Functional characterisation of *GmSALT3*, a candidate gene for conferring salt tolerance in soybean [*Glycine max* (L.) Merr.]

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A thesis submitted for the partial fulfilment of the requirements for the degree of Doctor of Philosophy

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2017

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Abstract

Soybean (*Glycine max* (L.) Merrill) is native to East Asia, which includes China that has a cultivation history stretching back at least 5,000 years. Now soybean is widely cultivated around the world as an important crop. It is an annual plant and its seeds are processed to produce two major products, oil and meal. Many biotic and abiotic stresses threaten soybean production in different areas of the world, such as fungal, bacterial and viral diseases; aluminium, drought, and salinity. In this thesis, the focus is on investigating the salinity stress responses in soybean and how *GmSALT3* (salt tolerance-associated gene on chromosome 3), a dominant gene that is associated with limiting the accumulation of sodium ions in shoots, contributes to soybean's salinity tolerance.

GmSALT3 was identified through fine-mapping; it encodes a protein from the cation/H^+ exchanger (CHX) family that I localized to the endoplasmic reticulum (ER) and which is preferentially expressed in the salt-tolerant parent Tiefeng 8 within root cells associated with phloem and xylem. In the salt-sensitive parent, 85-140, a 3.78-kb copia retrotransposon insertion in exon 3 of *Gmsalt3* was identified that truncates the transcript. In addition, nine haplotypes including two salt-tolerant haplotypes and seven salt-sensitive haplotypes were identified by sequencing 31 soybean landraces and 22 wild soybean (*Glycine soja*) cultivars in China. By analysing the distribution of haplotypes, it was found that haplotype 1 (H1, found in Tiefeng 8) was strongly associated with salt tolerance and is likely to be the ancestral allele. H1, unlike other alleles, has wide geographical range including saline areas, which indicates it is maintained when required but its potent stress tolerance can be lost during natural selection and domestication.

Then, I evaluated the impact of *GmSALT3* on soybean performance under saline or non-saline treatments, with both field and controlled conditions experiments being performed. Three sets of near isogenic lines (NILs), with genetic similarity of 95.6–99.3% between each pair of NIL-T (salt-tolerant) and NIL-S (salt-sensitive), were generated from a cross between 85–140 and Tiefeng 8 by using marker-assisted selection. It was shown that *GmSALT3* does not

contribute to an improvement in seedling emergence rate or early vigor under salt stress. However, when 12-day-old seedlings were exposed to NaCl stress, I found that the NIL-T lines accumulated significantly less leaf Na^+ and Cl^- compared with their corresponding NIL-S, while no significant difference of K^+ concentration was observed between NIL-T and NIL-S. In addition, I found that the NIL-T lines accumulated less Cl^- in the leaf and more in the root prior to any difference in Na^+; in the field, NIL-T accumulated less pod wall Cl^- than the corresponding NIL-S lines. Under non-saline field conditions, no significant differences were observed for yield related traits within each pair of NIL-T and NIL-S lines, indicating there was no observable yield penalty for having the *GmSALT3* gene. In contrast, under saline field conditions the NIL-T lines had significantly greater plant seed weight and 100-seed weight than the corresponding NIL-S lines, meaning *GmSALT3* conferred a yield advantage to soybean plants in salinized fields.

In addition to confirming that Cl^- exclusion occurs prior to Na^+ exclusion using a time course analysis, I found that stem secretion of Na^+ contributes to its exclusion from leaves; NIL-T also accumulated less K^+ in the leaf compared to NIL-S. I observed that Cl^- concentration is significantly higher in both the stem xylem and phloem sap of NIL-T. This likely means that whilst more Cl^- is transported from root-to-shoot more Cl^- is recirculated back to roots, and this contributes to a greater accumulation of Cl^- in NIL-T roots. Na^+ is significantly greater in concentration in NIL-S xylem sap but no differences were detected in phloem sap and roots between NILs, which indicates Na^+ is most likely regulated by exclusion at the root xylem, so in a different way in NIL-T compared to Cl^-. Plants with full-length *GmSALT3* maintain a significantly higher photosynthetic rate than NIL-S plants before and after salt treatment. In heterologous expression systems, GmSALT3 could restore bacterial growth of *E. coli* strain LB2003 (*trkAΔ, kup1Δ, kdpABCDEΔ*) that is defective in K^+ uptake systems; when expressed in *Xenopus laevis* oocytes, GmSALT3 contributes to higher accumulation of Na^+, K^+, and Cl^- and higher net influx of Na^+, K^+, and Cl^- (measured by MIFE, Microelectrode Ion Flux Estimation) compared to water-injected oocytes.

In an attempt to reveal new insights to the potential underlying mechanisms I used RNA-seq analysis of roots from soybean NIL (Near Isogenic Lines); NIL-S (salt-sensitive, *Gmsalt3*) and NIL-T (salt-tolerant, *GmSALT3*). Thirty RNA-seq libraries were constructed and sequenced, including NIL-T and -S roots from three time points of 14 day old plants, 0 hours, 6h, and 3d following salt-treatment (200mM NaCl) and their corresponding non-treatment controls. Gene ontology (GO) analysis showed that unique DEGs under salt treatment in NIL-T are clustered into GO terms such as response to biotic stimulus, oxidation reduction and oxidoredutase activity, while in NIL-S GO terms are more diverse including cell communication, signalling, and biological regulation. Accordingly, reactive oxygen species (ROS) generation and detoxification was measured and differed in NIL consistent with the RNA-seq data. As such, I propose that *GmSALT3* affects the ROS status of roots, which improves the ability of NIL-T to cope with stress.

