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

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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*.

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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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Chapter 1

Introduction

Overview

The adaptation of plants to less than ideal environmental conditions has evolved through necessity. As plants are sessile organisms, adaptation is the key to survival (Song and Matsuoka 2009). In agriculture, the sustainability of forage systems for high intensity grazing by stock, and the expansion of crop production to produce enough food to feed the constantly increasing world population, is dependent on the successful establishment of crop and pasture species. In times when the demand for high quality soils exceeds availability, forage and crop production has been relegated to environments with lower-fertility soils, which can also experience less than optimal drainage or periods of drought**.** Because of this, and an increase in competition for water as a resource for irrigation, the ability of plants to better tolerate environmental stresses or to grow on lower volumes of water, has become essential (Ge, et al. 2011). Thus in agricultural ecosystems, limited water availability is one of the most severely limiting factors of plant growth and overall crop yield. Thus the identification and development of genotypes that are proficient in making use of more limited available soil water, also described as high water-use efficiency (WUE), and perhaps any improved drought tolerance that result from these growth strategies, is of utmost importance (Inostroza and Acuña 2010).

The overexpression of the abscisic acid biosynthetic gene 9-*cis epoxycarotenoid dioxygenase* (NCED1) as a potential mechanism for increased water use efficiency was first proposed by Taylor and colleagues in 2000, after it was initially cloned in 1997 (Tan, et al. 1997). The role of *NCED1* in the upregulation of ABA biosynthesis in vegetative tissues as a response to a variety of environmental stresses, including drought, was originally identified from the ABA-deficient tomato mutant *notabilis*, which presented as the *wilty* phenotype due to inability to control stomatal conductance under environmental stress. Full complementation confirmed *SlNCED1* as the affected gene (Thompson, et al. 2004).

The Thompson group have overexpressed *SlNCED1* in tobacco leaves using a tetracycline inducible system and found a 10-*fold* increase in ABA. When constitutively overexpressed in tomato with the *CaMV 35S* promoter, ABA content was found to have increased in leaves, roots and xylem sap. However, physiological side effects associated with constitutive overexpression included increased seed dormancy, a delay in establishment, and an increased tendency for high humidity guttation and interveinal chlorosis. Reduced stomatal conductance was also found, and after a period of water deficit, plants appeared to exhibit enhanced drought tolerance which, when measured was associated with an improvement in water use efficiency. The group suggested that overexpression of *SlNCED1* under an inducible promoter could provide agronomic advantages associated with high ABA levels and increased WUE in a number of crop and forage species, with a reduction in negative effects on crop productivity linked to the constitutive overexpression of ABA (Taylor, et al. 2005, Thompson, et al. 2007).

Therefore, this thesis examines the direct overexpression of the *SlNCED1* gene (provided by Andrew J Thompson) in *Nicotiana tabacum* under the senescence induced promoters SAG_{13} and *SARK*. Plant transformation will be carried out in the model plant species *N. tabacum* (tobacco) as proof of concept, with the longer term aim of overexpression of the *SlNCED1* gene in the forage legume white clover (*Trifolium repens*). The two promoters were selected because senescent tissue and has been identified as where plants lose the most water, through increased transpiration rate. To date, the use of *SAG13* and *SARK* promoters to drive expression of *NCED1* to improve water use efficiency has not yet been evaluated in this way in plants. Overexpression of a gene that increases ABA levels within senescent or almost senescent leaves, improving stomatal control and allowing stomatal closure and therefore a reduction in transpiration should reduce water loss, and thus could improve whole plant water use efficiency. *Senescence-activated-gene 13* (SAG_{13}) was selected as it directs expression before the onset of visible leaf yellowing, and increases in senescent leaves (Swartzberg, et al. 2006). The *SARK* promoter was selected because it is a *senescence-associated-receptor-kinase*, which is activated by plants during late leaf maturation, but actively decreases during the development of senescence (Rivero, et al. 2007). Enhanced drought tolerance may also be a result of overexpression of *SlNCED1* using these

promoters, as expression should be enhanced in senescing and near senescent tissue, and the process of senescence is hastened in plants experiencing water deficit (Rivero, et al. 2007).

1.1 Water deficit stress in plants

Drought is used to describe an extended period where an area or region experiences a scarcity in atmospheric, surface, ground water, or a combination of all three. Water deficit is the biological term used to describe incomplete saturation of plant cells with water as a result of profound water loss by a plant, which is unable to be replenished through soil water absorption. Plants exposed to drought experience water deficit.

Drought, and therefore water deficit, is an abiotic stress that is the major limiting factor of plant growth and productivity around the world. While climate change is predicted to lead to greater extremes in terms of desertification and decreased levels of rainfall, increased temperatures will result in increased evapotranspiration.

To aid in the realization of the ultimate goal of breeding high water-use-efficiency plants, the Department of Primary Industries and Fisheries in Queensland (DPI Queensland) has classified a selection of vegetable crop plants according to physiological response to drying soils and subsequent water stress. These categories have been picked up by many other research groups including (Klein, et al. 2013). These classifications are based on the concept that stomatal conductance can be correlated to changes in soil water content of some plants experiencing water stress but not others, and that the water potential of leaves may have a very weak correlation to soil water, or none at all. The two categories designated by DPI Queensland are isohydric and anisohydric: isohydric plants being plants that have strong stomatal control and a minimum threshold of water potential for stomata to close, whereas anisohydric plants have little or no stomatal control, and no apparent threshold of water potential maintenance (Limpus 2009). It was noted that these classifications can change between cultivars of crops, and even within cultivars, when grown in extremely different environments. Therefore, in addition

to these classifications allowing growers to identify which crops require closer monitoring of leaf water potential, and plan irrigation schemes, particularly deficit irrigation, they also have the potential to identify desirable cultivars for breeding programmes.

In addition to this reduction in leaf water potential, reduced growth rates and leaf area, as well as an increased root:shoot ratio, inhibition of photosynthesis and premature leaf senescence is a common response to water-deficit which is induced by a reduction in CO_2 supply in intercellular spaces which again is caused by reduced stomatal conductance (Rivero, et al. 2007, Shardendu and Reddy 2011, Ballizany, et al. 2012, Snyman 2013).

The transpiration efficiency of a plant (at a whole plant level) is defined as the biomass gained per unit of water transpired (Thompson, et al. 2007). By increasing the amount of biomass gained per unit of water, plants become more efficient at using available soil water and the overall water requirements of a crop decrease, leading to improved water use efficiency. Drought tolerance demands an osmotic response, as it is the stomata that control the rate of transpiration, through which the greatest amount of water lost. This ability to control stomatal conductance and reduce plant water deficit would be a huge step towards optimizing the water-use efficiency (WUE) of plants (Song and Matsuoka 2009), as well as potentially conferring a degree of tolerance to drought conditions through delayed water deficit.

The use of agronomic cultivars with a high WUE in a non-stressed environment also has the potential to increase profitability as plant biomass should be equivalent to, or higher than, non-improved cultivars but with lower overall inputs, particularly water (Thompson, et al. 2007).

1.2 The Role of Abscisic Acid in water deficit stress

It is widely documented that the plant phytohormone abscisic acid (ABA) plays a regulatory role in higher-plant physiology, including seed development, dormancy and its responses to a variety of environmental conditions, including drought

(Taylor, et al. 2000, Thompson, et al. 2000). It has been found through biochemical approaches that ABA levels in vegetative tissue are elevated after exposure to water deficit, and the characterization of ABA deficient mutants has revealed that ABA decreases the amount of water lost from transpiration by reducing stomatal aperture (Liotenberg, et al. 1999, Taylor, et al. 2000).

Until the late 1990's, the only conceivable way to study the physiological role of ABA was through correlative investigation, use of ABA deficient mutants with external ABA application, or inhibition of ABA biosynthesis using Fluridone or nurfurazon (Tung, et al. 2008). However, with developments in the area of plant genetics and biotechnology, and a decrease in the cost of the technology (e.g. gene guns), genes of interest are now able to be inserted into, and expressed by plants.

A decrease in stomatal conductance (or aperture) has historically been identified as a stress response to drought conditions that is regulated by abscisic acid (ABA). The closure or reduction in the aperture of stomata provides protection against dehydration, delaying water deficit, and aiding in plant survival (Inostroza and Acuña 2010).

1.3 Biosynthesis and regulation of ABA

ABA synthesis is a small side branch of the carotenoid pathway, and the basic framework for the biosynthesis of ABA is well established (Taylor, et al. 2000) (Figure 1.1). ABA is synthesised from C_{40} epoxy carotenoid precursors via C_{15} intermediates. The first gene in the ABA pathway is zeaxanthin epoxidase (ZEP), which carries out the first two steps (Nambara and Marion-Poll 2005). The next steps involve the conversion of violaxanthin to neoxanthin and 9-*cis* neoxanthin, and violaxanthin to 9-*cis* violaxanthin. The genes responsible for these steps are still under scrutiny. The cleavage of 9-*cis* violaxanthin and 9-*cis* neoxanthin is performed by 9-*cis* epoxycarotenoid dioxygenase (NCED), which has been identified as a key, rate limiting step in the biosynthesis of ABA (Thompson, et al. 2000, Tan, et al. 2003). This is followed by two oxidation steps, carried out by ABA2 and Arabidopsis Aldehyde Oxidase 3 (AAO3) respectively, which result in

the final product ABA. (Thompson, et al. 2000, Tan, et al. 2003, Nambara and Marion-Poll 2005)

The mechanism behind stomata closing is believed to be regulated by transportation of ABA from mesophyll cells to the guard cells of stomata (Levchenko, et al. 2005, Melhorn, et al. 2008). It was discovered that the introduction of ABA biosynthetic genes (an *Arabidopsis* NCED gene in this case) to guard cells of broad bean (using a particle gun) stimulated a decrease in stomatal aperture, which suggests that the guard cells contain a complete set of ABA biosynthetic enzymes and intermediates (Melhorn, et al. 2008). Whether ABA is naturally synthesised inside guard cells remains to be seen.

Song et al (2009) identified the role of ABA in stomatal closure as creating a signal cascade in the guard cells of stomata via diffusion through cell membranes. The cascade is proposed to initiate the outflow of K^+ , Cl^- and other organic solutes from the guard cells, causing a decrease in the turgor of the cells and diminishing stomatal aperture which in turn slows transpiration (Song and Matsuoka 2009). The group only examined this process in rice and *Arabidopsis* and the paper focuses primarily on a secondary messenger in the signalling cascade in stomata: H_2O_2 - presenting their findings from the point of view that it is not specifically ABA that induces stomatal closure, a point of difference to others who have carried out research in the same area (Song and Matsuoka 2009). Further detail on the precise mechanism is beyond the scope of this thesis.

The key role of *NCED1* as one of the determinants in ABA biosynthesis arose from work on the ABA-deficient tomato mutant *notabilis*. Physiological analysis of *notabilis* found greatly reduced shoot and root development in comparison to the wild type, with dry matter weights of 54% and 52% respectively. Total leaf area, shoot fresh weight, and a reduction in stem length were also found, alongside leaf epinasty and the development of prolific adventitious roots. In the complemented mutant lines (with wild type *NCED1*) not_{comp13} and not_{comp1}, shoot and root growth was now found to be not significantly different to the wild-type, and the rate at which ethylene evolution occurred, was similar to that of the wild type, and about half that of *notabilis*. ABA levels of rapidly dehydrated leaves were also measured, and found to be decreased in the complementation lines when compared to the

wild type, and even further decreased in *notabilis* providing strong evidence for *SlNCED1* having a role in ABA biosynthesis and stress response (Thompson, et al. 2004).

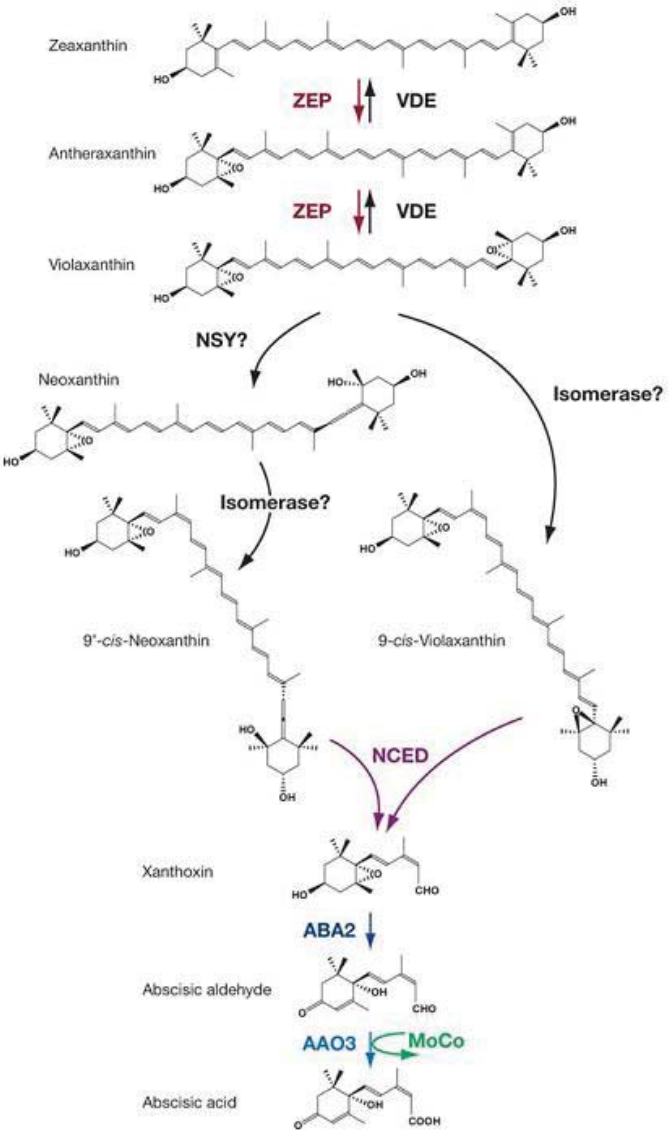


Figure 1.1 The Abscisic acid biosynthesis pathway. Modified from Nambara & Marion-Poll, 2005.

1.4 Does high NCED1 expression correlate with drought tolerant plants?

Drought tolerance refers to the degree to which plants can maintain growth and development, and most importantly in agriculture, yield, at times when accessibility to water is extremely limited.

The Marshall group discussed drought tolerance as a potential positive side-effect of breeding for more persistent, water-use efficient plants. However, this characteristic is yet to be achieved (Marshall, et al. 2001). Thompson et al (2007) found that once plants with high ABA contents from over-expression of the NCED gene were exposed to water deficit, the loss of turgor activated the inherent ABA biosynthetic pathway, greatly increasing the levels of ABA accumulation and drowning out the transgene effect displayed by the well-watered transgenic plants. Even though the *NCED* transformed plants displayed enhanced WUE under well watered conditions, their performance under water limited/stressed conditions was identical to that of the wild-type plants. They did however conclude that with refinement and specificity of NCED expression and therefore the amount of ABA produced, there is potential for enhanced drought tolerance to be a characteristic of plants with enhanced WUE (Thompson et al., 2007).

1.5 Molecular approaches to the conferral of increased water use efficiency

In addition to the work carried out by the Thompson group (see Section 1.4), a number of other groups have carried out research on a selection of plant species with the target of increasing or enhancing plant water use efficiency (WUE) to improve plant yields and reduce the pressure that agriculture places on water resources. The prevailing approach is the identification of traits that are related to drought tolerance, followed by manipulation of genes associated with those traits, through traditional breeding and crosses, or through transformation of target plants to overexpress these key genes to confer increased water use efficiency. In addition to *NCED1*, the ABA-responsive gene protein 28 (*RAB28*),

isopentenyltransferase (*IPT*), a specific type of C_2H_2-type zinc finger transcription factor known as *DST*, and *trans*-zeatin *O*-glucosyltranferase have all been identified as genes that have potential to improve plant WUE when manipulated (Rivero, et al. 2007, Haisel, et al. 2008, Song and Matsuoka 2009, Su, et al. 2010).

1.5.1 Selection of promoter to control gene expression in transgenic plants for improved water use efficiency.

In addition to water deficit responses in stomatal conductance (regulating a reduction in transpiration, and therefore increased efficiency of water use), differences in photosynthetic rate and senescence under well watered and water deficit conditions, and the mechanisms behind these changes, provide an alternate avenue for the development of plants with increased/higher water use efficiency. Increasing cytokinin content of plants through overexpression of isopentyl transferase (*IPT*), driven by senescence associated promoters such as the *Senescence-Activated-Gene* (SAG) and the *Senescence-Associated-Receptor-Kinase* (SARK) has been carried out by a number of research groups. The aim is to protect the biochemical processes associated with photosynthesis (which declines with a decrease in stomatal conductance), and induction of photorespiration during water stress without changing the source/sink relationships and nitrogen mobilisation (Swartzberg, et al. 2006, Rivero, et al. 2007, Rivero, et al. 2009, Rivero, et al. 2010).

The Swartzberg group examined overexpression of *IPT* in tomato plants, driven by *SAG12* and *SAG13* promoters, and while higher photosynthetic rates, alongside increased chlorophyll and carotenoid content when compared with wt controls were found, which is indicative of a delay in senescence, the loss of apical dominance combined with stunting and thickening of stems in plants transformed with *SAG13::IPT* mean much more refinement of the promoter:gene combination is required (Swartzberg, et al. 2006).

The Rivero group has found that overexpression of *IPT* driven by the *SARK* promoter in tobacco maintained similar levels i.e. did not cause any significant change of stomatal conductance, transpiration rate and photosynthetic rate under

well watered and water deficit conditions, compared to the well watered controls. They found that in the first 35 days of water deficit, WUE in the transformed plants was elevated when compared to the well watered wt controls, while from day 35-75 of water deficit, the WUE of the transformed plants decreased to match that of well watered wt controls (Rivero, et al. 2009). Transgenic plants also displayed minimal yield losses (Rivero, et al. 2007), although there are still some issues associated with elevated glycerate levels in transgenic plants under water deficit, which could lead to feedback regulation of photosynthesis, as well as inhibition of drought induced ABA responses (Rivero, et al. 2007, Rivero, et al. 2009, Rivero, et al. 2010).

Previous work on over-expression of *NCED1* used the CAMV 35S promoter to drive expression, which proved to be too strong, causing serious abnormalities in plant growth and development. (Thompson, et al. 2000, Thompson, et al. 2007, Tung, et al. 2008). More recent work, carried out in the same research lab as this thesis, examined over-expression of *NCED1* driven by the light inducible, RUBISCO small-subunit promoter (*SSUp*), with the expectation that the use of an inducible promoter would overcome growth abnormalities associated with elevated levels of ABA at key developmental stages. However, this promoter also proved to cause abnormalities in growth and development (Sixtus 2013). Due to these findings, it was decided that senescence induced promoters would be selected for the work in this thesis, as expression of the transgene would only occur in plant tissue that was beginning to senesce, not in young, developing tissues.

1.6 Drought and WUE studies in *Trifolium repens* (white clover)

1.6.1 White clover as a forage legume

The importance of the genus *Trifolium* as a forage species is compelling, with at least 16 species regularly grown worldwide as forage and green manure crops (Badr, et al. 2012). *T. repens* (white clover) is the most commonly grown, and is a genetically amphidiploid (2n = 4x = 32), nitrogen fixing, perennial forage legume of significant economic importance in temperate grasslands (Widdup and Williams 1982, Williams, et al. 2008, Hofmann and Jahufer 2011, Ballizany, et al. 2012).

Growth and persistence of white clover is *via* stolons, and while some white clover germplasm selections are able to adapt to dryland summer environments, the growth of usually high biomass cultivars is greatly reduced under summer drought (Hofmann and Jahufer 2011). The performance of white clover as a forage species is a key determinant of the quality, success, and therefore profitability of meat and dairy production. Improving production and realising the full genetic potential of white clover by overcoming limitations imposed by environmental stresses, including, but not limited to extended periods of drought, and also increasing WUE, should increase not only the quality of animal production, but also profitability (Jahufer, et al. 2012). The approach to breeding of improved white clover germplasm favours the utilisation of white clover ancestors and other *Trifolium* species as sources for the incorporation of desirable characteristics such as increased WUE into the white clover genome through crosses and traditional breeding practices, as well as more modern molecular techniques such as overexpression of key genes of interest.

1.6.2 White clover ancestry

A sister relationship between *T. nigrescens,* the non-stoloniferous annual, with a widespread distribution throughout the Mediterranean, northern Africa, Turkey, the Middle East and the Caucasus region, and *T. repens* has been identified through Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) analysis, suggesting that it may be the putative maternal progenitor. However, *T. pallescens* is a diploid member of the *Trifolium* genus, originating from the European alpine areas, found at altitudes above 1,800m and is predominately cross-pollinated, but self-fertile. This species has also been identified as having a sister relationship with white clover and to date, is considered the closest extant species to the maternal ancestor of white clover (Abberton 2007, Williams, et al. 2008, Williams, et al. 2012).

T. occidentale is a diploid plant species, arising from the saline coasts of Western Europe, and is classified as a creeping, clonal perennial. RAPD and AFLP analysis, combined with ribosomal DNA sequence analysis suggest that *T.* occidentale is the most likely direct male ancestor of *T. repens*. *T. occidentale,* interestingly, is self-

fertile and it is thought that previously, the geographical distribution was much wider.

The hybridization event that occurred to create the species that is known as *Trifolium repens* is speculated to have occurred 130,000-13,000 years ago (Abberton 2007, Badr, et al. 2012, Williams, et al. 2012). It is widely thought that the direct maternal ancestor of white clover became extinct in the time since this event.

1.6.3 Studies on drought tolerance in white clover

The Hoffman group in New Zealand is one of several that have carried out a considerable amount of research of the response of white clover to a selection of environmental stresses, including drought (Jahufer, et al. 2012, Nichols, et al. 2014). The group focused on the characterisation of performance of germplasm, and crosses between germplasm, currently or recently commercially available in Australasia, as well as new breeding lines that have been developed.

Research groups that have looked at plant performance under ideal growing conditions, and under a selection of environmental stresses. The examination of plants that are experiencing water deficit includes the analysis of physiological measurements such as root:shoot ratio, root and shoot dry matter, total dry matter, chlorophyll index, WUE, and changes in a selection of glycosides (Widdup and Williams 1982, Hofmann and Jahufer 2011, Ballizany, et al. 2012, Jahufer, et al. 2012). A selection of plant material from a single field trial that appeared promising in terms of improved WUE, carried out by the Hofmann group, was used for molecular analysis of *TrNCED1* in this thesis (see Section 3.1, 4.1).

1.6.4 Studies on WUE efficiency in white clover and the selection of germplasm

There is a small selection of researchers that have been investigating WUE in white clover, though no other group so far is considering manipulation of *NCED1* expression as an approach to improve WUE (Hou and Yan 2012, Ates, et al. 2013,

Black and Murdoch 2013, Forster, et al. 2013, Uliarte, et al. 2013, Da Silva, et al. 2014).

The majority of these groups have been assessing the water relations and overall plant WUE to identify material for traditional breeding programs as a way of improving WUE of white clover. However, Forster et al (2013) discuss three different transgenic mechanisms for improving plant water use efficiency in white clover. The inhibition of leaf senescence using the *SAG12* promoter to drive over-expression of *IPT,* and in parallel, agrobacterium mediated transformation with *A. thaliana MYB32* transcription factor has achieved some success. The third technique examined is the overexpression of *WXP1*, an *AP2/ERF* family transcription factor, which results in increased cuticle wax production. This has been found to enhance plant turgor, and hasten recovery from water stress (Forster, et al. 2013). While this last technique is not specifically looking at WUE, there is potential for enhanced WUE with the use of different promoters.

