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

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Abstract

In order to trial the concept of inducible overproduction of the plant hormone abscisic acid (ABA) to confer increased water use efficiency, the forage legume *Trifolium repens* (L.) (white clover) and the model plant species *Nicotiana tabaccum* (L.) (tobacco) were transformed with the construct *9-cis-epoxycarotenoid dioxgenase 1* (*NCED1*) gene from *Solanum lycopersicon* (*SlNCED1*) driven by the RUBISCO small subunit promoter (*SSUp*). For white clover, a total of 18 putatively genetically-independent transgenic lines were obtained through selection in tissue culture, and these were further cloned by vegetative propagation to give 56 plants in total. Ten of these tested positive for *NCED1* insertion using polymerase chain reaction (PCR) with genomic DNA. Establishment of transgenic clover on soil was problematic, but seven putatively transformed lines were established. Of these, only one line potentially expressed *SlNCED1,* but the transcriptional levels were too low, as determined by semi-quantitative reverse-transcriptase dependent PCR (sqRT-PCR), for any further analysis. This low expression, and the fact that only one line was identified, led to the decision to discontinue investigations with white clover.

In parallel, the more amenable tobacco transformation system was also used with the *SlSSUp::NCED1* construct to act as proof-of-concept. Fourteen putatively genetically independent transgenic lines of tobacco were obtained through tissue culture, six of which were successfully established onto soil. A range of integrated gene copy numbers and *NCED1* expression levels were identified in the T_o lines using genomic PCR and sqRT-PCR. Self-fertilised seed was collected from each transgenic line, but the germination rate from all of the transgenic lines was significantly lower than wild-type. Those lines that did germinate often displayed a range of aberrant growth phenotypes. After trialing methods to evaluate water use efficiency, a total of 47 T_1 seedlings

displaying a normal seedling phenotype were established on soil. A range of water use efficiencies were observed as determined by analysis of plant growth rates against water use, followed by a transpiration assay of plants deemed 'efficient' and 'poor' users of available water. No correlation of *SlNCED1* expression with the more efficient users of available water was observed, and so ultimately it was concluded that the transgene was ineffective at raising the water use efficiency of tobacco as determined by the parameters measured in thesis.

Acknowledgements

Abbreviations

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

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

RNA	ribose nucleic acid
RT-PCR	reverse-transcriptase-dependent polymerase chain reaction
RUBISCO	ribulose-1,5-bisphosphate carboxylase/oxidase enzyme,
SDS	sodium dodecylsulfate
Sln1	slender-1
SlNCED1	*Solanum lycopersicum* 9-*cis*-epoxycarotenoid dioxygenase
sqPCR	semi-quantitative polymerase chain reaction
SSC	saline sodium chloride (buffer)
SSUp	RUBISCO small subunit promoter
t-ABA	*trans*-abscisic acid
T_0	transgenic generation zero
T_1	transgenic generation 1
Tbsp	tablespoon
Tm	Melting temperature at which DNA strands separate in preparation for annealing
TR	relative transpiration rate.
Tris	tris(hydroxymethyl)aminomethane
Tween 20	polyoxyethylenesorbitan monolaurate
V	volts
v/v	volume per volume
Wt	wild type
WUE	water use efficiency
w/v	weight/volume
x *g*	acceleration due to gravity ($9.8 m\ s^{-2}$)
YEB	yeast extract broth

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1.   Introduction

Overview of thesis

The continued expansion of water intensive agricultural practices such as dairy farming to areas of lower water availability is exacerbating a system where fresh water is a major limiting factor in crop productivity. As such there is considerable research interest in increasing the water use efficiency (WUE) of many of the common agricultural crop species.

The enzyme 9-cis-epoxycarotenoid dioxgenase1 (NCED1) is the key rate limiting step in abscisic acid (ABA) biosynthesis in plants. Transgenic plant studies have demonstrated that the over-expression of *NCED1* can restore the wild type phenotype in ABA deficient mutants, as well as increase WUE in line with the role of ABA as a hormonal signal in plant water deficit stress. Simple upregulation of *NCED1* expression is not enough, however, as high expression or ectopic expression can have far reaching impacts on growth and development, if not carefully controlled.

The purpose of this thesis therefore was to assess the use of the transgene construct *pART-rbcS3Cp::SlNCED1* (here designated *SSUp::SlNCED1*) developed by Tung *et al* (2008) to drive light-dependent upregulation of ABA biosynthesis in the agronomically important crop white clover (*Trifolium repens* L.). The RUBISCO small subunit promoter (*SSUp*) was used in favour of a *CaMV35S* promoter in order to modulate gene expression to a time of the day when plants are most likely to experience water deficits while avoiding deleteriously high expression levels often seen with constitutive promoters. In parallel with the use of white clover, the model plant species *Nicotiana tabacum* (L.) (tobacco) was also used as a more reliable transformation system and to act as a proof-of-concept for the over-expression of the *SSUp::SlNCED1* construct.

The *SSUp::SlNCED1* construct was introduced into white clover and tobacco *via* *Agrobacterium*-mediated transformation, and the integration and expression of the transgene was evaluated in both species. For tobacco, a series of water use efficiency trials were conducted and the efficacy of the light-driven expression of *NCED1* is evaluated in terms of the conferment of improved water use efficiency in plants.

1.1 Abscisic acid

Abscisic acid (ABA) is a phytohormone produced and utilised by plants in order to mediate responses to abiotic stressors such as drought, salinity and low temperatures (Sharp 2002; Mulholland *et al*. 2003), a well as playing a role in developmental processes such as embryonic development, seed maturation and germination (Finkelstein *et al*. 2002). Under optimal conditions, ABA is synthesised at background levels and accumulates in small amounts (nM) *in vivo* and is degraded into less active phaseic acid which is in turn broken down to inactive dihydrophaseic acid (Cutler & Krochko 1999; Zeevaart & Creelman 1988). Under water limited conditions, such as drought, biosynthesis of ABA is up-regulated and the enzymes responsible for its degradation are down-regulated. The resulting increase in ABA concentration results in a cascade of effects ranging from loss of guard cell turgor to alterations in plant-wide gene expression that can affect growth and development, the net result being reduced water loss and an increase in plant water use efficiency (Thompson *et al*. 2007).

1.2 Physiological role of abscisic acid in higher plants

Abscisic acid is responsible for adjusting the physiology of plants to better withstand osmotically stressful stimuli, be they current or future stressors. To this end, ABA is involved in almost every stage of the life cycle, from flowering, seed maturation, and

germination to growth and development (Bray, 1997; Wang *et al*. 1998; Spollen *et al*. 2000; Razem *et al*. 2006 Tung *et al*. 2008).

1.2.1 Floral development

The transition of a plant from a vegetative state to a reproductive state is a potentially very costly period. The diversion of resources from general growth and storage in order to generate reproductive tissues such as flowers at a time when it could not be supported would result in a loss in valuable resources for little or no gain if the resulting progeny could not be carried to seed set by the parent plant. As such, the initiation of flowering is a very tightly regulated process. Razem *et al.* (2006) determined that ABA has a role in mediating this transition period in *Arabidopsis* by binding the RNA binding protein FCA, resulting in the delay of the onset of flowering.

FCA is a nuclear RNA binding protein that is plant-specific, and acts by repressing the accumulation of mRNA encoding Flowering Locus C (FLC), a powerful inhibitor of the floral transition (Michaels & Amasino 1999). For FCA to function, a second protein is also required for it to interact with RNA, FY, which is a 3′ end processing factor which binds to a tryptophan-tryptophan protein interaction domain on FCA to form a functional complex (Simpson *et al*. 2003). Without ABA, the FCA-FY complex autoregulates expression of *FCA* by promoting cleavage and polyadenylation of intron 3 of FCA precursor RNA (Simpson *et al*. 2003). Once functional, this complex will inhibit mRNA accumulation of FLC, thus relieving repression of the floral transition in the absence of ABA.

Razem *et al.* (2006) discovered that in *Arabidopsis*, FCA can bind ABA in a pH dependent manner, which will then disrupt the FCA-FY complex formation. This will inhibit the autoregulatory effect, and remove the repression of *FLC* mRNA

accumulation. In this manner ABA is able to disrupt the floral transition, thereby delaying flowering during unfavourable environmental conditions.

1.2.2 Seed maturation

During embryo development, ABA regulates several aspects of the developmental process such as the synthesis of seed storage proteins and lipids, the promotion of desiccation tolerance and dormancy, and the inhibition of the transition from embryonic to germinative growth (Finkelstein *et al*. 2002).

Seed maturation is characterised by a cessation of cell division and the initiation of cell enlargement as the storage reserves are accumulated (Finkelstein *et al*. 2002). Late Embryogenesis Active (LEA) proteins, as their name suggests, accumulate towards the end of embryogenesis and it is believed that they provide a protective role to certain cell structures, such as the mitochondria, as well as serving in a storage protein role (Nonogaki *et al*. 2010).

During seed maturation, two peaks of ABA accumulation are observed, the presence of which is broadly correlated with low germinability in some species (Finkelstein *et al*. 1985). The first peak was demonstrated to be maternally derived by genetic studies in *Arabidopsis* (Karssen *et al*. 1983). This peak signals the end of the cell division stage and is essential, in conjunction with the leafy-cotyledon *FUS3* and *LEC* genes, to prevent precocious germination of seedlings prior to the accumulation of energy reserves (Wang *et* al. 1998; Raz *et al*. 2001).

The second peak has been observed to be dependent on synthesis within the embryo itself and is required in order to induce dormancy in the seed (Karssen *et al*. 1983). As a part of this process in many species, the embryo must develop desiccation tolerance prior to seed drying. During this a seed will typically lower its metabolic activity to

levels incapable of supporting cell division and begin to dry to an eventual water content of approximately 7-14%, as it enters a dormant state (Nonogaki *et al*. 2010). Once dormancy has been achieved, it is maintained by low levels of ABA biosynthesis that act antagonistically with gibberellins to suppress germination until the correct environmental conditions have been met (Finkelstein *et al*. 2002).

1.2.3 Germination and seedling growth

When a seed is exposed to adequate light, temperature, and water availability, and sufficient time has elapsed to satisfy any other dormancy requirements such as alterations in the hormonal balance or breakdown of the testa and endosperm, germination will begin. By definition, germination is the uptake of water by the seed until such a point as the radicle emerges from the seed coat (Bewley, 1997). Anything after this point is termed seedling growth.

Imbibition is the first step in germination of seedlings and occurs in a largely passive manner as seeds in their dry state (7-14% moisture) are incapable of most metabolically active processes. As the tissues begin to rehydrate, cellular respiration begins to take place and is coupled with the repair and reactivation of damaged mitochondria and their duplication (Morohashi, 1986). Once cellular metabolism has commenced, various repair processes take place to repair any proteins, DNA and other cellular structures that may have been damaged during dehydration as part of maturation and the subsequent rehydration process.

During this period, mRNAs that were stored at the time of maturation are actively translated into proteins required for germination (Rajjou *et al*. 2004). This is accomplished while the DNA repair processes are taking place and transcription is otherwise compromised, and aids the embryo in restoring a baseline metabolism.

