

Copyright is owned by the Author of the thesis. Permission is given for a copy to be downloaded by an individual for the purpose of research and private study only. The thesis may not be reproduced elsewhere without the permission of the Author.

**Evaluation of the efficacy of the inducible over-expression of 9-*cis*-  
*epoxycarotenoid dioxygenase1 (NCED1)* to confer improved water use efficiency  
in transgenic plants**

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

**Caleb D. Sixtus**

**2013**



## Abstract

In order to trial the concept of inducible overproduction of the plant hormone abscisic acid (ABA) to confer increased water use efficiency, the forage legume *Trifolium repens* (L.) (white clover) and the model plant species *Nicotiana tabaccum* (L.) (tobacco) were transformed with the construct *9-cis-epoxycarotenoid dioxygenase 1* (*NCEDI*) gene from *Solanum lycopersicon* (*SINCEDI*) driven by the RUBISCO small subunit promoter (*SSUp*). For white clover, a total of 18 putatively genetically-independent transgenic lines were obtained through selection in tissue culture, and these were further cloned by vegetative propagation to give 56 plants in total. Ten of these tested positive for *NCEDI* insertion using polymerase chain reaction (PCR) with genomic DNA. Establishment of transgenic clover on soil was problematic, but seven putatively transformed lines were established. Of these, only one line potentially expressed *SINCEDI*, but the transcriptional levels were too low, as determined by semi-quantitative reverse-transcriptase dependent PCR (sqRT-PCR), for any further analysis. This low expression, and the fact that only one line was identified, led to the decision to discontinue investigations with white clover.

In parallel, the more amenable tobacco transformation system was also used with the *SISUp::NCEDI* construct to act as proof-of-concept. Fourteen putatively genetically independent transgenic lines of tobacco were obtained through tissue culture, six of which were successfully established onto soil. A range of integrated gene copy numbers and *NCEDI* expression levels were identified in the T<sub>0</sub> lines using genomic PCR and sqRT-PCR. Self-fertilised seed was collected from each transgenic line, but the germination rate from all of the transgenic lines was significantly lower than wild-type. Those lines that did germinate often displayed a range of aberrant growth phenotypes. After trialing methods to evaluate water use efficiency, a total of 47 T<sub>1</sub> seedlings

displaying a normal seedling phenotype were established on soil. A range of water use efficiencies were observed as determined by analysis of plant growth rates against water use, followed by a transpiration assay of plants deemed 'efficient' and 'poor' users of available water. No correlation of *SINCE1* expression with the more efficient users of available water was observed, and so ultimately it was concluded that the transgene was ineffective at raising the water use efficiency of tobacco as determined by the parameters measured in thesis.

## **Acknowledgements**

Without the aid of a great many people, this would never have been possible. So with the greatest amount of respect I would like to thank my supervisor Michael McManus for his wisdom and patience, as well as all the people in the C5.19 lab, not in the least Susanna Leung, who aided me since day one.

All those who assisted me; Afsana, Alvina, Srishti, Matt, Jibran, Sam, Paul Djikwel, Warren Williams, the folks at the lab in Canada, and Andrew Thompson and Ian Taylor for the original construct.

Lastly, I would like to thank my wife Kelly for standing by me and more than once telling me to; “Shut up, and do your work.”

## Abbreviations

°C	<i>degrees Celsius</i>
%	percent
µg	microgram
µL	microlitre
µM	microMolar
AAO	abscisic aldehyde oxidase
ABA	abscisic acid
ABA-GE	abscisic acid glucose ester
BAP	benzyl aminopurine
bp	base pair
CaMV 35S	cauliflower mosaic virus 35S promoter
Cef	cefotaxime
CR	clover regeneration (media)
DEPC	diethylpyrocarbonate
DIG	digoxigenin
DNA	deoxyribonucleic acid
DNAase	deoxyribonuclease
DTT	dithiothreitol
CTAB	cetyl trimethylammonium bromide
dNTP	deoxynucleotide triphosphate
DPA	dihydrophaseic acid
EDTA	ethylene diaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FAA	formalin acetic acid alcohol