The two varieties selected for WUE trials by Hofmann and Jahufer (2011) were *T. repens cv.Grasslands Kopu II* (Kopu II) and *T. repens* ecotype *Tien Shan* (Tien Shan). Kopu II is a cultivar bred by AgResearch Grasslands to produce a high biomass under normative field conditions throughout New Zealand (Hofmann and Jahufer 2011) whereas the Tien Shan cultivar originates from the Tien Shan mountain ranges in central Asia and while it produces a lower biomass than Kopu II, with smaller, waxier leaves (see figure 1.2) it has found to be more resilient and tolerant to drought conditions. It thus which raises the question of a mechanism of enhanced WUE. Both varieties have been identified by the Hofmann group as displaying compelling physiological response to water deficit, and potential for improved WUE (Ballizany, et al. 2012, Nichols, et al. 2014).

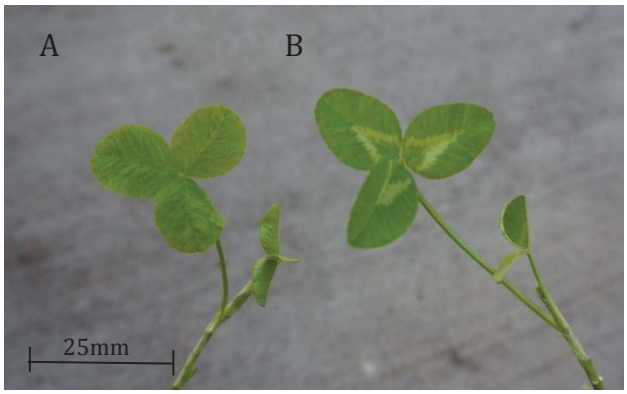


Figure 1.2 Comparison of the leaf size and colouration of the two *Trifolium repens* varieties Tien Shan (A) and Kopu II (B).

1.7 Aims of Thesis

The primary aim of this thesis is to determine whether overexpression of the ABA biosynthetic gene *SlNCED1* (*Solanum lycopersicum 9-cis-epoxycarotenoid dioxygenase 1*) directly in tobacco *(Nicotiana tabacum),* driven by the senescence associated promoters *SAG13* and *SARK*, is a suitable mechanism for production of germplasm that displays enhanced water use efficiency. The longer term aim of the research programme is to assess the overexpression of *NCED1* in the agronomically-important pasture legume white clover (*T. repens L.*)

To address this primary aim, the thesis was divided into three research objectives:

- To assess the constitutive expression of *NCED1* in germplasm of white clover and other *Trifolium* species to determine is any correlation exists between expression and germplasm with higher WUE.
- To assess the phenotype of genetically independent lines of tobacco transformed with *SlNCED1*, when driven by the senescence-associated *SAG13* and *SARK* promoters, in terms of any changes to the water relations parameters (stomatal conductance and transpiration rate) in the transformants.
- To examine the constitutive expression of *TrNCED1* in different tissues of white clover, but particularly in senescent tissue, prior to transformation with $SAG13_p$*::SlNCED1* or $SARK_p$*::SlNCED1*.

Chapter 2

General Materials and Methods

2.1 Chemicals and Media

2.1.1 Chemicals

Unless otherwise stated, all chemicals were sourced from Sigma Aldrich Company (St. Louis, Mo., USA), Duchefa Biochemie B.V (Haarlem, The Netherlands), Merck Ltd. (Darmstadt, Germany), Promega Corporation (Madison, WI. USA), QIAGEN GmBH (Hilden, Germany), Life Technologies Corp. (Grand Island, NY. USA), Roche Applied Sciences (Roche Diagnostics GmBH, Mannheim, Germany), and Bio-Rad Laboratories (Hercules, CA USA).

2.1.2 Media

Bacterial and tissue culture media were prepared using milli-Q water, and autoclaved at 121^0C for 15 min to sterilise before use. Solid media was allowed to cool to approximately 50^0C before the addition of antibiotics. Liquid media was cooled to room temperature before the addition of antibiotics.

2.1.3 Buffers and Solutions

Milli-Q water was used to prepare all buffers and solutions excluding those used for RNA work where DEPC treated or DNase free water was used. DEPC water was prepared by adding 1mL of DEPC into 1L of Milli-Q water, followed by stirring overnight at room temperature and then autoclaving at 121^0C for 15 min.

2.2 Plasmid cloning and Transformation

2.2.1 pGEM®-T Easy Vector Transformation

The inserts from two plasmids (Appendix 3) supplied by Professor Andrew Thompson, Cranfield University, UK, were ligated into the pGEM®-T Easy Vector (Promega) according to the protocol supplied with the kit, by Ms Susanna Leung, Massey University.

To ensure transformation was successful, the vectors were submitted to the DNA Analysis Facility, Massey Genome Service at Massey University for sequencing based on the standard protocol for automated capillary analysis on the ABI3730 DNA analyser (Applied Biosystems) by Ms Susanna Leung.

2.2.2 Electroporation of *Agrobacterium tumefaciens*

Electroporation of *Agrobacterium tumefaciens* was carried out by Ms Susanna Leung, following the protocol set out by (Franklin, et al. 1992). The details are provided in Appendix 10.

2.2.2.1 Preparation of electrocompetent cells

- A Fernblach flask containing 1.5 L of YEB Broth was inoculated with a 7.5 mL aliquot from the log phase culture of *A. tumefaciens*, followed by incubation at 30^0C overnight with shaking at 300 x *g* to a density of 5-10 x 10^7 cells/mL.
- Cells were decanted into sterile 500 mL centrifuge bottles and collected by centrifugation at 300 *x g* for 10 min at 4^0C
- The supernatant was carefully discarded and the bottles containing the pelleted cells placed on ice.
- Fifty mL of sterile, ice-cold 10% (v/v) glycerol was added to each bottle and the pellets resuspended by vortexing. The volume of each bottle was increased to 500 mL with more sterile, ice-cold 10% (v/v) glycerol to wash the cells that were collected again by centrifugation at 3000 *x g* for 5 min at

4^0C. The supernatant was carefully discarded, and the entire washing process repeated.

- The cells were then resuspended in 5 mL of sterile, ice-cold glycerol and transferred to a chilled 30 mL Oakridge tube. The cells were again collected by centrifugation at 3000 *x g* for 5 min at 4^0C, and the supernatant carefully discarded.
- The cell pellet was then resuspended in 0.5 mL or sterile, ice-cold 1 M sorbitol, to give a final cell volume of approximately 1.5 mL, and a cell concentration of approximately 5 x 10^{10} cells/mL.
- Two hundred µL aliquots were made into 1.5 mL microtubes, frozen in an isopropanol/dry ice bath then stored at -80^0C.

2.2.2.2 Electroporation

- Up to 5 µL of each DNA sample/plasmid to be electroporated was pipetted into sterile 1.5 mL microfuge tubes and placed on ice.
- For each DNA sample/plasmid to be electroporated, 1 mL of YEB broth was added to a 17 x 100 tube at room temperature, and a 0.2cm electroporation cuvette was placed on ice.
- The electrocompetent *A. tumefaciens* was thawed on ice, before 40 µL was added to each DNA sample/plasmid, and tapped to mix.
- The MicroPulser (BioRad, CA, USA) was set to "Agr" (time constant approximately 5 millisec), and the DNA/Plasmid-cell samples were transferred to the electroporation cuvettes and tapped down to the bottom of the tubes. The tubes were placed one –at-a-time into the chamber slide, slid into the chamber, and pulsed once
- The cuvettes were removed from the chamber and the cells immediately transferred into 17 x 100 tubes, using the YEB broth inside.
- The cells were then incubated at 30^0C for 3 hours with shaking at 250 x *g* before aliquots of the electroporated cells were plated onto YEB agar plates and incubated for 48 hours at 25^0C.

2.3 Plant Material

2.3.1 Lincoln Trials

Trials were designed to compare the drought response of two white clover varieties cv. Kopu II and the ecotype Tien Shan, against well watered (non-droughted) control plants. The trials and sampling were carried out by Dr Rainer Hofmann at Lincoln University, Canterbury, and the sampled material immediately frozen in liquid nitrogen and sent up to Massey University in Palmerston North on dry ice, for analysis of *TrNCED1* expression, (see section 2.3.1).

The first trial was carried out in a glasshouse in vermiculite and complete root systems for each plant were collected.

The second trial was designed to look at differences in *TrNCED1* in three tissue types: root tissue, the first fully expanded leaf, and shoot apical tissue both in well-watered tissues and in response to a drought. Plants were grown in soil, and the tissue split into first-fully-expanded leaves, apical structures, and complete root systems for each plant. The complete root systems in the Lincoln 2 trial were quickly rinsed with water and blotted dry before freezing. On receipt of Lincoln 1 and Lincoln 2 trials, samples were ground to a fine powder using a chilled mortar and pestle and liquid nitrogen in preparation for RNA extraction.

2.3.2 AgResearch Trials

Six clover species, including one white clover cultivar, and one ecotype, were selected by Mrs Isabelle Williams of AgResearch, for leaf and apex sampling: *T. ambiguum, T. pallesens, T. uniflorum, T. occidentale, T. repens* cv. Kopu II and *T. repens* ecotype Tien Shan*.* Three biological replicates, where a vegetatively propagated copy of the same parent plant was deemed to be a single biological replicate, were available for each species (excluding *T. repens* ecotype *Tien Shan* where only two copies were available for sampling). Three technical repeats were made for the collection of the first-fully-expanded leaf (FFEL), whereas five technical repeats were made for the collection of apical meristem (Apex), to ensure enough tissue for successful RNA extraction. A technical repeat is deemed to be

collection of tissue types from more than one stolon of the same vegetative replicate.

2.3.3 Generation of Transgenic tobacco plants

2.3.3.1 Preparation of Agrobacterium Broth

For plant transformation, aliquots of *Agrobacterium tumefaciens* strain LBA 4404, containing one strain of the plasmid vector pART-AtSAG13-NCED-35S (747), and a second strain containing the plasmid vector pART-AtSARK-NCED-35S (751), were streaked out onto plates of YEB agar containing 100 mg/L Spectinomycin ($YEB^{100Spec}$), and the incubated at 25^0C for 48 h. A loop of each culture was then inoculated into 100 mL of liquid $YEB^{100Spec}$ broth that was then incubated at 30^0C for 48 h with continuous shaking.

2.3.3.2 *Nicotiana tabacum* transformation

Prior to transformation, the Agrobacterium broths (see 2.3.3.1) were separated by centrifugation at 3000 x *g* for 5 min, and the pellet resuspended in 3 mL each of 10 mM $MgSO_4$. Tobacco leaves were excised from sterile 8 week old seedlings grown in tissue culture pottles separately, and cut into approximately 1 cm^2 discs, which were then soaked into the *Agrobacterium* suspension for 1 min. The discs were blotted dry on sterile cellulose filter paper, Grade 1 (Whatman), and placed on Nic I media (MS media including vitamins, 3% (w/v) sucrose, 1 mg/L BAP, 0.1 mg/L NAA, 0.8% (w/v) phytoagar, pH 5.7, no antibiotics; Appendix 2). The tissue was left to co-incubate at 23^0C under continuous light in a SANYO MLR-350H Plant Growth Chamber (SANYO, Panasonic Healthcare Company of North America, IL, USA) for two days before being transferred to fresh Nic I media containing Cefotaxime (100 mg/L) and Kanamycin (100 mg/L). Green tissue material was transferred to fresh Nic $I^{Cef100, Kan100}$ every week until callus formation began. After initiation of callus, the tissue was transferred to Nic $II^{Cef100, Kan100}$ media, which was refreshed each fortnight until shoots formed from the calli. A single shoot from each original leaf disc (to ensure the selection of genetically independent lines) was excised, incorporating the callus base

(after removal of as much surrounding callus as possible), and placed onto Nic III$^{Cef100, Kan100}$ media to induce root formation and growth.

2.3.4 Establishment of Transformed *Nicotiana tabacum* on soil.

Once a root system was established, and shoots were large enough (30-50 mm in height), the transgenic tobacco plants were potted out in soil (Premiere Potting Mix, Daltons, Matamata, New Zealand) in a glasshouse. The soil was first sterilised in an autoclave at 121^{0}C for 20 min, then left to dry for three days. Pots were filled with the sterilised, dry soil, and each was wetted with (1 Tbs/L) Thrive All-Purpose Plant Food (Yates New Zealand, Auckland, New Zealand) and placed in a tray that was 1/3 full of water until each was wet through. Each plant was transferred to a single pot and labelled with a unique identification code. The pots were then placed inside a large plastic bag with ventilation holes to harden off for three days. After hardening off, plants were placed in direct sunlight, watered regularly and given extra nutrients as required in the form of 1 Tbs/L Thrive All Purpose Plant Food.

2.3.5 *Trifolium repens* stolon trials

Ten stolons were taken from a single Kopu II White clover plant, and 10 from a single Tien Shan plant. Stolons were excised just below the fourth node from the apex, stripped of all leaves except the first emerged leaf, and washed in a gentle detergent solution (1/2 tsp of sunlight liquid soap in 1 L water) to remove any insects. These cuttings were placed in pottles containing vermiculite, and watered every other day with 0.5 X Hoagland's solution. The pottles were placed in labelled trays on top of black/white polythene sheeting, white side up and the stolons encouraged to grow horizontally over the white plastic. Any lateral stolons were removed as they formed.

When harvested, seven tissue types for each cultivar were selected (see Figure 3.4).

- Apex and emerging leaf

- First fully expanded leaf
- Mature leaf
- Onset senescence leaf
- Young stolon
- Older stolon
- Roots

2.4 Chlorophyll extraction and Absorbance measurements

Chlorophyll was extracted from the leaves of transgenic *N. tabacum* plants, as well as the leaves of *T. repens* following the protocol below (Ritchie 2008):

- Approximately 100 mg of fresh tissue was ground in liquid nitrogen.
- The tissue was resuspended in 1 mL of chilled 96% (v/v) EtOH, and the chlorophyll extracted at 4^0C overnight.
- After centrifuging at 14000 *x g*, 2 mL of the supernatant was removed and used to quantify Chlorophyll *a* and *b* using a Thermo Scientific NanoDrop ND 1000 (Thermo Scientific, Waltham, MA USA) to measure absorbance at 649 nm (Chlorophyll *b*) and 665 nm (Chlorophyll *a*).

The chlorophyll concentration was then calculated using the following equations:

$$\text{Ch}a = 13.70\ x\ \text{A665 nm} - 5.76\ x\ \text{A649 nm}$$

$$\text{Ch}b = 25.80\ x\ \text{A649nm} - 7.60\ x\ \text{A665nm}$$

$$\text{Total Chlorophyll } = 6.5\ x\ \text{A665nm} + 20.04\ x\ \text{A649nm}$$

Chlorophyll content is expressed as µg/mg fresh weight.

2.5 RNA extraction from *T. repens* and *N. tabacum*

Total RNA was extracted from a variety of tissue samples from *T. repens* and *N. tabacum* using the Hot Borate method used by Hunter and Reid (2001) and Moser (2004). All mortar and pestles, spatulas and glassware used for RNA extraction were wrapped in aluminium foil and baked at 180^0C for a minimum of 16 h. A 0.3% (v/v) hydrogen peroxide solution was used to sterilise plastic-ware and stir-bars overnight, followed by a rinse with DEPC-treated water before use. Microtubes were autoclaved to sterilise. All solutions required for the protocol were prepared to the required concentrations using DEPC-treated water. Reagents and chemicals used in the protocol were:

- Borate Buffer: 200 mM di-sodium tetraborate decahydrate (pH 9.0), 30 mM EGTA, 1% (w/v) SDS and 1% (w/v) sodium deoxycholate salt.
- Extraction Buffer: Borate buffer (pH 9.0), 10 mM DTT, 1% (w/v) IGEPAL CA-630 and 2% (w/v) PVP-40
- Proteinase-K (20 mg/mL)
- 2 M KCl
- 3 M Sodium acetate (pH 5.2)
- 4 M LiCl
- Chloroform:isoamyl-alcohol [24:1 (v/v)], Isopropanol, and 80% (v/v) Ethanol

Steps followed for total RNA extraction:

- To extract RNA from frozen apex, leaf and root tissues, typically 50 mg – 200 mg fresh weight was ground to a fine powder in a pre-chilled mortar and pestle with liquid nitrogen, or pre-chilled microtube with a micro pestle.
- The ground tissue was transferred to a microtube containing five volumes (w/v) of hot (80^0C) extraction buffer, and vortexed to resuspend the powder in the extraction buffer.
- Proteinase-K was added (0.75%; v/v) and the slurry was incubated at 42^0C with shaking for 90 min.

- Following incubation, 2 M KCl was added [0.08 v/v] to give a total concentration of 160 mM, the solution mixed by inversion then incubated in an ice bath with shaking for 30 min.
- The mixture was then centrifuged at 17,000 *x g* for 20 min at 4^0C, the supernatant transferred to a fresh microtube, to which an equal volume of cold 4 M LiCl (to give a final concentration of 2 M) was added to precipitate the RNA at 4^0C overnight.
- The next day the precipitate was collected by centrifugation at 17,000 *x g* at 4^0C for 30 min, and resuspended in 200 µL of DEPC-treated water before the addition of 20 µL of 3 M sodium acetate (to give a final concentration of 0.3 M) and 200 µL of chloroform:Isoamyl alcohol [24:1 (v/v)].
- The aqueous and organic phases were vortexed to mix, before separation by centrifugation at 17,000 *x g* at 4^0C for 5 min. The upper aqueous phase was carefully pipetted to a fresh microtube.
- One volume (200 µL) of chloroform:Isoamyl alcohol [24:1 (v/v)] was added, the tube vortexed to mix the aqueous and organic phases, followed by separation by centrifugation at 17,000 *x g* at 4^0C for 5 min. The upper aqueous phase was carefully pipetted to a fresh microtube.
- One volume (200 µL) of isopropanol was added, the contents mixed well and incubated on ice for 60 min to precipitate the RNA.
- The RNA was collected by centrifugation at 17,000 *x g* at 4^0C for 30 min, washed with ice-cold 80% (v/v) ethanol before collection at 17,000 *x g* at 4^0C for 10 min. The pellet was air dried for 10 min then resuspended in 500 µL of DEPC-treated water.
- To remove traces of genomic DNA, 0.8 x volume of cold 4 M LiCl was added to precipitate RNA at 4^0C overnight.
- The next day the RNA was collected by centrifugation at 17,000 *x g* at 4^0C for 30 min, and then washed with first 2 M LiCl, then 80% (v/v) ethanol at 17,000 *x g* at 4^0C for 10 min each.
- The pellet was air dried for 10 min then resuspended in 20 µL of DEPC-treated water.

Aliquots of RNA were stored at -80^0C until required.

2.6 DNase I Treatment

Genomic DNA-free RNA samples were prepared using an RNase-free recombinant DNase (Roche) treatment.

Total RNA (1 µg – 3 µg) extracted as described in section 2.5, was mixed with 5 µL of 10 x DNase reaction buffer and 1 µL (10 U) of DNase I to give a final volume of 50 µL.

The mixture was then incubated at 37^0C for 20 min before the reaction was stopped by adding 2 µL of 0.2 M EDTA (pH 8.0) and heat deactivation at 75^0C for 5 min.

2.7 Isolation of genomic DNA (gDNA) using the CTAB/Chloroform-Isoamyl Alcohol method.

Extraction of genomic DNA from tobacco was performed using the modified protocols of Doyle and Dickson (1987), Doyle (1991) and Cullings (1992). Reagents used for the protocol were:

- Extraction buffer: containing 0.1 M Tris (pH 8.0), 1.4 M NaCl, 20 mM EDTA, 2% (w/v) CTAB, 4% (w/v) PVP-40, and 83% (v/v) β-mercaptoethanol
- Chloroform:isoamyl-alcohol [24:1 (v/v)]
- 7.5 M Ammonium acetate
- Isopropanol
- 70% (v/v) and 95% (v/v) Ethanol

Steps followed for genomic DNA extraction:

- Frozen leaf tissue (50-100 mg) was ground in a pre-chilled mortar and pestle with liquid nitrogen, to which 500 µL of CTAB buffer was added before transfer to a microtube.
- The solution was vortexed, and then incubated at 55^0C for 60 min. Following incubation, 500 µL of chloroform: Isoamyl alcohol [24:1 (v/v)] was added and the slurry mixed well by inversion before separation by centrifugation at 17,000 *x g* for 10 min.
- The aqueous (top) phase (approximately 350 µL) was quickly and carefully pipetted into a fresh microtube, and 0.08 volumes of cold 7.5 M Ammonium acetate and 0.54 volumes of cold isopropanol were added. The solution was mixed well before incubation at -20^0C for 60 min.
- The pellet was separated by centrifugation at 17,000 *x g* for 3 min and the supernatant carefully pipetted off before the pellet was washed with first 700 µL of cold 70% (v/v) ethanol, then 95% (v/v) ethanol by a single tube inversion with centrifugation at 17,000 *x g* for 1 min after each wash.

- After removal of the ethanol *via* careful pipetting, the pellet was dried on a hot plate at 55^0C, and resuspended in 100µL of milli-Q water at 55^0C for 60 min before use.
- Genomic DNA was stored at 4^0C if being used within 2 weeks, and at -80^0C for long term storage.

2.8 Quantification of Nucleic Acids

2.8.1 Genomic DNA

The genomic DNA concentration was determined by measuring the absorbance at 260 nm (A_{260}) using a NanoDrop ND-1000 spectrophotometer V3.6 (Thermo Scientific, USA). The purity of the genomic DNA was determined using the A_{260}/A_{280} ratio, against a blank of milli-Q water. Relatively pure solutions were found to have a A_{260}/A_{280} ratio of >1.8 (Sambrook, et al. 1989)

2.8.2 Total RNA

Total RNA concentration was determined by measuring sample fluorescence against RNA standards. The value given by the Qubit® Fluorometer (Life Technologies, USA) is then put through the following calculation to determine sample concentration in ng/mL:

$$sample\ concentration = QF\ value \times \left(\frac{200}{x}\right)$$

Where:

QF = the value given by the Qubit® Fluorometer

X= the amount (µL) of sample added to the assay tube

2.9 Synthesis of cDNA

Synthesis of the first single-strand DNA was carried out using the Expand Reverse Transcriptase cDNA synthesis kit (Roche), with oligo (dT) primer. Reagents used:

- Expand Reverse Transcriptase (50 U/µL)
- Expand RT buffer (5 X): 250 mM Tris-HCL, 200 mM KCl, 25 mM $MgCl_2$, and 2.5% (v/v) Tween[2] 20 pH 8.3.
- Oligo $(dT)_{15}$ primer (final concentration 2.5 µM)
- RNase Inhibitor (40 U/µL)
- dNTP mixture (final concentration 1 mM
- Dithioerythritol (DTT) (final concentration 10 mM)

Programme used for cDNA synthesis:

Steps	Temperature	Time
Denaturation	65^0C	10 min
Incubation	43^0C	60 min
Deactivation	95^0C	2 min

Steps followed for cDNA synthesis:

- Total RNA (1 µg) was combined with the Oligo $(dT)_{15}$ primer in a 0.2 mL PCR tube, and the volume made up to 10.5 µL with PCR-grade water.
- The tube contents were denatured at 65^0C for 10 min in an Axygen MaxyGene™ thermocycler (Axygen Scientific, CA, USA) followed immediately by placement on ice.
- 9.5 µL of a Master Mix containing 5 X Expand RT buffer, 100 mM DTT, 10 mM dNTP mix, 40 U/µL RNase inhibitor and 50 U/µL Expand Reverse Transcriptase was then added to each tube.
- The tubes were returned to the Maxygene™ thermocycler and cDNA synthesis was carried out at 43^0C for 60 min, followed by heat-deactivation at 95^0C for 2 min.

- The cDNA was stored at 4^0C for short periods, and at -80^0C for long-term storage.

2.10 Polymerase Chain Reaction (PCR)

2.10.1 Primer Design

For Q-RT-PCR of the gene of interest from white clover, specific primers were designed using the conserved region sequences of *Medicargo truncatula*, *Phaseolus vulgaris*, *Glycine max*, *Cicer arietinum* and *Pisum sativum* (Appendix 7), in addition to the general requirements for melting temperature (T_M = 60^0C ±1^0C), minimal secondary structure, and inability to form stable diamers. All primers annealed to give similar size amplified products (140-170 nucleotides), and a standard curve was generated to determine primer efficiency. A stock solution of each primer (Sigma and Integrated DNA Technologies, IA, USA) was prepared by dissolving the primers in milli-Q water to give a final concentration of 1 mM. Working stocks of 10μM were then prepared by dissolving 1 μL of the stock in 99 μL of milli-Q water.