When the repair processes are complete, integration of environmental cues is possible and the breaking of dormancy can be achieved. If the environmental cues are favourable, transcription of genes responsible for breaking dormancy will occur. Simple oligosaccharides are mobilised and newly synthesised enzymes are used to alter the hormonal balance of the embryo (Seo *et al*. 2009). Existing ABA is degraded to phaseic acid and dihydrophaseic acid, relieving repression on the gibberellic acid (GA) biosynthesis pathway. The breakdown of ABA and the biosynthesis of GA have been shown to be aided by the presence of red light (Seo *et al*. 2006; Sawada *et al*. 2008), a powerful promoter of germination that acts through the red/far-red light phytochrome response pathway (Borthwick *et al*. 1952). Decreasing levels of ABA coupled with an increase in free GA acts as a stimulus for the rest of the germination processes. If the environmental conditions at the time are unfavourable for seedling growth, the concentration of ABA is maintained and is capable of sustaining dormancy until conditions are conducive to germination (Finkelstein *et al*. 2002).

If the conditions are favourable, cell division to support post-radicle radicle emergence begins to take place and major reserves are mobilised in order to maintain growth and enable seedling establishment. Once the seedling has begun to establish, ABA is important for the maintenance of seedling desiccation tolerance, as well as for maintaining root growth in the form of primary root elongation (Sharp *et al*. 1988).

1.2.4   ABA signalling during growth and development

Sometimes referred to as a stress hormone, ABA has been co-opted during plant evolution for use in signalling osmotic stress and promoting effective water use related responses (Hartung, 2010). During the vegetative stage, ABA is used to mediate responses to osmotically stressful stimuli such as drought, high salinity, and low

temperature (Sharp 2002; Mulholland *et al*. 2003), all of which can result in a decrease in cellular osmotic potential.

How plants sense a decreasing osmotic potential remains unclear, but current theories suggest that it may be based upon a similar system to that seen in budding yeast (*Saccharomyces cerevisiae*) whereby two transmembrane proteins, Sln1 and Sho1, act as sensors for changes in extracellular osmolarity in a redundant manner (Reiser, *et al*. 2005). Changes in turgor pressure of the cell results in a phosphorylation cascade through signalling pathways to initiate osmo-adaptation. Indeed, the plant histidine kinase cytokinin response 1 (Cre1), in the presence of cytokinin, has been observed to behave in a similar manner to the yeast osmosensor Sln1, leading to speculation that it functions as a plant analogue of Sln1 for osmosensing (Reiser, *et al*. 2005). However, to date, it is still unclear as to how plants sense osmotic stressors.

The most well documented effect of increased ABA content is that of stomatal closure which occurs when guard cell turgor is selectively lost in response to water deficit in order to limit the transpiration rate and thus decrease water loss from the plant. This has the flow-on effect of increasing plant water use efficiency (Iuchi *et al*, 2001) and, if maintained for an extended period of time, is associated with stunting of plant growth due to lowered diffusion of gasses through the stomata (Tung *et al*, 2008). The reduction in turgor pressure of guard cells to mediate stomatal closure, when influenced by ABA, is enabled by an efflux of K^+ and Cl^- ions, the removal of sucrose and the conversion of malate to osmotically less active starch (Schroeder *et al*. 2001). An increase in the level of free Ca^{2+} in the cytosol of guard cells is prompted by ABA, which regulates ion channel permeability by controlling ion efflux and influencing stomatal closure.

1.2.5 ABA receptors and perception

Until recently, no single receptor had been shown to bind ABA and mediate widespread ABA-related signalling. Various regulators that act downstream of ABA sensing have been identified, such as a sub-family of type 2C protein phosphatase enzymes (PP2C) that act as negative regulators of subclass III protein kinases of the SNF1-related protein kinase 2 (SnRK2) family. SnRK2s are autophosphorylating kinases that are responsible for phosphorylating downstream elements that switch on ABA-responsive gene expression or other ABA-related responses (Yoshida *et al*. 2002). Several positive and negative regulators of ABA signalling and their downstream effects on ABA-responsive gene expression have been studied extensively (Leung *et al*. 1994; Kobayashi *et al*. 2004; Furihata *et al*. 2006; Hirayama & Shinozaki, 2007; Nakashima *et al*. 2009), but none of these regulators has been shown to interact directly with ABA in order to generate a signal. Recently, two independent groups identified the *Arabidopsis* PYR/PYL/RCAR family of START proteins as ABA receptors that bind to and inhibit group-A PP2C's in response to ABA (Ma *et al*. 2009; Park *et al*. 2009).

Without ABA, the three proteins PYR, PYL, and RCAR are unable to form a long lasting stable complex and are unable to influence the activity of HAB1, ABI1 and ABI2, the group-A PP2C's involved in ABA signalling (for brevity only HAB1 will be discussed) . The absence of ABA allows HAB1 to prevent SnRK2 from successfully auto-phosphorylating; thereby inhibiting ABA related gene expression and responses (Melcher *et al*. 2009). When ABA is available, PYR, PYL and RCAR form a stable protein complex that competes with SnRK2 for the PP2C active site of HAB1 and inhibits activity via a gate-latch-lock mechanism (Melcher *et al*. 2009). With HAB1 inhibited, SnRK2 is activated and begins to phosphorylate ABA Binding Factors

(ABFs), leading to ABA mediated gene expression and responses through ABA Binding Response Elements (ABREs) (Sheard & Zheng, 2009).

Recently, two trans-membrane ABA receptors were identified which are involved in stomatal responses, among other ABA signalling events. These two proteins appear to be related to G-protein coupled receptors (GPCRs) which are widely found in vertebrates but are seldom found in plants or fungi (Offermanns, 2003; Jones & Assmann, 2004). Named GPCR-Type G proteins 1 and 2 (GTG1 and GTG2), these proteins have been demonstrated to possess classic G-protein GTP-binding/GTPase activity, and selectively bind ABA. However, because their loss does not eliminate ABA signalling it is believed that they are redundantly part of ABA signalling and/or components of ABA receptor complexes (Pandey *et al*. 2004; Pandey *et al*. 2006 Pandey *et al*. 2009).

GTGs bind GDP in order to be able to bind ABA and propagate the ABA signal and are subject to variable regulation by GPA1, a prototypical G_α subunit, which is also sensitive to the GTP/GDP ratio. When GPA1 binds GTP the binding of ABA to GTG1 and 2 is inhibited, and therefore ABA signal transduction is interrupted. Thus plants with the *gtg1 gtg2* double mutation are partially insensitive to ABA, and plants with the *gpa1* mutation are hypersensitive to ABA (Pandey *et al.* 2009). The presence of these proteins are only partially able to explain the broader ABA responses but do appear to play an important role in stomatal closure and the prevention of stomatal opening which are two separate responses. *gtg1 gtg2* double mutants are hyposensitive for ABA promotion of stomatal closure and are wild-type for stomatal opening. Conversely, *gpa1* mutants are wild type for stomatal closure and hypersensitive to ABA in the prevention of stomatal opening (Coursol *et al*. 2003). These findings indicate that there are further G-protein related receptor effects involved in ABA recognition and response that have

yet to be identified (Pandey *et al*. 2009), particularly in the prevention of stomatal opening.

The physiological effects of ABA signalling include an increased tolerance to cold and salt stresses, alongside physiological adaptations to decreasing water availability which include stomatal closure, regulation of root versus shoot growth, and osmotic adjustment of the plant. The cellular signalling involved downstream of ABA sensing and signalling in order to elicit these physiological responses is not well understood (Verslues and Zhu, 2005).

A common physiological response to the three stressors, low temperature, high salinity, and low water potential, is osmotic adjustment. Almost all organisms posses some capability to synthesise compatible osmolytes in response to osmotic stress (Burg *et* al. 1996). Plants produce a wide variety of solutes, with the type of osmolytes synthesised varying between species. Osmolytes can include sugars, such as fructans (Hoekstra *et al*. 2001), cyclitols, such as pinitol (Streeter *et al*. 2001), and quaternary ammonium compounds, such as glycine betaine (Rontein *et al*. 2002). The most commonly synthesised osmolytes are amino acids, with the most abundant and well studied of these being proline (McCue & Hanson, 1990; Delauney & Verma, 1993). Proline is synthesised in a two-step reaction (Yoshiba *et al*. 1995) and is able to diffuse throughout the cytosol and vasculature of the plant without adversely affecting native proteins (Bray, 1997).

While it is believed that all of these osmolytes have a role in turgor maintenance, many show involvement in the stabilisation of membranes and structural proteins (Yancey *et al*. 1982). Additionally, others may have a role in scavenging oxidative species to prevent oxidative damage from occurring in the dehydrated state (Shen *et al*. 1997; Akashi *et al*. 2001).

1.3 Biosynthesis of abscisic acid

Abscisic acid belongs to a class of metabolites known as the isoprenoids, which are also known as the terpenoids, and are derived from the common 5-carbon precursor isopentenyl pyrophosphate (IPP) (Nambara & Marion-Poll, 2005). IPP is utilised by plants to synthesise carotenoids *via* the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway and ABA is produced by successive oxidation, isomerisation and cleavage of these carotenoids (Hirai *et al*., 2000). The first committed step of ABA biosynthesis is the conversion of 9-*cis*-epoxyxanthophylls to xanthoxin by 9-*cis*-epoxycarotenoid dioxygenase (NCED) (Taylor *et al*. 2005). Xanthoxin is produced in the plastids and enters the cytosol where it is converted to abscisic aldehyde by a short-chain alcohol dehydrogenase (ABA2) before being converted to ABA by an abscisic aldehyde oxidase (AAO), with the aid of a molybdenum cobalt cofactor (Figure 1.1) (Seo *et al*. 2000).

With the detection of a change in osmotic potential, the biosynthesis of ABA is increased by the up-regulation of *NCED1*, which has been demonstrated to be a key rate limiting step for ABA production, and hence a major site of regulation at the level of gene expression (Tompson *et al*. 2000b; Iuchi *et al*. 2001; Qin & Zeevaart, 2002). In tomato, *NCED1* has been observed to be highly up-regulated in both leaf and root tissues during periods of water stress, and this results in subsequent increases in ABA biosynthesis (Thompson *et al*. 2000a). Ikegami *et al* (2009) demonstrated in *Arabidopsis* that ABA is predominantly synthesised in the above ground portions of a plant due to the availability of 9-*cis*-epoxyxanthophylls, as substrates for NCED1. Once synthesised, ABA travels via the vasculature to mediate plant wide responses and is also capable of acting locally by diffusing apoplastically through the cell wall (Weyers & Hillman, 1979).
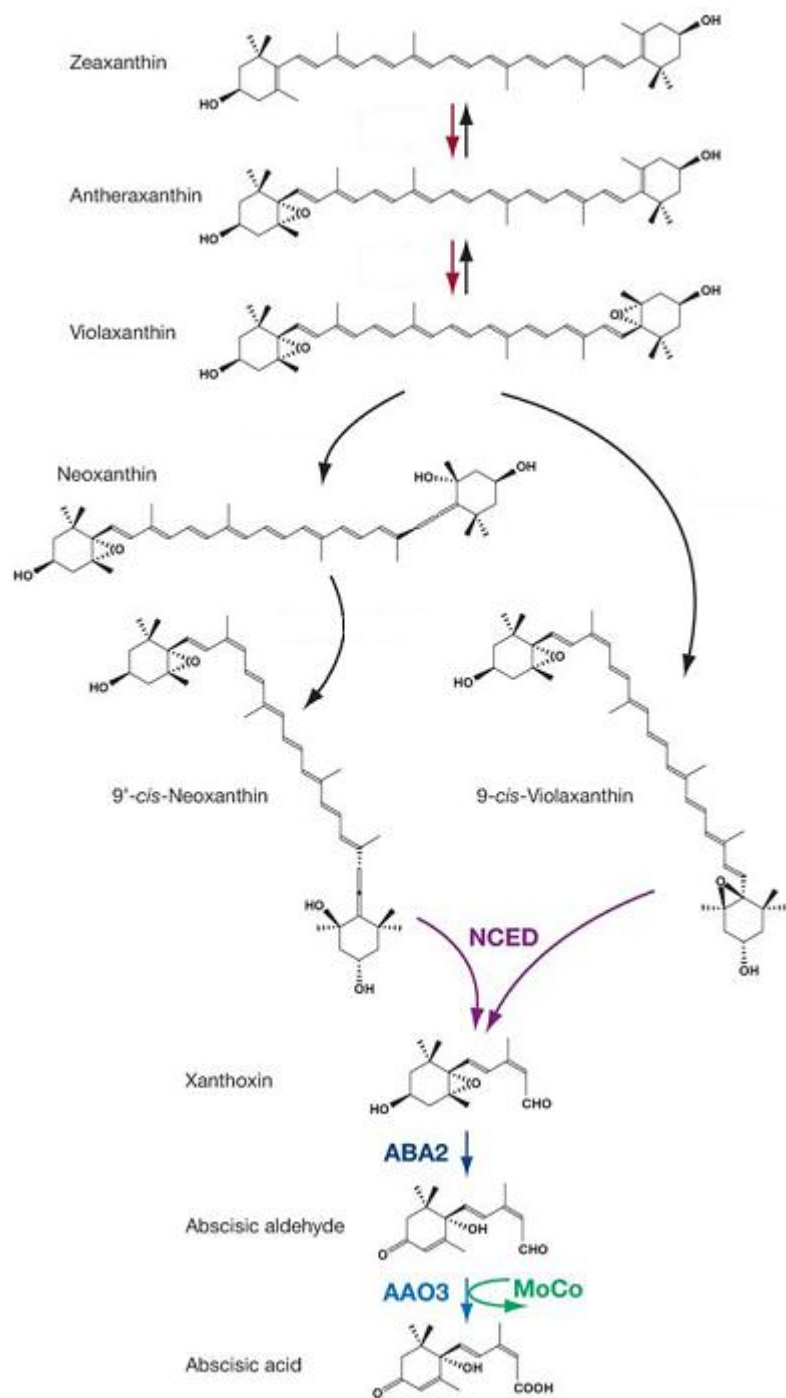
Figure 1.1: Abscisic acid biosynthesis pathway; sourced and modified from Nambara & Marion-Poll, 2005. NCED = NCED1

