004; Bansal and Sankararamakrishnan 2007). Many studies have established water permeability for PIPs, including those from maize, but experimental evidence for other subfamilies and ar/R filters is limited.

AQP1 was the first aquaporin shown to be water permeable through swelling assays performed on heterologous gene expression in *Xenopus laevis* oocytes (Preston et al. 1992). The first plant aquaporin, AtTIP1;1, was also characterised through heterologous expression in *Xenopus* oocytes (Maurel et al. 1993). Heterologous expression in *Xenopus* oocytes has been used to show many activities and regulatory measures of plant aquaporins, including PIP gating through increased cytosolic pH (Tournaire-Roux et al. 2003), PIP1 and PIP2 interactions (Fetter et al. 2004; Zelazny et al. 2007; Zelazny et al. 2009) and solute transport, such as boron (Schnurbusch et al. 2010). The set of experiments in this chapter were designed to clone aquaporins from maize (*Zea mays*) to compare sequences predicted by the published B73 genome and those previously known

(Chaumont et al. 2001; Deshmukh et al. 2015).The water channel activity and the effects of nitrate and low pH were determined through heterologous expression in *Xenopus laevis* oocytes.

3.2 Methods

3.2.1 Cloning maize aquaporins

3.2.1.1 cDNA synthesis

Maize RNA was isolated from root tissue and shoot tissue of three week old maize plants of the inbred line B73. Tissues were collected in liquid nitrogen and stored at -80°C. Tissues were ground in liquid nitrogen and powder stored at -80°C. 50-100mg of frozen tissue was combined with 1 mL of Trizol (Thermo Fisher Scientific, Waltham, MA USA), vortexed and rested at room temperature for 5 min. 200 µL chloroform was added, vortexed and allowed to sit at room temperature for 3 min. Samples were centrifuged at 14000g for 15 minutes. All centrifugation was done at 4°C. The aqueous top phase containing RNA was removed and transferred to a fresh tube. 500µL of isopropanol was added to precipitate the RNA and allowed to sit at room temperature for 15 min. The sample was centrifuged at 14000g to pellet the RNA, and the supernatant removed. RNA was then washed with 1mL of 75% RNase-free ethanol and centrifuged at 7500g for 5 minutes. The RNA pellet was washed again using 500 µL RNase-free water (treated with DEPC), 50 µL of 3M sodium acetate and 1 mL of ethanol. Mixture was vortexed and put in a -20°C freezer overnight. The samples were then centrifuged for 15 minutes at 14000g, discarding the supernatant. Pellet was washed with 75% RNase-free ethanol, vortexed, and centrifuged at 7500g for 5 minutes. The pellet was dried at 37°C, then dissolved in 50 µL DEPC-treated water. Samples were frozen at -20°C, defrosted, and checked for RNA amount using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham,

MA USA). RNA concentration was determined by measuring absorbance at 260nm and 280nm, then applying the formula: $A_{260} \cdot \text{dilution} \cdot 40$ to get µg/mL RNA. The absorbance ratio $A_{260/280}$ was between 1.80 and 2.0 for all samples.

The RNA was used to generate cDNA via reverse transcriptase using SuperScript III (Thermo Fisher Scientific, Waltham, MA USA). 1µg RNA was combined with 50 µM oligo$(dT)_{20}$ primer, 10 mM dNTP mix, then heated at 65°C for 5 min. 10 µL cDNA Synthesis mix was then added composed of 2 µL (10x) RT buffer, 4 µL (25 mM) $MgCl_2$, 2 µL (0.1 M) DTT, 1 µL (40units/µL) RNaseOUT, and 1 µL SuperScript III RT. Samples were gently mixed and incubated 50 minutes at 50°C. Reactions were terminated by heating at 85°C for 5 minutes, then chilled on ice. RNA templates were removed by adding 1 µL RNase H and incubating for 20 minutes at 37°C. cDNAs were stored at -20°C.

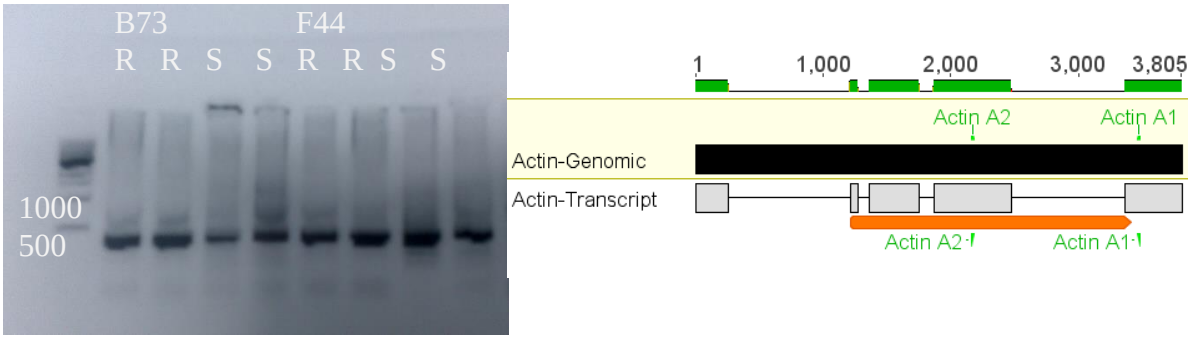


Figure 3.1 cDNA tested for genomic DNA contamination
Actin primers were designed to amplify across an intron (right). The cDNAs were amplified using the actin primers and run on an agarose gel (R=root, S=shoot). From the lack of band at 1325 and the presence of band just below 500bp, the cDNAs were assumed to be free of genomic DNA contamination. (Orange bar = coding region, green ticks = primers)

Table 3.1 Primers used to test quality of cDNA

Primers used to target an exon boarder in the actin gene. Both gene annotations, AGPv3 and AGPv4, were identical near where the primers bound. Both predicted identical three splice variants, but all transcripts were identical between the primer bind sites.

Primer Name	Sequence (5'→3')	Target	cDNA product length	Genomic DNA product length
ActinA1	ACGCCTGACGTACCATGTCGA	GRMZM2G126010 (AGPv3) Zm00001d01059 (AGPv4)	439 bp	1325 bp
ActinA2	AGGTTTAGGTGCCCCGAGGTG			

The cDNAs were tested for genomic DNA contamination by amplifying across an exon boarder in the gene encoding actin (GRMZM2G126010 in AGPv3, Zm00001d010159 in AGPv4). Actin primers (**Figure 3.1**) were designed to amplify across an 886 bp intron of genomic DNA. A PCR was performed using *Taq* DNA polymerase (New England BioLabs, Ipswich, MA USA) with primer annealing temperature of 58°C. cDNAs were determined to be free of genomic DNA contamination (band size in **Figure 3.1**).

3.2.1.2 Maize aquaporin cloning

Primers for maize aquaporins were designed, targeting just the open reading frame of the maize cDNA (**Table 3.5**). Due to poor primer options to include the open reading frame, several were first amplified using primers in the 5' and 3' UTRs (**Table 3.6**). These reactions were then used as the template to amplify the open reading frame of the aquaporin.

Primers were first checked for amplification product and tested at various annealing temperatures if needed using *Taq* DNA polymerase (New England BioLabs, Ipswich, MA USA) using the recommended protocol. When suitable primers and annealing temperatures were found, the primers were used with a high fidelity DNA polymerase: Platinum *Taq* High Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, MA USA) or Q5 High Fidelity DNA Polymerase (NEB). All amplifications were done using

the manufactures recommended protocols. As Platinum *Taq* High Fidelity DNA Polymerase left 5' dA overhangs, the reaction product could directly be used in a TOPO cloning reaction. For Q5 High Fidelity products, dA overhangs were added after removing the Q5 enzyme (due to its proofreading ability, Q5 degrades dA overhangs). First, the PCR product was precipitated (0.05 volume 0.5 M EDTA, 0.10 volume 3 M sodium acetate, 2 V 100% ethanol stored at -20° overnight), centrifuged (15 min maximum speed) and supernatant discarded, washed (30 µL 70% ethanol) and centrifuged, air dried, and finally reconstituted in water (20 µL). A overhangs were then added with *Taq* DNA polymerase (20 µL reconstituted PCR product, 1.5 µL water, 2.5 µL ThermoPol buffer, 0.5 µL 50 mM dATP, 1.0 µL *Taq* for 10 min at 68°C).

Table 3.2 Reaction conditions and PCR protocol for Taq DNA Polymerase

PCR conditions used for *Taq* DNA Polymerase. Reaction conditions and PCR cycle used. T_m is the annealing temperature found in **Table 3.5** or **Table 3.6**. Total reaction volume was 25 µL. Reagents were prepared as a master mix, and combined with primers and cDNA template.

Reagent	Volume	Final Concentration
H_2O	19.9 µL	-
10X ThermoPol Buffer	2.5 µL	1X
10 mM dNTP mix	0.5 µL	200 µM each
10 µM forward primer	0.5 µL	200 nM
10 µM reverse primer	0.5 µL	200 nM
cDNA template	1.0 µL	-
Taq DNA Polymerase	0.125 µL	0.625 U/rxn

Stage		Temperature (°C)	Time (mm:ss)
Initial Denaturation		95.0	00:30
35 x	Denature	95.0	00:30
	Anneal	T_m	00:30
	Extend	68.0	01:30
Final Extension		68.0	5:00

Table 3.3 Reaction conditions and PCR protocol for Platinum Taq DNA Polymerase High Fidelity

PCR conditions used for Platinum *Taq* DNA Polymerase High Fidelity. Reaction conditions and PCR cycle used. T_m is the annealing temperature found in **Table 3.5** or **Table 3.6** total reaction volume was 25 µL. Reagents were prepared as a master mix, and combined with primers and cDNA template.

Reagent	Volume	Final Concentration
H_2O	18.9 µL	-
10X High Fidelity Buffer	2.5 µL	1X
50 mM $MgSO_4$	1.0 µL	2.0 mM
10 mM dNTP mix	0.5 µL	0.2 mM each
10 µM forward primer	0.5 µL	0.2 µM
10 µM reverse primer	0.5 µL	0.2 µM
cDNA template	1.0 µL	-
Platinum *Taq* DNA Polymerase High Fidelity	0.1 µL	1 U/rxn

Stage		Temperature (°C)	Time (mm:ss)
Initial Denaturation		94.0	00:30
35 x	Denature	94.0	00:30
	Anneal	T_m	00:30
	Extend	68.0	01:00-01:30
Final Extension		68.0	5:00

Table 3.4 Reaction conditions and PCR protocol for Q5 High Fidelity DNA Polymerase

PCR conditions used for Q5 High Fidelity DNA Polymerase. Reaction conditions and PCR cycle used. T_m is the annealing temperature found in **Table 3.5** or **Table 3.6**. Total reaction volume was 25 µL. Reagents were prepared as a master mix, and combined with primers and cDNA template.

Reagent	Volume	Final Concentration
H_2O	16.75 µL	-
5X Q5 reaction buffer	5.0 µL	1X
10 mM dNTP mix	0.5 µL	0.2 mM each
10 µM forward primer	1.25 µL	0.5 µM
10 µM reverse primer	1.25 µL	0.5 µM
cDNA template	1.0 µL	-
Q5 High Fidelity DNA Polymerase	0.25 µL	0.5 U/rxn

Stage		Temperature (°C)	Time (mm:ss)
Initial Denaturation		98.0	00:30
35 x	Denature	98.0	00:15
	Anneal	T_m	00:30
	Extend	72.0	01:00
Final Extension		72.0	2:00

Table 3.5 Primer sequences and PCR condidtions used to amplify maize aquaporin open reading frames

Aquaporins were amplified from cDNA or purified PCR product of a previous reaction (**Table 3.6**). Primers (except ZmTIP4;4b) were tested for amplification using *Taq* DNA Polymerase (NEB). After verification that the produced the correct product size, a PCR using a high fidelity enzyme was used. Primers were selected that amplified from the start codon (first three nucleotides 5'→3') to the stop codon (first three codonds 5'→3' are antisense stop codon) so only the open reading frame was amplified. Band size for full length transcripts amplified by the primers as predicted by AGPv3 and AGPv4 genomes (only one listed if identical or AGPv3/AGPv4 if different). Platinum *Taq* = Platinum *Taq* High Fidelity (Invitrogen), Q5 HF = Q5 High Fidelity Polymerase (NEB)

#	Target	AGPv3 Target	AGPv4 target	Forward Primer (5'→3')	Reverse Primer (5'→3')	Taq Tm (°C)	High Fidelity DNA polymerase	HF Tm (°C)	Band Size (AGPv3/ AGPv4
1	ZmPIP1;2b	AC209208.3_FGT002	Zm00001d017526_T001.1	ATGGAGGGGAAGGAGGAGGAC	TTAAGACCTGCTCTTGAACGGGATG	55.0	Platinum *Taq*	55.0	870
2	ZmPIP1;4	GRMZM2G392975_T01	Zm00001d051403_T001.1	ATGGAGGGGAAGGAGGAGGAC	TTAAGACCTGCTCTTGAACGGGATG	55.0	Platinum *Taq*	55.0	879
3	ZmPIP1;5	GRMZM2G081843_T01	Zm00001d051872_T001.1	ATGGAGGGCAAGGAGGAGG	TCAGTCACGGCTCTTGAAGG	55.0	Platinum *Taq*	55.0	867
4	ZmPIP1;6b	GRMZM2G136032_T01	Zm00001d020383_T001.1	ATGGCAGGAGGCACGCT	CTAGTAGTGAGCGCTGGACTTGA	55.0	Platinum *Taq*	55.0	891/960
5	ZmPIP2;1	GRMZM2G014914_T02	Zm00001d019563_T006.1	ATGGGCAAGGACGACGTGA	TCACGCGTTGCTCCTGAA	55.0	Platinum *Taq*	55.0	873
6	ZmPIP2;2	GRMZM2G092125_T01	Zm00001d005421_T001.1	ATGGGCAAGGACGACGTG	TCACGCGTTGCTCCTGAA	55.0	Platinum *Taq*	55.0	879
7	ZmPIP2;3	GRMZM2G081192_T01	Zm00001d051174_T002.1	ATGGCGAAGCAGGACATCGAA	TTAGGCGTTGCTCCGGTAGG	55.0	Platinum *Taq*	55.0	870
8	ZmPIP2;4	GRMZM2G154628_T01	Zm00001d017288_T001.1	ATGGCGAAGGACATCGAGGCA	TTAGGCGTTGCTCCGGTAGGAC	55.0	Platinum *Taq*	55.0	867
9	ZmPIP2;5	GRMZM2G178693_T01	Zm00001d003006_T001.1	ATGGCCAAGGACATCGAGGC	CTAGCGGCTGAAGGAGGCAG	55.0	Platinum *Taq*	55.0	858
10	ZmPIP2;6	GRMZM2G047368_T02	Zm00001d019565_T001.1	ATGGGCAAGGAGGTGGATGTGTC	CTAGGCGTTGCTCCGGAAGG	55.0	Platinum *Taq*	55.0	867
11	ZmTIP1;2b	GRMZM2G168439_T01	Zm00001d011778_T001.1	ATGCCTGTGAGCAGGATCG	TCAGTAGTCTGCTGCCGTG	55.0	Platinum *Taq*	55.0	765
12	ZmTIP2;1	GRMZM2G027098_T01	Zm00001d051362_T001.1	ATGGTGAAGCTCGCCTTCGG	TTAGGCGTAGTCCTGGTCCGC	55.0	Platinum *Taq*	55.0	750
13	ZmTIP4;1b	GRMZM2G103945_T03	Zm00001d037779_T001.1	ATGGCGAAGCTCATGAACAA	TCAGACTTCCCCATTGAGCA	55.0	Platinum *Taq*	55.0	768
14	ZmTIP4;4b	GRMZM2G093090_T01	Zm00001d039293_T001.1	ATGTATCCGTCTCCCCCTCC	CTAGAAGTCGGTGTCATCCCTG	n/a	Q5 HF	68.0	864
15	ZmNIP1;1	GRMZM2G041980_T02	Zm00001d016237_T001.1	ATGGCCGGAGGCGGAG	TCAGGCGGAGGCGGC	60.0	Platinum *Taq*	64.0	849
16	ZmNIP2;1	GRMZM2G028325_T01	Zm00001d018037_T001.1	ATGTCGACCAACTCGAGGTCCAA	TCACACTTGGATGTGGTCGAGC	55.0	Platinum *Taq*	55.0	888
17	ZmNIP2;2	GRMZM2G137108_T01	Zm00001d037228_T001.1	ATGGCCGCCGCCTC	TTAGACGGTGTCGAACTCGT	55.0	Platinum *Taq*	55.0	885
18	ZmNIP3;1	GRMZM2G176209_T01	Zm00001d032461_T001.1	ATGGAGCCGGGGTCG	TCAGCGGCGGAAGCT	55.0	Platinum *Taq*	55.0	909
19	ZmSIP1;1	GRMZM2G113470_T01	Zm00001d048959_T002.1	ATGGCGATGGGAGCGA	TCATGCTTTCTTGGTCTTGGG	55.0	Platinum *Taq*	55.0	738/708
20	ZmSIP1;2	GRMZM2G060922_T01	Zm00001d010325_T001.1	ATGGCGATGGGGGAGG	TCATGCTTTCTTGGCCTTGG	63.0	Platinum *Taq*	63.0	732
21	ZmSIP2;1	GRMZM2G175038_T01	Zm00001d028951_T001.1	ATGTCACCGGCGCC	CTATTCCTTCTTTATCTTGTTTTCATCTAC	55.0	Platinum *Taq*	55.0	750

Table 3.6 Preliminary amplication of selected aquaporins from UTRs

Several primers listed in **Table 3.5** were not able to amplify the aquaporin open reading frame from the cDNA. A second primer pair was designed (above) to amplify in the 5' and 3' UTR (only 5' UTR for ZmPIP2;2). These primers were tested, then used to amplify with a High Fidelity DNA polymerase. These reactions were then used as the template to amplify the open reading frame (**Table 3.5**). For ZmSIP2;1, primers only targeted AGPv3, not AGPv4. Platinum Taq = Platinum Taq High Fidelity (Invitrogen), Q5 HF = Q5 High Fidelity Polymerase (NEB)

#	Target	AGPv3 Target	AGPv4 target	Forward Primer, 5'→3' (bp of 5' UTR amplified)	Reverse Primer , 5'→3' (bp of 3' UTR amplified)	*Taq* Tm	HF Polymerase	HF Tm	Band size (bp)
1	ZmPIP2;2	GRMZM2G092125_T01	Zm00001d005421_T001.1	TCACGCACTCATCCATTCGG (94)	TCACGCGTTGCTCCTGAA (0)	55.0	Q5 HF	68.0	973
2	ZmNIP1;1	GRMZM2G041980_T02	Zm00001d016237_T001.1	ACAGGGAGAGACGCAGAAGAGAC (44)	GGCTAAGCACAGTGATGCGTATG (294)	60.0	Platinum *Taq*	55.0	1187
3	ZmNIP3;1	GRMZM2G176209_T01	Zm00001d032461_T001.1	AGAGAGAGCTAGCAGTTACA (47)	GACAATGTCTCGCGCC (34)	55.0	Q5 HF	68.0	990
4	ZmSIP2;1	GRMZM2G175038_T01	Zm00001d028951_T001.1	TTGGTGGCTTACTGGCTTGC (99)	AACATTTCGCCTTGTGTGTAGC (79)	55.0	Q5 HF	68.0	928

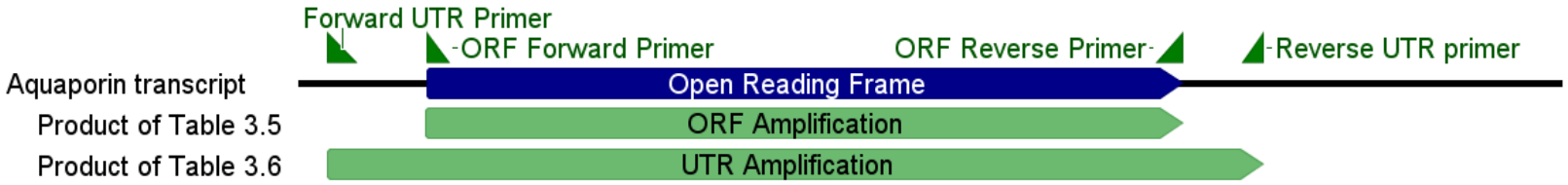


Figure 3.2 Location of primers used for amplication

Generic amplification of maize aquaporin transcripts. ORF primers (**Table 3.5**) amplified the open reading frame only of the aquaporin. The UTR (untranslated region) primers (**Table 3.6**) amplified the open reading frame and 5' and/or 3' UTR.

High fidelity PCR products with dA overhangs were cloned into a pCR8/GW/TOPO vector (Thermo Fisher Scientific, Waltham, MA USA) using a TOPO reaction. The pCR8/GW/TOPO vector contained a bacterial spectinomycin resistance gene. 0.5 µL of PCR product was combined with 1 µL salt solution, 4.0 µL water and 0.5 µL TOPO vector and reacted at room temperature 5-10 min. Half (3µL) of the reaction was immediately used to transform chemically competent *E. coli*, either OneShot Top10 Chemically competent *E. coli* (Thermo Fisher Scientific, Waltham, MA, USA) or XL1-blue *E. coli* (Agilent, Santa Clara, CA USA). Transformations were performed by placing 3 µL of the TOPO reaction into 50 µL *E. coli* on ice and heat shocking the bacteria at 42°C for ~40s. Bacteria were rested on ice for 15 minutes, combined with 250 mL SOC media (at room temperature) then incubated (shaking) for 1 hour at 37°C.

Bacteria were then plated onto a LB agar plates with 100µg/mL spectinomycin to select for transformed cells and grown overnight at 37°C. Single colonies were selected and grown in liquid LB media overnight also containing 100µg/mL spectinomycin.

Plasmids were harvested from the LB media using am EZ-10 Spin Column kit (Bio Basic, Markham, ON, Canada). A 2 mL aliquot of bacterial suspension was placed in a 2 mL microcentrifuge tube and spun at 12000 RPM for 2 min to pellet the cells. 100 µL of Solution I was added and vortexed to dissolve the pellet and allowed to rest for 1 minute. 200 µL of Solution II was added and mixed gently. Finally 350 µL of Solution was added and gently mixed. The solution was centrifuged at 12000 RPM. The supernatant was transferred to an EZ-10 Spin Column and spun at 10000 RPM for 2 minutes. The flow through was discarded the column twice washed with Wash Solution, discarding flow through and centrifuging at 10000 RPM for 2 min. A final spin was performed to remove residual wash solution for 1 min at 10000 RPM. The column was transferred to a fresh

1.5 mL microcentrifuge tube and the plasmid eluted with 50 µL Elution buffer (preheated to 60°C). A NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) was used to determine final plasmid concentration and purity. Plasmids were stored at -20°C.

Size and orientation of aquaporin gene inserts in plasmids were determined using restriction enzymes. A restriction enzyme was selected to cut the plasmid at least twice, once in the backbone and once in the insert such that two distinct patterns of digestion were possible. Protocols from the manufacturer were performed for each restriction enzyme. Fragments were run on a gel to determine orientation.

Plasmids with the correct size inserts in the correct orientation were sequenced to confirm target. 1,500 ng of plasmid and 0.96 µL of 10 mM primer in a volume of 12 µL were submitted to AGRF (Adelaide, Australia) to be sequenced using Sanger sequencing. Sequencing results were aligned with the expected insert to confirm sequence and orientation. If needed, additional sequencing reactions were performed using different primers to get complete coverage of the pCR8/GW vector insert.

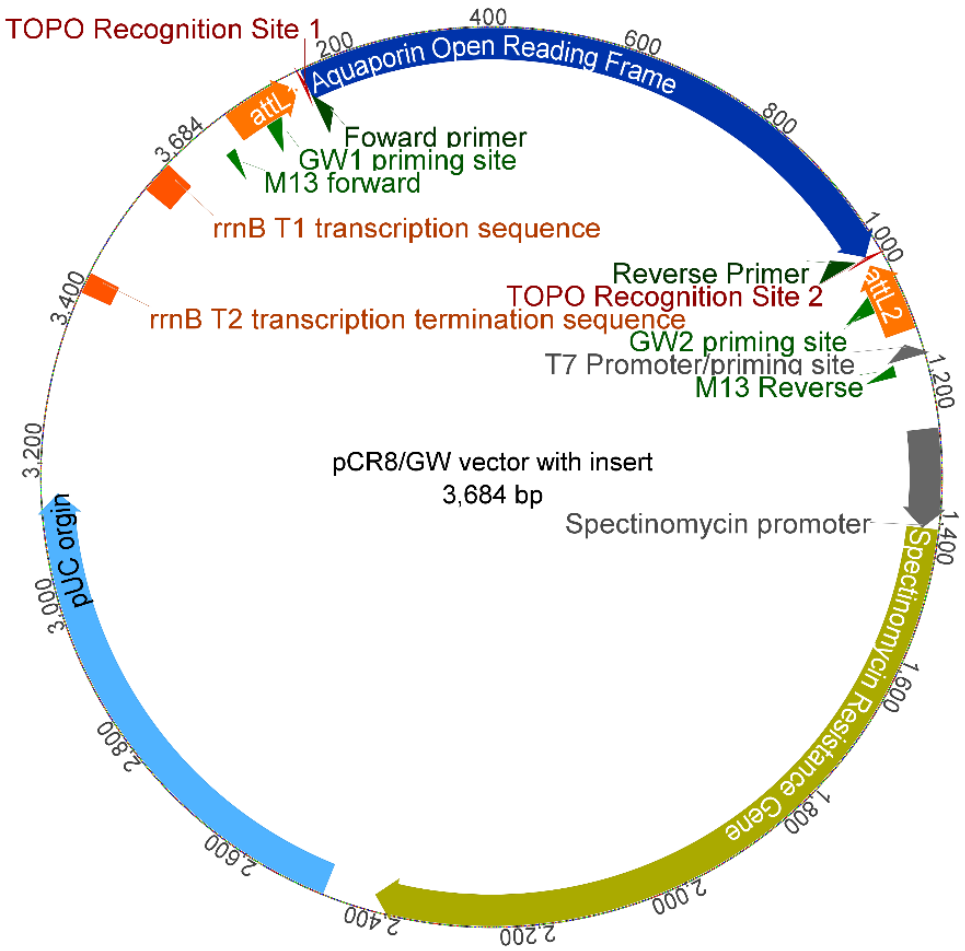


Figure 3.3 pCR8/GW vector with insert

Above is a representative pCR8/GW vector backbone with aquaporin open reading frame insert. Without the insert, the pCR8/GW/TOPO backbone is 2817bp. The insert if flanked by TOPO sites, dT 3' overhangs bound to a TOPO isomerase. During the TOPO reaction, the dT's align with the 5' dA overhangs of the insert and the TOPO isomerase ligates the ends. The vector includes AttL1 and AttL2 reverse combination sites for gateway cloning, and a spectinomycin resistance gene for selection.

3.2.1.3 cRNA synthesis of maize aquaporins for expression in *Xenopus* oocytes

The aquaporin insert in the pCR8/GW vector was recombined into a Gateway ready *Xenopus* expression vector, pGEMHE (Shelden et al. 2009), by LR recombination (Thermo Fisher Scientific, Waltham, MA USA), following manufacturer protocols. The pGEMHE vector contains the 5'and 3' UTRs for the *Xenopus* β-globin gene and has been shown to enhance expression of cRNAs in *Xenopus* oocytes (Liman et al. 1992). The reaction product was used to transform *E. coli* (Top10 or XL1Blue), selecting for the recombined vectors with 100 µg/mL carbenicillin. Plasmids were harvested and sequenced as above.

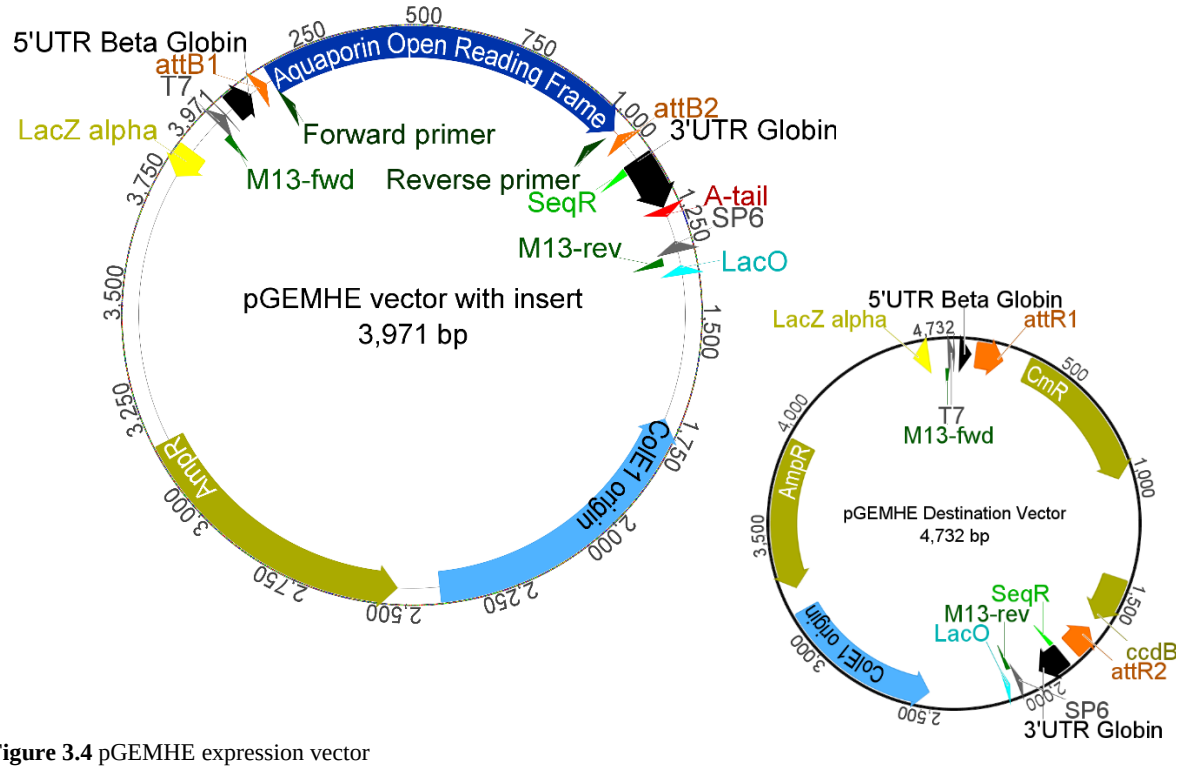


Figure 3.4 pGEMHE expression vector

A representative pGEMHE expression vector with aquaporin insert and the gateway enabled pGEMHE destination vector (Shelden et al. 2009). The Aquaporin insert from the pCR8/GW vector was inserted in the pGEMHE destination vector via gateway recombination. The insert is flanked by the 5' and 3' UTRs from the *Xenopus* β-globin gene. The pGEMHE vector contains an ampicillin resistance gene (AmpR) and a kanamycin resistance gene (CmR) as well as a ccdB toxin (both replaced after recombination).

pGEMHE vectors were linearized with restriction enzymes (5 µg plasmid in 50 µL reaction according to the manufacturer's protocol) and purified after Proteinase K treatment (SDS to final concentration of 0.5%, 50-100 µg/mL Proteinase K, incubate 2 hr at 50°C). Linearized plasmids were isolated with a phenol/chloroform extraction: 50.5 µL phenol/chloroform was added to Proteinase K treated linearized plasmids. Reactions were vortex vigorously and centrifuged 1 min at maximum speed. The aqueous phase was transferred to a new tube, combined with 5 µL 5M ammonium acetate and 100 µL 100% ethanol, and incubated overnight at -20°C. Linearized plasmids were pelleted via centrifugation (top speed 15 minutes), air dried, and resuspended in 10-20µL RNase free water. Concentration and purity was determined on a NanoDrop spectrophotometer as previously described.

Purified linearized plasmids were used as a template to *in vitro* synthesize cRNA using the mMessage mMachine T7 Transcription Kit (Thermo Fisher Scientific, Waltham, MA USA). cRNA was transcribed by combining 10 µL of 2X NTP/CAP was combined with 2 µL reaction buffer, 1 µg linearized plasmid, 2 µL enzyme mix and water (to 20 µL) and incubating for 2 hrs at 37°C. A DNase step was not performed as cRNA was in much greater concentration than template DNA. cRNA was purified using lithium chloride precipitation according to the manufactures recommendations. 30 µL RNase free water and 30 µL LiCl precipitation solution was added to each reaction and chilled at -80°C overnight. cRNA was pelleted through centrifugation, and washed with 70% RNase free ethanol. The wash was removed after centrifugation and the pellet air dried. cRNA was resuspended in 10.5 µL RNase free water. A 1:10 dilution was prepared and used to quantify on a NanoDrop spectrophotometer. 250 ng was run on an agarose gel (non-denaturing) to check for degradation with a ssRNA ladder (New England BioLabs,

Ipswich, MA USA), and approximate concentration. cRNA was diluted to 500 ng/µL and stored in aliquots at -80°C.

3.2.1.4 ZmNRT1.1B cRNA

ZmNRT1.1B (AGPv3: GRMZM2G161459_T02) cRNA was obtained from Zhengyu Wen of the Kaiser Laboratory (University of Sydney). Renamed ZmNPF6.6, it is a pH dependent high affinity nitrate transporter (Wen et al. 2017).

3.2.1.5 Expression in Xenopus oocytes

A Xenopus laevis frog colony was maintained by the University of Adelaide Animal Housing Facility at the Waite campus. Wendy Sullivan of the Tyerman lab surgically removed the oocytes from the animals without sacrificing them. Xenopus work was performed under Animal Ethics Approval (S-2014-192 from 01 Apr 2015 to 31 Mar 2018 and S2009-044B from 30 Mar 2009 to 31 Mar 2015) from the Office of Research Ethics, Compliance and Integrity at the University of Adelaide. The frogs and oocytes were under subject to Biosecurity Control (previously Quarantine). Frogs were used under Biosecurity Control Approved S1708 and experiments with oocytes under S1552. Oocytes were exempt from PC2 regulations. All users were approved under both Animal Ethics and Biosecurity Control.

Oocytes isolated from *X. laevis* were digested at room temperature for 70 minutes with 2 $mg \cdot mL^{-1}$ collagenase in ND96 (96 mM NaCl, 1 mM KCl, 1 mM $MgCl_2$, and 5 mM Hepes-NaOH, pH 7.5). Oocytes were de-folliculated with a hypotonic buffer (100 mM KH_2PH_4-NaOH, 0.1% BSA, pH 7.6), then washed twice with ND96 and once with calcium-free Ringer's solution (96 mM NaCl, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes-NaOH, pH 7.6). Oocytes were stored in working Ca-Ringer's solution (Ringer's with 0.6 mM $CaCl_2$,

supplemented with horse serum (5% v/v), 100 µg·mL^{-1} penicillin/streptomycin, and 2.5 mg tetracycline). Stage V or VI oocytes were selected for injection that had uniform shape and clearly defined hemispheres (a creamy vegetative hemisphere and a dark animal hemisphere).

Selected oocytes were injected with 46 nL of cRNA (total of 23 ng) or 46 nL of sterile water. Injection pipettes were prepared from (Drummond Scientific, item # 3-000-203-G/X, Broomall, PA USA) pipettes on a two stage capillary puller (Narishige Scientific, Tokyo, Japan) with Heater 1 set at 11.83 and Heater 2 at 9.00. The injection pipettes were then ground to a ~60 µm diameter at a 45° angle on a micro grinder EG400 (Narishige Scientific, Tokyo, Japan). Syringes were filled with silicon-water oil with a capillary syringe and attached to a Nanoject II microinjector (Drummond Scientific, Broomall, PA USA), avoiding the entrapment of air bubbles. The oil was displaced and filled with cRNA solution or sterile water. Oocytes were placed, in working Ca-Ringer's, on a mesh grate (to limit the movement of the oocytes) and injected at a (degree) angle. The injection pipette was aimed at the band separating the animal and vegetative hemispheres. Oocytes were injected, and checked that they swelled when injected. The syringe was removed and used on the next oocyte (each syringe could hold enough cRNA or water to inject 40-50 oocytes at 46 nL per injection). A new syringe was used each time it was loaded (for refilling or switching to new injection type). Oocytes were removed and incubated in working Ca-Ringer's solution (changed daily) for three days at 18°C.

3.2.2 Water transport assay

The water transport capacity of each *Xenopus* oocyte was determined by measuring the rate of swelling when placed in a hypotonic solution (Zhang et al. 1990). The rate was determined by video recording the swelling of each oocyte immediately after it was

placed in the hypotonic solution. The video file was processed to get the cross-sectional area, which was used to calculate the volume, assuming each oocyte was a sphere and swelled evenly. The volume rate of change was calculated from the volumes, and knowing the initial and hypotonic osmolalities, the osmotic permeability coefficient (P_f) of each oocyte was calculated.

The swelling solution was prepared from a five-fold dilution of the working Ringer's solution (from about 215 mOs to 43 mOs), with the osmolarity determined on a Fiske Micro-Sample Osmometer (Advanced Instruments, Norwood, MA USA) using freezing point depression. A swelling chamber was constructed so that the oocyte would be placed in the same position for recording. The end of a 10 mL test tube (with round bottom) was cut and embedded in a plastic petri dish with paraffin wax. This allowed a backlight to be used so that under a microscope, the oocyte would be a crisp dark sphere on a light background. The chamber was placed under a dissecting microscope (Nikon, SMZ800, Tokyo Japan) with 1.5x objective lens (Nikon, WD45, Tokyo, Japan). Oocytes were imaged with a Vicam colour camera (Pacific Communications, Port Melbourne, Vic Australia) at 2x magnification on a computer. The swelling chamber was filled with the swelling solution. An oocyte was selected and placed into the swelling chamber, and the video recording immediately started. At the end of the recording, the oocyte was discarded.

The video camera was connected to a computer running IC Capture 2.0 (Imaging Source Asia, Taipei, Taiwan). The camera sampled at 25 frames per second (fps). Each recording saved every 50^{th} frame (0.5 fps) for 1-5 minutes. The videos were analysed with ImageJ (Schneider et al. 2012) to get the area of each oocyte (macro available in Appendix 3.6.3.1). Briefly, for each video, a threshold was applied to each frame to separate light

background from the dark oocyte. The dark pixels of the oocyte in each frame were calculated using the "Analyse Particle…" tool in ImageJ, ignoring particles below 0.4 mm^2 (particulates), particles that touched the side of the frame (less than complete oocytes), or inconsistent particles. The area of each particle in the frame was recorded in an excel .xls file and saved for each oocyte.

Area files were analysed further with MATLAB (MathWorks, Natick, MA USA) using a custom macro (available in Appendix 3.6.3.2). By selecting lower and upper bounds of linearity, the macro modelled the relative volumes (from the initial volume), from the area files, calculated the rate of change during the video, and calculated P_{os} from the rate of change in volume, the initial and final osmolarities, the initial surface area (calculated from initial area), and the molar volume of water (V_w = 18 $mL \cdot mol^{-1}$). Volumes and P_{os} values were plotted and analysed in GraphPad Prism (GraphPad Software, La Jolla, CA USA).

$$P_{os} = \frac{V_0 \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}}{S_0 \cdot V_w \cdot \left(osm_f - osm_i\right)}$$

$$V_0 = initial\ volume, \qquad V_w = molar\ volume\ of\ water$$
$$V_t = volume\ at\ time\ t, \qquad osm_i = initial\ osmolaritiy$$
$$S_0 = initial\ surface\ area, \qquad osm_f = final\ osmolarity$$

$$A_t = \pi \cdot {r_t}^2 \quad \rightarrow \quad r_t = \sqrt{\frac{A_t}{\pi}}$$

$$V_t = \frac{4}{3} \cdot \pi \cdot {r_t}^3$$

$$V_t = \frac{4}{3} \cdot A_t \cdot \sqrt{\frac{A_t}{\pi}}$$

$$S_t = 4 \cdot \pi \cdot {r_t}^2$$

$$S_t = 4 \cdot A_t$$

$$A_t = area\ at\ time\ t, \quad r_t = radius\ at\ time\ t$$

Equation 3.1 Osmotic permeability calculation
Assuming each oocyte is a perfect sphere that swells equally, the area change is modelled as a sphere. Each area is assumed to be circular with radius r. The surface area and volume are calculated from the sphere of radius r as shown below. Analysis based on Zhang et al. (1990). V2 represents the partial molar volume of water (18 cm^3/mol).

The effect pH had on water transport in *Xenopus* oocytes was tested by incubating oocytes in a Ringer's pH solution (adjusted to pH 5.0 or pH 7.1) consisting of 46.0 mM NaCl, 50.0 mM sodium acetate, 2.0 mM KCl, 5.0 mM $MgCl_2$, and 5.0 mM HEPES, for 10 minutes. After 10 minutes, the oocyte was swelled as before in a fivefold dilution of pH adjusted Ringer's solution.

The effect of nitrate on osmotic water permeability was tested by incubating oocytes in N-Ringer's (96 mM $NaNO_3$, 2 mM KCl, 5 mM $MgCl_2$, 5 mM Hepes-NaOH, 0.6 mM $CaCl_2$, supplemented with horse serum (5% v/v), 100 $\mu g \cdot mL^{-1}$ penicillin/streptomycin, and 2.5 mg tetracycline pH 7.6) for 10 minutes and swelling, comparing to the swelling rate in Ca-Ringer's.

3.3 Results

3.3.1 Cloning maize aquaporins

The primers listed in **Table 3.5** and **Table 3.6** resulted in bands in both root and shoot tissues (**Figure 3.5**), although for several shoot samples the band was less intense. Except for ZmTIP4;1b, the PCR amplified from root cDNA was used for cloning purposes.

Together, 21 maize aquaporins from inbred line B73 were cloned from the root or shoot cDNA into pCR8/GW vectors (**Table 3.7**). Due to sequence similarity, the same primers were used to clone ZmPIP1;2b and ZmPIP1;4. Open reading frames were sequenced and compared to the predicted genomic sequences and the previously published versions (**Figure 3.11** to **Figure 3.31**). Where differences were observed, the sequenced clone matched the genomic prediction (ZmPIP1;2b, ZmPIP1;6b, ZmTIP1;2b, ZmTIP4;1b, and ZmTIP4;4b). ZmSIP1;1 and ZmPIP1;6b sequences aligned with AGPv3's genome annotation and were different from the AGPv4 genome annotation. Compared with AGPv3, the AGPv4 genome's ZmPIP1;6b had a very different exon structure and ZmSIP1;1 had 30 base pairs removed from an exon junction. For sequence alignments, see Appendix 3.6.2.

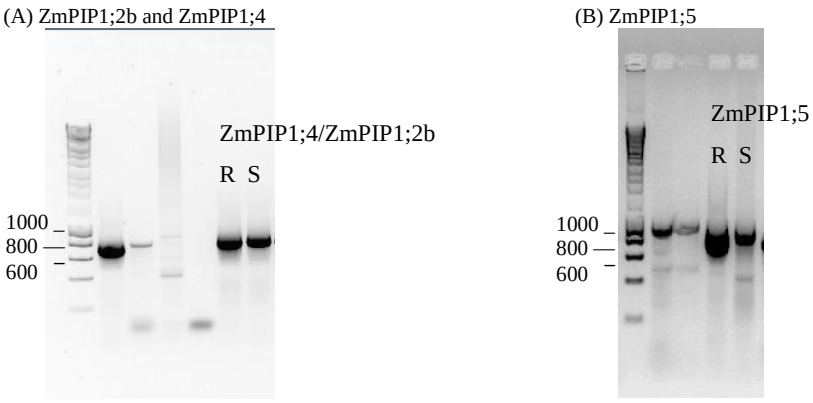
Table 3.7 Cloning maize aquaporins

21 maize aquaporin open reading frames were cloned from cDNA sourced from B73 roots and shoots (denoted in bold). The primers from ZmPIP1;2b and ZmPIP1;4 had products in both roots and shoots. As both were isolated from the root cDNA, it is unknown whether a band seen in the shoot is from ZmPIP1;2b, ZmPIP1;4 or both. Shoot bands labelled weak were of much less intensity than the band in the root sample. R= cDNA from whole root tissue, S = cDNA from shoot tissue

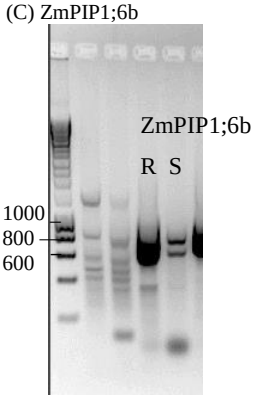
	Aquaporin	AGPv3	AGPv4	Amplification		Open Reading Frame length (bp including stop codon)	Amino acids	Predicted molecular weight (kDa)
				R	S			
1	ZmPIP1;2b	AC209208.3_FGT002	Zm00001d017526_T001.1	Y	Y*	870	289	30.81
2	ZmPIP1;4	GRMZM2G392975_T01	Zm00001d051403_T001.1	Y	Y*	879	292	31.00
3	ZmPIP1;5	GRMZM2G081843_T01	Zm00001d051872_T001.1	Y	Y	867	288	30.72
4	ZmPIP1;6b	GRMZM2G136032_T01	Zm00001d020383_T001.1	Y	weak	891	296	30.96
5	ZmPIP2;1	GRMZM2G014914_T02	Zm00001d019563_T006.1	Y	Y	873	290	30.21
6	ZmPIP2;2	GRMZM2G092125_T01	Zm00001d005421_T001.1	Y	Y	879	292	30.26
7	ZmPIP2;3	GRMZM2G081192_T01	Zm00001d051174_T002.1	Y	Y	870	289	30.42
8	ZmPIP2;4	GRMZM2G154628_T01	Zm00001d017288_T001.1	Y	Y	867	288	30.32
9	ZmPIP2;5	GRMZM2G178693_T01	Zm00001d003006_T001.1	Y	Y	858	285	29.84
10	ZmPIP2;6	GRMZM2G047368_T02	Zm00001d019565_T001.1	Y	Y	867	288	30.19
11	ZmTIP1;2b	GRMZM2G168439_T01	Zm00001d011778_T001.1	Y	Y	765	254	25.41
12	ZmTIP2;1	GRMZM2G027098_T01	Zm00001d051362_T001.1	Y	weak	750	249	24.87
13	ZmTIP4;1b	GRMZM2G103945_T03	Zm00001d037779_T001.1	Y	Y	768	255	26.54
14	ZmTIP4;4b	GRMZM2G093090_T01	Zm00001d039293_T001.1	Y	Y	864	287	29.32
15	ZmNIP1;1	GRMZM2G041980_T02	Zm00001d016237_T001.1	Y	Y	849	282	29.56
16	ZmNIP2;1	GRMZM2G028325_T01	Zm00001d018037_T001.1	Y	Y	888	295	31.39
17	ZmNIP2;2	GRMZM2G137108_T01	Zm00001d037228_T001.1	Y	Y	885	294	31.76
18	ZmNIP3;1	GRMZM2G176209_T01	Zm00001d032461_T001.1	Y	weak	909	302	31.16
19	ZmSIP1;1	GRMZM2G113470_T01	Zm00001d048959_T002.1	Y	Y	738	245	25.62
20	ZmSIP1;2	GRMZM2G060922_T01	Zm00001d010325_T001.1	Y	Y	732	243	25.70
21	ZmSIP2;1	GRMZM2G175038_T01	Zm00001d028951_T001.1	Y	Y	750	249	26.75

Figure 3.5 PCR amplificaiton of maize aquaporins

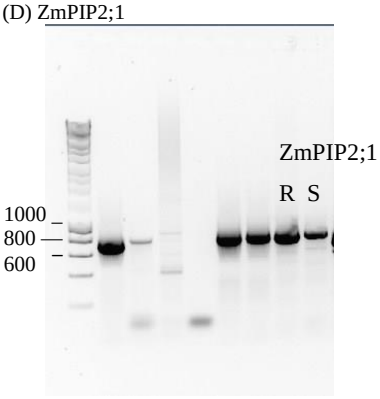
Amplification of Maize aquaporins. R=root, S=shoot cDNA. Amplification shown with ORF primers from **Table 3.5**, unless noted UTR in which they were amplified using primers in **Table 3.6**. A Primers designed for ZmPIP1;4 also amplified ZmPIP1;2b, and bands are a combination of PCR products.



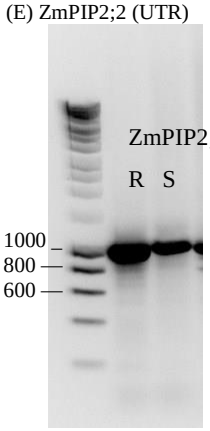
(C) ZmPIP1;6b



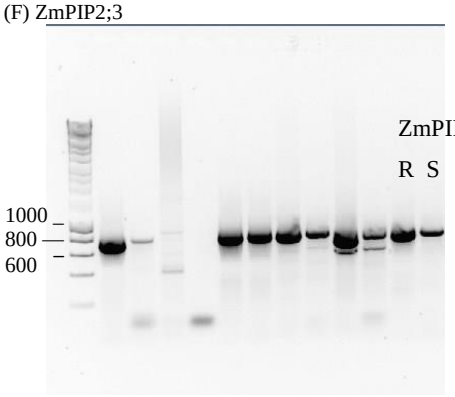
(D) ZmPIP2;1



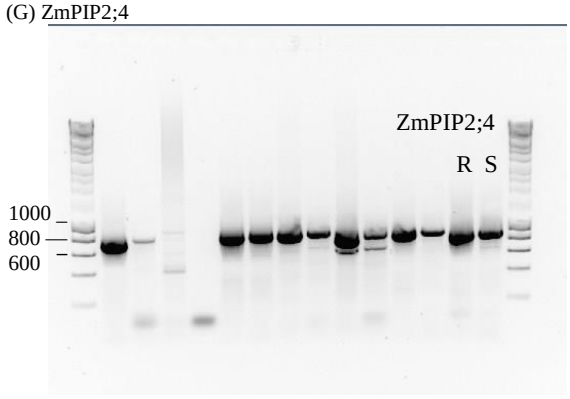
(E) ZmPIP2;2 (UTR)



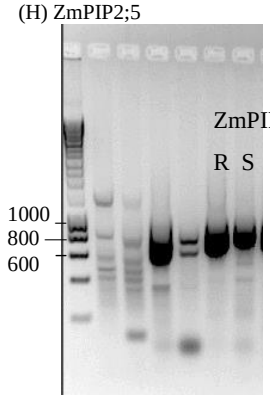
(F) ZmPIP2;3



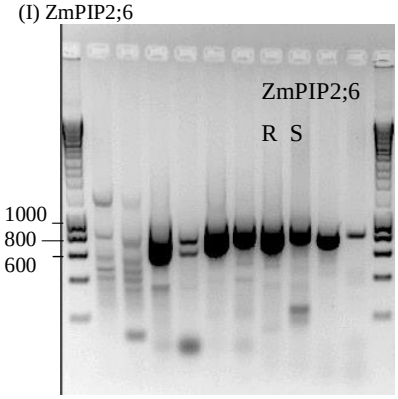
(G) ZmPIP2;4



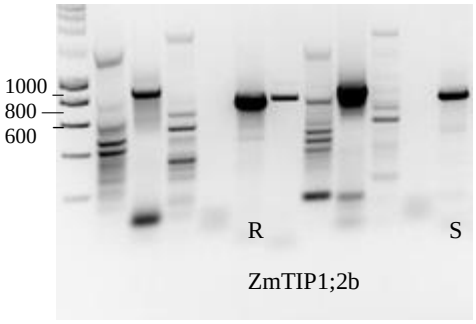
(H) ZmPIP2;5



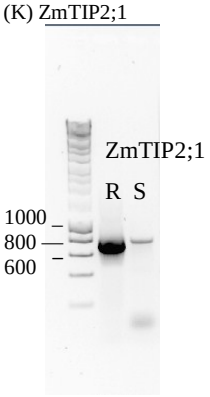
(I) ZmPIP2;6



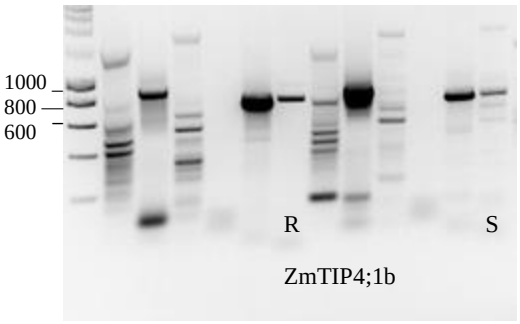
(J) ZmTIP1;2b



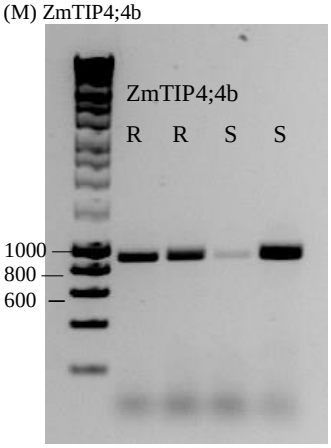
(K) ZmTIP2;1



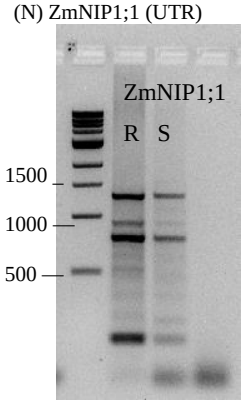
(L) ZmTIP4;1b



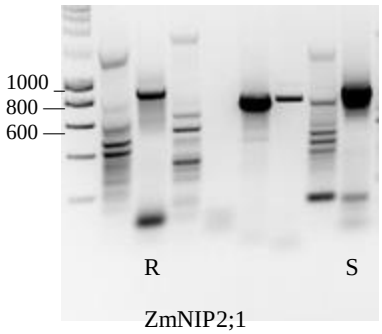
(M) ZmTIP4;4b



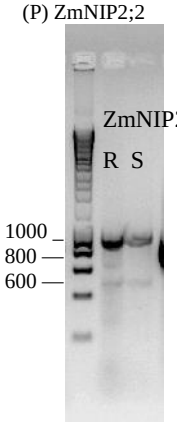
(N) ZmNIP1;1 (UTR)



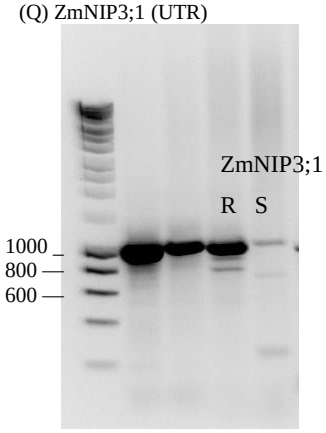
(O) ZmNIP2;1



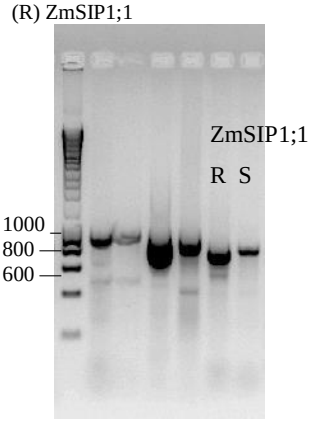
(P) ZmNIP2;2



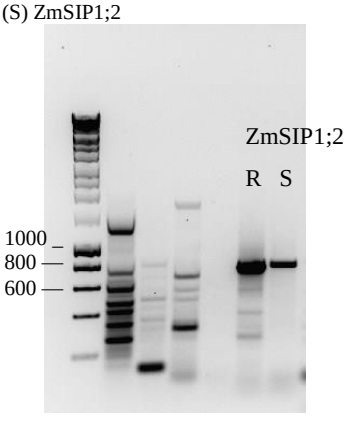
(Q) ZmNIP3;1 (UTR)



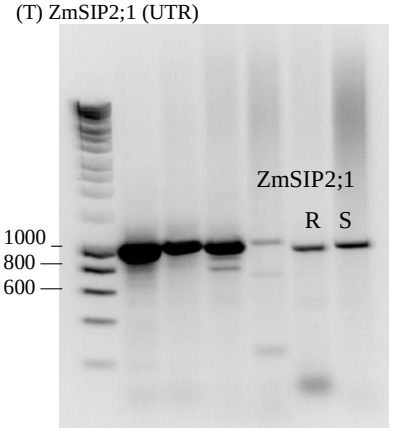
(R) ZmSIP1;1



(S) ZmSIP1;2



(T) ZmSIP2;1 (UTR)



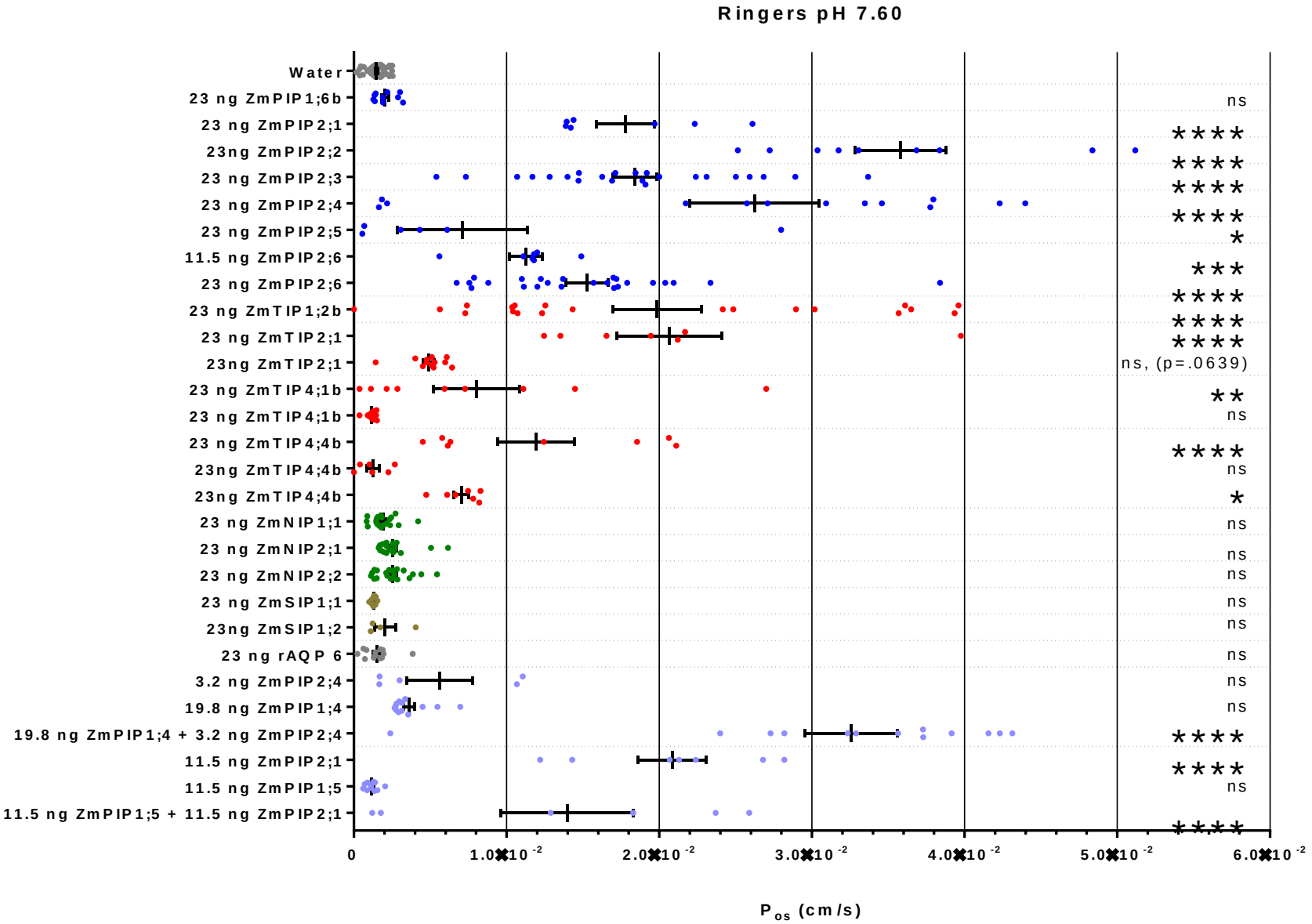

Ringers pH 7.60
Water
23 ng ZmPIP1;6b
23 ng ZmPIP2;1
23ng ZmPIP2;2
23 ng ZmPIP2;3
23 ng ZmPIP2;4
23 ng ZmPIP2;5
11.5 ng ZmPIP2;6
23 ng ZmPIP2;6
23 ng ZmTIP1;2b
23 ng ZmTIP2;1
23ng ZmTIP2;1
23 ng ZmTIP4;1b
23 ng ZmTIP4;1b
23 ng ZmTIP4;4b
23ng ZmTIP4;4b
23ng ZmTIP4;4b
23 ng ZmNIP1;1
23 ng ZmNIP2;1
23 ng ZmNIP2;2
23 ng ZmSIP1;1
23ng ZmSIP1;2
23 ng rAQP 6
3.2 ng ZmPIP2;4
19.8 ng ZmPIP1;4
19.8 ng ZmPIP1;4 + 3.2 ng ZmPIP2;4
11.5 ng ZmPIP2;1
11.5 ng ZmPIP1;5
11.5 ng ZmPIP1;5 + 11.5 ng ZmPIP2;1
ns
****
****
****
****
*
***
****
****
****
ns, (p=.0639)
**
ns
****
ns
*
ns
ns
ns
ns
ns
ns
ns
****
****
ns
****
0
1.0✖10 -2
2.0✖10 -2
3.0✖10 -2
4.0✖10 -2
5.0✖10 -2
6.0✖10 -2
P os (cm/s)

Figure 3.6 Swelling assay in 1:5 dilution of Ringer's solution at pH 7.60
Oocytes were injected with 46 nL of RNase free water with aquaporin cRNA (no cRNA for water control). The amounts of cRNA are shown on the left. Dots represent individual oocytes and bars represent mean with SEM. At least 6 oocytes were used per injection. Dots are coloured to distinguish subfamilies (blue – ZmPIPs, red – ZmTIPs, green – ZmNIPs, brown – ZmSIPs). Significance compared to water injected oocytes: ns (not significant)→$p>0.05$, ★→$p<0.05$, ★★→$p<0.01$, ★★★→$p<0.001$, ★★★★→$p<0.0001$

3.3.2 Water transport of maize aquaporins

To measure water transport activity, *Xenopus* oocytes were injected with cRNA (or water control) of aquaporins, incubated for three days, and swelled by placing oocytes from isotonic solution to hypotonic solution. The water permeability was calculated for each oocyte from the swelling video based on the relative rate of increase and initial oocyte dimensions. The permeability was calculated from at least five oocytes. As no data on protein expression levels was taken, results can only show water transport ability as compared to controls (water-injected oocytes).