2.10.2 General protocol for PCR amplification of cDNA

PCR set-up:

Forward primer (10 μM)	1 μL
Reverse primer (10 μM)	1 μL
2X PCR Master Mix (containing taq DNA polymerase, dNTP's, $MgCl_2$ and reaction buffer)	10 μL
cDNA	1 μL
Sterile water	7 μL
Total	20 μL

PCR programme:

Steps	**Temperature**	**Time**	**Cycle(s)**
Initialization	95^0C	3 min	1
Denaturation	95^0C	10 sec	

Annealing	60^0C	10 sec	(x35)
Extension/elongation	72^0C	10 sec	
Final extension	72^0C	10 min	1

2.10.3 Agarose Gel Electrophoresis

DNA fragments generated by PCR were separated using agarose gel electrophoresis. The reagents used for this process were:

- UltraPURE™ agarose (Life Technologies)
- 50 X TAE Buffer
- Loading Dye [10 X SUDS (0.1 M EDTA, pH 8.0, 50% (v/v) Glycerol, 1% (w/v) SDS, 0.025% (w/v) Bromophenol blue)]
- Ethidium Bromide 10 mg/mL
- HyperLadder™1 DNA ladder (Bioline, London, UK)

To prepare a 1% (w/v) gel, 1 g of powdered agarose was dissolved into 100mL of 1X TAE buffer, and the cooled agarose poured into an appropriately sized gel tray with a well-comb inserted. After the gel had set, 1X TAE running buffer was added and the comb carefully removed. The PCR products were mixed with 2µL of 0.1% (v/v) SUDS before loading into wells. PCR products were generally run at 90V for 45 min. Agarose gels (2%) were also used, which consisted of 2 g of powdered agarose dissolved into 100 mL of 1 X TAE buffer.

Following electrophoresis, the gel was developed with 0.1 µg/mL ethidium bromide for 10-15 min, and then de-stained in water for 5-10 min. The DNA fragments were visualised using a GelDoc 2000 Gel Documentation system from Bio-Rad Laboratories (USA).

For in-gel quantification, the DNA quantity was estimated by through comparison of the relative intensity the DNA band to the DNA ladder using a Gel Doc™ system (Bio-Rad Laboratories Inc. Hercules, CA USA).

2.10.4 Semi-quantitative RT-PCR

RNA was isolated and used for RT-PCR. The resulting RT-PCR products were used for SQ-PCR, using *SlNCED1* and *NtEF1*-α primer sets. The *NtEF1*-α was used as an internal control to compare expression levels of pART-AtSARK-NCED-35S (751 lines), and pART-AtSAG13-NCED-35S (747 lines).

After RT-PCR (2.10.2) and separation of bands through gel electrophoresis (2.10.3), the bands were quantified using Image lab software version 3.0 (Biorad, CA. USA). Bands were selected and the volume/intensity of each band was determined using the following formula:

$$\text{relative } LeNCED1 \text{ expression} = \frac{\text{volume of } LeNCED1 \text{ band}}{\text{volume of } NtEF1\alpha \text{ band}}$$

2.10.5 Gel extraction and Sequencing

PCR products were separated by gel electrophoresis and excised from 2% (w/v) agarose gels to prepare for sequencing, using a Thermo Scientific GeneJET Gel Extraction and DNA Clean-up Micro Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions.

Reaction Protocol:

- A gel slice containing the DNA fragment of interest, weighing up to 200 mg, was excised from a 2% (w/v) agarose TAE gel, and placed in a 1.5 mL tube.
- 200 µL of Extraction Buffer from the kit was added, and mixed thoroughly by pipetting.
- The mixture was incubated at 50-58^{0}C for 10 min, or until the gel slice is completely dissolved. The tubes were regularly mixed by inversion during this time.
- An Aliquot of 200 µL of 96% (v/v) ethanol was then added to each tube, and the contents mixed by pipetting.

- The mixture was then transferred to the DNA Purification Micro Column over a collection tube and centrifuged at 14 000 x *g* for 1 min.
- The flow-through was discarded and the collection tube replaced, before 200µL of Prewash Buffer from the kit was added to the purification column, and the tubes centrifuged at 14 000 *x g* for 1 min.
- The flow-through was discarded and the collection tube replaced, before 700 µL of Wash Buffer from the kit was added to the purification column and centrifuged for at 14 000 *x g* for 1 min.
- The previous step was repeated before the empty DNA Purification Micro Column was centrifuged for one additional minute to remove residual Wash Buffer.
- The column was transferred into a fresh 1.5 mL tube before the addition of 10 µL of elution buffer and centrifugation at 14 000 x *g* for 1 min to elute the DNA.
- The column was then able to be discarded, and the purified DNA stored at -20^{0}C.

2.10.6 DNA Sequencing

DNA sequencing was carried out on purified PCR products excised from agarose gels (Section 2.10.5). An aliquot of 500 ng of purified product was used per sequencing reaction with 3.2 pmol of qPCR primer. The diluted template and primer was sent to Massey Genome Service (Massey University) where the sequencing reads were carried out using the BigDye Terminator V3.1 chemistry. Sequence alignments were carried out using BLAST (Appendix 9).

2.10.7 Quantitative RT-PCR (qRT-PCR)

The LightCycler® 480 Real-Time PCR system (Roche) and software V1.7, was used to carry out qRT-PCR. Three technical repeats of each cDNA sample (10 fold dilution) and SYBER green was used to monitor the efficiency of DNA synthesis.

Reaction set-up:

Forward Primer	0.5 µL
Reverse Primer	0.5 µL
2X LightCycler®480 SYBER green I Master Mix	5 µL
cDNA	2.5 µL
Sterile Water	1.5 µL
Total	10 µL

Master Mix and cDNA were dispensed into 96-well plates, and underwent the following programme:

Steps		**Temperature**	**Time**	**Cycle(s)**
Pre-incubation		95^0C	5 min	1
Amplification	Denaturation	95^0C	10 sec	
	Annealing	60^0C	10 sec	45
	Extension	72^0C	10 sec	
Melting Curve		95^0C	5min	1
Cooling		40^0C		1

The relative abundance of each transcript was determined by comparative quantification to the geometric mean of the two reference genes, *Tr-GAPDH* and *β-Tr-Actin* (Pfaffl 2001). Fluorescence measurements were carried out at 72^0C for each cycle and continuously throughout final melting (see Appendix 8 for primer sequences).

2.11 Gas Exchange Measurements

Measurements were taken non-destructively, on three leaves that were judged to be young/recently emerged, mature, and onset senescent, once plants were

flowering. Measurements of conductance, transpiration, internal CO_2 and photosynthetic rate were carried out using a LI-6400 Infra-red gas analyser (Li-Cor Biosciences, Lincoln, NE, USA), under cuvette conditions of 100 µmol $m^{-2}s^{-1}$ PAR saturation, 75% relative humidity, 400 ppm CO_2, and a temperature of 20^0C, and PAR was provided by a Li-Cor RGB LED light source within the cuvette. This low PAR saturation level was selected to match the level in the growth room. Leaves were maintained within the cuvette for a minimum of 5 min, to stabilise before a measurement was recorded.

2.12 Statistical Analysis

Statistical analysis was carried out using Microsoft Office Excel 2010. For qPCR, the geomean of technical repeats was calculated for each sample, before calculation of the change in cq, and ratio. Standard error for each ratio was calculated, and the Student's T-test used to determine significance. All graphs were prepared using Origin 9.1 (OriginLab Corporation, Northampton, M.A, USA). Multiple sequence alignments were carried out using Clustal Omega (EMBL-EBI, Cambridgeshire, UK) and BLAST (National Centre for Biotechnology Information, U.S. National Library of Medicine, Bethesda M.D, USA).

Chapter 3

Results

3.1 Assessment of *NCED1* expression as a natural determinant of improved water use efficiency.

The allotetraploid (2n=4x=32) out-crossing plant species *T. repens* (white clover) has a broad adaptive range as a result of interspecific hybridisation between the diploid ancestors *T. occidentale* and *T. pallescens* proposed by Williams et al (2012). The difference in geographical origins of the two ancestors; *T. occidentale* originating from the saltine coasts of western Europe, and *T. repens* from alpine areas of Europe, and the characteristics evolved for optimal growth (salt and drought tolerance, and prolific flowering respectively) in the corresponding environments is thought to be responsible for the high productivity and suitability of white clover as a forage legume. Water-use-efficiency (WUE) however, among other characteristics, varies significantly between different cultivars of white clover, which impacts growth and performance under less than optimal environmental conditions.

To examine whether the up-regulated expression of *NCED1* is a natural determinant of germplasm with increased WUE, two approaches were used. The first involved examining the expression levels of *TrNCED1* in two varieties of white clover, the ecotype Tien Shan and the cultivar Kopu II that had been shown to display differences in terms of adaptations to dryland areas. The second approach expanded on this idea, investigating the natural expression levels of *TrNCED1* in a selection of *Trifolium* species that displayed some morphological characteristics that would support increased water use efficiency and/or drought tolerance.

3.1.1 Examination of *TrNCED1* expression in two different white clover varieties.

The expression of *TrNCED1* in the well watered Kopu II root tissue was more than *10-fold* higher (with respect to expression of the reference genes *Tr-Actin* and *Tr-GAPDH*) than expression of *TrNCED1* in well watered root tissue from Tien Shan. *TrNCED1* expression was also seen to be significantly lower (p=0.009 for Tien Shan; p=0.002 for Kopu II) in root tissue from the drought treatment, when compared with levels in the well watered tissue for both cultivars (Figure 3.1).

3.1.2 Analysis of *TrNCED1* expression in different tissue types.

In terms of well-watered comparisons between the two varieties, expression in the apex was *ca.* 5.9 *fold* higher in c.v Kopu II, while expression in the leaf tissue was *ca.* 1.6-*fold* higher. For the root tissue expression was *ca.* 2.4 *fold* lower in cv. Kopu II (see figure 3.2).

The expression level of *TrNCED1* in the apical tissue of both cultivars, in response to drought/water deficit was *ca. 100-fold* lower than in the other tissue types measured. There was a significant *ca. 12- fold* decrease in *TrNCED1* expression (p=0.008) between the well watered and drought treatments of Kopu II. There was no significant difference in expression for apical tissue was found for the Tien Shan cultivar (Figure 3.2) between the well watered and drought treatments.

The expression of *TrNCED1* (with respect to *Tr-Actin* and *Tr-GAPDH*) was highest the first fully expanded leaf tissue from both cultivars and in response to both treatments. In Tien Shan, there was higher *TrNCED1*expression (with respect to *Tr-Actin* and *Tr-GAPDH*) in tissue harvested from plants that had undergone the drought treatment when compared with expression in tissue that had undergone the well watered treatment. For Kopu II, there was *ca. 1.7- fold* lower expression of *TrNCED1* (p=0.011) found in tissue excised from plants that had undergone drought treatment, when compared with expression of leaf tissue from the well watered treatment (with respect to *Tr-Actin* and *Tr-GAPDH* expression).

The expression of *TrNCED1* in root tissue showed the opposite trend to that observed for the first fully expanded leaf tissue. In Tien Shan, expression was *ca. 17-fold* lower (p=0.008) in tissue from the drought treatment, when compared with expression root tissue from the well watered treatment (with respect to *Tr-Actin* and *Tr-GAPDH*). Comparatively, in Kopu II, expression of *TrNCED1* was *ca. 2.7- fold* higher (p=0.002) in tissue from the drought treatment than levels in tissue from the well watered treatment (with respect to *Tr-Actin* and *Tr-GAPDH*).

For comparisons of tissue that had been subjected to a period of water deficit, across the two varieties, expression in the apex was *ca.* 1.8 *fold* higher (p=0.269) in Tien Shan, whereas in the leaf tissue expression was *ca.* 1.5 *fold* higher (p=0.086). Expression in the root tissue was *ca.* 3.9 *fold* lower (p=0.001) in ecotype Tien Shan.

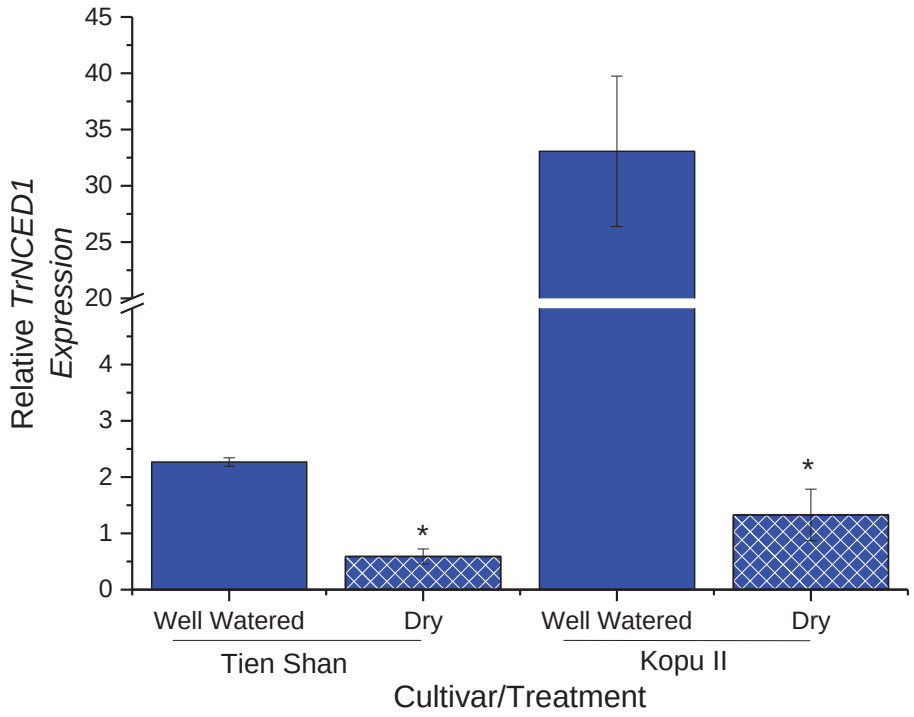


Figure 3.1 Relative expression of *TrNCED1* in roots of plants of the ecotype Tien Shan or cv. Kopu II, either well-watered or subjected to a water deficit, as indicated. q-RT PCR was carried out using three biological replicates, where pooled tissue from four plants represents one biological replicate, for each cultivar/treatment using *Tr-Actin* and *Tr-GAPDH* as internal controls. Each data point represents the mean value of biological replicates and the error bars represent mean ± SEM, n=3. Significant differences within each variety are calculated by a t-test to 95% confidence, which is indicated by a (*).

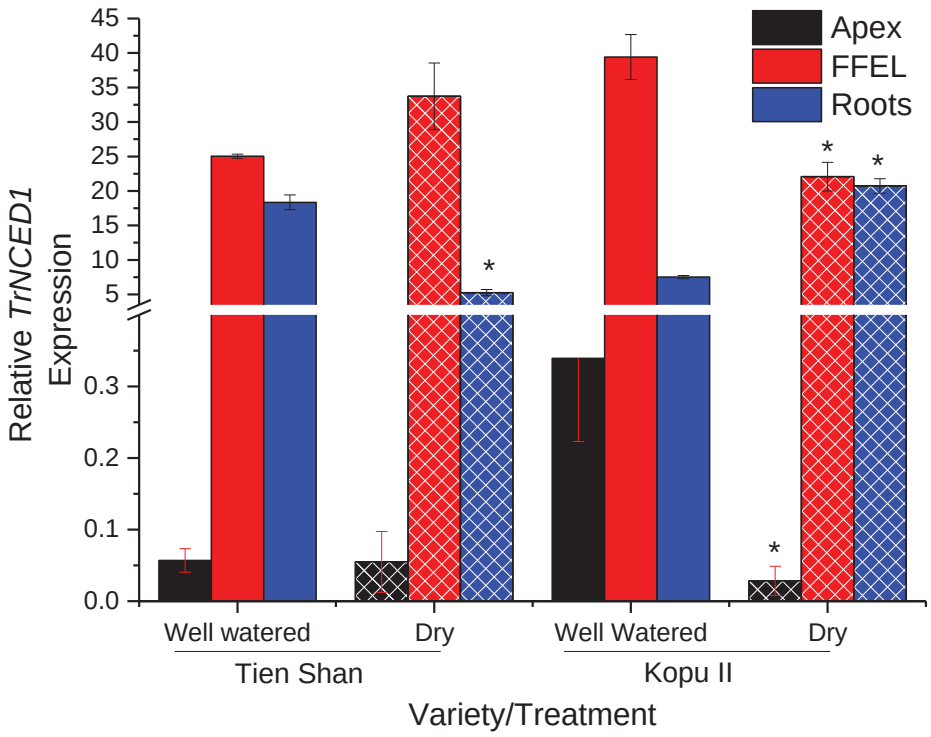


Figure 3.2 Relative expression of *TrNCED1* in apex, leaf and root tissues of cv. Kopu II and ecotype Tien Shan, as indicated. q-RT PCR was carried out using three technical replicates with pooled tissue from four plants for each cultivar/treatment using *Tr-Actin* and *Tr-GAPDH* as internal controls. Each data point represents the mean value of three technical replicates and the error bars represent mean ± SEM. Significance differences between treatments within a variety and tissue are calculated by a t-test, to 95% confidence, and is indicated by a (*)

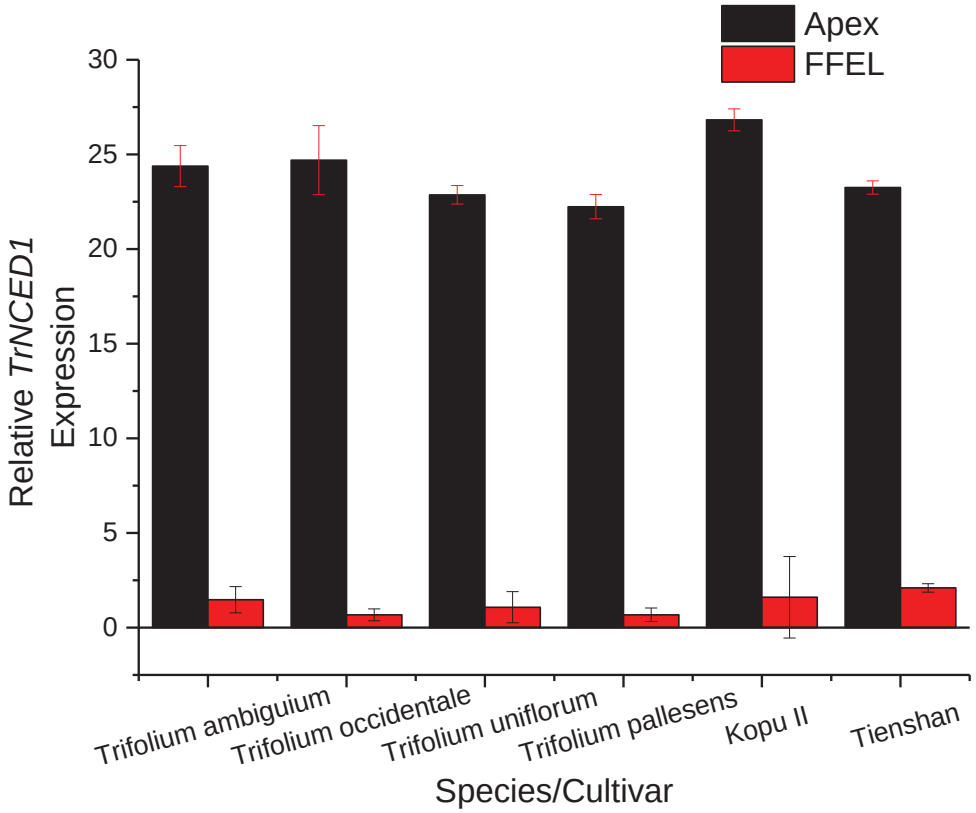


Figure 3.3 Relative expression of *TrNCED1* in a selection of *Trifolium* species as indicated. q-RT PCR was carried out using three biological replicates with pooled tissue from 3 plants for each cultivar/treatment using Tr-Actin and Tr-GAPDH as internal controls. Each data point represents the mean value of three biological replicates and the error bars represent mean, ± SEM.

3.1.3 Expression of *TrNCED1* across a selection of *Trifolium* species.

As differences in *TrNCED1* expression were found, particularly in terms of well watered gene expression (see section 3.1.2), between the two white clover varieties Tien Shan and Kopu II, it was decided that it would be of interest to investigate the relative expression of *TrNCED1* in a selection of closely related *Trifolium* species. The four species selected, in addition to the two varieties of *T. repens* already examined, also display a variety of physiological characteristics/ adaptive mechanisms that confer differences that may support differences in WUE.

In terms of the relative expression of *TrNCED1* with respect to expression of *Tr-Actin* and *Tr-GAPDH* in each background, it was observed that transcript abundance in leaf tissue was a minimum of *ca. 5-fold* lower than expression in the apical tissue. However, the amount of variation between the biological replicates for each species meant that no significant difference was found in *TrNCED1* expression across the different species/varieties (Figure 3.3).

3.2 Overexpression of *SlNCED*1 in *N. tabacum*.

As abscisic acid has been identified as playing a role in controlling stomatal conductance (see section 1.2) and therefore water loss, overexpression of the ABA biosynthetic gene *NCED1* in tissues that have been found to have low native expression, should assist in reducing water loss, by reducing the amount of stomata present or open in those tissues. The senescence-induced promoters *AtSAG13* and *AtSARK* were selected in an attempt to avoid overexpression of *NCED1* too early in plant development, where elevated ABA levels cause a variety of growth problems, including delayed germination and plant growth (Tung, Smeeton et al. 2008).

Senescent tissue has much lower levels of chlorophyll than younger tissue, as photosynthesis is slowing in the lead-up to cell death. Measuring chlorophyll content is therefore one way of monitoring senescence. Therefore, chlorophyll assays were carried out on a range of tobacco plants that had undergone transformation to equate expression of the transgene, and therefore activation of the promoter, with leaf senescence (as determined by chlorophyll content).

3.2.1 Confirmation of transformation of *N. tabacum* and expression of *SlNCED1*.

N. tabacum leaf discs were transformed with *Agrobacterium tumefaciens* containing either the *AtSAG13p::SlNCED1* (line 747), or *AtSARKp::SlNCED1* (line 751) constructs, and plants grown through tissue culture (Appendix 3). Once large enough, leaf tissue was sampled and underwent genomic DNA extraction. RNA extractions were carried out on tissue samples to determine whether those plants confirmed as being transformed were also expressing the transgene. For small plantlets still in tissue culture tubs, a single, green leaf from halfway down the plantlet was taken for sampling. For larger plants with developed roots, planted in soil, three leaves were taken at various developmental stages down the plant.

Three lines of the putative *AtSARKp::SlNCED1* transformants and one line of the putative *AtSAG13p::SlNCED1* transformants were found to have the transgene

integrated into the genome using qPCR (Figure 3.4). The *ca.* 650bp bands resolved by agarose gel electrophoresis were much larger than the *ca.* 148bp expected (see Appendix 4), but the bands were confirmed by sequencing to be fragments of *SlNCED1* (Appendix 7.1).

Of the three 751 lines, two were observed to be expressing *SlNCED1* through analysis using RT- PCR from leaf RNA as bands of the expected size (148 bp) could be detected. (Figure 3.5). To confirm this, the PCR products of the expected size (*ca.* 140bp) were excised from the gel and sequenced. Alignment of the sequences obtained confirmed them to be the expected fragments of *SlNCED1* (Appendix 7.2).

