Functional Characterization of Nitrate Transporters in Maize

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I. Abstract

Nitrate is an essential nutrient for plant growth. Nitrate acquisition by roots and its intercellular translocation is mediated by nitrate permeable transport proteins. Nitrate transporters have been extensively studied in the model plant, *Arabidopsis thaliana*. Nitrate transporters belong to three protein families: NPF (Nitrate Transporter 1/Peptide Transporter), NRT2 (Nitrate Transporter 2) and CLC (Chloride Channel) (Miller et al., 2007; Wang et al., 2012). However, there is little known about how these proteins orchestrate nitrate transport in maize.

Four putative nitrate transporter genes (*ZmNPF6.4*, *ZmNPF6.5*, *ZmNPF6.6*, and *ZmNPF7.10*) were cloned from a maize root cDNA population. Preliminary localization studies using C-terminal YFP-fusions showed maize NPF proteins targeting to the plasma membrane, with the exception of ZmNPF7.10, where targeting could not be resolved. Gene expression studies indicated *ZmNPF6.6* was induced strongly in roots by nitrate. Its shoot expression was mostly absent. In contrast, *ZmNPF6.4* exhibited a constitutive expression pattern in both root and shoot tissues and was not sensitive to nitrate. Both ZmNPF6.5 and ZmNPF7.10 showed little expression in either root or shoot tissues.

Functional characterization studies were conducted on ZmNPF6.4 and ZmNPF6.6 as there was no nitrate transport activity measured with ZmNPF6.5 and ZmNPF7.10 using a preliminary screening experiment in *Xenopus laevis* oocytes. Combining electrophysiology and chemical flux analysis, ZmNPF6.4 was characterized as a pH-dependent, low-affinity, non-selective nitrate and chloride transporter. On the other hand, *ZmNPF6.6* encoded a pH-dependent, dual-affinity, nitrate specific transporter, which was also permeable to chloride in the absence of nitrate. The functional differences between ZmNPF6.4 and ZmNPF6.6 were explored using site-directed mutagenesis experiments. The “affinity switch” Thr101 within the nitrate transceptor, AtNPF6.3, is conserved in ZmNPF6.6

(Thr104) (Liu, 2003). However, mutating ZmNPF6.6:Thr104 to alanine or aspartate (dephosphorylation and phosphorylation mimics, respectively), did not transform the dual-affinity transporter into either a high- or low-affinity monophasic transporter. Instead, both HATS and, predominantly, the LATS activities of ZmNPF6.6 were repressed by both T104A and T104D mutations. The equivalent of the predicted nitrate-binding residue in AtNPF6.3 (His356) was investigated in ZmNPF6.4 and ZmNPF6.6. In ZmNPF6.4, a tyrosine residue (Tyr370) is present instead of a histidine. Replacement of Y370 with histidine (ZmNPF6.4:Y370H) conferred dual-affinity nitrate transport and enhanced nitrate specificity over chloride. However, replacing His362 in ZmNPF6.6 with Tyr362 made the transporter non-functional.

A preliminary analysis of the high-affinity nitrate transport system was conducted by functionally characterizing *ZmNRT2.1* and *ZmNRT3.1A*. The plasma membrane targeting of ZmNRT2.1 required the presence of ZmNRT3.1A. This was confirmed using a C-terminal fusion of NRT2.1 with YFP. Signal was only detected in onion epidermal cells that were co-transformed with both *ZmNRT2.1* and *ZmNRT3.1A*. Gene expression analysis identified both a N-starvation induced expression and a nitrate induced expression pattern for *ZmNRT2.1*. In contrast, *ZmNRT3.1A* exhibited a constitutive expression in both roots and shoots. When ZmNRT2.1 and ZmNRT3.1A were co-injected into *Xenopus laevis* oocytes, high-affinity nitrate transport activity was measured. Single injections of either cRNA failed to elicit a nitrate transport phenotype.

II. Declaration

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

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Zhengyu Wen

III. Acknowledgements

Finally, I am at the very end of my PhD journey. When I look back, I see a path full of joy and happiness blended with sorrow and sadness. It was not an easy way and I do not think I can make this far without help from people around me. Therefore, I would like to sincerely acknowledge them here.

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IV. Abbreviations

~	Approximately
3'	Three prime of nucleic acid sequence
ABA	Abscisic acid
BLAST	Basic Local Alignment Search Tool
C-terminal	Carboxyl terminal
CBL	Calcineurin B-like molecule
cDNA	Complementary deoxyribonucleic acid
cHATS	Constitutive HATS
CIPK	CBL interacting protein kinase
cLATS	Constitutive LATS
CLC	Chloride Channel
cm	Centimeter
CRISPR	Clustered regularly interspaced short palindromic repeats
cRNA	Capped RNA
Ct	Threshold cycle
DNA	Deoxyribonucleic acid
DW	Dry weight
ECFP	Enhanced Cyan Fluorescent Protein
g	Grams
GOGAT	Glutamate-oxoglutarate aminotransferase
GS	Glutamine synthetase

h	Hour
HATS	High-affinity transport system
HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid
iHATS	Inducible HATS
iLATS	Inducible LATS
IRMS	Isotope Ratio Mass Spectrometer
kg	kilogram
LATS	Low-affinity transport system
M	Molar
MES	2-(N-Morpholino) ethanesulfonic acid, 4-morpholineethanesulfonic acid
MFS	Major Facilitator
mg	Milligram
Min	Minute
mM	Millimolar
mmol	millimole
mRNA	messenger RNA
N	Nitrogen
NPF	Nitrate Transporter 1/Peptide Transporter
NRT	Nitrate Transporter
NUE	Nitrogen Use Efficiency
NUtE	Nitrogen Utilization Efficiency
PCR	Polymerase Chain Reaction

qPCR	Quantitative PCR
RNA	Ribonucleic acid
RNaseA	Ribonuclease A
SDS	Sodium Dodecyl Sulphate
SEM	Standard error of the mean
TALENs	Transcription activator-like effector nucleases
TM	Transmembrane Domain
UTR	Untranslated region
v/v	volume/volume
w/v	weight/volume
YFP	Yellow Fluorescent Protein
ZFNs	Zinc finger nucleases
µM	Micromolar
µmol	Micromole