1.3.1 Manipulation of ABA levels to alter plant growth and development

Early manipulation of ABA involved exogenous applications of relatively high levels of the hormone, resulting in growth inhibition and growth cessation, and is the reason that ABA was originally considered to be a growth inhibitive hormone (Zeevaart & Creelman, 1988). More recently, ABA has been demonstrated to be involved in growth maintenance during periods of biotic and abiotic stress.

Through the use of knockout mutations and over-expressors, studies have shown that a rise in ABA concentration in plants is broadly correlated with increased drought, salinity and cold tolerance, disease resistance, and heightened expression of stress resistance genes (Shinozaki & Yamaguchi-Shinozaki, 2000; Zhu, 2002; Delessert *et al*. 2004). The increases in plant tolerance afforded by these changes in ABA allow plants to maintain growth and development under otherwise adverse biotic and abiotic conditions.

Manipulation of ABA levels also demonstrated that a peak in ABA can interrupt or delay the shift from one life phase to the next (i.e. from seed to germinating seedling, from seedling to vegetative growth, from vegetative growth to flowering, or from flowering to fruiting), with the strength and duration of the interruption being dependent on the level of ABA and the stage of the plants life cycle (Karssen *et al*. 1983; Finkelstein *et al*. 2002; Razem *et al*. 2006).

Direct manipulation of ABA to alter plant growth and development in specific areas is difficult due to its wide ranging effects on plant growth, as well as its interactions with other hormones, and as a result, there are few publications on its successful implementation.

1.4 Water use efficiency

The efficiency with which plants make use of the available soil water reserves is termed water use efficiency (WUE). This depends largely on the transpiration rate of the plant as a whole, as the greatest source of plant water loss is from the stomata and the rate of water uptake from soil reserves is reliant on the water status of the plant as well as the water content of the soil. Some plants are specifically adapted to make the greatest use of all available water (those adapted to arid conditions, for example) and often this is managed by limiting stomatal opening to periods of heightened water availability or to times of the day when transpiration is reduced. However, not all plants are well adapted to conserve water; bryophytes for example have poor control of their stomatal aperture and as such their growth is limited to areas of high water availability.

Beyond stomatal aperture, there are a range of physiological adaptations that enable greater water use efficiency and conservation of water. These include: cuticle thickness, rooting depth, rooting type (i.e. taproots versus fibrous root systems), and regulation of shoot versus root growth (Chaves *et al*. 2003; Jackson *et al*. 2000; Li *et al*. 2007; Zhu, 2002).

As long as the concept of WUE has existed, attempts have been made to investigate and improve it, particularly for important crop species. Traditionally, plant breeding has been seen as one of the more straight forward methods for improving plant WUE, often targeted in tandem with increasing the drought tolerance of the plant of interest. Much traditional breeding has focussed on introgressing greater drought resistance traits from landraces and ecotypes into high performing cultivars, which often have a low WUE. In many cases this requires a trade-off between cultivar performance and drought tolerance or WUE.

The cause of this trade-off becomes more obvious when looking at the phenotypes of the plants involved. High performing cultivars tend to have fast growing stems, large leaves and comparatively shallow root systems. In contrast to this, resilient plants are often slower growing, have smaller leaves and less stomata to limit transpirative water loss, and are often supported by deeper root systems. These resilient plants possess a genotype that is adapted to a low input environment and often are unable to respond effectively under 'ideal' conditions, whereas high performance cultivars are often bred for greater growth under such idyllic conditions and can suffer a loss in productivity outside of their optimal environment (Braun *et al.* 1996).

When the within-species gene pool fails to deliver the desired traits, attempts are made to widen the gene pool by introgressing genes from closely related species, either by direct breeding or by using a third species as a 'genetic bridge' (Harlan & de Wet, 1971).

Technological advances have also allowed for increases in plant WUE. Techniques such as partial root zone drying and drip-lines allow more tightly controlled rationing of water to the plant of interest (Kang & Zhang, 2004), and allow lessened evaporative loss from soil and evapo-transpirative loss from non-target species. Unfortunately, these techniques are largely limited to horticultural crops where the same plants may be harvested from for years and relatively complex semi-permanent irrigative systems can be set up but, tend to be of little use to cropping systems where soil tilling or grazing animals may damage the irrigative infrastructure. When such technological approaches are unable to increase plant WUE and traditional breeding is ineffective, transgenics can be used to introduce genes from an unrelated source and potentially provide a source for improving plant WUE.

1.4.1 Manipulation of ABA levels to alter water use efficiency

The manipulation of ABA to yield increased water use efficiency was best demonstrated by Thompson *et al* (2004) when the tomato *NCED1* gene and its native promoter were introduced to the wilty phenotype mutant *notabilis*. Introduction of the *SlNCED1* gene was able to yield several plants that grew with a non-wilty phenotype due to increased water use efficiency. Of course, this was the relatively simple restoration of a wild type phenotype into an ABA deficient mutant and is not the same as showing that an increase in the ABA level in wild type crop plants would lead to increased water use efficiency. However, it did go a long way in demonstrating that the manipulation of ABA could affect plant water use efficiency.

Further evidence of direct upregulation of ABA resulting in increased water use efficiency came from Thompson *et al* (2007) using a constitutive over-expressor to upregulate *SlNCED1*, a key rate-limiting step in ABA biosynthesis. The resulting plants contained more ABA than wild type plants under non-stressed conditions and showed greater transpiration efficiency, an efficient measure of water use efficiency. Under water stress conditions both wild type and transformed plants showed similar levels of ABA accumulation, while under well watered conditions the transformed plants displayed greater conservation of soil water reserves due to lowered stomatal conductance.

1.5 White clover (*Trifolium repens* L.)

White clover (*Trifolium repens* L. 2n=4X=32) is an allotetraploid legume native to Europe that is used extensively as a forage crop throughout temperate regions of the world for its nitrogen fixing capabilities (Seker *et al*. 2003). The ability to fix atmospheric nitrogen makes white clover, and other members of the legume family,

valuable as livestock feed and for improving soil without the drawbacks of fertiliser run-off. White clover is characterised by a prostrate stoloniferous growth habit, combined with a shallow root system, which results in the plant spreading swiftly *via* the formation of clonal colonies. However, this growth habit is more susceptible to water deficit compared to other members of the legume family such as lucerne (*Medicago sativa* L.) which has a robust tap root system. The agronomic practice of growing white clover with a companion grass species, such as perennial ryegrass (*Lolium perenne* L.) or tall fescue (*Festuca arundinacea* Schreb.), may confer additional water stress (Jiang *et al*. 2010). Further, due to its extensive usage as a forage crop there is increasing demand for the import of white clover to environments into which it is poorly adapted. It is thus expected that this adaptation will be accomplished through conventional breeding, genetic modification, or a combination of the two.

There are a number of areas of interest for genetic improvement that include modifying white clover for deeper growing roots, increased tolerance of abiotic stressors such as drought and low nutrients (Ma *et al.* 2009), and increased tolerance of biotic stressors from the multitude of pest species that target white clover as a nitrogen rich food source.

However, *Agrobacterium*-mediated transformation of white clover is not as simple and effective as that for *Arabidopsis*, which utilises inflorescence dipping (Bechtold, 1993). This technique is limited in its effectiveness in white clover, as an outcrossing species, thus confining transformation to variants of leaf discs and callus propagation. Traditionally white clover has been considered to be largely insensitive to crown gall formation, a simple measure of *Agrobacterium* susceptibility, and is incalcitrant to genetic modification by *Agrobacterium*-mediated transformation (Voisey *et al*. 1994). Advances in our understanding of clover transformation methods (Voisey *et al*. 1994)

and tissue culture have increased the rate of transgenic recovery from tissue culture to levels that make larger scale genetic modification of white clover possible (Ding *et al*. 2003). The rate of recovery of transgenics from tissue culture for white clover vary from around an average of about 0.3% to 6.0%, at best (Ding *et al*. 2003), whereas the minimum rate of recovery species such as *Arabidopsis thaliana* is approximately 1% (Bechtold, 1993), and is often closer to 10% on average.

1.5.1 Water relations studies in white clover

It is relatively well known that white clover does not perform well under poor water potentials. Understanding how and why this is allows for more focussed efforts for improvements in water use efficiency of white clover and as a result of this, water relations in white clover have been well studied. Hart (1987) identified that white clover has poor stomatal control over evapo-transpirative water loss, whereby stomatal closure is incomplete even under decreasing water potentials and thus allows for continued water loss, limiting water use efficiency at low water potentials.

Susceptibility to water deficit is often also associated with root structure in combination with vegetative growth. The shallow rooting depth of most white clover cultivars limits the amount of soil moisture available to the plant. Generally, white clover roots do not penetrate more than the top 20 cm of the soil profile, with more than 80% of the roots located within this zone. When grown with a typical companion species such as ryegrass (*Lolium perenne*), white clovers comparatively shallow root density presents a further barrier to collecting soil moisture (Caradus, 1990).

Several studies have been performed to assess the feasibility of white clover cultivars in environments where water deficit is the major determining factor in pasture crop survival. Clark & McFadden (1997) conducted a five year assessment of white clover

cultivars in south-western Victoria, Australia. During this study they determined that under periodically intensive grazing intermediate leaf sized cultivars with high stolon yield or a low stolon death rate were the most likely to be productive and to persist in a dryland environment with an average annual rainfall of 700mm. While plants that did not place sufficient photosynthetic reserves into their stolons were less able to survive the summer drought period.