FC	field capacity
FCW	field capacity weight
g	gram
GA	gibberellic acid
hr	hour
Kan	kanamycin sulfate
L	litre
LEA	late embryogenesis active
M	Molar (moles per litre)
mg	milligram
min	minute
mL	millilitre
mM	milliMolar
mRNA	messenger ribose nucleic acid
mQ	milliQ grade water
MS	Murashige and Skoog salt mix
NAA	1-naphthaleneacetic acid
ng	nanogram
nM	nanoMolar
Nic	Nicotiana transformation (media)
<i>NtGAPDH</i>	<i>Nicotiana tabacum</i> glyceraldehyde-3-phosphate dehydrogenase
PA	phaseic acid
PCR	polymerase chain reaction
PVP	polyvinylpyrrolidone
rbcS3Cp	RUBISCO small subunit promoter



RNA	ribose nucleic acid
RT-PCR	reverse-transcriptase-dependent polymerase chain reaction
RUBISCO	ribulose-1,5-bisphosphate carboxylase/oxidase enzyme,
SDS	sodium dodecylsulfate
Sln1	slender-1
<i>SINCED1</i>	<i>Solanum lycopersicum</i> 9- <i>cis</i> -epoxycarotenoid dioxygenase
sqPCR	semi-quantitative polymerase chain reaction
SSC	saline sodium chloride (buffer)
SSUp	RUBISCO small subunit promoter
<i>t</i> -ABA	<i>trans</i> -abscisic acid
T <sub>0</sub>	transgenic generation zero
T <sub>1</sub>	transgenic generation 1
Tbsp	tablespoon
T <sub>m</sub>	Melting temperature at which DNA strands separate in preparation for annealing
TR	relative transpiration rate.
Tris	tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
V	volts
v/v	volume per volume
Wt	wild type
WUE	water use efficiency
w/v	weight/volume
x <i>g</i>	acceleration due to gravity (9.8m s <sup>-2</sup> )
YEB	yeast extract broth

## Table of contents

List of figures	xii
List of tables	xv
1. Introduction, overview of thesis	1
1.1. Abscisic acid	2
1.2. Physiological role of abscisic acid in higher plants	3
1.2.1. Floral development	3
1.2.2. Seed maturation	4
1.2.3. Germination and seedling growth	5
1.2.4. ABA signalling during growth and development	7
1.2.5. ABA receptors and perception	8
1.3. Biosynthesis of abscisic acid	11
1.3.1. Manipulation of ABA levels to alter plant growth and development	13
1.4. Water use efficiency	14
1.4.1. Manipulation of ABA levels to alter plant growth and development	16
1.5. White clover ( <i>Trifolium repens</i> L.)	16
1.5.1. Water relations studies in white clover	18
1.5.2. Studies to improve water use efficiency in white clover	19
1.6. Improving water use efficiency by over-expression of <i>NCEDI</i>	20
1.7. Thesis aims	21
2. Methods	23
2.1. Plant transformation	23
2.1.1. <i>Agrobacterium tumefaciens</i> culture	23
2.1.2. Transformation and tissue culture of <i>Trifolium repens</i>	23

2.1.3.	Transformation and tissue culture of <i>Nicotiana tabacum</i>	25
2.1.4.	Establishment on soil	26
2.1.4.1.	Establishment of <i>Trifolium repens</i>	26
2.1.4.2.	Establishment of <i>Nicotiana tabacum</i>	27
2.1.5.	Obtaining seedlings for use in water use efficiency trials	28
2.2.	Assessment of transgenic plants	29
2.2.1.	Germination trials	29
2.2.2.	Microscopy methods	29
2.2.2.1.	Tissue fixation	29
2.2.2.2.	Tissue sectioning	31
2.2.3.	Water use efficiency trials	32
2.2.3.1.	Trial one	32
2.2.3.2.	Trial two	32
2.2.3.3.	Transpiration assay	32
2.3.	Molecular techniques	33
2.3.1.	Extraction of genomic DNA	33
2.3.2.	Extraction of RNA	34
2.3.3.	Southern analysis	36
2.3.3.1.	DNA transfer	36
2.3.3.2.	Probe preparation	39
2.3.3.3.	Probe hybridisation	40
2.3.4.	Polymerase chain reaction	41
2.3.4.1.	Genomic PCR	41
2.3.4.1.1.	Design of primers	42
2.3.4.2.	Reverse transcriptase-PCR	43