Overall, the collective findings of this thesis provide new insights into the transport activity of GmSALT3 and how *GmSALT3* contributes to salinity tolerance in soybean.

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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Acknowledgements

Firstly, I would like to thank my principal supervisor and mentor Professor Matthew Gilliham for the continuous support of my Ph.D research, for his patience, motivation, and guidance. Besides my principal supervisor, I also thank my co-supervisor Dr. Stefanie Wege for her critically reading of my work and providing research ideas; my postgraduate coordinator Associate Professor Ken Chalmers for his support; my independent advisor Dr. Tim Sutton for his research ideas and support.

I thank Professor Rongxia Guan, Professor Lijuan Qiu and their soybean research group members from Chinese Academy of Agricultural Sciences in Beijing, for donating soybean seeds and for research support through discussing results and further experiments; Dr. Gwenda Mayo for technical assistance with instruments in Adelaide Microscopy; Dr. Jayakumar Bose for assistance in MIFE experiments; Dr. Rakesh David for assistance in RNA-seq analysis; Dr. Bo Xu, Dr. Jiaen Qiu, Dr. Sam Henderson, Dr. Sunita Ramesh, Dr. Yu Long, Dr. Caitlin Byrt, Dr. Zhengyu Wen, Prof. Stephen Tyerman, Zeyu Xiao, and Siyang Liao for help and advice; Asmini Athman, Wendy Sullivan, Ali Mafakheri, and Dr. Rebecca Vandeleur for technical support.

I also thank Australian Research Centre, Plant Energy Biology Centre of Excellence, School of Agricultural, Food, and Wine, and the University of Adelaide for providing financial support and resources to this project, and the reviewers and Editors of *The Plant Journal*, *Journal of Experimental Botany* and *Frontiers in Plant Science* for their comments that helped to improve the manuscripts within this thesis.

Last but not the least, I would like to thank my family for supporting me throughout my study abroad and my life in general.

List of Abbreviations

APX	Ascorbate peroxidase
AsA	Ascorbic acid
Car	Carotenoid
CAX	Calcium/Proton antiporter
CCC	Cation-chloride cotransporter
CHI	Chalcone isomerase
CHS	Chalcone synthase
CHX	Cation/proton exchanger
Cl^-	Chloride ion
CLC	Chloride channel
CPA	Cation/proton antiporter
CPM	Cytochrome P450 monooxygenase
DAS	Days after sowing
DEG	Differentially expressed genes
DNA	Deoxyribonucleic acid
dS/m	decisiemens per metre
EC	Electrical Conductivity
ER	Endoplasmic Reticulum
FC	Fold change
FDR	False Discovery Rate
FL	First trifoliate leaf
GFP	Green fluorescent protein
GO	Gene ontology
GSH	Glutathione
H^+	Proton
HKT	High affinity K^+ transporter
HS	Higher stem
Hy	Hypocotyl
IRGA	Infrared gas analyzer
K^+	Potassium ion
KEGG	Kyoto Encyclopedia of Genes and Genomes
kg, g, µg, ng	kilograms, grams, micrograms, nanograms
L, mL, µL, nL	Litre, millilitre, microlitre, nanolitre
Li^+	Lithium ion

LS	Lower stem
LTR	Long terminal repeats
mM	Millimolar
mRNA	Messenger RNA
Na^+	Sodium ion
NILs	Near isogenic lines
PCA	Principal Component Analysis
PCR	Polymerase chain reaction
PR	Primary root
qRT-PCR	Quantitative real-time PCR
QTL	Quantitative trait loci
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SNP	Single-nucleotide polymorphism
SOD	Superoxide dismutase
SR	Secondary root
SSR	Simple sequence repeats
TEM	Transmission electron microscopy
TMDs	Transmembrane domains
UTR	Untranslated region
v/v	Volume per volume
w/v	Weight per volume
YFP	Yellow fluorescent protein
YL	Youngest trifoliate leaf

Preface

This project was initiated through a collaboration with the laboratories of Professor Rongxia Guan and Professor Lijuan Qiu from the Institute of Crop Sciences at the CAAS (Chinese Academy of Agricultural Science), Beijing, who identified a candidate salt tolerance gene from soybean (*GmSALT3*) via a fine mapping approach; they sought the assistance of my home lab (led by Professor Matthew Gilliham) in characterising the role of this gene in improving soybean salt tolerance. This aim was the focus of my PhD studies – how does GmSALT3 confer improved salt tolerance in soybean. As such an objective of my PhD studies was to contribute to our understanding of possible salt tolerance mechanisms in soybean. As the only fine-mapped gene from a salt tolerance QTL in soybean, this study has promise to improve the salt tolerance of soybean (and related species). Throughout the period of my thesis our collaborators, my supervisors and I have jointly planned experiments and discussed analysis; my principal supervisor and I have visited CAAS on 5 occasions (2014; 2015x2; 2016), and Prof. Guan (the lead CAAS researcher) has visited Adelaide (2017). We have also maintained regular email and videoconference contact throughout. This thesis contains 3 published manuscripts and 2 manuscripts that are intended for publication. It contains a minimal broad introduction to avoid repetition with the introductions of the manuscripts, and a general discussion. Each chapter, in addition to the manuscript includes a brief introduction to orientate the reader and provide some context to the study, and a conclusion with extended discussion to provide a clear link between the manuscripts.