1.5.2 Studies to improve water use efficiency in white clover

Historically the improvement of water use efficiency in white clover has relied exclusively on traditional plant breeding with the aim of generating plants with greater rooting potential as well as developing vegetative tissues more responsive to decreasing water potential. These efforts have utilised genetic stock isolated from areas of heat and water stress, such as ecotypes from the Mediterranean basin, for introgression of desirable genes into cultivars coupled with breeding programmes located in areas that will enable targeted selection of drought and heat stress tolerant lines.

More recently, attempts have been made to transgenically alter white clover and introduce desirable traits from the tertiary gene pool. However, as white clover is a recalcitrant species that is largely resistant to genetic manipulation, successful studies have been few.

The first study to demonstrate effective transgenic enhancement of water use in white clover was performed by Jiang *et al* (2010) by inserting the *Medicago truncatula* L. gene *WXP1* (wax production) into white clover, driven by the Arabidopsis epidermal cell promoter *CER6*, to increase cuticle thickness. Transgenic lines containing *WXP1* displayed greater photosynthetic activity, higher relative water contents and increased leaf water potential under water deficit conditions.

1.6 Improving water use efficiency by over-expression of *NCED1*

Initial use of the *NCED1* gene was performed as a means of determining what genes and enzymes were involved in the biosynthesis of ABA. The tomato mutant *notabilis*, being deficient in ABA biosynthesis, was used to generate transgenic plants with the insertion of the *SlNCED1* gene under the control of either a doubly enhanced CaMV 35S or a chimeric 'superpromoter' (Thompson *et al*. 2000b). Successfully transformed plants displayed increased guttation, seed dormancy and lowered stomatal conductance, a measure of water use efficiency. This study also helped to demonstrate that plant ABA content could be increased by manipulation of *NCED* genes.

Following the Thompson *et al*. (2000b) study was a Thompson *et al*. (2004) study aimed at complementation of the *notabilis* mutant with a section of wild type genomic material believed to be the mutated gene that results in the ABA deficient mutant, *SlNCED1*. Transgene insertion was able to restore the wild type phenotype under normal well watered conditions, improving growth and WUE and demonstrating the role of *SlNCED1* in managing water relations in tomato.

Constitutive over-expression of a gene of interest is a relatively simple way to measure its effectiveness in a transgenic organism. Thompson *et al*. (2007) used *SlNCED1* driven by the Gelvin superpromoter, a form of chimeric superpromoter, to upregulate ABA biosynthesis in transgenic tomato plants. During this study it was observed that plants with a heightened basal level of ABA biosynthesis, and a greater active ABA content, have a greater degree of WUE under well watered conditions due to greater transpiration efficiency and root hydraulic conductivity. This advantage in WUE was only apparent under well watered conditions and could be surpassed by native upregulation of ABA biosynthesis under water deficit conditions to the point that

there was no significant difference between transgenic plants and wild type under water stress.

Tung *et al*. (2008) used the RUBISCO small subunit promoter rbcS3C isolated from tomato to drive SlNCED1 expression in tomato in a light responsive circadian manner. This was intended to reduce the effects of constitutive expression such as increased seed dormancy, while maintaining increased WUE. This was successful in that plant WUE increases were maintained and seed dormancy was reduced however, severe symptoms were generated in transgenic plants and their progeny including; abnormal seedling development, substantial reductions in carotenoid content, interveinal leaf flooding and greatly reduced growth. All presumably due to a daily rise in ABA levels much greater than that observed with constitutive promoters.

This thesis aims to assess whether the use of the *SlrbcS3C::SlNCED1* construct was able to be applied in its current form in species other than *Solanum lycopersicum*. To this end it was decided that direct transformation of the agronomically important crop white clover (*Trifolium repens*) would be attempted in order to generate plants with a greater WUE, as a secondary objective it was decided that tobacco (*Nicotiana tabacum*) would also be attempted in order to test the effectiveness of the construct in a genetically more similar but still heterologous background.

1.7 Thesis aims

The primary aim of this thesis is to assess whether the direct transformation of white clover (*Trifolium repens* L.), with the tomato (*Solanum lycopersicum* L.) *9-cis-epoxycarotenoid dioxygenase 1* (*SlNCED1*) gene driven by the RUBISCO small unit (*rbcS3c*) promoter (designated *SSUp*), provides an avenue to produce germplasm that displays an increased water use efficiency. A secondary aim is to use a heterologous

genetic background (tobacco, *Nicotiana tabacum* L.) to further assess the effectiveness of the *SlNCED1* gene driven by the RUBISCO small unit promoter at conferring increased WUE to the transformants.

The thesis hypothesis states that:

Expression of *SlNCED1* will yield increased efficiency in the use of available water reserves for the same or greater accumulation of plant biomass.

To test the hypothesis, the following research objectives were devised:

(i)   Transformation of white clover with the *SSUp::SlNCED1* construct and identification of transformants expressing *SlNCED1,*

(ii)   Assessment of a sufficient number of independently transformed lines in terms of water use efficiency (maximum increase in biomass for less water uptake),

(iii)   In parallel with white clover, utilisation of the more facile transformation system using the model transformation species, *Nicotiana tabacum* with the *SSUp:: SlNCED1* construct,

(iv)   Evaluation of a sufficient number of independently transformed tobacco lines expressing *SlNCED1* in terms of increased WUE.

2.   Methods

2.1 Plant transformation

2.1.1 *Agrobacterium tumefaciens* culture

For transformation, an aliquot of *Agrobacterium tumefaciens* (strain LBA4404) containing the plasmid vector *pART-rbcS3Cp::SlNCED1* (Tung *et al*. 2008) (designated *SSUp::SlNCED1*) was streaked onto YEB agar containing 100 mg/L spectinomycin (YEB^{100Spec})(Appendix 5.1) and the culture incubated at 25°C for 48 hr. Prior to transformation, a loop of culture was inoculated into 100 mL of liquid YEB^{100Spec} broth (Appendix 5.1) that was then incubated at 30°C for 48 hr with continuous shaking.

2.1.2 Transformation and tissue culture of *Trifolium repens*

Approximately 12 hr prior to seed cutting, white clover seeds (cv. Huia) were sterilised in 70% (v/v) ethanol for 1 min, then in 1% (v/v) hypochlorite, 0.1% (v/v) Tween$^{®}$ 20 (Sigma-Aldrich. St Louis, MO, USA) solution for 3 min, before washing 5 times in sterile mQ water, with 3 min each wash. The seeds were then placed in a petri dish, covered with sterile mQ water, and wrapped in foil to imbibe overnight.

Prior to cutting, the seeds were re-sterilised in a solution of 6% (v/v) H_2O_2 and washed 5 more times in sterile mQ water with 3 min each wash, and (typically) 500 seeds were then cut to produce 1000 cotyledons. This was achieved by removing the testa and the endosperm, as well as any excess hypocotyl. The cotyledons were then divided and separated from each other before being placed onto sterile cellulose filter paper, Grade 1 (Whatman$^{®}$ PIC, Kent, UK) on CR7 media [MS media (Duchefa Biochemie B.V., Haarlem, The Netherlands) (Appendix 5.1) including vitamins, 30% (w/v) sucrose, 1 mg/L 6-benzyl aminopurine (BAP) (Duchefa), 0.1 mg/L 1-

napthaleneacetic acid (NAA) (Sigma-Aldrich), 0.8% (w/v) phytoagar (Duchefa), pH 5.7] containing no antibiotics (White & Voisey 1994; Voisey *et al*. 1994).

The *Agrobacterium* broth was centrifuged at 3000 x *g* for 5 min, the pellet re-suspended in 3 mL of 10 mM $MgSO_4$ and 3 µL was spread evenly per cotyledon. The tissues were then left to co-incubate at 25^oC under continuous light with the *Agrobacterium* for two days.

After two days of co-incubation with *Agrobacterium*, all green cotyledons were transferred to CR7 media (White & Voisey, 1994) containing kanamycin (Gibco®, Life Technologies, Carlsbad CA, USA) (50 mg/L) and cefotaxime (100 mg/L) [$CR7^{Kan50,Cef100}$]. Every two weeks, the surviving tissues were moved to fresh $CR7^{Kan50,Cef100}$ media, selecting all cotyledons that remained green. As the cotyledons progressed they were given more space with every change to fresh media. After approximately 2 months, cotyledons with shoots (plantlets) were transferred to $CR7^{Cef100}$ to induce callus formation.

The general failure to induce callus meant that all resulting shoots were moved to fresh $CR0^{Cef100}$ media [MS media including vitamins, 30% (w/v) sucrose, 0.8% (w/v) phytoagar (Duchefa), pH 5.7] for root formation to take place. In order to avoid non-transformed shoots, care had to be taken to avoid shoots that grew from the area near the original apical meristem, as sections of this could still be attached to the cotyledons. Plantlets, and eventually plants, were transferred to fresh $CR0^{Cef100}$ media every two weeks.

Plants were named according to the convention x-y.z, where x = batch number, y = line number and z = clone of original line progenitor.

2.1.3 Transformation and tissue culture of *Nicotiana tabacum*

Prior to transformation, the *Agrobacterium* broth (prepared as described in 2.1.1) was centrifuged at 3000 x *g* for 5 min at room temperature and the pellet suspended in 3 mL of 10 mM $MgSO_4$. Sections of tobacco (*Nicotiana tabacum*) leaf were cut from sterile healthy leaves and scored with tweezers before being dipped into the *Agrobacterium* suspension. The leaf pieces were left to soak for approximately 1 min before being blotted onto sterile cellulose filter paper, Grade 1 (Whatman) and placed on Nic I media [MS media including vitamins, 30% (w/v) sucrose, 1 mg/L BAP (Duchefa), 0.1 mg/L NAA (Sigma-Aldrich), 0.8% (w/v) phytoagar (Duchefa), pH 5.7; Appendix 5.1] containing no antibiotics. The tissues were then left to co-incubate with the *Agrobacterium* at $25^{o}C$ under continuous light for two days.

After two days of co-incubation with *Agrobacterium*, all the tobacco leaf pieces were transferred to fresh Nic I media containing kanamycin (Gibco$^{®}$) (50 mg/L) and cefotaxime (100 mg/L) [Nic I^{Kan50,Cef100}]. Every week subsequently green tissue material was transferred to fresh NicI^{Kan50,Cef100} media.

After callus formation had begun, the tobacco cuttings were transferred to Nic II^{Kan50,Cef100} media [MS media including vitamins, 30% (w/v) sucrose, 1 mg/L BAP (Duchefa), 0.2 mg/L NAA (Sigma-Aldrich) , 0.8% (w/v) phytoagar (Duchefa), pH5.7, 50 mg/L kanamycin (Gibco$^{®}$), 100 mg/L cefotaxime; Appendix 5.1] once the callus was sufficiently developed. The calli were then transferred every two weeks until shoots began to form, which were excised to include the base of callus they formed from. These were then placed on Nic III^{Kan50,Cef100} media [MS media including vitamins, 30% (w/v) sucrose, 0.8% (w/v) phytoagar (Duchefa), pH5.7, 50 mg/L kanamycin (Gibco$^{®}$), 100 mg/L cefotaxime; Appendix 5.1] to induce root formation and growth.

2.1.4 Establishment on soil

Due to the use of the *SSU* promoter to drive expression of the transgene construct, plants had to be removed from media that contained sucrose before transgene expression could be tested due to the possibility that sucrose in the media would inhibit the activity of the *SSU* promoter (Van Oosten & Besford. 1994). So, to ensure that there was adequate transgene expression, any plants of interest were transferred to soil prior to further study.