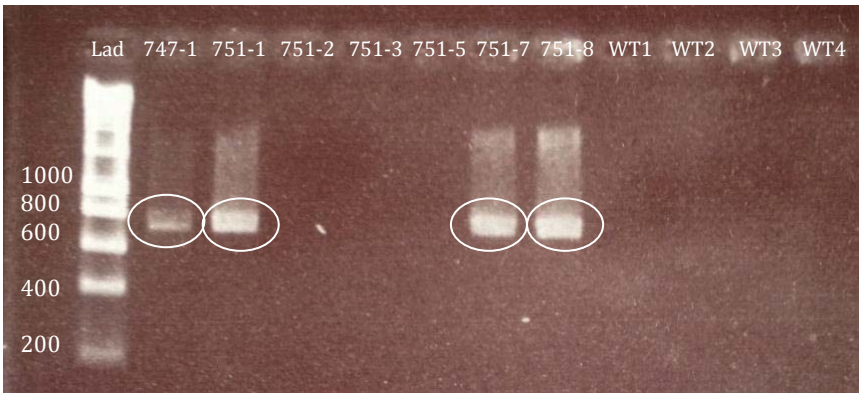
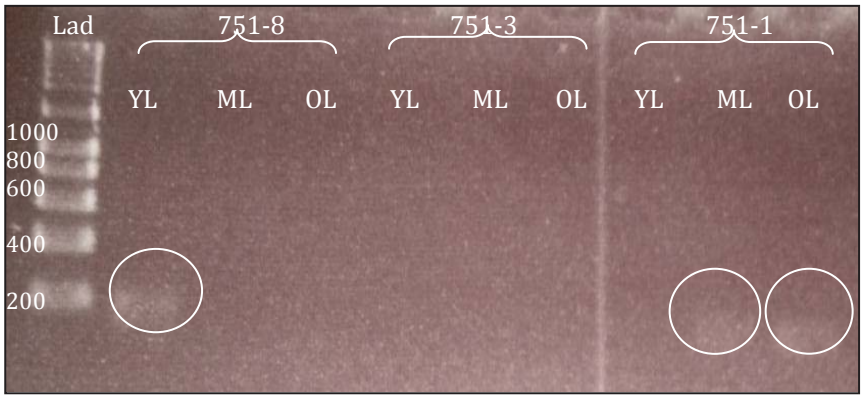


Figure 3.4 Separation of PCR products after gDNA PCR, with bands *ca.* 650bp, indicating incorporation of *AtSAG13p::NCED1* (line 747-1) and *AtSARKp::NCED1* (lines 751-1, 751-7 and 751-8) into the genome of the respective plant lines. Lad = Hyperladder I, with size markers indicated.

A



B

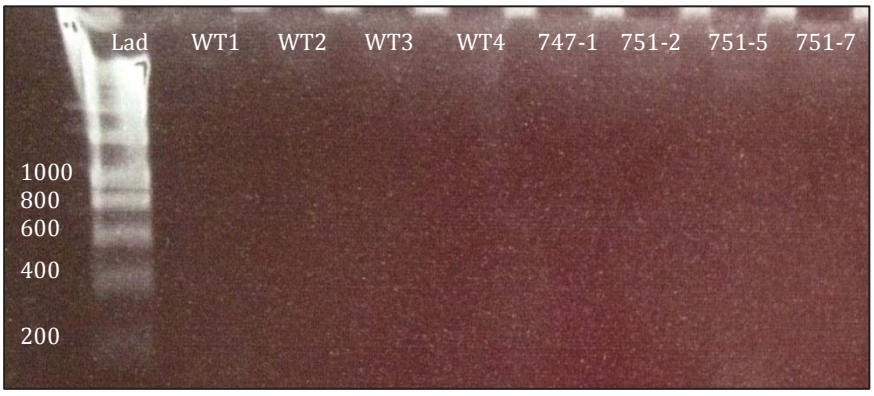


Figure 3.5 Separation of cDNA after RT-PCR of RNA isolated from the putative transformed lines and wild-type (wt) tobacco as indicated. The circles indicate products of *ca.* 148 bp. Lad=Hyperladder I with size markers indicated.

3.2.2 Measurements of plant water relations

To determine whether transformation of tobacco and expression of *SlNCED1* has any effect on traits associated with plant water relations, chlorophyll assays were first carried out on a selection of tobacco plants (Figure 3.8). Gas exchange measurements had been taken of the same tissue before sampling, to record photosynthetic rate, transpiration rate, stomatal conductance, and internal CO_2 levels (Figure 3.6). The chlorophyll measurements were then used in conjunction with gas exchange measurements taken on the same plant material to calculate gas exchange per unit chlorophyll (Figure 3.7).

In terms of overall trends, for the wild-type (wt) plants, the chlorophyll measurements showed that levels were higher in the mature leaf (ML), then the young leaf (YL) and the content was lowest in the older (senescent) leaf (OL) (Figure 3.7). In general then, the photosynthetic rate (A), transpiration rate (Tr) and the stomatal conductance (*gs*) followed the chlorophyll content with the lowest values recorded for the senescent leaves (OL) (Figure 3.6). For the transgenic lines measured, A, Tr, and *gs* were also lowest in the OL irrespective of whether a line had been shown to be expressing *SlNCED1*. However, overall the parameters measured were lower than the wt plants, such that the *gs* values were essentially at 0 (possibly too low to measure). Accordingly, the internal CO_2 levels were higher in these transgenic lines. For three transgenic lines (747-1, 751-1, 751-7) sampling occurred when the chlorophyll levels were much reduced when compared to 751-8 and the EVC, and so the decrease in A, Tr, and *gs* is not solely due to low chlorophyll.

More specifically, once these gas exchange measurements per unit chlorophyll had been calculated, it was clear that for all four measurements, the results for plant line 751-1, a confirmed expresser of *SlNCED*, were noticeably different to the results for the other three transformed lines and the control lines (EVC and wt). The transpiration rate for 751-1 ML tissue was *ca.* 2.4-*fold* higher than the transpiration rate for 751-7 ML (the next highest value), and *ca.* 16.4-*fold* higher than the transpiration rate for EVC ML. The internal CO_2 value recorded for 751-1 ML was *ca.* 5.6-*fold* higher than the rate for 747-1 ML (the next highest value), and *ca.* 40.9-*fold* higher than EVC ML. The conductance measurement for 751-1 ML was

ca. 1.7-*fold* lower than the value for 751-7, and *ca.* 16-*fold* lower than EVC ML. The photosynthetic rate for 751-1 was measured at 0 for the ML and OL tissues. All other plant lines and tissues did have measureable levels of photosynthesis occurring.

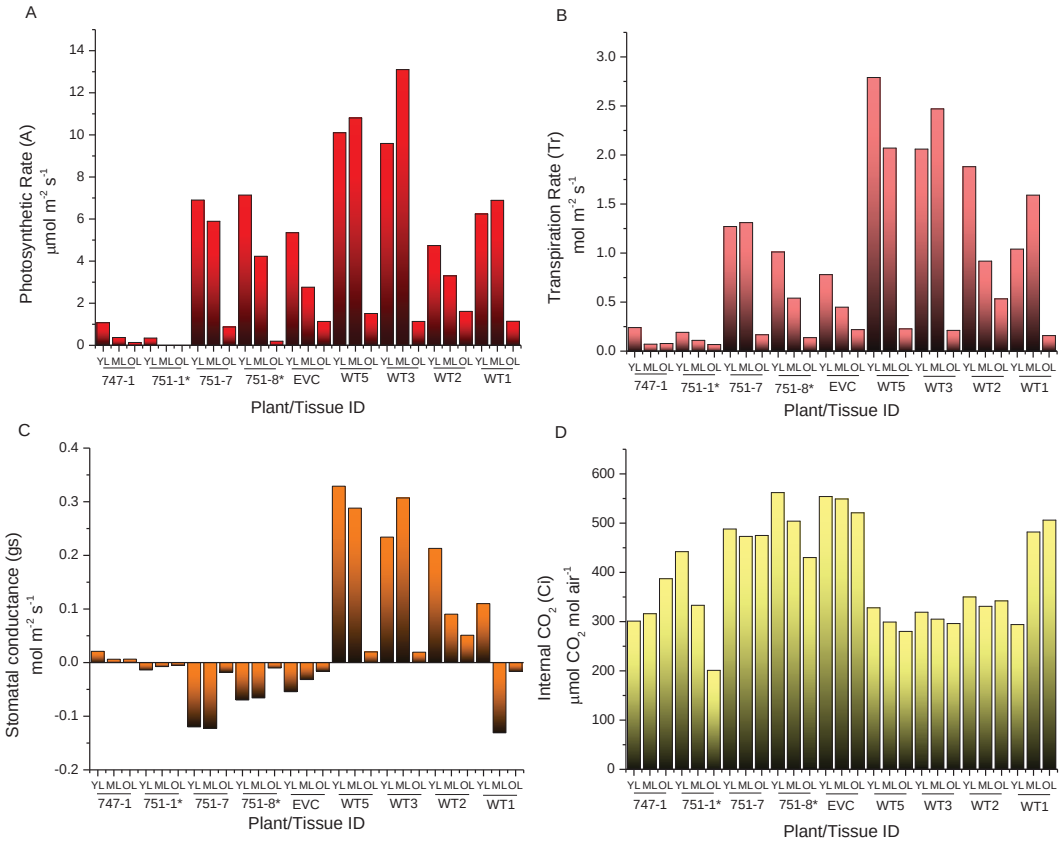


Figure 3.6 Measurements of photosynthetic rate (A), stomatal conductance (*gs*), internal CO_2 transpiration rate (Tr) and intrinsic water-use-efficiency (*A*/*gs*) of three tissue types (YL=young leaf, ML=mature leaf, OL=onset senescence leaf) from four lines of transformed tobacco (747 and 751), an empty vector control (EVC) and four wild type controls (wt), as indicated. Lines confirmed as expressing *SlNCED1* are indicated by a (*).

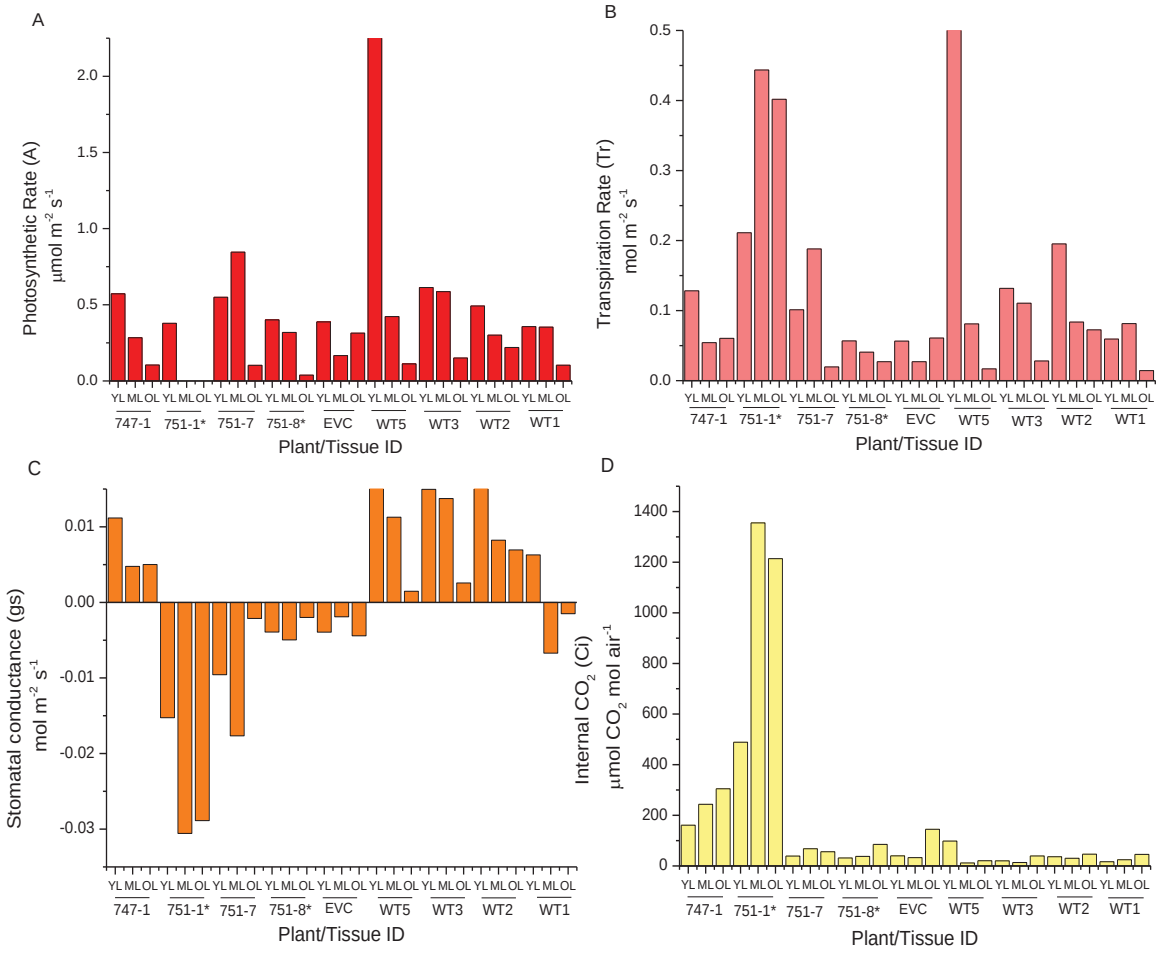


Figure 3.7 Measurements of photosynthetic rate (A), stomatal conductance, internal CO_2 transpiration (Tr) per unit of Chlorophyll from three tissue types (YL=young leaf, ML=mature leaf, OL=onset senescence leaf) of four lines of transformed tobacco (747 and 751), an empty vector control (EVC), and wild type controls (wt), as indicated. Lines confirmed as expressing *SlNCED1* are indicated by a (*).

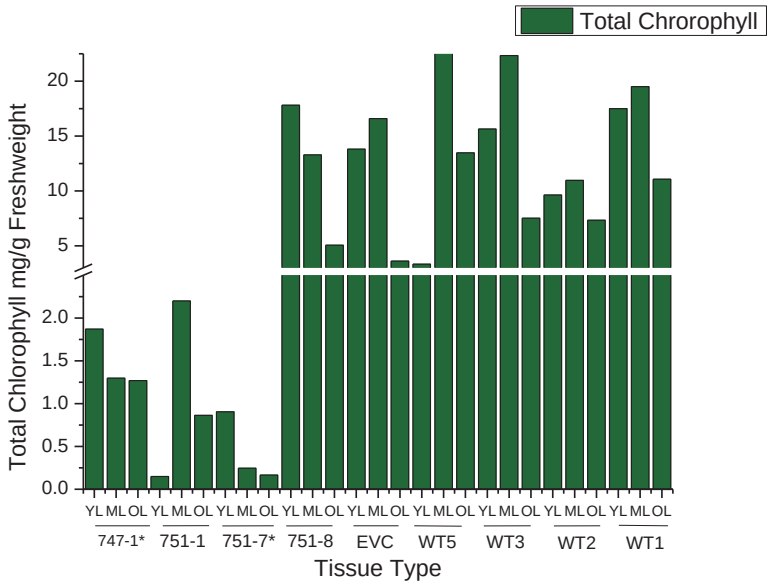


Figure 3.8 Chlorophyll content of four lines of transformed tobacco plants, and an empty vector control (EVC) and wt controls . The three tissues types are young leaf (YL), mature leaf (ML), and onset senescence leaf (OL). Plants that were completely yellow when sampled are indicated by a (*).

3.3 Expression of *TrNCED1* in different tissues of white clover

The *At-SAG13* and *At-SARK* promoters were selected to increase expression of *TrNCED1* in senescent tissue. As a longer term aim of the research programme is to express the *AtSAG13::TrNCED1* and *AtSARK::TrNCED1* in white clover, it was of interest to determine the constitutive expression of *TrNCED1* to gauge some insight into effects of over expression of the transgene. To determine the developmental stages of the tissues used, the chlorophyll content was also determined (Figure 3.11).

The expression levels of *TrNCED1* in six tissue types from two cultivars of white clover grown from stolon cuttings was determined (Figure 3.9). Expression of *TrNCED1* was found in all tissue types, although the levels detected varied greatly between tissue types. Highest expression of *TrNCED1* was found in root tissue, for both cultivars, followed by the first fully expanded leaf. For the cv. Kopu II, expression varied greatly within the biological replicates for the young stolon, older stolon and mature leaf tissue types, making it difficult to determine much significant differences in expression (Figure 3.10). However, expression in the FFEL was significantly higher ($p=0.057$) than in the apex, as was root expression ($p=0.016$), and O-S leaf tissue expression was significantly lower ($p=0.015$) than in the FFEL. For the Tien Shan ecotype, the biological variation within the young stolon tissue type also prevented determination of any statistical significance.

The expression of *TrNCED1* was observed to be lower in the onset of senescence leaf tissue, compared to the majority of other tissue types sampled, for both cultivars (with respect to *Tr-Actin* and *Tr-GAPDH*), but these differences were not significant.

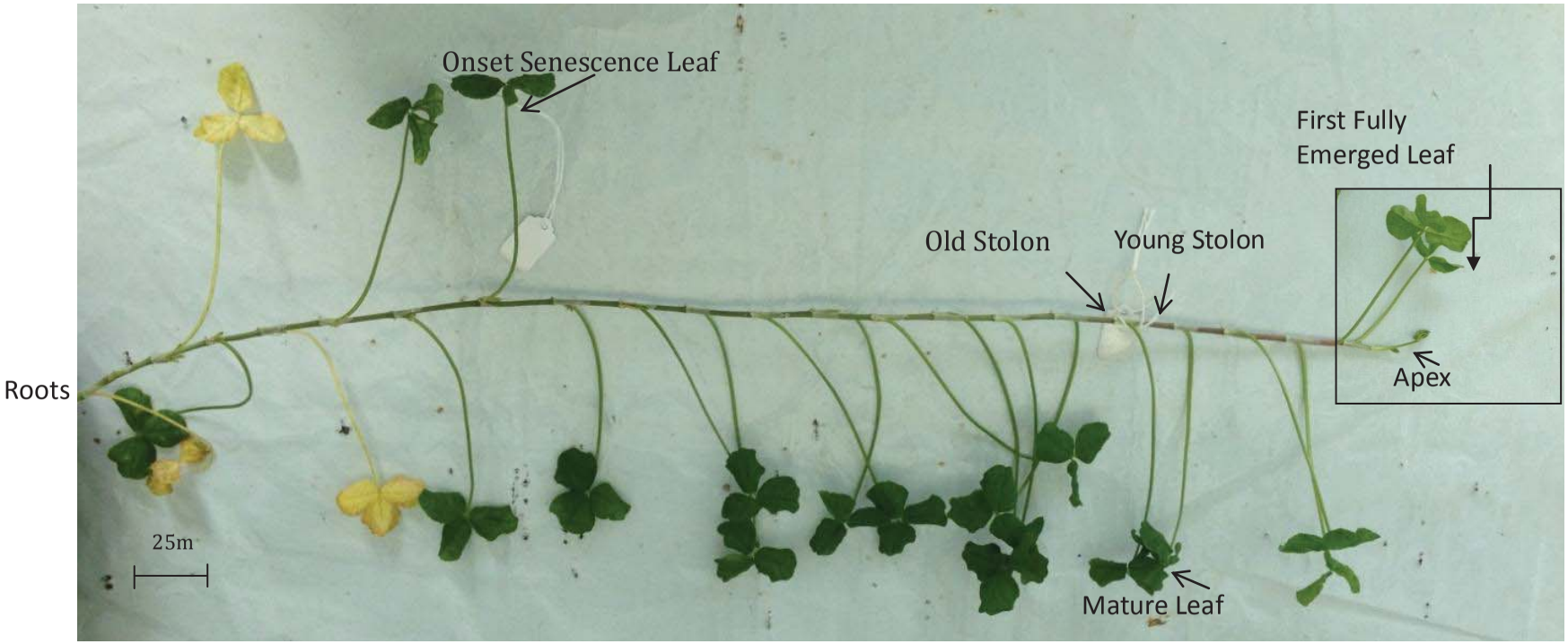


Figure 3.9 *Trifolium repens* stolon indicating tissue types sampled

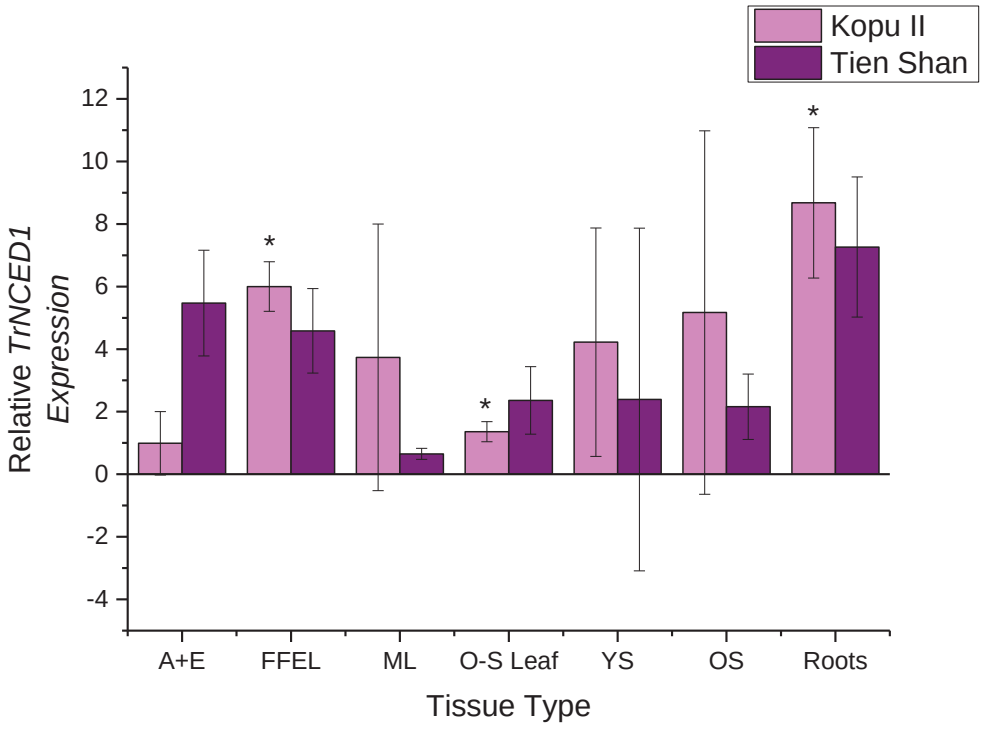


Figure 3.10 Expression of *TrNCED1* in a selection of white clover tissues as indicated. q-RT PCR was carried out using three technical replicates with pooled tissue from 9 plants for each variety/treatment using *Tr-Actin* and *Tr-GAPDH* as internal controls. Each data point represents the mean value of three technical replicates and the error bars represent mean ± SEM. Significance within varieties was calculated to 95% confidence using a t-test, and is indicated by a (*).