Each gene's osmotic permeability is plotted in **Figure 3.6**. For ZmPIPs, the ZmPIP1;4, ZmPIP1;5 and ZmPIP1;6b displayed no water conductivity when expressed alone. For the ZmPIP2s (ZmPIP2;1, ZmPIP2;2, ZmPIP2;3, ZmPIP2;4, ZmPIP2;5 and ZmPIP2;6), increased water permeability was seen, however for ZmPIP2;5 water permeability was lower than for the others. For ZmPIP2;4, injecting 23 ng of cRNA produced greater osmotic permeability than injecting just 3.2 ng. For ZmPIP2;1 and ZmPIP2;6, injecting 11.5ng or 23ng of cRNA produced similar results. Co-injecting ZmPIP1;5 (11.5 ng) and ZmPIP2;1 (11.5 ng), did not raise the P_{os} as compared to ZmPIP2;1 (11.5 ng) alone, however a small sample size was used (6 oocytes each). Injecting 19.8 ng of ZmPIP1;4 did not increase P_{os} compared to water control, but when co-injected with 3.2 ng ZmPIP2;4, a much greater increase in P_{os} was observed over 3.2 ng of ZmPIP2;4 alone.

For the ZmTIPs, ZmTIP1;2b increased P_{os} over water controls. For ZmTIP2;1, ZmTIP4;1b and ZmTIP4;4b, the results were mixed over several experiments. For ZmTIP2;1, two experiments (pooled above) showed very low P_{os}, while a third had increased P_{os} over water controls. Similarly, over two experiments for ZmTIP4;1b, in one experiment increased P_{os} was observed in some oocytes. Similarly, in ZmTIP4;4b, two experiments had increased P_{os} over water injected oocytes but the third did not.

For the ZmNIPs and ZmSIPs, there were not significant differences between water injected oocytes and ZmNIP1;1, ZmNIP2;1, ZmNIP2;2, ZmSIP1;1 or ZmSIP1;2.

3.3.3 Acid effects on maize aquaporin swelling rates

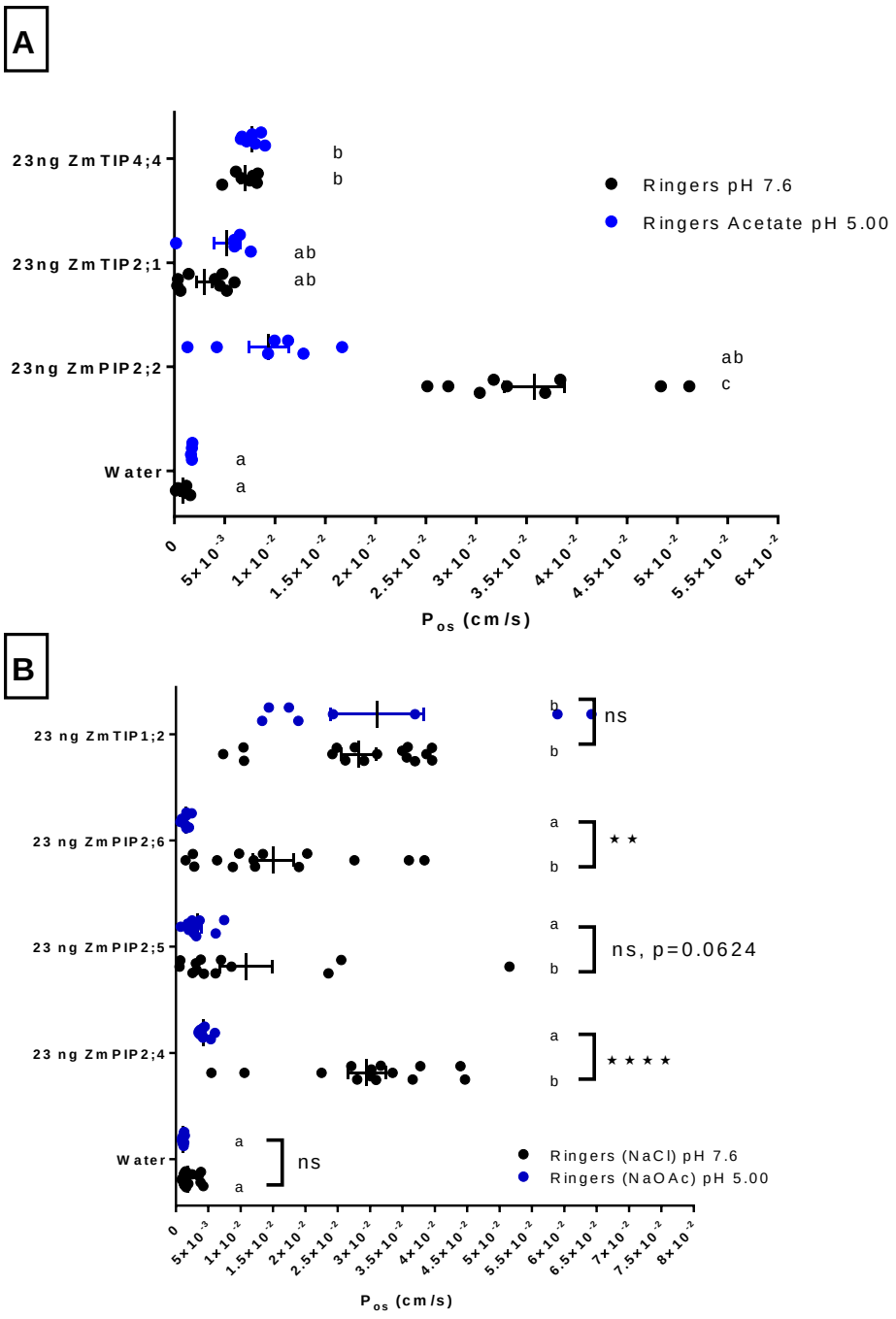


Figure 3.7 Acid effects on oocyte osmotic permeability

To test the effect of acid on the oocytes, oocytes were incubated in an isotonic solution buffered at 5.00 containing 50 mM sodium acetate for 10 minutes, then swelled as above. The rates were compared to oocytes incubated in isotonic solution at pH 7.1-7.6 . Treatments are displayed by colour: black Ringer's (96 mM NaCl) pH 7.08-7.6, blue Ringer's (46 mM NaCl, 50 mM sodium acetate) pH 5.00. (A) Injection of ZmTIP4;4, ZmTIP2;1 and ZmPIP2;2 compared with water control. (B) Injection of ZmTIP1;2, ZmPIP2;4, ZmPIP2;5 and ZmPIP2;6.

Acidification of the cytosol has been shown to modify water carring capacity of aquaporins by inducing conformational changes in the protein leading to the central pores closure (Hedfalk et al. 2006b; Törnroth-Horsefield et al. 2006). The acetate ion has been shown to induce the closure of aquaporins at low pH in *Xenopus* oocytes (Tournaire-Roux et al. 2003).To test the effects of acid on the water swelling rates, oocytes were incubated ten minutes in isotonic treatment solutions (Ringer's pH 7.60, with 50 mM NaCl replaced with 50 mM NaOAc) at pH 7.08 or 5.00 prior to swelling. ZmPIP and ZmTIP proteins were investigated as some show water channel activity.

For ZmPIP2;2, ZmPIP2;4, ZmPIP2;5 and ZmPIP2;6 were all sensitive to acetate, with treatment reducing water transport (**Figure 3.7**-A,B, **Figure 3.8**). Interestingly, ZmPIP2;5 had low water transport in most oocytes. ZmTIP1;2 (**Figure 3.7**-B) was insensitive to acetate. ZmTIP4;4b was also insensitive to acetate at low pH, though its transported water at lower rates compared with the ZmPIPs. In these experiments, ZmTIP2;1 was not permeable to water, and low pH did not raise water conductance. ZmSIP1;1 and ZmPIP1;6b were not water permeable in *Xenopus* oocytes with or without acetate.

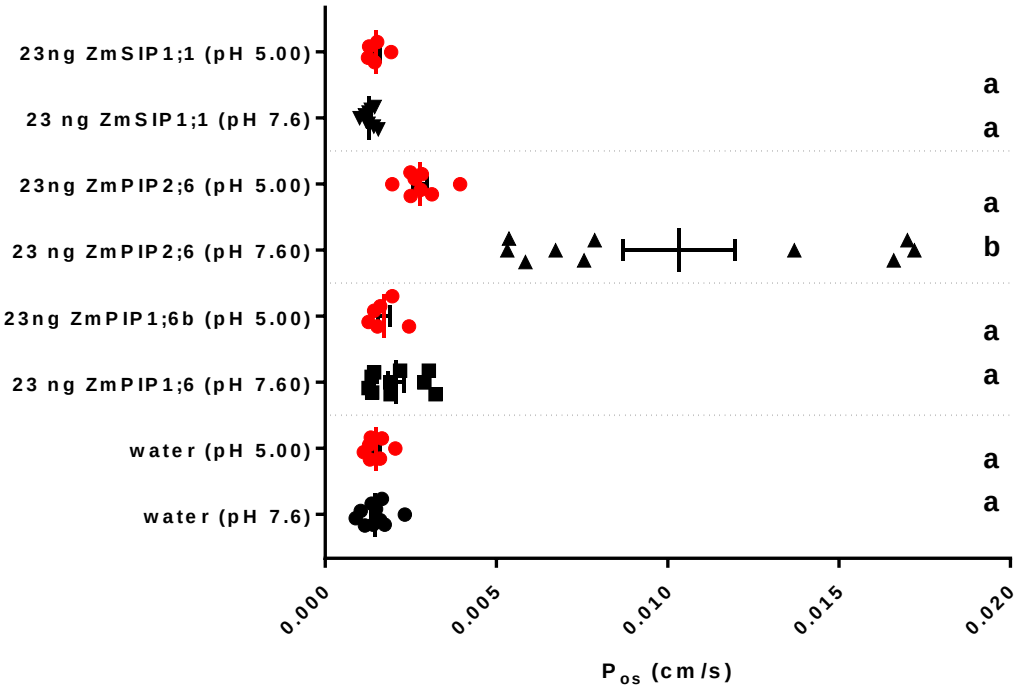


Figure 3.8 Acid sensitivity of maize aquaporins

Osmotic permeability coefficients for aquaporins pre-treated with acetate at pH 5.00. Bars denote mean and SEM. Letters denote significant difference compared to other groups at $p<0.05$. Only ZmPIP2;6 at neutral pH showed statistically significant increase in water conductance. ZmPIP2;6 was used as a positive control.

3.3.4 Nitrate and nitrate uptake effects on the water transport of ZmPIP2;6

To examine the potential effect of cytosolic acidification due to the effect of nitrate uptake, and to test the effect that nitrate had on ZmPIPs, ZmNRT1.1B was co-injected with ZmPIP2;6. ZmPIP2;6 was selected as it was a known root expressed aquaporin(Górska et al. 2008; Hachez et al. 2006). ZmNRT1.1B (ZmNPF6.6) is a pH dependent nitrate transporter (Wen et al. 2017). In low pH, ZmNRT1.1B transports nitrate into the cell. The aim of these experiments was to see if nitrate presence could inhibit PIP aquaporin activity either directly, or through the acidification of the cytosol from cotransport of nitrate. In a simple nitrate flux experiment (**Figure 3.9**-A), ZmPIP2;6 water transport was inhibited at pH 5.5 with no acetate. Surprisingly, ZmPIP2;6 showed

increased water conductance at neutral pH in the presence of nitrate (**Figure 3.9**-A, **Figure 3.9**-B, **Figure 3.10**). Interestingly, ZmPIP1;5 also showed increased water transport in the presence of nitrate at neutral pH when co-expressed with ZmPIP2;1. ZmPIP2;1 and ZmPIP2;3 were not sensitive to nitrate.

When co-injected, ZmPIP2;6 and ZmNRT1.1b displayed greater hydraulic conductance, but at both pHs were decreased compared to ZmPIP2;6 at neutral pH. Using Ringer's with chloride replaced with nitrate (96 mM) or partially with acetate (50mM), swelling was increased with ZmPIP2;6 (23 ng cRNA) with nitrate (**Figure 3.9**-B), but not when just 11.5 ng was injected. Co-injection with ZmNRT1.1 decreased the water permeability in both chloride and nitrate oocytes.

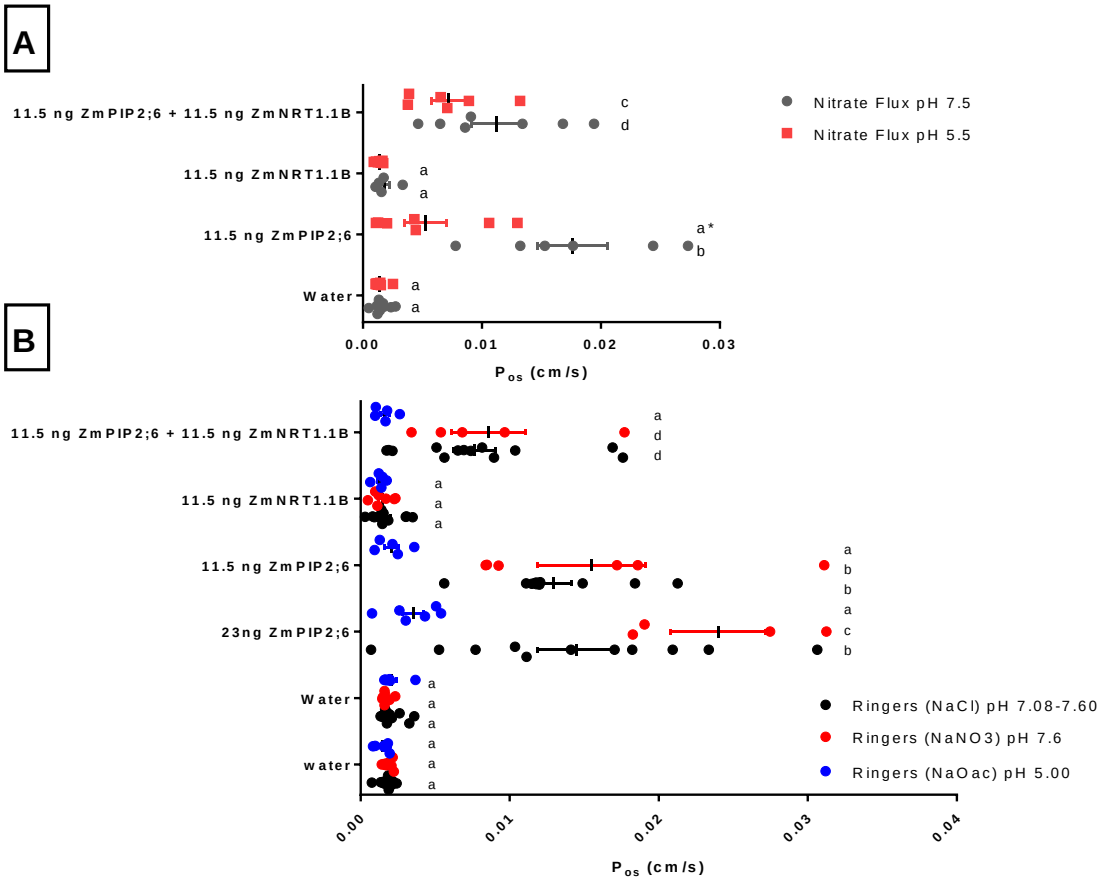


Figure 3.9 Nitrate uptake on water transport in ZmPIP2;6

(A) Oocytes injected with either ZmNRT1.1B, ZmPIP2;6 or both were treated with a nitrate flux solution at pH 7.5 (grey) or pH 5.5 (red) for ten minutes, then swelled in a 1:5 dilution of the treatment. The flux solution did not contain acetate, so was thought not to acidify the oocyte and inhibit ZmPIP2;6. However ZmPIP2;6 was impacted at low pH without the ZmNRT1.1B co-injection, masking any effects the co-injection may have had. Co-injected oocytes at in flux solution of Ringer's Nitrate swelling rates were not inhibited by the presence of nitrate at neutral pH values. (B) Oocytes exposed to a solution with either nitrate or low pH with acetate. Acetate inhibited all oocytes with ZmPIP2;6, and at neutral pH, oocytes with ZmPIP2;6 had greater water transport. When 11.5 ng of ZmPIP2;6 cRNA was injected, nitrate did not change water transport compared with chloride, but when 23 ng was injected, nitrate increased the water transport of ZmPIP2;6. Co-injected oocytes had increased water conductance than water controls, but were decreased from just ZmPIP2;6 in either nitrate or chloride. Different letters denote significance at $p<0.05$. In subfigure (A), a* denotes $0.04<p<0.07$.

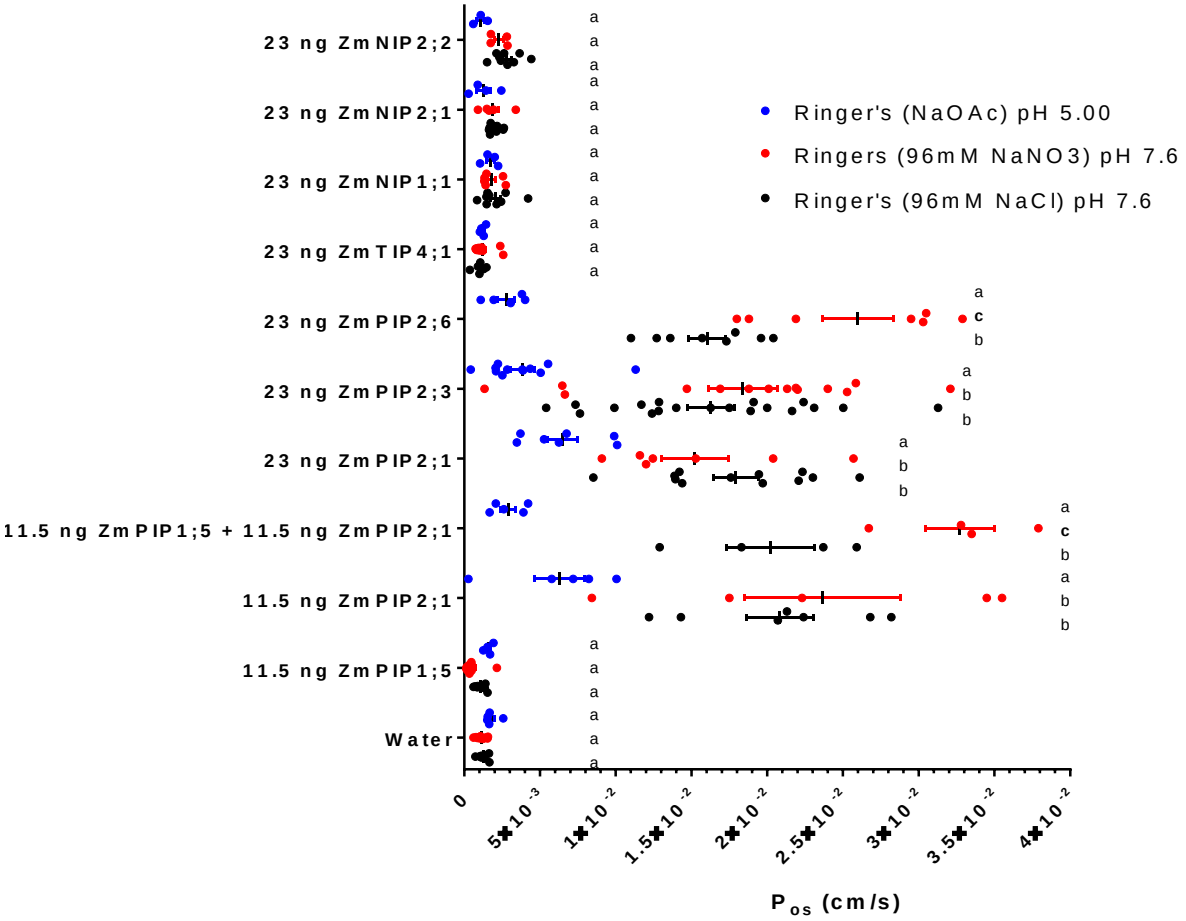


Figure 3.10 Nitrate and acid effect on oocytes expressing maize aquaporins
Maize aquaporins swelling in the presence of nitrate (pH 7.6) or acetate (pH 5.00). Bars represent mean and SEM. Letters denote significant differences ($p<0.05$) within each group,

3.4 Discussion

3.4.1 Maize aquaporin cloning

The cloning of 21 maize aquaporins from the inbred line B73 confirmed several sequence variations from Chaumont et al. (2001) and clarified which genome annotation (AGPv3 or AGPv4) was correct. For both ZmPIP1;6b (**Figure 3.14**) and ZmSIP1;1 (**Figure 3.29**), the sequenced versions aligned to the AGPv3 annotations. The ZmPIP1;2b (**Figure 3.11**), ZmPIP1;6b, ZmTIP1;2b (**Figure 3.21**), ZmTIP4;1b (**Figure 3.23**) and ZmTIP4;4b

(**Figure 3.24**), sequencing confirmed the amino acid sequence deviation from previously published (Chaumont et al. 2001) versions of the genes. Additionally, ZmPIP1;4 was confirmed (over ZmPIP1;3) which shares and identical protein sequence, differing by just 6 base pairs in the open reading frame. While the genome (only ZmPIP1;4 is predicted in either AGPv3 or AGPv4 genomes) only predicts this version, the screen used here was searching for a single clone, and did not intentionally compare multiple sequences obtained from identical clones. While clones for full length transcripts were achieved, the amplification patters of several (ZmNIP1;1-R,S and ZmPIP1;6b-S) appeared to have alternative splice variants.

The remaining 22 maize aquaporins were not attempted to be cloned. It is unknown under what conditions and tissues they are expressed. Five of these 22 sequences have some variation between the published version and the genome prediction (ZmPIP1;7, ZmTIP3;2b, ZmTIP4;2b, ZmTIP4;3b and ZmTIP5;1b), and nine are predicted in the genome, but have not been isolated in the literature (ZmPIP2;9, ZmPIP3;1, ZmTIP2;4, ZmTIP3;4, ZmNIP1;2, ZmNIP1;3, ZmNIP4;1, ZmNIP5;1, and ZmNIP5;2). Future work should investigate the presence and sequences of these aquaporins. These could be cloned in a similar manner as the 21 in this chapter, with open reading frame primers used, and expanding to UTR primers if needed.

Another future direction could investigate the diversity of maize aquaporins across the species. Maize is very genetically diverse, and so diversity in aquaporins may be expected. The differences in sequences seen in ZmPIP1;5b, ZmTIP1;2b, ZmTIP4;1b and ZmTIP4;4b and predicted for ZmPIP1;1/ZmPIP1;7, ZmTIP2;3b, ZmTIP4;2b, and ZmTIP4;3b with previously cloned and sequenced versions suggest that across the maize species, the aquaporin profile may vary slightly on sequences. It also hints there may be

some variation on the members of the aquaporin set. Chaumont et al. (2001) reported ZmPIP2;7, a sequence to predicted in either genome nor reported anywhere else. The effect of this is unknown, but could result in changes in regulation through promoter differences, gene copy number or an amino acid change to a residue affecting water or other solute transport.

3.4.2 Water permeation

The water permeation properties of ZmPIPs were as expected and supported previous results (Chaumont et al. 2000; Moshelion et al. 2004; Fetter et al. 2004; Zelazny et al. 2007; Van Wilder et al. 2008; Zelazny et al. 2009). For ZmPIP1 and ZmPIP2 coexpression, additional replicates would be needed to gain confidence. First however, a titration of cRNA amounts is needed. It is assumed that as the amount of cRNA that injected increases, the P_{os} would also increase up to a threshold. This threshold is likely reached before 23ng of cRNA (based on swelling rates from 11.5ng to 23ng of ZmPIP2;1 and ZmPIP2;6). This threshold is needed to allow ZmPIP1 coexpression to raise the P_{os} value above that of the ZmPIP2 alone (as was shown for ZmPIP1;4 and ZmPIP2;1).

The water permeation of ZmTIP, ZmNIP and ZmSIP were insightful as there are limited studies of the water transport capacity of these aquaporins. ZmTIP1;1 (ZmTIP1) was shown to increase water permeability in *Xenopus* oocytes and is inhibited by mercury (Chaumont et al. 1998). ZmTIP2;3 also displays water transport capacity (Lopez et al. 2004). ZmNIP2;1, ZmNIP2;4 and ZmTIP4;4 have been characterized, but not for water transport (Gu et al. 2012). For the ZmSIPs, there is no published data, however *Arabidopsis* AtSIP1;1 and AtSIP1;2 are water permeable and are localized to the ER membrane (Ishikawa et al. 2005). It is highly possible the ZmSIPs are also localized to the ER membrane and may have water transport capacity as well.

Homology models have been used to model and predict aquaporin function. A major determinant of aquaporin specificity is the aromatic/arginine (ar/R) filter composed of four amino acid residues: one on transmembrane helix 2, one on transmembrane helix 5, and two on loop E, and represent the narrowest part of the pore on each monomer (Wallace and Roberts 2004). Based on its ar/R filter (HIGR), ZmTIP2;1 would be expected to have water transport ability, as it shares the same ar/R as ZmTIP2;3 that has water transport ability (Lopez et al. 2004). In these experiments, ZmTIP2;1 had increased water permeability over that of water injected controls. Water permeation via ZmTIP1;2b is consistent with ZmTIP1 (Chaumont et al. 1998) as both share the ar/R filter HIAV. For ZmTIP4;4b, its ar/R (HVAR) is similar to ZmTIP2;3 (HIGR) and it has been grouped with ZmTIP2;1 (Bansal and Sankararamakrishnan 2007). These results suggest that ZmTIP4;4b has some water conductance as expected. However, ZmTIP4;4b has also been shown to be urea permeable (Gu et al. 2012). ZmTIP4;1b was given a separate classification based on its ar/R (HSAR) (Bansal and Sankararamakrishnan 2007). These results suggest that this ar/R filter may allow for some water transport.

The ZmNIPs tested (ZmNIP1;1, ZmNIP2;1 and ZmNIP2;2) displayed no water transport ability. This is not unexpected, as ZmNIPs have been shown to be permeable to other solutes with some showing low water permeation. ZmNIP2;1 (ZmLsi1) and ZmNIP2;2 (ZmLsi6) expressed in *Xenopus* oocytes facilitate the uptake of silicon (Mitani et al. 2009). ZmNIP2;1 has also been shown, along with ZmNIP2;4 to have urea transport capacity (Gu et al. 2012). Finally, ZmNIP3;1 is permeable to boric acid (Leonard et al. 2014; Durbak et al. 2014).

Cytoplasmic acidification via acetic acid treatment inhibited ZmPIPs, but did not inhibit ZmTIP1;2b. This also is consistent as the ZmPIPs, but not the ZmTIP1s, have a cytosolic

oriented loop D histidine, seen in *Arabidopsis* AtPIP2;2, that is necessary for low pH sensitivity (Tournaire-Roux et al. 2003).

Cytoplasmic acidification has implications in terms of nitrate uptake, as nitrate transporters are proton coupled (Tsay et al. 2007; Tsay et al. 1993). In maize, Espen et al. (2004) demonstrated that in roots treated with tungstate to block nitrate reductace activity, a decrease in cellular pH was observed when roots were exposed to nitrate. The authors noted that when nitrate reductase activity was not inhibited, a pH decrease was not observed, indicating the reduction of nitrate is important for maintaining cytosolic pH. In maize roots, Górska et al. (2008) found nitrate and nitrite concentrations rose in the presence of nitrate, but did not change when pre-incubated with tungstate to block NR activity. They suggested tungstate could have inhibited nitrate uptake, or that the unloading rate of nitrate into the xylem was near to the uptake rate of the root. Interestingly, these authors demonstrated an increase in water conductance in the presence of nitrate, but not when nitrate reductase activity was blocked with tungstate. These studies suggest that nitrate uptake works to lower cellular pH, closing aquaporins, but the cellular nitrate concentration stimulates water transport through mechanisms unknown.

The data here suggest a possible mechanism, that certain PIP aquaporins may be sensitive to nitrate and increase water conductance. Here ZmPIP2;6, and the coexpression of ZmPIP1;5 with ZmPIP2;1 are stimulated by nitrate. This represents a novel regulatory form of aquaporin water transport, though additional study is required. The dose effect of nitrate should be determined through changing the concentration of nitrate in the treatments solution, as well as the time of incubation. A dose effect of the amount of cRNA injected could also be useful in determining the sensitivity of ZmPIP2;6 to nitrate.

3.4.3 Errors in the calculation of water permeability

The P_{os} value modelled from the measurements is likely different than the true value because of three sources of errors. The P_{os} value was calculated from **Equation 3.1** and can be rewritten as:

$$P_{os} = \frac{V_0 \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}}{S_0 \cdot V_w \cdot \left(osm_f - osm_i\right)} = \frac{1}{3 \cdot \sqrt{\pi} \cdot v_w \cdot (Osm_{out} - Osm_{in})} \cdot \sqrt{A_0} \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}$$

Equation 3.2
Rearranging **Equation 3.1**

(see appendix for derivation), and shows the P_{os} value for the oocyte is dependent on the initial area (A_0) and the initial rate of swelling ($\frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}$). The values used for the initial area and initial swelling rate had several sources of error resulting from the experiment technique and equation used to model the relative swelling rate. The specific technique of swelling was crude: pipetting an oocyte from the initial solution, placing (and aligning) under the camera then pressing record. The first source of error arises from the δ-second delay between when the oocyte was placed in solution and video started. While not measured, this was less than 1s, but varied from oocyte to oocyte. A second technical error was due to the camera and computers method of recording frames. The camera sampled at a frame rate of 25 frames per second, and the video recording program sampled the n^{th} frame (from 25 to 300), meaning the first $\frac{(n-1)}{25}$ seconds (~1-6 s) of the swelling (from when the recording initialized) were not recorded. Taken together, this means the true initial area was $\frac{(n-1)}{25} + \delta$ seconds before the first frame recorded. The first frame recorded had already started to swell, so the initial area of the first frame was greater than the true initial area.

A second error in calculating P_{os} was due to the change in osmolarities. The model assumes an instantaneous change, but in practice a small amount of initial solution was introduced to the swelling solution when the oocyte was transferred. This would decrease the osmotic change as the solutions mixed and would lower, even if by a small amount, the end absolute change. The theory presented in Moshelion et al. (2004) and technique presented in Shatil-Cohen et al. (2014) would give a better approximation of P_{os} through the introduction of model for a controlled gradual shift from isotonic to hypertonic solutions. Their model and techniques utilise a flow of solution over the oocyte to better mix the solution near the oocyte.

A third error in calculating P_{os} arose from the calculation of the initial swelling rate: $\frac{\mathrm{d}}{\mathrm{dt}}\left(\frac{\mathrm{V_t}}{\mathrm{V_0}}\right)_{\mathrm{t=0}}$. A simple linear model was used to model $\frac{\mathrm{V_t}}{\mathrm{V_0}}$, specifically $\frac{\mathrm{V_t}}{\mathrm{V_0}} = m \cdot t + b$, and thus $\frac{\mathrm{d}}{\mathrm{dt}}\left(\frac{\mathrm{V_t}}{\mathrm{V_0}}\right) = \frac{\mathrm{d}}{\mathrm{dt}}(m \cdot t + b) = m$ at all times t. This was not a terrible approximation for the initial swelling rate, but was in reality the average rate of swelling over values listed instead of the real initial rate of swelling. Oocytes generally had a greater initial swelling rate that decreased as the internal oocyte osmolarity approached the external osmolarity. A better model could have been selected such as a quadratic: $\frac{\mathrm{V_t}}{\mathrm{V_0}} = A \cdot t^2 + B \cdot t + C$. This results in $\frac{\mathrm{d}}{\mathrm{dt}}\left(\frac{\mathrm{V_t}}{\mathrm{V_0}}\right)_{\mathrm{t=0}} = \frac{\mathrm{d}}{\mathrm{dt}}(A \cdot t^2 + B \cdot t + C)_{\mathrm{t=0}} = (2 \cdot A \cdot t + B)_{\mathrm{t=0}} = B$. While still a crude approximation of reality, it would have been as simple to calculate and given some room for the swelling rate to change.

While the measured osmotic permeability was not without error, it was useful to demonstrate water transport capacity of expressed aquaporins through comparison to the P_{os} value from water injected oocytes that gave some indication of the magnitude of change over water injected oocytes. However, while approximately equal amounts of

cRNA were injected, the P_{os} value is of limited value as it was not standardized against active protein in the plasma membrane. Neither the aquaporin's translational efficiency, nor the efficiency of inserting those channels into membranes, is known. This makes comparisons between aquaporins (especially divergent aquaporins like ZmPIPs vs ZmTIPs) based on relative swelling rates and osmotic permeability impossible without further data. Translational efficiency could be approximated through Western blots (absolute quantification or relative comparison to a reference protein) and insertional efficiency by GFP tagging the aquaporin and tracking its location in the oocyte. GFP tagging would also have allowed firmer negative conclusions to be made to oocytes that did not swell differently from water controls if the GFP tagged protein was present in the plasma membrane.

3.4.4 Future experiments

This research could be furthered by the cloning of the remaining 22 maize aquaporins, and comparing the sequences to those that are known. Additionally, performing swelling experiments on the remaining maize aquaporins to get an idea of those capable of water transport would allow a complete picture of the water carrying aquaporins in maize. As above, tagging the aquaporins with GFP and using confocal microscopy to image the localization of the clone would give increased credibility to negative swelling results in *Xenopus* oocytes by showing the localization of the aquaporin in the oocyte. If not in the plasma membrane (as expected with ZmPIP1s and ZmSIPs), a different way of showing swelling is needed. GFP-tagged aquaporins that appear to be localized to the plasma membrane with low swelling rates would have greater confidence in the lack of ability of the specific aquaporin isoform to transport water. A Western blot could be used to show protein amounts, and the GPF as a tag if needed.

Localization of the aquaporins in tissue is also desired. PIPs are established to be inserted into plant plasma membranes (PIP2s on their own, PIP1s with the help of PIP2s) (Fetter et al. 2004; Zelazny et al. 2009; Zelazny et al. 2007; Chevalier et al. 2014), but little is empirically known about localization of the ZmTIPs, ZmNIPs or ZmSIPs. Some ZmNIPs (also known as the ZmLSIs) have been shown to transport silicon across the PM (Mitani et al. 2009), but due to the variability in sequences, each ZmNIP should be analysed independently. This can be achieved by transiently expressing GPF tagged aquaporins in protoplasts, onion epidermal cells, or tobacco leaf cells and using confocal microscopy to establish localization patterns.

Finally, after confirmation of plasma membrane localization, additional swelling studies could be performed to establish substrate transport preferences, especially of the ZmTIPs and ZmNIPs. To enhance swelling rates, in an isotonic initial solution and final solution with diluted and rebalanced with solute of interest could be used in conjunction with a ZmPIP2 or other known water transporting aquaporin. This would facilitate both the solute entering through a ZmTIP or ZmNIP and water to balance through a ZmPIP2. A ZmPIP2 only injection would be necessary to monitor swelling due to small osmotic differences between solutions (should be no observed if identical osmolarities).

3.5 Conclusion

The results of these experiments confirm 21 of the 43 sequences predicted by the maize genome, the genome version and differences to published cDNAs. Expression in *Xenopus* confirmed water transport capacity of ZmPIPs and TIPs, and also showed nitrate may have increased ZmPIP;6 water transport. ZmTIPs were also water permeable and, lacking a loop D histidine, were not inhibited by cytosolic acidification assumed to have occurred

with acetic acid at low pH (Tournaire-Roux et al. 2003).The ZmNIPs and ZmSIPs did not show water transport when expressed in *Xenopus* oocytes.

3.6 Appendix

3.6.1 Deriving Equation 1.2

$$P_{os} = \frac{V_0 \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}}{S_0 \cdot V_w \cdot (osm_f - osm_i)}$$

$$P_{os} = \frac{\left(\frac{4}{3} \cdot \pi \cdot r^3\right) \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)}{(4 \cdot \pi \cdot r^2) \cdot V_w \cdot (osm_f - osm_i)}$$

$$P_{os} = \frac{r \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}}{3 \cdot v_w \cdot (osm_f - osm_i)}$$

$$P_{os} = \frac{\sqrt{\frac{A_0}{\pi}} \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}}{3 \cdot v_w \cdot (osm_f - osm_i)}$$

$$P_{os} = \frac{1}{3 \cdot \sqrt{\pi} \cdot v_w \cdot (Osm_{out} - Osm_{in})} \cdot \sqrt{A_0} \cdot \frac{d}{dt}\left(\frac{V_t}{V_0}\right)_{t=0}$$

3.6.2 Cloning alignments

This appendix contains individual alignments of the open reading frames of the sequenced aquaporin clone (top), the genomic prediction (second, possibly third as well), and the known transcripts (remaining). Dots represent agreements, and nucleotide differences are marked. Above the sequenced clone (yellow), the primers used in cloning (green), and the predicted exons (grey) are marked. Below it, are the amino acid sequence (black letters on coloured background), predicted transmembrane domains from Phyre2 (red), the ar/R residues (orange), the NPA domains (blue), and any amino acid changes from the previously published version (black, if present).

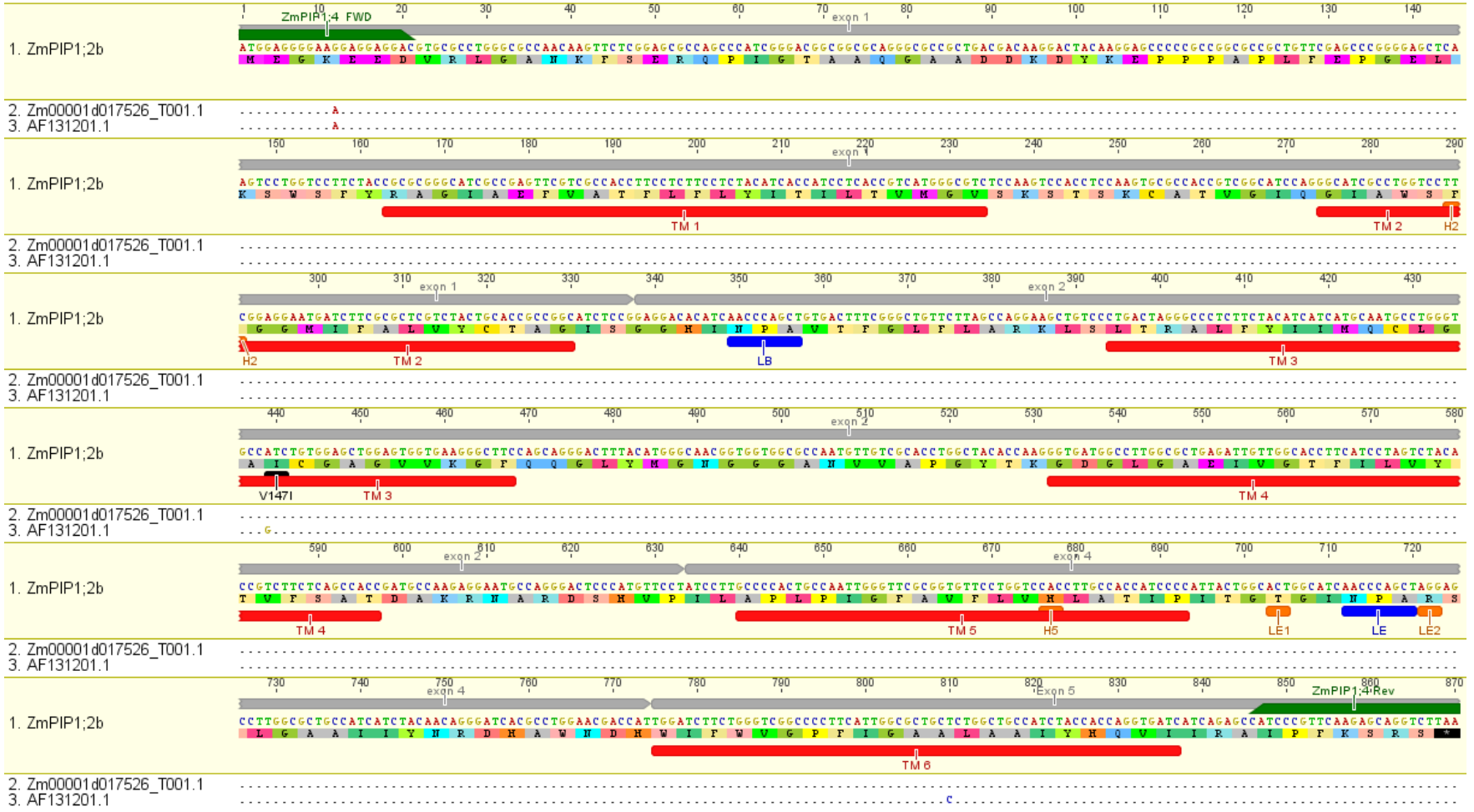


Figure 3.11 Alignments of the open reading frames (ORFs) of the sequenced ZmPIP1;2b clone, the genomic prediction (Zm00001d017256_T001.1) and published cDNA (AF131201.1)
The sequence of the open reading frame confirms two nucleotide differences (G439A and C810T) between the cDNA and genomic prediction that result in one amino acid change (V147I). A third base pair difference (A12G) is likely due to an artefact from the primers used for cloning. The primers used were specific to ZmPIP1;4, and have one mismatch (A12G) to ZmPIP1;2b.



Figure 3.12 Alignments of the ORFs of the sequenced ZmPIP1;4 clone, the genomic prediction (Zm00001d051403_T001.1) and published cDNAs (ZmPIP1;3 AF326487.1 and ZmPIP1;4 AF326488.1)

The sequence of the open reading frame confirms identity with the published cDNA ZmPIP1;4. In the open reading frame, there are six nucleotide differences to published ZmPIP1;3 cDNA, but no amino acid changes.

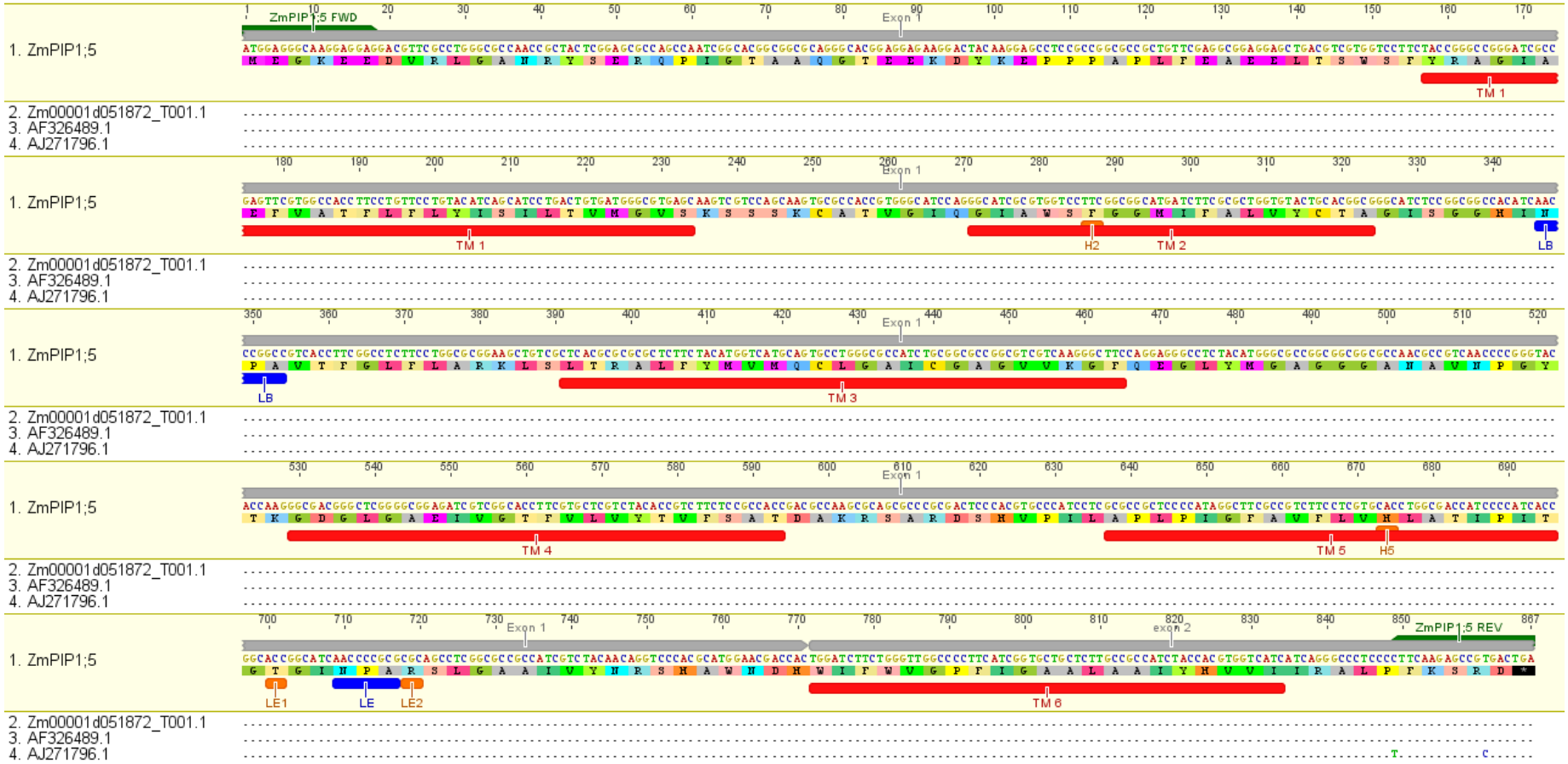


Figure 3.13 Alignments of the ORFs of the sequenced ZmPIP1;5 clone, the genomic prediction (Zm00001d051872_T001.1) and published cDNAs (ZmPIP1;5 AF326489.1 and ZmPIP1;5b AJ271796.1)

The sequence of the open reading frame confirms identity between the genomic prediction and ZmPIP1;5 cDNA. As predicted, ZmPIP1;5b contains two nucleotide differences (T849C and C861T).



Figure 3.14 Alignments of the ORFs of the sequenced ZmPIP1;6b clone, the genomic predictions (AGPv4 Zm0001d020383_T001.1 and AGPv3 GRMZM2G136032_T01) and published cDNA (AF236490.1)

The sequence of the open reading frame confirms ZmPIP1;6b aligns to the AGPv3 genomic prediction. The AGPv4 prediction does not contain a full length open reading frame. Between the AGPv3 and published cDNA, three base pair differences (A35C, A36C, and G219C) are confirmed resulting in one amino acid change (E12A).

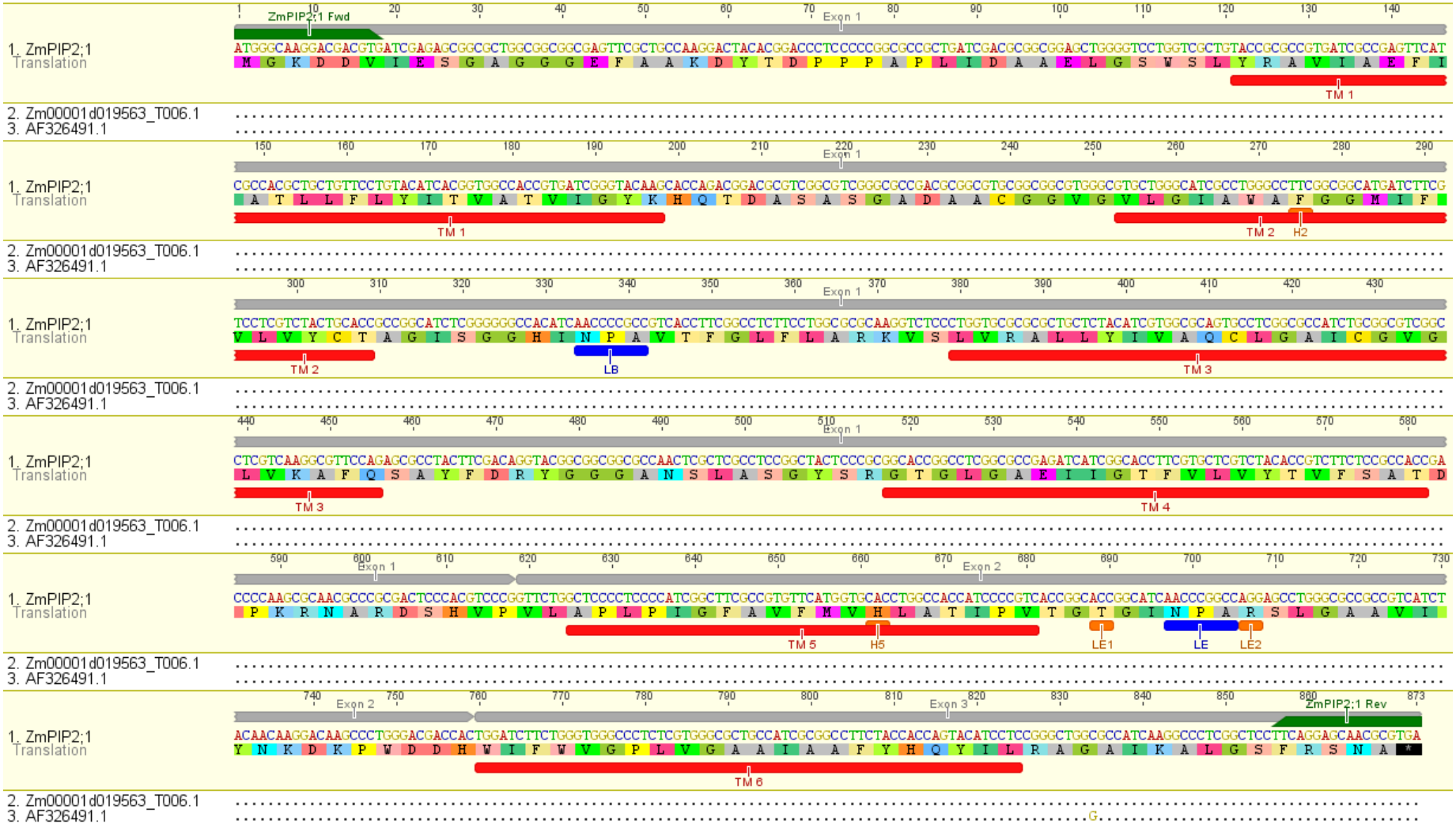


Figure 3.15 Alignments of the ORFs of the sequenced ZmPIP2;1 clone, the genomic predictions (Zm00001d019563_T006.1) and published cDNA (AF326491.1)

The sequence of the ORF confirms a one nucleotide difference (G834C) between the genomic prediction and cDNA of ZmPIP2;1. The amino acid sequences are identical.



Figure 3.16 Alignments of the ORFs for the sequenced ZmPIP2;2 clone, the genomic prediction (Zm00001d005421_T001.1,) and cDNA (AF326492.1)
The sequence of the open reading frame confirms identity between the genomic prediction and ZmPIP2;2 cDNA. There are no nucleotide differences.



Figure 3.17 Alignments of the ORFs for the sequenced ZmPIP2;3 clone, the genomic prediction (Zm00001d051174_T002.1) and published cDNA (AF326493.1)
The sequence of the open reading frame confirms identity between the genomic prediction and ZmPIP2;3 cDNA. There are no nucleotide differences.



Figure 3.18 Alignments of the ORFs for the sequenced ZmPIP2;4 clone, the genomic prediction (Zm00001d017288_T001.1) and published cDNA (AF326494.1)
The sequence of the open reading frame confirms identity between the genomic prediction and ZmPIP2;4 cDNA. There are no nucleotide differences.



Figure 3.19 Alignments of the ORFs for the sequenced ZmPIP2;5 clone, the genomic predication (Zm00001d003006_T001.1) and published cDNA (AF130975.1)
The sequence of the ORF confirms a one base pair difference (T144C) between the published cDNA and genomic prediction. There are no amino acid differences.



Figure 3.20 Alignments of the ORFs for the sequenced ZmPIP2;6 clone, the genomic prediction (Zm00001d019565_T001.1) and cDNA (AF326495.1)
The sequence of the open reading frame confirms identity between the genomic prediction and ZmPIP2;6 cDNA. There are no nucleotide differences.



Figure 3.21 Alignments of the ORFs for the sequenced ZmTIP1;2b clone, the genomic prediction (Zm00001d011778_T001.1) and published cDNA (AF326500.1)

The sequenced ORF of ZmTIP1;2b confirms eight nucleotide differences that result in three amino acid changes (T30M, A53T and L130I) between the genomic prediction and published cDNA.



Figure 3.22 Alignments of the ORFs for the sequenced ZmTIP2;1 clone, the genomic prediction (Zm00001d051362_T001.1) and published cDNA (AF326501.1)
The sequence of the open reading frame confirms identity between the genomic prediction and ZmTIP2;1 cDNA. There are no nucleotide differences.

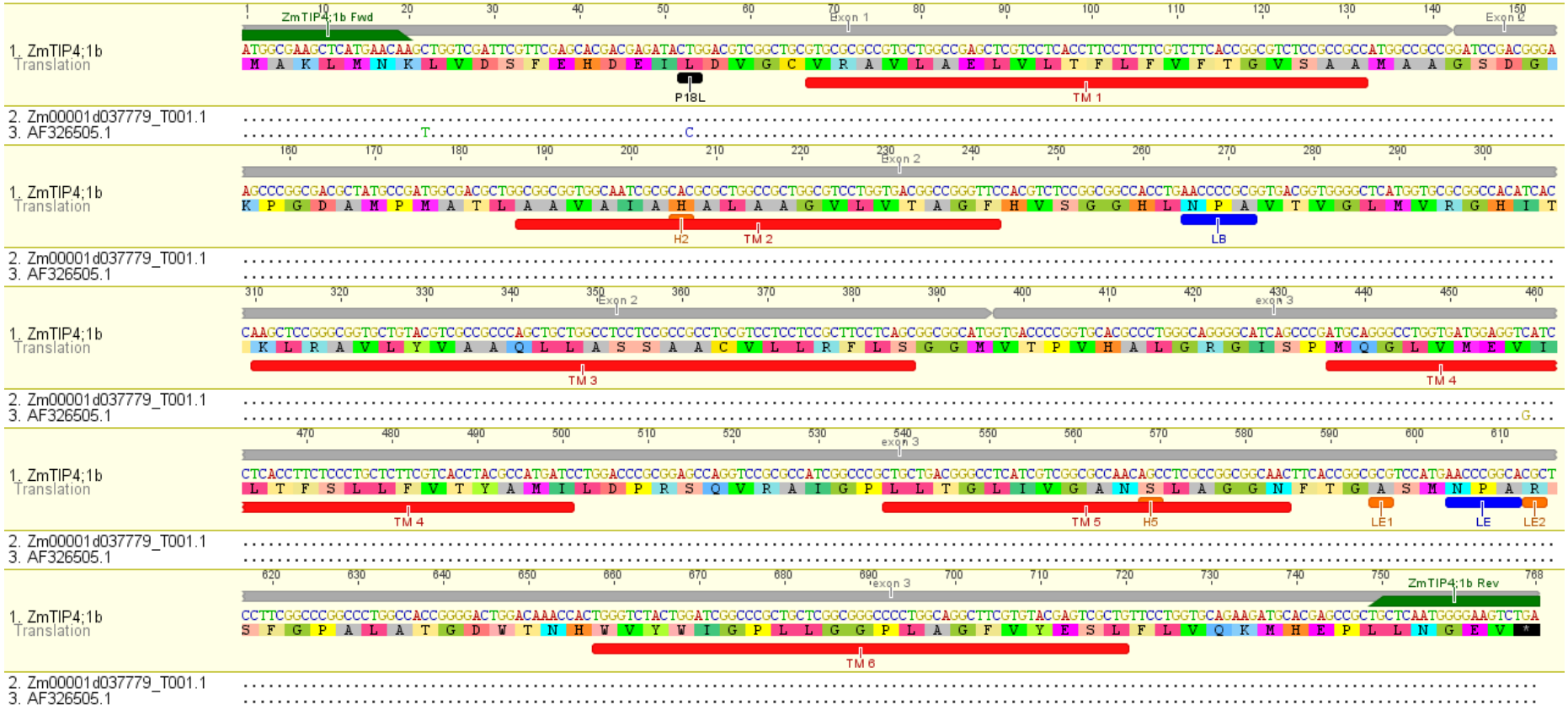


Figure 3.23 Alignments of the ORFs of the sequenced ZmTIP4;1b clone, the genomic prediction (Zm00001d037779_T001.1) and published cDNA (AF326505.1)

The sequenced ORF of ZmTIP4;1b confirms three nucleotide differences that result in one amino acid changes (P18L) between the genomic prediction and published cDNA of ZmTIP4;1b.