2.3.4.2.1.	DNAase treatment	.....	43
2.3.4.2.2.	Reverse transcription	.....	44
2.3.4.2.3.	Semi-quantitative PCR	.....	45
2.3.5.	Agarose gel electrophoresis	.....	47
2.3.6.	Confirmation of expression	.....	48
2.3.6.1.	Expression in <i>T. repens</i>	.....	48
2.3.6.2.	Expression in <i>N. tabacum</i>	.....	48
2.4.	Statistical analysis	.....	48
3.	Results	.....	50
3.1.	Production of transformed white clover lines expressing <i>SLNCED1</i>	....	50
3.1.1.	Overview of the tissue culture process used for white clover and nomenclature of different developmental stages used in this thesis	....	50
3.1.2.	<i>Agrobacterium</i> mediated transformation and tissue culture	....	51
3.1.3.	Confirmation of insertion of the <i>SSUp::SINCED1</i>	.....	54
3.1.4.	Establishment on soil	.....	58
3.1.5.	Confirmation of expression	.....	59
3.2.	Production and analysis of the transformed <i>Nictiana tabacum</i> lines expressing <i>SINCED1</i>	.....	61
3.2.1.	<i>Agrobacterium</i> mediated transformation and tissue culture	....	61
3.2.2.	Confirmation of insertion of the <i>SSUp::SINCED1</i> transgene	....	61
3.2.3.	Establishment on soil	.....	64
3.2.4.	Confirmation of expression	.....	64
3.3.	Phenotypic analysis of transformed tobacco lines transformed with <i>SSUp::NCED1</i>	.....	68
3.3.1.	Phenotypic analysis of primary (T <sub>0</sub> ) transformants	.....	68

3.3.2. Germination trials	.....68
3.3.2.1. Germination on MS media	.....68
3.3.2.2. Germination on gibberellic acid-containing media	....71
3.3.3. Description of aberrant seedlings	.....74
3.3.3.1. Aberration on MS media	.....74
3.3.3.2. Aberration on gibberellic acid-containing media	....77
3.3.4. Preliminary observations of the anatomy of aberrant seedlings	....82
3.3.5. Expression of <i>SINCED1</i> in aberrant seedlings	.....85
3.3.6. Evaluation of water use efficiency of <i>SINCED1</i> transformed tobacco	.....87
3.3.6.1. Trial one	.....87
3.3.6.2. Trial two	.....90
3.3.6.2.1. Transpiration assay	.....93
3.3.7. Expression of <i>SINCED1</i> in T <sub>1</sub> genotypes	.....96
4. Discussion	.....99
4.1. White clover ( <i>Trifolium repens</i> L.)	.....99
4.1.1. Agrobacterium mediated transformation of white clover	....99
4.1.2. Initial evaluation of primary white clover transformants	...102
4.1.3. Establishment of primary clover transformants on soil	...104
4.1.4. Final assessment of primary white clover transformants	...105
4.2. Tobacco ( <i>Nicotiana tabacum</i> )	.....107
4.2.1. Assessment of <i>SINCED1</i> relative expression in T <sub>0</sub> plants	...108
4.2.2. Relationship between NT5/7 and NT9/12	.....108
4.2.3. Progeny of transformed tobacco display altered germination rates	...109
4.2.4. Evaluation of aberrant seedlings in transformed tobacco progeny	...110

4.2.5. Evaluation of relative expression in aberrant seedlings	.....115
4.2.6. Evaluation of water use efficiency in T <sub>0</sub> progeny	.....116
4.2.7. Accumulation of ABA levels in transgenic plants	.....118
4.3. Conclusions	.....119
4.4. Future work	.....120
5. References	.....122
6. Appendix	.....135
6.1. Appendix 1: Media formulations	.....135
6.2. Appendix 2: Gene sequences	.....138
6.3. Appendix 3: Alternative measures of water use efficiency	.....144
6.4. Appendix 4: Additional water use efficiency testing	.....146
6.5. Appendix 5: Analysis of abscisic acid content	.....149

## List of figures

Figure 1.1: Abscisic acid biosynthesis pathway .....	12
Figure 2.1: Schematic for the set up for the Southern Blot DNA transfer .....	38
Figure 3.1: Development of plants in Batch 15 .....	53
Figure 3.2: <i>SINCE</i> DI expression in leaf tissue isolated in the morning (10:00am) or afternoon (2:00pm) .....	60
Figure 3.3: a) Southern blot analysis of transgenic T <sub>0</sub> tobacco lines, b) Schematic of Southern blot analysis shown in a) displaying all observable bands .....	65
Figure 3.4a, b: a) RT-PCR results for <i>SINCE</i> DI gene expression in the T <sub>0</sub> transgenic tobacco lines, b) The relative expression of the inserted <i>SINCE</i> D gene construct when compared to the housekeeping gene <i>EF1<math>\alpha</math></i> .....	67
Figure 3.5: Mean germination rate for progeny of T <sub>0</sub> transgenic tobacco (T <sub>1</sub> lines) ....	70
Figure 3.6a, b: Mean germination rate for progeny of T <sub>0</sub> transgenic tobacco (T <sub>1</sub> ) in a) gibberellic acid media containing 1 mL L <sup>-1</sup> ethanol) and, b) non-GA <sub>3</sub> media containing 1 mL L <sup>-1</sup> ethanol .....	72
Figure 3.7: Examples of the three types of aberrant growth behaviour observed in T <sub>1</sub> seedlings .....	76
Figure 3.8: Aberration rate and type of aberration for progeny of each tobacco T <sub>0</sub> line .....	78
Figure 3.9a, b: Aberration rate and type of aberration for progeny of each tobacco T <sub>0</sub> line on; a) gibberellic acid enhanced media (containing 1 mL L <sup>-1</sup> ethanol) and, b) media containing 1 mL L <sup>-1</sup> ethanol .....	80