2.1.4.1 Establishment of *Trifolium repens*

Prior to transfer to soil, all lines were successively cloned using stolon cuttings so that there were ample copies of each genotype. These were maintained aseptically on CR0 media in tissue culture pottles at 25ºC in continuous light. Stolon cuttings that had developed adequate root systems were transferred to potting mix (Daltons, Matamata, New Zealand) that had been autoclaved three times and left to dry. During transfer, each pot was wetted with Thrive® Soluble All Purpose Plant Food (Yates New Zealand, Auckland, NZ) (1 Tbsp/L) to supplement the nutrient balance of the soil before being covered with a transparent plastic bag. Each plant was then given an identifying number before being left to harden off for approximately two weeks. During hardening off, the bag was successively opened to increase air flow and to decrease the relative humidity and encourage hardening off.

Once the plants reached a stage where they produced two fully expanded leaves they were removed from the bag and placed under direct sunlight and given ample water weekly and nutrients as required.

For recalcitrant lines that failed to establish on soil, establishment trials were attempted with individual stolon cuttings being established in CR0 media that had the

sucrose component replaced with 16 g/L mannitol (-suc/+man) in order to maintain the same osmotic gradient. The pottles were then placed in the glasshouse and allowed to acclimatise to direct sunlight. Periodically the pottles were opened to the air to allow air flow for diffusion of gasses and to remove excess water that had accumulated. Plantlet clones were also sent to AgResearch (AgResearch Grasslands, Palmerston North, New Zealand) for establishment on soil using an intermediate step whereby the plantlets were first established on peat plugs that had been rehydrated using half strength CR0 media containing no phytoagar. The CR0 media was successively diluted during the establishment period until the now regenerated plants were able to survive on water alone.

These plantlets were allowed to establish in the peat plugs inside a growth chamber until their roots began to emerge from the plug. At this point they were transferred to a glasshouse and maintained inside an opaque container at high humidity and allowed to harden off. Once sufficiently hardened off, the plants, along with the plugs they were growing in, were transferred into pots containing soil and allowed ample room to grow under direct light conditions.

2.1.4.2 Establishment of *Nicotiana tabacum*

Plants that had developed adequate root systems in tissue culture were transferred to potting mix (Daltons) that had been autoclaved three times and left to dry. During transfer, each pot was wetted with Thrive® Soluble All Purpose Plant Food (1 Tbsp/L) before being covered with a transparent plastic bag. Each plant was then given an identifying number before being left to harden off for two weeks, as described in section 2.1.4.1.

Once hardened off, the plants were removed from the bag and placed under direct sunlight and given ample water and nutrients. Seed was collected from self fertilised inflorescences that had been allowed to dry naturally. Once the seed pod was fully browned and beginning to dehisce, the pod was collected and placed in a paper bag and allowed to drop seed. The loose seeds were collected from the bag and placed into 1.75 mL capacity Eppendorf tubes before storage at 4ºC until use in germination trials (section 2.2.1). For other experiments (Section 2.1.5), seeds were germinated asceptically by placing them on damp cellulose filter paper, Grade 1 (Whatman) and allowing them to imbibe for 36 hr at 25°C under continuous light.

2.1.5 Obtaining seedlings for use in water use efficiency trials

For the first water use efficiency (WUE) trial (3.3.6.1), selected plants grown from seed obtained from either transgenic or wild type tobacco (Section 2.1.4.2) were allowed to grow to a multi leaved size in aseptic NicIII media [MS media including vitamins, 30% (w/v) sucrose, 0.8% (w/v) phytoagar (Duchefa), pH5.7]. Prior to being established on soil, seedlings were stripped of all but the four most recently emerged leaves and the soil was covered with clear plastic to limit water loss.

For the second WUE trial (3.3.6.2), both transgenic and wild type seedlings were established in soil in 50 mL Light Protection Polypropylene Centrifuge tubes (these tubes were modified with a pinhole at the base to allow water to escape the tube). This trial consisted of 24 plants descended from T5, 16 descendants of T8, 15 plants descended from T12, and 12 control plants. Once established all 67 plants were assessed for size and leaf number, 13 were discarded and the remaining 54 plants had all but the four most recently emerged leaves removed. For the purpose of the trial any emerging leaf of less than 10 mm total length was ignored.

2.2 Assessment of transgenic plants

2.2.1 Germination trials

Seed from regenerated tobacco plants or a wild type control were used to determine the germination efficiency of the transgenic seed on MS media [MS media including vitamins, 30% (w/v) sucrose, 0.8% (w/v) phytoagar (Duchefa), pH5.7], MS media containing 1 mg/L GA_3 (Sigma-Aldrich) (2.89×10^{-6} mol/L) dissolved in absolute ethanol (GA_3 media) or control MS media containing 1 mL/L ethanol (non-GA_3 media).

Three lots of thirty seeds were placed on each type of media (MS media, GA_3 media, and non-GA_3 media). The growth rate of each line was monitored over a 33 day period for those on MS media, and 43 days for those on GA_3 or non-GA_3 media, with the number of germinated seedlings noted at appropriate time intervals, and the morphology of the seed recorded photographically.

At the end of each trial, a total count of all germinated seedlings and their growth type (wild type, type 1 aberrant, type 2 aberrant, type 3 aberrant and uncountable) were made (see section 3.3.3.1 for a description of each type).

2.2.2 Microscopy methods

2.2.2.1 Tissue fixation

Tissues must be fixed in a solution that removes all available water, then cleared of this solution prior to embedding in a substrate that will allow the tissue to be sectioned and stained. All of this must be performed before histological analyses can be performed.

The fixative formalin-acetic acid-alcohol (FAA) was prepared fresh each time samples were collected. Enough FAA was prepared so that the tissue does not take up more than 10-20% of the volume of the fixative to ensure proper penetration of the fixative.

FAA	volume (mL)
100% (v/v) ethanol	50
Glacial acetic acid	5
Formalin (37% (v/v) formaldehyde)	10
water	35

Excised tissues from plants of interest were vacuum infiltrated in fixative for 10-15 min at 15 psi. The tissues were not allowed to 'boil.' The vacuum was released slowly to avoid spillage, and the process repeated until the tissue sank. Tissues were incubated in fixative overnight at 4ºC, after which the tissues were washed with a small amount of 50% (v/v) ethanol then the solution was replaced with fresh 50% (v/v) ethanol. The tissues were left to soak for 1 hr before the 50% (v/v) ethanol was replaced with 70% (v/v) ethanol. Tissues were then stored overnight.

Tissues were permeated with increasing concentrations of Histo-clear II (National Diagnostics, Atlanta, GA, USA) prior to embedding. Each new solution consisted of increasing concentrations of Histo-clear II and decreasing concentrations of ethanol as follows: 25% Histo-clear II/75% ethanol (v/v); 50% Histo-clear II/50% ethanol (v/v); 75% Histo-clear II/25% ethanol (v/v) for 30 min each change, followed by incubation in 100% Histo-clear II for 1 hr. A 0.25 volume of Paraplast® Plus (McCormick Scientific LLC, St Louis, MO, USA) wax chips were then added to the 100% Histo-clear II and the mixture incubated overnight at room temperature. The next day, the solution was incubated at 42ºC until the chips melted completely (1-2 hr), after which further Paraplast® Plus wax chips were added until the vial was full. This process was repeated twice more after which the Paraplast® Plus/Histo-clear II was replaced with freshly

melted wax, and the tissues incubated in an oven at 60ºC overnight. After this, the

molten wax was changed twice daily (separated by several hr) for three days.

At the conclusion of this stage, portions of tissue were embedded in Paraplast® Plus

wax using a Modular Tissue Embedding Centre (Leica Microsystems, Wetziar,

Germany). When set, the tissues were stored at 4ºC until sectioned.

2.2.2.2 Tissue sectioning

Tissues were sectioned using a Rotary Microtome (Leica Microsystems), set to give 10

µm sections. Ribbons of tissue were floated on water at 42ºC and mounted on Polysine

slides by bringing the slide up under the ribbons floating on the bath surface. The slides

were tapped gently on a paper towel to get off the majority of water, with care taken to

not disturb the mounted tissues. Mounted sections were placed face up on slide warmer

overnight to allow for the evaporation of any remaining water.

Sections were stained with 0.05% (w/v) toludine blue (Sigma-Aldrich) for 15 min,

then rinsed with water and left to dry. Slides were deparaffinised by suspending the

slides in 100% Histo-clear II for 20 min, then transferring to fresh 100% Histo-clear II

for 20 min, 50% Histo-clear II/50% ethanol (v/v) for 2 min, and then 100% ethanol for

2 min. The slides were left to dry before having a small (~1 mL) amount of 30% (v/v)

glycerol solution placed on the tissues and a cover slip placed over them, before being

sealed with nail polish.

Sections were examined using a compound microscope (Leica Microsystems) at the

appropriate magnification. Images were captured using a Leica camera attachment

(Leica Microsystems).

2.2.3 Water use efficiency trials

2.2.3.1 Trial one

Each plant was measured for height, and total width at the widest point. Each pot was watered to field capacity weight (FCW; which for the purpose of this experiment was defined as the amount of water required to saturate the soil to the point where free water began to leak from the base of the pots) before being weighed. Over the next 72 hr, each plant was remeasured every 24 hr, with the volume of water required to reach FCW measured each time.

2.2.3.2 Trial two

Each leaf was measured for length and width at the widest point. All plants were then watered to FCW, the soil covered to prevent water loss *via* evaporation, and the weight of the plants recorded at FCW. Twenty four hr later each leaf was re-measured and each plant was re-watered to FCW, noting the volume of water required to do so. This was repeated for the next three days.

2.2.3.3 Transpiration assay

A transpiration assay was performed according to a modified method of that used by Green *et al* (1998). Due to the unavailability of 400-W sodium vapour bulbs, two 75-W bulbs were used along with reflective foil to decrease the amount of light lost to the surroundings. The treatment solution itself was distilled H_2O.

2.3 Molecular techniques

2.3.1 Extraction of genomic DNA

DNA was extracted by a modified CTAB extraction (Murray & Thompson, 1980) from previously collected leaf tissue samples that had been frozen in liquid nitrogen and stored at -80ºC.

Extraction buffer: 0.1 M Tris-HCl (pH 7.5)

                      0.35 M sorbitol

                      0.005 M EDTA

                      Adjust volume to 1 L

A fresh solution of 20 mM sodium bisulfate ($Na_2S_2O_3$) was prepared prior to use and added to give a final concentration of 0.038% (w/v) of extraction buffer.

Nucleus-lysis buffer: 0.2 M Tris-HCl (pH 7.5)

                      0.05 M EDTA

                      2 M NaCl

                      2% (w/v) CTAB

5% (w/v) Sarkosyl (N-lauroylsarcosine; Sigma-Aldrich)

Fifty mg of leaf tissue was ground with a micropestle in a 1.7 mL Eppendorf® tube before 0.5 mL of extraction buffer was added and the solution vortexed briefly. Tubes were kept on ice prior to being centrifuged for 1 hr at 20,000 x g at 4ºC. The supernatant was removed and 200 µL of fresh extraction buffer was added before the pellet was briefly reground. Following this, 300 µL of nucleus-lysis buffer was added

along with 100 µL of 5% (w/v) N-lauroylsarcosine (Sigma-Aldrich) and the samples

mixed by inversion prior to incubation for 1 hr at 65°C. After incubation, 500 µL of

chloroform : isoamyl-alcohol (24:1) was added and each tube vortexed. The samples

were centrifuged for 15 min at top speed (20,000 x g) at 25ºC, without using the brake.