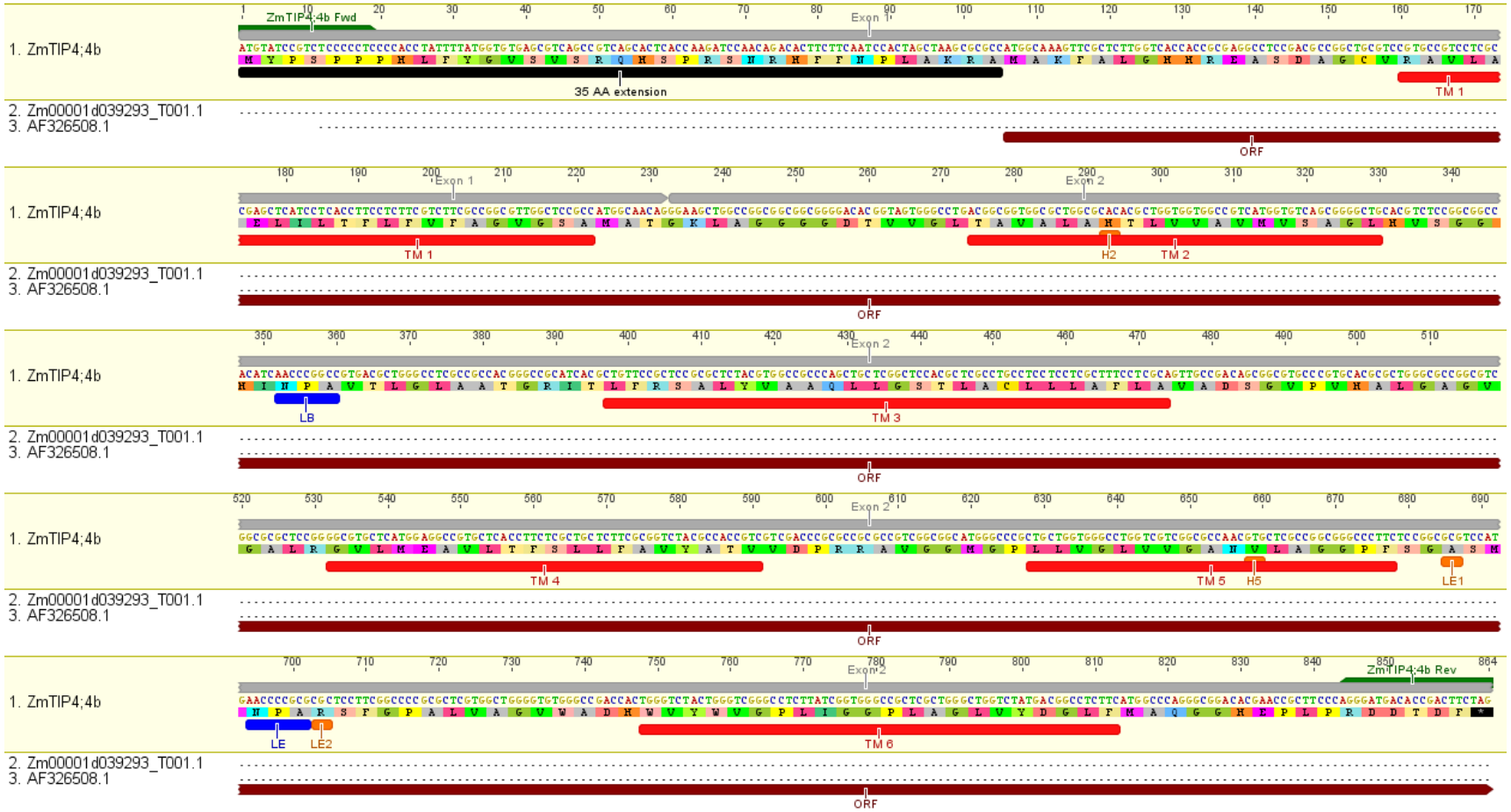


Figure 3.24 Alignments of the ORFs of the sequenced ZmTIP4;4b clone, the genomic prediction (Zm00001d039293_T001.1) and published cDNA (AF326508.1)

The sequenced ORF of ZmTIP4;4b confirms an extended 5' end resulting in earlier start codon and the addition of 35 amino acids to the N-terminus of the protein. The remainder is identical. The ORF of the published cDNA is highlighted in Red

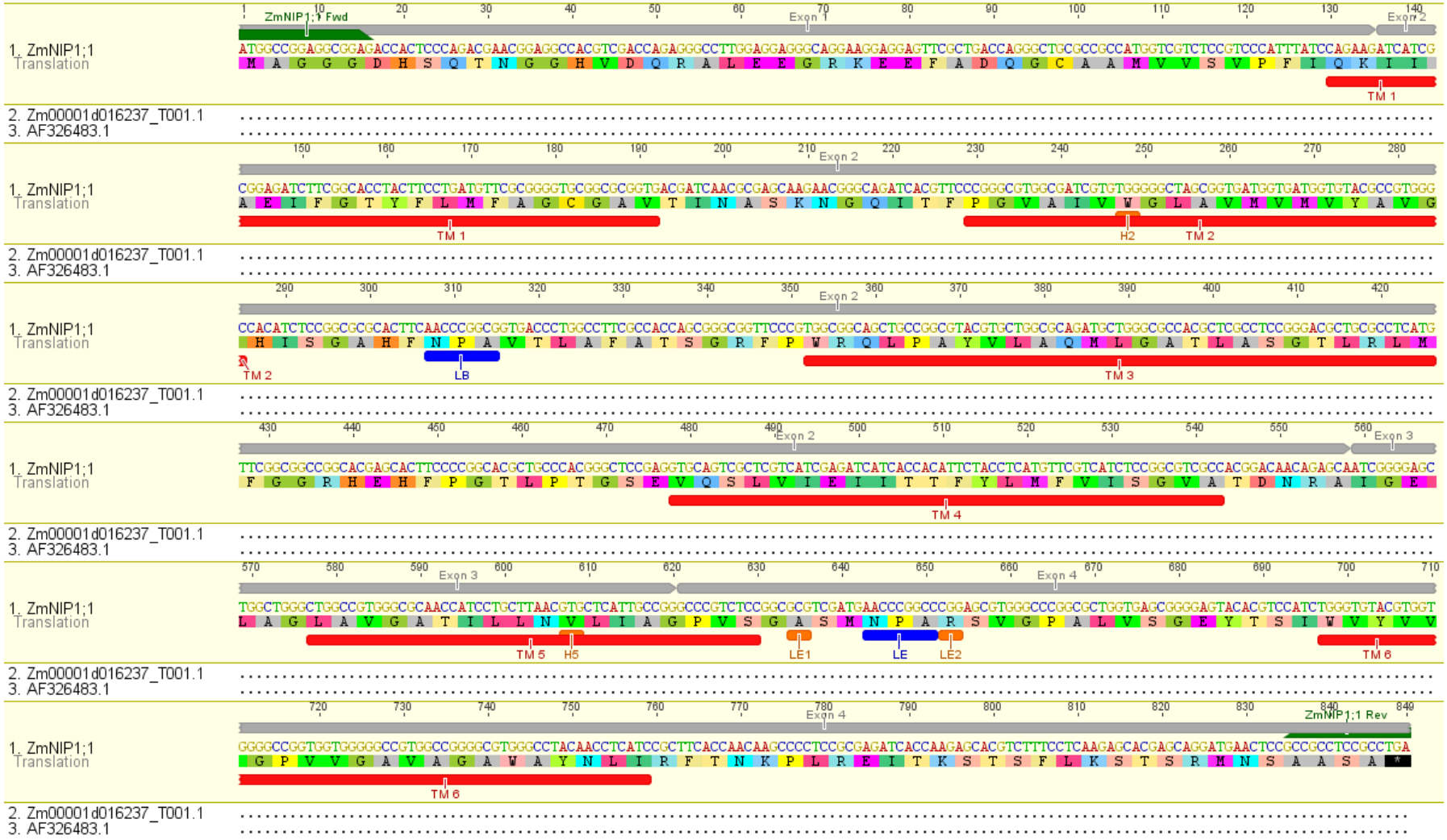


Figure 3.25 Alignments of the ORFs of the sequenced ZmNIP1;1 clone, the genomic prediction (Zm00001d016237_T001.1) and the published cDNA (AF326483.1)
The sequence of ZmNIP1;1 ORF confirms the identity of the ORFs for the genomic prediction and published cDNA.

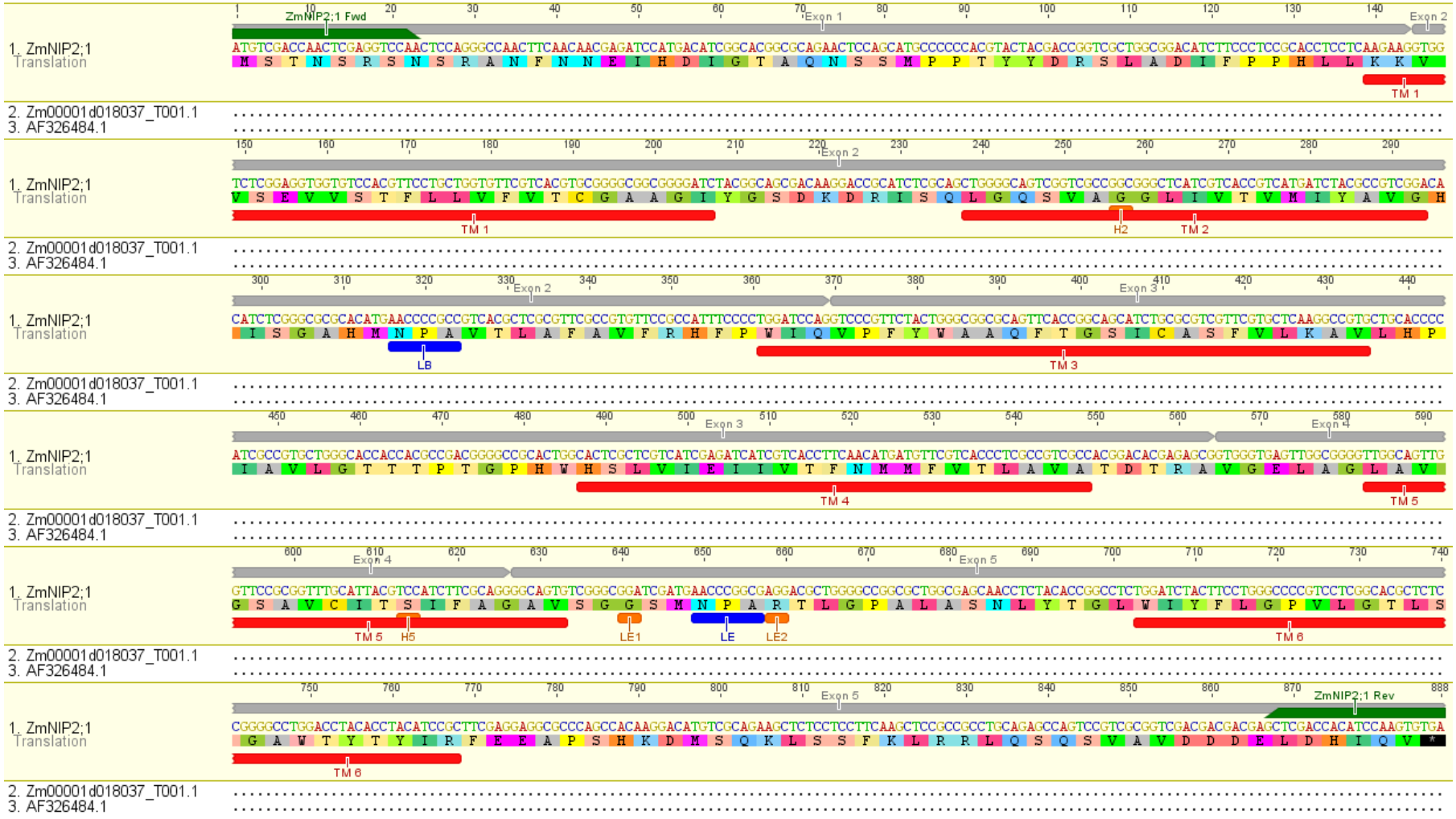


Figure 3.26 Alignments of the ORFs of the sequenced ZmNIP2;1 clone, the genomic prediction (Zm00001d018037_T001.1) and published cDNA (AF326484.1)

The sequence of ZmNIP2;1 ORF confirms the identity of the ORFs for the genomic prediction and published cDNA.

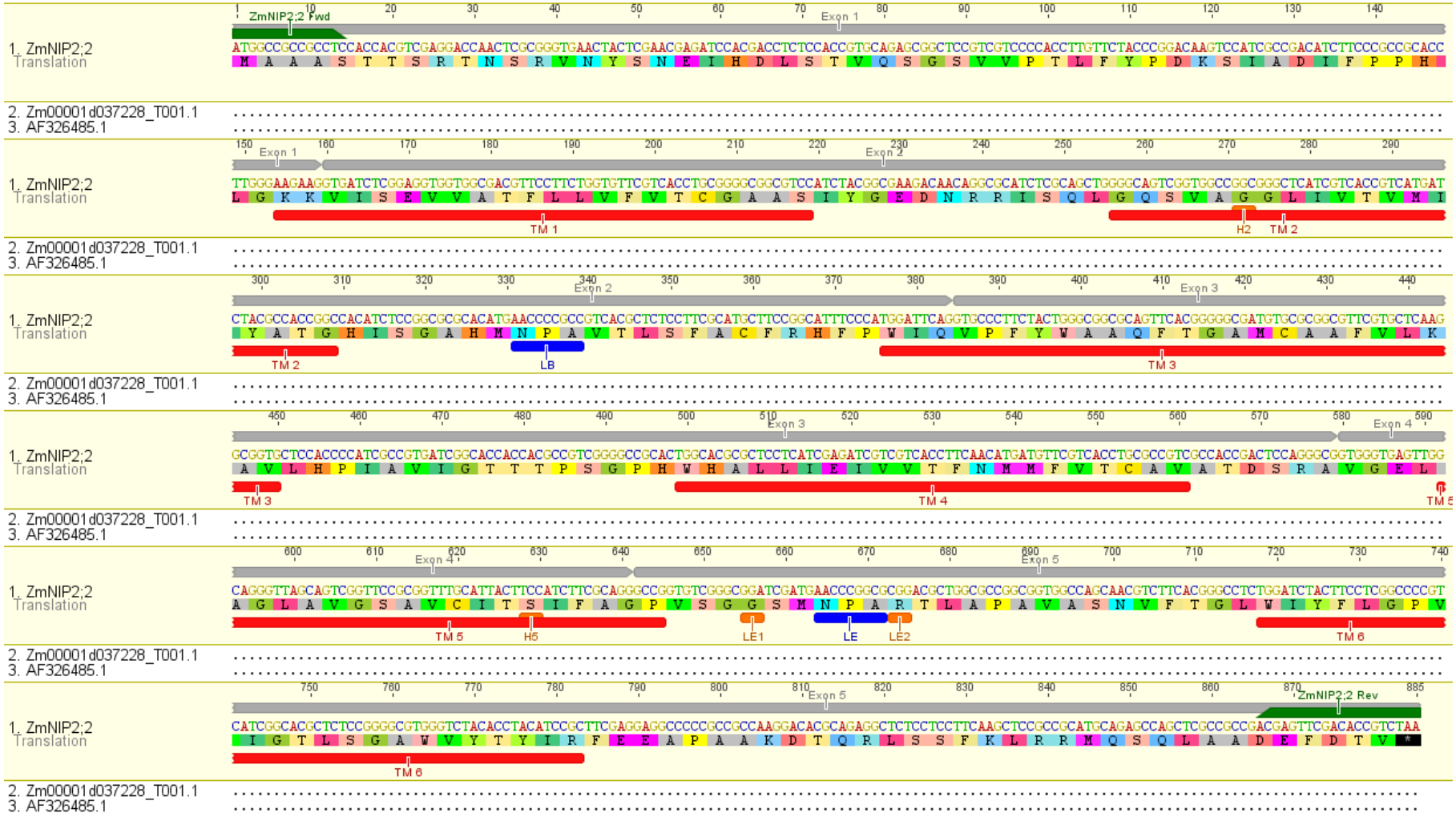


Figure 3.27 Alignments of the ORFs of the sequenced ZmNIP2;2 clone, the genomic prediction (Zm00001d037228_T001.1) and published cDNA (AF326485.1)

The sequence of ZmNIP2;2 ORF confirms the identity of the ORFs for the genomic prediction and published cDNA

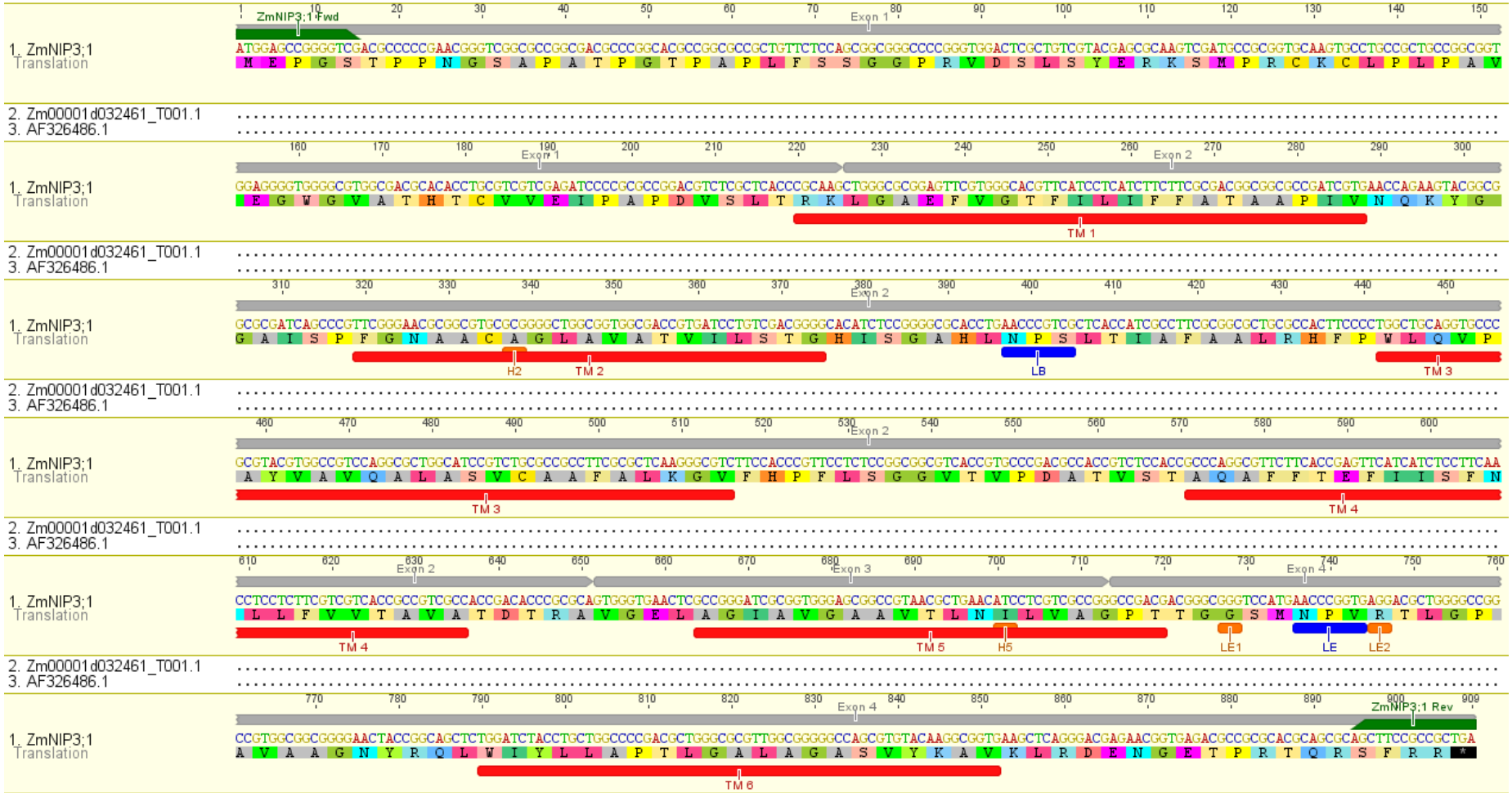


Figure 3.28 Alignments of the ORFs of the sequenced ZmNIP3;1 clone, the genomic prediction (Zm00001d032461_T001.1) and published cDNA (AF326486.1)
The sequence of ZmNIP3;1 ORF confirms the identity of the ORFs for the genomic prediction and published cDNA

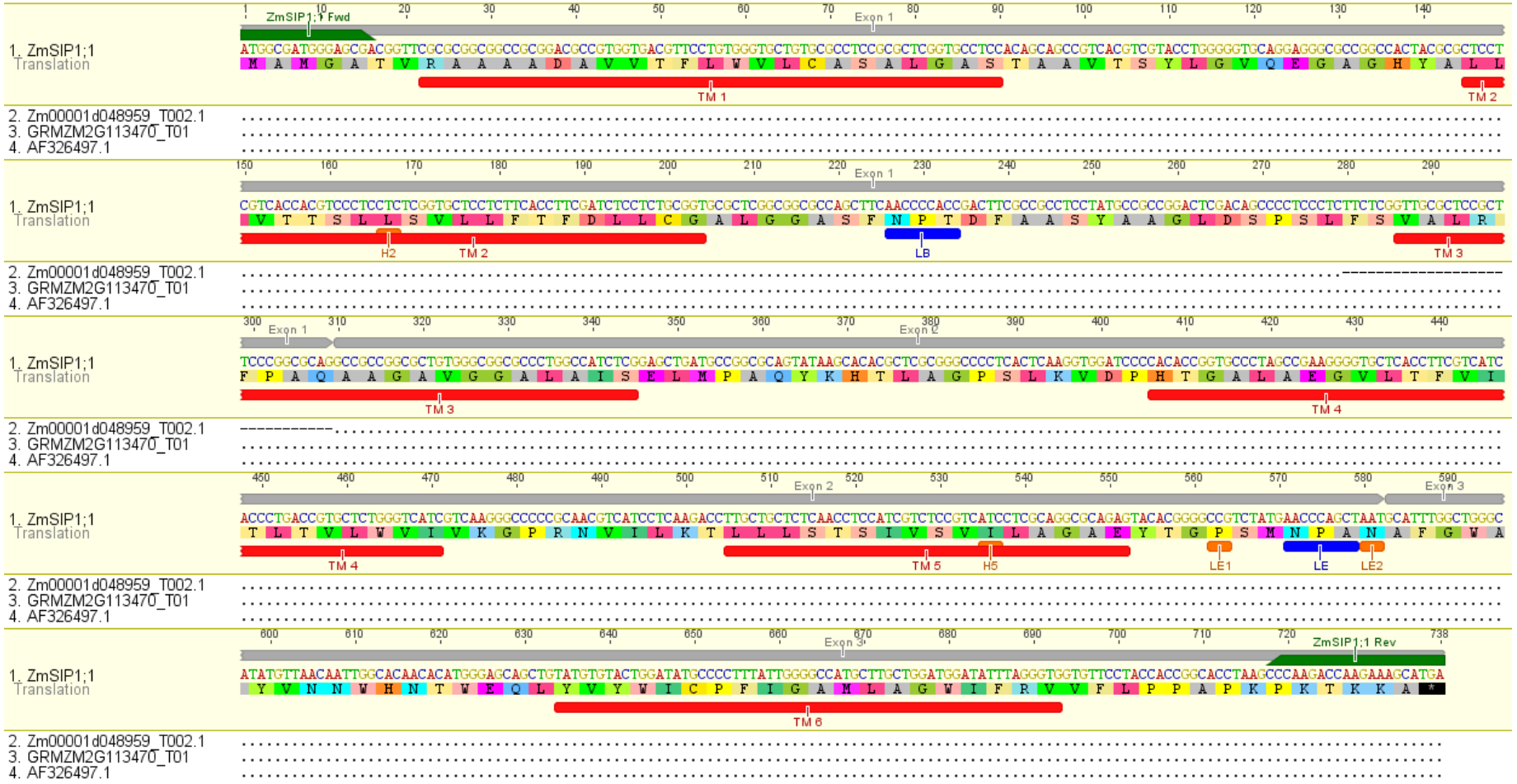


Figure 3.29 Alignments of the ORFs of the sequenced ZmSIP1;1 clone, the genomic predictions (APGv4 Zm00001d048959_T002.1 and AGPv3 GRMZM2G113470_T01) and published cDNA (AF326497.1)

The sequence of the ZmSIP1;1 ORF confirms the AGPv3 notation and its complete identity to the published cDNA. The APGv4 prediction lacks 30 base pairs at the end of exon 1 resulting in a ten amino acid truncation.



Figure 3.30 Alignments of the ORFs of the sequenced ZmSIP1;2 clone, the genomic prediction (Zm00001d010325_T001.1) and published cDNA (AF326498.1)
The sequence of ZmSIP1;2 ORF confirms the identity of the ORFs for the genomic prediction and published cDNA.



Figure 3.31 Alignments of the ORFs of the sequenced ZmSIP2;1 clone, the genomic prediction (Zm00001d028951_T001.1) and published cDNA (AF326499.1)

The sequence of ZmSIP2;1 ORF confirms the three base pair differences between the genomic predication and published cDNA. The amino acid sequences are identical.

3.6.3 Macros used for image processing and swelling assay

3.6.3.1 ImageJ macro for image processing

This macro for ImageJ was used to convert video files to area files. Text can be copied to a text file and saved "oocyte_area_measurement.ijm.txt" then run in ImageJ. This macro was run on a PC running Microsoft Windows 7.

```
// Oocyte Area Measurement //

//
// Description:       This is an ImageJ macro used to turn oocyte video files (.avi) to areas
//             (in mm^3 as a .csv file). These areas can be used to calculate the volume
//             changes over time and the osmotic permeability coefficients.
//
// Input:      A directory (folder) where .avi files of the swelling experiment are stored,
//             The experimental conditions including frame capture rate, and experimental
//             conditions.
//
// Output:     A new folder in the parent directory of the video files containing the .csv
//             files with areas. A new file labelled info.csv is also created which maps the
//             video file names to the injection type as well as stores the experimental
//             conditions.
//
// Author:     Jonathan Wignes, Steve Tyerman 29/09/2013
//             Updated 25/05/2015
//


// Choose the Directory to analyse

dirInput = getDirectory("Choose video directory: ");
filename = File.getName(dirInput);
print("Input Directory Path: ", dirInput);

parent = File.getParent(dirInput);
dirOutput = parent + File.separator + filename + " Area Files" + File.separator;


// The macro does not overwrite very well, before a new output folder is created
// user must remove prior analysis attempts by removing or renaming old output folders.

while(File.exists(dirOutput)){
       Dialog.create("Directory Error");
       Dialog.addMessage("Output directories already exist.");
       Dialog.addMessage("Please delete, move, or rename prior analysis attempt, then press
'ok' to continue, or click 'cancel' to exit macro.");
       Dialog.show();
}


// Create Ouput Folder in Input folder parent directory
File.makeDirectory(dirOutput);
print("Output Directory Path: ", dirOutput);


// Create a list of files to process and to show progress

listInput = getFileList(dirInput);
listOutput = getFileList(dirOutput);


//Prompt for experimental conditions, not necessary and easily edited later, but helpful for
downstream //processing

Dialog.create("Experimental Conditions and Video Attributes");
Dialog.addMessage("Experimental Conditions");
```

```
Dialog.addMessage("(These are stored in info.csv and can be adjusted later if needed)");
Dialog.addString("Swelling Type", "Water conductivity");
Dialog.addNumber("Initial Osmolarity", 240);
Dialog.addNumber("Final Osmolarity", 45);
Dialog.addMessage("Video Attributes");
Dialog.addNumber("Camera Frame Rate", 25);
Dialog.addNumber("Nth Frame Recorded", 50);
Dialog.addNumber("Maximum Frames Recorded", 180);
Dialog.show();

swellingType = Dialog.getString();
osmI = Dialog.getNumber();
osmF = Dialog.getNumber();
fpsC = Dialog.getNumber();
nframe = Dialog.getNumber();
secondsBetweenFrames = nframe/fpsC;
maxLength = Dialog.getNumber();


// Configure Output Settings

run("Input/Output...","jpeg=100 gif=-1 file=.csv use_file copy_row save_row");


// Set to run as batch mode (behind the scenes). Change to False to see swelling (a bit slower)

setBatchMode(true);


//For each video, open video, calculate swelling and save areas as .csv

for (index=0; index<listInput.length; index++){

        showProgress(index, listInput.length);
        path = dirInput + listInput[index];
        print("Current Video: ", path);

        //Open video, get name and set scale
        run("AVI...", "select=path first=1 last=maxLength convert");
        videoName = File.getName(path);
        List.set(index,listInput[index]);
        run("Set Scale...", "distance=222.11 known=1 pixel=1 unit=mm global");

        listInput = getFileList(dirInput);
        listOutput = getFileList(dirOutput);

        //Set and Run Threshold
        //This setting may need to be adjusted if threshold does not exactly mirror the oocyte.
        setThreshold(0,62);
        run("Threshold...");

        //Analyse Areas of Threshold images
        run("Set Measurements...", "area limit display redirect=None decimal=4");
        run("Analyze  Particles...",  "size=0.350-Infinity  circularity=0.00-1.00  show=Masks
display exclude clear stack");

        //Save results
        nameOutput = replace(videoName,"avi","csv");

        saveAs("Results", dirOutput + File.separator + nameOutput);
        close();
        close();
        print("Completed " + index+1 + " out of " + listInput.length + " files.");
}


//clear results
run("Clear Results");


//Create map for video file names with images
for (j=0; j<listInput.length; j++){
        setResult("Video Number", j, List.get(j));
}
updateResults();
```

```
//Organize Injections

Dialog.create("Injections");
Dialog.addNumber("Number of Injections ",0);
Dialog.show();
injectionNumber = Dialog.getNumber();

Dialog.create("Injection labels");
for(n=0; n<injectionNumber; n++){
        Dialog.addString("Injection Type " + n+1, "");
}
Dialog.show();

item0 = "n/a  ";
item1 = Dialog.getString() + " ";
array = newArray(item0, item1);

for(m=1; m<injectionNumber; m++){
        nextItem = Dialog.getString() + "   ";
        array = Array.concat(array, nextItem);
}


//goes through index, will present in patterns of injection number
for(index=0; index<listInput.length; index+=injectionNumber){
        Dialog.create("Label Injections");
        Dialog.addMessage("Select the injection label for each video");

        num2Show = injectionNumber;
        if(listInput.length - index < injectionNumber) num2Show = listInput.length-index;
        if(listInput.length<9) num2Show = listInput.length;

        for(z=index; z<index+num2Show; z++){
                label = List.get(z);
                Dialog.addRadioButtonGroup(label, array, 1, injectionNumber, "No Label");
        }
        Dialog.show();

        for(z=index; z<index+num2Show; z++){
                videoID = Dialog.getRadioButton();
                setResult("Injection Type", z, videoID);
        }

        if(listInput.length<9) index = listInput.length;
}



// Put experimental Conditions into results and save

for(x=0; x<listInput.length; x++){
        setResult("Swelling Type", x, swellingType);
        setResult("Initial Osmolarity", x, osmI);
        setResult("Final Osmolarity", x, osmF);
        setResult("Camera Frames per Second", x, fpsC);
        setResult("Nth frame recorded", x, nframe);
        setResult("Seconds Between Frames", x, secondsBetweenFrames);
        setResult("Maximum Video Length (s)", x, maxLength);
}
updateResults();

run("Input/Output...", "file=.csv copy_column save_column");
infoFile = dirOutput +  "info.csv";
selectWindow("Results");
saveAs("results", infoFile);
print("Experimental Conditions and Injection Type info saved as: ", infoFile);

exit("Finished!");
```

3.6.3.2 MATLAB macro for processing area files

This macro can be saved as "OocyteSwellingAnalyser.m" and run from the MATLAB command by calling "OocyteSwellingAnalyser()". The program takes the directory containing the .xls files containing areas, reads and copies each to a single file, and calculates the volumes, relative volumes, and surface areas for each oocyte. It plots the relative volume change (V_t/V_0) and prompts the user to select bounds where it is linear. These bounds are used, along with the data from the swelling experiment, to calculate the osmotic permeability coefficient for each oocyte using **Equation 3.1**. Results and all data steps are saved in an .xls file. This macro was run on a PC running Microsoft Windows 7.

```
function [ string ] = OocyteSwellingAnalyser()
%OocyteSwellingAnalyzer will analyse oocyte swelling data from areas
%   OocyteSwellingAnalyzer asks user to select directory of area analysis
%   files. In this folder must be a file called 'info.csv' that contains a
%   video to injection map, osmolarity changes, and the frame rate of the
%   videos in the file. It prompts for user to point out on plot of rel.
%   vol. vs time what bounds for calculations should be used. It outputs an
%   array in MATLAB containing all relevant data and saves this in the
%   directory as an .xls file

%   Select Directory to analyse that contain area files and 'info.xls'
directory = uigetdir('','Please select directory to analzye: ');
directoryitems = dir(fullfile(directory,'*.csv'));
directoryitems = {directoryitems.name};
%videoInfoFile = fullfile(directory,'info.csv');

numcols = length(directoryitems);
k=1;
for i = 1:numcols
    if not(strcmp(directoryitems{i},'info.csv'))
        vidfilenames{k}=directoryitems{i};
        k=k+1;
    end
end

numgvids = length(vidfilenames);

%   Construct final array structure
results{1,1}  = 'Assay type';
results{2,1}  = 'Initial osmolarity';
results{3,1}  = 'Final osmolarity';
results{4,1}  = 'Camera speed (fps)';
results{5,1}  = 'nth frame recorded';
results{6,1}  = 'Maximum number of frames recorded';
results{8,1}  = 'Video name';
results{9,1}  = 'Injection type';
results{10,1} = 'Osmotic water permeability (Pf in mm/s)';
results{11,1} = 'Lower bound (s)';
results{12,1} = 'Upper bound (s)';
results{13,1} = 'time (s)';
results{2,4}  = 'Water concentration';
results{2,5}  = 1.8*10^-5;
results{13,2} = 'Linear Relative Volume';

%   Place Video file names into array
for i=1:numgvids
    results{8,i+1} = vidfilenames{i};
```

```
end

%   Find and read video attributes
%[ndata, text, videoInfo] = xlsread(videoInfoFile);
videoInfor = {100,100}
results{1,2}  = videoInfo{2,3};
results{2,2}  = videoInfo{2,4};
results{3,2}  = videoInfo{2,5};
results{4,2}  = videoInfo{2,6};
results{5,2}  = videoInfo{2,7};
results{6,2}  = videoInfo{2,9};

[vidrs vidcs] = size(videoInfo);
for i = 2:vidrs
    for j=2:numgvids+1
        name1ext = videoInfo{i,1};
        name2ext = results{8,j};
        name1lngth = length(name1ext);
        name2lngth = length(name2ext);
        name1 = name1ext(1:name1lngth-4);
        name2 = name2ext(1:name2lngth-4);
        if strcmp(name1,name2)
            results{9,j} = videoInfo{i,2};
        end
    end
end

%   Make Time Matrix and place in results table
fps = results{4,2};
nframe = results{5,2};
maxframes = results{6,2};
spf = nframe/fps;

timeM = zeros(maxframes,1);
for i = 1:maxframes
       timeM(i,1) = (i-1)*spf;
end

for i=1:maxframes
    results{13+i,1} = timeM(i,1);
end

%   Make area matrix from areas of each csv file
areaM = zeros(maxframes,numgvids);

for j = 1:numgvids
    file = fullfile(directory,vidfilenames{j});
    temparea = csvread(file,0,2);
    [tempareaR tempareaC] = size(temparea);
    for i = 1:tempareaR
        areaM(i,j) = temparea(i,1);
    end
end

%   Calculate Vol from area
[areaR areaC] = size(areaM);
volumeM = zeros(areaR,areaC);
for j = 1:areaC
    for i = 1:areaR
        volumeM(i,j) = areaM(i,j)*4/3*sqrt(areaM(i,j)/pi);
    end
end

%   Calculate Relative Volume from vol.
relvolM = zeros(areaR,areaC);
for j=1:areaC
    for i=1:areaR
        relvolM(i,j) = volumeM(i,j)/volumeM(1,j);
    end
end

%   Calculate initial surface area for each oocyte
insurfareM = zeros(1,areaC);
for i = 1:areaC
    insurfareM(1,i) = 4*areaM(1,i);
end
```

```
%   Prepare a plotting matrix for Relative Volume
RelVol =zeros(areaR,areaC+1);
for i=1:areaR
    RelVol(i,1) = timeM(i,1);
end
for i=1:areaR
    for j=2:areaC+1
        RelVol(i,j) = relvolM(i,j-1);
    end
end

%   Create a temporary bounds matrix.
bounds = zeros(2,numgvids);

%   Plot each and get bounds of linearity
for currentvideo = 1:numgvids
    x = zeros(maxframes,1);
    y = zeros(maxframes,1);

    for i = 1:maxframes
        x(i,1) = RelVol(i,1);
        y(i,1) = RelVol(i,currentvideo+1);
    end

    ymin = min(y);
    ymax = max(y);
    index = find(ymax==y);

    if length(index)>1
        index = index(1);
    end
    if index<=4
        index = 5;
    end

    xmin =0;
    xmax = x(index);
    if ymin==0
        ymin = 1-.01*ymax;
    end
    if ymin==ymax
        ymax = ymax*1.10;
    end

    relvolPlot                          =                          plot(x(1:index),y(1:index),'-
ro','MarkerEdgeColor','k','MarkerFaceColor','b','MarkerSize',5);
    axis([xmin xmax ymin ymax]);
    [xbounds ybounds] = ginput(2);

    lowertbound = timeM(maxframes,1);
    uppertbound = timeM(1,1);

    difference1 = abs(lowertbound - xbounds(1,1));
    for i = 1:maxframes
            difference2 = abs(xbounds(1,1) - timeM(i,1));
            if difference2<=difference1
                lowertbound = timeM(i,1);
                difference1 = difference2;
            end
    end

    differencea = abs(uppertbound - xbounds(2,1));
    for i = 1: maxframes
        differenceb = abs(xbounds(2,1) - timeM(i,1));
        if differenceb<=differencea
            uppertbound = timeM(i,1);
            differencea = differenceb;
        end
    end

    bounds(1,currentvideo) = lowertbound;
    bounds(2,currentvideo) = uppertbound;
    results{11,currentvideo+1}=lowertbound;
    results{12,currentvideo+1}=uppertbound;
    close(gcf);
end
```

```
%   Calculate initial slope of each graph.
slope = zeros(1,numgvids);
for currentvideo = 1: numgvids
    lbindex = find(timeM==bounds(1,currentvideo));
    ubindex = find(timeM==bounds(2,currentvideo));
    x = RelVol(lbindex:ubindex,1);
    y = RelVol(lbindex:ubindex,currentvideo+1);
    line = polyfit(x,y,1);
    slope(1,currentvideo) = line(1);
    for j = 1:length(x)
        results{13+j,currentvideo+1}=y(j);
    end
end

%   Calculate osmotic permeability and place in results
for currentvideo = 1:numgvids
    initialVolume = volumeM(1,currentvideo);
    initialSlope = slope(1,currentvideo);
    initialSurfaceArea = insurfareM(1,currentvideo);
    Vwater = 1.80 * 10^(-5);
    osmi = results{2,2};
    osmf = results{3,2};
    Pos = (initialVolume*initialSlope) / (initialSurfaceArea*Vwater*(osmi-osmf));
    results{10,currentvideo+1}=Pos;
end
%   Organize new arrays for various information
template = results(8:9,1:numgvids+1);
template{3,1} = 'time (s)';
for i = 1:length(timeM)
    template{3+i,1}=timeM(i,1);
end

%   Area
Areas = template;
Areas{3,2}= 'Raw Area in mm^2';
for j=1:numgvids
    [a b] = size(areaM);
    for i=1:a
        Areas{i+3,j+1} = areaM(i,j);
    end
end

%   Volumes
Volumes = template;
Volumes{3,2}= 'Raw Volumes in mm^3';
for j=1:numgvids
    [a b] = size(volumeM);
    for i=1:a
        Volumes{i+3,j+1} = volumeM(i,j);
    end
end

%   Relative Volumes
RelVolumes = template;
RelVolumes{3,2} = 'Relative Volumes from t=0';
for j=1:numgvids
    [a b] =size(relvolM);
    for i=1:a
        RelVolumes{i+3,j+1}=relvolM(i,j);
    end
end

%   Initial Surface Area, slope
Others = results(8:9,1:numgvids+1);
Others{3,1} = 'Initial Surface Area';
Others{4,1} = 'Initial Slope';
for j=1:numgvids
    Others{3,j+1}=insurfareM(j);
    Others{4,j+1}=slope(j);
end

%   Export all results to new excel file 'results.xlsx' in directory
exportFileName = fullfile(directory,'results.xlsx');
sheet = 2;
xlswrite(exportFileName,Areas,sheet);
sheet = 3;
xlswrite(exportFileName,Volumes,sheet);
```

```
sheet = 4;
xlswrite(exportFileName,RelVolumes,sheet);
sheet = 5;
xlswrite(exportFileName,Others,sheet);
sheet = 1;
xlswrite(exportFileName,results,sheet);

e = actxserver('Excel.Application');
ewb = e.Workbooks.Open(exportFileName);
ewb.Worksheets.Item(2).Name = 'Raw Area';
ewb.Worksheets.Item(3).Name = 'Raw Volume';
ewb.Worksheets.Item(4).Name = 'Relative Volume';
ewb.Worksheets.Item(5).Name = 'Initial SA and Slope';
ewb.Worksheets.Item(1).Name = 'Results';
ewb.Save;
ewb.Close(false);
e.Quit;

string = strcat('Analysis of directory',' ',directory,' is complete');

end
```

Chapter 4: Maize root hydraulic responses to nitrogen

4.1 Introduction

Water flow through the root depends on the relative resistances to water flow and solute selectivities of the pathways as developed in the composite transport model of the root (Steudle 2001). There are two major components for the whole root: axial flow through open xylem vessels and radial flow across the root perpendicular to the xylem. The radial flow is dependent on the relative resistances of the apoplastic pathway and cell to cell pathway and the type of gradient that generates water flow (i.e. hydrostatic and/or osmotic) (Steudle 2000; Steudle and Peterson 1998). In roots, apoplastic barriers (Schreiber et al. 2005; Schreiber et al. 1999) can limit the flow of solutes (Sattelmacher 2001) and water (Zimmermann et al. 2000) through the apoplastic pathway by greatly raising the resistance to diffusion. The resistance of the cell to cell pathway can be lowered through the activity of aquaporins.

Hydraulic conductance, defined as a flow rate per unit differential pressure, is the inverse of a hydraulic resistance. Water flow is dependent on not only the resistance, but also the area of coverage, and can be normalised to per unit surface area to give the hydraulic conductivity. In many cases, including in maize roots (Frensch and Steudle 1989), the axial resistance is much less than the radial resistance, providing a mechanism for aquaporins to modulate whole root water transport. Root water flow is complex and is affected by many factors such as low temperature (Lee and Chung 2005), diurnally (Henzler et al. 1999), drought stress (Vandeleur et al. 2009), salt stress (Boursiac et al. 2005), anoxia from flooding (Tournaire-Roux et al. 2003), shoot wounding (Vandeleur et al. 2014) as well as many other factors (Gambetta et al. 2017; Aroca et al. 2012).

Nitrogen nutrition can have an effect on plant root hydraulic properties, and does so in maize. Barthes et al. (1996a) supplied nitrogen as nitrate, urea, and amino acids and found that after 16 hours (h), all had raised the exudation rate (water flow) in low N grown maize (inbred line F7F2) seedlings. The exudate (xylem sap) osmotic concentration change was not sufficient to explain the increased water flow, indicating that there was increased root hydraulic conductivity (Barthes et al. 1996a). A time course experiment showed the exudation rate of nitrate supplied plants was increased within 2 h, while that of urea supplied plants increased over 6 h of treatment to a value less than the initial increase seen with nitrate (Barthes et al. 1996a). This indicates that in maize, hydraulic conductance is sensitive to the form of nitrogen supplied. The exudation rate was also increased over –N plants after three days. An increase in exudation rate that was not fully explained by increased osmotic gradient was also seen by Hoarau et al. (1996) in the inbred line F7F2 after 16 h nitrate resupply. It should be noted that measuring exudation rates to assess hydraulic conductance of roots can be subject to artefacts as a result of root excision for long periods, which has been shown to lower hydraulic conductance (Vandeleur et al. 2014), and internal gradients in osmotic pressure within the xylem sap (Fritz et al. 2010).

Górska et al. (2008), performed a similar experiment (using the hybrid 'White sweet') but examined the effect of nitrate resupply on individual roots over four h. Seedlings were de-topped and placed in an aerated pressure chamber and the de-topped root attached to a container on a balance. This allowed the authors to apply a pressure and observe the exudation in real time. Plants were attached and a baseline flow rate established. Plants were then resupplied with nitrate, monitoring the flow rate relative to the baseline. They showed nitrate induced an increase in flow rate above the initial (not observed when nitrate was not added on control plants), with a delay of ~1 h. The flow rate increased for

at least four h where it was +50% of the initial flow rate. The osmotic gradient across the roots increased, but could only account for ~7% of the increased flow, leaving the remainder of the increase to be from increased hydraulic conductivity. Furthermore, in both control (-N) roots and the nitrate fed roots, flow rates were reduced to less than half the initial baseline rate in 1.5 h of anoxia treatment, with a rapid initial decline over the first 20 min, suggesting pH gating of aquaporins that occurs under anoxia (Tournaire-Roux et al. 2003). Within 4 h, neither urea nor ammonium (both nitrate assimilation products) induced an increased flow rate. Finally treating plants with tungstate (to inhibit nitrate reductase - NR) did not induce increased flow in –N plants after nitrate addition. However, NR inhibited roots did not accumulate nitrate (nor nitrite), suggesting either uptake was also inhibited or that the rate at which nitrate was unloaded to the xylem was similar to the uptake rate. In roots with NR activity, concentrations of both nitrate and nitrite increased. The authors concluded that increased cellular nitrate concentrations (or nitrite) are necessary for an increase in the hydraulic conductivity to occur. In later research, they showed the magnitude of the nitrate induced flow across species to be dependent on the nitrate uptake rate of the species (Górska et al. 2010) further supporting their hypothesis that increased tissue nitrate concentrations are required in signalling for nitrate-induced increase in hydraulic conductivity.

These previous experiments looked at resupply, over short to long term (0-72 h), but did not examine the effect that starvation had on the hydraulic conductivity of maize plants in either a short or long term. In addition, they utilised inbred and hybrid maize lines that may be different from the response of inbred line with a sequenced genome, B73. The aims of the experiments in this chapter were to test the hypothesis that nitrate starvation leads to a decrease in the hydraulic conductivity of maize plants that can be rescued with nitrate resupply, and to characterise the hydraulic response to nitrate. Maize inbred lines

B73 and F44 were used to investigate the hydraulic response to nitrogen. B73 is an inbred line that was selected as its genome serves as the reference genome for maize (Schnable et al. 2009; Jiao et al. 2017) and has been used in many studies. B73 and F44 are longstanding inbred lines and are parents of many other maize (Inbred Releases By Year: From 1974 to 2011). In a genetic analysis of 260 inbred lines based on polymorphisms of microsatellite loci, B73 and F44 were grouped to distinct (Stiff Stalk (SS) and Non-Stiff Stalk (NSS), respectively) subgroups and have a minimum genetic distance (Nei's minimum genetic distance) of 0.32 (Liu et al. 2003a).

4.2 Materials and methods

4.2.1 Plant materials and germination

Seeds were obtained from Pioneer Seeds (Toowoomba, Qld, Australia) and stored at 4°C. Seeds were surface sterilized in 4-6% bleach with 0.05% Tween 20 for 5 min then rinsed with RO (reverse osmosis) water. A plastic tray with holes in the bottom (to drain excess water) was lined with approximately 10 mm of small diatomite (roughly 5 mm in diameter). Seeds were placed, then covered with 20-30mm of diatomite and watered and allowed to drain. Trays were covered and placed at 25°C for 3-4 days until shoots emerged. Trays were kept moist.

After shoots emerged, plants were carefully removed and rinsed to remove diatomite from roots. Plants with fungal infections were discarded. Plants were placed into individual trays consisting of a hollow tray covered with mesh. For stability, a short PVC section was attached. Shoots were carefully threaded through a small hole in the mesh to the start of the nodal roots. Wet diatomite was placed around the shoot for stability and to hold the

seedling and tray together. As shoots grew, pressure from the expanding shoot on the diatomite and PVC would keep the plant from falling out of the tray.

4.2.2 Growth conditions

4.2.2.1 Low light growth room hydroponics

A hydroponics system was set up in 13L plastic containers (about 150 mm deep) (**Figure 4.1**). Nutrient solution (**Table 4.1**) was placed in the container and aerated with an aquarium pump with aeration stone continuously. Solution was topped off every second to third day and changed weekly. A plastic lid was placed such that it was at the level with the top of the solution and had holes to support the individual plant trays. Empty spaces were kept covered to limit light entering the hydroponic solution. The growth room was set to 22°C during the day (12 h) and 18°C night (12 h) due to restrictions from other growth room users.

Table 4.1 Nutrient solutions for hydroponics and aeroponics

Nutrient solutions used for maize in hydroponic systems and aeroponic system. All solutions were maintained at pH 5.7-6.3 using KOH or H_2SO_4.

Ion	Hydroponics					Aeroponics	
	Ammonium and Nitrate		Ammonium or Nitrate			Ammonium or Nitrate	
	+AN (5 mM)	-N	+A (5 mM)	+N (5 mM)	-N	+N (15 mM)	-N
NH_4^+	2.5 mM	0 mM	5 mM	0 mM	0 mM	0 mM	0 mM
NO_3^-	2.5 mM	0 mM	0 mM	5 mM	0 mM	15 mM	0 mM
PO_4^{3-}	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM	1.4 mM	1.4 mM
K^+	4.05 mM	4.05 mM	4.05 mM	4.05 mM	4.05 mM	6.4 mM	6.4 mM
Mg^{2+}	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.5 mM	0.75 mM	0.75 mM
Cl^-	1.55 mM	1.55 mM	1.55 mM	1.55 mM	1.55 mM	2.0 mM	13 mM
SO_4^{2-}	3.0545 mM	3.0545 mM	6.0045 mM	1.0045 mM	1.0045 mM	0.7625 mM	2.7625 mM
Ca^{2+}	2.00 mM	2.00 mM	2.00 mM	2.00 mM	2.00 mM	6.0 mM	6.0 mM
Fe^{3+}	0.20 mM	0.20 mM	0.20 mM	0.20 mM	0.20 mM	0.20 mM	0.20 mM
MnO_4^{2-}	2 µM	2 µM	2 µM	2 µM	2 µM	10 µM	10 µM
ZnO_4^{2-}	2 µM	2 µM	2 µM	2 µM	2 µM	2 µM	2 µM
H_3BO_3	25 µM	25 µM	25 µM	25 µM	25 µM	20 µM	20 µM
$CuSO_4^{2-}$	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM
MoO_4^{2-}	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM	0.5 µM
Na^+	1 µM	1 µM	1 µM	1 µM	1 µM	1 µM	1 µM

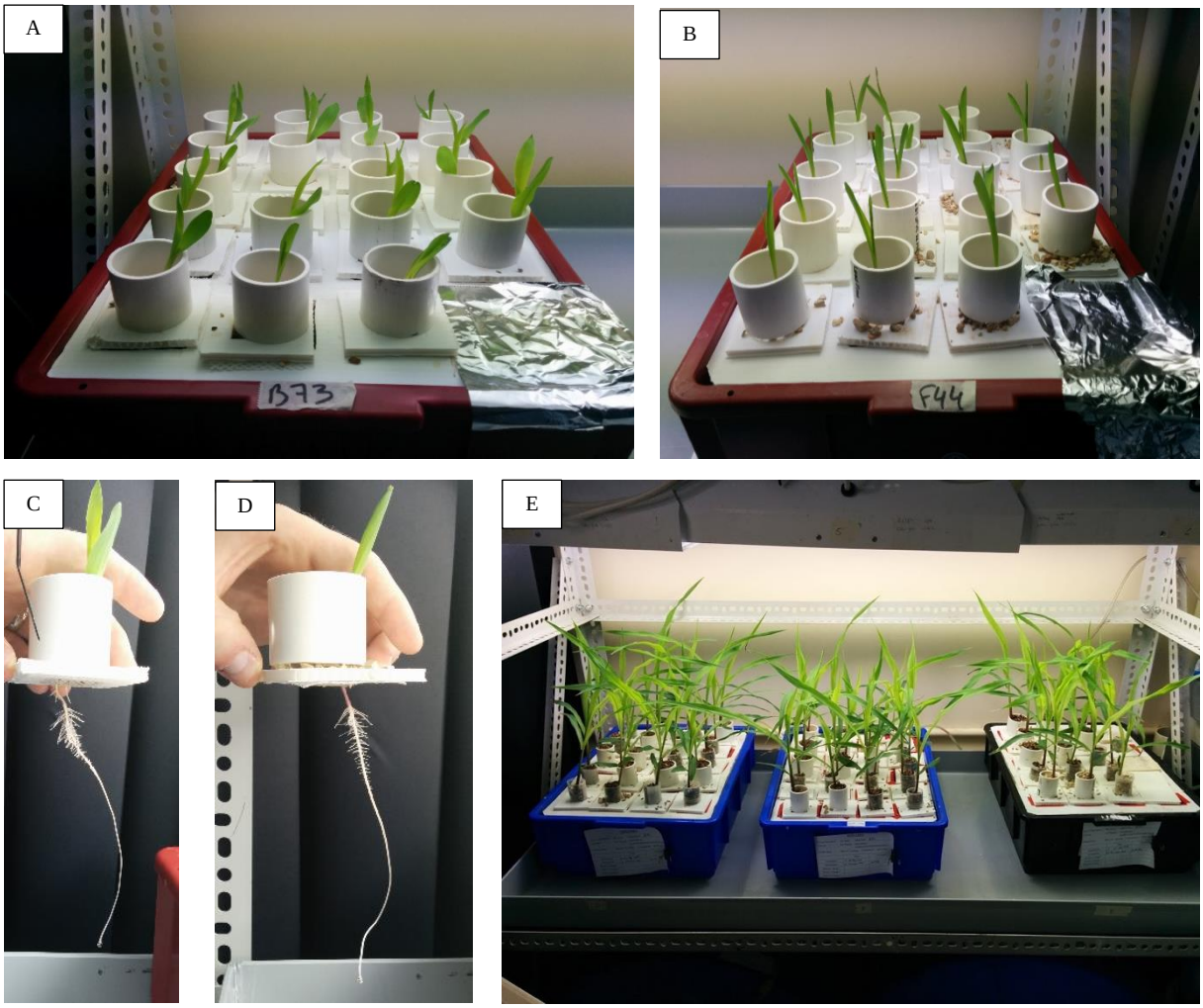


Figure 4.1 Maize hydroponic system
(A) B73 seedlings in hydroponic system. System consisted of trays with mesh bottoms and flat base and a PVC section. Shoots were threaded through a hole cut in the mesh, then surrounded by wet diatomite for support. Trays rested on a lid at the solution surface level in a of a 13 L plastic container (about 15 cm deep). The tank was aerated with an aquarium air pump continuously (not shown). Tanks were placed under growth room lights with 12 light cycles at 20°C. (B) Side on view of a representative B73 maize seedling after 5 days of hydroponic growth. (C) F44 seedlings in hydroponic system. (D) A representative F44 primary root after five days of hydroponic growth. At five days, the major root for either B73 or F44 was the primary root. (E) Maize seedlings after 14 days in hydroponics.

A baseline light condition was established by measuring light intensity with an ICT Photosynthetically Active Radiation (PAR) meter. A planar photometer was placed in plane with the bottom of the tray parallel to solution level and a reading taken. Measurements were taken from different positions on the container as a proxy of light available to the plant for photosynthesis (**Figure 4.2**). Intensities ranged from 37 to 95 µmol m^{-2} s^{-1} with a mean of 69.85 µmol m^{-2} s^{-1} (SD=14.31, SEM=1.947, CV=20.49%).

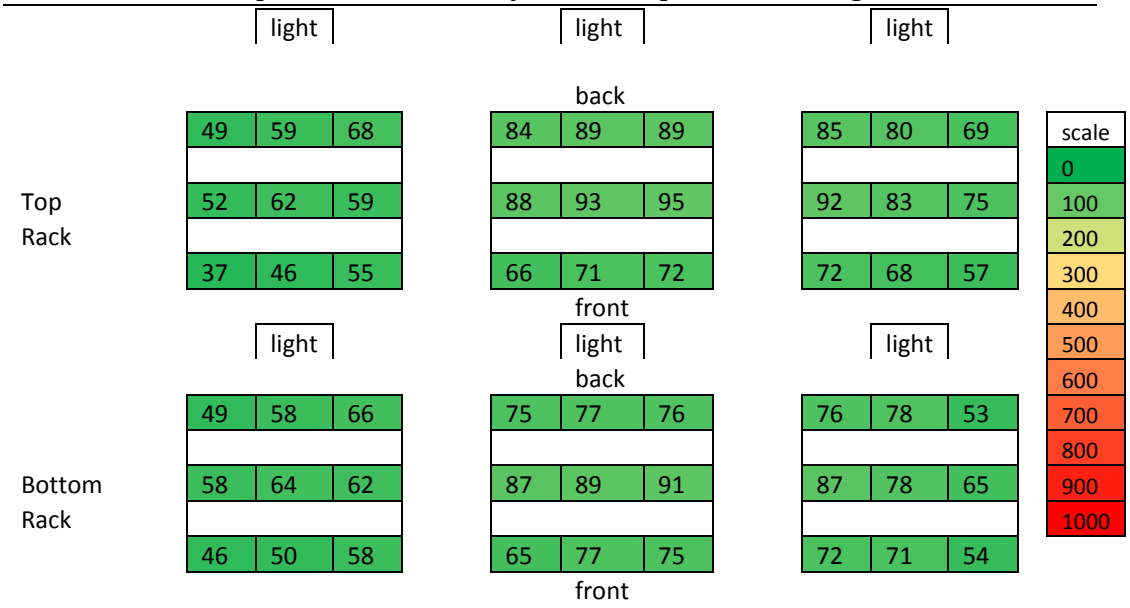


Figure 4.2 Light conditions in growth room
Light conditions of the growth room, as seen in **Figure 4.1**.

4.2.2.2 Medium light growth chamber hydroponics

The hydroponic system (**Figure 4.1**) and nutrient solutions (**Table 4.1**) used in the low light was also used to grow maize under medium light in a growth chamber. Light conditions were determined as above and are shown in **Figure 4.3**. The light intensities had a mean value of 295.6 µmol m^{-2} s^{-1} (SD=56.58, SEM=6.668, CV=19.14%). Plants were kept at 26°C during the day (18 h) and 18°C at night (6 h). Ultimatley, three items were changed: increased day temperatures (same night), increased light:dark period, and increased light.

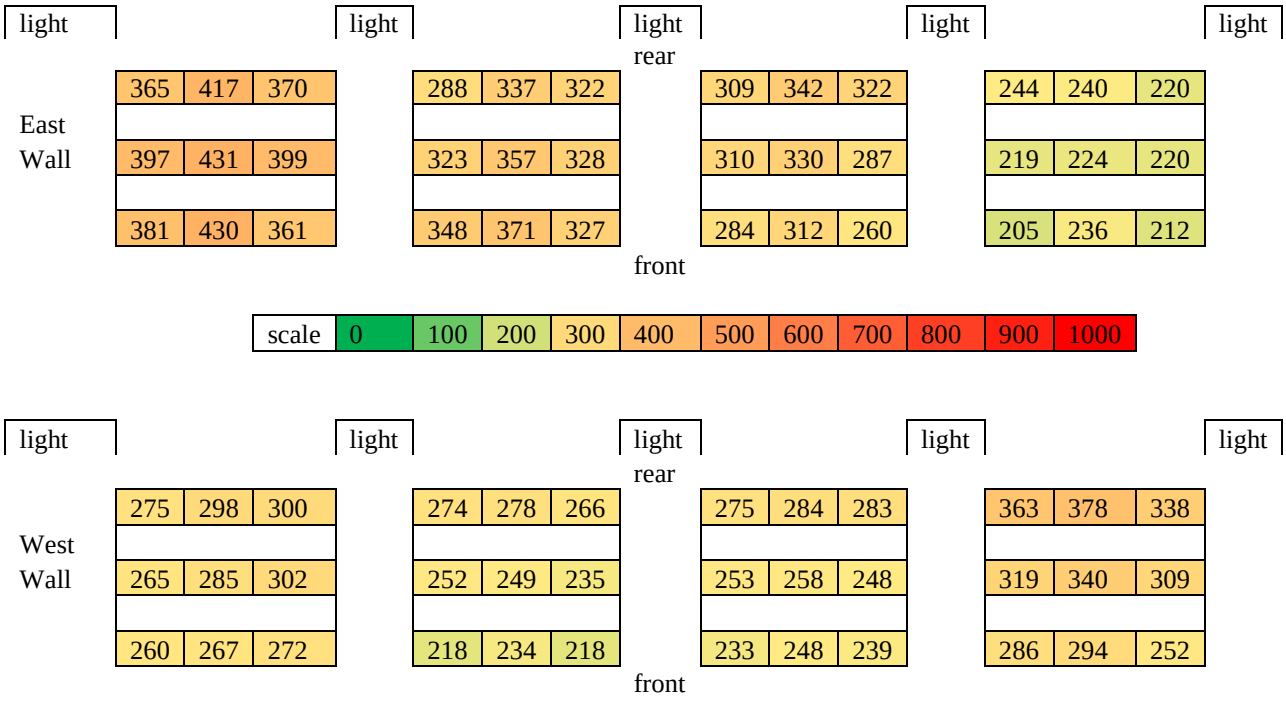


Figure 4.3 Light conditions in growth chamber

Hydroponic systems were set up in a growth chamber with larger lights. These lights had greater PAR output at the maize plant level, but were still well below the photosynthetic saturation intensity for maize. Fletcher et al. (2008) showed that the photosynthetic capacity for maize was not saturated until at least 1500-2000 µmol·m^{-2}·s^{-1}. The growth was set up with two rows of plants each under 5 lights (labelled east and west). All units µmol·m^{-2}·s^{-1} with minimum value of 205 µmol·m^{-2}·s^{-1} and maximum of 431 µmol·m^{-2}·s^{-1}.

4.2.2.3 High light glass house aeroponics

Maize plants were grown in an aeroponic system in a glass house (**Figure 4.4**). The aeroponic system, inspired by Gaudin et al. (2011), was constructed in a glasshouse consisting of three root growth chambers connected to a nutrient reservoir. The root growth chambers were constructed from dark green 240 L wheelie bins (Adelaide Merchandising, Adelaide, SA Australia) and were placed on a raised platform. The lid had 13 holes cut to support the seedling trays used in the hydroponics system, and were about 1.05 m tall. A main line made of 19 mm polyethylene (PE) tubing (Holdman

Industries, Osbourne Park, WA Australia) supplied nutrient solution pumped from the nutrient reservoir to 11 micro jet spray misters (Pope, Beverley, SA Australia) connected via 4 mm PE tubing (Neta Australia, Tuggerah, NSW Australia). Spray misters were installed into the sides of the root growth chamber and positioned so all roots would receive mist. The growth chamber had a hole drilled and fitted with a rubber grommet to allow solution to gravitationally return (through 13-19 mm PE tubing (Holdman)) to the nutrient reservoir. The nutrient reservoir (a 76 L plastic bin) contained two submersible pumps (EHEIM #1262 or #1260, Deizisau, Germany) at 2400-3400 L/h connected in series to pump solution to the micro jets. A third pump was used to mix the nutrient solution in the tank. The pumps were controlled by a time relay (SuperPro Hydroponics, Xiamen, China) set to 10 s on, 50 s off. Two systems were constructed. The nutrient solution (**Table 4.1**) was topped up 2-3 times per week (due to evaporation and leakages) and replaced with fresh solution weekly. Experiments were carried out in the Australian summer and spring from December 2016 to March 2017. In the sun, the surface of the tanks received 1000-1100 µmol m^{-2} s^{-1} light and was reduced to 250 µmol m^{-2} s^{-1} when overcast. Experiments were carried out on sunny days.