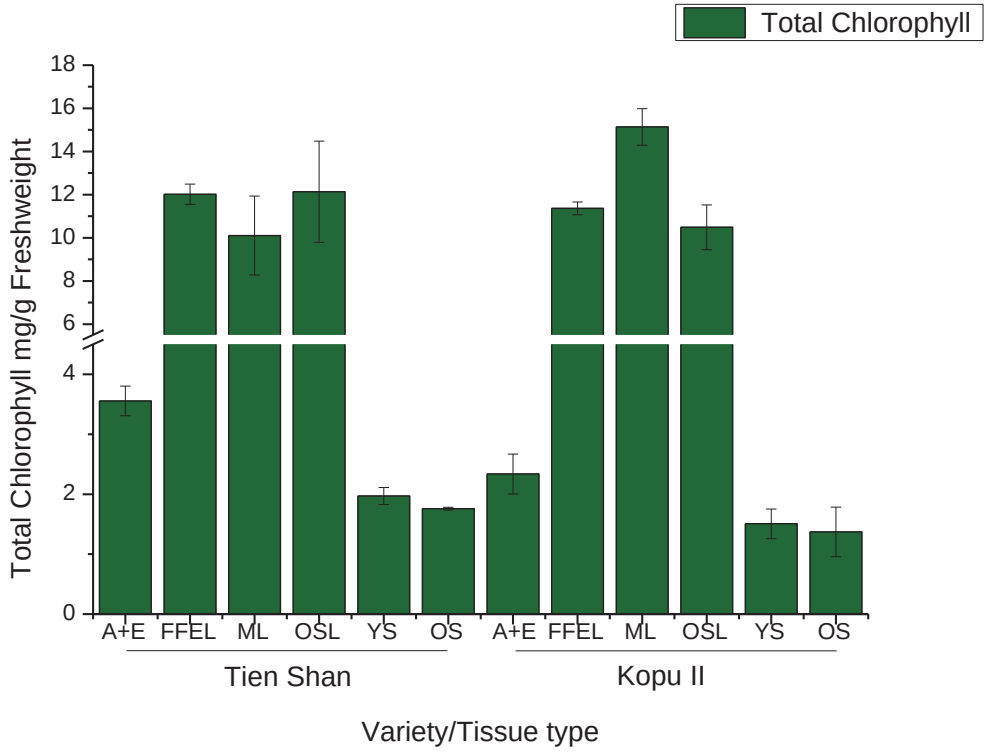


Figure 3.11 Chlorophyll content of six tissue types from two varieties of white clover, as indicated. Apex and emerging leaves (A+E), first fully emerged leaf (FFEL), mature leaf (ML), onset-senescence leaf (OSL), young stolon (YS), older stolon (OS) and root tissue (Roots). Each sample contained pooled tissue from nine plants for each cultivar. Each data point represents the mean value of three technical replicates and the error bars represent mean ± SE

Chapter 4

Discussion

4.1 Assessment of *NCED1* expression as a natural determinant of improved water use efficiency.

The aims of this research were to assess the expression of *NCED1*, an ABA biosynthetic gene that had been proposed by Thompson et al (2007) to potentially increase plant water use efficiency. Two major approaches have been used. The first was to determine whether increased expression of *NCED1* is a determinant of increased water use efficiency, as suggested by Sharp (2002), Thompson, et al. (2004), Taylor, et al. (2005), Kitahata, et al. (2006) and Xian, et al. (2014). To show this, it first needed to be determined whether this gene is up-regulated in germplasm that has been identified as displaying anatomical and morphological characteristics that support water conservation and confer the ability to grow in drier areas.

The second approach was to over-express *NCED1,* firstly in tobacco as proof of concept, then eventually in white clover to examine whether higher levels of ABA biosynthesis do in fact improve water use efficiency when compared to untransformed plants that are otherwise genetically the same. Over the time-course of this thesis, transformation of tobacco was successfully achieved and the expression of *SlNCED* observed in terms of change to plant water relations. As a prelude to over-expression of the gene in white clover, the expression of the endogenous *TrNCED1* was also assessed.

4.1.1 Comparison of Tien Shan and Kopu II

For the first aim of the thesis, two varieties of white clover were compared. The white clover cultivar Kopu II, and ecotype Tien Shan, are both *T. repens* species. However, they differ with regard to drought tolerance (Hofmann and Jahufer 2011,

Ballizany, et al. 2012). Physiological differences between the two populations have been relatively well scrutinized, and it is well documented that Kopu II is a larger, more productive plant than Tien Shan, provided sufficient water is available, with an average shoot weight twice that of Tien Shan, and an average leaf diameter 20% wider than that of Tien Shan. However, the root:shoot ratio of Tien Shan is 0.44, when compared with Kopu II which has a root:shoot ratio of 0.39 (Hofmann and Jahufer 2011) under well watered conditions. Under water deficit conditions, this has been found to increase for Kopu II and decrease for Tien Shan (Ballizany, et al. 2012). The same group found that WUE was increased by water deficit (values averaged across Tien Shan, Kopu II and their F1 progeny (Ballizany, et al. 2012). Thus examination of the relationship between expression of *TrNCED1* in well-watered Tien Shan and Kopu II would be interesting to determine if there is any correlation between transcript abundance and the reported differences in the two varieties in terms of water use efficiency. Likewise, examination of *NCED1* expression in response to a water deficit may also contribute some insight into the mechanism of drought tolerance displayed by the two varieties.

In terms of differences in responses to drought for this first field trial on Kopu II and Tien Shan, root expression of *TrNCED1* dropped in both cultivars/ecotypes with respect to the housekeeping genes in tissue that had been subjected to a water deficit. It should be noted that in the first field trial (Figure 3.1) sampling of the plant material may have been inadequate for molecular work. Leaf material was bulked together, and not separated into FFEL and apical tissue, and so was unable to be used. The root tissue received was not sampled quickly enough, and contained a high level of water and vermiculite contamination which interfered with the RNA extractions and q-RT PCR results. This was primarily due to RNA degradation and foreign matter that was unable to be completely removed from the tissue. Because of these factors, the results from Figure 3.1 cannot be taken alone as a true indication of *TrNCED1* expression.

For the second field trial, plant material was sampled in a manner more conducive to examination at a molecular level. Tissue harvesting and manipulations were rapid and the plant material was frozen in liquid nitrogen within the minimum timeframe. The results showed that Tien Shan root tissue expressed *TrNCED1* at a

level 17- *fold* higher under well watered conditions, than under water deficit, with respect to *Tr-Actin* and *Tr-GAPDH* expression, whereas expression was 2.7-*Fold* lower in well watered Kopu II, compared to water deficit *TrNCED1* expression, with respect to the house-keeping genes (Figure 3.2). It is worthy of note that the trend shown by Tien Shan is similar to that found in Figure 3.1, but the Kopu II results differ.

These results from the second trial may indicate that Tien Shan has moved into a "conservation mode" when experiencing water deficit, that is conserving energy by minimising transcriptional activity. The magnitude of the difference also suggests that this ecotype is 'prepared' for water-deficit, through the higher relative expression of *NCED1* in the well watered plants. Further, there must be a mechanism for transport, or signalling of ABA from the roots up the plant to leaf tissue where it is well documented that high ABA levels decrease transpiration through stomatal closure (Kitahata, et al. 2006, Speirs, et al. 2013).

For Kopu II, the 12-*fold* and 1.7-*fold* decreases in *TrNCED1* expression in Apex and FFEL tissues respectively in response to a water deficit, support the idea of a switch from plant growth to plant survival, which is reinforced by the 2.7-*fold* increase in root expression of *TrNCED1* with respect to *Tr-Actin* and *Tr-GAPDH*. This may suggest transport or signalling of ABA up the plant into the leaves (Ghanem, et al. 2011, Hu, et al. 2013).

The distinct morphologies of the two varieties, particularly in terms of leaf size and root size/structure (Ballizany, et al. 2012), pose the idea of separate mechanisms for drought response and tolerance for each variety. Examination of the expression data from Figure 3.2 proposes a root response to water deficit for Tien Shan, and a leaf response to water deficit for Kopu II. The role that *TrNCED1* plays in each of these responses is likely to vary in importance and it is unlikely to be the sole determinant of any differences in response to water deficit. During a drought, the physiological measurements recorded for each variety (Appendix 5), under well watered and water deficit conditions support the idea of different mechanisms for drought response in each cultivar, particularly as the root:shoot ratio actually increases in Tien Shan seedlings when plants are subjected to water deficit. In contrast, it does not notably change for Kopu II, though the total biomass of Kopu II

grown under water deficit is approximately half that of the same variety grown under well watered conditions (a *ca.* 50% decrease in both shoot and root biomasses) (Hofmann 2014). Measurement of ABA levels would provide more definitive evidence as to whether differences in *NCED1*expression are a key determinant of these changes or not.

Plants that do grow in dryland areas do display well known morphological adaptations that principally conserve water and allow growth in environments with more limited soil moisture (Muthuri, et al. 2009, Snyman 2013). The distinct morphologies between the two varieties of *T. repens* does support these differences. Kopu II is an improved cultivar that has been bred for increased dry matter accumulation in well watered environments. In contrast, the ecotype Tien Shan was collected from a dryland habitat. These differences did lead to a central question of this thesis as to whether an increased constitutive expression of *NCED1* also contributes to this dryland adaptation. Comparison of *NCED1* expression between well-watered Tien Shan and Kopu II showed higher expression in Apex and FFEL tissue of Kopu II than in Tien Shan, but lower expression in the root tissue of Kopu II than Tien Shan. In common with the response to a water deficit, higher expression of *NCED1* in Tien Shan occurred in the roots, the primary origin of water capture, and where the ABA produced could be contributing to an increase in root growth in the drier habitat.

A role for ABA in contributing to root growth in water deficit has been proposed by Sharp (2002). Again, the actual levels of ABA in the root and shoot tissues, and any compartmentalisation of the hormone, will give a clearer picture.

When comparing expression of *TrNCED1* within a variety, or between cv. Kopu II and the ecotype Tien Shan, it is important to point out one qualification. The *TrNCED1* gene (and also the two house-keeping genes) used to design primers for these two experiments (and all q-RT-PCR experiments on white clover and other *Trifolium* species) was originally cloned from *Trifolium repens* cv. Huia, and a BLAST search and alignment against *NCED1* genes from other species was used to assist in primer design (Appendix 3). Using these resources, one primer was designed in the conserved region of the *NCED1* gene, and the other was designed to be specific to the *TrNCED1* gene from cv. Huia. There was no evidence of split

peaks in q-RT PCR raw data, indicating that the primers were not efficient, even for q RT-PCR on the selection of *Trifolium* species, suggesting that there is probably very little diversity over the sequences of *NCED1* in the *Trifolium* genome. In a similar study with ACC oxidase genes from *T. occidentale* and *T. pallescens*, very high gene identity (98%) was observed (Du, et al. 2011). *Tr-Actin* and *Tr-GAPDH* were selected as house-keeping genes because of conservation of these genes across the plant kingdom (Pfaffl, et al. 2004), and a conserved primer set was used to amplify these genes. Thus is it likely that the primer sets are giving an accurate identification of transcript abundance in cv. Kopu II and the Tien Shan ecotype. However, to be more certain the *NCED1* transcripts could be quantified with the aide of new technologies such as nanostring, which uses barcodes to detect and count unique transcripts without any amplification steps (NanoString Technologies, Seattle, WA. USA).

4.1.2 Expression of *TrNCED1* homologues across a selection of *Trifolium* species.

Traditional breeding programs around the world, for a variety of plants not simply limited to forage and arable species, regularly turn to closely related or ancestral species to increase genetic variation *via* inter-specific hybridisation for trait improvement of a target species (Jahufer, et al. 2012).

With recent developments in phylogenetics, and the refinement of marker assisted breeding, quantitative trait loci (QTL's) have been developed for seed yield traits in key regions of the white clover genome, and it is hoped that the same can be done for increased biomass accumulation with less water (greater water-use-efficiency) and also improved drought tolerance using ecotypes and species closely related to white clover (Ballizany, et al. 2012, Jahufer, et al. 2012, Nichols, et al. 2014).

The variety of root systems and growth habits of the four *Trifolium* species examined in this thesis, along with *T. repens* cv. Kopu II and ecotype Tien Shan thus raises the possibility of multiple water use efficiency strategies.

For example, the tap root of *T. uniflorum* grows much deeper than the roots of the stoloniferous *Trifolium* species (including *T. repens*), providing access to water that

other plants are unable to reach. The extensive network of rhizomes found in *T. ambiguum* could act in a similar manner, facilitating the use of water that is inaccessible to more shallow-rooted, stoloniferous plant species. In these species, it would not be unexpected to find lower expression of *TrNCED1* homologues under both well watered, and water deficit conditions. Thus the constitutive expression of *TrNCED1* was examined in a range of *Trifolium* species to determine if up-regulation (or down-regulation) was an additional determinant to the morphological adaptations in terms of efficient use of water.

As shown in Figure 3.3, results from qRT-PCR of four different *Trifolium* species indicate that expression was higher in Apex tissue than in the FFEL, but no significant differences were observed in expression levels of *NCED1* homologues across the different *Trifolium* species. A possible explanation for the lack of significant difference in *NCED1* homologue expression in these *Trifolium* species may be found in the broad geographical origins of the species sampled.

T. occidentale, a diploid species of clover, originates from the saline coasts of Western Europe, and is classified as a creeping, clonal perennial, with known tolerance to salt and drought, and has been described as the most likely direct male ancestor of *T. repens* (Badr, et al. 2012, Williams, et al. 2012, Williams, et al. 2012). *T. pallescens* is, to date, the closest extant species to the female ancestor of *T. repens* after a hybridisation even with *T. occidentale*. It is also diploid, and originates from European alpine regions from 1,800m above sea level, and is a prolific flowerer (Williams, Ellison et al. 2012). Both the above *Trifolium* species have stolons. The fact that both these species are natural diploids means that transcript abundance will be much lower than for species that are polyploids. However, when synthetic polyploids are formed, chromosomal pairing does occur, so transcript abundance would, in theory, increase in proportion to the ploidy level.

The other two *Trifolium* species examined were *T. ambiguum* and *T. uniflorum. T. ambiguum*, also known as Kura or Caucasian clover, originates from the eastern shores of the black sea and Caucasus region. It has a high ratio of below ground biomass made up of thick roots and rhizomes (Abberton 2007) and varies in its ploidy level (can be diploid, tetraploid or hexaploid). Due to the extensive root

system, *T. ambiguum*, once established, shows considerable tolerance to drought. However, establishment can be very slow. *T. uniflorum* is a tetraploid originating from the Mediterranean. Named after the single flower it produces from floral primordia, *T. uniflorum* has a smaller habit than the other *Trifolium* species studied, with small, thick leaves, and a tap root has also been found to display tolerance to drought (Nichols, et al. 2014). Thus all of these species do display adaptations that support greater water use efficiency. The exception is cv. Kopu II, but expression of *TrNCED1* in the tissues sampled was not significantly lower or higher that the other *Trifolium* species.

This suggests that *NCED1* expression, at least in well-watered plants, may not be a large contributor to water use efficiency. Future experiments where the plants are maintained using deficit irrigation may reveal more as to whether *NCED1* expression is a primary determinant.

It should be noted that this result is at odds to that found in Figure 3.2, where apex expression was the lowest of all three tissue type. Had time permitted, these measurements would have been repeated, to determine if this difference may reflect differences in the physiological state of the tissue.

The tissue sampled for RNA extraction was collected from plants that were much more established that *T. repens* plants in the first two field trials, i.e. several years old, rather than several months. Because of this, the plants were under the constraints of the pots, limiting active growth. The plants had been grown in a glasshouse, under well-watered conditions for the duration of their lifecycle, which may also have influenced native expression levels of the *NCED1* homologue, as the plants had never experienced any type of environmental stress, though it is water use efficiency, in plants that are well watered, that is of particular interest.

The developmental stage under which the plants were sampled also differed from the other clover trials. Tissue was collected when plants were flowering, and it was difficult to distinguish between shoot apical meristems and floral meristems. Because ABA is involved in so many different developmental processes, (Liotenberg, et al. 1999, Taylor, et al. 2000) the expression levels of any *NCED1*

homologues would be expected to differ significantly in floral meristems when compared with shoot apical meristems.

4.2 Overexpression of *SlNCED1* in *Nicotiana tabacum.*

Several groups have suggested that plant water use efficiency could possibly be increased through overproduction of ABA by over-expression of the ABA biosynthetic gene, *NCED1* (see Section 1.4). Thus the second aim of the thesis was to over-express *NCED1* and then determine if any differences in water use efficiency could be observed.

The selection of two senescence associated promoters was made in an attempt to avoid delays in plant development, and also to target overexpression of *SlNCED* to mature tissues that would otherwise be responsible for the highest levels of water loss. It is important to note that the two promoters are senescence-associated, a process that begins in the mature leaf. Thus transgene expression will be first switched on in mature leaf tissue, prior to any chlorophyll loss.

Previous research carried out in the same lab where the research for this thesis was carried out, by Sixtus (2013) looked at over-expression of *SlNCED1* driven by the light inducible RUBISCO Small Subunit (*SSU)* promoter. While no detectable increase in plant water-use-efficiency was found in this work, a selection of growth abnormalities that can be attributed to up-regulation of ABA at key stages in plant growth and development were identified. An elongated hypercotyl and underdeveloped cotyledons, accompanied by the failure to shed a seed coat, was the most common aberration found. Two other classes of abnormal seedlings were also described: mildly aberrant seedlings had normal cotyledons and the presence of shoots, though failed to develop beyond this point, whereas the most severe aberration consisted of underdeveloped cotyledons, swollen hypercotyl, and the presence of callus like tissue at the base of the hypercotyl just above the roots (Sixtus 2013). These abnormalities, in addition to the finding that mature tissues have a higher transpiration rate and therefore a higher rate of water loss (Swartzberg, Dai et al. 2006, Rivero, Kojima et al. 2007) were instrumental in the decision to use senescence-associated promoters to drive over-expression of *SlNCED1.*

4.2.1 Direct assessment of *SlNCED1* overexpression in *Nicotiana tabacum*

After transformation with the *AtSAG13p::SlNCED1*construct (line 747, See Appendix 3), only one plant survived to grow to maturity. Incorporation of the construct into the genome was confirmed by block PCR (Figure 3.7), but only after two rounds of 45-cycles. However, expression of the *NCED1* gene was not found by block PCR. It is not clear why very few transformants were obtained using the *$AtSAG13_p$:SlNCED1* construct. The gene, *SAG13* is known to be expressed at detectable levels in leaf tissue up to two days before the onset of visible senescence, and increases during senescence (Swartzberg, et al. 2006), and other researchers have produced transformants with *$AtSAG13_p$:GUS* constructs (Swartzberg, et al. 2006). However, within the time-frame of this thesis, a reason for this lack of transformants could not be investigated further.

Of the eight lines putatively transformed with the *AtSARKp::SlNCED1* construct, three had the construct successfully integrated in the genome. Of these three, two were also determined to be expressing, again only after two rounds of 45-cycle PCR. However, when examining the literature, several groups (using constructs containing GUS, driven by the P_{SARK} and P_{SAG13} promoters) had found that expression of constructs driven by both of these senescence induced promoters (but SARK in particular) were difficult to detect during vegetative growth stages, but increased once transformed plants began to flower, and also after periods of drought (Swartzberg, et al. 2006, Rivero, et al. 2007, Delatorre, et al. 2012).

Of the lines that were shown to be expressing, only line 751-1 had expression in the ML and OL, as expected (Figure 3.5). In 751-8, expression was detected in the YL. To confirm this, a second RNA extraction would need to be undertaken and the PCR repeated. If it is confirmed then it is possibly a transgene (T-DNA) insertion effect, where the normal developmental cues that determine expression of the promoter are interrupted.

No expression was detected in lines 747-1, 751-2, 751-5 and 751-7. Note that tissue was sampled just after transfer out of tissue culture, when all tissue was very young, and so it is not surprising that these plants do not accumulate the *NCED1* mRNA.

The bands in Figure 3.5 are very faint even after such a large number of PCR cycles. As the leaf tissue was sampled before plants began flowering, this corresponds to similar findings in the literature of lower expression prior to flowering (Swartzberg, et al. 2006, Rivero, et al. 2007, Rivero, et al. 2009, Rivero, et al. 2010). However, within the time-frame of this thesis, this was not examined further.

It is also possible that qRT-PCR may have been able to pick up higher expression levels, but previous work encountered issues with the ability of primers to distinguish between *SlNCED1* and *NtNCED1* (Sixtus 2013) as the transgene is expressed in the tobacco background. However, sequencing was carried out on the bands excised from the gel in Figure 3.5, and alignments run against *SlNCED1*, confirming that the expected product size of *ca.* 140 bp is in fact a fragment of *SlNCED1(*Appendix 7.3).

The size of the bands of genomic PCR product visualised in Figure 3.4 is also noteworthy. The same pair of specifically-designed *SlNCED1* primers (see Appendix 4), and therefore the same PCR programme, was used for block PCR of both RNA and cDNA preparations of the tobacco plants. However, the product size using gDNA as template (Figure 3.4) was *ca.* 640 bp, much larger than the 120bp size expected. This was of particular concern because the construct used for plant transformation did not contain any introns. Therefore sequencing was carried out to determine the identity of the PCR product, and alignments of the sequence were run against the *SlNCED1* sequence that had been used for primer design, which confirmed the PCR product to be a large fragment of *SlNCED1* (Appendix 7.1 and Appendix 7.2). BLAST searches also found *SlNCED1* to be the top hit for both the *ca.* 640 bp and *ca.* 140 bp PCR products (See Appendix 10). It is proposed that the fragment size was due to the reverse primer binding further along the gene than it was designed to (binding non-specifically); though why exactly this has occurred is unknown as alignments that have been carried out do show the primers binding specifically in the designed locations.

4.2.2 Phenotypic Analysis of Transformants

Because senescence-induced promoters have been selected to drive the expression of *SlNCED1* in tobacco, it is important to be able to determine what effect on plant transpiration and photosynthesis overexpression of the transgene may have. The over-expression of *NCED1*, and the assumed downstream increase in ABA levels were predicted to reduce stomatal conductance and transpiration in the older leaves. To assess this, an Infrared Gas Analyser (IRGA) was selected to conduct these measurements as it is able to record internal CO_2 levels, stomatal conductance, photosynthetic rate, and transpiration rate (Figure 3.6). Where possible, transformants and wild-type plants were compared. No abnormalities associated with growth and development were observed in any of the plant lines that came through tissue culture, regardless of individual transformation status. Chlorophyll assays, to measure levels of chlorophyll a, b, and total chlorophyll to assess the developmental stage of the leaf tissue, were also carried out. However, because transformants were coming through tissue culture at different rates and at different times from the wild-type controls, it was not possible to directly compare a sufficient number of plants at the same developmental stage. Therefore, the IRGA measurements were also compared per unit total chlorophyll to gain some equivalence of the plant populations (Figure 3.7).

Comparison of the transpiration of the wt lines confirmed a higher rate in the ML, but not so in the OL. A significantly higher rate was also observed in the young leaf (YL) tissue (Figure 3.6). The higher transcription rate also correlated with a higher stomatal conductance. Therefore, it is expected that expression of *SlNCED1* under control of the *SAG13* and *SARK* promoters would reduce water loss from mature leaves, but sufficient transpiration through the younger leaves would ensure continued water uptake by the plant (as predicted by the Cohesion-Tension mechanism of water movement).

Interestingly, all of the transgenic lines displayed similar plant water parameters, including the EVC line. It would be expected that the EVC should be quite similar to the wt lines, and so it is unclear yet why this difference exists.

Nevertheless, the single transgenic line, which was confirmed to be expressing, did show a very low transpiration rate and stomatal conductance. Correspondingly,

the photosynthetic rate and the internal CO_2 content were also reduced. The other line that also showed similar changes was 747-1. It is yet to be determined in older plants whether this line is expressing.

Three of the transgenic lines displayed very low chlorophyll levels and therefore gas exchange values were expressed per unit chlorophyll. Here, the actual differences in line 751-1 could be seen more clearly, suggesting that, for this line at least, expression of the transgene has altered plant water relations. The next step for this line is to determine whether this has increased water use efficiency by comparing growth rate with water uptake.

However, it is of equal importance to be able to attribute the trends observed to transgene expression with more certainty. Many more transgenic plants must be measured and at the same developmental stage. While the phenotypes of the over-expression of *NCED1* are being assessed, these are transgenic lines and it is possible that the T-DNA has inserted in the many genes that determine changes in transcription and stomatal conductance. Thus, at least 12 genetically independent lines are recommended (Jackson and Linskens 2003). Alternatively, analysis of F1 progeny in this manner would further confirm whether this specific set of gas exchange measurements is characteristic of transgene expression. Here, progeny can be selected that are either positive, or null segregates, and these populations are then compared. Thus the comparisons are made with very similar genetic backgrounds. Had time permitted, this is what would have been done.