The top layer was transferred to a clean 1.7 mL Eppendorf® tube, 500 µL of cold (-

20°C) isopropanol added and the tubes mixed by inversion before being placed on ice

for 15 min. After this they were spun at 4°C for 20 min (20,000 x g), the supernatant

was carefully removed and the pellets washed with 70% (v/v) ethanol then air dried for

10 min before being resuspended in 20 µL of 10 mM Tris-HCl (pH8.0).

2.3.2 Extraction of RNA

RNA was extracted from tissue samples using the hot borate method (Hunter & Reid,

2001).

Buffer XT: 0.2 M sodium borate

               0.03 M EGTA

               1% (w/v) SDS

               1% (w/v) sodium deoxycholate

Extraction Buffer: Buffer XT

                       10 mM DTT

                       1% (w/v) Nonidet^{TM} P-40 (Merck KGaA, Darmstadt, Germany)

                       2% (w/v) PVP-40 (Sigma-Aldrich)

Extraction buffer was prepared fresh and pre-warmed to 80°C. Approximately 0.1-0.2 g

of tissue was ground in a 1.7 mL Eppendorf® tube. One mL of extraction buffer was

added to the ground tissue and vortexed. Proteinase K (7.5 μL; PCR Grade, Roche Applied Science, Mannheim, Germany) was then added and the slurry incubated with shaking at 42°C for 1.5 hr prior to the addition of 80 μL of 2M KCl before the mixture was gently shaken on ice for 0.5 hr. The resuspension was then centrifuged at 20,000 x *g* for 20 min at 4°C, the supernatant was transferred to a fresh 2 mL centrifuge tube and 500 μL of cold (4°C) 4M LiCl added. The RNA was precipitated at 4°C for 16 hr.

The precipitate was collected by centrifugation at 20,000 x *g* at 4°C for 30 min and the supernatant discarded and the pellet was re-suspended in 200 μL of DEPC treated water. Twenty μL of 3M sodium acetate (pH 5.2) was then added before 200 μL of chloroform/isoamyl alcohol (24:1) and the resulting solution was mixed by vortex before being centrifuged at 20,000 x *g* for 5 min at 4°C. The aqueous phase (top layer) was transferred to a fresh 1.7 mL Eppendorf$^{®}$ and another 200 μL of chloroform/isoamyl alcohol (24:1) was added, the solution was then mixed by vortex before being centrifuged at 20,000x *g* for 5 min at 4°C. The aqueous phase was transferred to a fresh 1.7 mL Eppendorf$^{®}$ before 200 μL of isopropanol was added, and the RNA was precipitated on ice for 1 hr. The precipitated RNA was collected by centrifuging at 20,000 x *g* for 30 min at 4°C before being washed with 80% (v/v) ethanol and then re-centrifuged at 20,000 x *g* for 10 min. The supernatant was decanted and the pellet air-dried for 10 min, after which the RNA was re-suspended in 500 μL of DEPC treated water and 500 μL of cold 4M LiCl was added and the RNA precipitated overnight at 4°C.

The precipitate was pelleted by centrifugation at 20,000 x *g* for 30 min at 4°C and the supernatant discarded. The pellet was washed with 2M LiCl before being centrifuged at 20,000 x *g* for 10 min. The supernatant was discarded and the pellet was washed with 80% (v/v) ethanol before being re-centrifuged at 20,000 x *g* for 10 min and the

supernatant discarded. The pellet was air-dried for 10 min before being resuspended in

20 µL of DEPC treated water.

Ten-fold dilutions of all RNA samples were prepared and quantified using

NanoDrop® (NanoDrop Technologies, Wilmington, DE, USA), prior to being stored at -

80ºC.

2.3.3 Southern analysis

2.3.3.1 DNA transfer

Genomic DNA was extracted from the plants of interest using the CTAB DNA

extraction method (section 2.3.1). For each DNA sample (100 µg), 10 µL of 1 mg/mL

RNase A (Sigma) was added prior to incubation at 37ºC for 1 hr and quantification of

DNA was performed using Nano-Drop®. This was followed by the enzymatic digestion

and gel electrophoresis of genomic DNA. For this, 60 µg of genomic DNA was

combined with 50 µL of 10x SuRE Cut Buffer H (Roche) and 10 µL of *Eco* RI (Roche),

the volume adjusted to 500 µL with PCR grade water and the solution incubated at 37ºC

for 16 hr before heat inactivation at 65ºC for 15 min. The Speed Vac® DNA

Concentrator (Savant, Holbrook, NY, USA) was used to reduce the volume to around

30 µL after which the products were separated on a 0.8% (w/v) agarose gel in 1x Tris-

EDTA (TAE) buffer in a SubCell® GT Cell (BioRad, Hercules, CA, USA) at 20V for

20 hr. The gel was then stained with 1 µg/mL of ethidium bromide for 20 min, then

destained in MQ water for 10 min.

After gel electrophoresis and staining, the separated DNA was depurinated in 250

mM HCl with shaking for 15 min at room temperature. After two rinses with mQ water,

the gel was submerged in denaturation solution (0.5 M NaOH, 1.5 M NaCl) at room

temperature for 15 min, again with two changes of solution. After rinsing with mQ

water, the gel was neutralised in Neutralisation solution (0.5 M Tris-HCl, pH 7.50, 1.5 M NaCl), by incubation at room temperature for 15 min with two changes of solution. After this, the gel was equilibrated in 20 x SSC ($\underline{S}$aline $\underline{S}$odium $\underline{C}$itrate buffer comprising 3 M NaCl, 300 mM Sodium Citrate, pH 7.0 with HCl) for 30 min. During this time, Hybond^{TM} N$^+$ nylon membrane (GE Healthcare) (16 cm x16 cm) was pre-wet in 20 x SSC and allowed to soak for 5 min. The blot was then assembled as shown in Figure 2.1, and transfer occurred over 24 hr whilst completely covered. After transfer, the blot was disassembled and a piece of 3MM Whatman$^®$ paper, wetted with 10 x SSC, was used to keep the membrane moist while the DNA strands were cross-linked using UV light with an energy of 120 mjoules for 1.2 min using a Stratalinker$^®$ 2400 (Stratagene, USA).
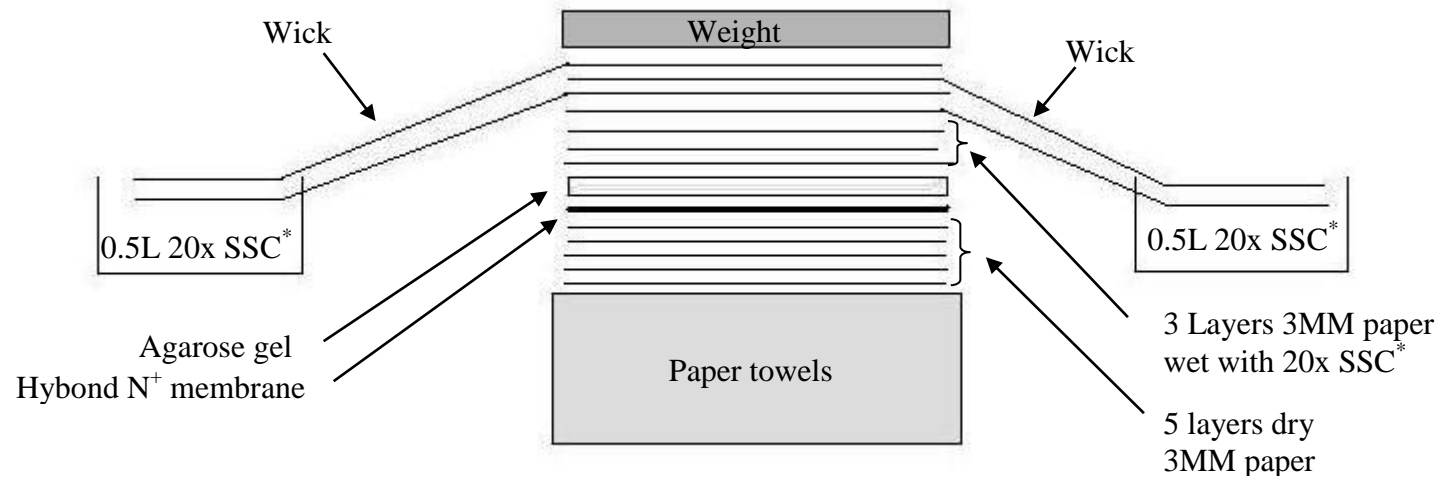
Figure 2.1: Schematic for the set up for the Southern Blot DNA transfer

2.3.3.2 Probe preparation

A probe was prepared using a DNA fragment amplified from the *SSUp::SlNCED1* gene construct using the following primers:

pSSU-SlNCED1 F1: GTG GCC ATT GAT TTT GTA AT;   Tm = 58.8

SlNCED1-qPCR-R1: AG GTG GAG CTT GAA GAG AGG; Tm=62.5

After PCR, the amplified products were separated using agarose gel electrophoresis, and the band of interest (336 bp) purified using the QIAquick® Gel Extraction Kit (QIAGEN, GmbH, Hilden, Germany) , resulting in a DNA concentration of approximately 175 ng/µL. The DNA probe was labelled and the blot was detected using DIG High Prime Labelling and Detection Starter Kit 1 (Roche). To label the probe, 4 µL of DIG High-Prime (Roche) was added to 16 µL of the DNA solution (~2800 ng) and the mixture incubated at 37ºC overnight. The next day, 2 µL of 0.2 M EDTA was added and the solution was heat inactivated at 65ºC for 10 min. The labelling efficiency was checked according to the instruction manual with the kit (Version 1, August 2011). To do this the supplied control DIG labelled DNA was diluted to a concentration of 1 ng/µL, then diluted further as follows:

Tube	Dilution Buffer	DNA	Dilution	Final conc
1				1 ng/µL
2	99 µL	1 µL of Tube 1	1:100	10 pg/µL
3	7 µL	3 µL of Tube 2	1:3.3	3 pg/µL
4	9 µL	1 µL of Tube 2	1:10	1 pg/µL
5	9 µL	1 µL of Tube 3	1:10	0.3 pg/µL
6	9 µL	1 µL of Tube 4	1:10	0.1 pg/µL
7	9 µL	1 µL of Tube 5	1:10	0.03 pg/µL
8	9 µL	1 µL of Tube 6	1:10	0.01 pg/µL
9	10 µL			

One μL of solution from tubes 1-9 from the DIG labelled probe and the control DNA was spotted onto a Hybond™ N⁺ nylon membrane (GE Healthcare). The DNA was crosslinked as described (section 2.3.3.1) and the membrane incubated in a maleic acid buffer [0.1 M maleic acid, pH 7.5, 0.15 M NaCl] for 2 min at room temperature. The maleic acid buffer was discarded and replaced with the supplied blocking solution (Roche), and the membrane incubated for 30 min before incubation in 10 mL of antibody solution (1:5000 anti-DIG-AP conjugate in 1 x blocking buffer) at room temperature for 30 min prior to two 15 min washes with wash buffer [maleic acid buffer with 0.3% (v/v) Tween 20]. The membrane was then equilibrated in detection buffer [0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl] for 2-5 min prior to overnight incubation in 2 mL of freshly prepared colour substrate buffer [40 μL of NBT/BCIP (20 mM NBT/20 mM BCIP stock, supplied in the DIG High Prime Kit) in 2 mL detection buffer] in the dark.