A

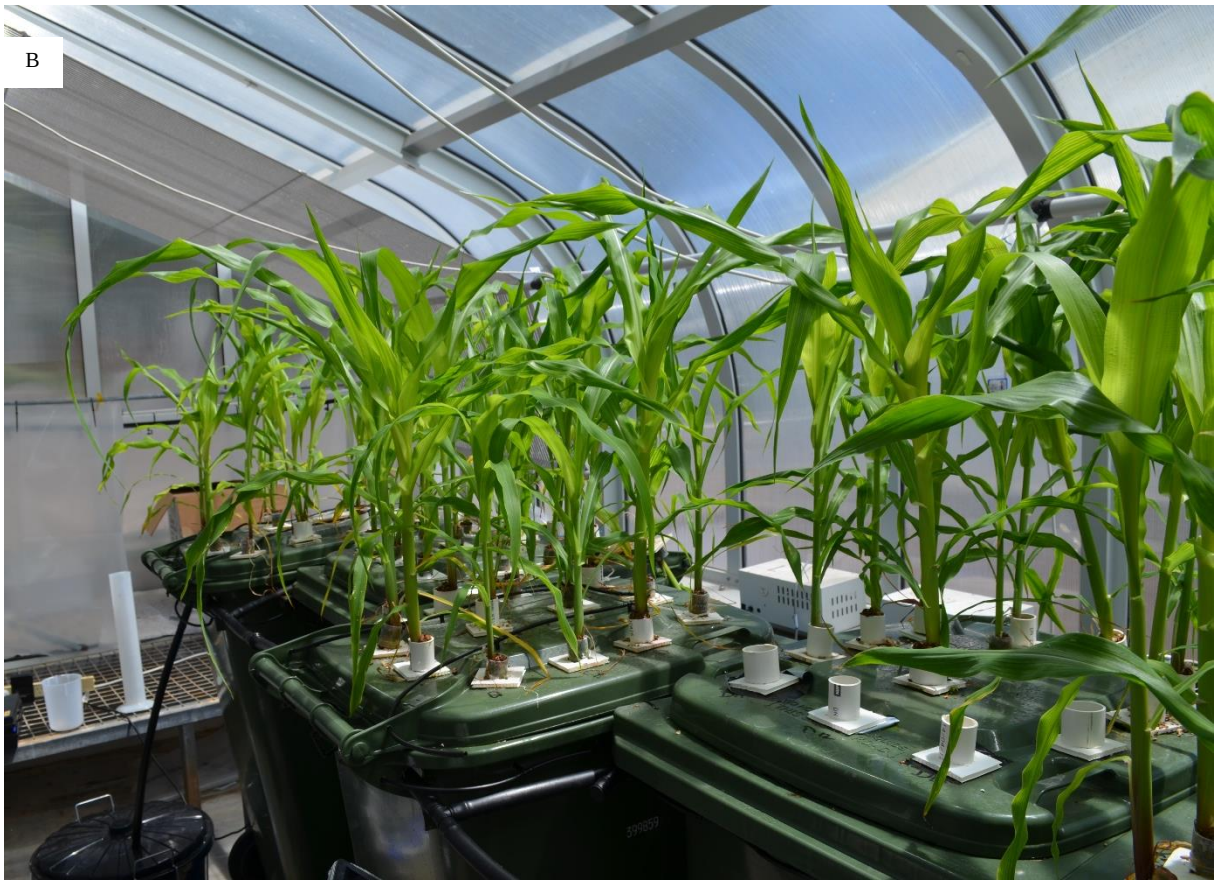

B







Figure 4.4 Aeroponic system

(A) Two aeroponic systems set up on a raised bench with 7 day old plants. The top of one nutrient reservoir located on the bottom left. Visible is the 19mm line carrying nutrient solution from the pumps (not shown) to the 4mm tubing supplying the micro jets. Pumps were controlled by a relay timer (not shown) set to 10s on, 50s off with ~4 week old plants. Nutrient reservoir is the black tank on the bottom left. (C) Representative root s of F44 plant after three weeks. (D) Representative roots of B73 plant after 4 weeks. (E) Roots of F44 plant after 4 weeks. Plants were grown and experiments performed between the end of spring (November 2016) to the start of autumn (March 2017). Light intensity was variable, but peaked above 1000 µmol m^{-2} s^{-1} during sunny conditions and could be reduced to 250 µmol m^{-2} s^{-1} when overcast. Experiments were performed in sunny conditions.

4.2.3 Plant measurements

Plants were removed from the growth solution (hydroponics) or growth chamber (aeroponics). For plants grown in aeroponics, an image of the whole plant with shoot and roots system was taken with a Nikon DSLR camera. A single root segment, either primary or nodal, was selected and excised from the plant as close as possible to the base (near seed for primary roots and near base of plant for nodal roots). For aeroponics, the plant was returned to the growth chamber while hydraulic measurements were taken on the first root. A second root was then sampled.

4.2.3.1 Hydraulic measurements

Hydraulic measurements were carried out using a Xyl'em Flow meter (Instrutec, Montigny-lès-Cormeilles, France) or a high pressure flow meter (HCFM) (Dynamax, Houston, TX USA). In both systems, conductance was inferred from the slope from the linear regression of the flow rate of water into roots against the applied pressure. The excised root was quickly (<60 s) attached to HPLC tubing using a seal (cast from dental impression paste) and was made water tight by using a compression fitting on the end of the tubing. Care was taken to not introduce air to the coupling. Once attached, the system was depressurized between measurements. During measurements, roots were kept moist by placing in a container with a small amount (2-5 ml) of nutrient solution.

For hydraulic conductance measurements with the Xyl'em flow meter, the initial pressure was set near 0.200 MPa. The flow was allowed to reach a steady state and recorded. The pressure was reduced (0.03-0.05 MPa) and the flow rate again allowed to reach a steady state (always high to low pressure). This was repeated three times more on the same root to get a total of four measurements per root. The time from excising the root to the end of the final conductance for each root was 8-10 min. Flow rates were plotted against

pressure, and fit with a linear regression, the slope of which representing the hydraulic conductance and the x-intercept, the root pressure. Measurements taken on the Xyl'em flow meter were performed with an external flow sensor (<5 g/h).

Hydraulic conductance measurements taken with the HCFM (Hydraulic Conductance Flow Meter) originally designed by Mel Tyree (Tyree et al. 1995; Tsuda and Tyree 2000) were done using the transient runs feature. The HCFM determined the flow rate by using the differential pressure across two pressure transducers connected by capillary tube of known hydraulic resistance. For each piece of tubing the resistance was linear for a set of flow ranges. These were ranges and calibrations set for the HCFM by Dynamax. Calibrations were checked independently by measuring flow rate with a flowmeter for each range. The HCFM had a tank separated by a diaphragm. One side was pressurized from a gas cylinder. A needle valve was used to adjust the rate that pressure increased. The other side of the diaphragm contained degassed water with algaecide (Dynamax, Houston, TX USA). Water to fill the HCFM was vacuum degassed by passing through a vacuum degasser three times. For transient measurements, pressures were increased from 0 MPa to 0.55 MPa at a rate of ~0.01 MPa s^{-1} and the flow rate determined from the pressure differential (3-4 points per second). A linear regression was fit to the linear part of the graph (usually between 0.200 MPa and 0.500 MPa) and the hydraulic conductance taken as the slope (in units $kg \cdot s^{-1} \cdot MPa^{-1}$). These raw conductance values were adjusted for the flow attributed to expansion of the system under pressure. This was determined by subtracting the conductance of the closed system from the conductance from the root segment. The closed system conductance was determined by placing a steel rod with approximately the same diameter as the roots being measured into the HCFM outlet. For HCFM measurements three technical replicate measurements were obtained in quick

succession and, if consistent were averaged. These three transient runs were completed within 4-5 min of root excision.

4.2.3.2 Determination of open xylem

Open xylem was determined two ways using the HCFM. In the first, a root was attached to the HCFM and transient measurements performed to establish the conductance of the whole root. Segments of known length were then removed from the distil end (root tip) and additional transient measurements made. The location where the xylem opened was determined by plotting conductance against root length removed. Where xylem was closed, there was no difference in conductance as root length was decreased. When an open xylem vessel was reached, resistance was decreased and an increased flow rate and conductance observed.

A second method of determining open xylem was performed using the quasi-steady state setting of the HCFM. The root was attached to the HCFM and set to 0.500 MPa constant pressure. The flow rate was allowed to reach a steady state. Starting near the tip, root sections were excised (5-50 mm) until there was an increase in the flow rate. The increase in flow rate was the maximum distance from the base of the shoot with a continuous open network of conducting xylem vessels.

4.2.3.3 Root properties

After measurements, root segments were blotted to remove surface water and weighed for fresh weight, then dried by placing in an oven (65°C) for three days and weighed to get dry weight. A rough total length was calculated by gently stretching the length of the root against a tape measure. Distance from the tip to the start of first obvious lateral root emergence was also measured using tape measure (+/- 1 mm). Root surface area and

number of root tips were determined by scanning the roots and analysing the images with WhinRhizo software (Regent Instruments, Québec, Canada).

4.3 Results

The results and methology of this chapter are summarized in **Figure 4.5**. It outlines the steps taken in this chapter.

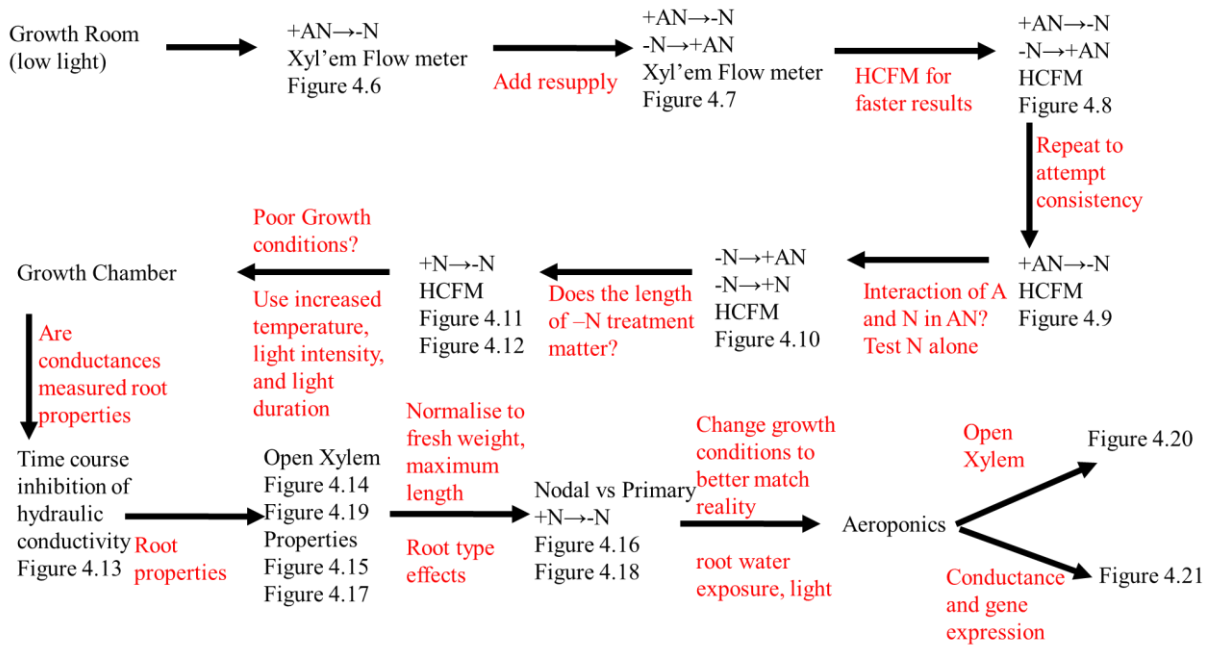


Figure 4.5 Experimental conditions summary

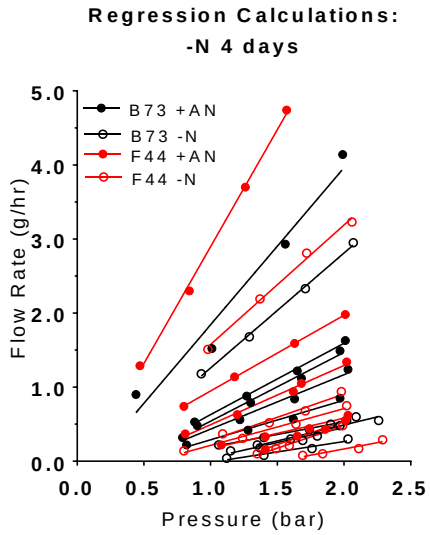
Experimental conditions and rational for changing conditions. Red arrows describe a brief justification for the next step. Results can be found in the figures indicated. Briefly, attempts were made to measure maize hydraulic properties. Xyl'em was a valid method, but took ~10-20 min per plant to attach and collect data. Checked to see if single maize roots could be measured on HCFM, then attempt to get consistency. Effects of ammonium nitrate (AN) and nitrate (N) were separated, as well as observing how long under –N it took to reduce hydraulic conductivity. It was thought that the growth conditions may not have been optimal, so plants moved to better conditions in a growth room (highter temperature, greater light intensity and greater light duration). Test out other properties to see if hydraulic conductivity normalised to different root properties. Attempted again in an aeroponic system to better match natural growth conditions.

4.3.1 Hydraulic measurements in a low light growth room

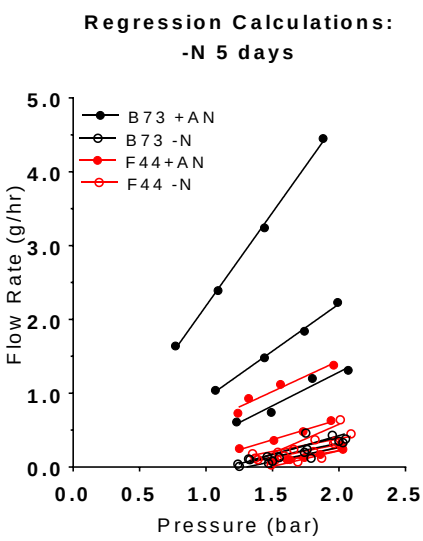
4.3.1.1 Hydraulic conductance measurements measured with the Xyl'em Flow Meter

Maize seedlings were grown hydroponically for 11 days in 2.5 mM NH_4NO_3, then either continued (+AN) or starved of nitrogen (-N) for 4-5 d in low light conditions. Hydraulic conductance values were determined using the Xyl'em flow meter and normalized to root dry weight (**Figure 4.6**-D,E,F). However, conductance in this case did not scale with dry weight as expected (larger roots would be expected to have a greater surface area and ability to conduct water), as the slopes of regressions were not significantly different from zero (**Figure 4.6**-C). For dry weight normalized conductances (L_o), at four days (**Figure 4.6**-D), N starved plants (-N) did not have significantly different L_o from +AN, however the variances did differ by Bartlett's test ($p=0.0440$) (**Figure 4.6**-D). After five days starvation, L_o means were significantly different ($p=0.0078$) with B73+AN being significantly higher than the others ($p<0.0001$) (**Figure 4.6**-F). For the combined data, B73+AN had significantly higher L_o compared to F44 and N starved plants (**Figure 4.6**-F). The variance was also larger of B73 +AN (**Figure 4.6**-F) ($p<0.0001$). There was no significant difference in the Lo response to N for F44 (**Figure 4.6**-D,E,F). From the linear regression of flow rate against pressure, the pressure at zero flow was determined (x-intercept). This should correspond to the root pressure. The B73 +AN root pressure was significantly less than that of the other treatments.

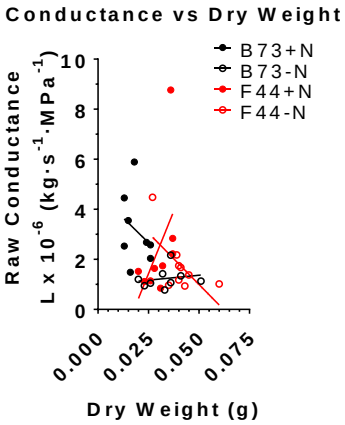
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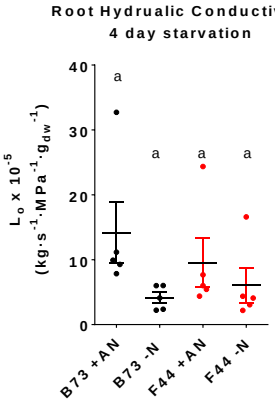
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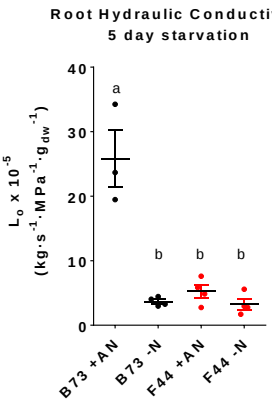
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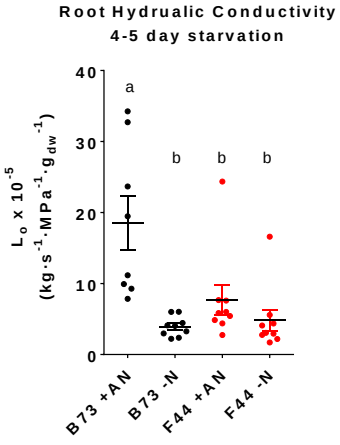
D



E



F



G

Root Pressure						
Treatment	Mean (MPa)	SD (MPa)	SEM (MPa)	CV (%)	n	p
B73 +AN	0.03451	0.01587	0.00561	45.99	8	0.004
B73 –N	0.09082	0.03693	0.01231	40.66	9	
F44 +AN	0.06636	0.05016	0.01672	75.59	9	0.031
F44 -N	0.09609	0.04948	0.01649	51.50	8	

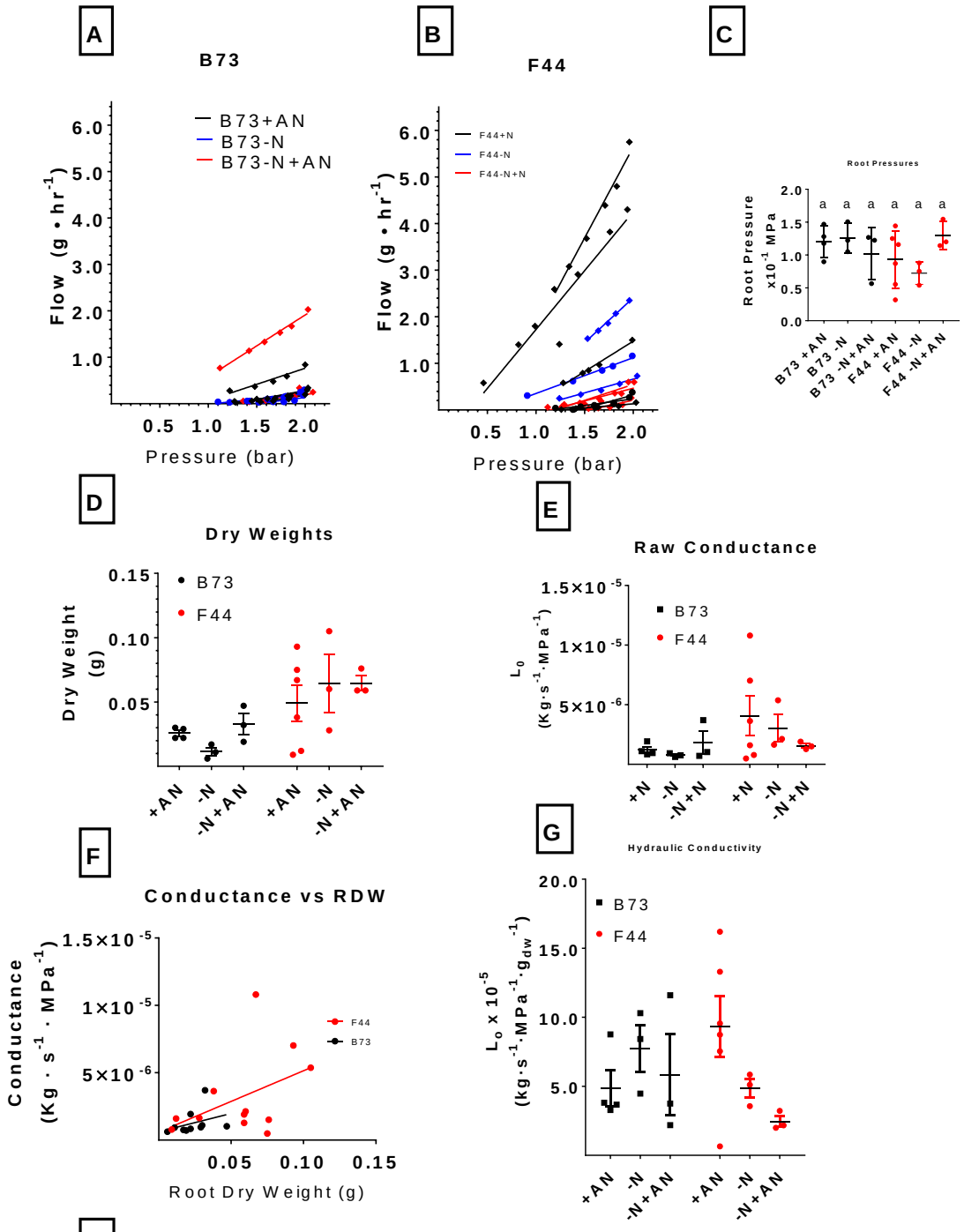
H

	Hydroponics		Treatment
Treatment	+AN	+AN	B73+AN, F44+AN
		-N	B73-N, F44-N
Length in treatment	11 days	4-5 days	

Figure 4.6 Maize seedling hydraulic responses to ammonium nitrate

Maize primary root grown hydroponically in low light (A,B) Xyl'em measurements of steady state flow rate vs pressure. The slope of the regression represented the hydraulic conductance and the x-intercept the root pressure. (C) Raw conductance values compared to dry weight. The slopes of the linear regressions were not significantly different from zero. Units were original units of measurements. (D,E) Hydraulic Conductivity normalized to root dry weight. (F) Pooled dry weight normalized hydraulic conductivity. (G) Root pressures derived from the linear regressions of (A,B). (H) Treatment overview. +AN and –N seedlings were germinated then grown hydroponically for 11 days on. 2.5 mM NH_4NO_3. –N plants were switched to nitrogen free media for 4-5 day prior to measurement. Red shading denotes –N treatment.

The hydraulic conductance of maize primary root from two-week old seedlings resupplied with nitrate was then tested (**Figure 4.9**) with the Xyl'em flow meter. Conductance values were again determined from a linear regression of flow rate against pressure and root pressures derived from the x-intercept (**Figure 4.7**-A,B). In this case, root pressures nor their variance differed between treatments (**Figure 4.7**-C). Again, hydraulic conductance did not correlate with root dry weight (**Figure 4.7**-F) (regression slopes were not significantly different from zero). A two-way ANOVA analysis revealed no effect on L_o of nitrate treatment or inbred line (**Figure 4.7**-G). This is possibly due to the low sample size. The variance between the normalized conductivities was not significantly different.



H

	Hydroponics			Treatment
Treatment	+AN	+AN		B73+AN F44+AN
		-N		B73-N F44-N
		-N	+AN	B73-N+AN F44-N+AN
Length in treatment	11 days	4 days	4 hrs	

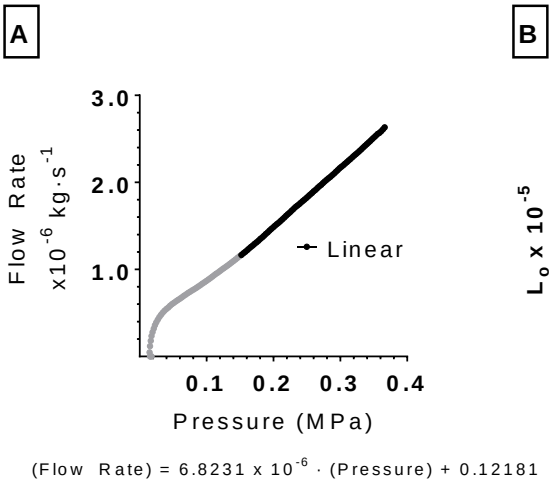
Figure 4.7 Maize seedling hydraulic responses to ammonium nitrate
Maize seedlings were grown in hydroponics as in **Figure 4.6**, but a second N condition was added: resupply for four hours. The primary root was used for measurements. (A,B) Linear regressions to determine hydraulic conductance and root pressure for B73 and F44. (C) Root pressures did not differ from one another. (D) Dry weights of roots, which were not significantly different, likely due to low sample sizes (min n=3). (E) Raw hydraulic conductance values. These were not significantly different. (F) Plot of root dry weight vs conductance. The regression was not significantly different from zero. (G) Conductance normalized to dry weight. These values were also not significantly different. (H) Experimental design. Seedlings were grown on +AN for 11 days. –N plants were switched to –N for four days. –N+N plants were then resupplied with +AN for four hours. Red shading denotes –N treatment

While the variances were not statistically different from one another, they were still large in magnitude. The coefficient of variation (CV) for Hydraulic conductance in **Figure 4.9**-G ranged between 24% (F44-N) and 86% (B73-N+AN). A concern was the time it took (8-10 minutes per plant) to record the steady state flow rates at four pressures that may have been confounded by a loss of hydraulic conductance seen in maize and other plants after excision of the roots from the shoot for measurement (Vandeleur et al. 2014). To decrease time between root excision and completion of hydraulic measurements, the HCFM was selected to measure conductance of the roots, since the transient method was able to be performed rapidly and within a short time after excision.

4.3.1.2 Hydraulic conductivity with HCFM in low light conditions in the growth room

The HCFM was selected to measure hydraulic conductance on maize primary roots in low light conditions. It took 4-5 min to excise root and finish collecting hydraulic data on the HCFM. Experiments were carried out as in **Figure 4.10**A with hydraulic measurements on the HCFM however it was not possible to obtain the pressure at zero flow. F44 had significantly higher L_0 than B73 ($p<0.0001$) (**Figure 4.8**-B), however treatments within each inbred line were not significantly different, nor were the variances among them. The variances however were still high (CV values - B73+AN: 86.57%, B73-

N: 88.43%, B73-N+AN: 82.54%, F44+AN: 74.18%, F44-N: 57.72%, F44-N+AN: 51.12%).



C

	Hydroponics			Treatment
Treatment	+AN	+AN		B73+AN F44+AN
		-N		B73-N F44-N
		-N	+AN	B73-N+AN F44-N+N
Length in treatment	11 days	4 days	4 hrs	

Figure 4.8 HCFM measurements of maize seedlings

Hydroponically grown maize seedlings in low light L_0 as measured by HCFM. (A) HCFM measurements included measuring flow rate against pressure. HCFM settings were set to collect maximum number of points. A linear regression was performed to get the hydraulic conductance, in this example $6.8231 \cdot 10^{-6}$ $kg \cdot s^{-1} \cdot MPa^{-1}$. This conductance was partially due to the expansion of the instrument and tubing under pressure. This was calculated and removed (not shown) to get a raw conductance value. In this example, 175 individual points are plotted. (B) Maize primary root hydraulic conductance normalized to root dry weight. Nitrogen treatments did not have an effect within inbred lines, but F44 had greater ($p<0.0001$) normalized conductance values than B73. (C) Experimental Conditions. These are the same as in **Figure 4.7**.

To attempt to separate the effects that ammonium and nitrate may be having on L_o, nutrients were switched to a single source of N: either 5 mM NO_3^- (cations balanced with SO_4^{2-}) or 5mM NH_4^+ (by addition of $(NH_4)_2SO_4$). For nitrate supplied maize seedlings (**Figure 4.9**-A), 3 days nitrogen starvation led to significant decreases in the dry root weight of F44 ($p=0.0079$), but not in B73. Increasing root dry weight did not correlate with an increase in conductance (slopes of regression were not significantly different from zero for any treatment nor when all samples were pooled together) (**Figure 4.9**-C). B73

had an increased normalized hydraulic conductance under –N conditions (p=0.0235), while –N treatment did not affect the hydraulic conductance in F44 (p=0.9918) (**Figure 4.9**-D). In both F44 and B73, -N treatment did increase the variance (CV – B73: 58.2%→110.77%, F44:36.81%→129.86%).

The effect of nitrate resupply was then analysed, along with ammonium resupply. Plants were grown in low light hydroponics for 7 days on +N, then switched to –N for 7 days, and then resupplied as shown in **Figure 4.10**. Resupplying with nitrate did not affect hydraulic conductivity values from –N treatments after 2 h (p = 0.520-B73, 0.9484-F44), 3 d (p=0.8314-B73, p=0.3378-F44), 4 d (p=0.6134-B73, 0.1454-F44), or 5 d (p=0.9093-B73, 0.5831-F44) within inbred lines. Resupply with ammonium after four days also had no effect on hydraulic conductivity (p=0.8314-B73, 0.2939-F44).

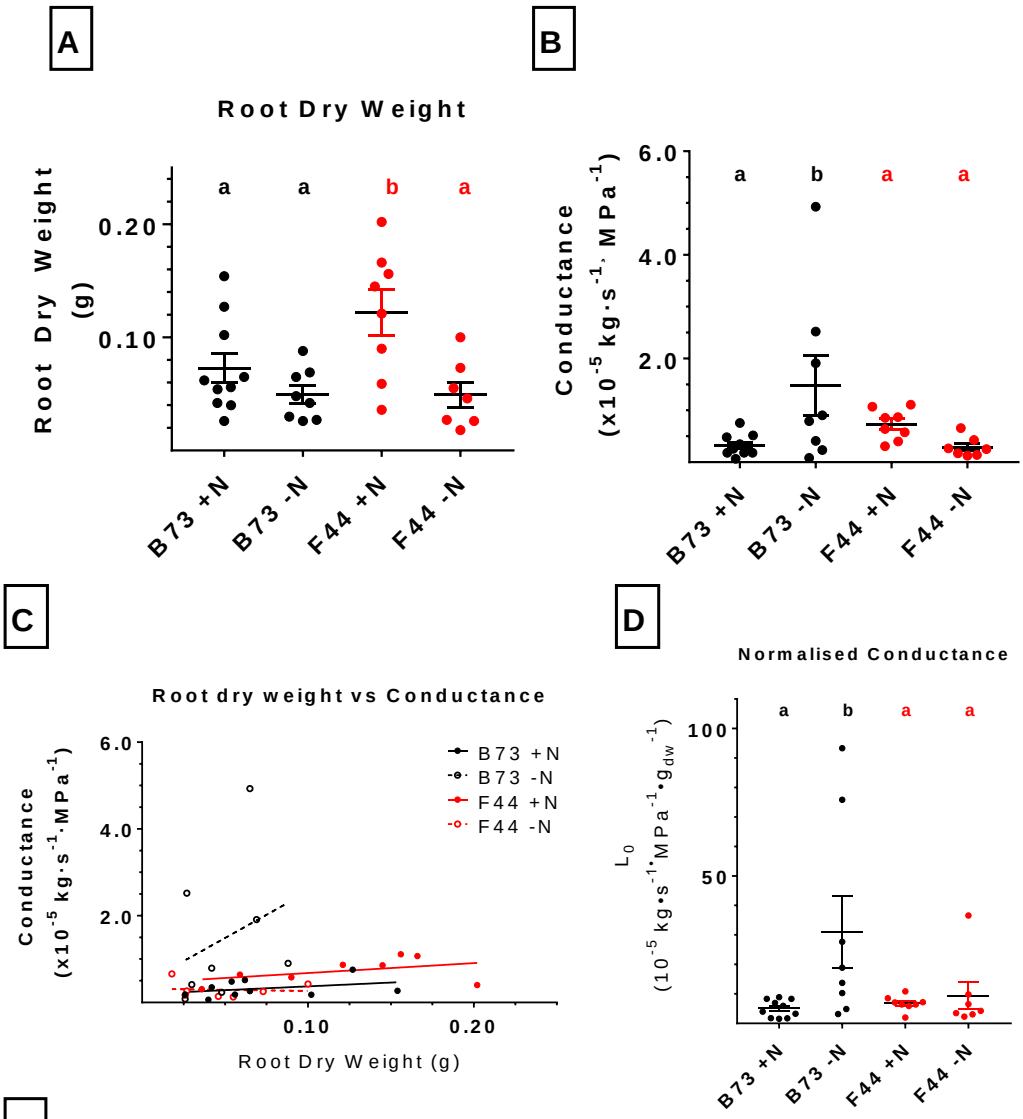
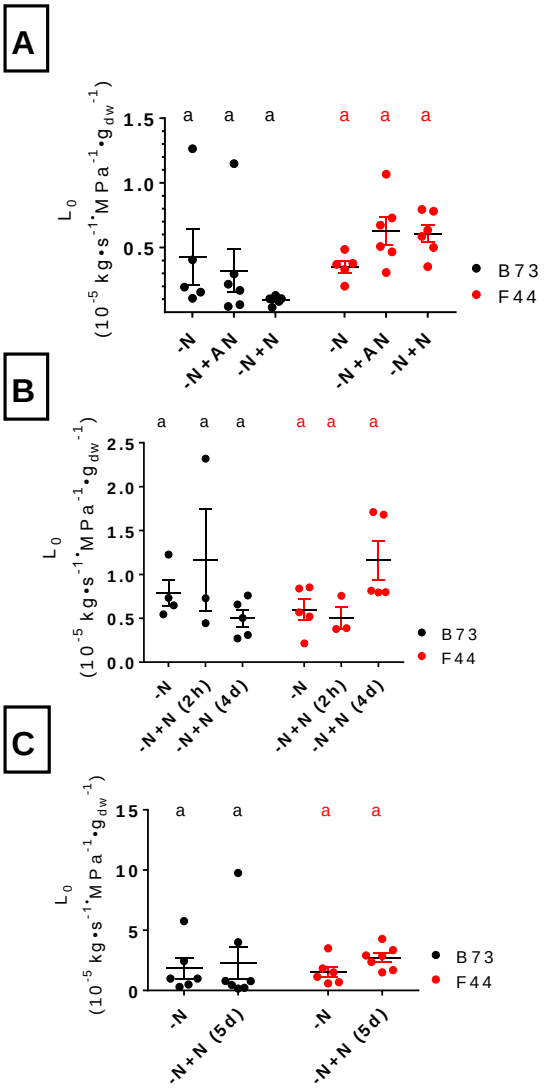


Figure 4.9 Low light growth room water conductance measured with HCFM

Maize grown hydroponically in low light. Nitrogen was supplied at 5 mM NO_3^- and –N plants were had cations balanced with 2.5 mM SO_4^{2-}. Hydraulic measurements taken with HCFM on the primary root. (A) Primary root dry weights under +N and –N for 3 days. (B) Raw hydraulic conductance values. B73 –N hydraulic conductivity was increased, but not F44 (C) Plot of raw conductance against root dry weight and regressions. The slopes of the regression (or regression of pooled values) were not significantly different than zero. (D) Normalized conductance values. Normalised hydraulic conductance values. B73 –N hydraulic conductivity was increased, but not F44 (E) Treatment outline letters on graph represent significant differences ($p<0.05$).



	Hydroponics			Treatment
Treatment	+N	-N	-N	-N
			+A	-N+A
			+N	-N+N
Length in treatment	11 days	7 days	3 days	

	Hydroponics				Treatment
Treatment	+N	-N	-N		-N
			-N	+N	-N+N (2h)
			+N		-N+N (4d)
Length in treatment	13 days	7 days	4 days	2 hr	

	Hydroponics			Treatment
Treatment	+N	-N	-N	-N
			+N	-N+N (5d)
Length in treatment	12 days	7 days	5 days	

Figure 4.10 Maize seedling hydraulic response to N resupply

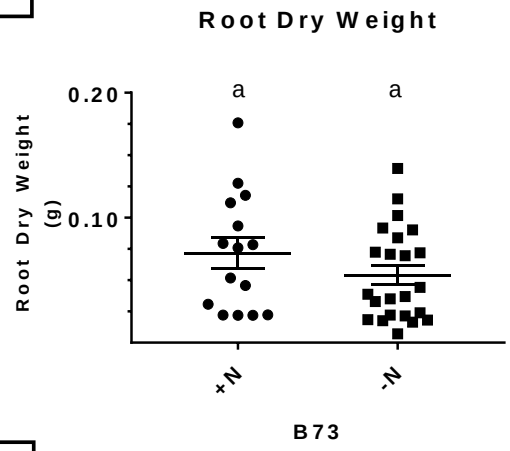
Maize seedlings grown hydroponically in low light. Primary root tested in three resupply experiments. After germination, plants were grown on +N for 7 days, then starved for 7 days on –N. Plants were then resupplied as (A) ammonium or nitrate for three days, (B) nitrate for two hours of four days, (C) nitrate for five days. In all cases, resupply did not affect hydraulic conductivity of the primary root.

The effect of starvation was further investigated (**Figure 4.11**). Plants were grown on +N for about two weeks, then starved on N for 1-4 d. In this experiment, a linear regression showed there was an increase in conductance with an increase in root dry weight for –N and +N plants (**Figure 4.11**-C). L_0 was increased 4.7x after one day and 10x after four

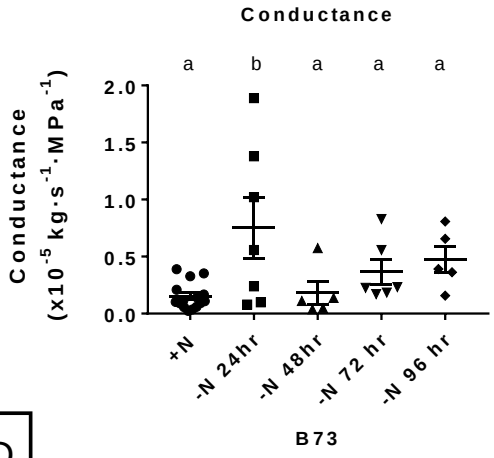
days starvation, but was not different from +N plants at 2 or 3 d starvation (**Figure 4.11**-D). Diurnal variation of hydraulic conductance has been reported in other plants (Henzler et al. 1999). However, in this experiment, there was no relationship for either +N or-N based on the time of day the experiment was performed.

A second experiment was performed with the same conditions (**Figure 4.12**). Again, root dry weights were not significantly different (**Figure 4.12**A). An analysis of variance on the raw conductance values showed there was increased conductance values and variation under nitrate withdrawal (**Figure 4.12**-D). During this experiment however, there was not an increase in hydraulic conductivity with increasing root weight (slopes were not different than zero) (**Figure 4.12**C), and the intercepts were also unchanged. For Lo the ANOVA showed no difference between means, but did show the variances were increased with N starvation. Likewise the pooled –N samples were not statistically different, but the variance was increased (**Figure 4.12** E).

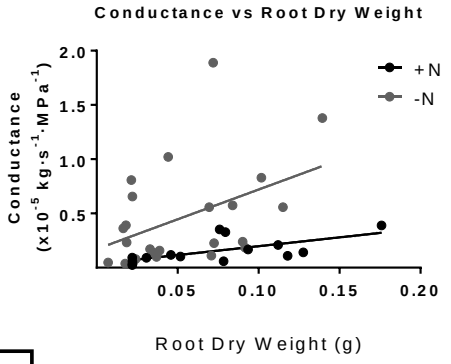
A



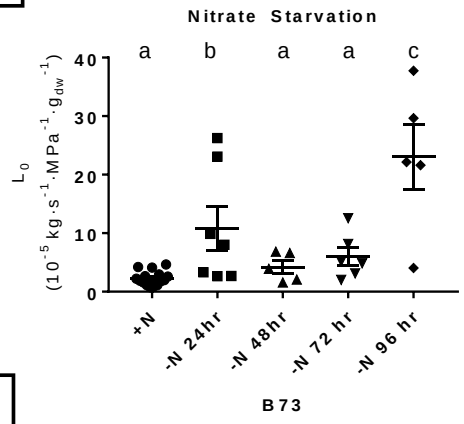
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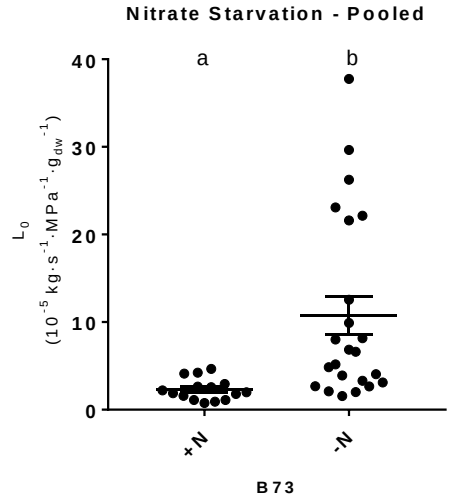
C



D



E



F

	Hydroponics		Treatment
Treatment	+N	+N	+N
		-N	-N (h)
Length in treatment	16 days	1-4 days	

Figure 4.11 Hydraulic response of hydroponic B73 seedlings in low light response to nitrate starvation over four days B73 maize plants grown in hydroponics with low light then starved of nitrogen. Primary root used for conductance measurements. (A) Root dry weights (p=.22) nor their variances (p=.30) were not different between the treatments. (B) Raw conductance values. Bartlett's test showed the SD values to be significantly greater in –N plants (p=0.022). –N treatment after 24 h resulted in a 5x increase in raw L_0 (p=0.0052). (C) Linear regression of root dry weight and conductance. Both +N (Y=1.63x+0.3477, p=0.0078) and pooled –N values (y=5.486x+0.1713, p=.0388) slopes were significantly different than zero, and, while the slopes of the regressions were not different (p=0.1748), the intercepts were (p=0.0029). (D) L_0 was increased 4.7x from +N to –N 24h (p=0.0441) and was increased 10x to –N 96 h (p<0.0001). At 48 and 96 hours the differences were not significant. (E) When –N values were pooled, starvation led to an increase of L_0 by 4.7, and led to a significant increase in variance (CV = 53.56% to 97.19%) (F) Experiment Timeframe. Each dot represents a single primary root. Error bars are mean and SEM. Letters denote statistical differences (p<0.05)

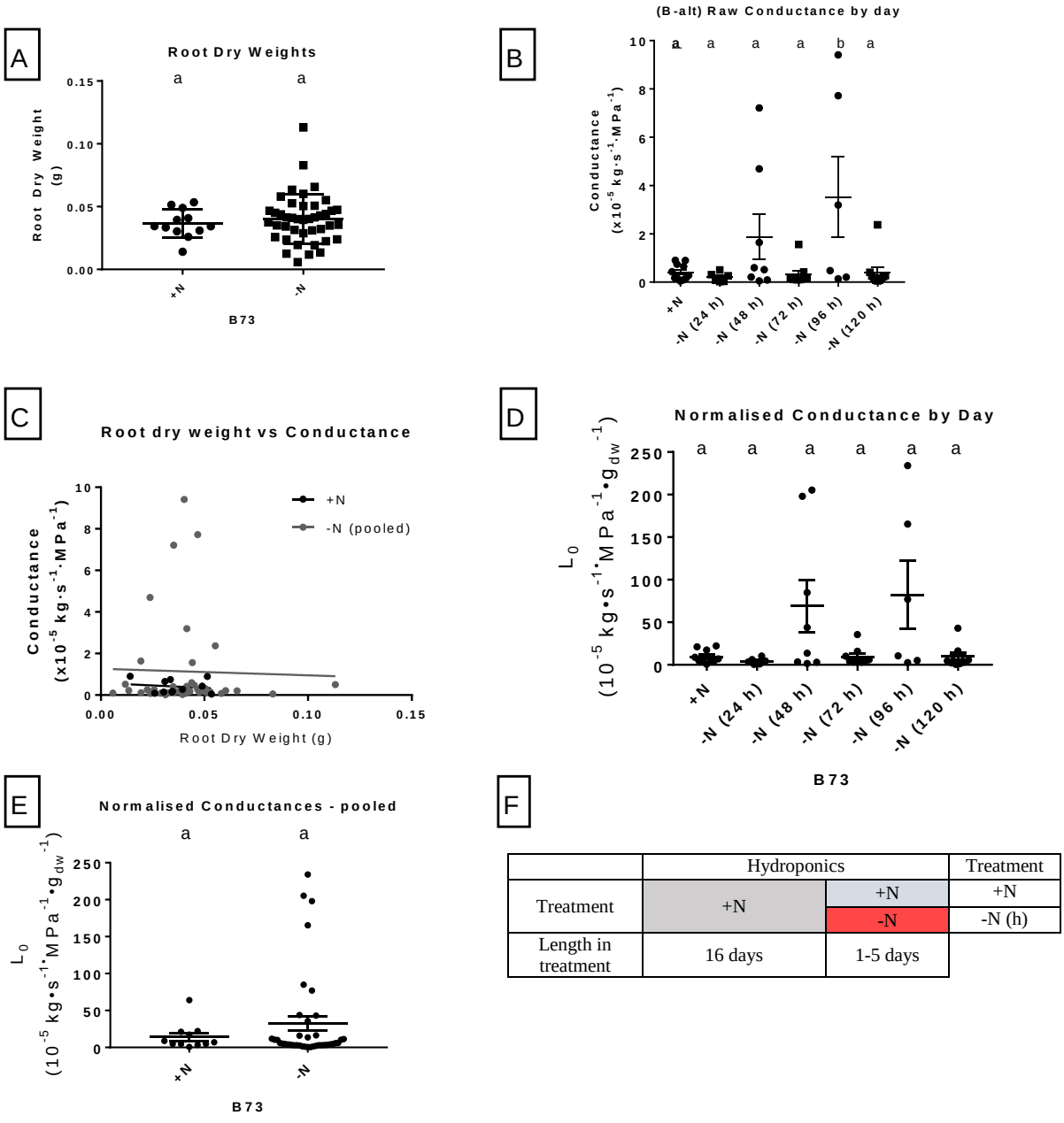


Figure 4.12 Hydraulic response of hydroponic B73 seedlings in low light response to nitrate starvation over five days B73 maize seedlings were grown hydroponically in low light conditions for two weeks on 5 mM nitrate. N starvation was imposed by replacing nitrate with sulfate. Conductance was measured on the primary root over five days. (A) Nitrate starvation over five days did not lead to a change in root dry weight. (B) Raw conductance values were significantly raised in –N plants, but the (C) linear regression of root dry weight to conductance was not significant (slopes were not different than zero and intercepts not different). (D) Normalized to root dry weight conductances were not different from +N, nor when (E) –N plant normalised hydraulic conductances were pooled. (F) Overview of treatments. Each dot represents a single primary root. Error bars are mean and SEM. Letters denote statistical differences ($p<0.05$). Statistics for each chart are available in the appendix of this chapter.

4.3.2 Maize root hydraulics in medium light grown in hydroponics

4.3.2.1 Time after excision inhibits root hydraulic conductance

The results of the low light were interpreted to mean that there may have been some external variable other than nitrogen concentration that may have been influencing the results. One potential variable was the light conditions (**Figure 4.2**). The mean light in this growth room was 69.85 ± 14.31 μmol·m^{-2}·s^{-1} (mean ± SD). The net assimilation saturation point for B73 has been shown to be greater than 1000 μmol·m^{-2}·s^{-1} (Pengelly et al. 2011). To increase the light available to the plants, the hydroponics system was moved to a growth room with light intensity of 295.6 ± 56.58 μmol·m^{-2}·s^{-1} (**Figure 4.3**). While still lower than the saturation point, it was a four-fold increase over the intensity in the low-light growth room.

One source of variation is a possible response to excision since the conductances may decrease over time after excision as seen in shoot de-topped B73 plants in Vandeleur et al. (2014). Plant roots were excised (t=0) and attached to the HCFM. Triplicate measurements (taken as replicates for the time point of the second transient run) were plotted against time after excision (**Figure 4.13**). From the plots, a decrease with time was observed, so each plot was fitted with an exponential decay curve:

$$L(t) = (L_{t=0} - L_{t=\infty}) \cdot e^{-k \cdot t} + L_{t=\infty} \qquad \text{Equation 4.1}$$

where $L(t)$ is the conductance at time t, $L_{t=0}$ is the initial conductance, $L_{t=\infty}$ the final conductance, and k the exponential rate constant. Decay models modelled the decay, with a half time of 26.08±9.817 min (mean ± SD) (**Figure 4.12**-B). The models predicted final

measurements above zero, and were independent of the initial measurement (p=0.207) (**Figure 4.12**-A). The range from initial conductance of the model to final predicted scaled linearly with initial conductance (**Figure 4.12**-E). Taken together, this indicates the variation observed may be due to a factor controlling water flow in the roots, and may be indicative of variable aquaporin activity.

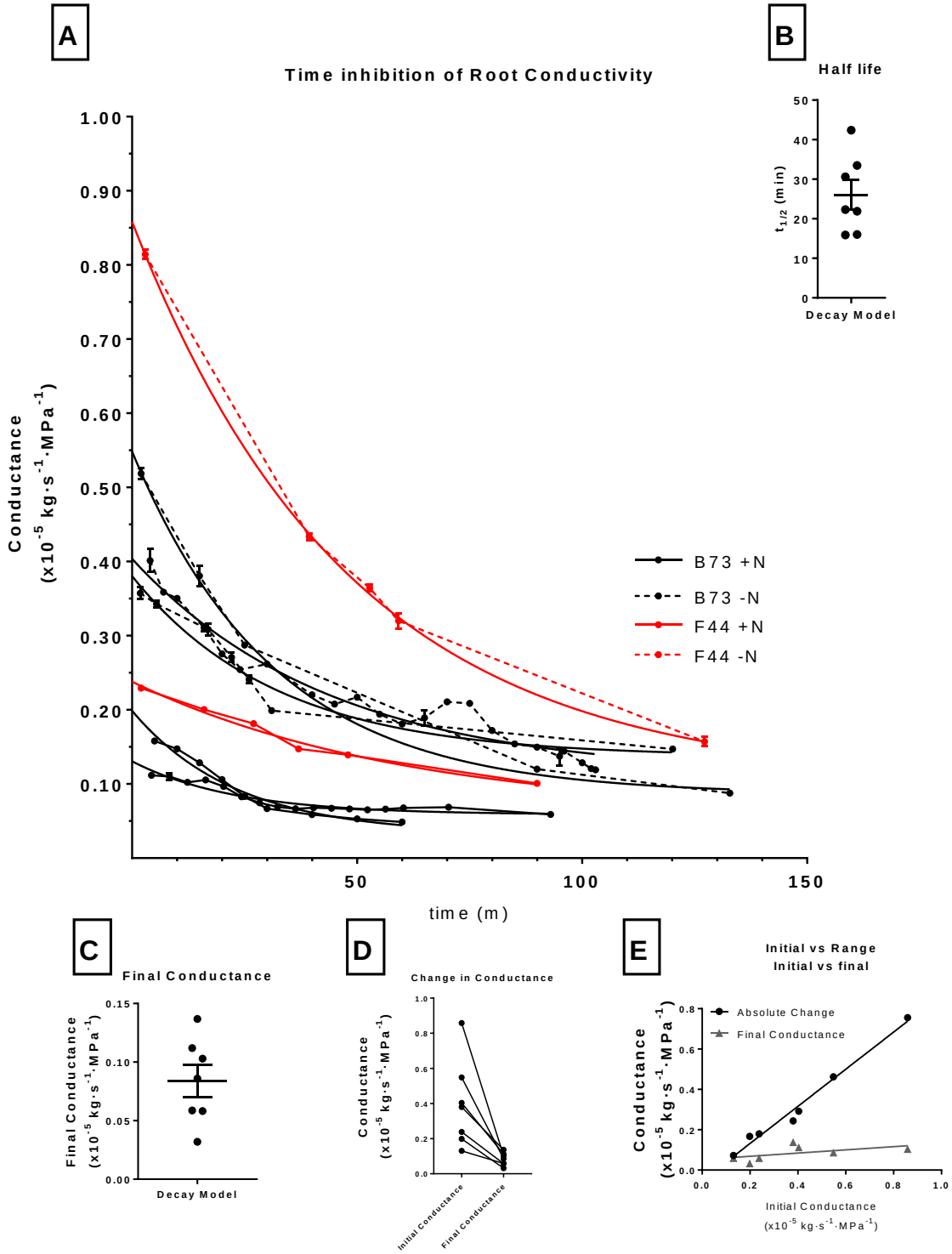

A
Time inhibition of Root Conductivity
Conductance (x10-5 kg·s-1·MPa-1)
time (m)
B73 +N
B73 -N
F44 +N
F44 -N
B
Half life
t1/2 (min)
Decay Model
C
Final Conductance
Final Conductance (x10-5 kg·s-1·MPa-1)
Decay Model
D
Change in Conductance
Conductance (x10-5 kg·s-1·MPa-1)
Initial Conductance
Final Conductance
E
Initial vs Range
Initial vs final
Absolute Change
Final Conductance
Conductance (x10-5 kg·s-1·MPa-1)
Initial Conductance (x10-5 kg·s-1·MPa-1)

Figure 4.13 Time inhibition of root hydraulic conductivity
Maize plants grown in medium light growth chamber. Primary root was excised at t=0 and attached to HCFM. (A) Conductance measurements plotted against time excised from plant. Three HCFM measurements were made at each point (shown mean +SEM). Decays were then modelled with Equation 4.1. (B) Half times ($\frac{\ln(2)}{k}$) for the decay models. $t_{1/2}$ =26.08±9.817 (SD). (C) Final conductance predicted by model (L as $t \to \infty$). (D) Mapping of Initial and Final Conductance values (E) Linear regression of initial conductance from model (t=0) compared with final conductance and the range change (L_i-L_f). The initial and final conductance values were not linearly related (slope not different from zero, p=0.2073), while the linear regression of the absolute change to initial conductance were very related (y=0.9205x-0.05243, r^2 = 0.9825, p =0.001).

4.3.2.2 Open xylem vessels in hydroponically grown maize

A second potential source of variation is variable distances along the root to when the xylem vessels become open. Therefore, measurements were performed to determine when the axial (xylem vessels) conductance became open. The conductance measured was assumed to be composed of two components – the radial component arising from the cell to cell pathway and the apoplastic conductance values, and the longitudinal conductance from the xylem vessels. The axial resistance would mean that the realized pressure would decrease to some degree along the root from excision to root tip, as seen when root modelled as electrical circuit, with axial resistance being dependent on length (Knipfer and Fricke 2011). As water did not flow out of the root tip, either the xylem vessels were closed somewhere along the longitudinal axis of the root, or flow in the xylem vessel was zero at some distance from the excision point. In either case, removing tissue from the root tip at some point would increase the flow rate, and be observed as an increase in conductivity. With closed xylem, cutting back to an open xylem vessel would allow water to flow at a much increased rate due to the greater conductance of xylem over the cell-cell and apoplastic pathways.

Figure 4.14 shows a plot of conductance against the distance removed from the root tip. It demonstrates first that conductance measured is dependent on the root, and second, that there is a component of the longitudinal axis from the tip to a location that does not contribute to the conductance (seen in the lack of change in conductance as sections were excised). This will be explored further later in the chapter, and the implications of this in the discussion section.

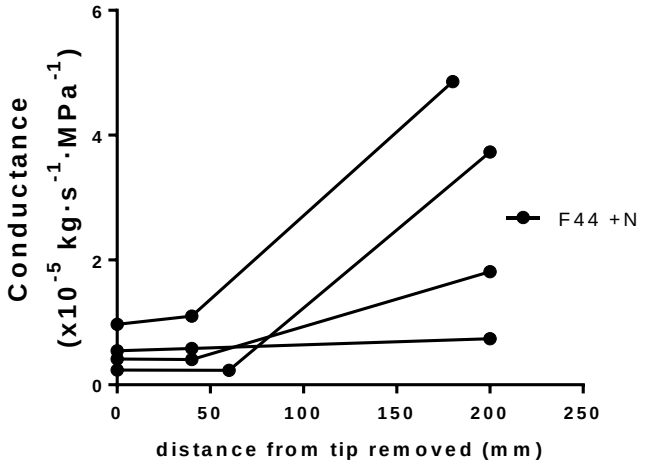


Figure 4.14 Open xylem determination
F44 primary roots were attached to the HCFM. A whole root conductance was determined, then sections of root cut back. Three conductance values were obtained and shown with SEM for each distance. Lines denote roots, and are not meant to indicate a model for conductance. In general conductance values did not change in the first 50 mm from the root tip. Somewhere between 50 and 200, the xylem opened.

4.3.2.3 Nitrate effects on root properties of maize plants grown in a medium light growth chamber

Given that the root hydraulic conductance was not artefactual, additional root properties were determined for plants grown in medium light in the growth chamber. F44 was selected and correlations examined between: root fresh weight, root surface area, and the total number of root tips (**Figure 4.15**). There was no difference observed between fresh

weight, surface area nor the number of root tips in +N or –N for F44 plants. There was a strong correlation between fresh weight and surface (**Figure 4.15**-F). Due to high correlations (**Figure 4.15**-D,E,F), it is expected that normalising hydraulic conductance to any of these properties would have the same comparative results.

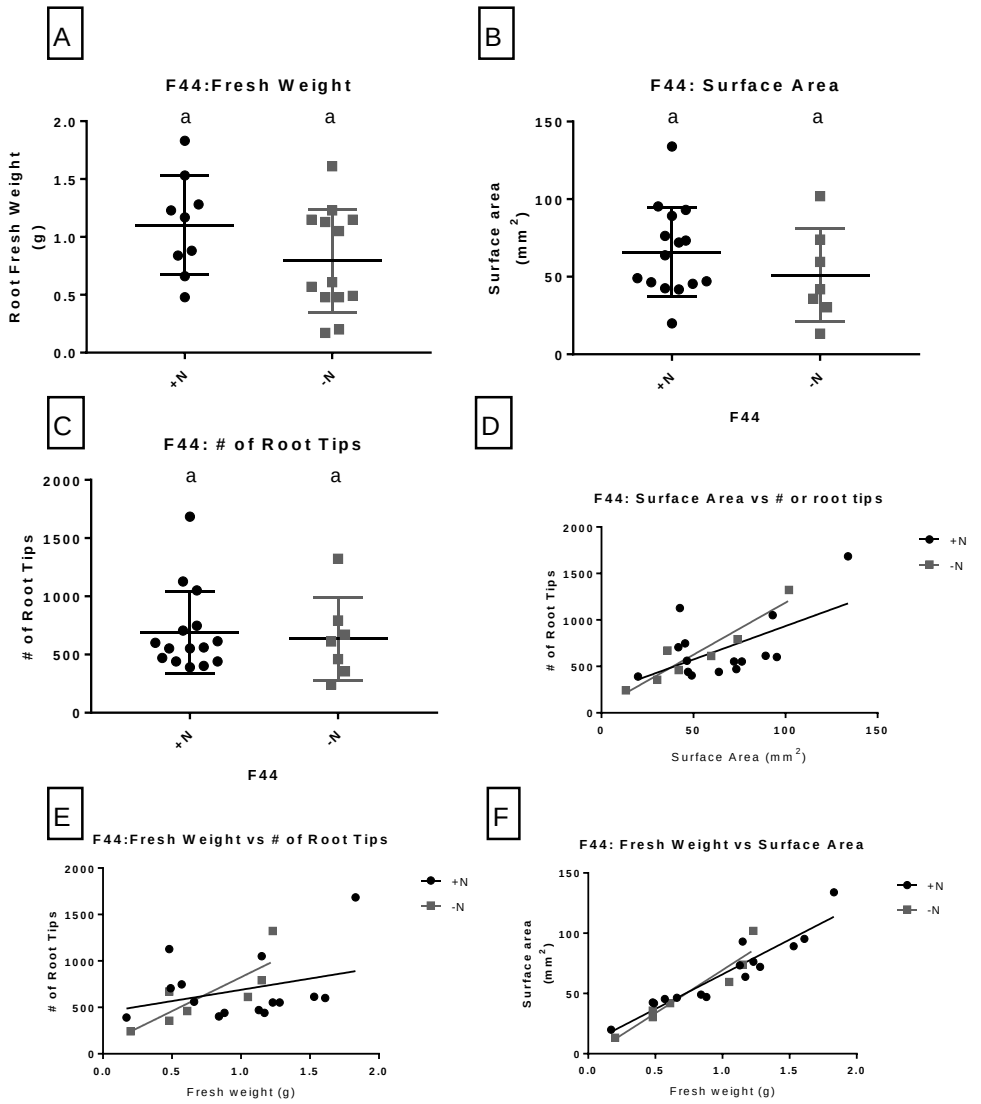


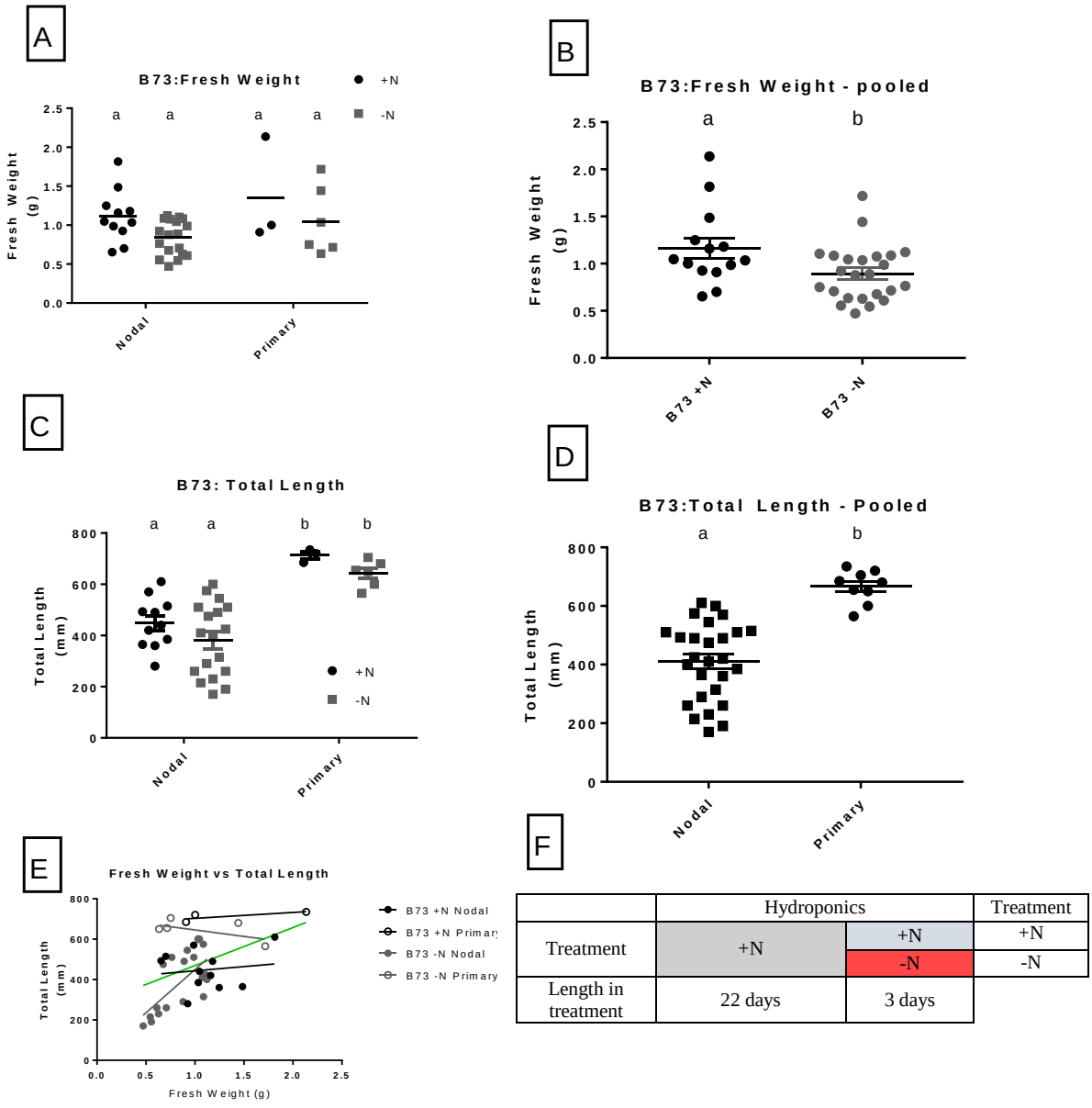
Figure 4.15 Regression analysis of physical root properties in F44

F44 plants grown in medium light growth chamber. Plants grown either +N or –N for three days. (A) There was not a difference in root fresh weight (B) There was not a difference in root surface area. (C) There was no difference in the number of root tips. (D) Linear regression between the surface area and number of root tips (+N: y=7.1885x+215.3, r^2 = 0.3484, p=0.205 | -N: y=11.21x+64.86, r^2 = 0.8836, p=0.0016). (E) Linear regression between number of root tips and fresh weight (+N y=242x+446.4, r^2 = .01055, p=0.2376 | -N: y=730.6x=93.7, r^2 = 0.6641, p =0.0255). (F) Linear regression of fresh weight and surface area (+N: y=57.57x+8.299, r^2 = 0.8816, p<.0001 | -N: y=71.26x-2.224, r^2 = 0.908, p=0.0009).

