Breeding and transgenic approaches to improving water use efficiency in white clover (*Trifolium repens L.*)

A thesis presented in partial fulfilment of the requirements for the degree of

Master of Science

In

Plant Biology

At Massey University, Palmerston North

New Zealand

Julia Sarah Margaret Raynes

2014

Abstract

This thesis investigates the potential role of the ABA biosynthetic gene *9-cis epoxycarotenoid dioxygenase 1*(*NCED1*) as a determinant of water-use-efficiency (WUE) in plants as part of a longer term aim to confer improved WUE to the forage legume white clover (*Trifolium repens* L.). Two experimental approaches have been used. The first looked at the expression of *NCED1* in a range of *Trifolium* species that display anatomical and morphological traits that confer some adaptations to growth and survival in dryland habitats. The second approach involved over-expression of *NCED1* in the model species tobacco (*Nicotiana tabacum*) in proof-of-concept experiments to directly determine if any changes in plant water relations can be measured.

Initially the constitutive expression of *TrNCED1* was assessed, using the quantitative real-time polymerase chain reaction (q-RT-PCR), in two varieties of white clover. The first is an agronomically elite cultivar with a higher water requirement for optimal growth, cv. Grasslands Kopu II, and the second a dryland-adapted ecotype, Tien Shan. It was found that for both varieties, *TrNCED1* expression was highest in the younger (first-fully-expanded) leaf (FFEL) tissue, followed by root tissue, then lastly apical tissue. When comparing the two varieties under well-watered conditions, the relative expression of *TrNCED1* was higher in aerial parts of the cv. Kopu, but in the roots of the Tien Shan ecotype with respect to the reference genes, *TrActin* and *TrGAPDH.* Further, in the high biomass cv. Grasslands Kopu II, expression of *TrNCED1* decreased in the apical and FFEL tissues of plants experiencing a water deficit, and decreased while for the dryland ecotype Tien Shan, *TrNCED1* expression did not change in the apex reguardless of plant water status. Expression in the FFEL increased in tissue experiencing water deficit, and decreased in roots, suggesting different mechanisms for drought tolerance and response in the two varieties. For the range of *Trifolium* species assessed, constitutive expression of the *TrNCED1* homologue was measured under well watered conditions in the FFEL and apical tissues. Essentially no significant difference in expression in either tissue between species was detected, with respect to the reference genes, *TrActin* and *TrGAPDH.*

As the second experimental approach, *NCED1* was over-expressed in tobacco as a model species. In other studies, over-expression of *NCED1* has been found to confer some characteristics associated with increased WUE, although abnormal growth associated with high levels of ABA at key developmental stages has proved problematic. For this thesis, the two senescence associated promoters, *Senescence-Activated-Gene* (*SAG13*) and *Senescence-Associated-Receptor-Kinase* (*SARK*), were selected to drive over-expression of *NCED1* from *Solanum lycopersicum* in tobacco. Of those plants that came through tissue culture and were successfully established in soil, a single line transformed with $SAG13_p$*::SlNCED1*, and three plants transformed with $SARK_p$*::SlNCED1*, were shown to be positive for transgene insertion using a polymerase chain reaction (PCR) with genomic DNA. Of these, two plants, both transformed with $SARK_p$*::SlNCED1,* were found to express *SlNCED1* when tested using PCR with cDNA from isolated RNA. Water relations measurements performed on all four plants that were positive for the transgene, an empty vector control, a selection of plants that were negative for transgene insertion, and some wild-type controls, found that one of the lines confirmed as expressing the transgene, line 751-1, had a very low transpiration rate and low level of stomatal conductance. To extend these measurements to determine and increase in water-use-efficiency, a comparison must be made between growth rate and water uptake, and many more transgenic plant lines must be analysed.

Finally, to determine whether the eventual transformation of white clover with $SAG13_p$*::SlNCED1* and $SARK_p$*::SlNCED1* would result in co-suppression of both types of transgene and the constitutive *NCED1*, expression of *TrNCED1* in seven tissue types, from two varieties was measured. Highest expression was determined in the root tissue and in the younger leaf tissue, but was lower in the mature tissue examined. This suggests that transformation with *SlNCED1* and expression in the mature tissues is not likely to be influenced by the constitutively expressed *TrNCED1*.

Acknowledgements

First of all, it is with fervent respect that I wish to thank my supervisor Professor Michael McManus for his continued wisdom, support, patience, encouragement and helpful criticism throughout the course of my study and the writing process. I cannot thank you enough.