2.3.3.3 Probe hybridisation

The membrane containing the separated DNA fragments (section 2.3.3.1) was rolled up with the nucleic acids facing inwards and placed into a Hybaid™ glass tube (Amersham) to which 15 mL of DIG Easy Hyb buffer (Roche), pre-warmed to 38ºC, was added. The hybridisation temperature was calculated using the following formula:

$$Tm = 49.82 + 0.41(GC\%) - (600/Length)$$
$$T_{opt} = Tm - 24$$
$$GC\% = 38\% \quad Length = 336 \text{ bp}$$

The optimised DIG-labelled *SlNCED1* probe (78 ng; as determined in 2.3.3.2) was boiled for 5 min, and chilled on ice before being added to 3.5 mL of the pre-warmed DIG Easy Hyb buffer, to give a probe concentration of 22.3 ng/mL and this was then

added to the Hybaid^{TM} glass tube. The membrane was hybridised for 40 hr at 38ºC with gentle agitation, after which the Easy Hyb solution with the *SlNCED1* probe was poured off before the membrane was washed twice at room temperature with low stringency solution [2 x SSC, 0.1% (w/v) SDS] for 5 min each wash. It was then washed twice more at 35ºC with high stringency solution [0.5 x SSC, 0.1% (w/v) SDS] for 15 min each wash, before being rinsed in wash buffer [0.1 M maleic acid, 0.15 M NaCl, 0.3% (w/v) Tween 20] for 2 min. The membrane was blocked for 1 hr at room temperature with Blocking Solution (Blocking Solution supplied in the kit 1:10 diluted with maleic acid buffer), 20 mL of antibody solution was prepared by diluting anti-DIG-AP conjugate in blocking solution to 1:10000 dilution, and the mixture then incubated with the membrane at room temperature for 45 min. The membrane was then washed twice for 15 min in wash buffer and rinsed with detection buffer [0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl] for 2 min. The CDP-Star substrate solution (Roche) was prepared and was diluted 1:50 using detection buffer, and 2 mL of the diluted CDP-Star substrate solution transferred to the membrane and incubated for 5 min. The image was developed on Biomax® XAR X-ray film (Kodak, Rochester, NY, USA) for 1 min.

2.3.4 Polymerase chain reaction

2.3.4.1 Genomic PCR

Genomic PCR was performed using two rounds of PCR with 1 μL of 100-fold diluted DNA for the first round and 1 μL of first round PCR product for the second round of PCR. All PCRs were performed using the following reactions.

(~10 ng) Genomic DNA	1 μL	1) 95°C – 3 min
Forward primer (0.5 μM)	1 μL	2) 95°C – 45 sec
Reverse primer (0.5 μM)	1 μL	57°C – 45 sec x35 cycles
PCR Master mix *	10 μL	72°C – 1 min
PCR grade water	7 μL	3) 72°C – 10 min

* 0.025 U/μL Taq, 1.5 mM $MgCl_2$, 200 μM dNTP (Promega Corporation, Madison, WI, USA)

2.3.4.1.1 Design of primers

Three separate primer pairs were used at different stages to confirm transformation: (i) a primer set anchored in the *SSU* promoter region of the *SSUp::SlNCED1* construct to amplify an expected 600 bp product (*SSUp* F1/R1), (ii) one set with the forward primer anchored in the *SSUp* promoter region and the reverse primer anchored in the *SlNCED1* gene to amplify an expected 250 bp product (*SSUp::SlNCED1* F1/R1), and (iii) a *nptII* primer pair to amplify an expected 400 bp product (*NptII* F1/R1) (Table 2.1).

Table 2.1: Primer sequences for all primers used for the confirmation of transformation of putative transgenic white clover and tobacco.

a) SSUp	F1	GATCTAAAGCAATATGTC
	R1	AATTGGTTACTAAGAGG
b) SSUp::SlNCED1	F1	GTGGCCATTGATTTTGTAAT
	R1	ACCAAACTCCTTTGATGATG
c) NptII	F1	TATCCGCAACTTCTTTACC
	R1	AAGGTGAGAGATGACAGGAGA

2.3.4.2 Reverse transcription PCR

2.3.4.2.1 DNAase treatment

A 10-fold dilution of RNA samples was prepared and quantified using NanoDrop®, as described in section 2.3.2. The volume of sample required to yield 2 µg of RNA was identified and any genomic DNA was removed using recombinant RNase free DNAase I (Roche) in the following reaction mixture:

```
                                      Final Concentration:
RNA (2 µg)            x µL            2 µg
10 x DNase I buffer   5 µL            1 x
DNase I               1 µL            10 µL
DEPC-treated water    44 - x µL       -
------------------------------------
        TOTAL         50 µL
```

The solution was incubated at 37ºC for 15 min before 8 mM EDTA was added and the DNase I heat-inactivated at 75ºC for 5 min. DEPC-treated water (450 µL) was added to each sample followed by 400 µL of cold (4ºC) 4 M LiCl and the RNA allowed to precipitate at 4ºC overnight.

The precipitate was collected by centrifuging at 20,800 x g for 30 min at 4ºC. The supernatant was removed and the pellet was washed with 200 µL of 80% (v/v) ethanol and then air-dried for 10 min before being re-suspended in 10 µL of DEPC-treated water, to give an RNA concentration of 0.2 µg/µL.

At this point, the RNA was checked to ensure that it was free of DNA using agarose gel electrophoresis (section 2.3.5). One µL of RNA (0.2 µg/µL) was diluted 20-fold and used for the following PCR.

```
                              Final Conc.
RNA (0.01 µg)            1 µL    0.01 µg      1) 95°C – 3 min
10 µM NtGAPDH-F1         1 µL    0.5 µM       2) 95°C – 10 sec  ⎤
10 µM NtGAPDH-R1         1 µL    0.5 µM          60°C – 10 sec  ⎬ x40 cycles
2 x PCR Master mix*      10 µL   1 x             72°C – 10 sec  ⎦
Water                    7 µL    -            3) 72°C – 5 min
-------------------------------------------
       TOTAL             20µL
```
* 0.025 U/µL Taq, 1.5 mM $MgCl_2$, 200 µM dNTP (Promega)

The products were then separated using gel electrophoresis to check whether any DNA

was amplified.

2.3.4.2.2 Reverse transcription

Reverse transcription (RT) of RNA was performed using Transcriptor reverse

transcriptase (Roche) for clover and primary (T_0) transformants of tobacco, using the

following reactions:

```
                                      Final Conc.
RNA (1 µg)                  5 µL      1.0 µg
50 µM Anchor Oligo dT       1 µL      2.5 µM
DEPC-treated water          7 µL      -
------------------------------------------------------
                            13 uL  Heat denatured at 65°C for 10 min
```

The following reaction mixture was then added:

```
                                          Final Conc.
5 x Transcriptor Buffer           4 µL    1 x
20 mM dNTP                        2 µL    1 µM
PROTECTOR RNase Inhibitor         0.5 µL  20 U
Transcriptor Reverse transciptase 0.5 µL  10 U
------------------------------------------------------
                                  20 µL
```
Incubated at 55°C for 0.5 hr.
Heat inactivated at 85°C for 5 min.

Reverse transcription was achieved for RNA isolated from tobacco progeny (T_1

genotypes) using Expand reverse transcriptase (Roche) by performing the following

reactions:

		Final Conc.
RNA	5 µL	1 µg
25 µM Anchor Oligo dT	1 µL	2.5 µM
DEPC-treated water	4.5 µL	-

10.5 µL Heat denatured at 65°C for 10 min

The following reaction mixture was then added:

		Final Conc.
5 x Expand RT Buffer	4 µL	1 x
100 mM DTT	2 µL	10 mM
10 mM dNTP	2 µL	1 mM
PROTECTOR RNase Inhibitor	0.5 µL	20 U
Expand Reverse transciptase	1 µL	25 U

----

20 µL

Incubated at 43°C for 1 hr.

Heat inactivated at 85°C for 5 min.

PCR was performed to confirm successful reverse transcription of the RNA samples,

using the following reaction mixture and PCR reaction:

		Final Conc.	
cDNA	1 µL	-	1) 95°C – 3 min
2 x PCR Master mix*	10 µL	1 x	2) 95°C – 10 sec
10 µM *NtGAPDH*-qPCR-F1	0.5 µL	0.5 µM	60°C – 10 sec ⎫ x35 cycles
10 µM Nt*GAPDH*-qPCR-R1	0.5 µL	0.5 µM	72°C – 10 sec ⎬
PCR water	8 µL	-	3) 72°C – 5 min

----

20 µL

* 0.025 U/µL Taq, 1.5 mM $MgCl_2$, 200 µM dNTP (Promega)

2.3.4.2.3 Semi-quantitative RT-PCR

RNA was isolated and RT-PCR was performed on the samples of interest. The resulting

RT-PCR products were used for sqPCR. Three primer sets were used. *SlNCED1*

primers, *NtGAPDH*-qPCR primers, and *NtEF1α* primers (Table 2.2).

The *SlNCED1* primer set was used in all sqPCRs, whereas the *NtGAPDH*-qPCR

primers were only used as an internal control to test for successful DNase treatment and

reverse transcription (2.3.4.2.1, and 2.3.4.2.2). The *NtEF1α* primers were used as an

internal control to test the expression levels of *SSUp::SlNCED1* in the progeny of the

primary transformants (Figs. 3.11 and 3.16). The PCR reactions were set up and run as

follows:

		Final Conc.	
cDNA	1 µL	-	1) 95°C – 3 min
2 x PCR master mix	10 µL	1 x	2) 95°C – 10 sec
10 µM *SlNCED1*-F1	0.5 µL	0.25 µM	60°C – 10 sec } x30 cycles
10 µM *SlNCED1*-R1	0.5 µL	0.25 µM	72°C – 10 sec
PCR water	8 µL	-	3) 72°C – 5 min
---			
	20 µL		

		Final Conc.	
cDNA	1 µL	-	1) 95°C – 3 min
2 x PCR master mix	10 µL	1 x	2) 95°C – 10 sec
10 µM *NtEF1α* -F1	0.5 µL	0.25 µM	63°C – 10 sec } x26 cycles
10 µM *NtEF1α* -R1	0.5 µL	0.25 µM	72°C – 10 sec
PCR water	8 µL	-	3) 72°C – 5 min
---			
	20 µL		

The PCR products were separated using gel electrophoresis and the gel bands of interest

were quantified using Image Lab Software version 3.0 (BioRad). To do this, bands were

selected and the volume (intensity) of each band was determined using the following

formula: The relative *SlNCED1* expression = the volume of *SlNCED1* band / the

volume of *NtNtEF1α* band.

Table 2.2: Primer sequences used for semi-quantitative PCR of tobacco plants[1]

a) *SlNCED1*	F1	ACT TCA CAT GCC ACA AAT AC
	R2	CGA GCG TAA AAC AGC ATA A
b) *GAPDH-qPCR*	F1	TGC TGC TGT GAG GAG TCT GT
	R1	GAC TGG GTC TCG AAT GTT GT
c) *NtEF1α*	F1	CAA GGC TGC TCA GAA GAA GA
	R1	TGA GAG CTG GTT CCA GAC AT

[1]The site of these primers is indicated on the full gene sequence, as shown in Appendix 5.2. The *SlNCED1* primer set was selected to amplify a unique region of the *SlNCED1* gene to differentiate it from the *NtNCED1* gene (Appendix 5.2).

2.3.5 Agarose gel electrophoresis

PCR products or plasmid DNA was separated on a 1.5% (w/v) agarose gel [20 mM acetic acid, 10 mM EDTA dissolved in 1 x TAE (40 mM Tris, pH 8.0)] at 100V for 1 hr.

At the conclusion of the electrophoresis the gels were then soaked in a 0.1 µg/mL ethidium bromide solution for 15 min and then destained in mQ water for 10 min, before recording the separation by exposing the gel to UV light for 0.04 sec using a gel doc (BioRad Laboratories). The digital image was captured using the Universal Hood with the attached digital camera (BioRad) and the Quantity One Software version 4.40 (BioRad Laboratories).