4.3.2.4 Physical properties of nodal roots

While the primary root was the dominant root, nodal roots began to emerge around ten days after germination for B73, and began to decrease the fraction of total root composed of the primary root. Due to age, primary roots at 3 weeks were longer than nodal roots (**Figure 4.16**-C), but there was no difference in fresh weight (**Figure 4.16**-A,B) indicating either nodal roots had more branching, larger diameters, a greater density. Interestingly, only the nodal roots in –N treatment had fresh weight that scaled with total length (**Figure 4.16**-E).

Lateral root growth patterns on nodal roots was similar to that for the primary root. The distance from the root tip to the emergence of lateral roots was not changed by root type nor by nitrogen treatment (**Figure 4.17**). This distance was also independent of root length (**Figure 4.17**-B).



	Hydroponics		Treatment
Treatment	+N	+N	+N
		-N	-N
Length in treatment	22 days	3 days	

Figure 4.16 Root properties

B73 maize seedlings four weeks old were grown in +N then switched to –N for three days. Each point represents one root with error bars denoting mean and SEM. Root properties were obtained for different root types. Main root was defined as the dominant root emerging from the seed. The nodal roots were roots that came off the base of the plant. (A) Fresh weights of primary and nodal roots under +N or-N treatment. A two-way ANOVA showed the interaction of root type and nitrogen treatment, and root type was not significant (p=0.9166, p=0.1101 respectively) while N has a significant source of variation (p=0.0428), however all multiple comparisons were not significant. (B) As root type nor its interaction was a source of variation, the results were pooled, and the difference was significant (p=0.0244). (C) Total length. A two way ANOVA demonstrated the variance was due to root type (p<0.0001), but not nitrogen treatment or their interaction (p=0.1493, 0.9617 respectively). (D) Pooled based on root type, the significance was high (p<0.0001). (E) Linear regressions of root fresh weight and total length. For –N nodal roots, there was a relationship (x=428.5x+20.94, r^2 = 0.4576, p=0.0021), but for the +N nodal roots and –N or +N primary roots, there was no relation (p-0.6801, 0.4345, 0.2616). (F) Treatment overview.

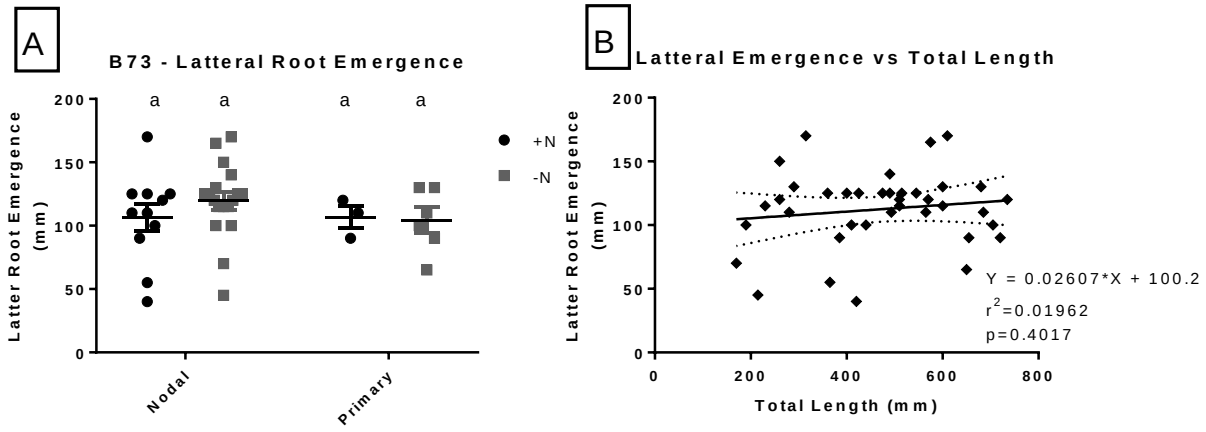
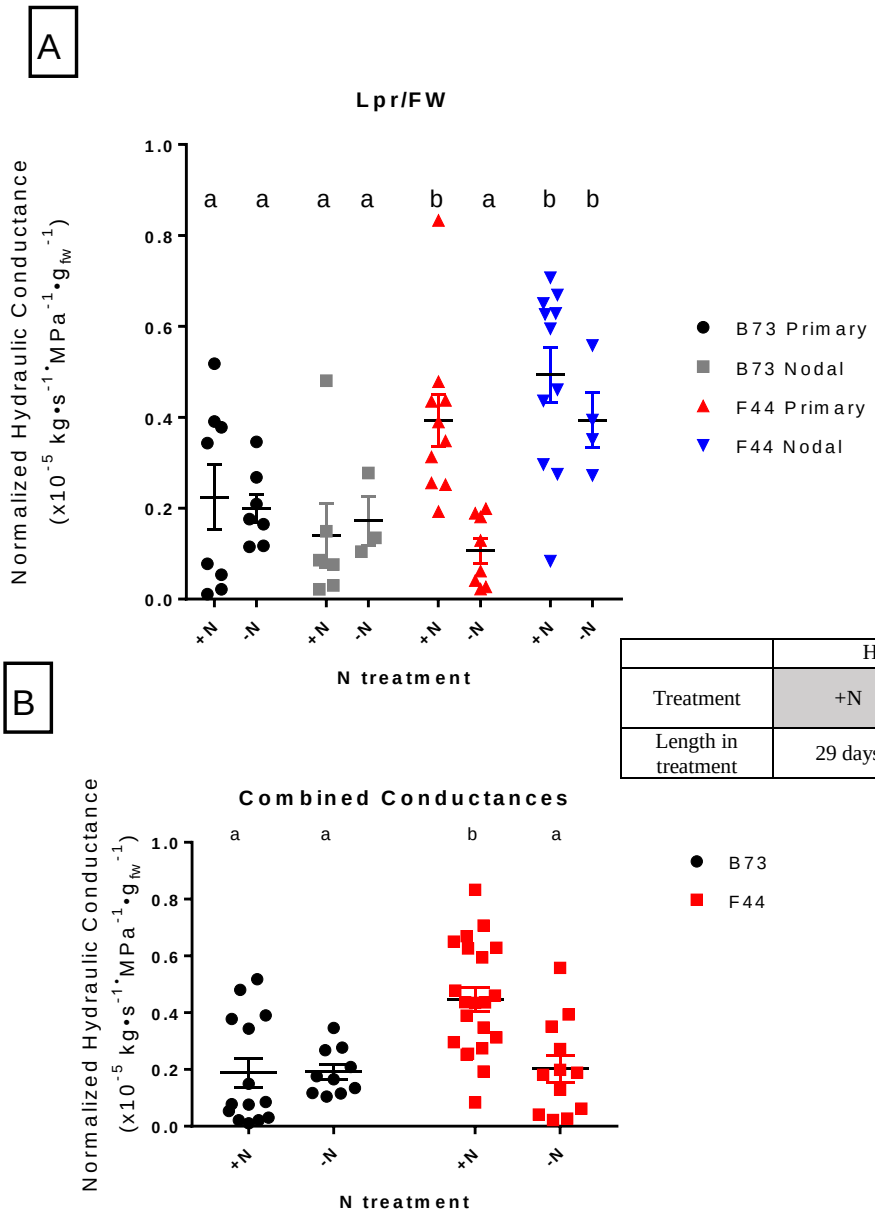


Figure 4.17 Lateral root emergence in B73

Lateral roots as measured from the base of the tip. Treatment was the same as that in **Figure 4.16**. (A) Lateral root emergence was not dependent on nitrogen treatment of root type. The average lateral root emergence from all values was 112.4±29.93 mm (SD). (B) Lateral root emergence plotted against total length. Linear regression (with 95% confidence interval) indicated lateral root emergence was independent of total root length (p=0.4017)

4.3.2.5 Hydraulic conductance in response to nitrogen starvation in medium light

Fresh weight was chosen to normalise root conductance as it had a linear relation with surface area. Also examined were the water relations of the nodal roots (**Figure 4.18**). In F44, nitrate starvation led to decreased conductivity in the primary root, but not in the nodal root (**Figure 4.18**A). There were no differences between treatments for B73 roots. There were large variances for L_o, with coefficients of variation ranging from 40-108%.



	Hydroponics		Treatment
Treatment	+N	+N	+N
		-N	-N
Length in treatment	29 days	3 days	

Figure 4.18 Hydraulic conductance measurements on maize plants in medium light

Hydraulic conductance normalized to root fresh weight for maize primary and nodal roots of F44 and B73 (A) Two way ANOVA showed variation with F44, but not B73. The –N value was reduced from primary roots, but not in nodal roots. (B) Combined normalized conductances, inbred line, the N treatment and their interaction were all significant (p=0.01420.066/.0121). F44+N plants had greater conductances than the +N or –N B73 and the –N F44. Each point represents the mean of the transient runs done within 5 minutes of root excision. Bars represent mean and SEM. Letters denote significantly different means p<0.05.

4.3.2.6 Open xylem determination in maize hydroponics

The variation in conductance was still high in the medium-light growth chamber. It was hypothesised that the variation may be real, and may be due to a property in the root. A factor investigated was the position at which open xylem occurred along the root. Normalization with surface area, dry weight or fresh weight all assume that the whole root contributes equally, or at least in an efficiency that is static between plants. To test the validity of this assumption, the root was simplified to two conductance – an axial conductance along the length of the root that decreased due to friction, and a radial conductance through the cell-cell and apoplastic pathways. It was assumed the axial conductance was greater than the radial conductance. As water was not observed to pour out of the root tip, at some point the xylem was closed (due to blockage or the effect of friction within the vessels). Cutting from the root tip would have no effect on the measured flow rate until the xylem opened and would be allowed to flow. While root traits are complex, it would not be expected that the proportion of root capable of transporting water would change for properties such as surface area and dry or fresh weights to be sufficient normalising standards between roots.

The open xylem was tested in maize plants with a quasi-steady state experiment on the HCFM. Roots were excised and attached and came to a relative steady state flow rate. The tip was removed in 20 mm sections until an increase in flow was observed, which was recorded as the location of the xylem opened.

In B73 roots the distance to open xylem vessels was unaffected by nitrogen treatment nor by root type (**Figure 4.19**). The distance from the tip to open xylem was correlated with the total root length however ($p=0.009$) (**Figure 4.19**-C). This also meant, that as a root lengthened, the ratio (as a percent of the total root length) that had open xylem also

increased at a rate of 0.86%/cm, a non-trivial amount (**Figure 4.19**-D), resulting in a wide range of open xylem normalised to root length (20% to 80%). These also show that the mean distance to open xylem is greater than the distance to the start of lateral roots, suggesting a non-trivial amount of root surface area is not involved in hydraulic conductance. In a complete root, where the xylem vessel closes, the flow of water into the xylem vessel is thought to be through the radial axis. This shows, that radial axis length with open xylem vessels is dependent on the total length of the root. Normalising roots to the surface area, root dry weight or root fresh weight, means normalising to the whole root, and is likely masked by the changing open xylem distance. This does suggest that normalising roots, at least for maize roots grown hydroponically, the length of roots with open xylem vessels may be a better normalising standard.

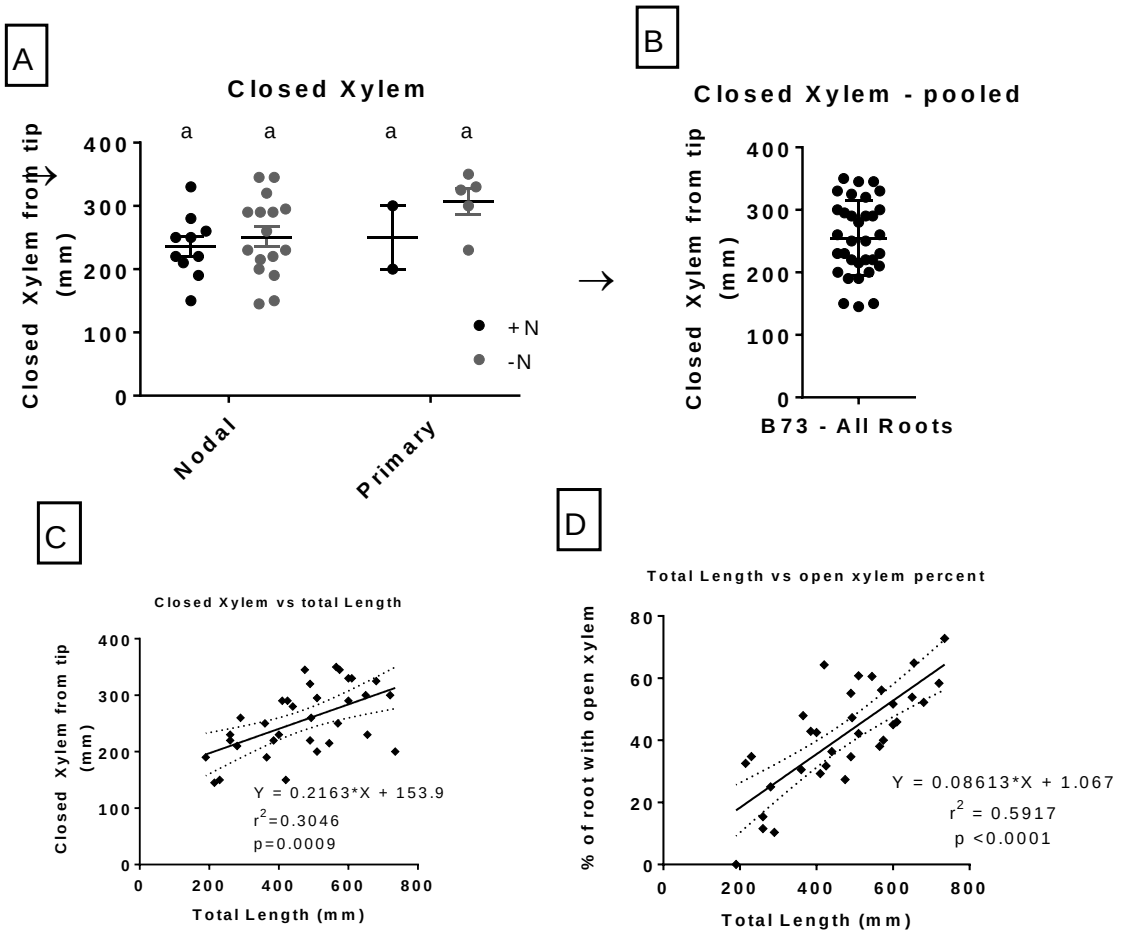


Figure 4.19 Distance to open xylem from root tip

Maize plants (B73) were grown as in **Figure 4.16**. Open xylem determined by monitoring quasi-steady state with HCFM for a jump, indicating an increased conductance though a xylem vessel open. (A) Distance xylem was closed form the root tip. N treatment nor root type effected the distance to open xylem. (B) Pooled data from (A). The mean is 254.8±59.55 mm (SD). (C) Distance to open xylem from the tip vs total root length were linearly related. (D) Percent of root with open xylem vessels compared to the total root length. Regression and 95% confidence intervals plotted. Equations listed on the figure.

4.4 Aeroponics experiments with HCFM

Hachez et al. (2012) demonstrated that compared to hydroponics, plants grown in aeroponics had greater endodermis suberisation, and may impact water transport properties of the root. As hydroponics was having mixed results on the nitrogen effect on hydraulic conductivity, an aeroponics system was built, inspired by Gaudin et al. (2011).

This was done in a glasshouse during the late spring to early autumn (Adelaide Australia, November 2016 – March 2017). On sunny days, light intensity was between 1000 – 1200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 12:00 pm at the base of the top of the root chamber, and was reduced to ~250 $\mu mol \cdot m^{-2} \cdot s^{-1}$ when overcast. All conductivity measurements were performed on sunny days, with light intensities above 1000 $\mu mol \cdot m^{-2} \cdot s^{-1}$. The light intensity was slightly reduced compared to the exterior light intensity on those days (1300-1500 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

The open xylem location of F44 roots was investigated (**Figure 4.19**). Roots were attached to the HCFM and transient measurements performed to establish the length of root with closed xylem (**Figure 4.19**-A). Subtracting from the total length gave the length of the root with open xylem. The length with open xylem correlated with the total length with a slope of 0.64 (p=0.0377), and an x-intercept of 151.5mm, the predicted minimum length for open xylem (**Figure 4.19**-B). F44 roots had 49.08%±4.981 (mean ± SEM, n=7) of the root with open xylem. Initial hydraulic conductance did not correlate with total length, but did correlate with the length of root with open xylem (p=0.0688) (**Figure 4.19**-D). The regression has a y-intercept of 202.2 mm, which would be the length of root with zero conductance. This is ~50mm off from the predicted length of root with open xylem. With a larger number of replicates and with a larger distribution of initial conductance, this relationship would be better deduced. Normalising the initial conductance reduced the coefficient of variance from 66.5% for the conductance measurement to 50.6% when normalised to total length, and to 41% when normalised to the length with open xylem (**Figure 4.19**-E).

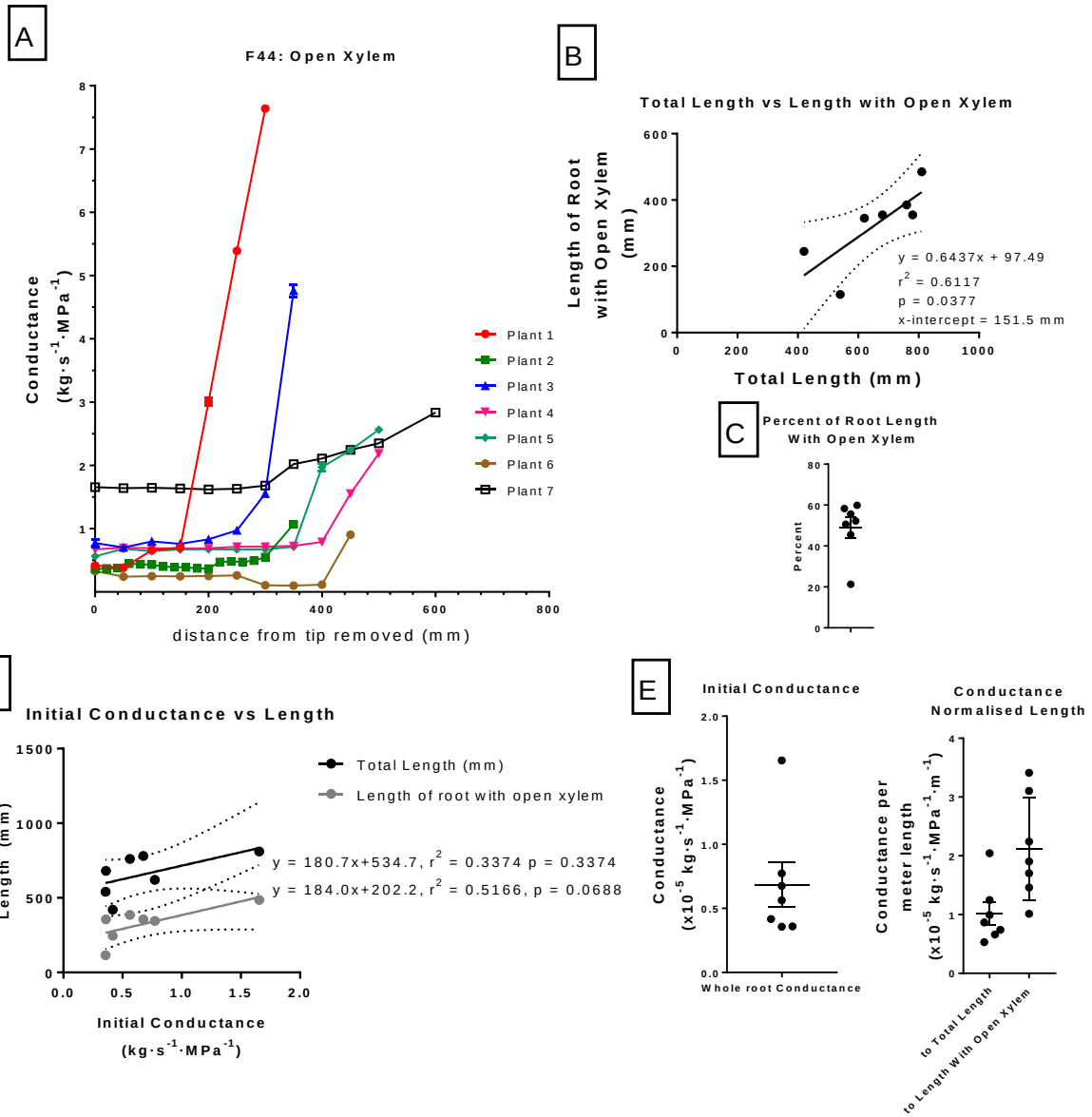

A
F44: Open Xylem
Conductance (kg·s-1·MPa-1)
distance from tip removed (mm)
Plant 1
Plant 2
Plant 3
Plant 4
Plant 5
Plant 6
Plant 7
B
Total Length vs Length with Open Xylem
Length of Root with Open Xylem (mm)
Total Length (mm)
y = 0.6437x + 97.49
r2 = 0.6117
p = 0.0377
x-intercept = 151.5 mm
C
Percent of Root Length With Open Xylem
Percent
D
Initial Conductance vs Length
Length (mm)
Initial Conductance (kg·s-1·MPa-1)
Total Length (mm)
Length of root with open xylem
y = 180.7x+534.7, r2 = 0.3374 p = 0.3374
y = 184.0x+202.2, r2 = 0.5166, p = 0.0688
E
Initial Conductance
Conductance (x10-5 kg·s-1·MPa-1)
Whole root Conductance
Conductance Normalised Length
Conductance per meter length (x10-5 kg·s-1·MPa-1·m-1)
to Total Length
to Length With Open Xylem

Figure 4.20 Open xylem determination in F44

Nodal roots of F44 plants grown on 15 mM nitrate in an aeroponic system with high light (<1000 µmol·m^{-2}·s^{-1}). (A) Plot of conductance values as tissue was excised from the root tip. Plot was flat, then increased. The midpoint between the previous flat and first point of the jump was taken as the distance from tip that the xylem was closed. (B) Length of whole root *vs* length with open xylem (total-xylem closed). x-intercept would be the minimum length to have open xylem. (C) Percent of root with open xylem (open xylem length / total length x 100). Mean and SEM: 49.08%±4.981. (D) Plots of conductance *vs* lengths. The relationship between total length and conductance was not significant, but was nearly significant at p=0.0688 for length to open xylem *vs* conductance (n=7). (E) Comparison of the conductance and normalized to total length or length open xylem. Normalising to the length of open xylem lowered the coefficient of variance from 50.6% to 41%. The initial variance of the conductance was 66.5%. Each plot has an n=7 roots, denoted by points. Bars represent mean and SEM.

The effect on the root fresh weight and conductance of 3 days of nitrate starvation was investigated on nodal roots of 4-week old B73 and F44 plants grown in aeroponics (**Figure 4.21**) +N treatment was increased from 5 mM to 15 mM. For each plant, two nodal roots were sampled. The conductance values were normalized to root length, and not to root fresh weight. The intra-plant root hydraulic conductance was not uniform when normalised to fresh weight or total length, and there was no trend between first and second roots from the same plant. This indicated that there is a large intra-root variability within a plant.

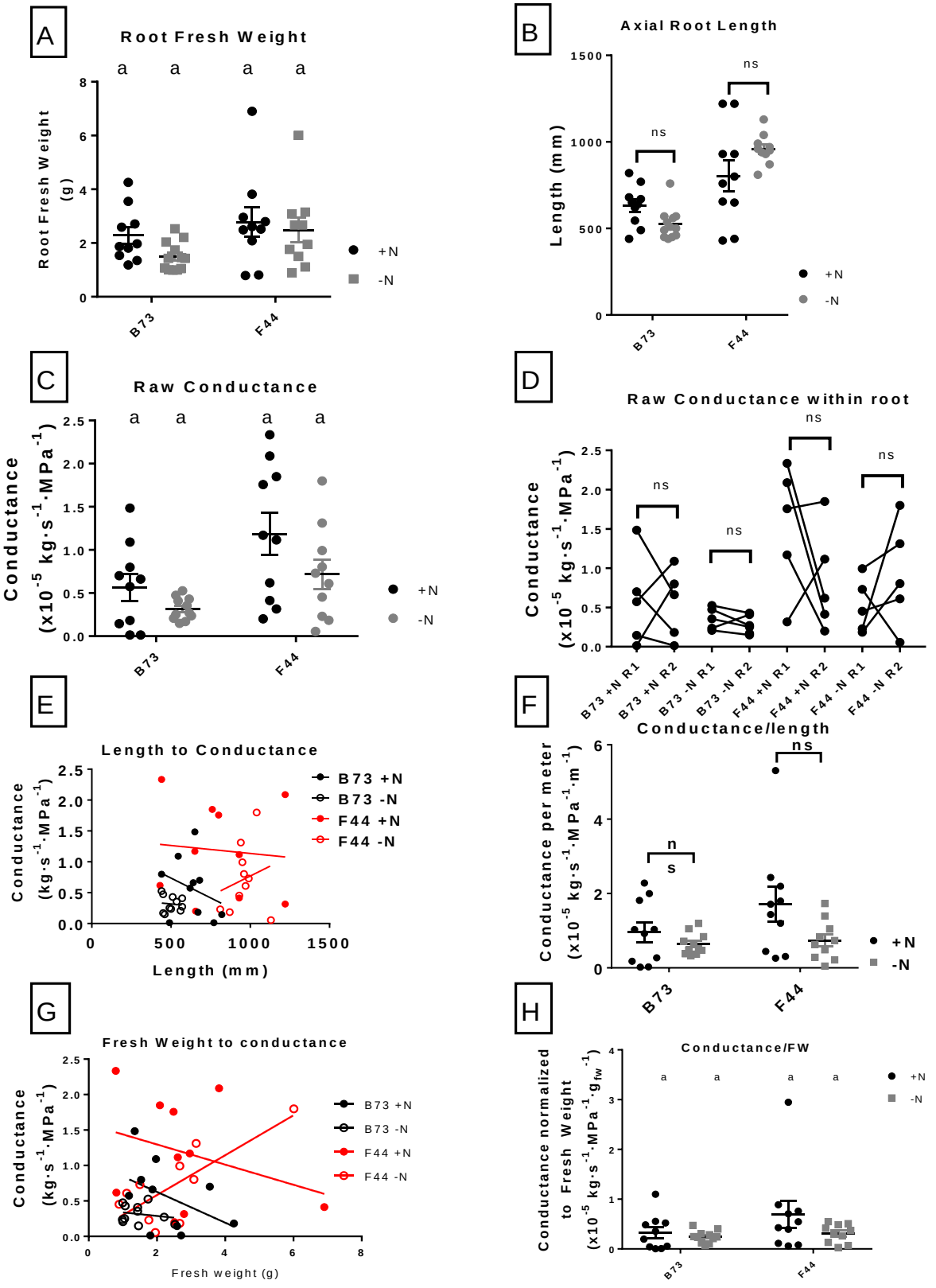

A
Root Fresh Weight
a a a a
Root Fresh Weight (g)
B73
F44
+N
-N
B
Axial Root Length
ns
Length (mm)
C
Raw Conductance
Conductance (x10-5 kg·s-1·MPa-1)
D
Raw Conductance within root
B73 +N R1
B73 +N R2
B73 -N R1
B73 -N R2
F44 +N R1
F44 +N R2
F44 -N R1
F44 -N R2
E
Length to Conductance
B73 +N
B73 -N
F44 +N
F44 -N
Length (mm)
F
Conductance/length
Conductance per meter (x10-5 kg·s-1·MPa-1·m-1)
G
Fresh Weight to conductance
Fresh weight (g)
H
Conductance/FW
Conductance normalized to Fresh Weight (x10-5 kg·s-1·MPa-1·gfw-1)

Figure 4.21 Hydraulic conductance of maize roots grown in high light (glass house) in an aeroponics system
Maize plants grown in aeroponics on 15 mM nitrate for ~4 weeks, to growth stage v3. –n plants were starved of nitrate (see **Table 4.1**) (A) Root fresh weight. There was no difference by two-way ANOVA between inbred line (p=0.0607), nitrogen nutrition (p=0.1645), nor their interaction (0.5284). (B) Total axial root length. Two-way ANOVA showed the variation was due to inbred line (44%, p<0.0001), and the interaction (p=0.115), but not to N treatment (0.p=0.6341). (D) Raw conductances comparing roots of the same plant. Connected lines represent roots from the same plant. Paired t-tests were not significant with any treatment. (E) Regression of the total axial root length to the hydraulic conductance. Regression slopes were not different from zero (p values: B73+N 0.3856, B73-N 0.7687, F44+N 0.8016, F44-N 0.5688). (F) Conductances normalized to axial root length. Intra-root paired t-tests showed no variation between 1st and 2nd roots measured for any treatment (not shown) (G) Regression of conductance and fresh weight. Regression was only significant for F44 –N ($y=0.2811\cdot10^{-5}x+0.02015$, $r^2=0.5811$, p=0.0104). Regressions of the others were not significant (p values: B73 +N 0.1992, B73-N 0.5452, F44+N 0.3814). (H) Conductances normalized to fresh weight were not significantly different. Intra-root paired t-tests showed no variation between 1st and 2nd roots measured for any treatment (not shown).

4.5 Discussion

4.5.1 Maize hydraulic responses to nitrogen

In previous studies, nitrate induced increased water flow through root systems of maize plants. Barthes et al. (1996a) and Hoarau et al. (1996) demonstrated that giving nitrate to nitrogen starved plants resulted in a 2-fold induction in sap exudation from de-topped maize plants in the first 16 h, and attributed the increased flow to increases in root hydraulic conductance. More recently, Górska et al. (2008) showed that, after a 60 min delay, nitrate induced a gradual increase in water flow through maize roots of plants grown on low nitrogen (min 4 days) , and reached a plateau sometime after 4 h. The change in xylem-sap osmotic pressure accounted for 7% of the increased flow, indicating that the rest was due to changes in hydraulic conductivity. These studies only relied on resupply of nitrate to nitrogen starved plants on relatively short time scales. They did not investigate the conductance after several days of resupply, nor the effect that starvation had on nitrogen grown plants. Schraut et al. (2005) investigated radial water flow in maize

roots after 7 days of nitrate starvation, and found a greater than 50% reduction in root water flow.

The work reported in this chapter sought to expand the knowledge of the effect of nitrate resupply and starvation on maize root hydraulic conductivity. With that intention, the results were less clear cut. Generally, starvation did not induce a multi-fold reduction in conductivity, and resupply did not result in a massive increase in conductivity across experiments. Several possibilities exist that may help understand the variation and deviation from the previously reported trends.

4.5.2 Tracking down the source of variance

Firstly, the studies listed above utilized water flow in the positive direction to its normal flow, where as HCFM and Xyl'em both measure flow in the negative flow direction. Additionally the previous studies utilized water flow from whole root systems, where as measurements reported here were performed on a subset of the total root systems.

A more complete study may have included a comparison of the hydraulic conductance of a root with the HCFM and through sap exudation rates. While a direct comparison cannot be made, the HCFM measurements are likely measuring the conductance of the root segments attached to the instrument. First, the root segments experienced a decrease in conductance over time, seen previously for maize plants (Vandeleur et al. 2014), and models predicted positive and somewhat consistent final values of conductance, suggesting the variation in initial conductance was due to conductivity differences, likely through aquaporin activity. A control could have been added such as a set of plants with the addition of an aquaporin inhibitor such as mercury, low pH (with acetate) or azide. Comparing the final value of the inhibited roots *versus* that of the excised only roots would have given a better indication whether the decay was due to aquaporin activity, or

to other factors such as root-to-root variability in apoplastic barriers, pasmodesmata, metabolic state (influencing energy available for solute transport), turgor ro water flow through cells. Additionally, if they were different, it may indicate the root hairs and lateral roots were undamaged. A test for damaged roots could have been done with an acid fuchin stain to stain injured root areas as done in Fritz et al. (2010).

Another consideration for the mixed response could be due to the choice in starvation and resupply conditions. For resupply experiments, a minimum for four hours was used, and may have been too short. However, Górska et al. (2008) demonstrated that while there is a delay in onset of nitrate induced hydraulic conductance increases, it was significantly raised around 1 hour post nitrate addition and was increasing for at least the first four hours post treatment. Barthes et al. (1996a) also showed that increased exudation rates were present at 16 hours after treatment in nitrate resupplied maize. Together, these suggest that hour hours should have been long enough to see a response.

The determination of the proportion of root with open xylem is consistent with consistent with the hypthysis that measured conductances were real. It indicates that conductances increased with effective root length. The conductances shown in **Figure 4.14** and **Figure 4.20** with change of flow rates in **Figure 4.19**, were all performed with the root attached, meaning the observed changes in conductance resulted from cutting the root.

Being convinced, or perhaps hopeful, the HCFM measurements represent real root segment hydraulic conductance, the variability becomes more interesting. Much of the chapter was devoted to understanding and attempting to limit the variability of the normalised hydraulic conductance measurements in an attempt to reproduce previously reported effects of N. The variability may be in part to the normalisation choice. In many cases, conductance did not increase with root dry weight or root fresh weight.

Normalising by these factors statistically did little to decrease the variance, and, as it was correlated, may have increased it in some cases. At least two other sources of the variance may exist: differential open xylem proportions, and true root segment variability.

4.5.3 The open xylem problem

The open xylem calculations suggested the xylem vessels in maize grown in a hydroponic or aeroponic system occur several centimetres from the root tip. Hachez et al. (2006) examined root structure (suberisation), ZmPIP aquaporin localization and ZmPIP aquaporin expression along the primary maize root in 7 d old maize (B73) grown in an aeroponic system. For gene expression and protein quantification, the authors focused on the first 60 mm from the tip, with a later measurement at 110 mm. For maize grown hydroponically, open xylem was determined to be 255±59 mm, and in all samples, the xylem did not open until after 110 mm. This suggests that for maize roots, either root water through radial absorption is not significant in a large part of the root, or that radial absorption to the open xylem is supported by an axial water flow that is not related to increased conductance.

Currently, this is unanswered, but hydraulic measurements are often normalized to a physical root property, such as fresh weight, dry weight, or surface area, and assumes that the proportion of root active in uptake does not change. The open xylem measurements challenge this assumption by showing the proportion of open xylem increases at physiologically meaningful rate with root length. Observed values ranged from ~20 to ~80% of the root containing open xylem vessels. As Hachez et al. (2012) demonstrated, the growth conditions of hydroponics and aeroponics alter the root suberisation (increased in exodermis in aeroponics) and may also influence where xylem becomes open for axial flow. A study with a small sample size (n=7) is seen in **Figure 4.20**, which measures open

xylem in aeroponics. It suggests the xylem also opens above 200 mm, but a larger sample size is needed to confirm. A further study should investigate the function of root surface area from the root tip. This could help account for the root surface area available for uptake with active xylem vessels. Perhaps this value, the amount of root surface area with open xylem vessels, would be a more accurate normalisation for maize hydraulic measurements, and would reduce the variability seen in the results.

This technique of measuring open xylem may also be expanded. During most measurements, the collection of data ended when open xylem was reached. However, continuing to cut and measure the root past the open xylem, such as in the sample in **Figure 4.20**, the decline in axial conductance for the root segment could be calculated. Frensch and Steudle (1989) demonstrated axial resistances in a similar manner in maize roots, showing ~2cm from the tip did not have axial conductance, but did not look at the effect nitrogen or other stresses had on this property. Bramley et al. (2009) measured axial and radial conductances and showed variation in barley and lupin root structure. In wheat, axial conductance is maintained along a greater distance of the root than compared with lupin, which indicated wheat absorbed a greater amount of water near the tips, whereas lupin water absorbance was spread across the length of the root. In barley, water transport was also seen to be differnet in seminal and adventitious roots, with open xylem vessels starting at different positions in different root types (Knipfer et al. 2011; Knipfer and Fricke 2011) and was related to aquaporin activity (Knipfer and Fricke 2010).

4.5.4 Individual root contributions to total root conductivity

Figure 4.21 examined the conductance of two nodal roots from the same plant. These roots did not have the same normalised conductance values, suggesting the whole root conductance may not be distributed equally among the individual root segments even

after normalisation. A simple explanation is that the normalisation method used (total root length) is still not an adequate normalisation factor. However, it can't be ruled out that in root segments exhibit a range of normalised conductances. In barley, Suku et al. (2014) showed variance within root types, between seminal and adventitious roots and that the net water flow through a root system was not equal to the sum of its individual root segments. With split root experiments, Gorska et al. (2008) showed water was preferentially and reversibly absorbed from the root side exposed to nitrate. In theory, roots grown in aeroponics or hydroponics should be in a homogeneous nitrate environment, but other factors may lead to a range of normalised conductances. It may be that the overall root conductance varies, but that each root has a wide range of segment conductance. Statistically, measuring one sample per root, and with small n, would give variabilities of the root segment population that would exhibit a range of conductance. This would give N and other effectors of hydraulic conductivity another mechanism of action – controlling the conductance of individual root segments. Treatments could affect change by altering the size of the distribution (increasing or decreasing the SD) and/or by shifting the distribution (shift left or right). For the data in this chapter, it could mean that N had an effect on whole root conductance, but was masked by a large distribution of conductance values under sampled. This could mask the effect N had on the hydraulic conductance among sampled roots. A better experimental approach would have to measure the total root hydraulic conductivity, not a single nodal root. The effect of nitrate on hydraulic conductance could have been masked by this variability.

A further test could be done to examine the individual components of conductance in relation to the whole root. This setup would work best through calculating the conductivity through the exudation (rate and concentration of exudate) to a negative pressure applied with a vacuum. A manifold could be added such that the exudates of

many could be collected at once. This would allow the whole root to be calculated, then excise the individual roots and attach and measure exudate through each, similar to the approach done in barley by Suku et al. (2014). Summing the values of exudate of the individuals should be close, especially if done relatively quickly, to the whole root conductance, and after normalising, compare the contributions of the various root segments to see a distribution. This could also be compared across treatments to see if how the segment and whole root conductance vary (do the distributions SD increase, or does the distribution shift with the same SD).

4.5.5 Data that would be useful for further interpretation

A better picture of root anatomy would assist in the interpretation of the experiments in this chapter. This would include collecting data on the number and types of roots present at various ages, and the properties (dry weight, fresh weight, surface area, and number of root tips) for both inbred lines under various nitrogen treatments. Additionally, as open xylem scales with root length, a clearer picture of how root surface area scales from the tip would be useful in knowing the proportion of root surface area corresponds to root with open xylem. Also, knowing the total N and nitrate concentrations of the roots and shoots could be useful in correlating conductance.

Additional work with the open xylem is needed to see if the percent of root with open xylem as a function of root length are consistent across growing conditions in aeroponics and in solid media. An additional follow up would be to perform cutting along the whole root axis under aquaporin inhibition to calculate the conductance of the xylem vessels. The time inhibition of root conductivity should also be compared with aquaporin inhibitors, and values taken until the plateau is reached to get better exponential curves.

Finally, better control over N resupply could be monitored. After three days, variability was present (although there did not seem to be a difference after 4 h where Górska et al. (2008) observed changes after 1 h). The authors measured water exudate of the whole root system, and did see variation (as well as diurnal confounding), but had statistically significant differences after ~60 minutes of N addition. In these experiments, it is possible that the increase was present, but had declined after 3 d or was masked by variation among individual root segments.

4.5.6 Conclusions

Both Xyl'em and HCFM measured hydraulic conductances of roots. The HCFM measurments declined over time post excision, suggesting measured conductances were root hydraulic conductances that decreased over time and are likely due to aquaporin activity (Vandeleur et al. 2014). Based on the data in this chapter, there were some differences in magnitude of response between maize lines B73 and F44. Hoverver variability prevents strong conclusions from being made. These results did not show a strong correlation between nitrate and hydraulic conductance as seen by others (Pan et al. 1985; Barthes et al. 1995; Barthes et al. 1996a; Schraut et al. 2005; Górska et al. 2010; Górska et al. 2008).

Chapter 5: Gene expression in response to nitrate starvation

5.1 Introduction

A mechanism for the short term modulation of water flow through roots is by altering the hydraulic conductivity, which can be influenced by the activity of aquaporins. There are many complex mechanisms for changing the activity of aquaporins that alters the amount of water permeating via the aquaporin proteins in a membrane. This can be done by mechanisms that act on existing aquaporins by increasing or decreasing activity though gating, such as with protonation (Tournaire-Roux et al. 2003), or with heavy metals (Maurel et al. 1993; Niemietz and Tyerman 2002). Phosphorylation has also a been shown to modify aquaporin water activity (Johansson et al. 1998). Other post-translational modifications have been observed, but their effect on water transport remains to be described.

The activity of aquaporins can also be changed by adjusting the amount of active protein in a membrane though modulating the rates of insertion and removal. Increasing the membrane insertion is seen in the interaction of ZmPIP2s to form heteromers with ZmPIP1s that translocate to the plasma membrane (Fetter et al. 2004; Zelazny et al. 2009; Zelazny et al. 2007). Aquaporins can also be removed from the plasma membrane as is seen in *Arabidopsis* during salt stress (Boursiac et al. 2005). Polar localization can also affect aquaporin activity (Boursiac et al. 2005; Sakurai-Ishikawa et al. 2011; Hachez et al. 2008; Hachez et al. 2006).

Increasing protein amount is another mechanism, and may be done through increases in gene expression, though its relation with protein amount is not always clear. Aquaporin

transcripts are seen to be regulated during stress (examples below, and see Chapter 1).Maize aquaporin gene expression is largely unknown. Relative gene expression of many of the ZmPIPs has been seen along the longitudinal root axis (Hachez et al. 2006) and along the leaf (Hachez et al. 2008). ZmPIP2;8 expression in roots has been monitored under corn borer infestation as well (Lawrence et al. 2012; Lawrence et al. 2013). Maize PIP gene expression varies diurnally, and aligns with diurnal regulation of maize root water conductivity (Lopez et al. 2003). ZmTIP2-3 also has diurnal regulation (Lopez et al. 2004) and water transport capacity. ZmNIP2;1 (ZmLsi1) and ZmNIP2;2 (ZmLsi2) expression has been investigated in tissue as they transport silicon (Mitani et al. 2009). Urea transporters ZmNIP2;1, and ZmNIP2;4 and ZmTIP4;4 expression were examined across different maize tissues (Gu et al. 2012), as well as the boron transporter ZmNIP3;1 (Leonard et al. 2014). Maize PIP gene expression has also been examined in maize roots under short term (0-4hr) nitrate resupply, where it was concluded that increased hydraulic conductivity was not due to increased abundance of ZmPIP transcripts (Górska et al. 2008).

For most ZmTIPs, ZmNIPs and ZmSIPs, there is no direct data on gene expression in either roots or shoots. Except for a subset of the ZmPIPs during the first four hours of nitrate resupply (Górska et al. 2008), there is no data on aquaporin gene expression, and no data on aquaporin gene expression in the shoot or root during nitrate starvation. The aim of the experiments performed in this chapter was to understand the gene expression of the full set of maize aquaporins, along with nitrate uptake and assimilation genes, in roots and leaves of maize plants grown on nitrate or after 5 days of nitrate starvation.

5.2 Materials and methods

5.2.1 Plant materials and tissue harvest

Tissues were sampled from B73 and F44 roots and shoots grown in an aeroponic system from Chapter 4. During the transient runs of the hydraulic conductance measurements with the HCFM, a shoot sample was obtained by using a razor to excise the middle third of the fourth mature leaf between 4-8 hrs into the photoperiod (10am – 2pm) . This sample was placed in a conical tube and frozen in liquid nitrogen. After measuring the hydraulic conductance (Chapter 4), maize roots were rinsed in purified (reverse osmosis) water. From the root tip, the first 10 cm of root material along the central axis (coinciding to the start of lateral root formation) was removed and discarded. The remaining root tissue was placed in a conical tube and frozen in liquid nitrogen. The time from the removal of the root from the plant, completing HCFM measurements and submerging in liquid nitrogen was under eight minutes. Tissue samples were stored at -80°C, then were ground in liquid nitrogen with a porcelain mortar and pestle to a fine powder. Ground tissue was stored at -80°C until RNA extraction.

5.2.2 RNA extraction and processing

RNA isolation was performed using an acid guanidinium thiocyanate-phenol-chloroform extraction protocol (Chomczynski and Sacchi 1987) using Trizol (Thermo Fisher Scientific, Waltham, MA, USA). Unless noted, steps were carried out at room temperature and centrifuge steps at 4°C. 75 ± 25 mg frozen tissue was placed in a 2 mL microcentrifuge tube, combined with 1 mL Trizol and vortexed for a few seconds. The mixture was left to incubate at room temperature, then 200 µL chloroform was added and vortexed to mix. Samples were then centrifuged at 14,000 g for 15 min (at 4°C). The aqueous phase (the clear upper phase) was transferred to a fresh 1.6 mL microcentrifuge

tube. 500 µL isopropanol was added to the aqueous phase, mixed by vortexing and incubated at room temperature for 15 minutes. Samples were then centrifuged to 14,000 g for 15 minutes to pellet the RNA. The supernatant was discarded and the pellet was washed with RNase free ethanol (80%) and vortexed. RNA was pelleted again by centrifuge 7,500 g for 5 minutes. The supernatant was discarded. The pellet was washed again with a combination of 500 µL DEPC-treated water, 50 µL sodium acetate (3M), and ethanol (100%). The mixture was vortexed and incubated at -20°C overnight. The samples were then centrifuged at 14,000 g for 15 minutes and the supernatant discarded. The RNA pellet was washed with 1 mL of RNase free ethanol (80%), vortexed, and centrifuged at 7,500 g for 5 minutes. The supernatant was discarded and the RNA pellet dried in a 37° C oven. The pellet was resuspended in 50 µL DEPC-treated water and frozen overnight. The RNA quantity (absorbance at 260 nm) and purity (ratios of absorbance at 280 nm for protein and 230 nm for residual solvents) was checked with a NanoDrop (Thermo Fisher Scientific, Waltham, MA, USA) spectrophotometer.

RNA's (if needed) were diluted to 200 ng/µL and treated with DNase to remove residual genomic DNA using the TURBO DNA free kit (Thermo Fisher Scientific, Waltham, MA USA). RNA's were then checked for quantity and quality using the Nanodrop. 500 ng of RNA was run on a non-denaturing agarose gel to check integrity.

For some root RNA samples, due to multiple poor cDNA synthesis reactions, a phenol-chloroform extraction was performed after the TURBO DNase treatment. Equal volume of acidic phenol (pH 4.3) was added in equal volume to the RNA, then an equal volume of chloroform. Samples were vortexed, then centrifuged (15 min, maximum speed). The aqueous phase was removed and the RNA precipitated with 1/10 volume 3M sodium acetate and 2.5 volumes of 100% ethanol. Samples were stored at -20°C for 2 h. The

supernatant was discarded, and the RNA pellet washed twice with 70% ethanol. The pellet was air dried, then resuspended in 20 µL RNase free water. RNA's were then checked for quantity and quality using the Nanodrop. 500 ng of RNA was run on a non-denaturing agarose gel to check integrity.

5.2.3 cDNA synthesis

RNAs were reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA USA). 1 µg of RNA was reverse transcribed using iScript in a 20 µL reaction, and the RT enzyme was inactivated according the manufacturer's recommendations. The iScript cDNA Synthesis Kit uses blend of oligo$(dT)_{20}$ and random hexamer primers in a master mix.

The success of the RT reaction and presence of genomic DNA was determined by the PCR amplification across an exon junction on the actin gene. Amplification from the acting gene resulted in a 439 bp product for and a 1,325 bp product from genomic DNA. PCR products were run on a 1% agarose gel. The cDNA was checked for cDNA amplification and no genomic DNA amplification. Following manufacturer recommendations, no PCR clean up step was performed to isolate the cDNA from the RT-PCR prior to gene expression analysis.

5.2.4 qPCR on the OpenArray 12k Flex

5.2.4.1 Overview

The OpenArray (Thermo Fisher Scientific, Waltham, MA, USA) system was selected to monitor gene expression of 112 genes per sample. Each OpenArray plate consisted of 48 subarrays, with each subarray containing 56 individual reaction wells with pre-loaded TaqMan primers and probes. Each plate contained two sets of subarrays for a total of 112

genes. A loading robot was used to transfer cDNA mixed with a DNA polymerase for a total reaction volume of 33 nL per well.

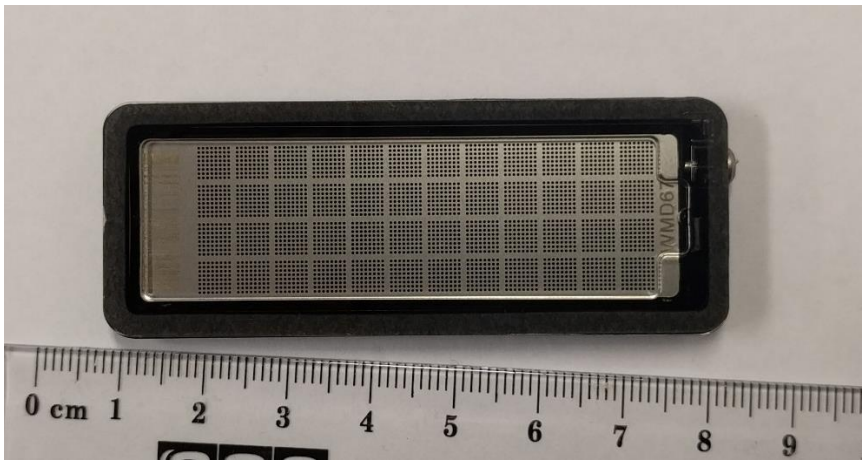


Figure 5.1 OpenArray plate
The array has 48 subarrays, each with 64 wells (but only have 56 have assays). Each well was preloaded with TaqMan primers and probes. Each sample was loaded onto two subarrays (33 nL per well) and were examined for 112 genes.

5.2.4.2 OpenArray design

The 112 gene format was chosen for the OpenArray. Aquaporin genes (Chapter 2) along with genes for nitrogen uptake and assimilation were chosen for the array (One OpenArray plate run included expression monitoring for 112 genes for 24 samples). For each target, specific TaqMan primers and probes were designed by Thermo Fisher Scientific (see Appendix 5.3.1 and 5.3.2 for sequences[2]). Each probe utilized FAM as the reporter dye that was quenched by NFQ. Preference was given first to TaqMan probes specific to the target cDNA, then to sets that spanned an exon junction. A set of reference genes was selected and included ZmFPGS (folylpolyglutamate synthase), ZmUBQc (ubiquitin-conjugating enzyme), ZmSIN3 (Sin3-histone deacetylase), ZmCul (cullin),

[2] While all were designed, only some were validated by Thermo Fisher Scientific. These primer and probe combinations were proprietary information and the specific sequences not provided. A context sequence within each target gene which was amplified was given instead.

ZmLUG (Leunig), and ZmMEP (membrane protein PB1A10.07c). Manoli et al. (2012) showed ZmFPGS, ZmMEP, ZmUBQc, ZmLug and ZmCul to be stabaly expressed genes suitable for use as reference genes. They found ZmMEP and ZmLug to be the most stably expressed, and showed actin to be less stable than the five selected. ZmSIN3 was suggested to be a stable reference gene from previous unpublished studies. Assay information, including primers and probes and transcriptional target are listed in **Table 5.8** and **Table 5.9**.

5.2.4.3 OpenArray loading

Samples of cDNA were loaded onto a 96-well plate, then to a 384-well plate and combined with the PCR master mix. Each well of the 384-well plate was used to load a single subarray on the OpenArray plate. OpenArray Sample Tracking software (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to track each sample. Undiluted cDNA's were loaded onto a 96-well plate (5 µL). Three technical replicates were run for each sample (total of eight samples per OpenArray plate). Working TaqMan master mix was prepared by adding 68.8 µL nuclease free water to 132.0 µL 2x TaqMan OpenArray Real-Time PCR Master Mix. To ensure identical loading of each subarray, 2x reactions were assembled and mixed, then split between wells on the 384-well plate. 7.6 µL of working TaqMan master mix was pipetted to the odd rows on the 384-well plate. 2.4 µL of each undiluted cDNA was transferred to the odd rows using a multichannel pipette and mixed with the working TaqMan master mix. Samples were mixed by pipetting, then centrifuged briefly to remove air bubbles. 5 µL of each was then transferred to the even

rows on the 384-well plate.[3] The 384-well plate was sealed using foil and centrifuged to remove bubbles.

An OpenArray plate (stored at -20°C) was thawed to room temperature. Reactions from the 384-well plate were loaded on the OpenArray plate using the QuantStudio OpenArray AccuFill System (Thermo Fisher Scientific, Waltham, MA, USA) with corresponding software. The AccuFill system loaded reactions from the 384-well plate into pipette tips using the water-adhesion properties of each reaction. The AccuFill System then touched the tip to the OpenArray plate, and loaded each well of the designated subarray with 33 nL. Once loaded, the plate lid was fitted to the OpenArray and the plate was filled with the provided oil and sealed. Sealed plates were wiped with ethanol and were ready for the qPCR run.

5.2.4.4 Instrument Run

Loaded OpenArray plates were wiped with 75% ethanol and loaded onto the QuantStudio 12K Flex Real-Time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). The qPCR protocol was run for 40 cycles with fluorescence data recorded after each cycle. Results were transferred to a computer for analysis.

[3] Each well on the 384-well plate corresponded to an individual subarray on the OpenArray plate. Each sample utilized two subarrays. To ensure identical reaction mixtures were added to both subarrays for each sample, reactions were assembled, mixed and then evenly split.

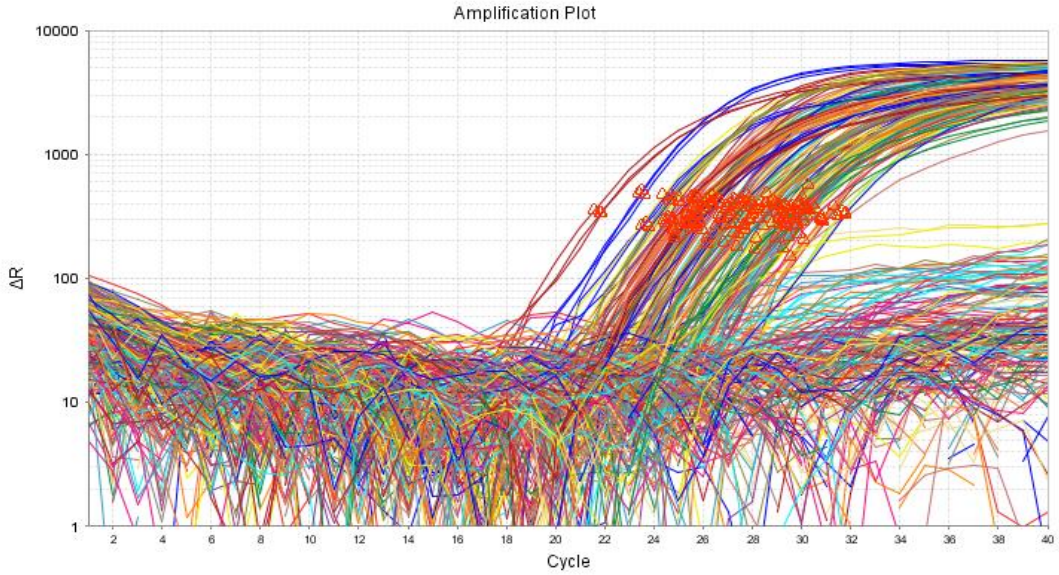


Figure 5.2 Example OpenArray amplification plot for one sample
An example OpenArray amplification for one sample. Each OpenArray assayed 24 samples against 112 genes. Red triangles represent C_{RT} values determined by the QuantStudio 12k Flex software.

5.2.4.5 TaqMan primer/probe quasi-validation

A simplified validation was performed for the TaqMan primers and probes. A single OpenArray plate was utilised, loaded with serially diluted cDNAs. The cDNAs used for validation were an equal volume mixture of B73+N root and B73+N shoot samples. A 1:10 serial dilution was performed, and the results run reps of 4 on the OpenArray plate (6 dilution steps). The plate was run and analysed as below and a standard curve plotted (C_{RT} value to dilution). The slope of the standard curve was taken as the efficiency of the TaqMan probe.

5.2.5 Analysis of qPCR data

The QuantStudio 12K Flex Real-Time PCR system software determined if amplification occurred in each well and calculated a C_{RT} value for the well. The C_{RT} value was similar to a C_T value, but was not fixed to a specific threshold of fluorescence (although wells of the same gene used similar fluorescence levels). Six potential reference genes were included on the OpenArray plate: ZmCul, ZmFPGS, ZmLUG, ZmMEP, ZmSIN3 and ZmUBQc, Stability was checked by plotting C_{RT} values and checking for differences between tissue and inbred line. A reference level for each sample was determined as the geometric mean of the stable reference genes. Relative expression was analysed via the ΔC_{RT} method (Pfaffl 2001). Briefly, a ΔC_{RT} values was calculated by subtracting the geometric C_{RT} mean of the reference genes from each gene.

$$\Delta C_{RT} = C_{RT}(Gene) - C_{RT}(Ref)$$

Undetermined C_{RT} values were ignored. For relative expression plots, efficiency of each assay was assumed to be equal (100%), and relative expression was set as $2^{\Delta CRT}$.

$$Rel.Expression = \frac{2^{C_{RT}(Gene)}}{2^{C_{RT}(Ref)}} = 2^{C_{RT}(Gene) - C_{RT}(Ref)} = 2^{\Delta C_{RT}}$$

To test the effect of nitrate starvation had on gene expression, a t-test was performed on the ΔC_{RT} values of +N and –N plants, and a $\Delta\Delta C_{RT}$ value determined. The changes presented represent changes due to N starvation. Statistics and data were performed using R (R Core Team 2017) with modified code provided by Johannes Scharwies (Tyerman Lab, University of Adelaide).

5.3 Results

5.3.1 Isolating RNA and generating cDNA from root and shoot samples

Total RNA isolation was done through Trizol extraction and was treated with TURBO DNase. The DNase treatment was followed by the removal of DNase by precipitating with a slurry mixture (TURBO DNA free kit). Running on a non-denaturing agarose gel, the RNA for all samples appeared to be intact. RNA was reverse-transcribed to cDNA using iScript (BioRad), and the quality was tested by amplifying a short actin fragment across an exon. For all shoot samples, actin amplified strongly. For root samples, results were problematic, with many samples not amplifying actin. Repeating reverse transcription on several independent RNA isolations from root tissues, actin was amplified in 7 out of 12 (7/12) B73 +N, 0/12 B73-N samples, 8/8 F44 +N samples, and 5/11 F44-N. Performing an extra phenol:chloroform extraction on the RNA after treatment of TURBO DNase, then RT-PCR with iScript led to amplification of 9/12 B73+N, 4/12 B73-N, 8/8 F44+N and 7/11 F44-N samples.