4.3 Expression of *TrNCED1* in different tissues of white clover.

The longer term aim of the research programme is to assess whether over-expression of *NCED1*, when driven from a senescence specific promoter will confer increased water-use-efficiency in white clover. The use of this promoter is based on the observation that mature leaves transpire more frequently than young tissues. Before the transgenic plants could be examined, it was necessary to examine the constitutive expression of *TrNCED1,* particularly due to the wide variety of roles that ABA plays in plant growth and development. It was expected that the expression levels of *TrNCED1* will differ across tissue types, a prediction supported by Figure 3.10.

Root tissue had the highest level of expression of all the tissue types sampled, followed by the first fully expanded leaf (FFEL) and the apex. Expression of *TrNCED1* was higher in FFEL tissue, than in the Apex and ML tissue types. This is to be expected as the apical tissue is also supported by rest of plant until fully emerged, therefore having lower ABA requirements than other tissues sampled. ABA can inhibit growth and development if expressed too highly at this developmental stage, as has been found by groups over-expressing *TrNCED1* homologues driven by constitutive and even light-induced promoters (Thompson, et al. 2000, Thompson, et al. 2007, Tung, et al. 2008, Sixtus 2013). Thus the use of a senescence-associated promoter may reduce this problem. In terms of the expression levels of *TrNCED1* found in the mature leaf tissue, levels were measured to be between those recorded for A+E and FEEL. This is the only documented example where expression of *NCED1* in ML was measured, so there is no other data to compare it to so as to determine whether these results are within the normal range.

The onset-senescence leaf tissue had a very low constitutive expression level of *TrNCED1*. However, the error bars from the other tissue types, particularly those for expression in the two different ages of stolon tissue, mean this is unable to be classified as significant. The error bars for the two ages of stolon tissue indicate great variation of *TrNCED1* expression, irrespective of tissue age. This could be suggestive of a mechanism for transport of *TrNCED1* mRNA transcript up the plant from the roots, though it would be more logical for the end product of the pathway,

ABA, to be transported up the plant rather than the gene responsible for the first dedicated step of ABA biosynthesis. Thus the results obtained from the work carried out so far suggests that transformation of white clover to overexpress *SlNCED1* under P_{SAG13} and P_{SARK} should not interfere with the constitutive expression of *TrNCED1*. In transgene biology, the phenomenon of co-suppression has long been known to exist, whereby transgenic expression of mRNA in a cell that already has a high population of mRNA from endogenous gene expression results in silencing of both genes (Napoli, et al. 1990). Thus it is important to monitor the expression of the endogenous gene in the tissues targeted for transgene expression.

4.4 Future Work

Direct testing of water-use-efficiency of the transformants obtained through the measurement of growth against water taken up to determine whether there is an increase in WUE as a result of *NCED1* over-expression should be carried out. Even though the expression levels of the transcript appear to be low, it is the total ABA levels that confer increased water-use efficiency, which are also able to be measured in a variety of variety of tissues.

Germination tests on F1 generation of tobacco from 747, 751-1, 751-7, 751-8 and wild-type controls would show whether there are any delays in germination, or developmental mutations associated with transgene expression, like those found by several other research groups (Thompson, et al. 2000, Thompson, et al. 2007, Tung, et al. 2008, Sixtus 2013).

Following germination testing, growth of germinated seedlings to the flowering stage where gas exchange measurements could be made on well watered plants, as well as plants that have experienced a period of water deficit should be carried out. This would confirm whether or not the trends discussed in section 4.2.3 are reproducible characteristics of transgene expression, and whether there are any differences in response to water deficit, as an end to proof of concept that overexpression of *NCED1* conveys higher water-use efficiency.

Transformation of *Trifolium repens* with *At*SAG13p::SlNCED1 (line 747) and AtSARKp::SlNCED1 (line 751) constructs, followed by germination trials, growth to maturity, then gas exchange measurements on well watered plants, and plants that had experienced water deficit, to examine whether the transgene conveys similar properties in *Trifolium repens* as it does in *Nicotiana tabacum*. Direct testing of water-use-efficiency and ABA levels in transformed white clover would complete this research project.

Chapter 5

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Chapter 6 Appendices

Appendix 1: Hoaglands Medium (Gibeaut et al., 1997)

Macronutrients:

Macronutrients	Working conc. (mM)	Weight (g)/L for 10X
KNO_3	1.25	1.264
$Ca(NO_3)_2$	1.5	3.543
$MgSO_4$	0.75	1.849
KH_2PO_4	1.0	1.360

Micronutrients:

Micronutrients	Working conc. (µM)	Weight (mg)/L for 100X
KCL	50.0	372.8
H_3BO_3	50.0	309.2
$MnSO_4$	10.0	151.0
$ZnSO_4$	2.0	57.5
$CuSO_4$	1.5	37.5
$(NH_4)_6Mo_7O_{24}$	0.075	9.3
Na_2O_3Si	0.1	2.1
FeNaEDTA	72.0	2642.8

Appendix 2: Tissue culture Media recipes

Nic I Media:

Murashige and Skoog powder(including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)
Phyto Agar	0.8% (w/v)
Adjust to pH 5.7 with 2M KOH before autoclaving at 121^{0}C and 15 psi for 20 min	
BAP	1mg/L
NAA	100µg/L

Nic II Media:

Murashige and Skoog powder(including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)
Phyto Agar	0.8% (w/v)
Adjust to pH 5.7 with 2M KOH before autoclaving at 121^{0}C and 15 psi for 20 min	
BAP	1mg/L
NAA	100µg/L
Cef	100mg/L
Kan	100mg/L

Nic III Media:

Murashige and Skoog powder(including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)
Phyto Agar	0.8% (w/v)
Adjust to pH 5.7 with 2M KOH before autoclaving at 121^{0}C and 15 psi for 20 min	
Cef	100mg/L
Kan	100mg/L

Appendix 3: Plasmids

Figure A3.1 *SAG13$_p$::SlNCED1* (747 Line):

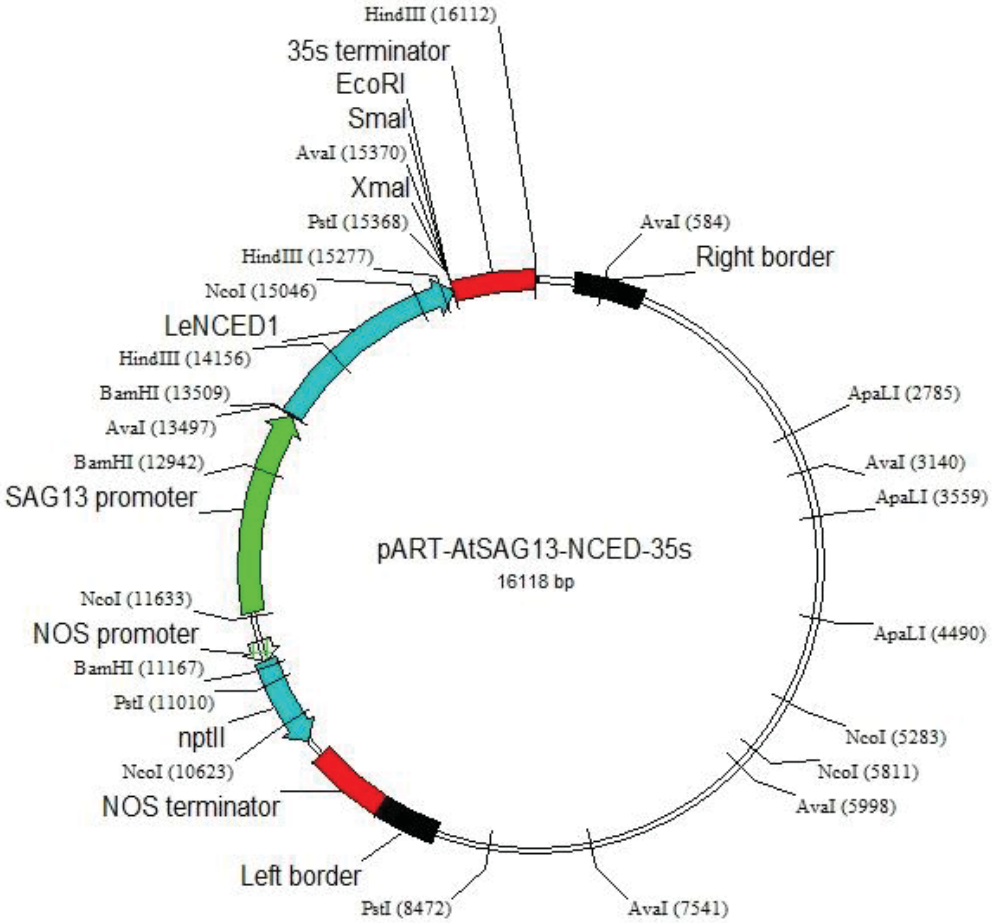
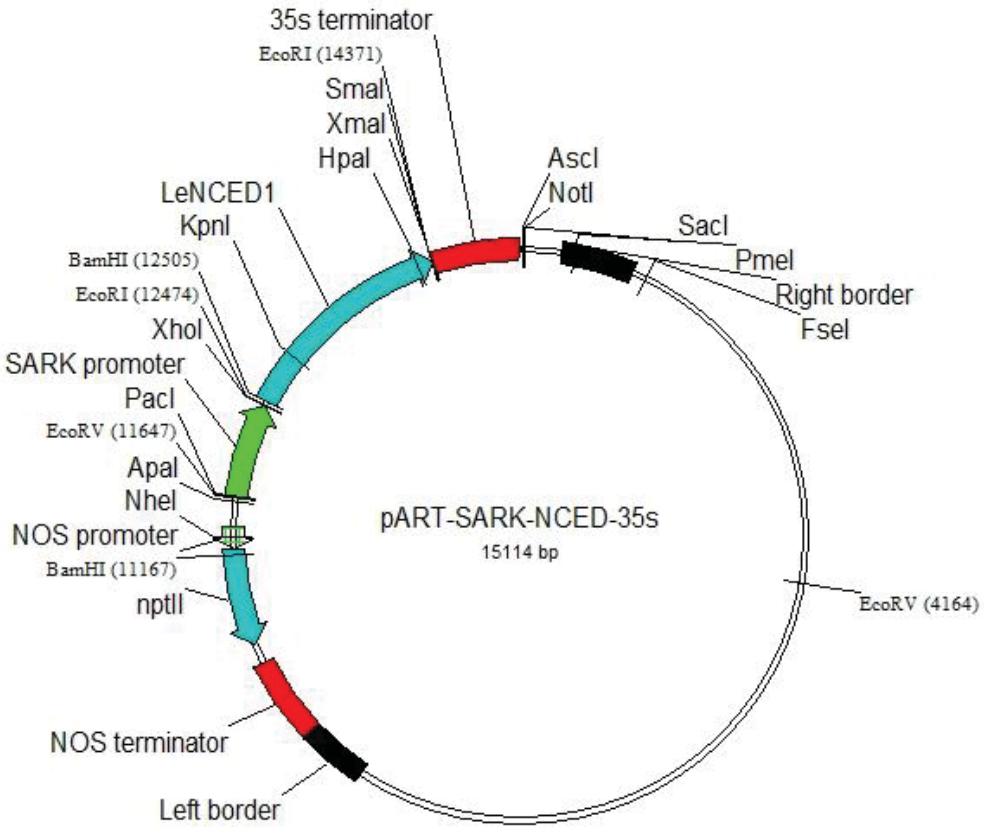


Figure A3.2 *SARK$_p$::SlNCED1* (751 Line):



Appendix 4: RNA Qubit Quantification

4.1 Lincoln 2 White clover Apex:

Table A4.1

Sample ID	QF Value	Conc. (ng/mL)	Conc. (ng/µL)
4A	288	57,600	57.6
9A	800	160,000	160.0
11A	154	30,800	30.8
15A	296	59,200	59.2
52A	150	30,000	30.0
54A	406	81,200	81.2
59A	520	104,000	104.0
61A	451	90,200	90.2
63A	440	88,000	88.0
68A	342	68,400	68.4
71A	273	54,600	54.6
75A	641	128,200	128.2

4.2 Lincoln 2 White Clover First-fully-expanded leaf:

Table A4.2

Sample ID	QF Value	Conc. (ng/mL)	Conc. (ng/µL)
4L	155	31,000	31
9L	407	81,400	81.4
11L	138	27,600	27.6
15L	630	126,000	126
52L	403	80,600	80.6
54L	187	37,400	37.4
59L	223	44,600	44.6
61L	283	56,600	56.6
63L	246	49,200	49.2
68L	220	44,000	44.0

71L	160	32,000	32.9
75L	437	87,400	87.4

4.3 Lincoln 2 White clover Roots:

Table A4.3

Sample ID	QF Value	Conc. (ng/mL)	Conc. (ng/µL)
4R	199	39,800	39.8
11R	382	76,400	76.4
15R	104	20,800	20.8
54R	258	51,600	51.6
59R	241	48,200	48.2
61R	235	47,000	47.0
63R	425	85,000	85.0
68R	640	128,000	128.0
71R	206	41,200	41.2
75R	419	83,800	83.8

4.4 AgResearch Clover Leaves:

Table A4.4

Sample ID	QF Value	Conc. (ng/mL)	Conc. (ng/µL)
T.a L1	151	30,200	30.2
T.a L2	730	146,000	146.0
T.a L3	1000	200,000	200.0
T.u L1	1000	200,000	200.0
T.u L2	283	56,600	56.6
T.u L3	241	48,200	48.2
T.o L1	117	23,400	23.4
T.o L2	1000	200,000	200.0
T.o L3	900	180,000	180.0
T.p L1	830	166,000	166.0
T.p L2	620	124,000	124.0

T.p L3	860	172,000	172.0
Kopu II L1	1000	200,000	200.0
Kopu II L2	215	43,000	43.0
Kopu II L3	880	176.000	176.0
Tien Shan L1	1000	200,000	200.0
Tien Shan L2	810	162,000	162.0

4.5 AgResearch Clover Apexes:

Table A4.5

Sample ID	QF Value	Conc (ng/mL)	Conc (ng/µL)
T.a A1	91.0	18,000	180
T.a A2	26.7	5,340	5.3
T.a A3	27.0	5,400	5.4
T.o A1	47.7	9,540	9.5
T.o A2	39.5	7,900	7.9
T.o A3	680	136,000	136
T.u A1	32.8	7,640	7.6
T.u A2	700	140,000	140
T.u A3	24.0	4,920	4.9
T.p A1	441	88,200	88.2
T.p A2	396	79,200	79.2
T.p A3	280	56,000	56
Kopu II A1	93.2	18,640	18.6
Kopu II A2	321	64,200	64.2
Tien Shan A1	461	92,200	92.2
Tien Shan A2	312	62,400	62.4

Key:

T.a = *Trifolium ambiguum*

T.o = *Trifolium occidentale*

T.u = *Trifolium uniflorum*

T.p = *Trifolium pallescens*

Appendix 5: Hofmann Field Trials: Physiological Data

Table A5.1

Water potential							
Cultivar	Acrylic						
	well watering				Drought		
	water potential	solute potential	pressure potential		water potential	solute potential	pressure potential
	(MPa)	(MPa)	(MPa)		(MPa)	(MPa)	(MPa)
K1	-6.0	-0.965	-5.035		-5.0	-1.348	-3.652
	-6.0	-1.006	-4.994		-7.0	-1.438	-5.562
	-4.5	-1.053	-3.447		-9.5	-1.248	-8.252
K2	-5.5				-9.5		
	-7.5						
T1	-6.5	-1.123	-5.377		-10.0	-1.2380	-8.7620
	-8.0	-1.087	-6.913		-6.0	-1.2551	-4.7449
	-6.0	-1.194	-4.806		-6.0	-1.2307	-4.7693
T2	-7.5				-5.0		
Ks	-6.0	-0.838	-5.162		-7.5	-1.418	-6.082
	-5.0	-1.067	-3.933		-5.0	-1.233	-3.767
		-0.975	-0.975		-6.0	-1.426	-4.574
Ts	-5.0	-0.963	-4.037		-6.5	-1.194	-5.306
	-4.5	-0.892	-3.608				
	-6.5	-1.121	-5.379		-6.0	-1.372	-4.628
Cultivar	Polycarbonate						
	well watering				Drought		
	water potential	solute potential	pressure potential		water potential	solute potential	pressure potential

	(MPa)	(MPa)	(MPa)		(MPa)	(MPa)	(MPa)
K1	-6.0	-1.067	-4.933		-6.0	-1.338	-4.662
	-5.0	-1.365	-3.635		-7.0	-1.194	-5.806
	-4.5	-1.172	-3.328		-8.0	-1.496	-6.504
K2	-7.0						
T1	-4.5	-1.353	-3.147		-6.0	-1.1893	-4.8107
	-6.0	-1.335	-4.665		-7.0	-1.1430	-5.8570
	-7.0	-0.943	-6.057		-6.0	-1.1503	-4.8497
T2	-4.5						
Ks	-6.0	-1.067	-4.933		-8.0	-1.301	-6.699
	-4.5	-0.989	-3.511		-5.5	-1.404	-4.096
	-6	-1.036	-4.964		-6.5	-1.209	-5.291
Ts	-5.0	-1.053	-3.947		-7.0	-1.389	-5.611
	-6.0	-0.875	-5.125		-6.0	-1.401	-4.599
	-4.5	-0.997	-3.503		-6.5	-1.472	-5.028

Table A5.2

Relative water content (RWC)								
Cultivar	Acrylic							
	well watering					drought		
	FM (g)	TM (g)	DM (g)	RWC (%)		FM (g)	TM (g)	DM (g)	RWC (%)
K1	0.5185	0.5849	0.1249	85.57		0.2182	0.2492	0.0494	84.48
	0.3503	0.3617	0.0654	96.15		0.2705	0.2913	0.0607	90.98
	0.4347	0.4537	0.0785	94.94		0.3693	0.3997	0.0679	90.84

K2									
T1	0.1447	0.1770	0.0340	77.41		0.0938	0.1196	0.0224	73.46
	0.1156	0.1207	0.0214	94.86		0.0759	0.0836	0.0150	88.78
	0.1125	0.1189	0.0181	93.65		0.0979	0.1034	0.0179	93.57
T2									
Ks	0.5826	0.6083	0.1007	94.94		0.2361	0.2538	0.0470	91.44
	0.3109	0.3271	0.0711	93.67		0.2578	0.2781	0.0466	91.23
	0.4373	0.4595	0.0900	93.99		0.2247	0.2431	0.0446	90.73
Ts	0.1840	0.1943	0.0405	93.30		0.1230	0.1351	0.0241	89.10
	0.2088	0.2193	0.0373	94.23					
	0.2024	0.2129	0.0393	93.95		0.1261	0.1399	0.0256	87.93
Cultivar	Acrylic								
	well watering					drought			
	FM (g)	TM (g)	DM (g)	RWC (%)		FM (g)	TM (g)	DM (g)	RWC (%)
K1	0.3200	0.3660	0.0741	84.24		0.2139	0.2380	0.0476	87.34
	0.5405	0.5662	0.1079	94.39		0.3109	0.3381	0.0581	90.29

	0.3821	0.3993	0.0771	94.66		0.2104	0.2310	0.0454	88.90
K2									
T1	0.1617	0.1863	0.0362	83.61		0.0772	0.0911	0.0184	80.88
	0.1149	0.1246	0.0221	90.54		0.0874	0.0953	0.0185	89.71
	0.0907	0.0946	0.0166	95.00		0.0803	0.0880	0.0149	89.47
T2									
Ks	0.5498	0.5738	0.0979	94.96		0.2617	0.2811	0.0496	91.62
	0.4542	0.4684	0.0796	96.35		0.2732	0.2880	0.0589	93.54
	0.6201	0.6514	0.1115	94.20		0.3653	0.3940	0.0681	91.19
Ts	0.1693	0.1748	0.0352	96.06		0.0958	0.1033	0.0193	91.07
	0.1774	0.1827	0.0302	96.52		0.0992	0.1061	0.0204	91.95
	0.2372	0.2487	0.0498	94.22		0.1738	0.1983	0.0322	85.25

Table A5.3

Shoot biomass, root biomass, total biomass and root:shoot ratio	
Cultivar	Acrylic

	well watering					drought			
	shoot biomass	root biomass	total biomass	root:shoot		shoot biomass	root biomass	total biomass	root:shoot
	(g)	(g)	(g)	ratio		(g)	(g)	(g)	ratio
K1	102.37	52.53	154.90	0.51		59.40	30.24	89.64	0.51
	100.84	46.75	147.59	0.46		58.97	27.11	86.08	0.46
	116.97	44.40	161.37	0.38		50.22	25.31	75.53	0.50
K2	88.06	38.63	126.69	0.44		60.26	24.02	84.28	0.40
	92.73	43.94	136.67	0.47		54.76	25.65	80.41	0.47
	100.82	39.61	140.43	0.39		55.39	21.11	76.50	0.38
T1	47.27	36.89	84.16	0.78		38.95	27.23	66.18	0.70
	58.35	27.17	85.52	0.47		36.28	23.34	59.62	0.64
	24.26	19.81	44.07	0.82					
T2	61.06	26.54	87.60	0.43		40.40	33.82	74.22	0.84
						31.74	32.64	64.38	1.03
	47.67	28.48	76.15	0.60		34.11	23.89	58.00	0.70
Ks	71.67	33.80	105.47	0.47		44.46	21.50	65.96	0.48
	55.51	28.91	84.42	0.52		49.00	22.06	71.06	0.45
	83.36	39.20	122.56	0.47		29.75	18.73	48.48	0.63
Ts	26.81	22.12	48.93	0.83		29.87	19.74	49.61	0.66
	51.05	32.88	83.93	0.64					
	52.03	29.42	81.45	0.57		29.63	19.21	48.84	0.65
Cultiv	Polycarbonate								

ar									
	well watering					Drought			
	shoot biomass	root biomass	total biomass	root:shoot		shoot biomass	root biomass	total biomass	root:shoot
	(g)	(g)	(g)	ratio		(g)	(g)	(g)	ratio
K1	106.04	47.62	153.66	0.45		56.85	28.16	85.01	0.50
	102.31	49.34	151.65	0.48		52.91	28.75	81.66	0.54
	115.47	45.12	160.59	0.39		42.12	28.13	70.25	0.67
K2	107.88	47.54	155.42	0.44		61.96	25.98	87.94	0.42
	101.94	47.82	149.76	0.47		65.49	20.64	86.13	0.32
	108.20	50.19	158.39	0.46		57.2	23.82	81.02	0.42
T1	23.24	18.40	41.64	0.79		35.92	27.23	63.15	0.76
	64.05	39.84	103.89	0.62		40.66	32.10	72.76	0.79
	66.47	37.78	104.25	0.57		45.88	30.38	76.26	0.66
T2	47.96	38.21	86.17	0.80		43.31	25.76	69.07	0.59
	74.71	30.34	105.05	0.41		47.95	28.24	76.19	0.59
	60.80	27.23	88.03	0.45		39.04	31.12	70.16	0.80
Ks	86.59	44.88	131.47	0.52		40.92	22.10	63.02	0.54
	77.81	38.83	116.64	0.50		45.51	21.97	67.48	0.48
	49.70	29.83	79.53	0.60		47.28	23.89	71.17	0.51

Ts	59.09	30.39	89.48	0.51		26.55	18.84	45.39	0.71
	52.10	30.11	82.21	0.58		36.63	24.38	61.01	0.67
	45.66	35.73	81.39	0.78		23.89	20.38	44.27	0.85

Table A5.4

Leaf damage and leaf senescence					
Cultivar	Acrylic				
	well watering			drought	
	Leaf damage	Leaf senescence		Leaf damage	Leaf senescence
	(%)	(%)		(%)	(%)
K1	5	1		55	20
	40	3		40	40
	40	0		60	10
K2	10	3		45	47
	45	3		50	10
	30	3		65	10
T1	35	7		40	45
	40	3		30	40
	10	3		35	40
T2	40	32		10	25
				30	50
	30	3		60	25
Ks	3	0		40	20
	35	10		15	3
	30			60	5
Ts	10	0		25	10
	20	3			
	60	5		65	5