Figure 3.10 a, b, c: Sections (10 $\mu\text{m}$ ) of seedlings from a) type 1 aberrants; b) type 2 aberrants and; c) a wild type growth habit seedling	83, 84
Figure 3.11: Relative expression of the <i>SINCE1</i> gene in bulked T <sub>1</sub> aberrant seedlings	86
Figure 3.12a, b: Mean changes in plant width (a) and plant height (b) against water use per day for transgenic T <sub>1</sub> seedlings of tobacco	89
Figure 3.13: Change in the rate of extension of the second emerged leaf against mean daily water use of transgenic T <sub>1</sub> lines	92
Figure 3.14: Relative transpiration rate of all T <sub>1</sub> transgenic plants tested	94
Figure 3.15: Relative transpiration compared with relative growth for the T <sub>1</sub> transgenic plants classified as efficient, average or poor users of available water	95
Figure 2.16: Relative expression of <i>SINCE1</i> in T <sub>1</sub> transgenic plants deemed efficient (E series), average (A series), and poor (P series) users of water	97
Figure A2.1: <i>SINCE1</i> gene sequence	138, 139
Figure A2.2: <i>NtNptII</i> gene sequence	140, 141
Figure A2.3: <i>NtEF1<math>\alpha</math></i> gene sequence	142, 143
Figure A3.1: Comparison of mean daily leaf growth ( $\text{mm}^2$ ) plotted against average daily water use (mL)	144
Figure A3.2: Comparison of total leaf growth for all four days of experimentation ( $\text{mm}^2$ ) plotted against total amount of water used (mL)	145
Figure A3.3: Comparison of total mean daily leaf growth ( $\text{mm}^2/\text{day}$ ) for all four leaves over all days of experimentation plotted against mean daily water use per day (mL/day)	145
Figure A4.1: Water use efficiency of bulked T <sub>0</sub> line progeny compared with wild type control	146



Figure A4.2: Photosynthetic rate of bulked T <sub>0</sub> line progeny compared with wild type control .....	147
Figure A4.3: Stomatal conductance of bulked T <sub>0</sub> line progeny compared with wild type control .....	147
Figure A4.4: Internal CO <sub>2</sub> concentrations of bulked T <sub>0</sub> line progeny compared with wild type control .....	148
Figure A4.5: Transpiration rate of bulked T <sub>0</sub> line progeny compared with wild type control .....	148
Figure A5.1: ABA and ABA catabolites content in T <sub>0</sub> tobacco .....	149

## List of tables

Table 2.1: Primer sequences for all primers used for the confirmation of transformation of putative transgenic white clover and tobacco	42
Table 2.2: Primer sequences used for semi-quantitative PCR of tobacco plants	47
Table 3.2: Screening by PCR of all surviving regenerated clover plants	56, 57
Table 3.2: Positive genomic PCR band results for tobacco (batch) 1 and tobacco (batch) 2	63
Table 3.3: Comparative germination rates for progeny of T <sub>0</sub> transgenic tobacco (T <sub>1</sub> lines)	70
Table 3.4: Summary of germination rates for progeny of T <sub>0</sub> transgenic tobacco (T <sub>1</sub> lines)	73
Table 3.5: Numerical summary of aberration rate for seed collected from each tobacco T <sub>0</sub> line	78
Table 3.6: Numerical summary of aberration rate for seed collected from tobacco T <sub>0</sub> lines when sown on GA <sub>3</sub> media and non-GA <sub>3</sub> media respectively	81
Table A5.1: Analysis ABA and ABA catabolites content in T <sub>0</sub> tobacco	149