5.3.2 Quality of reference genes

Normalising to a reference gene requires stable expression of the reference genes. To determine stability, it was assumed that the reverse-transcription efficiency was the same for all samples (independent of tissue type, inbred line, or N treatment). As input RNA amounts were the same, it was assumed that cDNA amounts were the same and direct comparisons of the expression levels (C_{RT} values) could be made between samples for each reference gene.

Analysis of the expression (**Figure 5.3** and **Table 5.1**) across inbred lines, tissue types, and N treatment showed the expression and variation of the reference genes selected;

ZmUBQc, ZmSIN3, ZmFPGS, ZmCul, ZmMEP and ZmLug. Of the six selected as potential reference genes, only ZmLug and ZmFPGS showed stable expression across all the factors. For expression normalisation, ZmLug and ZmFPGS were selected as the reference genes.

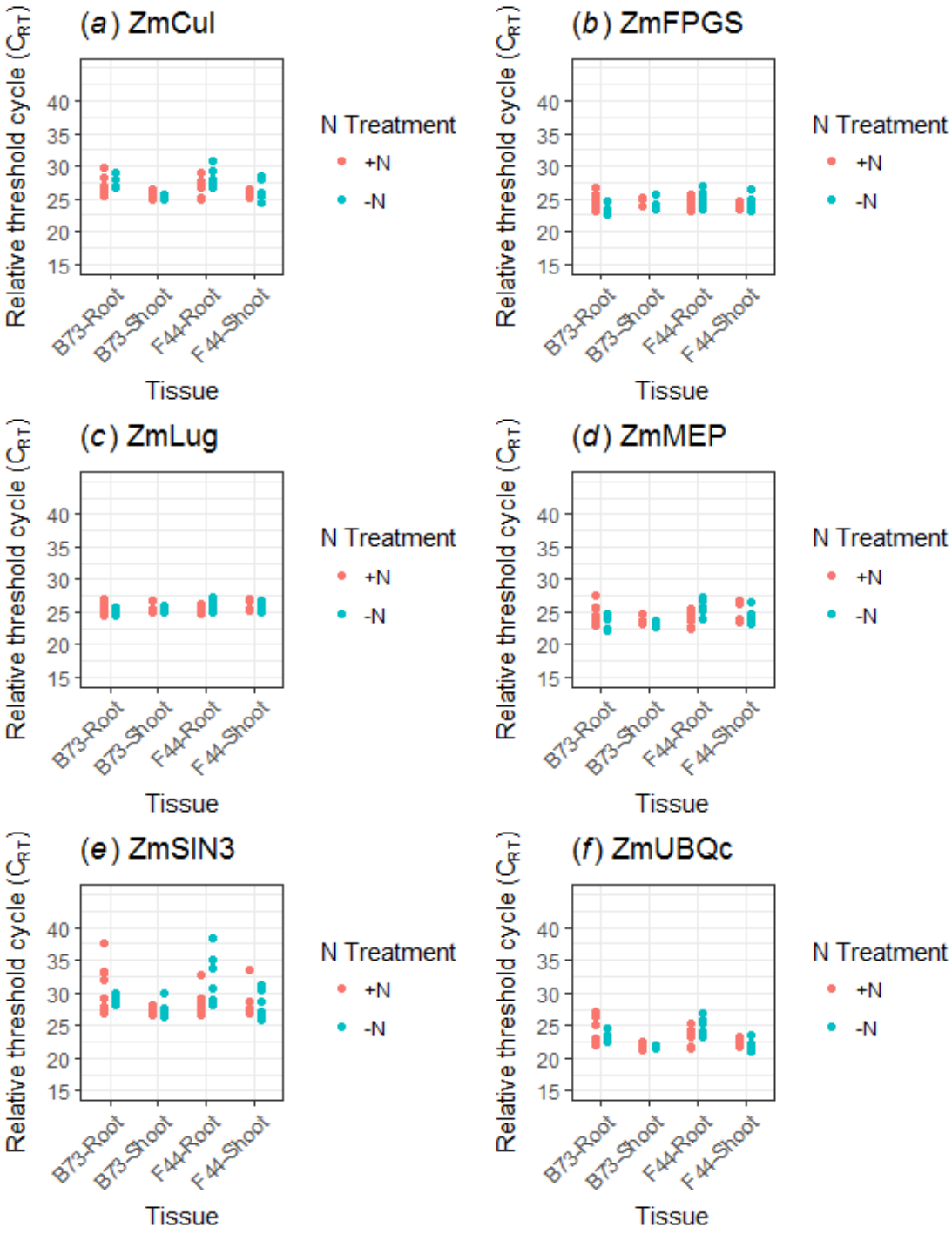


Figure 5.3 Expression levels of the reference genes

Plots of mean C_{RT} values for samples across treatment types: inbred line, tissue type and N treatment for each of the reference genes on the OpenArray plate: (a) ZmCul (b) ZmFPGS (c) ZmLUG (d) ZmMEP, (e) ZmSIN3 and (d) ZmUBQc. Only ZmLug and ZmFPGS showed stable expression across N treatment, tissue type and inbred line.

Table 5.1 C_{RT} values of the reference genes

Statistics for reference gene expression. Only ZmLug and ZmFPGS had stable across inbred lines, tissue types, and treatments. On its own, N treatment did not affect reference gene stability in ZmCul, ZmMEP, ZmSIN3 or ZmUBQc.

Gene Name	Inbred Line	Tissue	N Treatment	CRT Mean	CRT SD	CRT SEM	n			
ZmCul	B73	Root	+N	27.097	1.461	0.517	8			
ZmCul	B73	Root	-N	27.689	1.077	0.539	4	Source of Variation	% of total variation	P value
ZmCul	B73	Shoot	+N	25.553	0.592	0.224	7	Interaction	5.695	0.2144
ZmCul	B73	Shoot	-N	25.453	0.376	0.154	6	Inbred Line + Tissue	36.86	<0.0001
ZmCul	F44	Root	+N	26.898	1.356	0.479	8	N treatment	4.907	0.0514
ZmCul	F44	Root	-N	28.813	1.593	0.602	7			
ZmCul	F44	Shoot	+N	25.905	0.447	0.200	5			
ZmCul	F44	Shoot	-N	26.274	1.579	0.645	6			
ZmFPGS	B73	Root	+N	24.683	1.078	0.359	9			
ZmFPGS	B73	Root	-N	23.348	0.928	0.464	4	Source of Variation	% of total variation	P value
ZmFPGS	B73	Shoot	+N	24.197	0.528	0.200	7	Interaction	12.72	0.0921
ZmFPGS	B73	Shoot	-N	24.117	0.838	0.342	6	Inbred Line + Tissue	4.856	0.4631
ZmFPGS	F44	Root	+N	24.320	0.891	0.315	8	N treatment	0.7049	0.541
ZmFPGS	F44	Root	-N	24.845	1.260	0.476	7			
ZmFPGS	F44	Shoot	+N	24.290	0.561	0.251	5			
ZmFPGS	F44	Shoot	-N	24.513	1.100	0.449	6			
ZmLUG	B73	Root	+N	25.687	0.892	0.297	9			
ZmLUG	B73	Root	-N	25.107	0.535	0.267	4	Source of Variation	% of total variation	P value
ZmLUG	B73	Shoot	+N	25.430	0.648	0.245	7	Interaction	5.327	0.4357
ZmLUG	B73	Shoot	-N	25.358	0.456	0.186	6	Inbred Line + Tissue	10.16	0.1674
ZmLUG	F44	Root	+N	25.558	0.514	0.182	8	N treatment	0.216	0.7386
ZmLUG	F44	Root	-N	25.922	0.838	0.317	7			
ZmLUG	F44	Shoot	+N	25.931	0.809	0.362	5			
ZmLUG	F44	Shoot	-N	25.947	0.727	0.297	6			
ZmMEP	B73	Root	+N	24.708	1.518	0.506	9			
ZmMEP	B73	Root	-N	23.375	1.196	0.598	4			
ZmMEP	B73	Shoot	+N	23.710	0.461	0.174	7	Source of Variation	% of total variation	P value
ZmMEP	B73	Shoot	-N	23.391	0.414	0.169	6	Interaction	14.03	0.0365
ZmMEP	F44	Root	+N	24.282	1.224	0.433	8	Inbred Line + Tissue	19.45	0.0097
ZmMEP	F44	Root	-N	25.854	1.199	0.453	7	N treatment	0.000187	0.9912
ZmMEP	F44	Shoot	+N	24.817	1.593	0.712	5			
ZmMEP	F44	Shoot	-N	24.912	1.357	0.554	6			
ZmSIN3	B73	Root	+N	30.468	3.689	1.230	9			
ZmSIN3	B73	Root	-N	29.049	0.699	0.350	4	Source of Variation	% of total variation	P value
ZmSIN3	B73	Shoot	+N	27.117	0.566	0.214	7	Interaction	12.69	0.0606
ZmSIN3	B73	Shoot	-N	27.549	1.181	0.482	6	Inbred Line + Tissue	17.57	0.0193
ZmSIN3	F44	Root	+N	28.516	1.856	0.656	8	N treatment	1.153	0.3998
ZmSIN3	F44	Root	-N	32.486	3.900	1.592	6			
ZmSIN3	F44	Shoot	+N	28.834	2.684	1.200	5			
ZmSIN3	F44	Shoot	-N	28.316	2.170	0.886	6			
ZmUBQc	B73	Root	+N	24.139	1.799	0.600	9			
ZmUBQc	B73	Root	-N	23.314	0.878	0.439	4	Source of Variation	% of total variation	P value
ZmUBQc	B73	Shoot	+N	21.978	0.458	0.173	7	Interaction	7.75	0.1193
ZmUBQc	B73	Shoot	-N	21.855	0.164	0.067	6	Inbred Line + Tissue	37.03	<0.0001
ZmUBQc	F44	Root	+N	23.311	1.304	0.461	8	N treatment	0.03501	0.8681
ZmUBQc	F44	Root	-N	24.605	1.375	0.520	7			
ZmUBQc	F44	Shoot	+N	22.516	0.683	0.306	5			
ZmUBQc	F44	Shoot	-N	21.949	0.867	0.354	6			

5.3.3 Relative expression of genes in tissue types

5.3.3.1 Maize aquaporin relative gene expression

To assess genes that were highly expressed in tissues, relative gene expression was plotted for +N and –N conditions. Gene expression was relative to the reference genes ZmLUG and ZmFPGS. Maize aquaporin transcripts were detected in both roots and shoots. In general, aquaporins had the highest relevant expression of the genes on the Open Array (scales on **Figure 5.2-5.10**). **Figure 5.2** and **Figure 5.3** show B73 and F44 root (a) and shoot (b) relative expression. In B73 roots (**Figure 5.2**-A), the highest expressed aquaporins were the ZmPIP1s (ZmPIP1;7>>ZmPIP1;2a, ZmPIP1;4), ZmPIP2s (ZmPIP2;6, ZmPIP2;3, ZmPIP2;4) and ZmTIP2s (ZmTIP2;1, ZmTIP2;2, ZmTIP2;3). In F44 roots (**Figure 5.3**-A), the highest expressed aquaporins were ZmPIP2;6 (near same level as B73 roots), and the ZmTIP2s (ZmTIP2;2, ZmTIP2;3). In shoots ZmPIP1;7 and ZmTIP4;1b were the highest expressed in both B73 (**Figure 5.2**-B) and F44 (**Figure 5.3**-B). The open array detected most, but not all of the aquaporin gene transcripts (**Table 5.3**).

Table 5.2 Aquaporin gene transcripts detected in each tissue type

	B73		F44	
Aquaporin	Root	Shoot	Root	Shoot
ZmNIP1;1	x	x	x	x
ZmNIP1;2	x	x		x
ZmNIP1;3				
ZmNIP2;1	x	x	x	x
ZmNIP2;2		x	x	x
ZmNIP2;3				
ZmNIP2;4	x	x	x	x
ZmNIP3;1	x		x	
ZmNIP4;1				
ZmNIP5;1				
ZmNIP5;2				
ZmPIP1;2b	x	x	x	x
ZmPIP1;4	x	x	x	x
ZmPIP1;5	x	x	x	x
ZmPIP1;6b	x	x	x	x
ZmPIP1;7	x	x	x	x
ZmPIP2;1	x	x	x	x
ZmPIP2;2	x	x	x	x
ZmPIP2;3	x	x	x	x
ZmPIP2;4	x	x	x	x
ZmPIP2;5	x	x	x	x
ZmPIP2;6	x	x	x	x
ZmPIP2;7				
ZmPIP2;8				
ZmPIP2;9	x	x	x	x
ZmPIP3;1		x		x
ZmSIP1;1	x	x	x	x
ZmSIP1;2	x	x	x	x
ZmSIP2;1	x	x	x	x
ZmTIP1;1	x	x	x	x
ZmTIP1;2b	x	x	x	x
ZmTIP2;1	x	x	x	x
ZmTIP2;2	x	x	x	x
ZmTIP2;3	x	x	x	x
ZmTIP2;4	x	x	x	x
ZmTIP3;1	x	x	x	
ZmTIP3;2b				
ZmTIP3;3				
ZmTIP3;4				
ZmTIP4;1b	x	x	x	x
ZmTIP4;2b	x	x	x	x
ZmTIP4;3b				
ZmTIP4;4b				
ZmTIP5;1b				

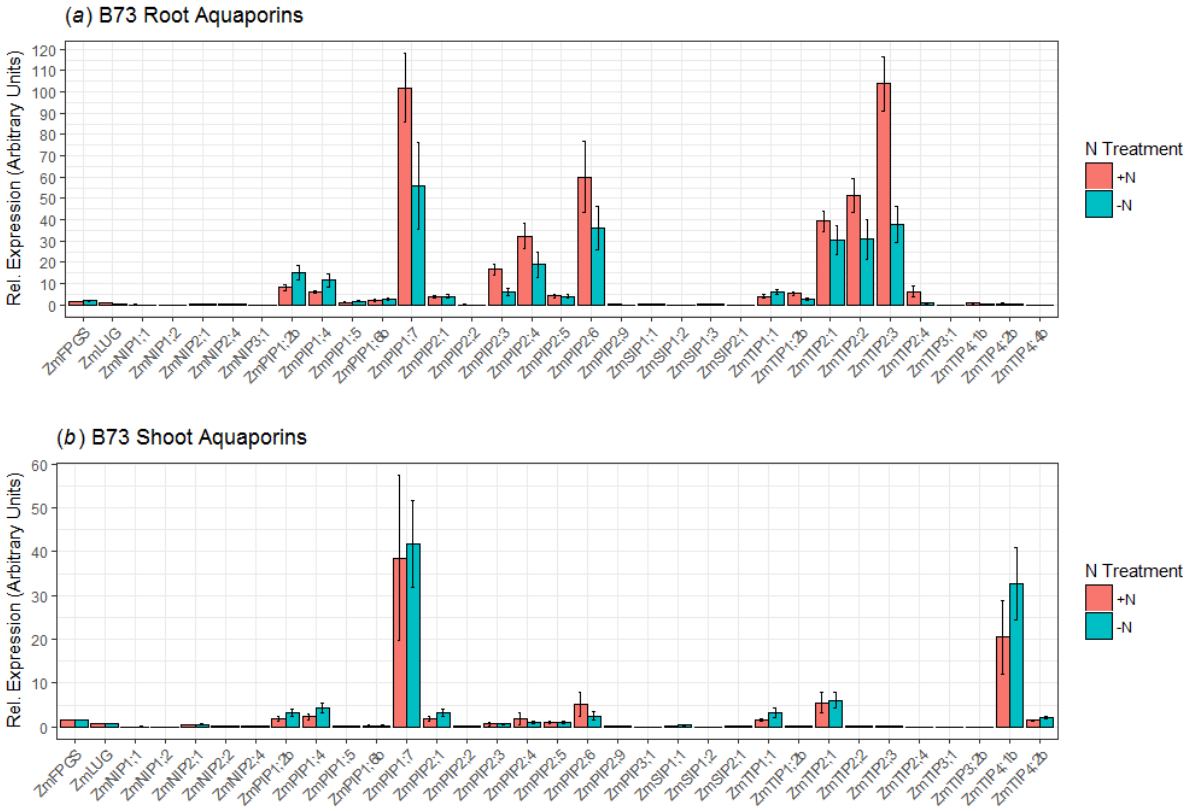


Figure 5.4 Aquaporin expression in maize roots and shoots of B73

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of aquaporins in maize roots (a) or shoots (b) of inbred line B73. Data are plotted Mean + SEM. See **Table 5.3** and **Table 5.5** for statistical significances.

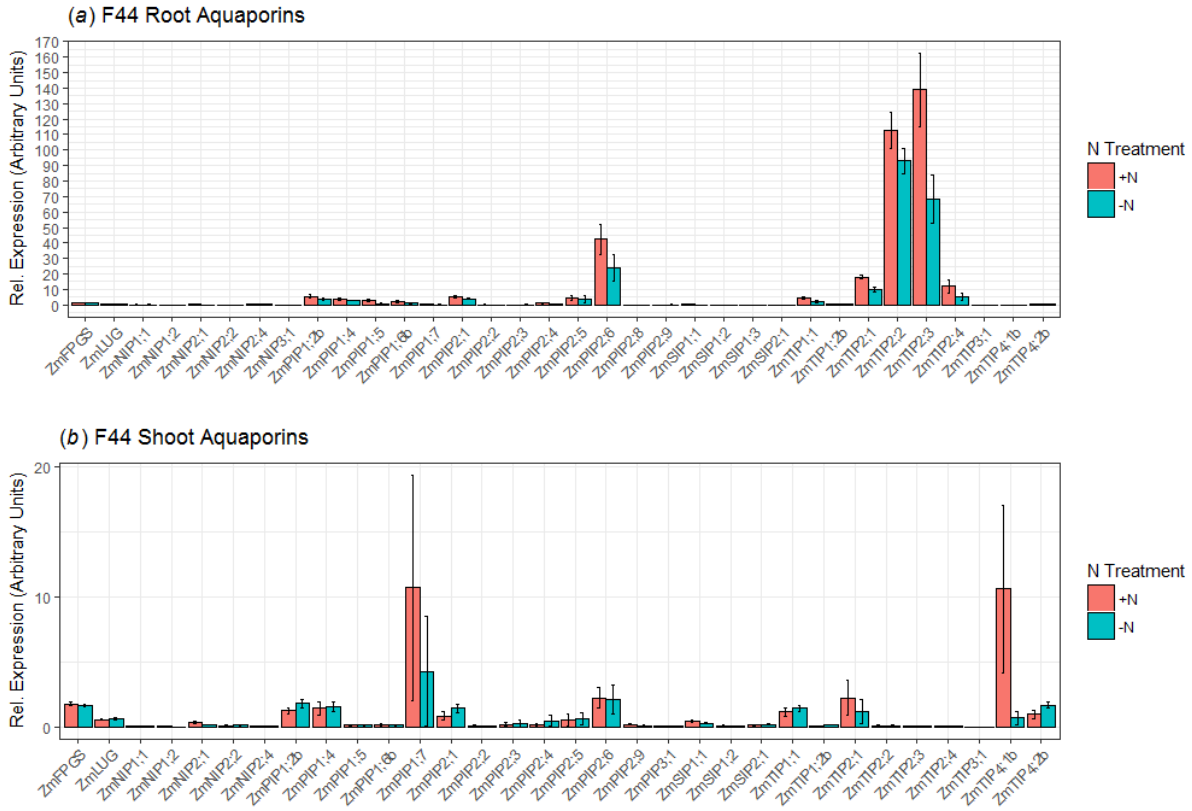


Figure 5.5 Maize aquaporins expressed in root and shoot tissue of F44

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of aquaporins in maize roots (a) or shoots (b) of inbred line F44. Data are plotted Mean + SEM. See **Table 5.4** and **Table 5.6** for statistical significances.

5.3.3.2 Expression of genes for Nitrogen uptake, assimilation and regulation

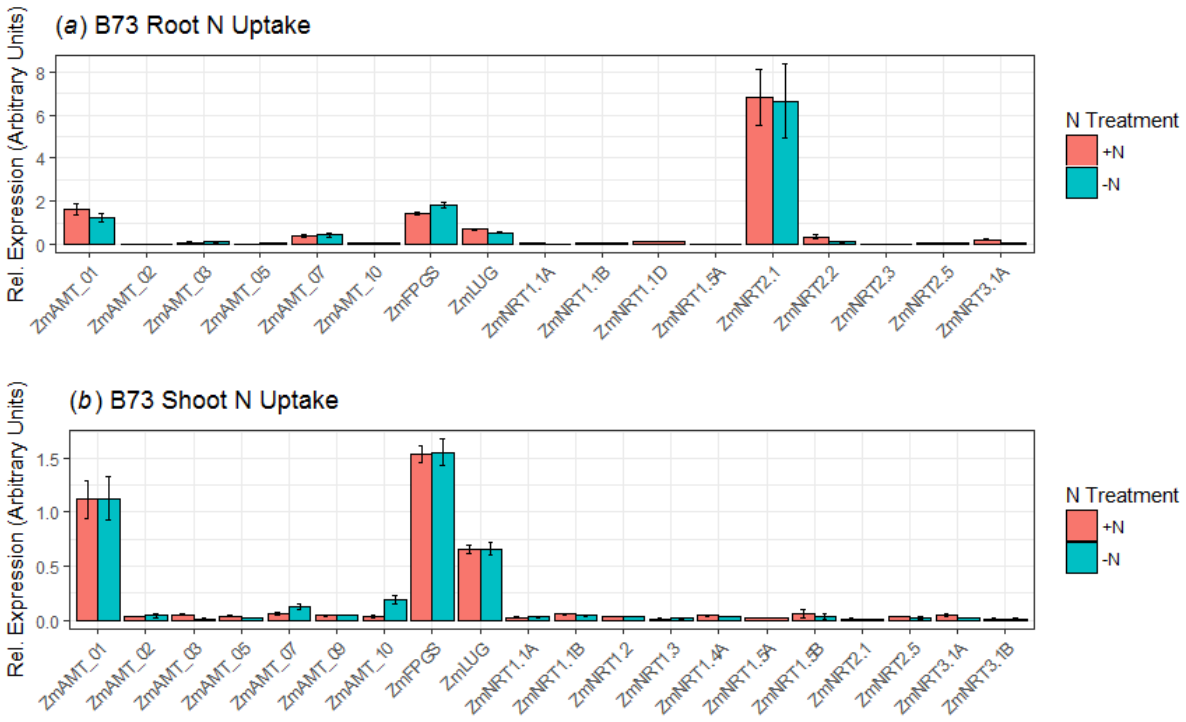


Figure 5.6 B73 root and shoot relative gene expression for N uptake genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of genes involved in N uptake in maize roots (a) or shoots (b) of inbred line B73. Data are plotted Mean + SEM. See **Table 5.3** and **Table 5.5** for statistical significances.

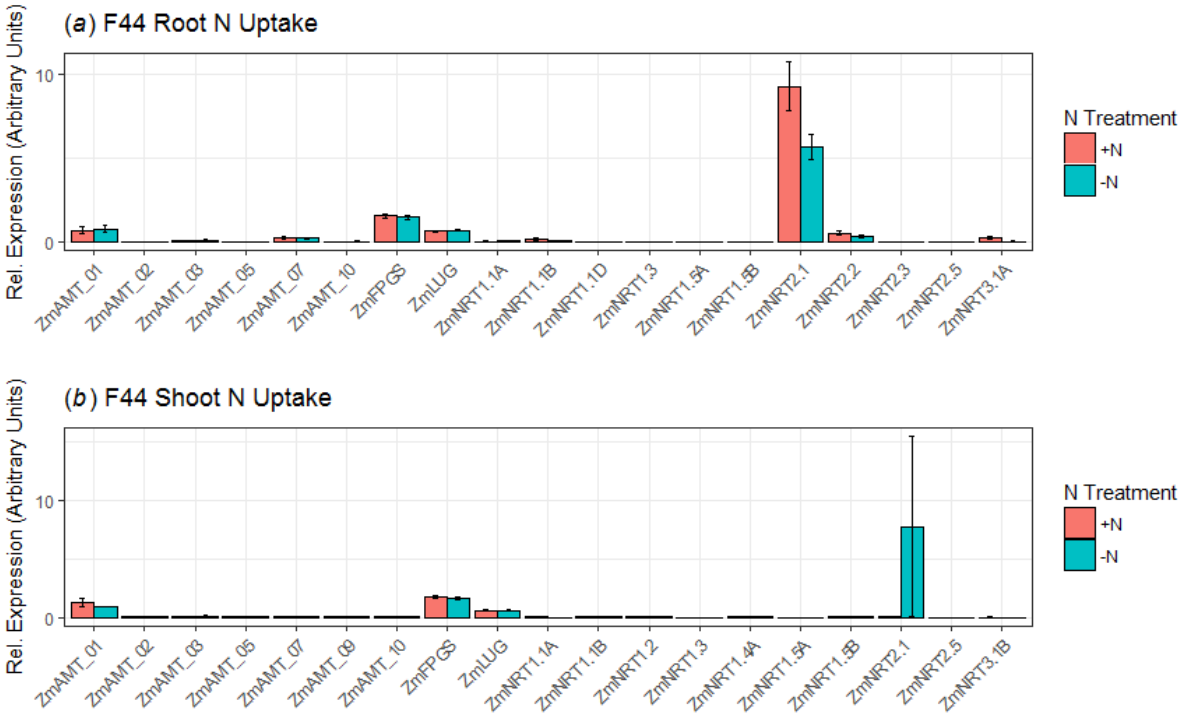


Figure 5.7 F44 root and shoot relative gene expression for N uptake genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of genes involved in N uptake in maize roots (a) or shoots (b) of inbred line F44. Data are plotted Mean + SEM. See **Table 5.4** and **Table 5.6** for statistical significances.

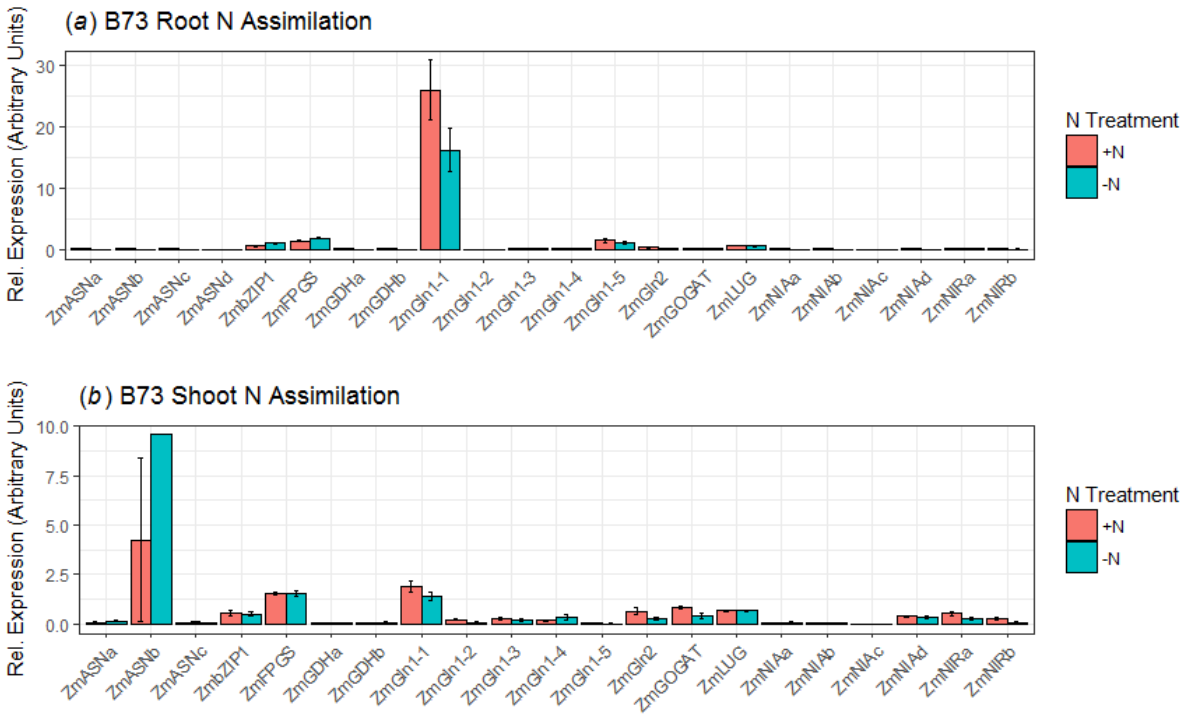


Figure 5.8 B73 root and shoot relative gene expression for N assimilation genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of genes involved in N assimilation in maize roots (a) or shoots (b) of inbred line B73. Data are plotted Mean + SEM. See **Table 5.3** and **Table 5.5** for statistical significances.

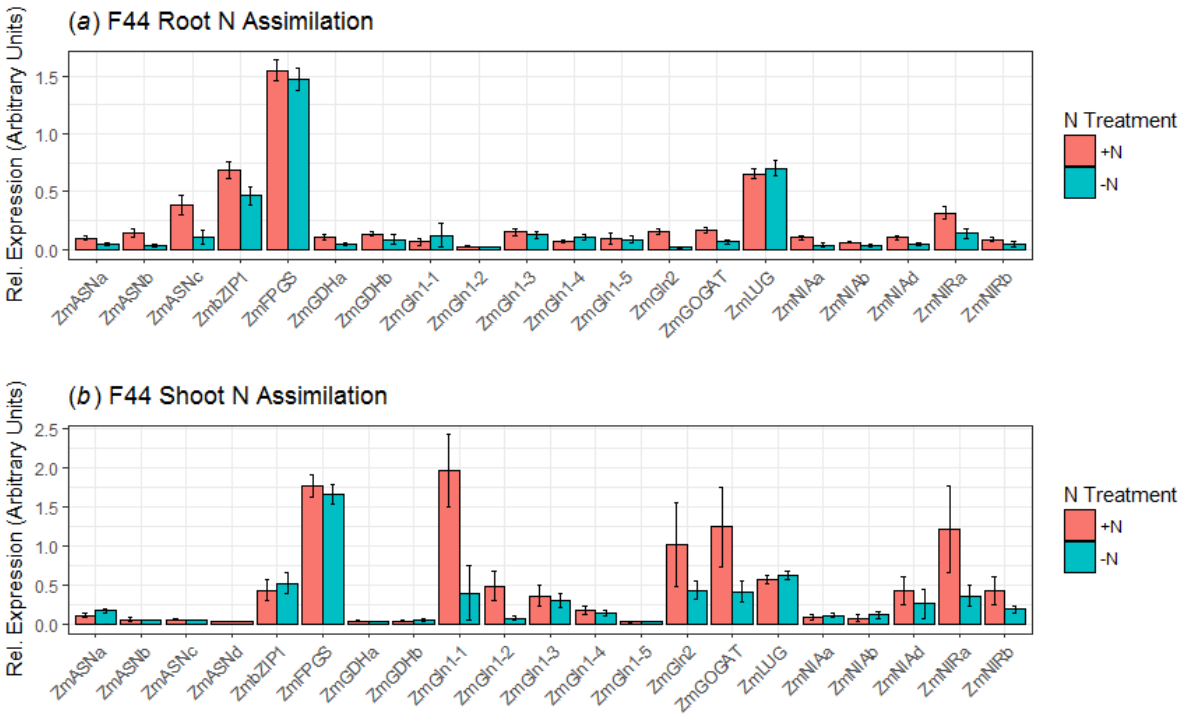


Figure 5.9 F44 root and shoot relative gene expression for N assimilation genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of genes involved in N assimilation in maize roots (a) or shoots (b) of inbred line F44. Data are plotted Mean + SEM. See **Table 5.4** and **Table 5.6** for statistical significances.



Figure 5.10 B73 relative root and shoot expression of N related regulatory genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of genes involved in N regulation in maize roots (a) or shoots (b) of inbred line B73. Data are plotted Mean + SEM. See **Table 5.3** and **Table 5.5** for statistical significances.

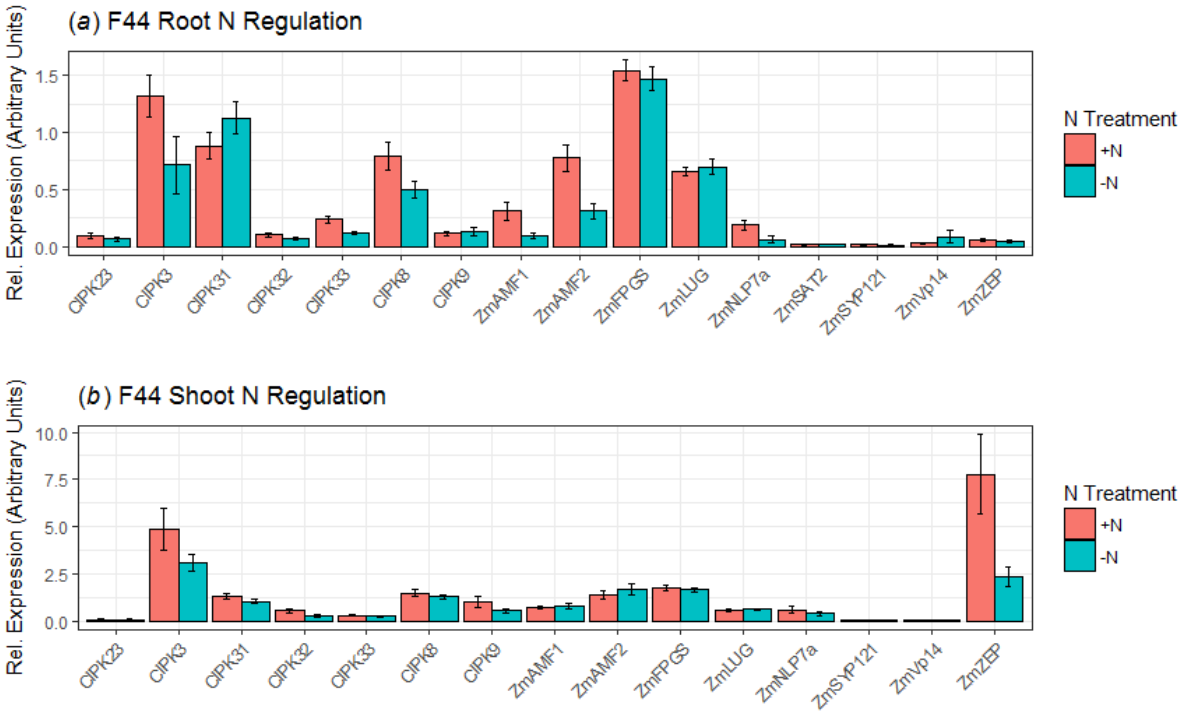


Figure 5.11 F44 relative expression of N related regulatory genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of genes involved in N regulation in maize roots (a) or shoots (b) of inbred line F44. Data are plotted Mean + SEM. See **Table 5.4** and **Table 5.6** for statistical significances.

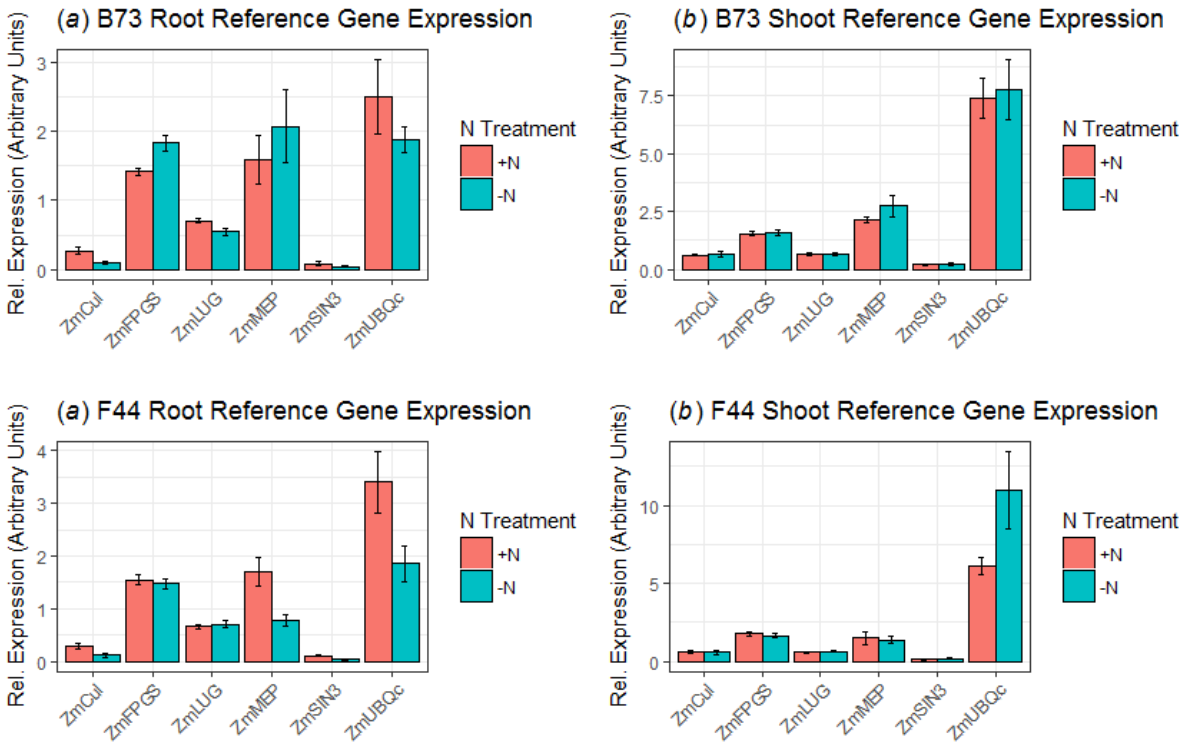


Figure 5.12 Relative expression of reference genes

Relative gene expression (normalised to the expression of the geometric mean of ZmFPGS and ZmLug) of reference genes in maize roots (a) or shoots (b) of inbred lines B73 or F44. Data are plotted Mean + SEM. See **Table 5.3**, **Table 5.4**, **Table 5.5** and **Table 5.6** for statistical significances.

5.3.4 Gene expression changes in response to nitrogen treatment

To monitor changes in gene expression, a ΔC_{RT} analysis was performed. Only genes with detected expression in both +N and –N conditions were analysed. In B73 roots, just six genes were significantly down regulated in response to nitrate starvation (none showed significant increases) (**Table 5.3**), and included a ~6 fold decrease in the expression of ZmTIP2;4. For F44 roots, 18 genes were significantly down regulated in response to nitrate starvation and one, ZmTIP4;2b (~3 fold increase), was upregulated (**Table 5.4**). The down regulated aquaporins in response to N starvation: ZmNIP1;1 (~4.5 fold

decrease), ZmNIP2;1 (~3 fold decrease), ZmPIP1;5 (~5 fold decrease), ZmPIP1;6b (~2.5 fold decrease), and ZmTIP2;4 (~3 fold decrease).

In shoot tissue, N starvation in B73 led to 10 genes with significant changes in expression. These included a three-fold increase in ZmPIP1;7, a 2.5 fold increase in ZmPIP2;2, a >5.5 fold increase in ZmTIP4;1b, and a 4 fold decrease in ZmPIP2;2 (**Table 5.5**). In F44, just six genes had significant changes in expression due to N starvation, and included a ~3 fold decrease in ZmTIP2;1 expression and ~4 fold decrease in ZmTIP4;1b expression (**Table 5.6**).

Table 5.3 B73 root gene expression changes in response to nitrate starvation

ΔC_{RT} values for genes of +N and –N. Green represents genes with expression significantly reduced due to nitrate starvation. Samples missing either +N or –N expression are listed at the end of the table. n=number of replicates.

B73 Root								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmASNa	3.374	5.145	1.771	0.2930	0.8214	8	4	0.0315
ZmGDHa	3.639	6.339	2.7	0.1539	0.8214	8	4	0.0011
ZmGln2	2.242	4.257	2.015	0.2474	0.8214	8	4	0.0144
ZmNIAd	3.24	8.24	5	0.0313	0.8942	9	3	<0.0001
ZmNRT3.1A	2.568	5.114	2.546	0.1712	0.8214	8	4	0.002
ZmTIP2;4	-2.047	0.6219	2.6689	0.1572	0.8061	9	4	0.001
CIPK23	3.872	4.445	0.573	0.6722	0.8214	8	4	0.4854
CIPK3	0.3463	0.2747	-0.0716	1.0509	0.8061	9	4	0.9292
CIPK31	-0.00270	0.5912	0.593896	0.6626	0.8061	9	4	0.4615
CIPK32	3.253	3.579	0.326	0.7977	0.8214	8	4	0.6917
CIPK33	1.333	1.004	-0.329	1.2561	0.8061	9	4	0.683
CIPK8	0.7843	0.8472	0.0629	0.9573	0.8061	9	4	0.9378
CIPK9	2.169	1.498	-0.671	1.5922	0.8061	9	4	0.4056
ZmAMF1	2.768	2.323	-0.445	1.3613	0.8061	9	4	0.5809
ZmAMF2	0.8287	0.5836	-0.2451	1.1852	0.8061	9	4	0.7612
ZmAMT_01	-0.5245	-0.2259	0.2986	0.8130	0.8061	9	4	0.7111
ZmAMT_03	3.712	3.227	-0.485	1.3996	0.8061	9	4	0.5474
ZmAMT_05	5.496	4.922	-0.574	1.4886	0.8998	5	4	0.5238
ZmAMT_07	1.519	1.282	-0.237	1.1785	0.8061	9	4	0.7695
ZmAMT_10	5.122	5.103	-0.019	1.0133	0.8658	6	4	0.983
ZmASNb	4.542	6.089	1.547	0.3422	0.9256	7	3	0.095
ZmASNc	3.898	5.209	1.311	0.4030	1.095	6	2	0.2319

B73 Root								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmbZIP1	0.9776	0.01615	-0.96145	1.9473	0.8061	9	4	0.2333
ZmCul	2.127	3.479	1.352	0.3917	0.8214	8	4	0.1002
ZmFPGS	-0.4964	-0.8629	-0.3665	1.2892	0.8061	9	4	0.6494
ZmGDHb	4.628	4.506	-0.122	1.0882	0.8658	6	4	0.8882
ZmGln1-1	-3.754	-3.878	-0.124	1.0898	0.8061	9	4	0.8769
ZmGln1-3	3.007	2.633	-0.374	1.2959	0.8214	8	4	0.6485
ZmGln1-4	3.522	3.554	0.032	0.9781	0.8061	9	4	0.9684
ZmGln1-5	0.07701	-0.1453	-0.22231	1.1666	0.8061	9	4	0.7828
ZmGOGAT	3.217	4.133	0.916	0.5300	0.9081	8	3	0.3135
ZmLUG	0.5075	0.8966	0.3891	0.7636	0.8061	9	4	0.6294
ZmMEP	-0.4713	-0.8354	-0.3641	1.2871	0.8061	9	4	0.6517
ZmNIAa	4.307	4.794	0.487	0.7135	1.095	6	2	0.6568
ZmNIAb	4.532	5.751	1.219	0.4296	1.095	6	2	0.2663
ZmNIAc	4.904	6.161	1.257	0.4184	1.549	1	3	0.4174
ZmNIP1;1	4.007	5.717	1.71	0.3057	1.434	7	1	0.2334
ZmNIP1;2	5.503	6.446	0.943	0.5202	1.122	5	2	0.4008
ZmNIP2;1	1.569	2.563	0.994	0.5021	0.8061	9	4	0.2179
ZmNIP2;4	2.636	1.707	-0.929	1.9040	0.8061	9	4	0.2495
ZmNIP3;1	6.082	6.748	0.666	0.6303	1.643	1	2	0.6856
ZmNIRa	2.294	2.752	0.458	0.7280	0.8061	9	4	0.5695
ZmNIRb	4.041	4.3	0.259	0.8357	1.122	5	2	0.8176
ZmNLP7a	3.038	4.605	1.567	0.3375	0.8214	8	4	0.0569
ZmNRT1.1A	4.871	5.496	0.625	0.6484	1.469	5	1	0.6708
ZmNRT1.5A	6.318	7.712	1.394	0.3805	1.643	2	1	0.3966
ZmNRT2.1	-2.554	-2.601	-0.047	1.0331	0.8061	9	4	0.9531
ZmNRT2.2	1.931	3.359	1.428	0.3716	0.8061	9	4	0.0769
ZmNRT2.3	6.898	7.536	0.638	0.6426	1.469	5	1	0.6641
ZmNRT2.5	5.024	4.271	-0.753	1.6853	1.224	2	3	0.5387
ZmPIP1;2b	-2.881	-3.835	-0.954	1.9372	0.8061	9	4	0.2372
ZmPIP1;4	-2.486	-3.353	-0.867	1.8239	0.8061	9	4	0.2826
ZmPIP1;5	-0.05845	-0.7089	-0.65045	1.5697	0.8061	9	4	0.42
ZmPIP1;6	-0.7233	-1.118	-0.3947	1.3147	0.8061	9	4	0.6247
ZmPIP1;7	-5.855	-5.459	0.396	0.7600	0.8061	9	4	0.6241
ZmPIP2;1	-1.815	-1.911	-0.096	1.0688	0.8061	9	4	0.9054
ZmPIP2;2	4.025	5.251	1.226	0.4275	1.049	9	2	0.2425
ZmPIP2;3	-2.128	-2.321	-0.193	1.1431	0.8061	9	4	0.8104
ZmPIP2;4	-4.423	-3.981	0.442	0.7361	0.8061	9	4	0.5837
ZmPIP2;5	-1.788	-1.841	-0.053	1.0374	0.8061	9	4	0.9472
ZmPIP2;6	-5.484	-4.951	0.533	0.6911	0.8061	9	4	0.5092
ZmPIP2;9	3.532	4.904	1.372	0.3864	0.8942	9	3	0.1253
ZmSAT1	5.225	4.426	-0.799	1.7399	1.024	4	3	0.4352
ZmSAT2	6.194	6.279	0.085	0.9428	1.122	5	2	0.9401
ZmSIN3	5.289	4.838	-0.451	1.3670	0.8061	9	4	0.5766
ZmSIP1;1	2.373	2.395	0.022	0.9849	0.8061	9	4	0.9785
ZmSIP1;2	6.233	6.977	0.744	0.5971	1.549	3	1	0.6312
ZmSIP2;1	4.173	5.111	0.938	0.5220	0.8407	7	4	0.265
ZmSYP121	8.788	8.849	0.061	0.9586	1.024	4	3	0.9529
ZmTIP1;1	-1.771	-2.473	-0.702	1.6268	0.8061	9	4	0.3839
ZmTIP1;2b	-2.166	-1.276	0.89	0.5396	0.8061	9	4	0.2697
ZmTIP2;1	-5.198	-4.814	0.384	0.7663	0.8061	9	4	0.6339
ZmTIP2;2	-5.522	-4.727	0.795	0.5763	0.8061	9	4	0.324
ZmTIP2;3	-6.595	-5.069	1.526	0.3472	0.8061	9	4	0.0588
ZmTIP3;1	5.537	5.928	0.391	0.7626	1.024	4	3	0.7034
ZmTIP4;1b	1.702	3.145	1.443	0.3678	0.8061	9	4	0.0739

B73 Root								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmTIP4;2b	1.262	2.252	0.99	0.5035	0.8407	7	4	0.2393
ZmUBQc	-1.04	-0.8967	0.1433	0.9054	0.8061	9	4	0.8587
ZmZEP	2.526	3.249	0.723	0.6058	0.8061	9	4	0.37
ZmNRT1.1B	3.875	n/a				8		
ZmGln1-2	5.034	n/a				5		
ZmAMT_02								
ZmAMT_08								
ZmAMT_09								
ZmASNd								
ZmNIP1;3								
ZmNIP2;2								
ZmNIP2;3								
ZmNIP4;1								
ZmNIP5;1								
ZmNIP5;2								
ZmNRT1.1C								
ZmNRT1.1D								
ZmNRT1.2								
ZmNRT1.3								
ZmNRT1.4A								
ZmNRT1.4B								
ZmNRT1.5B								
ZmNRT3.1B								
ZmPIP2;7								
ZmPIP2;8								
ZmPIP3;1								
ZmSIP1;3								
ZmSIP1;4								
ZmSIP1;5								
ZmTIP3;2b								
ZmTIP3;3								
ZmTIP3;4								
ZmTIP4;3b								
ZmTIP4;4b								
ZmTIP5;1b								
ZmVp14								

Table 5.4 F44 root gene expression changes in response to nitrate starvation

ΔC_{RT} values for genes of +N and –N. Green represents genes with expression significantly reduced due to nitrate starvation. Samples missing either +N or –N expression are listed at the end of the table and coloured grey.

F44 Root								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmAMF1	2.083	3.859	1.776	0.2920	0.6127	8	7	0.0038
ZmAMF2	0.5192	1.889	1.3698	0.3869	0.6127	8	7	0.0256
ZmASNa	3.207	4.868	1.661	0.3162	0.6587	6	7	0.0118
ZmASNb	3.357	5.112	1.755	0.2963	0.725	8	4	0.0157
ZmASNc	1.638	4.256	2.618	0.1629	0.6749	8	5	0.0001
ZmCul	1.967	3.437	1.47	0.3610	0.6127	8	7	0.0167
ZmGln2	3.014	6.137	3.123	0.1148	0.725	8	4	<0.0001
ZmGOGAT	2.701	4.232	1.531	0.3460	0.6394	8	6	0.0169
ZmNIAa	3.599	5.221	1.622	0.3249	0.725	8	4	0.0255
ZmNIP1;1	3.323	5.584	2.261	0.2086	0.936	8	2	0.0159
ZmNIP2;1	2.916	4.511	1.595	0.3310	0.6127	8	7	0.0094
ZmNIRa	1.883	3.248	1.365	0.3882	0.6127	8	7	0.0262
ZmNLP7a	2.722	4.8	2.078	0.2368	0.6394	8	6	0.0012
ZmNRT3.1A	2.761	4.72	1.959	0.2572	0.6749	8	5	0.0038
ZmPIP1;5	-0.784	1.525	2.3088	0.2018	0.6127	8	7	0.0002
ZmPIP1;6b	-0.789	0.6501	1.4393	0.3687	0.6127	8	7	0.0191
ZmSIN3	3.586	7.148	3.562	0.0847	0.6394	8	6	<0.0001
ZmTIP2;4	-3.200	-1.622	1.578	0.3349	0.6127	8	7	0.0102
ZmTIP4;2b	2.267	0.5831	-1.684	3.2130	0.6127	8	7	0.0061
CIPK23	3.643	4.217	0.574	0.6718	0.6394	8	6	0.3692
CIPK3	-0.297	0.8911	1.1884	0.4388	0.6127	8	7	0.0528
CIPK31	0.2687	-0.105	-0.373	1.2952	0.6127	8	7	0.5426
CIPK32	3.44	4.042	0.602	0.6588	0.6394	8	6	0.3468
CIPK33	2.206	3.196	0.99	0.5035	0.6127	8	7	0.1064
CIPK8	0.4268	1.172	0.7452	0.5966	0.6127	8	7	0.2242
CIPK9	3.336	3.375	0.039	0.9733	0.6328	7	7	0.9512
ZmAMT_01	0.9164	0.6615	-0.255	1.1933	0.6127	8	7	0.6775
ZmAMT_03	4.353	4.414	0.061	0.9586	0.5753	8	9	0.9152
ZmAMT_05	6.505	5.022	-1.483	2.7953	1.184	2	2	0.211
ZmAMT_07	2.421	2.23	-0.191	1.1416	0.6127	8	7	0.755
ZmAMT_10	5.718	5.057	-0.661	1.5812	0.6587	6	7	0.3158
ZmbZIP1	0.6029	1.304	0.7011	0.6151	0.6127	8	7	0.2529
ZmFPGS	-0.610	-0.531	0.0794	0.9465	0.6127	8	7	0.897
ZmGDHa	3.488	4.569	1.081	0.4727	0.6127	8	7	0.0782
ZmGDHb	3.015	3.865	0.85	0.5548	0.725	8	4	0.2414
ZmGln1-1	4.854	4.537	-0.317	1.2457	0.725	8	4	0.6614
ZmGln1-2	5.481	5.66	0.179	0.8833	0.9906	5	2	0.8568
ZmGln1-3	3.101	3.309	0.208	0.8657	0.6127	8	7	0.7348

F44 Root								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmGln1-4	4.063	3.524	-0.539	1.4530	0.6127	8	7	0.3793
ZmGln1-5	4.156	4.753	0.597	0.6611	0.6127	8	7	0.3303
ZmLUG	0.6277	0.5457	-0.082	1.0585	0.6127	8	7	0.8936
ZmMEP	-0.648	0.4775	1.1259	0.4582	0.6127	8	7	0.0665
ZmNIAb	4.039	5.079	1.04	0.4863	0.9493	7	2	0.2738
ZmNIAd	3.628	4.654	1.026	0.4911	0.936	8	2	0.2732
ZmNIP2;4	1.46	2.574	1.114	0.4620	0.6127	8	7	0.0692
ZmNIP3;1	6.198	6.87	0.672	0.6276	1.674	1	1	0.6882
ZmNIRb	3.902	4.985	1.083	0.4720	0.8015	8	3	0.1768
ZmNRT1.1A	4.807	3.446	-1.361	2.5686	0.7642	6	4	0.0753
ZmNRT1.1B	3.343	4.519	1.176	0.4426	0.817	7	3	0.1503
ZmNRT2.1	-3.108	-2.433	0.675	0.6263	0.6127	8	7	0.2706
ZmNRT2.2	1.14	2.027	0.887	0.5407	0.6127	8	7	0.1477
ZmNRT2.3	7.068	6.805	-0.263	1.2000	1.297	5	1	0.8393
ZmPIP1;2b	-2.205	-1.746	0.459	0.7275	0.6127	8	7	0.4539
ZmPIP1;4	-1.729	-1.518	0.211	0.8639	0.6127	8	7	0.7318
ZmPIP1;7	3.078	3.738	0.66	0.6329	0.6394	8	6	0.3023
ZmPIP2;1	-2.199	-1.837	0.362	0.7781	0.6127	8	7	0.5541
ZmPIP2;2	3.016	4.013	0.997	0.5010	1.297	5	1	0.4424
ZmPIP2;3	4.354	2.755	-1.599	0.3301	1.279	3	1	0.2115
ZmPIP2;4	1.346	2.041	0.695	0.6177	0.936	8	2	0.4581
ZmPIP2;5	-1.683	-0.635	1.0481	0.4836	0.6127	8	7	0.0877
ZmPIP2;6	-5.191	-4.186	1.005	0.4983	0.6127	8	7	0.1013
ZmPIP2;9	4.197	4.006	-0.191	1.1416	0.6749	8	5	0.7765
ZmSAT2	6.135	5.547	-0.588	1.5032	1.324	4	1	0.6568
ZmSIP1;1	2.167	3.056	0.889	0.5400	0.6127	8	7	0.1473
ZmSIP1;2	6.721	5.389	-1.332	2.5175	0.9667	3	3	0.1686
ZmSIP2;1	4.751	5.637	0.886	0.5411	0.9667	6	2	0.3599
ZmSYP121	6.177	6.407	0.23	0.8526	0.9493	7	2	0.8091
ZmTIP1;1	-1.943	-0.752	1.1912	0.4379	0.6127	8	7	0.0522
ZmTIP1;2b	1.033	2.224	1.191	0.4380	0.6127	8	7	0.0523
ZmTIP2;1	-4.144	-3.234	0.91	0.5322	0.6127	8	7	0.1378
ZmTIP2;2	-6.768	-6.508	0.26	0.8351	0.6127	8	7	0.6723
ZmTIP2;3	-7.014	-5.891	1.123	0.4591	0.6127	8	7	0.0672
ZmTIP3;1	5.833	4.777	-1.056	2.0792	0.7642	6	4	0.1677
ZmTIP4;1b	9.139	9.608	0.469	0.7225	0.6127	8	7	0.4446
ZmUBQc	-1.619	-0.771	0.8479	0.5556	0.6127	8	7	0.1666
ZmVP14	5.12	4.502	-0.618	1.5347	0.7942	5	4	0.4366
ZmZEP	4.243	4.6	0.357	0.7808	0.6749	8	5	0.5976
ZmNIP2;2	5.874	n/a				3		
ZmAMT_02								

F44 Root								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmAMT_08								
ZmAMT_09								
ZmNIAc								
ZmNIP1;2								
ZmNIP1;3								
ZmNIP2;3								
ZmNIP4;1								
ZmNIP5;1								
ZmNIP5;2								
ZmNRT1.1C								
ZmNRT1.1D								
ZmNRT1.2								
ZmNRT1.3								
ZmNRT1.4A								
ZmNRT1.4B								
ZmNRT1.5B								
ZmNRT2.5								
ZmPIP2;7								
ZmPIP2;8								
ZmPIP3;1								
ZmSIP1;3								
ZmSIP1;4								
ZmSIP1;5								
ZmTIP3;2b								
ZmTIP3;3								
ZmTIP3;4								
ZmTIP4;3b								
ZmTIP4;4b								
ZmTIP5;1b								

Table 5.5 B73 shoot gene expression changes in response to nitrate starvation

ΔC_{RT} values for genes of +N and –N. Green represents genes with expression significantly reduced due to nitrate starvation. Samples missing either +N or –N expression are listed at the end of the table and coloured grey.