Polycarbonate					
	well watering			drought	
Cultivar	Leaf damage	Leaf senescence		Leaf damage	Leaf senescence
	(%)	(%)		(%)	(%)
	5	0		20	20
	35	1		45	35
K1	35	0		25	10
	15	0		35	3
	30	3		25	5
K2	40	0		30	15
	30	3		30	15
	45	5		45	35
T1	65	5		25	10
	50	10		25	10
	50	15		50	15
T2	35	5		35	25
	20	1		60	10
	20	0		40	10
Ks	10	1		15	5
	25	3		50	3
	20	3		10	10
Ts	40	1		15	5

Table A5.5

Leaf area, leaf dry mass and specific leaf mass (SLA)							
Cultivar	Acrylic						
	well watering				drought		
	Leaf area	Dry mass	SLA		Leaf area	Dry mass	SLA
	(cm2)	(mg)	(cm2/ mg)		(cm2)	(mg)	(cm2/ mg)

K1	35.61	124.90	0.285		24.32	49.40	0.492
	24.60	65.40	0.376		37.04	60.70	0.610
	36.93	78.50	0.470		37.76	67.90	0.556
K2							
T1	22.79	34.00	0.670		24.05	22.40	1.074
	24.79	21.40	1.158		20.97	15.00	1.398
	18.03	18.10	0.996		26.67	17.90	1.490
T2							
Ks	64.65	100.70	0.642		14.00	47.00	0.298
	33.24	71.10	0.468		26.57	46.60	0.570
	54.27	90.00	0.603		31.09	44.60	0.697
Ts	27.83	40.50	0.687		17.79	24.10	0.738
	31.43	37.30	0.843				
	23.57	39.30	0.600		13.26	25.60	0.518
Cultivar	Polycarbonate						
	well watering				drought		
	Leaf area	Dry mass	SLA		Leaf area	Dry mass	SLA
	(cm2)	(mg)	(cm2/ mg)		(cm2)	(mg)	(cm2/ mg)
K1	51.22	74.10	0.691		31.58	47.60	0.663
	39.54	107.90	0.366		37.31	58.10	0.642
	41.33	77.10	0.536		11.3	45.40	0.249
K2							

T1	30.46	36.20	0.841		22.87	18.40	1.243
	30.72	22.10	1.390		20.5	18.50	1.108
	10.11	16.60	0.609		27.13	14.90	1.821
T2							
Ks	44.69	97.90	0.456		23.73	49.60	0.478
	49.1	79.60	0.617		38.84	58.90	0.659
	60.73	111.50	0.545		20.74	68.10	0.305
Ts	26.87	35.20	0.763		16.14	19.30	0.836
	21.05	30.20	0.697		17.63	20.40	0.864
	25.22	49.80	0.506		15.29	32.20	0.475

Table A5.6

Number of inflorescence and petiole length					
Cultivar	Acrylic				
	well watering			drought	
	no.	petiole		no.	petiole
	inflorescence	length (cm)		inflorescence	length (cm)
K1	17	12.7		8	2.8
	34	18.9		0	8.2
	16	16.7		0	4.8
K2	3	11.5		1	4.6
	2	18.0		0	6.6
	5	18.6		1	7.7
T1	9	8.8		0	1.5
	4	7.6		0	2.6
	0	4.6		0	2.0
T2	4	8.0		0	1.5
				0	2.5

	25	7.6		0	6.5
Ks	33	12.9		0	4.6
	3	14.3		36	7.9
	5	11.4		4	6.0
Ts	0	7.1		8	3.3
	2	12.3			
	0	7.4		10	4.0
Cultivar	Polycarbonate				
	well watering			drought	
	no.	petiole		no.	petiole
	inflorescence	length (cm)		inflorescence	length (cm)
K1	53	17.0		7	5.2
	15	18.7		0	6.8
	39	17.6		0	3.1
K2	6	16.9		0	9.8
	4	20.1		0	8.3
	2	14.3		12	9.0
T1	0	7.5		0	5.0
	0	6.1		0	5.3
	0	7.5		0	6.4
T2	0	6.7		38	3.9
	22	5.4		21	3.6
	9	7.9		0	5.4
Ks	0	17.0		0	6.1
	25	16.1		0	6.8
	9	6.9		0	6.7
Ts	53	13.0		0	3.7
	46	10.2		33	4.0
	3	7.5		0	3.5

Table A5.7

Soil moisture content					
Cultivar	Acrylic			Polycarbonate	
	soil moisture content (%)			soil moisture content (%)	
	well watering	drought		well watering	drought
K1	16	16		16	15
	17	18		15	13
	18	15		21	12
K2	16	10		18	15
	19	15		17	17
	16			16	9
T1	29	15		26	18
	24	16		21	15
	41	29		15	22
T2	20	13		24	16
		22		30	22
	21	13		21	14
Ks	13	10		19	13
	26	14		15	22
	16	24		25	10
Ts	34	21		20	22
	21			19	19
	18	28		31	17

K1 & K2 : *T. repens* cv. Kopu II (cutting)
T1 & T2 : *T. repens* Tienshan (cutting)
Ks : *T. repens* cv. Kopu II (seedling)
Ts : *T. repens* Tien Shan (seedling)

Appendix 6: Chlorophyll Contents

Appendix 6.1 Chlorophyll content of tobacco plants (refer Figure 3.8)

Figure A6.1

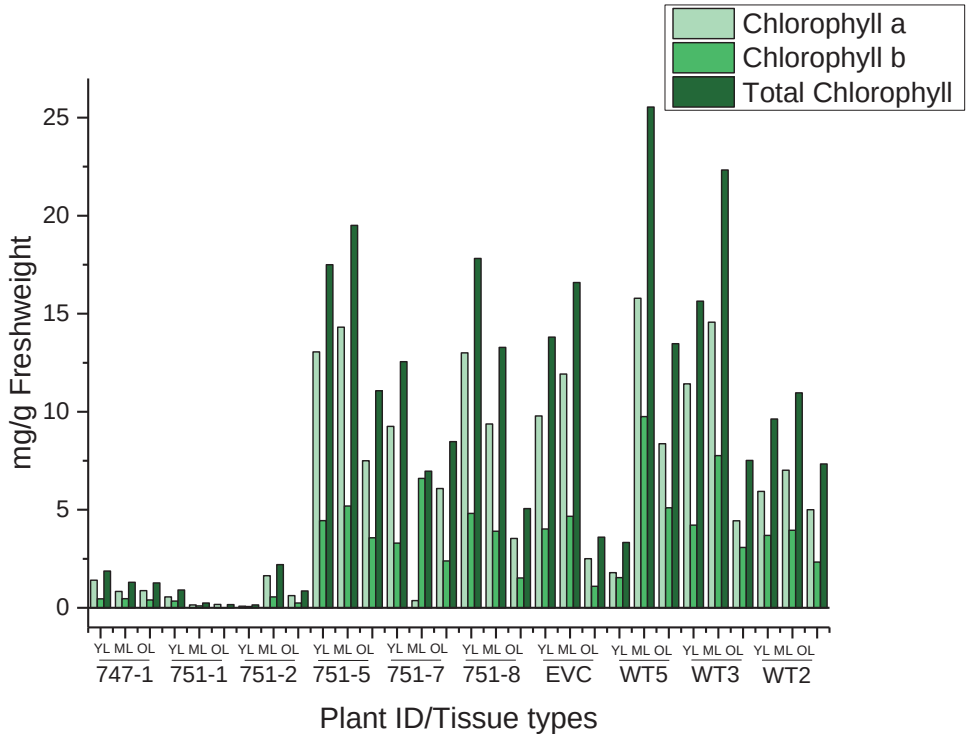
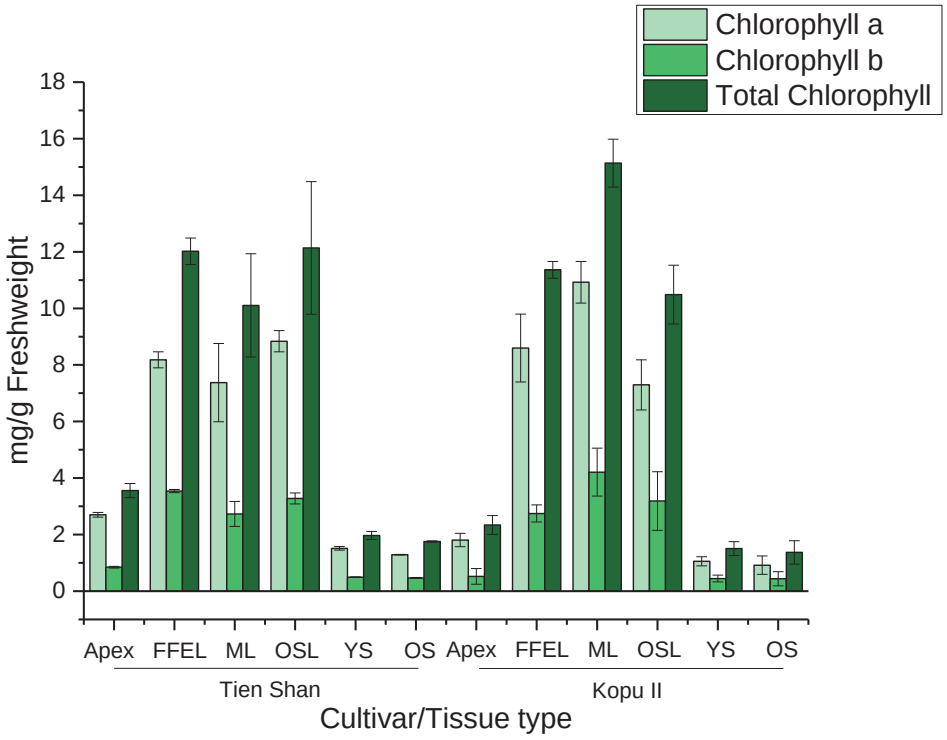


Figure A6.2

Appendix 6.2 Chlorophyll content of white clover stolon (refer Figure 3.11)



Appendix 7: Sequence alignments of legume *NCED1* genes for primer design for q-RT PCR

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CLUSTAL O(1.2.0) multiple sequence alignment

Glycine         ----------------------CAAAATGA---------------------------TCAA 12
Phaseolus       ------------------------------------------------------------ 0
Pisum           ------------------------------------------------------------ 0
Medicago        ------------------------------------------------------------ 0
Cicer           AGGGAGGGGATGTTTCCAAGTAGAAGTTATAATTTGCATACTATGGTAACCACGTGTCAG 60
TrNCED1         ------------------------------------------------------------ 0

Glycine         ATACACGTAATAACAATAAATAATCATAATAGTGGCACACTAATTCACCACTTTCCACAC 72
Phaseolus       ------------------------------------------------------------ 0
Pisum           ------------------------------------------------------------ 0
Medicago        -------GCCAGCTATGCCACTATGGAAGTTAAACCATACAAAACACAACATTTCCACAA 53
Cicer           TTCCTAGGCCAGCGAAGCCACTACGGTAGTTGAACCATACAAAAATCAACAACATCACTC 120
TrNCED1         ------------------------------------------------------------ 0

Glycine         CCAACTGCGTAAAGCTCAAAGCTTTT-TGACAGTTTGAGCATTTCAGACAAACATGGGGG 131
Phaseolus       ----------------------GGCAC-GAGGGGCAGTTTCATCATTCTGACAAATGGGGG 38
Pisum           ---------------------------------------GCAT---TCACGTGAATCTCCA 19
Medicago        ACAAATCCTCCATTTATTTACGTAAATCTCAACAATTT--CT---AACACAA---ACTGA 105
Cicer           ATCACTCATTTTTACACCACTATCGTCCCAAATCCCTTCTCT---GACACACACCCAACA 177
TrNCED1         ------------------------------------------------------------ 0

Glycine         ATGATGGAAAGAAGATTAGTGGAGAGGGGGGGTTAGTGAAGGTTGAGCCAAAACCCAGCA 191
Phaseolus       ATGATGGAAAGAAGAACGGTGCAGAGGGGGGGTTGGTGAAGGTGGACCCAAAACCCACCA 98
Pisum           AAATGGGTTCTGAGAAGAAGGAAAATGGAGTGATTTTGGAGGTGGAACCAAAACCTAGCA 79
Medicago        AAAAAATGGAGTCTGAGAAAATAGGAGGAGGAATTGTGAAAGTGGAACCAAAACCCAGTA 165
Cicer           TGGAGACTGAGAATAATGTAAAAGGATCAGGGATTTTGAATGTGGAACCAAAACCCAGTA 237
TrNCED1         ------------------------------------------------------------ 0

Glycine         ATGGTTTCACCTCAAAAGTGGTTGATTTGTTGGAGAAATTGGTGGTGAAGTTCTTGTATG 251
Phaseolus       ATGGCTTCTCCTCAAAAGTCATTGATTTGTTGGAGAAGCTGCTGGTGAAGTTTTTGTATG 158
Pisum           ATGGGTTCACTTCAAAAGCCGTTGATTTGTTGGAGAAGATTATTGTTAAGCTCTTCTATG 139
Medicago        ATGGTTTCACTTCAAAAGCTGTTGATTTATTGGAGAAGATTATTGTGAAGTTGTTTTATG 225
Cicer           ATGGCTTCACTTCAAAAGTGGTTGATTTGTTAGAGAAGGTTGTTGTCAAGTTGTTTTATG 297
TrNCED1         ------------------------------------------------------------ 0

Glycine         ATTCTTCACTGCCCCATCACTACCTCACTGGTAATTTTGCTCCCGTGAGT---GAGACCC 308
Phaseolus       ATTCTTCACTGCCCCACCACTACCTCACTGGTAATTTTGGTCCTGTTACT---GAGACCC 215
Pisum           ATTCTTCACTTCCTCATCACTGGCTTTCTGGTAATTTTGCACCTGTCAAAGACGAGACGC 199
Medicago        ATTCTTCGCTTCCTCATCATTGGCTTGCTGGTAATTTTGCACCTGTTAAGGATGAAACAC 285
Cicer           ATTCTTCACTTCCTCATCATTGGCTTGCTGGTAATTTCGCACCTGTTCAAGATGAAACCC 357
TrNCED1         ------------------------------------------------------------ 0

Glycine         CTCCAACCAAGGACCTTCCTGTCAAAGGGTACCTTCCGGATTGCTTGAATGGGGAGTTTG 368
Phaseolus       CTCCCACCAAGGATCTTCCTGTCAAAGGGCACCTCCCTGATTGCTTGAACGGGGAGTTTG 275
Pisum           CTCCTGTTAAGGATCTTACCGTGCAAGGCCACCTTCCGGATTGCTTGAATGGAGAGTTTG 259
Medicago        CTCCTATTAAGGATCTTCCTATTAAAGGACACCTTCCGGATTGCTTGAATGGAGAGTTTG 345
Cicer           CTCCCACTACAGACCTAACTGTTAAAGGCTTCCTCCCGGATTGCTTGAATGGAGAGTTTG 417
TrNCED1         ------------------------------------------------------------ 0

Glycine         TCAGAGTTGGGCCTAATCCGAAGTTTGCTCCTGTAGCTGGATATCACTGGTTTGATGGAG 428
Phaseolus       TCAGAGTTGGACCTAATCCAAAGTTTGCTCCTGTTGCTGGATATCACTGGTTTGATGGAG 335
Pisum           TTAGGGTGGGGCCCAATCCAAAGTTTTCTCCCGTGGCAGGATATCACTGGTTTGACGGAG 319
Medicago        TTAGGGTGGGACCAAATTCGAAGTTTGCTCCGGTGGCTGGATATCACTGGTTTGATGGAG 405
Cicer           TTAGGGTGGGACCCAATCCGAAGTTTGCTCCTGTGGCTGGATATCACTGGTTTGACGGAG 477
TrNCED1         ------------------------------------------------------------ 0
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Glycine         ATGGAATGATTCATGGCTTGCGCATCAAAGATGGAAAAGCTACATATGTTTCCCGTTTTG 488
Phaseolus       ATGGAATGATTCATGGTTTGCGTATCAAAGATGGAAAAGCTACATATGTTTCACGTTTTG 395
Pisum           ATGGAATGATCCATGGTTTGCGTATCAAAGATGGAAAAGCTTCTTATGTTTCCCGCTTCG 379
Medicago        ATGGAATGATTCATGGTTTGCGTATCAAAGATGGAAAAGCTACATATGTTTCCCGCTTCG 465
Cicer           ATGGAATGATTCACGGTTTGCGTATCAAAGATGGAAAAGCTACATATGTTTCACGTTTTG 537
TrNCED1         ------------------------------------------------------------ 0

Glycine         TGAGAACTTCTCGCCTTAAACAAGAAGAATACTTTGGAGGCTCCAAATTTATGAAGATTG 548
Phaseolus       TGGAAACTTCTCGTCTTAAACAAGAAGAATACTTTGGACGGTCCAAATTTATGAAGATTG 455
Pisum           TGAAAACTTCTCGTTTTAAACAAGAAGAATACTTTAATGGCTCTAAATTTATGAAGATTG 439
Medicago        TGAAAACTTCTCGTCTTAAACAAGAAGAATACTTTGGAGGCTCTAAATTTATGAAGATTG 525
Cicer           TGAAAACTTCTCGTCTTAAACAAGAAGAATACTTTGGAGGCTCTAAATTTATGAAGATTG 597
TrNCED1         ------------------------------------------------------------ 0

Glycine         GAGATCTCAAAGGTCTATTCGGACTTTTAATGGTTAACATACATATGCTGAGAACTAAAT 608
Phaseolus       GAGATCTCAAGGGTCTATTTGGACTTTTAATGGTCAACATTCATATGCTGAGAACTAAAT 515
Pisum           GAGATCTCAAAGGTCTATTTGGACTGTTAATGGTTAACATGCAAATGTTAAGAGCTAAAT 499
Medicago        GAGATATGAAAGGTCTATTTGGACTGTTAATGGTTAACATGCAAATGTTGCGAGCTAAAT 585
Cicer           GAGATCTCAAAGGTCTACTTGGACTTTTAATGGTTAACGTACAAATGTTGCGAGCTAAAT 657
TrNCED1         ------------------------------------------------------------ 0

Glycine         GGAAAGTGCTGGATGCTTCTTATGGAACTGGAACAGCTAATACTGCTCTCGTATATCACC 668
Phaseolus       TGAAAGTGTTGGATCTTTCTTATGGAGGTGGAACAACCAATACTGCTCTCGTATATCATC 575
Pisum           TGAAAATACTGGATGTTTCTTATGGACACGGAACAGCTAATACAGCTCTTGTATATCATC 559
Medicago        TGAAAGTAGTGGACGTTTCTTATGGACATGGAACAGCTAACACAGCTCTTGTATATCACC 645
Cicer           TGAAAGTACTGGATGTTTCTTATGGAACTGGAACAGCTAATACAGCTCTTGTATACCACC 717
TrNCED1         ------------------------------------------------------------ 0

Glycine         ATGGGAAACTTCTAGCACTCTCAGAAGCAGATAAACCCTATGCTATTAAAGTTTTTGAAG 728
Phaseolus       ACGGGAAGCTTCTAGCTCTCTCAGAAGCAGATAAACCCTATGCTATTAAAGTTTTTGAAG 635
Pisum           ACCAGAAGCTTCTAGCACTCTCGGAAGGAGACAAACCTTATGCTATTAAAGTTTTTGAAG 619
Medicago        ATCAGAAGCTTCTAGCACTCTCAGAAGGAGACAAACCTTATGCTATTAAAGTATTTGAAG 705
Cicer           ATCGGAAGCTTCTAGCACTCTCAGAAGGAGACAAACCTTATGCTATTAAAGTTTTTGAAG 777
TrNCED1         ------------------------------------------------------------ 0

Glycine         ATGGTGATTTGCAGACACTTGGCATGCTAGATTATGACAAGAGATTGGGCCACTCCTTCA 788
Phaseolus       ATGGTGATTTGCAGACACTTGGAATGCTAGATTATGATAAAAGATTGGGTCACTCCTTCA 695
Pisum           ACGGTGATTTGCAGACACTTGGCATGCTAGATTATGACAAGAGATTAGGCCATAACTTCA 679
Medicago        ACGGTGATTTGCAGACACTTGGCATGCTAGATTATGACAAGAGATTGGGCCATAACTTCA 765
Cicer           ATGGTGATTTGCAGACACTTGGCTTGCTAGACTATGACAAGAGATTAGGCCATTCCTTCA 837
TrNCED1         ------------------------------------------------------------ 0

Glycine         CTGCTCATCCAAAAGTTGACCCATTTACTGGGGAGATGTTTACATTTGGCTATGCGCATA 848
Phaseolus       CTGCTCATCCCAAAGTTGACCCTTTTACTGGGGAGATGTTTTCATTTGGCTATGCACATA 755
Pisum           CAGCTCATCCAAAAGTTGACCCATTTACTGGGGAGATGTTTACATTTGGATATTCACATA 739
Medicago        CCGCTCATCCAAAAGTTGATCCATTTACTGGAGAGATGTTTACATTTGGATATTCACATA 825
Cicer           CTGCTCATCCAAAAGTTGACCCATTTACTGGGGAGATGTTTACATTTGGATATTCACATA 897
TrNCED1         ------------------------------------------------------------ 0

Glycine         CACCACCATATATCACATACAGAGTAATTTCAAAGGATGGTTATATGCATGATCCTGTAC 908
Phaseolus       CACCACCATATATCACATACAGAGTAATTTCAAAGGATGGTTATATGCATGATCCTGTAC 815
Pisum           CAGCACCATATGTCACATACAGAGTAATATCAAAGGATGGTTTTATGCAAGATCCTGTTC 799
Medicago        CACCCCCATATATCACATACAGAGTAATTTCAAAGGACGGTTTTATGCATGATCCTGTTC 885
Cicer           CACCACCATATATCACATACAGAGTAATTTCAAAGGATGGTTACATGCATGATCCTGTAC 957
TrNCED1         ------------------------------------------------------------ 0

Glycine         CCATAACAGTATCAGATCCCATCATGATGCACGACTTTGCCATCACAGAGAATTATGCAA 968
Phaseolus       CCATAACAATTTCAGATCCCATCATGATGCATGACTTTGCCATCACAGAGAATTATGCAG 875
Pisum           CCATAACAATATCAGATCCCGTCATGATGCATGACTTTGCCATCACAGAGAATTATTCGA 859
Medicago        CCATAACAATATCAGAGCCAATCATGATGCATGACTTTGCCATCACAGAGAACTATTCAA 945
Cicer           CCATAACAATATCCGATCCCATCATGATGCATGACTTTGCCATCACAGAGAATTATGCAA 1017
TrNCED1         ------------------------------------------------------------ 0

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Phaseolus       TATTCATGGATCTTCCTCTGATTTTTAGGCCAAAGGAAATGGTGAAGAATAAGACATTGA 935
Pisum           TATTTATGGACCTTCCTCTGTACTTTAGGCCAAAGGAAATGGTGAAGAATAAGACCTTGA 919
Medicago        TATTTATGGATCTTCCTCTGTACTTTAGGCCAAAGGAAATGGTGAAGAATAAGACACTGA 1005
Cicer           TATTTATGGATCTTCCTCTGTACTTTAGGCCAAAGGAAATGGTGAAGAATAAGACATTGA 1077
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TrNCED1         ------------------------------------------------------------ 0


Glycine         TATTCTCATTTGATTCAACCAAGAAAGCTCGTTTTGGTGTGCTACCTCGGTATGCTAAGG 1088
Phaseolus       TATTCTCATTTGATTCAACCAAGAAAGCCCGTTTCGGTGTGCTACCTCGGTATGCTAAGG 995
Pisum           TATTCTCATTTGATTCAACCAAGAAAGCTCGTTTTGGTGTCTTACCTCGGTATGCTAAGG 979
Medicago        TATTCTCTTTTGATTCGACCAAGAAAGCTCGTTTCGGTGTTCTACCCCGCTATGCTAAGG 1065
Cicer           TATTCTCTTTTGATTCAACCAAAAAAGCTCGTTTTGGTGTCCTTCCTCGCTATGCTAAGG 1137
TrNCED1         ------------------------------------------------------------ 0