Thank you to my C5.19 lab mates: Susanna, Srishti, and Matt - your help and advice has been invaluable from the start, Shane – for always pointing out the many different ways things could have gone worse, Afsana, Diantha, Sam, Willemein, Jennifer, Jibran, Jay, Faisal, Tina, Rachel and Rama – for your companionship, and to Briana and Hua for help with the final stages, and continuation of my research. Also to Ann Truter and Cynthia Cresswell for all their help behind the scenes.

For the provision of plant material and plasmids I wish to thank Dr Rainer Hofmann of Lincoln University, Canterbury, Professor Warren Williams from AgResearch Grasslands, Palmerston North, and Dr Andrew J Thompson of Cranfield University, Cranfield, England.

I convey special thanks and acknowledgement to the Foundation for Arable Research, L A Alexander Agricultural College Trust Board, and George Mason for scholarships and financial support to attend conferences and workshops throughout the course of my study.

Last, but not at all least, I wish to thank my parents, my brothers, and my friends for their ongoing encouragement and support, and Henry and Miumiu – for finding somewhere else to sit long enough for me to write this thesis!

Abbreviations

ABA	**-**	**abscisic acid**
AFLP	**-**	**amplified fragment length polymorphism**
BLAST	**-**	**basic local alignment search tool**
BAP	**-**	**6-benzyl amino purine**
bp	**-**	**base pairs**
Cef	**-**	**cefotaxime**
cDNA	**-**	**complementary deoxyribonucleic acid**
cm	**-**	**centimetre**
^{o}C	**-**	**degrees Celsius**
d	**-**	**days**
DMF	**-**	**di-methyl-formamide**
DMSO	**-**	**di-methyl-sulfoxide**
DNA	**-**	**deoxyribonucleic acid**
DNase	**-**	**deoxyribonuclease**
dNTP	**-**	**deoxyribonucleotide triphosphate**
DTT	**-**	**dithiolthreitol**
DW	**-**	**dry weight**
EDTA	**-**	**ethylene diamine tetra-acetic acid**
EGTA	**-**	**ethylene glycol tetra-acetic acid**
EPA	**-**	**Environmental Protection Authority**

EVC	**-**	**empty vector control**
FW	**-**	**fresh weight**
g	**-**	**gram**
g	**-**	**gravity, or g-force**
GMO	**-**	**genetically modified organism**
h	**-**	**hours**
Kan	**-**	**kanamycin**
kb	**-**	**kilobases**
LB	**-**	**Luria-Bertani broth or agar**
M	**-**	**Molar: moles per litre**
min	**-**	**minute**
milliQ	**-**	**water purified using the Milli-Q Ultrapure system**
µg	**-**	**microgram**
µL	**-**	**microlitre**
µM	**-**	**micromolar**
mg	**-**	**milligram**
mL	**-**	**millilitre**
mM	**-**	**millimolar**
NaOAc	**-**	**sodium acetate**
ng	**-**	**nanograms**
nm	**-**	**nanometre**

Nic I	**-**	**Murashige and Skoog media, mineral salts, vitamins, 3% weight per volume sucrose, 6-benzyl aminopurine (1 mg/L) and NAA (0.1 mg/L)**
Nic II	**-**	**Nic I media supplemented with Kanamycin (100 µg/mL) and Cefotaxime (100 µg/mL)**
Nic III	**-**	**Murashige and Skoog media,**
OE	**-**	**overexpression**
PCR	**-**	**polymerase chain reaction**
q-RT PCR	**-**	**quantitative reverse transcription PCR *syn.* qPCR, QPCR**
p	**-**	**pico**
PVP	**-**	**polyvinyl pyrrolidone**
RAPD	**-**	**Random Amplified Polymorphic DNA**
RNA	**-**	**ribonucleic acid**
RNase	**-**	**ribonuclease**
rpm	**-**	**revolutions per minute**
RT	**-**	**reverse transcription**
SDS	**-**	**sodium dodecyl sulphate**
s	**-**	**second**
Spec	**-**	**spectinomycin**
Strep	**-**	**streptomycin**
TAE	**-**	**tris – acetate EDTA buffer**
TBE	**-**	**tris – borate EDTA buffer**
TE	**-**	**tris EDTA buffer**
Tm	**-**	**melting temperature**

Tris	**-**	**tris(hydroxymethyl)aminomethane**
TrNCED1	**-**	***Trifolium repens 9-cis-epoxycarotenoid dioxygenase 1***
U	**-**	**enzyme units**
UC	**-**	**untransformed control**
UV	**-**	**ultra violet**
V	**-**	**volt**
v/v	**-**	**volume per volume**
WT	**-**	**wild type**
WUE	**-**	**water use efficiency**
w/v	**-**	**weight/volume**
YEB	**-**	***Agrobacterium tumefaciens* growth medium broth/agar**

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