B73 Shoot								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmAMT_03	4.448	7.078	2.63	0.16154	0.7432	7	4	0.0004
ZmAMT_10	5.333	2.559	-2.774	6.84002	0.6597	7	6	<0.0001
ZmGln1-2	2.485	4.313	1.828	0.28165	0.6597	7	6	0.0057
ZmGln2	0.8086	2.54	1.7314	0.30116	0.6597	7	6	0.0088
ZmNIRa	1.084	2.57	1.486	0.357	0.6597	7	6	0.0246
ZmNIRb	2.288	3.666	1.378	0.38475	0.6943	7	5	0.0476
ZmPIP1;7	-3.574	-5.161	-1.587	3.00424	0.6943	7	5	0.0225
ZmPIP2;2	4.389	6.328	1.939	0.2608	0.7954	5	4	0.015
ZmPIP2;4	1.637	0.2962	-1.3408	2.53292	0.6597	7	6	0.0425
ZmTIP4;1b	-2.357	-4.848	-2.491	5.62167	0.6597	7	6	0.0002
CIPK23	4.486	3.418	-1.068	2.09652	0.6597	7	6	0.1059
CIPK3	-1.935	-1.627	0.308	0.80776	0.6597	7	6	0.6402
CIPK31	0.000812	0.04831	0.0475	0.96761	0.6597	7	6	0.9426
CIPK32	0.9971	1.083	0.0859	0.9422	0.6597	7	6	0.8966
CIPK33	1.403	1.414	0.011	0.9924	0.6597	7	6	0.9864
CIPK8	-0.5497	-0.3332	0.2165	0.86065	0.6597	7	6	0.7428
CIPK9	-0.2729	-0.8817	-0.6088	1.52499	0.6597	7	6	0.3563
ZmAMF1	0.8206	0.426	-0.3946	1.31458	0.6597	7	6	0.5498
ZmAMF2	-0.3485	-0.8034	-0.4549	1.37069	0.6597	7	6	0.4907
ZmAMT_01	-0.05702	-0.0450	0.012	0.99172	0.6597	7	6	0.9855
ZmAMT_02	4.965	5.075	0.11	0.92659	0.718	5	6	0.8781
ZmAMT_05	4.874	5.639	0.765	0.58845	0.6597	7	6	0.2465
ZmAMT_07	4.381	3.172	-1.209	2.31177	0.6597	7	6	0.0672
ZmAMT_09	4.591	4.287	-0.304	1.23456	1.452	2	1	0.8346
ZmASNa	3.719	2.925	-0.794	1.73388	0.6597	7	6	0.229
ZmbZIP1	1.063	1.094	0.031	0.97874	0.6597	7	6	0.962
ZmCul	0.7479	0.7245	-0.0234	1.01635	0.6597	7	6	0.9717
ZmFPGS	-0.6083	-0.6109	-0.0026	1.0018	0.6597	7	6	0.9969
ZmGDHa	5.007	5.595	0.588	0.66526	0.6597	7	6	0.3727
ZmGDHb	5.112	4.27	-0.842	1.79253	0.6943	7	5	0.2254
ZmGln1-1	-0.8354	-0.377	0.4584	0.72779	0.718	5	6	0.5233
ZmGln1-3	2.216	2.759	0.543	0.68634	0.6597	7	6	0.4104
ZmGln1-4	2.906	2.11	-0.796	1.73628	0.6597	7	6	0.2279
ZmGln1-5	5.183	6.129	0.946	0.51907	0.6846	6	6	0.1671
ZmGOGAT	0.3283	1.532	1.2037	0.43416	0.6597	7	6	0.0684
ZmLUG	0.6251	0.6296	0.0045	0.99689	0.6597	7	6	0.9946
ZmMEP	-1.095	-1.337	-0.242	1.18263	0.6597	7	6	0.7137
ZmNIAa	4.095	4.589	0.494	0.71005	0.8659	3	5	0.5689

B73 Shoot								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmNIAb	4.506	4.741	0.235	0.84968	1.027	2	4	0.8191
ZmNIAd	1.558	1.858	0.3	0.81225	0.6597	7	6	0.6499
ZmNIP1;1	5.65	5.863	0.213	0.86274	0.9681	3	3	0.8259
ZmNIP1;2	6.446	5.846	-0.6	1.51572	0.7954	4	5	0.4507
ZmNIP2;1	1.329	1.205	-0.124	1.08975	0.6597	7	6	0.8499
ZmNIP2;2	3.26	2.434	-0.826	1.77276	0.6597	7	6	0.2109
ZmNIP2;4	4.265	4.88	0.615	0.65293	0.718	6	5	0.392
ZmNLP7a	0.537	1.084	0.547	0.68444	0.6597	7	6	0.4074
ZmNRT1.1A	5.677	5.135	-0.542	1.45599	0.718	5	6	0.4499
ZmNRT1.1B	4.216	4.625	0.409	0.75315	0.6597	7	6	0.535
ZmNRT1.2	4.856	4.915	0.059	0.95993	1.452	2	1	0.9678
ZmNRT1.3	6.017	6.062	0.045	0.96929	1.452	1	2	0.9753
ZmNRT1.5A	4.733	5.311	0.578	0.66989	1.027	4	2	0.5732
ZmNRT2.1	6.165	6.33	0.165	0.89193	1.452	2	1	0.9097
ZmNRT2.5	4.809	5.913	1.104	0.46522	1.082	3	2	0.3079
ZmNRT3.1A	4.331	5.703	1.372	0.38636	1.452	2	1	0.3452
ZmNRT3.1B	6.339	6.205	-0.134	1.09733	0.7499	5	5	0.8587
ZmPIP1;2b	-0.5474	-1.446	-0.8986	1.86426	0.6597	7	6	0.1735
ZmPIP1;4	-0.9113	-1.948	-1.0367	2.05153	0.6597	7	6	0.1163
ZmPIP1;5	3.112	3.239	0.127	0.91573	0.6597	7	6	0.847
ZmPIP1;6	2.103	2.169	0.066	0.95528	0.6597	7	6	0.9205
ZmPIP2;1	-0.7032	-1.404	-0.7008	1.62541	0.6597	7	6	0.2885
ZmPIP2;3	2.057	0.8751	-1.1819	2.26875	0.6846	6	6	0.0847
ZmPIP2;5	0.6094	0.4379	-0.1715	1.12623	0.6597	7	6	0.795
ZmPIP2;6	-1.411	-0.8657	0.5453	0.68525	0.6597	7	6	0.4086
ZmPIP2;9	4.278	4.375	0.097	0.93498	0.7654	6	4	0.8988
ZmPIP3;1	6.26	6.898	0.638	0.6426	1.082	2	3	0.5558
ZmSAT1	6.061	4.946	-1.115	2.16595	1.452	1	2	0.4428
ZmSIN3	2.312	2.821	0.509	0.70271	0.6597	7	6	0.4406
ZmSIP1;1	1.89	1.613	-0.277	1.21167	0.6597	7	6	0.6746
ZmSIP1;2	5.388	5.929	0.541	0.68729	0.9056	3	4	0.5503
ZmSIP2;1	2.915	2.771	-0.144	1.10496	0.6597	7	6	0.8275
ZmSYP121	7.077	5.687	-1.39	2.62079	0.718	5	6	0.0532
ZmTIP1;1	-0.4243	-1.339	-0.9147	1.88518	0.6597	7	6	0.1661
ZmTIP1;2b	4.189	4.17	-0.019	1.01326	0.6943	7	5	0.9783
ZmTIP2;1	-1.26	-2.231	-0.971	1.9602	0.6597	7	6	0.1417
ZmTIP2;2	4.831	4.457	-0.374	1.29594	0.8384	4	4	0.6551
ZmTIP2;3	3.96	4.46	0.5	0.70711	0.6943	7	5	0.4719
ZmTIP2;4	5.527	6.479	0.952	0.51692	1.369	3	1	0.4872
ZmTIP3;1	4.824	5.423	0.599	0.66021	1.452	1	2	0.6799
ZmTIP4;2b	-0.4545	-1.04	-0.5855	1.50056	0.6597	7	6	0.3753

B73 Shoot								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmUBQc	-2.827	-2.873	-0.046	1.0324	0.6597	7	6	0.9445
ZmVP14	5.778	5.673	-0.105	1.07549	0.8384	6	3	0.9001
ZmZEP	-3.166	-2.86	0.306	0.80888	0.6597	7	6	0.6437
ZmNRT1.4A	4.819	n/a				7		
ZmASNb	1.022	n/a				3		
ZmASNc	3.9826	n/a				3		
ZmAMT_08								
ZmASNd								
ZmNIAc								
ZmNIP1;3								
ZmNIP2;3								
ZmNIP3;1								
ZmNIP4;1								
ZmNIP5;1								
ZmNIP5;2								
ZmNRT1.1C								
ZmNRT1.1D								
ZmNRT1.4B								
ZmNRT1.5B								
ZmNRT2.2								
ZmNRT2.3								
ZmPIP2;7								
ZmPIP2;8								
ZmSAT2								
ZmSIP1;3								
ZmSIP1;4								
ZmSIP1;5								
ZmTIP3;2b								
ZmTIP3;3								
ZmTIP3;4								
ZmTIP4;3b								
ZmTIP4;4b								
ZmTIP5;1b								

Table 5.6 F44 shoot gene expression changes in response to nitrate starvation

ΔC_{RT} values for genes of +N and –N. Green represents genes with expression significantly reduced due to nitrate starvation. Samples missing either +N or –N expression are listed at the end of the table and coloured grey.

F44 Shoot								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmGln1-1	-0.927	3.069	3.996	0.063	1.273	2	3	0.0018
ZmGln1-2	0.747	3.941	3.194	0.109	1.065	3	4	0.0028
ZmNRT2.1	5.496	2.439	-3.057	8.322	1.166	2	5	0.009
ZmTIP2;1	-0.186	1.574	1.760	0.295	0.844	5	6	0.0375
ZmTIP4;1b	0.190	2.285	2.095	0.234	0.844	5	6	0.0134
ZmZEP	-2.761	-1.031	1.730	0.301	0.844	5	6	0.0409
CIPK23	4.193	3.740	-0.453	1.369	1.018	3	5	0.6561
CIPK3	-2.116	-1.505	0.611	0.655	0.844	5	6	0.4698
CIPK31	-0.327	-0.006	0.321	0.801	0.844	5	6	0.704
CIPK32	0.978	1.913	0.935	0.523	0.844	5	6	0.2685
CIPK33	1.868	2.016	0.148	0.903	0.844	5	6	0.8608
CIPK8	-0.533	-0.332	0.201	0.870	0.844	5	6	0.8122
CIPK9	0.138	1.062	0.924	0.527	0.844	5	6	0.2739
ZmAMF1	0.553	0.532	-0.021	1.015	0.844	5	6	0.98
ZmAMF2	-0.420	-0.619	-0.199	1.148	0.844	5	6	0.814
ZmAMT_01	-0.126	0.126	0.253	0.839	0.844	5	6	0.765
ZmAMT_02*	4.702	5.073	0.371	0.773	1.065	3	4	0.7274
ZmAMT_03	4.704	4.458	-0.246	1.186	1.166	5	2	0.8326
ZmAMT_05	5.507	5.341	-0.166	1.122	0.844	5	6	0.8436
ZmAMT_07	4.023	4.378	0.355	0.782	0.900	4	6	0.6934
ZmAMT_10	5.273	5.044	-0.229	1.172	1.018	3	5	0.8219
ZmASNa	3.662	2.699	-0.963	1.949	0.844	5	6	0.2544
ZmASNb	4.338	4.189	-0.149	1.109	1.707	2	1	0.9304
ZmASNc	4.365	4.567	0.202	0.869	1.610	3	1	0.9002
ZmbZIP1	1.540	1.432	-0.108	1.078	0.844	5	6	0.8986
ZmCul	0.809	1.056	0.247	0.842	0.844	5	6	0.7697
ZmFPGS	-0.806	-0.706	0.101	0.933	0.844	5	6	0.9052
ZmGDHa	4.527	5.056	0.529	0.693	0.900	4	6	0.5566
ZmGDHb	4.470	4.550	0.080	0.946	0.935	4	5	0.9324
ZmGln1-3	1.969	2.127	0.158	0.896	0.882	5	5	0.8585
ZmGln1-4	2.356	3.035	0.679	0.625	0.900	4	6	0.4506
ZmGln1-5	5.229	5.438	0.209	0.865	1.707	2	1	0.9025
ZmGln2	0.834	1.617	0.783	0.581	0.844	5	6	0.3537
ZmGOGAT	0.502	1.790	1.288	0.410	0.844	5	6	0.1276
ZmLUG	0.835	0.729	-0.106	1.076	0.844	5	6	0.8998
ZmMEP	-0.279	-0.306	-0.027	1.019	0.844	5	6	0.9744
ZmNIAa	3.780	3.364	-0.416	1.334	0.986	3	6	0.6728
ZmNIAb	4.064	3.344	-0.720	1.647	1.207	2	4	0.551
ZmNIAd	2.132	3.185	1.053	0.482	0.844	5	6	0.2126
ZmNIP1;1	3.272	5.312	2.040	0.243	1.707	1	2	0.2327
ZmNIP1;2	5.678	6.113	0.435	0.740	1.558	4	1	0.7804
ZmNIP2;1	2.104	3.175	1.071	0.476	0.844	5	6	0.205
ZmNIP2;2	3.716	3.790	0.074	0.950	0.900	4	6	0.9344
ZmNIP2;4	4.802	4.745	-0.057	1.040	0.935	4	5	0.9513
ZmNIRa	0.630	1.945	1.315	0.402	0.844	5	6	0.1199

F44 Shoot								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmNIRb	2.014	2.632	0.618	0.652	0.882	5	5	0.4836
ZmNLP7a	1.098	1.589	0.491	0.712	0.844	5	6	0.5609
ZmNRT1.1A	4.686	6.002	1.316	0.402	1.707	2	1	0.4412
ZmNRT1.1B	4.228	3.736	-0.492	1.406	1.065	4	3	0.6442
ZmNRT1.5B	5.478	5.448	-0.030	1.021	1.394	2	2	0.983
ZmNRT3.1B	6.017	5.895	-0.122	1.088	1.065	3	4	0.909
ZmPIP1;2b	-0.226	-0.709	-0.483	1.398	0.844	5	6	0.5672
ZmPIP1;4	-0.230	-0.334	-0.104	1.075	0.844	5	6	0.9022
ZmPIP1;5	3.400	3.372	-0.028	1.020	0.844	5	6	0.9739
ZmPIP1;6	2.961	3.116	0.155	0.898	0.844	5	6	0.8545
ZmPIP1;7	0.423	2.255	1.832	0.281	0.986	4	4	0.0636
ZmPIP2;1	0.674	-0.321	-0.994	1.992	0.844	5	6	0.2393
ZmPIP2;2	3.676	5.083	1.407	0.377	1.273	3	2	0.2694
ZmPIP2;3	4.059	3.430	-0.629	1.546	1.273	3	2	0.6218
ZmPIP2;4	3.221	2.935	-0.286	1.219	1.138	3	3	0.8017
ZmPIP2;5	2.312	2.370	0.058	0.961	1.018	3	5	0.9549
ZmPIP2;6	-0.660	0.142	0.802	0.573	0.844	5	6	0.3423
ZmPIP2;9	2.718	4.269	1.551	0.341	1.065	4	3	0.1458
ZmPIP3;1	5.645	4.554	-1.091	2.130	1.166	2	5	0.3498
ZmSIN3	3.738	3.097	-0.641	1.559	0.844	5	6	0.448
ZmSIP1;1	1.406	1.968	0.562	0.677	0.844	5	6	0.5061
ZmSIP1;2	2.835	5.078	2.243	0.211	1.610	1	3	0.1641
ZmSIP2;1	3.263	2.412	-0.851	1.804	0.882	5	5	0.335
ZmSYP121	6.084	5.572	-0.512	1.426	0.986	4	4	0.6032
ZmTIP1;1	-0.056	-0.372	-0.317	1.245	0.844	5	6	0.7079
ZmTIP1;2b	4.725	2.657	-2.068	4.193	1.527	5	1	0.1763
ZmTIP2;2	3.712	3.639	-0.073	1.052	0.882	5	5	0.9339
ZmTIP2;3	4.239	3.619	-0.620	1.537	0.935	4	5	0.5072
ZmTIP2;4	5.380	5.077	-0.303	1.234	1.018	3	5	0.7655
ZmTIP4;2b	0.297	-0.649	-0.946	1.926	0.844	5	6	0.2632
ZmUBQc	-2.581	-3.269	-0.688	1.611	0.844	5	6	0.415
ZmVP14	5.671	4.855	-0.816	1.761	0.900	4	6	0.3648
ZmNRT1.4A	4.82					2		
ZmAMT_08								
ZmAMT_09								
ZmASNd								
ZmNIAc								
ZmNIP1;3								
ZmNIP2;3								
ZmNIP3;1								
ZmNIP4;1								
ZmNIP5;1								
ZmNIP5;2								
ZmNRT1.1C								
ZmNRT1.1D								
ZmNRT1.2								
ZmNRT1.3								
ZmNRT1.4B								
ZmNRT1.5A								

F44 Shoot								
Gene	**ΔC_{RT} (+N)**	**ΔC_{RT} (-N)**	**$\Delta\Delta C_{RT}$**	**fold change ($2^{-\Delta\Delta Crt}$)**	**SE of diff.**	**n_{+N}**	**n_{-N}**	**p val.**
ZmNRT2.2								
ZmNRT2.3								
ZmNRT2.5								
ZmNRT3.1A								
ZmPIP2;7								
ZmPIP2;8								
ZmSAT1								
ZmSAT2								
ZmSIP1;3								
ZmSIP1;4								
ZmSIP1;5								
ZmTIP3;1								
ZmTIP3;2b								
ZmTIP3;3								
ZmTIP3;4								
ZmTIP4;3b								
ZmTIP4;4b								
ZmTIP5;1b								

5.3.5 Validity of TaqMan probes

A serial dilution of pooled cDNA from B73+N roots and shoots was performed and run on an OpenArray chip. A regression was performed and the slope obtained from the regression of C_{RT} value against the log([cDNA]). Unfortunately, it appeared that there

was a PCR inhibitor present, as PCR primer efficiencies were calculated to be over 100% (**Table 5.7**). Despite the OpenArray system running for 40 cycles, very few C_{RT} values were above 30. This resulted in very few data points for the end of the serial dilutions, and meant that initial C_{RT} value had to be below ~27 for amplification to be observed in two dilution points. The calibration of the pipettes used was examined. The desired dilution factor was 0.100, but the actual dilution factor was 0.1071. **Table 5.7** accounts for the actual dilution factor.

Table 5.7 TaqMan probe quasi-validation

TaqMan probes efficiency determined on OpenArray with serial dilution of pooled cDNA from root and shoot of B73+N tissues. Regression points are the number of dilutions that where amplification was observed. Slope is the slope of the linear regression. Efficiency is calculated $E = \left(10^{\left(-1/slope\right)} - 1\right) \cdot 100\%$. r^2 is the regression coefficient and p value the significance from zero slope.

#	Assay	Gene	Regression Points	Slope	Efficiency	r^2	p value
1	Zm04068994_g1	CIPK23	3	-1.89	237.81%	0.899	1.40E-02
2	Zm04019411_g1	CIPK3	3	-3.33	99.65%	0.964	1.61E-08
3	Zm04065752_g1	CIPK31	3	-3.48	93.84%	0.963	5.11E-07
4	AIS08E8	CIPK32	2	-2.79	127.98%	0.952	3.47E-05
5	Zm04062942_g1	CIPK33	2	-3.26	102.73%	0.958	2.31E-05
6	AIT96LG	CIPK8	3	-2.67	136.75%	0.971	2.95E-08
7	AIRR980	CIPK9	3	-3.05	112.60%	0.922	1.07E-05
8	Zm04051459_g1	ZmAMF1	3	-2.91	120.87%	0.897	3.04E-06
9	Zm04051460_g1	ZmAMF2	3	-3.40	96.97%	0.995	2.09E-10
10	Zm04035819_s1	ZmAMT_01	3	-2.98	116.83%	0.974	2.98E-09
11	Zm04061849_s1	ZmAMT_03	2	-2.71	134.22%	0.804	1.54E-02
12	Zm04079618_s1	ZmAMT_07	3	-1.06	780.19%	0.324	1.10E-01
13	Zm04052438_g1	ZmASNa	2	-3.84	82.17%	0.980	1.21E-03
14	Zm04074254_gH	ZmASNc	2	-1.77	265.99%	0.627	6.04E-02
15	Zm04059958_s1	ZmbZIP1	3	-2.80	127.91%	0.846	4.41E-04
16	AIQJB2S	ZmCul	3	-3.05	112.54%	0.953	1.36E-06
17	AIAA1IR	ZmFPGS	3	-2.54	147.43%	0.971	3.02E-08
18	Zm03999812_m1	ZmGDHa	2	-3.94	79.45%	0.920	6.36E-04
19	Zm04076215_s1	ZmGln1-1	4	-2.92	119.88%	0.985	2.91E-12
20	Zm04059228_g1	ZmGln1-2	2	-2.09	200.38%	0.764	1.00E-02
21	AIVI4RO	ZmGln1-3	3	-2.87	123.26%	0.924	9.27E-06
22	AIWR2XW	ZmGln1-4	2	-3.75	84.70%	0.870	2.18E-03
23	Zm04078790_s1	ZmGln1-5	3	-3.80	83.30%	0.955	5.78E-06
24	Zm04060104_g1	ZmGln2	3	-2.59	143.56%	0.933	1.36E-06
25	Zm04060019_g1	ZmGOGAT	3	-2.69	135.36%	0.904	2.44E-05
26	Zm04073882_g1	ZmLUG	3	-2.58	144.52%	0.888	1.42E-04
27	Zm04002140_g1	ZmMEP	3	-3.46	94.50%	0.983	3.13E-09
28	AI1RVMS	ZmNIAd	2	-4.21	72.82%	0.978	3.42E-06
29	Zm04060081_g1	ZmNIP1;1	2	-1.72	280.76%	0.792	1.10E-01
30	Zm04078818_sH	ZmNIP2;1	4	-1.43	398.83%	0.817	3.33E-04
31	Zm04077200_g1	ZmNIP2;4	2	-2.85	124.29%	0.585	7.65E-02
32	AI20TS0	ZmNIRa	2	-3.27	102.03%	0.897	3.58E-04

#	Assay	Gene	Regression Points	Slope	Efficiency	r^2	p value
33	AI39RY8	ZmNIRb	2	-2.87	123.12%	0.924	2.20E-03
34	AI6ROBO	ZmNLP7a	3	-1.44	396.49%	0.709	4.40E-03
35	Zm04004739_g1	ZmNRT1.1A	2	-2.19	185.79%	0.891	1.59E-02
36	Zm04052214_s1	ZmNRT2.1	4	-3.37	97.87%	0.947	1.15E-09
37	AI20TSZ	ZmNRT2.2	3	-2.10	199.40%	0.836	1.47E-03
38	*Zm04060753_g1*	*ZmNRT3.1A*	*3*	*-0.65*	*3345.26%*	*0.234*	*2.71E-01*
39	AII1OP9	ZmPIP1;2b	3	-3.33	99.52%	0.978	9.04E-09
40	AIKAMWH	ZmPIP1;4	3	-3.43	95.80%	0.980	4.35E-08
41	AI89KN3	ZmPIP1;5	4	-3.23	104.00%	0.948	1.98E-06
42	AILJK2P	ZmPIP1;6b	4	-2.91	120.60%	0.924	4.51E-08
43	AIMSI8X	ZmPIP1;7	4	-3.71	85.93%	0.981	2.04E-13
44	AIPAFLD	ZmPIP2;1	3	-4.15	74.19%	0.989	4.61E-11
45	Zm04075546_s1	ZmPIP2;3	4	-3.06	112.33%	0.961	1.22E-07
46	Zm04075896_s1	ZmPIP2;4	5	-2.52	149.25%	0.947	1.10E-09
47	Zm04059736_gH	ZmPIP2;5	3	-3.23	103.99%	0.938	4.07E-06
48	AIQJDRL	ZmPIP2;6	4	-3.44	95.31%	0.987	1.49E-13
49	AIRSBXT	ZmPIP2;9	2	-1.68	294.20%	0.785	4.52E-02
50	AIN1FQC	ZmSIN3	2	-3.11	109.50%	0.968	6.07E-05
51	Zm04059399_m1	ZmSIP1;1	3	-2.49	151.76%	0.918	1.76E-04
52	Zm04052636_m1	ZmSIP2;1	2	-3.36	98.24%	0.887	4.94E-03
53	AIX02SX	ZmTIP1;1	3	-2.76	130.15%	0.990	2.88E-08
54	AIY90Y5	ZmTIP1;2b	3	-2.51	150.69%	0.922	1.05E-05
55	AI0IY5D	ZmTIP2;1	4	-3.94	79.28%	0.984	5.95E-14
56	Zm04075902_s1	ZmTIP2;2	5	-3.23	103.92%	0.969	6.34E-12
57	AI1RXBL	ZmTIP2;3	4	-3.52	92.44%	0.990	1.95E-15
58	AI20VHT	ZmTIP2;4	3	-3.51	92.71%	0.961	2.38E-08
59	Zm04076072_s1	ZmTIP4;1b	4	-3.03	113.79%	0.979	2.05E-11
60	AI39TN1	ZmTIP4;2b	3	-2.62	140.86%	0.949	8.84E-06
61	AIMSHJ4	ZmUBQc	4	-3.09	110.45%	0.986	1.40E-12
62	Zm04015536_g1	ZmZEP	4	-3.11	109.83%	0.979	1.53E-10

5.4 Discussion

5.4.1 C_{RT} and the validity of the results

The OpenArray results were less than ideal due to problems with efficiency, but some conclusions from the results are still possible. The assay efficiencies appeared to be above 100%. This could arise for several reasons. A technical source of error could be the dilution factor, changing the distance between points would change the slope of the regression. This was checked, and likely had some variation (was 0.1071 instead of

0.1000), but that did not changed the regression efficiencies significantly. A second possibility could be the presence of PCR inhibitors in the reactions. This would proportionally increase C_{RT} values for the first few dilutions, and reduce the slope. This could result in perceived increased efficiencies. Another issue seems to be the sensitivity of the OpenArray system. Initial C_{RT} values were near 18 for highly expressed genes, with maximum C_{RT} valses around 30. This greatly limited the number of points, with regressions for some made on just two dilution points. For some genes, the initial C_{RT} value was near 30, so no regression could be made. The difficulty in amplifying actin in root cDNA, especially of –N plants, but also some +N plants, can also be explained by a PCR inhibitor. Performing a phenol:chloroform extraction after the DNase step reduced the effect on some samples.

5.4.2 Maize aquaporin gene expression

The expression profiles of the maize aquaporins in roots and shoots are presented in **Figure 5.2** and **Figure 5.3**. For the ZmPIPs, expression profiles along root lengths and leaves were determined by Hachez et al. (2006) and Hachez et al. (2008). In the roots, the authors determined expression along the root at specific distances, the largest at 100-110 mm from the root tip. Here, the first 100mm was removed before RNA extraction (as xylem did not open this close to the tip). At distances from the tip between 100-100mm, the authors showed that the most expressed aquaporins were ZmPIP1;1 (13% of PIP aquaporin expression), ZmPIP1;5 (23%), ZmPIP2;1 (14.1%) and ZmPIP2;5 (27.2%). All others except ZmPIP2;7 were detected, but each only had a contribution of <=5.1%. The expression here was not quantified absolutely, so making direct comparisons is difficult. High expression was seen similarly for ZmPIP1;7 (highly similar to ZmPIP1;1). However, ZmPIP2;4 and ZmPIP2;6 had greater expression than ZmPIP2;5 (**Figure**

5.2(a)), and ZmPIP1;5 had low relative expression compared to other ZmPIP1s. The authors did not find expression for ZmPIP2;7, and did not screen for ZmPIP2;8, ZmPIP2;9 nor ZmPIP3;1. Here, ZmPIP2;7, ZmPIP2;8 and ZmPIP3;1 were not detected in maize roots. ZmPIP2;9 was expressed, though did not contribute greatly to the total amount of aquaporins expressed. Surprisingly, ZmPIP2;8 was not detected. ZmPIP2;8 was cloned from B73 roots (Lawrence et al. 2013) and was shown to have measureable expression. The assay used on this OpenArray was the same assay used by Lawrence et al. (2013), so some expression was expected from root samples.

This chapter also suggests that some ZmTIPs, especially the ZmTIP2 subfamily, are highly expressed in roots. These may have a significant role in root water transport, and may be involved in hydraulic changes to nitrogen status. In both B73 and F44, ZmTIP2;4 was down-regulated with N starvation.

Between F44 and B73, the relative root expression profiles of aquaporins were quite different (**Figure 5.2**-A, **Figure 5.3**-B). In general, F44 had greater ZmTIP2 expression, while B73 had greater PIP expression. In B73, ZmPIP1;7 was highly expressed, with large expressions also seen in ZmPIP2;6, ZmPIP2;4 and ZmPIP2;5. In F44, ZmPIP2;6 had similar relative expression, but the expression of ZmPIP1;7 was greatly reduced. F44 had larger expression of ZmTIP2;2 and ZmTIP2;3. This could indicate different preferred uptake methods for water in the roots, but could also be indicative of different growth, structural or developmental stages between lines.

Within 4hr of N resupply Górska et al. (2008) observed no changes in the gene expression of ZmPIPs, suggesting that increases in hydraulic conductivity were instead due to post-secondary modifications to aquaporin proteins. Here, gene expression was monitored going to starvation, and over a longer period: 3 days. A three days, hydraulic data

(Chapter 4) was variable and inconsistent, however gene expression was reduced for several aquaporins. There were no increases in aquaporin gene expression due to nitrate starvation.

5.4.3 Further experiments

There are several possible explanations as to why only some of the TaqMan probes were quasi-validated. First, it could be that the probes designed by Thermo Fisher Scientific were not very good. In this case, further experimentation would not solve these sample expression issues. Alternatively, there could be a PCR inhibitor within the sample. This is possible, and consistent with the difficulty seen in amplifying actin from cDNAs, especially in root samples. Additionally, using pooled cDNA may not have been the best way to build standard curves. Probes should be validated with known concentrations of the target. If these known references did not amplify efficiently, it could be concluded the probe was inadequate.

In theory, the OpenArray system is a good option to monitor gene expression for many genes at once, but practical limitations reduce its cost-efficiency. Here a PCR inhibitor may have masked some samples, especially in roots. And taking concrete conclusions is difficult with only some probes giving acceptable efficiencies. Further steps should be followed if used for future experiments, including better validation of the TaqMan probes, and better testing of cDNA quality before loading.

5.4.4 Conclusion

The custom designed OpenArray plate monitored expression of genes in plants grown in 15 mM nitrate and after 3 days N starvation. The +N plants revealed a baseline aquaporin profile that showed high relative expression for ZmPIP1s, ZmPIP2s, and ZmTIP2s. In

addition, the aquaporin transcript expression profiles differened between the two lines, with B73 having relative root expression composed of ZmPIP1, ZmPIP2 and ZmTIPs and F44 having mostly just root ZmTIP expression. N starvation led to differential gene expression in both roots and shoots of B73 and F44. These responses included the down regulation of aquaporins.

5.5 Appendix

5.5.1 Custom TaqMan probes with sequences available

Table 5.8 TaqMan primer and probe sequences available

Sequences used for TaqMan primers and probes and their transcript target. Orientation is 5'→3'.

Assay ID	Gene Name	AGPv3/AGPv4 target	Forward Primer Seq.	Reverse Primer Seq.	Reporter 1 Sequence
AI0IXGJ	ZmNRT1.5A	GRMZM2G044851	ACACGCAAGGGTTCAGTTTCTT	CACCGAGCACAGCTTCCA	ACTGACTCCAAGCTCG
AI0IXGK	ZmNIAc	GRMZM2G076723	TGTTTTCATCTGTTCCTGGTGGATT	GCCAAACAGCGAAACAGTTGT	TCGAGCGCCATCGTC
AI0IY5D	ZmTIP2;1	Zm00001d051362_T001.1	GTCGCGGACCAGGACTAC	CGACGACGGTTTTGAATCCATTC	ATGGAGTGGTGAATAAA
AI1RVMR	ZmNRT1.5B	GRMZM2G061303	GCAGGAGAGGACCCTAAGGT	GAGCCCGTCGCTGTGAA	CATCCGCAAGATCC
AI1RVMS	ZmNIAd	GRMZM2G568636	GTGCCGTCCGCACAAG	GCGTCGGGTGCTCGAA	CACCAGCCCGATCTC
AI1RXBL	ZmTIP2;3	Zm00001d026177_T001.1	CGTCGTCGTCGTGTCATCT	GGAAATGCAAACAGGACAAAACATG	ACAGCACGAAGCGCC
AI20TS0	ZmNIRa	GRMZM2G079381	TGCCGAGGAAATGGAACGT	GATGTGCGGGTGCTCGTA	TCGGCTCGCATGACCT
AI20TSZ	ZmNRT2.2	GRMZM2G010251	CCTCCTCTACGGCTACTGCAT	GGTCGAAGCGGTCGTACAT	CACCACCGACAACGTC
AI20VHT	ZmTIP2;4	Zm00001d002738_T001.1	TCACCATCGTCGTCATGTCATC	ACCAGAAGGGAAATGCAAATAAGATCA	CCTTGTGCTTGTGAAGTT
AI39RY7	ZmNRT2.3	GRMZM2G163866	GCAGCCTCCGGAAGCA	GTCCCTGCGGCAGCA	ATCGCCGCCGTCACC
AI39RY8	ZmNIRb	GRMZM2G102959	GCCGGCCGTCAAGGA	GATGAACCCGCCCACCA	TTCGGCTTCAACCTTC
AI39TN1	ZmTIP4;2b	Zm00001d010410_T001.1	GCTGCTCAATGGAGACATCTGA	CATCATGCTTGTAGGTAACGAACTATTTT	CCGTGCTCACTGCCCTG
AI5IP5F	ZmNRT2.5	GRMZM2G455124	CGGCAACTACCGCAAGCT	GCCGAAGCTGTCCTTGTG	CAGGTCCGGCGACATG
AI5IP5G	ZmASNb	GRMZM2G053669	CGGCTACCTCTACTTCCACTTC	GTGCAGGGCCTTCACCTT	CCCTGTGGAACTCCTC
AI5IRT9	ZmTIP3;4	Zm00001d002952_T001.1	CGCGTCGTTGCTGCTAAC	GGACGAGCCCGAAGCC	TCCGGCGGCACGCG
AI6ROBN	ZmAMT_02	GRMZM2G043193	CCTCACCGCCGCTTACT	CAGCACCAGCAGTATGTTGTTG	CCGACGCCGACCTTG
AI6ROBO	ZmNLP7a	GRMZM2G375675	GGTGTCTTCGTAGCCTAAAGCT	GGATTCTTCATTCTCAAGTTTTAGCACATT	ACCCCACTCAAATCAT
AI6RP0H	ZmTIP5;1b	Zm00001d026178_T001.1	TGCCTCTCCCTCAACCTCTTATC	CGCCACCGCGATCCT	TCGGCACCTCCTCGCC
AI70MHV	ZmSAT1	GRMZM2G019806	GGCCGACGACGACGAG	GCCTCGGTGGCAACGA	TCCTGCGACGAGAACT
AI89KN3	ZmPIP1;5	Zm00001d051872_T001.1	CAACCCCGGGTACACCAA	GCGGAGAAGACGGTGTAGAC	TCGTCGGCACCTTCG
AIAA1IR	ZmFPGS	GRMZM2G393334_T01	GGCTCCCTGCATCTCGTT	ACCATAGATCATAGTCCAGTTCCAGTT	TAATCAAGAAGTGATACGCCGCTC
AIBJXZ4	ZmTIP4;3b	Zm00001d039713_T001.1	CAGCCTGCTCTTCGTCATCT	GCCCGGGAGCAGCTT	ACGCCACCATCCTGG
AICSV6D	ZmAMT_08	GRMZM2G473697	GGGTTGGACGGGCTTCAA	GCACAGGTGCGTGTTCAC	TACGCCGCCAACATC
AID1UCL	ZmAMT_09	GRMZM2G335218	GGCCCAGGCTCACCAA	GGCGCCGGTGAGCAT	TTCCCGCCAAACAAC
AID1V1D	ZmNIP1;3	Zm00001d046303_T001.1	CTTCTACCTCATGTTCGTCGTCAT	CGCGTTGAGCATGATGGTT	CTGCCCGACCGCTCG
AIFASIT	ZmAMT_10	GRMZM2G338809	GCCTCGACGTCGTCTTCTT	CAGACGAGTCCGGTGATCATG	ACGGAGGGCTTCTTG
AIFAT7L	ZmNIP2;3	Zm00001d044826_T001.1	CGAGTTCGACACCGTCTGA	TCCCACCTGAGATCTTGAACAGA	CACAGTAGTACTCGATCTCT
AIGJSDT	ZmNIP5;1	Zm00001d049578_T001.1	GCGCTTGCACTCACCAA	GTGAAGACCAGCAGGAACGT	CCCCAGGAGCTCTGC
AIHSQJ1	ZmNIP5;2	Zm00001d024634_T001.1	GTTCGTGCTCATGTTCGTCATC	GGCCACCAGCTCTTTGCT	TCGGCAACCTTGGAAAA
AII1OP9	ZmPIP1;2b	Zm00001d017526_T001.1	CGCTGCCATCATCTACAACAG	GCCGACCCAGAAGATCCA	ACGCCTGGAACGACCAT
AIKAMWH	ZmPIP1;4	Zm00001d051403_T001.1	CCATCATCTACAACCGGGATCAT	GCGGCGCCGATGAAG	ATCCAATGGTCGCTCCACG
AILJK2P	ZmPIP1;6b	GRMZM2G136032_T01*	ACGGCCACTGGATCTTCTG	GGACGACCTGGTGGTACAC	CCGCTCCGGCGAACG
AIMSHJ4	ZmUBQc	GRMZM2G027378	GCTCGAGCGTGGAGAGAA	CATCATCCATTCCATAGCTCACTGT	CCATCACCAATGCCC
AIMSI8X	ZmPIP1;7	Zm00001d002690_T001.1	GGTCTTAAAGGAGCCGATGCT	ACAGCCACGAATCCATCCTTTC	AAGACCGGCAGCATCT

(*Continued*)

Assay ID	Gene Name	AGPv3/AGPv4 target	Forward Primer Seq.	Reverse Primer Seq.	Reporter 1 Sequence
AIN1FQC	ZmSIN3	GRMZM2G334457	AGAAGAGGGTGAATTATCGCCAAAC	GAACCTTCCTTTCCTTTAGATGTTCCAT	CCTCATCAAAATCTCC
AIN1HE5	ZmPIP2;7	AF326496	GGAACGCTAGGGACTCTCATG	GAAGACGGCAAACCCAATGG	AAGTGGTGCTAACACAGGAA
AIPAFLD	ZmPIP2;1	Zm00001d019563_T006.1	CAATGGCGGGCGACCTA	ACCCAAGTCGATCGATGAATGATG	TCCTCGCTCACGGCCC
AIQJB2S	ZmCul	GRMZM2G166694	GCAGTTCGAATGCTGAGTTGTTG	CTTTTCACTGCAGCCTTTCTTCA	TTCTGCGACAATATTC
AIQJDRL	ZmPIP2;6	Zm00001d019565_T001.1	GGGTACGGAGCAACGTCTAG	AAAGCTACTGCTGCTGTGGAT	TCACCTCGCCTTTGCC
AIRR980	CIPK9	GRMZM2G055575	GATGATGCCAATCTGATGAGACTGT	AGAAAACCATGGTGGGCAAGA	CTGCCATGCAACTTTT
AIRR9R3	ZmPIP2;8	Zm00001d014285_T001.1	CGCGCCGCGCTCTA	CGGCGCCGCACATG	CACTGCGCGACCACG
AIRSBXT	ZmPIP2;9	Zm00001d005410_T001.1	CCCTGCCGGAGTTTAAGTTGAA	CCTGACTGAAAGCTACTAATACTGCAT	CCGGTGCTCACCCTTTC
AIS08E7	ZmNRT1.1B	GRMZM2G161459	GGCGGCGAGTCGTCA	TCCGTCCGCGTGCA	CAGCGCCCACTTGCT
AIS08E8	CIPK32	GRMZM2G051103	GATGATGCCAATCTGATGAGACTGT	AGAAAACCATGGTGGGCAAGA	CTGCCAAGCATATTTT
AIS0931	ZmPIP3;1	Zm00001d022608_T001.1	GCGCTGCCGTCCTCTA	CCAACCCAGAAGATCCAATGGT	ATGCGTCGTGTTGGTTG
AIT96LF	ZmNRT1.1C	GRMZM2G112154	CTCTACGACGTGGACGATGTG	GGTCAAGCAATCGGCATTCC	CCGGCTCCGACGCCAA
AIT96LG	CIPK8	GRMZM2G021517	CCTTTATGGAAAGATTGAGAGTGCAGAA	TGTCTCTGGATTTGGGTCAAGAATT	CACCACCTGAAAACC
AIT9799	ZmSIP1;3	Zm00001d032015_T001.1	TCTATTGCGCTCCGCTTCTC	CCTGCATCAGCTCCGAGAT	CAGGCCGTCGGTGTTG
AIVI4RN	ZmNRT1.1D	GRMZM2G161483	TGCTGTACGACGTCCAAGAC	GGCATCTTTTGCTTTCCCTTGAC	CTCTCCGGCGGCCAC
AIVI4RO	ZmGln1	GRMZM5G872068	TGCGGGCATCAACATCAGT	AAGGCCCGACTTGGAACTC	CCACTGCCCTGGCATC
AIVI6GH	ZmSIP1;4	Zm00001d017903_T001.1	GATAGCCCCTCCCTCTTCTCT	AGGGCACCACCCACAAC	CCTGCACCGAGAAGC
AIWR2XV	ZmNRT1.2	GRMZM2G137421	GGTATGGGTCGAGGACAACAG	GCCCGCGGCGAAGA	CCGTCGCCATCCTGC
AIWR2XW	ZmGln1	GRMZM2G036464	GGGCAGTGGGAGTTCCA	CGAGCAACCCACACCTGAT	CCGTCGGCATCTCTT
AIWR4MP	ZmSIP1;5	Zm00001d044543_T001.1	GCCCCTCCCTCTTCTCTGTT	CAGGGCACCACCCACAA	CCTGCACCAAGAAAC
AIX0033	ZmNRT1.4A	GRMZM2G064091	AGGGCGGCGGTGATG	CACGGAGCACAGCTTCCA	CCCGTCGTGCCCCACC
AIX0034	ZmNIAa	GRMZM2G428027	ACTGGTGCTGGTGCTTCTG	CGCGGACGGCGATTT	CCTTGGCCCCGAGCAC
AIX02SX	ZmTIP1;1	Zm00001d027652_T001.1	CATCTACGAGCTGCTCTTCATCTC	CGGTCGGCTTCGGCTTT	CCCCTCCACCGACTACT
AIY90Y5	ZmTIP1;2b	Zm00001d011778_T001.1	CCAGCAGCTGCCAACCA	TGGTCTGGTCTTAATTATTCAGTGCAT	CAGCAGACTACTGATGACCAT
AIY9ZAB	ZmNRT1.4B	GRMZM2G476069	TTCCCGTGCCTGGACAG	TGGCCCACCTCGTTGTC	TCGTCGCCAGCCATC
AIY9ZAC	ZmNIAb	GRMZM5G878558	GCCGGCCGCACAAG	CCGCCCGGCTGGTT	ACACCATGCCGATCTC

* AGPv3 used for design

5.5.2 Custom TaqMan primer and probes validated by Thermo Fisher Scientific (no sequences available)

Table 5.9 Custom TaqMan primer and probe sets with target and context sequence

Several probes were designed and validated by Thermo Fisher Scientific. Probes that were validated sequences were not available as it was protected intellectual property of Thermo Fisher Scientific. Listed are the probes, context sequence the probe set was designed around and the gene references.

Assay ID	Gene Name	AGPv3/AGPv4 target	Context Sequence	Gene Symbol	NCBI Gene Reference
Zm03999812_m1	ZmGDHa	GRMZM2G178415	TCAGATACCACCATGAGGTTGATCC	gdh1	NM_001111831.1;AY106054.1;U93560.1;U93561.1;D49475.1
Zm04002140_g1	ZmMEP	GRMZM2G018103_T01	CACCACGTGGGACCGACAAGCCATT	LOC100191588	NM_001137018.1;BT033719.1;BT064246.1
Zm04004176_g1	ZmASNd	GRMZM2G093175	GTGTAGGAAGGTTAAGGCTCTGCAT	asn2	NM_001137541.1;BT064177.1;FM211452.1;BT083882.1; FM212634.1;BT063342.1
Zm04004739_g1	ZmNRT1.1A	GRMZM2G086496	GTCCAGGCCTCGGGCGTGACGATCC	pco098705(612)	NM_001152263.1;BT053880.1
Zm04015536_g1	ZmZEP	Grmzm2g127139	GGAAGAAGCTATGGGTGGAGAATGG	LOC100285076	NM_001157971.1;EU970775.1
Zm04016516_s1	ZmAMT_05	GRMZM2G118950	GCGAGCAGCAACCGGGTGTAAAATT	LOC100273369	NM_001147808.1;BT040944.1
Zm04019411_g1	CIPK3	GRMZM2G174896	TTCAGAAGACCGGCATGTGAAAGAG	cipk31	NM_001154385.1;EU873319.1;EU956793.1
Zm04019827_g1	ZmNIP4;1	Zm00001d040108_T001.1	CGTACGGGAGCTGATGGTGGAGGGC	LOC100282312	NM_001155224.1;BT039406.1;EU960448.1
Zm04024497_g1	ZmNIP1;2	Zm00001d037733_T001.1	GGACAACAGAGCCATCGGCGAACTC	si605050a10(177)	NM_001158475.1;EU972991.1
Zm04024890_g1	ZmTIP3;2b	Zm00001d032610_T003.1	CGTCCTCTCGCTCGGGAAGATGTAC	tip3b	NM_001111575.1;AF342809.1
Zm04035819_s1	ZmAMT_01	GRMZM2G175140	ACAACAAGTGGACGATTCTGTCCTG	LOC100272903	NM_001147356.1;BT039740.1
Zm04040927_g1	ZmVp14	Grmzm2g014392	CACGGGCGAGCTCACCAAGTTCGAG	vp14	NM_001112432.2;U95953.2
Zm04044298_g1	ZmNRT1.3	GRMZM2G176253	ATCGCCGCCACGGGCGTGAGCTTGC	LOC100281564	NM_001154483.1;EU957112.1
Zm04050980_s1	ZmSYP121	GRMZM2G414540	CGAGTCCGGCGGCGTGGAGATGTCT	LOC100284409	NM_001157304.1;EU968220.1
Zm04051459_g1	ZmAMF1	GRMZM2G062024	AATGCCTTCAAGCTTCTTTCGGGTG	LOC100285732	NM_001158623.1;EU973642.1
Zm04051460_g1	ZmAMF2	GRMZM2G164743	CAACTCAGGCACCTGTAAATTTTGT	LOC100285732 LOC100381734	NM_001158623.1;NM_001174540.1;EU973642.1;BT062131.1
Zm04052214_s1	ZmNRT2.1	GRMZM2G010280	TAAGTCTACTACTAAGATGGATCGA	nrt2	NM_001111725.1;AY129953.1
Zm04052438_g1	ZmASNa	GRMZM2G074589	TACTCCAGCAAGACAGGTGGTCTAA	asn1	NM_001111997.1;BT035878.1;FM212633.1; FM211451.1;X82849.1
Zm04052636_m1	ZmSIP2;1	Zm00001d028951_T001.1	CGAATCCCAGCTCAGGTGATTGGGG	sbip2a	NM_001112170.1;AF326499.1;EU962597.1
Zm04052638_g1	ZmTIP4;4b	Zm00001d039293_T001.1	CCATGGCAACAGGGAAGCTGGCCGG	tip4d	NM_001112171.1;AF326508.1
Zm04057741_m1	ZmNIP3;1	Zm00001d032461_T001.1	GCTCACCCGCAAGCTGGGCGCGGAG	nip3a	NM_001111551.1;AF326486.1
Zm04057756_g1	ZmSIP1;2	Zm00001d010325_T001.1	GATTACCGGCACAGGCTGCCGGCGC	sbip1b	NM_001111558.1;AF326498.1;BT088172.1
Zm04059228_g1	ZmGln1-2	GRMZM2G024104	AAGGGGCAGAACATACTGGTCATGT	gln2	NM_001111973.1;AY104270.1;X65927.1
Zm04059399_m1	ZmSIP1;1	GRMZM2G113470_T01*	TGAACCCAGCTAATGCATTTGGCTG	sbip1a	NM_001112044.1;AF326497.1
Zm04059736_gH	ZmPIP2;5	Zm00001d003006_T001.1	GTCCCCGTGCTTGCCCCTCTCCCCA	pip2-5	NM_001112146.1;AF130975.1;AY243802.1
Zm04059958_s1	ZmbZIP1	GRMZM2G448607	CCGGCGCCGCCGTCGACATCCCGGA	lip15	NM_001112214.1;AY112511.1;D26563.1
Zm04060019_g1	ZmGOGAT	GRMZM2G036609	TTATGACCTCCATCAGATAAATCCT	fgs1	NM_001112223.1;M59190.1
Zm04060081_g1	ZmNIP1;1	Zm00001d016237_T001.1	TTTATCCAGAAGATCATCGCGGAGA	nip1a	NM_001112251.1;EU964298.1;BT068485.1;AF326483.1
Zm04060104_g1	ZmGln2	GRMZM2G098290	GTCATGCCTGGTCAGTGGGAGTACC	gln1	NM_001112255.1;AY110796.1;X65931.1
Zm04060734_m1	ZmNRT3.1B	GRMZM2G163494	CCGAAGGCCGGACAAGTCCTGCACG	NAR2.2	NM_001112454.1;AY968679.1

(*Continued*)

Assay ID	Gene Name	AGPv3/AGPv4 target	Context Sequence	Gene Symbol	NCBI Gene Reference
Zm04060753_g1	ZmNRT3.1A	GRMZM2G179294	CAAACCCGGCCAAGTCCTGCACGCC	NAR2.1	NM_001112459.1;AY968678.1
Zm04061849_s1	ZmAMT_03	GRMZM2G028736	GGTTAGCGTCACCATGGCTCCGCTG	LOC100191554	NM_001136984.1;BT033666.1;BT033667.1
Zm04062942_g1	CIPK33	GRMZM2G146553	GACTCAGAGCAGGAGTTCAAAAGAG	LOC100193758	NM_001138844.1;BT036109.1
Zm04065752_g1	CIPK31	GRMZM2G383240	AGGATACCCCGGTTATCAAAGTCTC	LOC100193471	NM_001138587.1;BT035739.1
Zm04066155_g1	ZmGDHb	GRMZM2G427097	ATGGGGACCAATGCACAAACCATGG	LOC100193614	NM_001138715.1;BT035930.1
Zm04068994_g1	CIPK23	GRMZM2G013236	CGGATGTATGAGGTGATGGCCAGCA	pco144723	NM_001138053.1;BT040687.1;BT034960.1
Zm04073882_g1	ZmLUG	GRMZM2G425377_t01	TACGAGTCTTTGGAACTTTGGGACA	LOC100217300	NM_001143648.1;BT038300.1
Zm04073970_g1	ZmTIP3;3	Zm00001d048520_T001.1	CCACGCTCTCTCTCGGGAGGATGCA	cl46210_1	NM_001153458.1;EU952374.1
Zm04074254_gH	ZmASNc	GRMZM2G078472	TTACCTGCCCGAGCATATTCTGTAC	asn4	NM_001137543.1;FM212636.1;FM211454.1
Zm04075541_sH	ZmNIP2;2	Zm00001d037228_T001.1	ACAACAGGCGCATCTCGCAGCTGGG	nip2b	NM_001111550.1;BT063648.1;EU956287.1;AF326485.1
Zm04075546_s1	ZmPIP2;3	Zm00001d051174_T002.1	CACATCTGCCACCACAGTCCCGTCC	pip2c	NM_001111555.1;BT087943.1;AF326493.1
Zm04075896_s1	ZmPIP2;4	Zm00001d017288_T001.1	CTCCGCTCGCTCGCTGCTGTTTTCT	pip2d	NM_001111556.1;BT063385.1;AF326494.1
Zm04075902_s1	ZmTIP2;2	Zm00001d017485_T002.1	TCGTCCTGGTCTGTTCCATTCCGTC	tip2b	NM_001111561.1;AF326502.1
Zm04075906_sH	ZmTIP3;1	Zm00001d013956_T003.1	TAGCTTGAAAATTGTATTGTGGGGT	tip3a	NM_001111562.1;AF326504.1
Zm04076072_s1	ZmTIP4;1b	Zm00001d037779_T001.1	GTAGAAATTGTTGATGGGCTTTTTT	tip4a	NM_001111563.1;AF326505.1
Zm04076215_s1	ZmGln1-1	GRMZM2G050514	CCTAAAATTTATCACTACGGTCTCC	LOC542520	NM_001112068.1;BT039599.1;D14579.1
Zm04077200_g1	ZmNIP2;4	GRMZM2G082184_T01*	CGTCGTCGTCGCACTCGCAGAAGCT	LOC100192638	NM_001137852.1;BT034672.1
Zm04078790_s1	ZmGln1-5	GRMZM2G046601	TCCCGTCCTAAAGTTTGTTACCACT	gln3	NM_001111827.1;D14578.1
Zm04078818_sH	ZmNIP2;1	Zm00001d018037_T001.1	CCGGGGCCTGGACCTACACCTACAT	NIP2-1	NM_001112167.1;AF326484.1
Zm04078826_sH	ZmPIP2;2	Zm00001d005421_T001.1	TCGGGGGCCGGGGCGGACGCGGCGT	pip2b	NM_001112168.1;EU967848.1;BT065379.1;AF326492.1
Zm04079618_s1	ZmAMT_07	GRMZM2G080045	CCGGCGTCATGGATAGAGAAGGGTC	LOC100279304	NM_001152325.1;BT054001.1
Zm04082247_s1	ZmSAT2	GRMZM2G082343	GCCTCCGCCACGCTTCTGCCGGAGA	LOC100281729	NM_001154649.1;EU957679.1

* AGPv3 gene used for design

Chapter 6: General discussion and conclusions

The original aim of these experiments was to examine root hydraulic conductivity changes in B73 and F44 under nitrogen starvation and resupply, and to link these changes to aquaporin activity. This research increased insight into maize aquaporins – the isoforms, water conductance, gene expression, but had unclear results in linking hydraulic changes to nitrogen treatment consistently.

Changes in maize root hydraulic conductance were measurable with the HCFM, but differences as a result of N treatment were difficult to correlate. Many factors affect water flow in roots: root architecture (length, thickness, branching etc.), axial conductance of xylem vessels, radial conductance (arising from resistances such as endodermis, exodermis, cortex, etc.), and osmolarity changes. The experiments here focused on the hydraulic conductance as measured with the HCFM. In maize, radial conductance is much greater than axial conductance (Frensch and Steudle 1989), so it was believed the hydraulic conductance measurements was primarily the radial conductance. While firm conclusions are difficult to make, the variability and inconsistency observed may be explained and point to challenges to assumptions made about these experiments and raise additional questions related to how water is transported through plants.

6.1 Maize aquaporin genes and gene expression

The set of maize aquaporins contained in the fourth generation (AGPv4) of the B73 genome was very similar to the set of aquaporins found previously through a cDNA library screen (Chaumont et al. 2001) and the third generation (AGPv3) genome (Deshmukh et al. 2015). Compared with AGPv3, AGPv4 has a few differences in

aquaporin profile. The true aquaporin profile is likely in between the two. ZmPIP1;6b and ZmSIP1;1 were present in both genomes, but both were truncated in AGPv4. Cloning confirmed that the AGPv3 annotation was correct. The AGPv4 genome also predicted ZmTIP2;3 and ZmTIP5;1b to be separate clones, as well as a full length ZmTIP1;1 that was disrupted in AGPv3. Several ZmNIPs (ZmNIP4;1, ZmNIP5;1 and ZmNIP5;2) had variation, however cloning data is not available to suggest one form over another. The AGPv4 genome was improved through the use of SMRT sequencing that closed the number of gaps between contigs. Gene prediction was also improved through mapping full length cDNA reads to the genome to predict and annotate exon structure (Jiao et al. 2017). These results show that while it may be improved, for aquaporins ZmPIP1;6b and ZmSIP1;1, the aquaporin predictions are not perfect.

The AGPv4 genome predicts several aquaporins that have not been described previously, including ZmPIP3;1, ZmNIP1;2, ZmNIP1;3, ZmNIP4;1, ZmNIP5;1 and ZmNIP5;2 have not been described (though ZmNIP5;1 was mentioned as not having any expression detected in an RNA-Seq experiment (Yue et al. 2012)). OpenArray analysis did not detect expression for ZmPIP2;7, ZmPIP2;8, ZmTIP3;2b, ZmTIP3;3, ZmTIP3;4, ZmTIP4;3b, ZmTIP4;4b, ZmTIP5;1b, ZmNIP1;3, ZmNIP2;3, ZmNIP4;1, ZmNIP5;1, or ZmNIP5;2. The lack of expression for ZmPIP2;7 was expected, as it was not present in the B73 genome. For ZmPIP2;8, the lack of expression was not expected. ZmPIP2;8 was cloned from B73 by Lawrence et al. (2013). The authors also monitored its gene expression using a TaqMan probe. The OpenArray here utilised the same primer set and probe. One possibility is the OpenArray may not accurately detect very low levels of expression, and could explain why other genes lacked expression. In chapter 3, ZmTIP4;4b was cloned from B73 tissue, and cloning primers designed to be specific for it showed amplification in both root and shoot cDNA. This indicates that ZmTIP4;4b has some expression, but

may be too low to detect with the OpenAarray platform. **Table 6.1** combines the expression monitored on the open array with the open reading frame cloned. Like ZmTIP4;4b, ZmNIP2;2 was amplified in roots, but was not detected on the OpenArray. Similarly, ZmNIP3;1 showed weak amplification from shoot cDNA, but was not detected on the OpenArray. Continued cloning could further establish if the predicted aquaporins are expressed.

Table 6.1 Cloned aquaporins and gene expression on the OpenArray

Open Array expression (**Table 5.3**) combined with cloning information from root and shoot tissue (**Table 3.7**)

	B73 Gene Expression		Cloning Amplification	
Aquaporin	Root	Shoot	Root	Shoot
ZmNIP1;1	x	x	x	x
ZmNIP1;2	x	x		
ZmNIP1;3				
ZmNIP2;1	x	x	x	x
ZmNIP2;2		x	x	x
ZmNIP2;3				
ZmNIP2;4	x	x		
ZmNIP3;1	x		x	x
ZmNIP4;1				
ZmNIP5;1				
ZmNIP5;2				
ZmPIP1;2b	x	x	x	x
ZmPIP1;4	x	x	x	x
ZmPIP1;5	x	x	x	x
ZmPIP1;6b	x	x	x	x
ZmPIP1;7	x	x		
ZmPIP2;1	x	x	x	x
ZmPIP2;2	x	x	x	x
ZmPIP2;3	x	x	x	x
ZmPIP2;4	x	x	x	x
ZmPIP2;5	x	x	x	x
ZmPIP2;6	x	x	x	x
ZmPIP2;7				
ZmPIP2;8				
ZmPIP2;9	x	x		
ZmPIP3;1		x		
ZmSIP1;1	x	x	x	x
ZmSIP1;2	x	x	x	x
ZmSIP2;1	x	x	x	x
ZmTIP1;1	x	x		
ZmTIP1;2b	x	x	x	x
ZmTIP2;1	x	x	x	x
ZmTIP2;2	x	x		
ZmTIP2;3	x	x		
ZmTIP2;4	x	x		
ZmTIP3;1	x	x		
ZmTIP3;2b				
ZmTIP3;3				
ZmTIP3;4				
ZmTIP4;1b	x	x	x	x
ZmTIP4;2b	x	x		
ZmTIP4;3b				
ZmTIP4;4b			x	x
ZmTIP5;1b				

The gene expression data presented here is complicated by efficiency curves. Many probes showed greater than 100% efficiency. While the manufacturer (Thermo Fisher Scientific) claims the TaqMan probes it designs actually work, this cannot be guaranteed, so the observed expression, and lack of expression should not be assumed to be correct until further validation is performed. Two potential sources of error could result in poor standard curve preparation; through miss-calibrated pipettes or variable solution delivery.

Pipetting to a balance suggests that the calibration may have been slightly off (a dilution factor of 0.1070 instead of 0.1000) but not enough to explain the varied efficiencies. Another source of error could be the presence of PCR inhibitors in the samples. This would increase the C_{RT} value (as amplification would be less efficient), but the effect would decrease as the inhibitor was diluted in the process of making the standard curve. In effect, the spacing of concentrations would decrease, which would increase the slope of the regression line. This is even more likely as amplification was missing for Actin to test for cDNA presence, especially in root samples. As RNA appeared to be intact on a gel, the lack of amplification was likely due to PCR inhibition of the actin amplification, or due to lack of cDNA through the inhibition of the reverse-transcription reaction.

The efficiency curve was also limited by the effective range. Many of the cDNA samples had relatively high C_{RT} values. Highly expressed genes were C_{RT} of 18, while less expressed fell below detection around a C_{RT} of 30. For some curves, only two dilution points were available, and for low expressed genes, only one point was present. To further validate the TaqMan probes, the target sequences should be obtained and validated with a standard curve. This may reveal that some amplifications were inhibited, or may reveal the probes themselves were poorly designed. An accurate standard curve would also allow better statistics based on copy number determination instead of relative expression.

The genome analysis, cloning work and comparisons with past studies suggest a further mechanism for hydraulic variation between inbred lines. It is known that aquaporin isoforms differ in nucleotide and amino acid sequence between plant species. In describing the set of cloned maize aquaporins, Chaumont et al. (2001) showed the protein sequences of the maize aquaporins were different from the *Arabidopsis* aquaporins (Johanson et al. 2001), and that they tended to group based on species (that is the ZmPIP1s

grouped together, and ZmPIP1;4 had no direct homolog with any AtPIP1). The maize genome aquaporins and those proposed by Chaumont et al. (2001) were not identical, and had nucleotide and amino acid changes. Some variation, as seen in ZmTIP4;1b, is in an N-terminal extension, explained by the published clone being sequenced from a midpoint of the transcript. Others, such as ZmPIP1;1/ZmPIP1;7, had point mutations that led to amino acid changes. Chaumont et al. (2001) sequenced many clones in their screen, suggesting that the variation in sequence is not due to sequencing errors. Variation in aquaporin sequences between inbred lines or varieties of maize raises potential mechanisms for changes in hydraulic responses (regulation changes in expression from promoter changes to functional changes in the amino acid sequence), but also presents a challenge in making direct comparisons between different varieties. With increasing genome technology, the *de novo* assemblies of the genomes for different inbred lines may become available and make direct comparisons between them possible. Current sequencing technologies use the B73 genome as a guide during assembly which may miss some of the diversity.

6.2 Maize aquaporin activity

Cloning of maize aquaporins and expression in *Xenopus* oocytes revealed their water transport capacity. The ZmPIP water transport was consistent with that previously seen (Fetter et al. 2004; Zelazny et al. 2007; Zelazny et al. 2009). Here, a subset of the ZmTIPs were also cloned and demonstrated water conductance. Combined with expression data, it shows that the ZmTIPs are highly expressed in maize roots, may be regulated with nitrate availability, and have water transport capacity, and have a potential role to play in root hydraulic conductivity.

Interestingly, ZmPIP2;6 and co-injected ZmPIP1;5-ZmPIP2;1 showed increased conductance to nitrate. There are several factors that impact these results. In terms for mass flow, a positive feedback look is created, where nitrate directly stimulates water flow, which in turn brings more nitrate (Miller and Cramer 2004; Cramer et al. 2009). This is countered by the nitrate uptake process which drives acidification (Tsay et al. 1993; Tsay et al. 2007; Espen et al. 2004) and can gate PIP aquaporins (Tournaire-Roux et al. 2003).

6.3 Maize root hydraulic conductivity

In the literature, maize displays the usual stimulation of hydraulics when N is present. In studying N effects on root exudation Pan et al. (1985) commented that rate of exudation of +N plants was significantly more than in –N plants. Barthes et al. (1995) and Barthes et al. (1996a) quantified this response, and determined that changes in xylem sap osmolarities were not sufficient to explain the increased exudation rate, and proposed that the increase in exudation rate was due to an increase in root hydraulic conductivity. Barthes et al. (1996a) also showed that tungstate, a nitrate reductase inhibitor and competitive inhibition of glutamate synthetase (via PPT or MSO competitive inhibition) reduced the response, suggesting it was a down-stream product of nitrogen assimilation that was responsible. Hoarau et al. (1996) also showed an increased exudation rate from increased root hydraulic conductance, and showed that while osmolarity did not change, the concentration of nitrate in the exudate increased in $+NO_3^-$ grown plants. Górska et al. (2008) also examined exudatation rate from applied pressure with high resolution and precision using a balance. They also showed an increased exudation rate, only partially explained by changes in osmolarity of the exudate solution. Nitrate reductase inhibition also eliminated the hydraulic response, but they showed that inhibitor-treated plants did

not accumulate nitrate as a result. They hypothesised that nitrate uptake was also inhibited, or that uptake rates were similar to unloading nitrate to the xylem. In cucumber, Gorska et al. (2008) suggested that the increase in hydraulic conductance was due to increased nitrate concentrations in the cell, as injecting nitrate into root cells reduced half-time pressure-relaxations when measured with a pressure probe.