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Phaseolus       ATGAACAGCATATTAGATGGTTTGAGCTACCAAACTGCTTCATATTTCACAATGCCAATG 1055
Pisum           ATGATAAGCATATCAGATGGTTTGAACTACCAAATTGCTTCATTTTCCACAATGCCAATG 1039
Medicago        ATGAAAAACATATTAGATGGTTCGAACTACCAAACTGCTTCATATTCCACAATGCCAATG 1125
Cicer           ATGAGAAGCTCATTAGATGGTTTGAACTACCAAACTGCTTCATATTCCACAATGCCAATG 1197
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Phaseolus       CATGGGAGGAGGAAGATGAAGTTGTTCTGATCACATGCCGCCTTCAGAATCCAAAGTTGG 1115
Pisum           CTTGGGAGGAGGAGGATGAAATTGTTTTGATCACATGCCGCTTGGAGAATCCAAATTTGG 1099
Medicago        CTTGGGAGGAGGAGGACGAAATTGTTTTGATCACATGCCGCCTGGAGAATCCAAATTTGG 1185
Cicer           CATGGGAGGAGGAGGATGAAATTGTTTTGATTACGTGCCGCTTGGAGAATCCAGATTTGG 1257
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Phaseolus       ACAATGTTGGTGGGACTGTCCAGGAAAAGCTTGAAAATTTCTCAAATGAGCTGTATGAAA 1175
Pisum           ACGTGGTCGGCGGGGCTGTCAAGGAAAAGCTTGATAATTTCTCAAATGAGCTGTATGAGA 1159
Medicago        ACATGGTCGGCGGAGCTGTCAAGGAAAAACTTGATAATTTTGCAAATGAGCTGTATGAAA 1245
Cicer           ACATGGTCAGTGGGGCTGTCAAGGAAAAGCTTGAAAATTTCAACAATGAGCTGTATGAAA 1317
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Phaseolus       TGAGATTCAACATGAAAACTGGTGAAGCTTCTCAAAAGAAACTATCAGCATCTACTGTGG 1235
Pisum           TGAGATTTAACATGAAAACCGGTGAAGCTTCTCAAAAGAAACTATCCGCGTCTACTGTAG 1219
Medicago        TGAGATTTAACATGAAAACCGGTGAAGCTTCTCAAAAGAAGCTATCAGCATCTGCTGTAG 1305
Cicer           TGAGATTTAACATTAAAAGTGGTGAAGCTTCTCAAAAGAAACTATCAGCATCTGCTGTTG 1377
TrNCED1         TGAGGTTTAACATGAAAACTGGCGAAGCTTCTCAAAAGAAACTATCAGCATCTGCTGTAG 115
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Phaseolus       ATTTTCCCAGGGTGAATGAAAACTACACTGGCAGGAAGCAGCGATATGTGTATGGGACCA 1295
Pisum           ATTTTCCTAGGGTGAATGAAAGCTACACTGGCAGGAAGCAACGATACGTGTATGGAACCA 1279
Medicago        ATTTCCCTAGGGTGAATGAAAGCTACACTGGCAGGAAACAACGATATGTATATGGGACTG 1365
Cicer           ATTTTCCTAGGGTGAATGAAAGCTACACTGGCAGGAAACAGCGATATGTATATGGAACCA 1437
TrNCED1         ATTTCCCTAGGGTGAATGACAGCTACACTGGCAGGAAACAACGATATGTATATGGAACCA 175
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Phaseolus       CATTAGACAGCATTGCAAAAGTTACTGGTATTATTAAATTTGATTTGCATGCTGAACCAG 1355
Pisum           CATTAGACAGCATTGCTAAAGTTACTGGGATTATTAAATTTGATTTGCACGCTGAGCCGG 1339
Medicago        AATTAGACAGCATTGCTAAAGTTACTGGGATTGTTAAATTTGATTTGCACGCCAAACCGG 1425
Cicer           CATTAGACAGCATTGCTAAAGTTACTGGAATTATTAAATTTGATTTGCATGCGAAACCAG 1497
TrNCED1         CATTAGACAGCATTGCTAAAGTTACTGGGATTATTAAATTTGATTTGCACGCCAAACCGG 235
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Phaseolus       ATCATGGAAAAGAAAAACTTGAAGTTGGAGGAAATGTTCAGGGTCTCTATGACTTAGGAC 1415
Pisum           ATTCTGGAAAAACAAAGCTTGAAGTTGGAGGAAATGTTCAAGGTCTTTATGACTTGGGAC 1399
Medicago        ATTCTGGAAAAACGAAGCTTGAAGTTGGAGGAAATGTCGTGGGTCACTATGACTTGGGAC 1485
Cicer           ATTCTGGAAAAACAAAGCTTGAAGTTGGAGGAAATGTTGAGGGTCTCTATGACTTGGGAC 1557
TrNCED1         ATTTGGGAAAAGCTAAGCTTGAAGTTGGAGGAAATGTTCAAGGTCTCTATGACTTGGGAC 295
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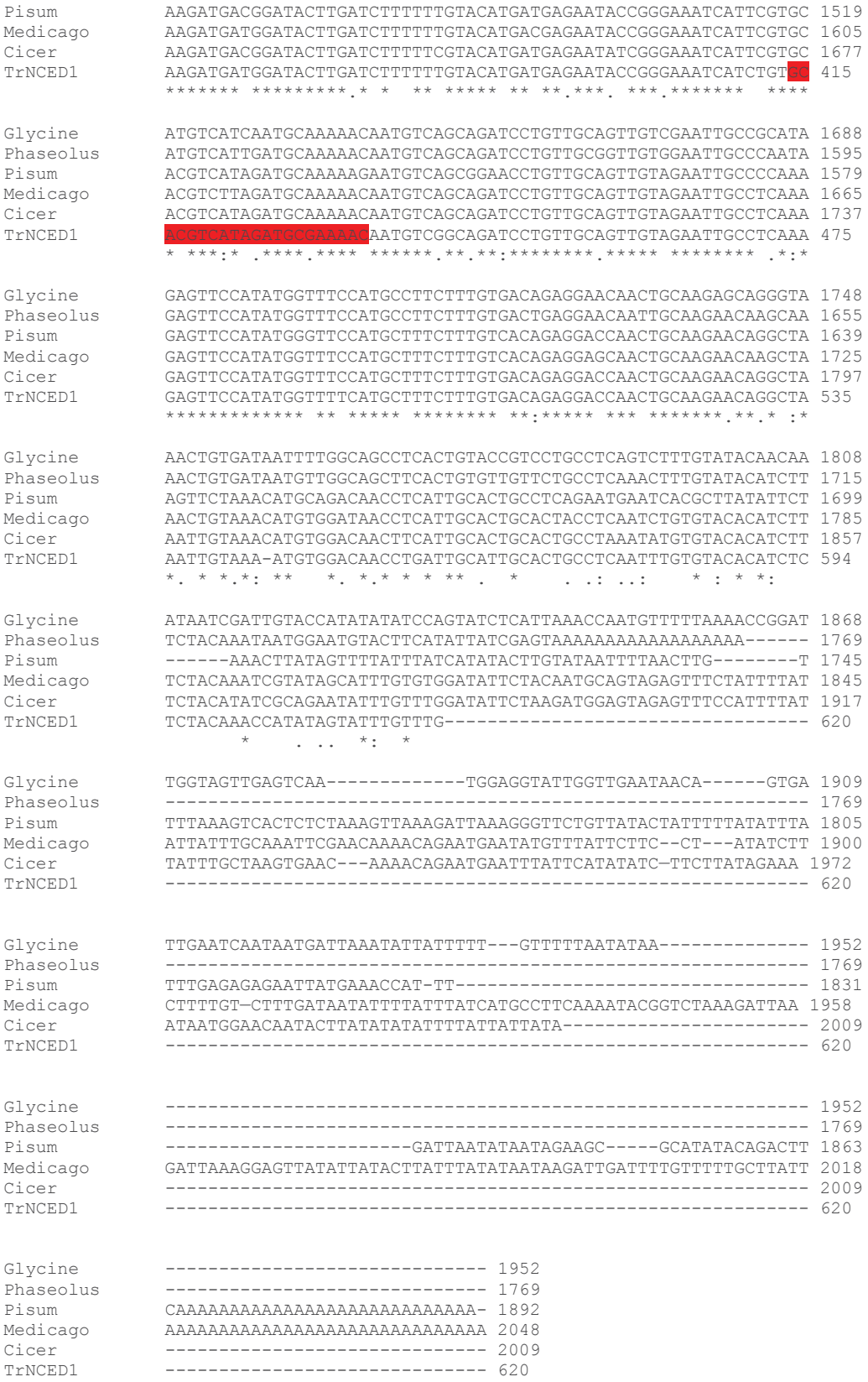
Phaseolus       CAGGAAAATTTGGCTCTGAAGCTGTTTATATTCCTCGTGTCCCCGGAATTGAGTCTGAAG 1475
Pisum           CAGGAAGGTTTGGCTCTGAGGCTGTTTATGTCCCACGTGTCCCTGGCACCGATTCTGAAG 1459
Medicago        CAGGAAGGTTTGGCTCTGAGGCTGTTTATGTTCCGCGTGTCCCTGGCACCGATTCTGAAG 1545
Cicer           CAGGAAGGTTTGGCTCTGAGGCTGTTTATGTCCCACGCATCCCAGGCACTGATTCTGAAG 1617
TrNCED1         CAGGAAGGTTCGGCTCCGAGGCTGTTTTTGTTCCACGCGTCCCTGGCACTGATTCTAAAG 355
                ****.*..*: ***** **.*******:*.* ** ** .**** ** *  ** ***.***


Glycine         AAGATGATGGATACTTGATTTGTTTTGTACACGATGAAAATACCGGAAAATCATTTGTGC 1628
Phaseolus       AAGATGATGGATACTTGGTGTTGTTTGTACATGATGAAAATGCCGGAAAATCATTTGTGC 1535
```



TrNCED1 forward primer indicated in red.

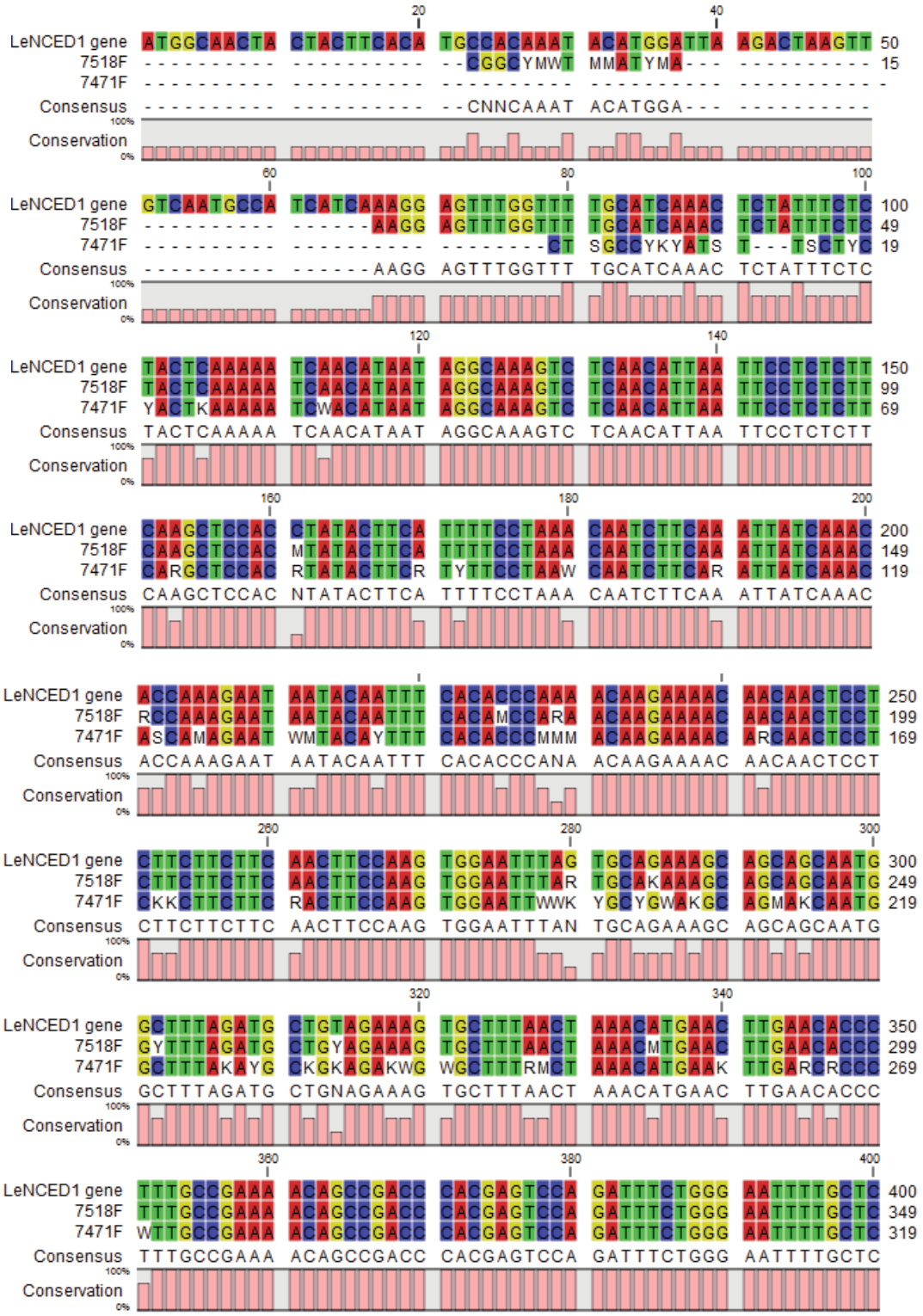
Appendix 8: Sequences of qPCR primers used to determine expression levels of the indicated genes.

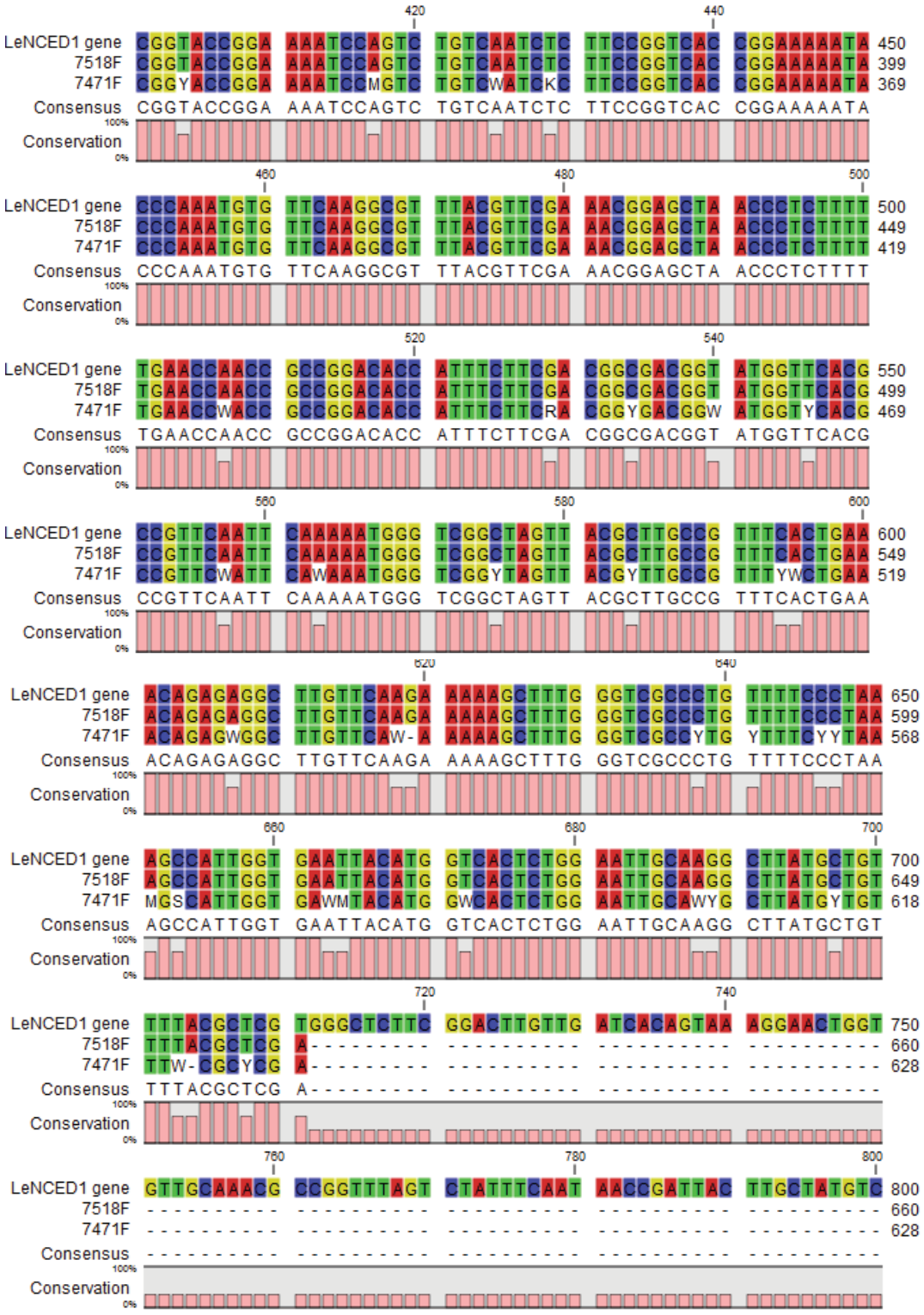
Table A8.1

Gene	Forward Primer	Reverse Primer	Size
TrActin	CGTATGAGCAAGGAGATCACATG	CATCTGCTGGAAGGTGCT	132bp
TrGAPDH	TCCAGTATTGAACGGTAAATTGAC	TCTGATTCCTCCTTGATAGCAG	138bp
TrNCED1	GCA CGT CAT AGA TGC GAA AAC	GTT CCT CTG TCA CAA AGA	104bp
NtGAPDH	TGC TGC TGT GAG GAG TCT GT	GAC TGG GTC TCG GAA TGT GT	178bp
NtEF1-α	CAA GGC TGC TCA GAA GAA GA	TGA GAG CTG GTT CCA GAC AT	180bp
SlNCED1	TTC ACA TGC CAC AAA TAC AT	GT GGA GCT TGA AGA GAG GA	148bp
NptII	GAA TAT CAT GGT GGA AAA TG	CAG AAG AAC TCG TCA AGA AG	207bp

Appendix 9: Sequencing Alignments

Appendix 9.1 Alignment of *SlNCED1* with gDNA PCR products



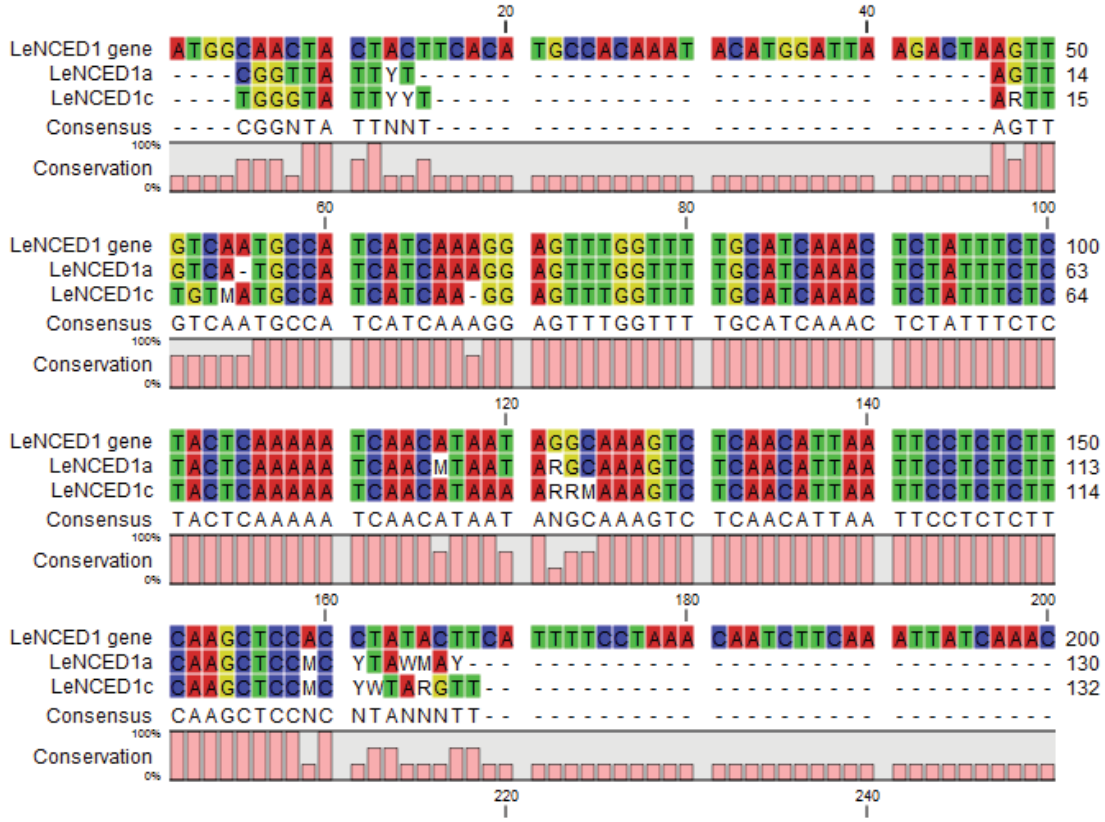


LeNCED1= SlNCED1

7518F=Line 751-8 gDNA PCR product

7471F=Line 747-1 gDNA PCR product

Appendix 9.2 Alignment of *SlNCED1* with cDNA PCR products



LeNCED1=SlNCED1

LeNCED1a=PCR product of 751-8 YL

LeNCED1c=PCR product of 751-1 ML

Appendix 10: Electroporation Protocol as set out by Franklin et al (1992). (refer section 2.2.2)

Carrot cell culture, protoplast preparation and electroporation conditions

- Carrot cells were chosen because of their high yield of protoplasts (approximately 3.0-5.0 x $1O^7$ protoplasts per 45 ml of carrot cell suspension culture).
- The carrot cells were cultured under ambient light and temperature on a gyrorotary shaker at approximately 190 r.p.m. (45 ml cells per 250 ml flask) in a medium consisting of 4.3 g/L Murashige and Skoog salts, 2 mg/L α-naphthalene acetic acid (NAA), 4 mg/L kinetin, 1 mg/L each of thiamine, pyridoxine, nicotinic acid, 4 mg L^{-1} glycine, 4% (w/v) sucrose (adjusted to pH 5.8 with KOH).
- Following subculture for 4 days, cells were centrifuged at 2800 *x g* for 5 min and resuspended in an enzyme solution consisting of 2% (w/v) driselase, 0.4 M sorbitol, and 5 mM 2[N-morpholino] ethanesulfonic acid (MES), pH 5.0, for 4-6 h at room temperature on a gyrorotary shaker at approximately 50 r.p.m.
- Protoplasts were pelleted at 60 *x g* for 5 min and then washed in 40 ml electroporation buffer (10 mM Hepes pH 7.1, 150 mM NaCI, 5 mM $CaCl_2.2H_2O$, and 300 mM sorbitol).
- They were counted in a Fuchs Rosenthal chamber, pelleted at 60 *x g* and resuspended in electroporation buffer at a density of 3.6 x 10^6 protoplasts per ml.
- One millilitre of protoplasts was aliquoted into a 5 ml scintillation vial 80 pL of DNA added, and electroporation carried out at 350 V cm^{-1}, 1000 pF for 9.9 msec (Fromm et al, 1987).
- The protoplasts were incubated for at least 10 min on ice at which time 4 ml of hormone-free medium were added, and they were gently transferred to 60 mm Petri plates, sealed with parafilm and allowed to incubate for the indicated time.