The hydraulic response measured in Chapter 4 did not reliably confirm these literature observations with maize. Several factors may be involved in this apparent contradiction. First, the maize lines used in Chapter 4 were B73 and F44, while previous studies used varieties F7F2 (Barthes et al. 1995; Barthes et al. 1996a; Hoarau et al. 1996), 'Helix' (Schraut et al. 2005) and "Hybrid Corn – White Sweet" (Górska et al. 2010; Górska et al. 2008). With known maize diversity, it is possible that B73 and F44 have alternative hydraulic responses to nitrogen treatment.

Table 6.2 Maize hydraulic response to nitrogen
Maize hydraulic responses in the literature.

Reference	Maize Line/Variety	N experiment	Method of measuring root hydraulic properties
Pan et al. (1985)	'Tanker'	Resupply	Exudation
Barthes et al. (1995)	F7F2	Resupply	Exudation, sap osmolarity
Barthes et al. (1996a)	F7F2	Resupply	Exudation, sap osmolarity
Hoarau et al. (1996)	F7F2	Resupply	Exudation, sap osmolarity
Schraut et al. (2005)	'Helix'	Starvation	Exudation from applied vacuum, sap osmolarity
Górska et al. (2008)	'Hybrid Corn – White Sweet'	Resupply	Exudation from applied pressure, sap osmolarity
Górska et al. (2010)	'Hybrid Corn – White Sweet'	Resupply	Exudation from applied pressure, sap osmolarity

Another effect could be a result of the method of hydraulic conductance determination. All the previous studies measured exudation rates and xylem sap osmolarity to deduce root hydraulic conductance. As a result, these studies measured the net response of the whole root system. In Chapter 4, hydraulic conductance was measured with the HCFM, measuring the flow of water pushed into the root, and was performed on a single primary

or nodal root. In designing the experiment, it was assumed, perhaps incorrectly, that the whole root response would be observed in individual roots or root segments. The results in Chapter 4 may be explained by challenging this assumption. Gorska et al. (2008) demonstrated in a split root system that roots preferentially absorb water where nitrate is present, reversibly modulating water uptake in different parts of the root. In Chapter 4, hydroponics and aeroponics systems were designed to give the root system a homogenous N treatment, however, there still may be variation with water uptake not uniform across root segments. Sampling individual roots with a diverse hydraulic conductance may mask the net water uptake by the whole root system. A simple experiment could be used, measuring the net water uptake by the whole root system instead of on individual root segments in B73 and F44 under N starvation or resupply.

Monitoring individual roots may also have masked the hydraulic response through incorrect normalisation methods for the measured conductance. **Figure 4.19**-D demonstrated that the proportion of the root with open xylem elements was very diverse in B73 grown in hydroponics. While this is just one condition, it raises several concerns in drawing conclusions from the results. First, whole root surface area may not be the appropriate normalisation standard for the hydraulic conductance when measured by the HCFM. As principally the radial conductance is measured, changing proportion of open xylem in the root changes the amount of radial conductance surface area. A potentially better method would be determining root surface area of the portion of the root where open xylem vessels were detected. In maize, not much is known about root xylem vessels opening. Frensch and Steudle (1989) indirectly indicated that in hydroponically grown maize (cv 'Tanker'), the first ~2cm are not significantly involved in axial water uptake. Between species, there is variation in the primary location of water uptake, as seen between wheat and lupin roots (Bramley et al. 2009). It remains to be seen if this can be

changed within a plant through environmental effects such as N treatment, or if it is variable within a species as it is between species. In terms of root water uptake, increasing the proportion of root with open xylem increases the effect that increases in root hydraulic conductivity have on total root water uptake. Additionally, variation could exist between root types (crown, nodal, primary, and secondary) and at different ages, at different stages of development. Maize roots between varieties are known to have different rates of elongation during water stress (Leach et al. 2011), highlighting the existence of diversity in maize root properties. Hydraulics may be another trait that displays variation.

Further work should be done to link the hydraulic properties and variation in Chapter 4, with changes in root and shoot gene expression. The OpenArray provides a useful tool to screen many genes at once, and could allow a detailed gene expression response profile over time (hours – days), to starvation or resupply, and with different forms of nitrogen.

6.4 Concluding remarks

This research uncovered the set of aquaporins in maize, and showed reduced expression of aquaporins to nitrate starvation after three days. Cloning confirmed genomic predictions and allowed the water transport capacity of ZmTIPs to be explored, suggesting the ZmTIP2 subfamily is water permeable. Maize ZmPIP2;6 may have greater water transport in the presence of nitrate, and nitrate transport can reduce cytosolic pH in *Xenopus* oocytes. Examining root hydraulics of individual roots segments produced variation that was difficult to explain, but may hint at diversity within seemingly homogeneous root systems. While the physiological response was not conclusive, both B73 and F44 had reduced aquaporin expression in response to nitrate starvation.

Appendix

The following pages present the book chapter the Introduction was based on.

Root hydraulic and aquaporin responses to N availability

2017

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Pages 207-236

in

Plant Aquaporins

Eds: F. Chaumont, S. Tyerman

Signaling and Communicaitons in Plants.

Springer, Cham

ISBN: 978-3-319-49393-0

DOI: 10.1007/978-3-319-49395-4_10

Root Hydraulic and Aquaporin Responses to N Availability

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Abstract Nitrogen (N) uptake in most plants is positively correlated to water flow in and through roots. This allows transpiration to drive convection of mobile N sources in the soil to the root surface. Generally N starvation suppresses root hydraulic conductivity (Lp_r) while resupply stimulates. However, ammonium and nitrate can give different responses depending on the species, and this may be associated with the form of N that different species prefer to transport and assimilate. Responses in Lp_r in the short to medium term are largely explained by aquaporin regulation at both the transcript and post-translational level. Local and systemic signalling is indicated in the regulation of aquaporins. A direct role for NO_3 sensing has been shown as well as a role of a high-affinity NO_3^- transporter (NRT2.1), but the mode of action in post-translational modification is not known. Transcripts of the aquaporins are also altered by N treatments, yet the regulations of these transcripts and the function of the proteins in N transport remain unclear. Further research is required to uncover the signalling and regulation of aquaporins in relation to N transport since modifications to this process may improve N use efficiency.

1 Introduction

Nitrogen (N) is essential for plant survival, being vital for cell chemistry and processes, and is the macronutrient taken up in the greatest quantity (80 % of total ion uptake) with the greatest energy requirement for transport and assimilation (Marschner 1995). While 78 % of the atmosphere is nitrogen gas, it is unavailable

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F. Chaumont, S.D. Tyerman (eds.), *Plant Aquaporins*, Signaling and Communication in Plants, DOI 10.1007/978-3-319-49395-4_10

for direct use by most plants except those that enter into symbiosis with N_2-fixing bacteria, where nitrogen gas (N_2) is converted to inorganic ammonium (NH_4^+). Once reduced, NH_4^+ can be assimilated into organic (amino acids, organic molecules) and inorganic (nitrate NO_3^-) forms by plants and soil microbes, respectively. These forms of N often present within the root rhizosphere have large effects on plant growth and development (Miller and Cramer 2004). In this chapter, the root hydraulic responses to N concentration and form across many species are analysed and discussed in terms of the roles played by aquaporins in this process. The possible sensors involved in NO_3^- detection and signal transduction to regulate aquaporins will be examined, and we will attempt to integrate the various signals and responses that have been shown to regulate N transport and aquaporins.

2 Forms of N and the Water Connection

The two major forms of available N in the soil are NO_3^- and NH_4^+, the availability of which depends on soil structure and composition, microbial activity, temperature and water (Glass 2003; Warren 2009). Under conditions of poor aeration, low temperatures and/or acidity, nitrifying bacteria become inactivated and the less mobile NH_4^+ increases relative to that of the more mobile NO_3^- (Marschner 1995; Laanbroek 1990). In contrast, in well-aerated pH-neutral soils, activity of nitrifying bacteria ensures NO_3^- is the predominant form of available N (Marschner 1995). Most plants have a preference for NO_3^- despite the overall energy costs of NO_3^- assimilation relative to that of NH_4^+ being higher (5–12 %) taking into account pH regulation (Raven 1985). The water costs per mole of N assimilated are also higher for NO_3^- by up to 12 % depending on where the NO_3^- is reduced and how pH is regulated (Raven 1985). There is a strong interaction between N uptake and water content of the soil, since this can have diverse effects on N availability through its effect on the degree of aeration and in the dissolution and transport of available N to the root surface (discussed below) (Moyano et al. 2013; Gonzalez-Dugo et al. 2010).

2.1 Soil N Availability and Mass Flow

N availability has interacting effects on many physiological and developmental processes in plants including water relations, and there is cross talk in the regulation of N and water relations (Easlon and Bloom 2013). The link between N and water movement is also important in biogeochemical models that influence predictions from global climate models (Simunek and Hopmans 2009; Seneviratne et al. 2010). Optimisation of water and NO_3^- uptake by root structure and architecture are also closely linked (Lynch 2013). In addition to supplying water for growth and nutrient movement in the xylem, water flow through plants has a role in nutrient acquisition and delivery from the soil.

Mass flow in the soil has been long observed (Barber 1962) and is the process by which water flow to the root brings soluble and mobile molecules to the root surface. In the absence of mass flow or for nutrients that are poorly mobile in soil (e.g. inorganic phosphate), uptake of nutrients by the root cells reduces the surface concentration and creates a localised diffusion gradient to the root so that the rate of uptake may be limited by the rate of diffusion to the root surface (or hyphae of mycorrhizae) (Nye 1977). Mass flow of water during transpiration can convey (via convection) dissolved and mobile nutrients to the surface of the root (Cramer et al. 2008; Chapman et al. 2012) thus reducing or possibly reversing the concentration gradient and assisting the membrane transport systems by increasing the external concentration at the sites of uptake. The degree of "assistance" will depend on the flow rate to the root surface; concentration kinetics of the transport systems, i.e. high, low or dual affinity (HATS, LATS, dual); and the type of transport systems engaged (i.e. AMTs and AMFs for NH_4^+ (Ludewig et al. 2007; Chiasson et al. 2014) or NRT2 or NPF (NRT1) for NO_3^- (Tsay et al. 2007)), which in turn depends on the preference for different forms of N by the plant and the relative availability of these N forms in the soil solution. NH_4^+ and NO_3^- uptake kinetics are complicated since there are also inducible components, e.g. iHATS. Most of the components recognised from physiological studies can be accounted for by specific transporters in *Arabidopsis*, e.g. AtNRT2.1, AtNRT2.2, AtNRT2.4 and AtNRT2.5 as HATs NO_3^- transporters (Li et al. 2007; O'Brien et al. 2016) and AtNRT1.1 and AtNRT1.2 as LATS NO_3^- transporters (Léran et al. 2014). The NRT2s interact with a nitrate assimilation- related protein (AtNAR2.1) to be active (Kotur et al. 2012).

A whole plant model for the acquisition of NO_3^- by mass flow has been proposed (Cramer et al. 2009b). By increasing total water flow through the plant when NO_3^- is sensed, more NO_3^- may be brought to the root surface. NO_3^- reduction to NH_4^+ is shifted from the root to the shoot as NO_3^- acquisition rises and closes stomata as it is reduced, providing a feedback mechanism to slow water uptake when NO_3^- levels are sufficient. NO_3^- at concentrations above 2 mM in the xylem reduces stomatal conductance in maize in an ABA-dependent manner (Wilkinson et al. 2007). The Cramer et al. (2009b) model necessitates a root NO_3^- sensor and a hydraulic response triggered by NO_3^-, but not by NH_4^+.

2.2 Transpiration and Stomatal Control

Transpiration is considered to be an essential driver of various forms of N uptake by roots by increasing both mass flow and diffusive fluxes of N to the root surface (Oyewole et al. 2014). Stomatal control of transpiration is regulated by N availability (Matimati et al. 2014; Cramer et al. 2008), and NO_3^- in the xylem in concert with ABA and xylem pH is a signal from roots that regulates stomatal conductance (Wilkinson et al. 2007). By inference from recent studies, stomatal regulation in response to NO_3^- is likely to involve internal hydraulic conductivities in the leaf bundle sheath regulated by plasma membrane intrinsic proteins (PIP) aquaporins

(Shatil-Cohen et al. 2011) as well as particular PIPs in the guard cell membrane (Wang et al. 2016; Grondin et al. 2015). In turn it is likely that transpiration itself regulates root hydraulic conductivity via root aquaporins (Vandeleur et al. 2014; Laur and Hacke 2013; Tardieu et al. 2015) thus providing a rather complex feedback regulation in concert with N availability. These factors add to the plethora of factors that must be integrated by the plant in regulating transpiration and nutrient uptake (Raven 2008).

3 Linking N and Water Movement: Basic Root Characteristics and Environmental Effects

3.1 Measurement of Root Hydraulic Properties

Root hydraulic properties can be measured using several methods, and these are important in the interpretation of the data obtained and the links with N transport. They apply to different root dimensions, tissue scales and rapidity of measurement at the level of single cells; root segments; whole roots in soil, intact or excised; and direction of flow. Each has advantages and disadvantages in interpretation of physiological significance. The water carrying capacity of the roots is generally measured as the hydraulic conductance variously normalised to account for root size (Lo) or hydraulic conductivity (Lp) in the strict sense when normalised to root surface area or root length. In this chapter we will use the term root hydraulic conductivity (Lp_r) rather loosely to indicate that conductance has been normalised to some component of root size.

Total flow of water through plants can be determined by observing the loss of water over time. This method is useful, since it avoids any wounding effects, but is unable to isolate root versus shoot responses or the effects of hydraulic conductance changes in different parts of the plant versus changes in water potential gradients unless these are measured (e.g. Franks et al. 2007). Isolating the effect of the roots can be done by excising the shoot and observing only the root system. This opens the xylem to the atmosphere and may interfere with signals from the shoot (Vandeleur et al. 2014). Simple observations are done by determining the rate and volume of sap exuded over time at atmospheric or applied suction to the root system (e.g. Hachez et al. 2012). Hydraulic conductivity and solute effects are determined from measurement of the osmotic concentration of the exuded sap and that of the nutrient solution supplied. Hydraulic conductivity can also be determined by observing the rate of solution flow through the root at varying applied pressures to the whole root system forcing water to flow in the direction of the shoot (e.g. Gorska et al. 2008a). Alternatively pressure variations can be applied to the excised root system (high-pressure flowmeter, HPFM (Tyree et al. 1995)) or single root segments (root pressure probe, e.g. Lee et al. 2004b)), and from relaxation kinetics or steady-state volume flow, the hydraulic conductance can be determined. These do

not always give the same answer when compared and depending on driving force (osmotic or hydrostatic) (Bramley et al. 2007b). Some techniques can give conductances very rapidly after root system excision, for example, the transient method using the HPFM, and although water flow is in the reverse direction to normal transpirational flow, the values obtained correlate well with measurements of flow in the normal direction (Tsuda and Tyree 2000). While many have used excised root systems or roots for measurement of hydraulic properties, it has been reported that shoot excision itself causes a decrease in hydraulic conductivity of grapevine, soybean and maize (Vandeleur et al. 2014). In soybean the reduction after excision is large and rapid (half-time ≈ 5 min).

Root hydraulics can also be observed on a cellular basis using the cell pressure probe (Tomos and Leigh 1999). The hydraulic conductivity of individual plant cells can be determined from the elasticity of the cell wall and the half-time of water flow from pressure pulse relaxations after the cell volume is rapidly changed (Hüsken et al. 1978). This is useful to attempt to reconstruct the components of radial flow across the root and to determine potential cells that could govern whole root hydraulic conductance (Bramley et al. 2009). It also allows a more direct link between root hydraulic conductance and the molecular components that determine cell membrane water permeability, i.e. the aquaporins.

3.2 Root Components That Influence Water Movement

Like N and other inorganic nutrients, water acquisition occurs via the root in nearly all plants. In the root, water is absorbed from the soil by flowing down its free energy gradient, the components of which in the plant can be modified through changing solute concentration and the rate of transpiration (Kramer and Boyer 1995). The free energy gradient (water potential gradient) for water to flow to roots through the soil, radially across roots, and then up to the shoot via the xylem, is generated by hydrostatic pressure and osmotic gradients, osmotic gradients only being effective when across a semipermeable membrane. Osmotic gradients may dominate when there is no transpiration, while pressure gradients established in the soil-root-xylem via the cohesion tension mechanism dominate during transpiration (Steudle 2001; Sperry et al. 2002). Given the relatively large energy requirements for the uptake of NO_3^- compared to other nutrients (Cannell and Thornley 2000; Poorter et al. 1991), it is not surprising that there may have been selection pressure to evolve mechanisms to assist the process through coupling with the energetically downhill flow of water in the soil-plant-atmosphere continuum.

Plants can modify the rate that water flows down the water potential gradient via the hydraulic conductivity in the flow pathways. In roots, water can enter the root through the apoplast and a parallel cell-to-cell pathway to reach the xylem (Steudle and Peterson 1998). In many systems, barriers interrupt the apoplastic water flow, forcing water to use the cell-to-cell pathway (Steudle 2001). The *sgn3* mutant of *Arabidopsis* that has compromised development of the Casparian strip due to the

lack of deposition of lignin has increased Lp_r but only shows signs of compromised nutrient transport for potassium (Pfister et al. 2014). Suberin is deposited later in development to surround the endodermal cells in *Arabidopsis*, and its deposition is dependent (reversibly) on nutrient and ionic stress (Barberon et al. 2016). This is mediated by ethylene (reduces suberisation) and ABA (increases suberisation), but of the nutrients examined by Barberon et al. (2016), NO_3^- or NH_4^+ were not included and should be examined in the future. Root ABA and ethylene are reported to be involved in N signalling (Kudoyarova et al. 2015; Ondzighi-Assoume et al. 2016; O'Brien et al. 2016), so it is possible that N nutrition could regulate suberisation of the endodermis and hence influence both water and N transport into the pericycle or the movement of a diffusible chemical signal from the pericycle out to the cortex and epidermis.

3.3 Aquaporins

Membrane water permeability of roots is modulated by the activity of aquaporins, certainly in the plasma membrane of certain cell layers, but probably also in the tonoplast, such that, depending on species, more than 50 % of water flow through roots may occur via aquaporins as determined by inhibitor effects (Chaumont and Tyerman 2014; Maurel et al. 2015) (see also chapter "Aquaporins and Root Water Uptake"). Higher plants have many aquaporin isoforms that can be classified into five main types (see also chapter "Structural Basis of the Permeation Function of Plant Aquaporins"). The plasma membrane intrinsic proteins (PIPs) will be largely discussed in this chapter related to root water movement, but tonoplast intrinsic proteins (TIPS) and nodulin intrinsic proteins (NIPs, see also chapter "Plant Aquaporins and Metalloids") are also relevant to interactions with N transport.

The PIPs are regulated on many levels (transcription, protein amount, protein location and by gating (Boursiac et al. 2008; Chaumont and Tyerman 2014; Maurel et al. 2015) (see also chapters "Structural Basis of the Permeation Function of Plant Aquaporins", "Heteromerization of Plant Aquaporins", "Plant Aquaporin Trafficking" and "Plant Aquaporin Posttranslational Regulation"), and this can account for considerable variation in hydraulic conductivity, sometimes by an order of magnitude in roots depending on species (Vandeleur et al. 2014). In maize roots, aquaporin expression has been seen to change diurnally and circadially (Lopez et al. 2003; Caldeira et al. 2014), in different developmental stages (Hachez et al. 2006) and during water stress (Hachez et al. 2012). Aquaporin protein activity can be modified in several ways including protein-protein interactions (Fetter et al. 2004; Zelazny et al. 2007), pH gating (Hedfalk et al. 2006; Tornroth-Horsefield et al. 2006; Nyblom et al. 2009), blocking with certain metals (Niemietz and Tyerman 2002; Verdoucq et al. 2008), ubiquitination (Lee et al. 2009), amidation (Pietro et al. 2013), phosphorylation (Van Wilder et al. 2008; Grondin et al. 2015) and membrane trafficking (Chevalier and Chaumont 2015) (see also chapters "Structural Basis of the Permeation Function of Plant Aquaporins", "Heteromerization of Plant Aquaporins", "Plant Aquaporin

Trafficking" and "Plant Aquaporin Posttranslational Regulation"). Any one or a combination of these including different combinations of isoforms could be involved in regulating water flow through roots to influence N uptake.

Apart from water, certain aquaporins also facilitate passive transport of small solutes, including N-containing molecules. Some members of the *Arabidopsis* TIP family have been shown to be permeable to urea in yeast complementation assays, and their expression can increase during N starvation (Liu et al. 2003). Other *Arabidopsis* TIP members, maize NIP and TIP, and a cucumber NIP, have been found to complement yeast for growth on urea (Klebl et al. 2003; Gu et al. 2012). ZmPIP1;5-b is induced with NO_3^- application and has been suggested to be water and urea permeable when expressed in *Xenopus* oocytes for 5 days (Gaspar et al. 2003). Wheat TaTIP2;1 and TaTIP2;2 have NH_4^+ permeability in yeast or *Xenopus* (Jahn et al. 2004; Holm et al. 2005; Bertl and Kaldenhoff 2007), and AtTIP2;1 protein structure indicates a unique side pore through which protons can be extracted to deprotonate NH_4^+ and allow NH_3 to permeate (Kirscht et al. 2016). Currently no plant aquaporins have been found to be NO_3^- permeable, but a NO_3^- permeable mammalian aquaporin is known – AQP6 (Yasui et al. 1999; Hazama et al. 2002; Liu et al. 2005).

3.4 Diurnal Variations

Root hydraulic conductivity can change diurnally with higher conductivity observed in the daytime, which has been correlated with expression patterns of PIP aquaporins (Henzler et al. 1999). This has been shown for a number of species (Vandeleur et al. 2009; Almeida-Rodriguez et al. 2011; Hachez et al. 2008) and in some cases related to circadian oscillation in aquaporin activity or expression (Caldeira et al. 2014; Takase et al. 2011; Lopez et al. 2003) and additionally via a transpiration signal from the shoots to the root (Sakurai-Ishikawa et al. 2011; Almeida-Rodriguez et al. 2011; Vandeleur et al. 2014). Though contrary to this, transition from shade to light for several tropical tree species over a long period caused a reduction in Lp_r despite increased transpirational demand (Shimizu et al. 2005). This was correlated with a reduced inhibition by mercury indicating that root aquaporins had a diminished role after the transition to light and further growth.

It is not surprising that NO_3^- or NH_4^+ transport by roots also varies diurnally with higher rates observed in the day or light period (e.g. in soybean (Delhon et al. 1995) and citrus (Camanes et al. 2007)). In citrus the expression of a high-affinity NH_4^+ transporter in the root is regulated by sugar, independently of a circadian control (Camanes et al. 2007). In *Arabidopsis* both NH_4^+ and NO_3^- uptake by roots appears to be regulated via sugar from photosynthesis. In addition to the NH_4^+ transporters (*AMT1.1, 1.2, 1.3*), *NRT1.1* (a NO_3^- transceptor, now called NPF6.3 (O'Brien et al. 2016)) and the high-affinity NO_3^- transporter and transceptor AtNRT2.1 (see below) are both sugar (sucrose and glucose) inducible, and deletion of AtNRT2.1 results in the loss of the regulation of root NO_3^- uptake by light and sugar (Lejay et al. 2003).

In rice NRT transcripts are also upregulated by light and sugar (Feng et al. 2011). Aquaporins can be upregulated by a protein kinase sugar sensor in seedlings (see below) (Wu et al. 2013) that could lead to entrainment of root water movement to N transport and shoot photosynthesis in the light. Recently a shoot-to-root mobile bZIP transcription factor activated by light (HY5) that interacts with sugar signalling in *Arabidopsis* increased expression of *AtNRT2.1* in roots (Chen et al. 2016). It is not known if this changes expression of any aquaporins.

3.5 Temperature

Root hydraulic conductivity is also sensitive to temperature. Cucumber roots experience a decline in both root pressure and hydraulic conductivity in response to temperature reductions below 25 °C (Lee et al. 2004a). In temperature-resistant rice (Ahamed et al. 2012) and maize (Aroca et al. 2005), aquaporin activity is linked with maintenance of hydraulic conductivity and recovery. In cucumber roots exposed to lowered temperature, there was a larger reduction in the osmotic component of water flow compared with the hydrostatic component. This was associated with an affect on root aquaporins (specific type not identified), while the reduction in root pressure was associated with reduced activity of the plasma membrane proton pump (Lee et al. 2004b). The kinetics of NH_4^+ uptake across the plasma membrane of rice roots also differs with temperature where the high-affinity transport system (HATS) is more strongly reduced by low temperature than that of the low-affinity transport system (LATS) (Wang et al. 1993). The relative uptake of NH_4^+ and NO_3^- also changes at low temperature due to different temperature effects on NO_3^- and NH_4^+ transport in ryegrass (Clarkson and Warner 1979). The interaction between soil temperature, water movement and N transport is poorly understood (Pregitzer et al. 2000; Kreuzwieser and Gessler 2010), and this is important in the context of global warming and predictions of biogeochemical fluxes (Davidson et al. 2006).

3.6 Anoxia and Hypoxia

Anoxia or hypoxia in the soil as a result of soil water logging also influences water flux through roots, leading to an immediate decrease in water flux and Lp_r (Vandeleur et al. 2005), in a species-dependent way (Bramley et al. 2010), and this again can have ramifications for predications of fluxes at ecosystem scales (Shaw et al. 2013). Anoxia reduced the hydraulic conductivity of *Arabidopsis* roots, which coincided with cytosolic acidification (Tournaire-Roux et al. 2003). This acidification caused protonation of a histidine on loop D of PIP proteins, which led to a conformational change that reduced water movement. Anoxia greatly reduced the water flow of NO_3^- fed tomato, cucumber (Gorska et al. 2008a), maize (Gorska et al. 2008b) and

sunflower indicating that aquaporins are important in hydraulic responses to N in some species. Increase in reactive oxygen species under hypoxia (van Dongen and Licausi 2015; Shabala et al. 2014) may also inhibit aquaporin activity (Wudick et al. 2015). Several aquaporin transcripts are increased under anoxia/hypoxia (Bramley et al. 2007a) that may be linked to tolerance mechanisms related to gas transport (CO_2 or O_2) or signalling (Bramley and Tyerman 2010) or release of a fermentation product (Choi and Roberts 2007).

Despite being passive transporters, it is interesting to note how dependent aquaporin function is on cell energy status as indicated by one of the best inhibitors of aquaporins, azide (Postaire et al. 2010; Grondin et al. 2016), which inhibits mitochondrial respiration (Hodges and Elzam 1967). Since the uptake of NH_4^+ and NO_3^- are highly energy-dependent processes requiring a proton electrochemical gradient established by the H^+-ATPase to drive their influx, it is not surprising that anoxia and soil water logging have profound effects on N uptake, e.g. wheat (Herzog et al. 2016; Robertson et al. 2009), probably due to collapse of pH gradients (Felle 2005). There are however differences between species in N uptake under flooding and in the amount of NH_4^+ versus NO_3^- taken up (Kreuzwieser et al. 2002) partly related to NO_3^- becoming less available relative to NH_4^+ in water logged soils due to decreased nitrification (Laanbroek 1990). An acidification of root cell cytoplasm, that can result from anoxia (Felle 2005), could increase NO_3^- efflux via the AtNAXT NO_3^- transporter (Segonzac et al. 2007) exacerbating the reduced availability in the soil. NO_3^- is particularly interesting in relation to the integration of water and N fluxes because of its stimulatory effect on root water movement (examined below) (Gloser et al. 2007) via aquaporins (Gorska et al. 2008b) and because of the ameliorative effects of NO_3^- and nitrite on cytosolic pH regulation under anoxia (Libourel et al. 2006). Recently it was shown that the *Arabidopsis* NRT1.1 is required for acid tolerance, and when knocked out, the roots were unable to increase the pH of the external medium when acidic. This was linked to NO_3^- uptake rather than NO_3^- sensing (Fang et al. 2016).

3.7 Energy and Nutrient Signalling

Energy and nutrient sensing in plant cells is also an area of potential overlap between NO_3^- transport and assimilation and aquaporin activity, especially since PIP aquaporins seem to be energy dependent. The TOR (target of rapamycin) and SnRK1 (sucrose non-fermenting-1 (SNF1)-related kinase 1) signalling pathways interact to regulate metabolism and development in response to energy supply and nutrition (Robaglia et al. 2012). SnRK1 is a central energy sensor important for metabolic homeostasis in plants (Baena-Gonzalez et al. 2007; Baena-González and Sheen 2008) and is partly controlled via trehalose-6-phosphate sensing among other signals (Jossier et al. 2009; Emanuelle et al. 2016). It is activated under energy-deficient conditions, e.g. by hypoxia and carbon starvation (Cho et al. 2016; O'Hara et al. 2013), and inhibits NO_3^- reductase activity via

phosphorylation (Sugden et al. 1999). Out of the 1,021 genes transcripts reported to be upregulated by SnRK1.1 in *Arabidopsis*, only 16 are channels or transporters, and three of these are aquaporins (AtTIP2;1, AtTIP1;3 and AtPIP1;3) (Baena-Gonzalez et al. 2007). AtTIP2;1 is of special interest because of its ability to facilitate the diffusion of NH_4^+ directly or indirectly as NH_3 (Holm et al. 2005; Kirscht et al. 2016) and its correlation to stomatal conductance (Pou et al. 2013). The TOR protein kinase on the other hand is a regulatory hub involved in nutrient sensing (Dobrenel et al. 2016). The TOR pathway is proposed to increase N assimilation under energy and nutrient-replete conditions (Dobrenel et al. 2016; Lillo et al. 2014). Protein phosphatase 2A is a component of the TOR pathway via the regulatory subunit (Tap46). When Tap46 is overexpressed in *Arabidopsis*, this results in growth stimulation, increased NO_3^- reductase activity and induction of transcription of genes involved in N transport including AtNRT1.1 (AtNPF6.3) and AtNRT2.1 (Ahn et al. 2015). Both are components of the NO_3^- transceptor signalling cascade, which regulates the primary nitrate response in plant roots (O'Brien et al. 2016) with AtNRT2.1 proposed to have a role in influencing root aquaporin activity in response to NO_3^- (see below) (Li et al. 2016).

Sugar, and hence energy supply, also regulates aquaporins in *Arabidopsis* via a sucrose-activated receptor kinase (SIRK1) in whole seedling plasma membrane, able to phosphorylate aquaporins and thereby positively affecting their activity (Wu et al. 2013). Several PIP aquaporins were shown to interact with SIRK1 including those that have been shown to be associated with Lp_r (e.g. AtPIP2;1) (Wudick et al. 2015; Sutka et al. 2011). Sugar signalling in plants is linked to C:N balance and root/shoot coordination with cross talk with hormone signalling (Wang and Ruan 2016). Altogether these data suggest that aquaporins could be added as an important component of nutrient signalling pathways, but location in roots needs to be determined for SIRK1.

3.8 Co-location of Water and N Uptake

For mass flow to have an effect on NO_3^- transport to the root, it would be expected that the location of NO_3^- influx along the axis of the root or in different root classes should correspond to the location of the maximum volume-flux density of water inflow (Gorska et al. 2008b). It is not a trivial exercise to determine where along the axis of roots or the type of roots where water inflow is maximal. Work with lupin and wheat has indicated contrasting locations for water flow based on the scaling of root hydraulic conductance with root length (Bramley et al. 2009). Wheat tends to have a greater concentration of water flow near the root tip and is more dependent on aquaporins than that for lupins (Bramley et al. 2009). Using neutron radiography to image deuterated water movement in and through roots in soil during transpiration with a model to separate convection from diffusion, Zarebanadkouki et al. (2014) concluded for lupin roots that radial flow into roots was maximal 12–16 cm from the root tip of lateral roots. For maize roots lateral roots show a greater uptake

of water relative to that of primary and seminal roots as determined by the same technique (Ahmed et al. 2016). It was concluded that lateral roots were the primary sites of water absorption and that primary and seminal roots functioned to axially transport water to the shoot. Since whole maize roots have been shown to have a positive response of water movement to NO_3^- (Gorska et al. 2008b), it would be expected from the results of Ahmed et al. (2016) that NO_3^- uptake would occur mainly through lateral roots. NO_3^- uptake kinetics measured for different root classes at two plant ages and in response to NO_3^- deprivation showed large differences, with lateral roots giving the highest affinity (lowest Michaelis-Menten constant, K_m) and highest maximum rate of uptake, which depended on plant age (York et al. 2016). Interestingly linear uptake kinetics indicating LATS transport of NO_3^- were not observed for laterals but occurred in all other root classes. Modelling of plant growth based on these kinetics and taking into account mass flow to the roots indicated that the maximum rate of influx was important in determining plant growth rather than the concentration kinetics (York et al. 2016).

Ion sensitive electrodes have been used to scan NH_4^+ and NO_3^- fluxes at finer scale along roots. In young maize roots, NO_3^- influx reached a maximum at 3.5–8.5 mm from the root tip and then remained more or less constant with distance to 21–61 mm (limit of measurements) (Taylor and Bloom 1998). NH_4^+ influx was high at the root tip and increased with distance but was about 1/3 that of NO_3^-. It was concluded that NH_4^+ was most actively taken up at the root tip reflecting the energy advantage over NO_3^-. In fine wheat roots, highest net fluxes of NH_4^+ and NO_3^- occurred at 20–25 mm from the root tip (Zhong et al. 2014), which would correspond to maximum hydraulic conductivity measured by Bramley et al. (2009). In rice, maximum net influx of NH_4^+ and NO_3^- occurred at 10–20 mm from the apex and then declined (Colmer and Bloom 1998). This was different from maize since maize did not show a decline with distance. It was concluded that sclerenchymatous fibres on the outer side of the cortex of older parts of the rice root could impede influx. However, later measurements of water flow across this region indicated that it was largely determined by apoplastic flow (Ranathunge et al. 2004). Examination of NO_3^- uptake kinetics, expression of *ZmNRT2.1* and H^+-ATPase activity along maize primary roots using a compartmental system showed that the root tip (0–4 cm) had higher capacity to take up NO_3^- due to higher maximum influx and more rapid induction of a high-affinity transport system (Sorgona et al. 2011). This did not correlate with the expression of *ZmNRT2.1*, which was highest at 4–6 cm from the root tip. To date there are no studies that we are aware of that have attempted to define water and NO_3^- or NH_4^+ fluxes simultaneously with root position or root type, and this data is required to better understand the link between water and N uptake.

In *Arabidopsis* specific NRT2-HATS transporters are associated with root development, plant age and specific cell types (Kiba and Krapp 2016). AtNRT2.4 and AtNRT2.5 are expressed in a polarised manner in epidermal cells (facing the external medium) with a greater level of expression of AtNRT2.4 in young plants and AtNRT2.5 in mature plants (Lezhneva et al. 2014; Kiba et al. 2012). *AtNRT2.4* is lowly expressed with high NO_3^- and is only increased to levels equivalent to *AtNRT2.1* without NO_3^- in young plants (Kiba et al. 2012). In mature plants

AtNRT2.1 has higher expression than both *AtNRT2.4* and *AtNRT2.5* after 1 day of N starvation but then declines with *AtNRT2.5* and *AtNRT2.4* expression increasing to dominate by 10 days (Lezhneva et al. 2014). Many experiments investigating the link between root water transport and NO_3^- transport initially starve plants of N or have them grown in low NO_3^- for various periods, then resupply with NO_3^- to examine the response in water transport. In this case HATS NRT2s would be induced and may account for a portion of the NO_3^- uptake when NO_3^- is introduced to the roots. However, the concentrations of NO_3^- used are generally well above the HATS concentration range and would require NRT1-LATs transporters or anion channels to carry the load of NO_3^- uptake. In roots of *Arabidopsis*, these would be AtNRT1.1, AtNRT1.2 (AtNPF4.6) and AtNAXT1 (AtNPF2.7) (O'Brien et al. 2016) or possibly an anion channel (initially before cytoplasmic NO_3^- increases) (Skerrett and Tyerman 1994). The location of the inducible LATS transporter *AtNRT1.1* mRNA is in the epidermis near the root tip and in the cortex or endodermis in more mature regions (Huang et al. 1996). Likewise the constitutively expressed *AtNRT1:2* (*AtNP4.6*) is located in root hairs and epidermis in mature regions of roots.

The location of AtNRT2.1 as a NO_3^- sensor that regulates PIP activity in *Arabidopsis* (Li et al. 2016) makes sense since mass flow of water to the root would be expected to build NO_3^- concentration in the apoplast of cortex cells due to endodermal barriers blocking apoplastic movement. However, being a high-affinity transporter with saturation occurring around 0.5 mM NO_3^-, one would expect that mass flow under conditions of NO_3^- resupply (often several millimolar NO_3^-) would require a LATS transport or dual-affinity transporter to do most of the influx, i.e. AtNRT1.1 and AtNRT1.2. The locations of these transporters (discussed above) would match with our present view of high water transport intensity, particularly in the endodermis and epidermis.

4 N Form and Amount Affects Root Hydraulic Properties

4.1 Growth and Development

Over the long term, N affects both root growth and development (Wilson 1988; Robinson 1994; Lopez-Bucio et al. 2003; Guo et al. 2007c; Desnos 2008). In *Arabidopsis* local patches of NO_3^- promote lateral root outgrowth via NO_3^--inducible MADS-box gene (ANR1) and coordinated by a systemic signal dependent on the plant's N status (Zhang and Forde 2000). N responses on root growth can affect different properties in different species. Górska et al. (2010) showed that cucumber, tomato, maize, *Arabidopsis* and *Festuca arundinacea* have increased relative root growth on 2 mM NO_3^- compared to 0.2 mM, whereas *Populus trichocarpa* and *Nephrolepis exaltata* do not have a relative root growth response. Tomato, maize, *Arabidopsis* and *Festuca arundinacea* also have increased root/shoot ratios on low NO_3^- nutrition, whereas cucumber, *Populus trichocarpa* and *Nephrolepis exaltata* do not.

The form of N available to roots impacts growth and development as well. French bean grown in NO_3^- as sole N source has larger roots and shoots than when grown on NH_4^+ (Guo et al. 2002). These results were repeated with split root experiments where one half of the root was grown on NO_3^- and the other NH_4^+ (Guo et al. 2007b). This demonstrated a repression of growth of roots grown in NH_4^+. In experiments where half the roots were grown on N (NH_4^+ or NO_3^-) and the other half starved of N, either form resulted in increased growth. Still NO_3^--grown roots had increased growth. In tomato, roots grown in NH_4^+ have stagnated growth over 3 weeks compared to plants grown in NO_3^- (Pill and Lambeth 1977).

Stimulation of lateral root growth in response to NO_3^- is regulated by multiple pathways related to initiation of the lateral root, early development and elongation of lateral root primordia (Forde 2014). There is an interaction between auxin and NO_3^- signalling in this process. Aquaporins also play a role in lateral root outgrowth. Auxin-mediated repression of AtPIP2;1 accounts for reduced polarised water flow into overlying tissue for optimum extension (Peret et al. 2012), while the tonoplast located aquaporins AtTIP1;1, AtTIP1;2 and AtTIP2;1 are also involved in the development of lateral root primordia in a complex manner, each isoform having different spatio-temporal expression patterns (Reinhardt et al. 2016). It is yet unknown if these aquaporins also play similar roles in lateral root outgrowth under NO_3^- stimulation. AtTIP2;1 is particularly interesting given that it complemented the triple *tip1;1/1;2/2;1* knockouts (Reinhardt et al. 2016) and that its expression can be linked to N and energy signalling (Baena-Gonzalez et al. 2007). However, neither *AtTIP2;1* nor *AtPIP2;1* comes up as regulated genes in local NO_3^- stimulation in wild-type *Arabidopsis* or the NO_3^- signalling mutant of NRG2 (Xu et al. 2016), while *AtTIP1;1, 2;2* and *2;3* are subject to changes in expression under local NO_3^- supply (Li et al. 2014; Ruffel et al. 2011).

Maize cultivars show large differences in the response to NO_3^- of lateral root outgrowth both in terms of homogeneous and locally high NO_3^- (Yu et al. 2016). In this study pericycle cell transcriptomes were examined for all root classes and compared between locally high NO_3^- and uniform low NO_3^-. The transcript responses were root-class dependent with brace roots showing the greatest response of some 2,740 NO_3^--regulated genes with unique cell-cycle control genes being strongly represented. Brace roots would be worthy of further physiological studies to examine the water: NO_3^- link.

4.2 Root Hydraulics

Root hydraulic properties are sensitive to N form and concentration and are dependent on species and length of treatment. For many species, an increase in water flow and hydraulic conductivity are observed. For example, in wheat, plants grown on NO_3^- 0.5–16 mM, de-topped root exudation rates are increased in a dose-dependent manner with increasing NO_3^- (Barthes et al. 1996). When starved of NO_3^- for 5 days, wheat plants showed a decrease in hydraulic conductivity and water flow

(Carvajal et al. 1996a). Resupplying NO_3^- to N-starved wheat caused quick increase in flow and hydraulic conductivity, resulting in a return to control values by 24 h (Carvajal et al. 1996b). A rapid stimulation of Lp_r by NO_3^- is not always observed (Górska et al. 2010), for example, in the tree *Eucalyptus grandis*, no root response was observed, though in this species there was a longer-term increase in whole plant hydraulic conductance when gown on high N (Graciano et al. 2016).

It may be that the root cellular concentration of NO_3^- plays an important role in these responses. Tomato and cucumber both increased water flow through roots in response to NO_3^- (Gorska et al. 2008a). This was observed at the cellular level as well using a cell pressure probe to measure the half-time of water flow to applied pressure relaxations. The half-time fell when roots were exposed to NO_3^-, indicating water flowed with smaller resistance in these cells. In the presence of tungstate, a NO_3^- reductase inhibitor, tomato root responses at the cellular and whole root levels were unaffected, but the response of cucumber roots and cells was eliminated. It was shown that tomato roots maintained NO_3^- uptake, but tungstate treatment inhibited uptake of NO_3^- by cucumber roots. Using the cell pressure probe, injecting NO_3^- directly into cucumber root cells treated with NO_3^- reductase recovered the cellular response. In maize, a delay is observed between NO_3^- application and increase in hydraulic flow (Gorska et al. 2008b). Tungstate application to maize roots results in a decreased NO_3^- uptake rate over 4 h and also eliminates the hydraulic response to NO_3^-. Tungstate application greatly inhibited *PIP* expression not seen in starved plants, indicating tungstate may disrupt many cellular pathways (Gorska et al. 2008b). In this case, diurnal patterns in maize root sap flow and osmolality (Lopez et al. 2003) or de-topping effects may have confounded the hydraulic response to increased cellular NO_3^- levels.

Observations over several species show that a species' NO_3^- uptake rate is linearly correlated with the magnitude of the hydraulic response (Górska et al. 2010). In these experiments, species with high NO_3^- uptake rates had large increases in water flow after NO_3^- application, while species with low NO_3^- uptake rates lacked a hydraulic response to NO_3^- (Górska et al. 2010). Cellular NO_3^- levels may have been important in the hydraulic response to NO_3^- in these plants. The link between NO_3^- uptake rate and hydraulic response also supports the mass flow hypothesis for the basic hydraulic response described in Cramer et al. (2009a). Larger NO_3^- uptake rates are quicker to deplete the root zone of NO_3^- , and mass flow could be a mechanism to quickly resupply NO_3^- pools near the root surface when NO_3^- is present (Gorska et al. 2008b).

4.3 NO_3^- Versus NH_4^+ in Regulation of Root Hydraulics

The form of N supplied affects root hydraulic responses and is species dependent. In French bean, roots exposed to NO_3^- (whole root or in split root) have increased water uptake compared to roots exposed to NH_4^+. Water uptake in root systems split between NO_3^- and zero N has more total uptake from the NO_3^- fed roots, but when normalised by root volume, starved roots absorb more water per root volume. Under

NH_4^+ or starvation, roots absorb more water and more water per unit root volume on the starvation side. Root volume and dry mass are larger when in NO_3^- or NH_4^+ compared to starvation and was more pronounced during the day than at night (Guo et al. 2002, 2007a, b).

Ammonium nutrition has mixed results in other species. Like French bean, maize NO_3^- resupply led to an increase in water flow through the root in N starved plants, whereas urea and NH_4^+ had slightly negative effects on water flow (Gorska et al. 2008b). In tomato, application of either NO_3^- , NH_4^+ or $NH_4^+ + NO_3^-$ increased the total exudate from de-topped plants, with NH_4^+ having a slightly larger effect than NO_3^- (Minshall 1964). Clover plants grown in 5 mM NO_3^- had a slight increase in Lp_r compared to 0.5 mM, but no increase was seen with NH_4^+. Contrasting to this, ryegrass had a 300 % increase to either 5 mM NO_3^- or NH_4^+ compared to 0.5 mM (Belastegui-Macadam et al. 2007).

In rice, NO_3^- and NH_4^+ have mixed effects on plant hydraulics. In cultivar "Shanyou63" hybrid *indica*, root dry weights between NO_3^- and NH_4^+ fed plants are not different (Li et al. 2009; Yang et al. 2012), whereas root fresh weight is significantly greater for NO_3^- grown plants (Gao et al. 2010). When normalised to fresh weight, the water uptake was found to be significantly greater for NH_4^+ grown plants in two out of three of the studies (Li et al. 2009; Yang et al. 2012) and that total root uptake of water (not normalised) was greatest for $NH_4^+ + NO_3^-$ fed plants, intermediate for NO_3^- and least with NH_4^+ (Gao et al. 2010). All three of these studies show that for NO_3^- fed plants, xylem water flow and total water uptake (normalised or not) decrease by greater amounts during water stress (PEG). These studies used the same rice cultivar and were treated with different N forms for up to 2 weeks potentially masking the hydraulic effects behind growth changes. In mixed N (NH_4^+ NO_3^-) treatment, lowering N from 20 to 0.2 ppm led to a decrease in xylem sap flow and hydraulic conductivity over 3 days (Ishikawa-Sakurai et al. 2014). Upon resupply, xylem sap flow rate and hydraulic conductivity were raised. Interestingly, the initial response for starvation or resupply was opposite from the final response. The first 6 h of starvation saw an increase in the hydraulic conductivity and xylem flow rate in N-starved plants. At 24 h, rates began to fall compared to plants in full N treatment. Between 0 and 6 h, N-fed plants showed a slight increase in xylem sap flow but a decrease in Lp_r. By the next light period, a large increase in both root xylem sap flow and Lp_r were observed. This highlights that the magnitude of hydraulic response to N is time dependent and suggests that the direction of change may be time dependent as well probably linked to circadian and diurnal regulation.

Further study on rice comparing two cultivars that have different water relations responses to N supply during growth showed a strong increase in Lp_r with increasing N supply (as equal NH_4^+ and NO_3^-) with larger responses observed for Yangdao6 than Shanyou63 (Ren et al. 2015). This difference was reflected in the response of leaf water potential, which became more negative in Shanyou63 and was constant in Yangdao6 with increasing N. It was concluded that the increase in Lp_r was due to a combination of reduced aerenchyma and lignin as well as increased PIP aquaporin expression in roots (see below). The greater response of Yangdao6 was linked to aquaporin expression and more reduced aerenchyma (Ren et al. 2015).

4.4 Transcriptional Responses of Aquaporins in Response to N

Aquaporins undoubtedly play a role in the N responses as indicated above. Transcriptional responses of aquaporins to changes in external N status, if they occur, tend to take days to become evident. Caution must be used, as transcription and protein amount are not always correlated (Hachez et al. 2012). In tomato, increased hydraulic conductivity to N is seen quickly (Gorska et al. 2008a, 2010), but aquaporin gene responses were not seen before 48 h of treatment (Wang et al. 2001). Exposing *Arabidopsis* to NO_3^- from NH_4^+ growth for 20 min only repressed *AtNIP2;1* expression (see also chapter "Plant Aquaporins and Metalloids") but left all other aquaporin levels the same (Wang et al. 2003). In *Arabidopsis* resupply of NO_3^- to N-stressed plants strongly induced a *TIP* member, and several others weakly (Scheible et al. 2004). In maize, NO_3^- addition was not reported to change *PIP* gene expression within 4 h, while tungstate treatment greatly inhibited the expression of most *PIP* genes (Gorska et al. 2008b). Eight hours after NO_3^- addition, *ZmPIP1-5b* transcript level was strongly induced (Gaspar et al. 2003). In French bean, NO_3^--induced *PIP1* expression occurred around 4 days after addition, but no *PIP1* gene induction was seen with NH_4^+ (Guo et al. 2007a). In rice, switching from 10 ppm NH_4^+ + NO_3^- to 0.5 ppm led to a slight repression of *OsPIP1;1*, *OsPIP2;3*, *OsTIP1;1* and *OsTIP2;2* expression. Major reduction in expression was seen for *OsPIP2;4* and *OsPIP2;5* with induction for *OsTIP2;1* and *OsPIP2;6*. During resupply, *OsPIP2;4*, *OsPIP2;5* and *OsTIP2;2* gene transcription was largely induced. Several other genes were slightly induced. This correlated with decreased hydraulic conductivity during starvation and increased during resupply (Ishikawa-Sakurai et al. 2014).

5 Post-translational Regulation of Aquaporins in Response to N

Aquaporin blockers result in reduced hydraulic conductivity under N treatments of wheat (Carvajal et al. 1996b), barley (Ruggiero and Angelino 2007) and figleaf gourd (Rhee et al. 2011). An investigation of the link between Lp_r and aquaporin protein abundance and post-translational responses in *Arabidopsis* revealed that AtPIP2 protein amount correlated with Lp_r responses to various treatments (Pietro et al. 2013). A higher correlation was observed with the PIP phosphorylated peptides. In contrast PIP1 and TIP abundance did not correlate with Lp_r. N starvation tended to have the largest impact on PIP protein abundance. They also observed differences in deamination in PIP2 protein fragments, suggesting a role in post-secondary modification of aquaporins during the response. Largely, however, these pathways, including pH-mediated gating, localization and phosphorylation, have remained unexplored in N regulation of plant hydraulics (Pietro et al. 2013). Other connections in this regard between NO_3^- signalling and factors that regulate

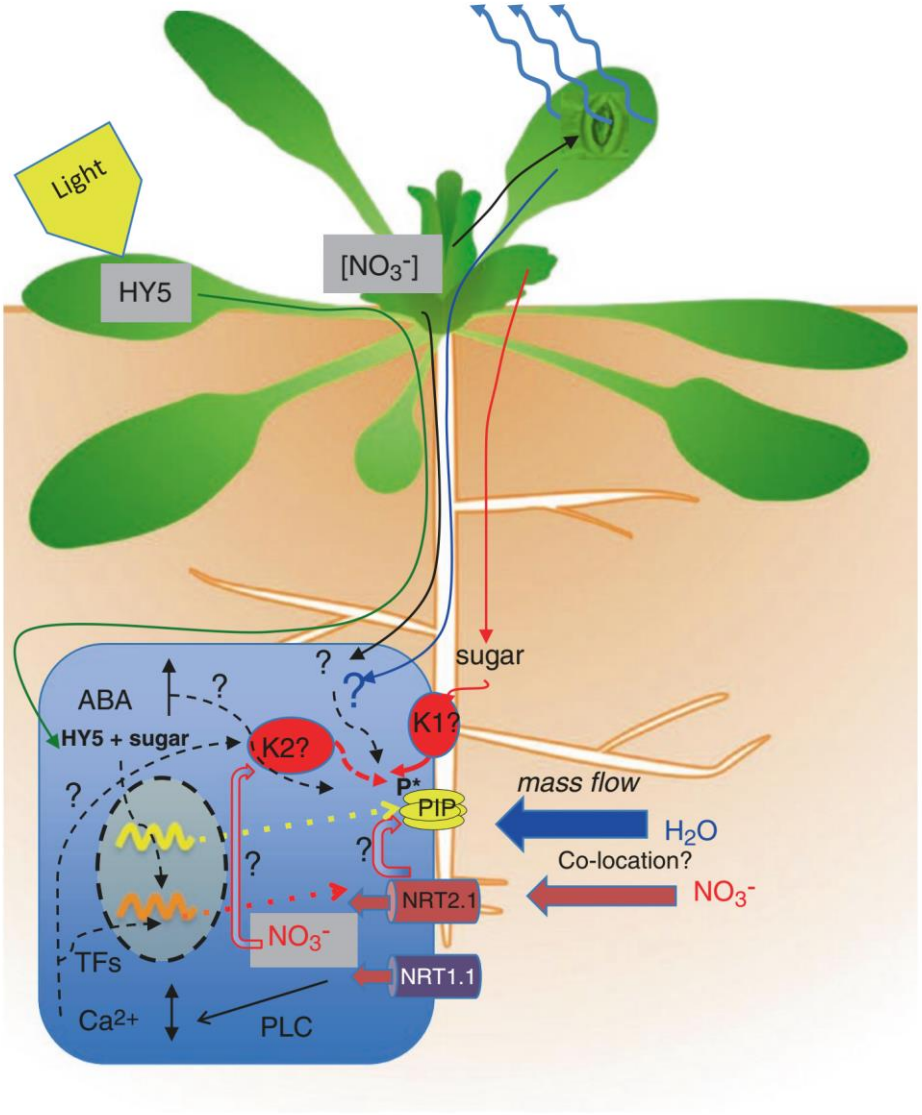
aquaporins include elevation of ABA in certain cells (Ondzighi-Assoume et al. 2016) and transient increased cytosolic Ca^{2+} concentration (Riveras et al. 2015) in response to NO_3^-. The Ca^{2+} response was abolished in mutants of NRT1.1 (NPF6.3). Both cytosolic Ca^{2+} and ABA are known to regulate aquaporins (Wan et al. 2004; Alleva et al. 2006).

Global protein phosphorylation patterns have been examined in N-starved *Arabidopsis* seedlings when resupplied with either NO_3^- or NH_4^+ compared with potassium chloride after 30 min (Engelsberger and Schulze 2012). Distinct phosphorylation patterns were observed for proteins that have signalling functions and transporters/channels. Phosphopeptides from aquaporins were identified, and these were rapidly dephosphorylated in response to KCl and NH_4Cl, but KNO_3 produced a weaker effect, and there was a subsequent increase in phosphorylation over time.

Recently a significant advance was made in understanding the regulation by NO_3^- of Lp_r and aquaporins in *Arabidopsis* (Li et al. 2016). Examination of the Lp_r of NO_3^- transporter mutants including the transceptors NRT1.1 (NPF6.3) (dual HATS LATS) and NRT2.1 (iHATS) showed that only the NRT2.1 knockout had reduced Lp_r. Though Lp_r was lowered in the NRT2.1 mutant, it still responded to reduced NO_3^- supply. Overall there was a better correlation between Lp_r and shoot NO_3^- concentration than root NO_3^- across all mutants and WT. Although NRT1.1 mutant was not considered further in the work, it did appear that the correlation between Lp_r and shoot NO_3^- was depressed in this mutant compared to WT. Transcripts of both *PIP1;1, PIP1;2, PIP2;1* and *PIP2;3* correlated with changes in Lp_r for NRT2.1 mutants under different NO_3^- concentrations, and both PIP1 and PIP2 protein abundance were also correlated with Lp_r. In WT, however, the regulation appeared to be mostly at the post-translational level. The conclusion from this study was that root aquaporins were regulated by a shoot-to-root signal communicating shoot NO_3^- status as well as the function of NRT2.1 (Fig. 1). It would be worthwhile examining a split root protocol to distinguish between local and systemic signals to test this model, especially since NO_3^- initially upregulates the level of the *NRT2.1* transcript after starvation and split root experiments indicate that a shoot-to-root signal is involved in transmitting shoot N-demand to changes in *NRT2.1* expression (Kudoyarova et al. 2015).

6 Conclusions and Research Questions

While not uniform, plant hydraulics are modified depending on N form and amount. This involves changes in membrane water permeability mediated by PIP aquaporins as well as root architectural modifications to facilitate N foraging in the soil. Cellular mechanisms of signalling of NO_3^- and transduction to aquaporin activity to regulate Lp_r are emerging and indicate that NO_3^- concentration in cells is a signal rather than the assimilated products. Both local and systemic (shoot-to-root) signalling are involved, but the identity of the systemic signal is unknown. NRT2.1



appears to be an important component in the signalling of NO_3^- to aquaporin responses resulting in transcriptional changes in aquaporin genes, but mainly post-translational changes affecting aquaporin activity (Fig. 1). There are parallel regulation paths via translocated sugar from the shoot on both NO_3^- transporters and probably aquaporins. If mass flow of NO_3^- to the root surface is the functional outcome of increased aquaporin activity, then major NO_3^- transporters and aquaporins involved need to be co-located in the root. This co-location need not be in the same cell type but in the relevant root class (i.e. laterals) and similar position along the root axis. Single-cell transcriptomics combined with sensitive techniques to resolve fine spatial and kinetic responses in NO_3^- and NH_4^+ transport and water

Fig. 1 Regulation of root PIP aquaporins involves specific and probably parallel pathways of local and systemic signalling. Local: The presence of NRT2.1 is required for maintaining a high Lp_r in *Arabidopsis* (Li et al. 2016), and while the signalling cascade to PIP aquaporins is unknown, it is likely to be via phosphorylation by an unidentified kinase(s) (denoted K2) (Engelsberger and Schulze 2012). NRT1.1 is also required for nitrate signalling and regulation of expression of NRT2.1 (Bouguyon et al. 2015) (short-term upregulation and long-term downregulation) via a cytosolic Ca^{2+} signal involving phospholipase C (PLC), and various transcription factors are activated (TFs) (O'Brien et al. 2016; Riveras et al. 2015). Although Ca^{2+} is known to directly regulate PIP aquaporins via gating (Verdoucq et al. 2008), the presence of NRT1.1 was not required for Lp_r responses to NO_3^- in *Arabidopsis* (Li et al. 2016). Abscisic acid (ABA) is also elevated in certain cells (endodermis) of the root tip of *Arabidopsis* in response to NO_3^- (Ondzighi-Assoume et al. 2016). ABA has been shown to have a positive effect on root cell water permeability by an unknown but rapid mechanism (Wan et al. 2004; Olaetxea et al. 2015). Transcription and translation to increase protein abundance are indicated as *dotted arrows* to PIPs and NRTs. *Systemic*: Shoot NO_3^- positively correlates with Lp_r in *Arabidopsis*, and unknown shoot-to-root signal was suggested (*black arrow*) (Li et al. 2016). Sugar has been shown to activate a kinase (K1? = SIRK1), which phosphorylates PIP aquaporins to activate them in *Arabidopsis* seedlings (Wu et al. 2013), and it is assumed that this occurs in roots in the diagram (red arrow, K1?). Sugar signals also regulate root nitrate uptake and NRT2.1 (Wang and Ruan 2016). A shoot-to-root mobile bZIP transcription factor activated by light (HY5) that interacts with sugar signalling in *Arabidopsis* increases expression of *AtNRT2.1* in roots (*green arrow*) (Chen et al. 2016). NO_3^- is also a signal to control stomatal aperture (Wilkinson et al. 2007), which will impact on transpiration and thence mass flow to the root. Also an unidentified shoot-to-root signal (*blue arrow*) conveying transpiration or leaf water potential regulates Lp_r in rice (Sakurai-Ishikawa et al. 2011) and probably other plants (Vandeleur et al. 2014); this could be ABA in wheat (Kudoyarova et al. 2011). Not shown in the diagram is the probable signalling involving NH_4^+, the role of certain aquaporins in lateral root initiation in response to NO_3^- and outgrowth that is important for NO_3^- foraging in soil, nor the relative expression patterns of NO_3^- transporters in the root that may influence the efficiency of coupling of water flow with PIP-activated increase in Lp_r. Other hormones involved in N signalling are also not included, and some of these, e.g. auxin, have also been demonstrated to have effects on aquaporin regulation (Peret et al. 2012)

movement will potentially resolve the location issue and genes involved in signalling. Research questions include:

- How is the cellular NO_3^- signal perceived and transduced to give relatively rapid changes in kinase and phosphatases that target PIP aquaporins?
- Is there a direct effect of NO_3^- and/or NH_4^+ on PIP and TIP aquaporins? Given that an animal aquaporin can transport NO_3^- and one TIP aquaporin (AtTIP2;1) has a structure to facilitate NH_4^+ transport, it would be wise to consider that both NH_4^+ and NO_3^- may have direct effects to regulate water flow through some aquaporins that could be tested in heterologous expression systems. Responses via changes in cytosolic pH have also not been thoroughly explored.
- What is the systemic signal that regulates aquaporin response to shoot N? For example, do shoot-derived microRNAs such as miR172c (Wang et al. 2014) or shoot-derived transcription factors such as HY5 (Chen et al. 2016) have a role in root aquaporin expression?
- Is there a control via NO_3^- signalling on the key PIP and TIP involved in lateral root growth in response to local NO_3^- concentration?

- Fine scale mapping is required of water flow along a root that could be used in conjunction with ion flux measurement and gene expression to correlate with NO_3^- and NH_4^+ fluxes. Even more interesting would be to map this with energy consumption during the process of N and water uptake.
- The role of the root rhizosphere including its physical, chemical and microbial characteristics needs to be investigated in relation to N and aquaporin signalling and the effective delivery of both water and solutes to the root surface.

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