2.3.6 Confirmation of expression

2.3.6.1 Expression in *T. repens*

Samples from each of the seven putative transgenic genotypes and six control plants were collected at 10:00 am and 2:00 pm to facilitate identifying any circadian rhythms in transgene expression. The mRNA was extracted from the samples using the RNA Extraction method described in section 2.3.2. The RNA was reverse transcribed to cDNA using Transcriptor Reverse Transcriptase (Roche) as described in section 2.3.4.2 and this cDNA was then used in a PCR using the *SlNCED1* F1/R2 primer pair (Table 2.2) to identify any transgene expression.

Collection, extraction and PCR were repeated to ensure the accuracy of the results.

2.3.6.2 Expression in *N. tabacum*

Confirmation of expression was performed by RT-PCR. The mRNA was extracted from the samples using the RNA Extraction method described in section 2.3.2. The RNA was reverse transcribed to cDNA using Transcriptor Reverse Transcriptase (Roche) as described in section 2.3.4.2. This cDNA was then used in a PCR using the *SlNCED1* F1/R2 primer pair (Table 2.2).

2.4 Statistical analysis

Due to the limitations of this thesis, statistical analysis was largely constrained to determination of the mean and standard deviation of collected data for use in determining whether there was any statistical difference between data points. Determination of sample sizes for water use efficiency trials was hampered by the standard deviation (σ) for the value of WUE being unknown. So for the calculation of sample size a value of 0.5 was assumed for the standard deviation. The following

calculation was used to determine the necessary sample size, based on a normal distribution: $n = (Z_{confidence} \times \sigma / \text{margin of error})^2$, where Z is 1.96 (95% confidence), σ is 0.5, and margin of error is 0.05 (5%). This gives a sample size of 385 plants each for two populations, one for the transgenic plants and one for the wild type control. Reassessment of population sample size with a confidence interval of 90% (1.645), and a margin of error of 10% (0.1), gives a sample size of 68 each for the transgenic plants and the wild type controls. Unfortunately, due to low regeneration rates on tissue culture, these numbers could not be matched for either the transgenic plants or for the wild type controls, meaning data acquired from the trials is not expected to be representational of the total population.

3.   Results

3.1 Production of transformed white clover lines expressing *SlNCED1*

3.1.1 Overview of the tissue culture process used for white clover and nomenclature of different developmental stages used in this thesis

The transformation of white clover begins with co-cultivation of excised cotyledons with *Agrobacterium* containing the vector of interest, and after resting on selection free media for 2 days, the cotyledons are placed on selection. In this case Kanamycin was used to select for the transgene construct and Cefotaxime was used to eliminate any *Agrobacterium* still present. Using this method, the progress of regeneration involves the dedifferentiation of the cotyledons to callus (on CR7 media), from which the formation of shoots is induced (on CR5 media), and at this stage these are designated as plantlets. The plantlets are then moved to a new media (CR0) to form roots and these are designated as regenerated plants when they consist of a plant with functional leaves, shoot, and roots supported by a stem. For the purposes of this thesis, the following terminology is also used:

Line (lineage): A genetically distinct individual, or progeny that share a common ancestor.

Genotype: A genetically distinct individual, or clones (can be the representative of a line), often used to distinguish siblings of a line from one-another.

Clone: A genetically identical copy, or copies, of a single genotype.

Plantlet: Partially regenerated plant consisting of shoots and stem in tissue culture (often accompanied by callus) that lacks roots.

3.1.2 *Agrobacterium* mediated transformation and tissue culture

White clover cotyledons were exposed to *A. tumefaciens* containing the plasmid vector *pART-SSUp::SlNCED1* (Section 2.1.1). After two days co-incubation the cotyledons were placed on CR7 media to begin the regeneration process of plants, from cotyledon to callus, to plantlets with shoots, to fully regenerated plants with both shoots and roots attached to a stem. However, in this thesis, few shoots actually formed from any cotyledon derived callus and the majority of plants arose from shoots growing directly from the original cotyledons. Because of this, the use of CR5 media was not required for the induction of shoot growth as this unexpectedly occurred on the preceding CR7 media. As a result, plants were transferred directly from CR7 media to hormone free CR0 media for the generation of roots and the final regeneration of the plantlets to functional plants.

Over the course of the transformation and regeneration process a batch effect was noticed. For batches 1 and 2 initial successes with regeneration of shoots all failed when the plantlets succumbed to antibiotics present in the media. The next several rounds of seed cutting (Batches 3 – 14) failed in the early callus stages of tissue culture due to an apparent seasonality of clover tissue regeneration, with the best regeneration of tissue occurring during late winter through spring (July-October, Southern hemisphere). Outside of this time window, tissue regeneration was very poor, with no cotyledons surviving long enough to regenerate plants successfully. To illustrate this, batches 15 (7/8/09) through 18 (25/9/09) had varied success, with batches 15 and 16 (21/8/09) being the most successful in regenerating shoots (Figure 3.1), while batch 17 (27/8/09) eventually failed and batch 18 was reduced to a single specimen that arrested as callus and was unable to regenerate any shoots until spring the following year.

Of a total 6250 cotyledons, constituting 18 rounds (batches) of seed cutting, 18

unrelated and genetically distinct lineages were obtained through tissue culture, giving a

regeneration rate of 0.29%.
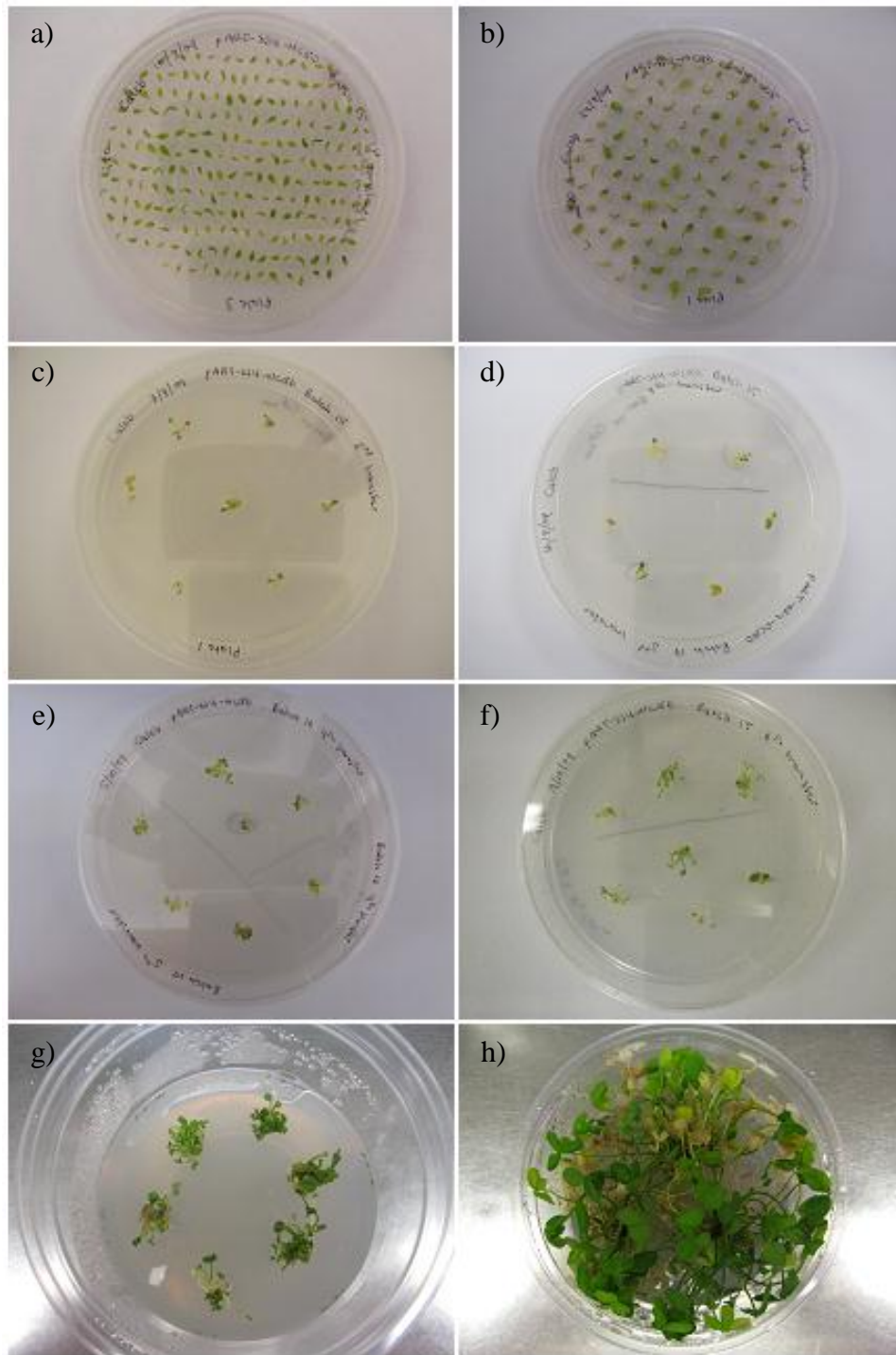
Figure 3.1: Development of plants in Batch 15; a) cotyledons 2 days post co-incubation with *Agrobacterium tumefaciens*, b) cotyledons 16 days post co-incubation, c) 30 days post co-incubation, d) 44 days post co-incubation, e) 56 days post co-incubation, f) 78 days post co-incubation, g) 93 days post co-incubation, h) 150 days post co-incubation (plant 15-5.6). Figures e) through g) are examples of plantlets undergoing regeneration. Figure h) is of fully regenerated plants. Stages a) through f) occurred on CR7 media, while CR0 media was utilised for stages g) and h).

3.1.3 Confirmation of insertion of the *SSUp::SlNCED1* transgene

Despite the low frequency of regeneration, a total of 18 genetically distinct plants were

initially obtained and named according to the nomenclature x-y.z, where x = batch

number, y = line number and z = clone of original line progenitor. Of the initial 18

plants, 3 lines died due to media contamination, leaving 15 lines (designated 15-1, 15-2,

15-4, 15-5, 16-3, 16-4, 16-5, 16-6, 16-7, 16-8, 16-9, 16-10, 16-11, 17-1 and 18-1) that

were then cloned further to give 56 plants. Further media contamination reduced the

number of available plants to a total of 39 (now designated 15-1.1, 15-1.2, etc).

To determine if there were any insertion events of the *SSUp::SlNCED1* transgene

into regenerated *T. repens* plants, polymerase chain reactions (PCRs) using genomic

DNA as a template were performed on the surviving 39 plants with three primer sets

[*SSUp* (expected band size, 600 bp); *SSUp::SlNCED1* (expected band size, 300 bp) and

NptII (expected band size, 400 bp)] (Table 3.1). PCR results for the *SSUp* primer pair

showed a range of bands from ˜ 200 bp to ˜ 800 bp, but re-extraction of DNA and

repeating the DNA extraction and PCR failed to produce bands of a consistent size (ie.

600 bp). Subsequently all bands not of the expected size (600 bp) were ignored. The

SSUp::SlNCED1 primer pair yielded more consistent band sizes than the *SSUp* primer

pair alone, but irregular band sizes were still observed. Because of this, all bands not of

the expected 300 bp size were ignored. The *NptII* primer pair always yielded a

consistent band size of 400 bp, the expected size (Table 3.1).

Using these three primer pairs, a total of 10 plants, comprising 8 lines were selected

based on their testing positive for the expected band size for more than one primer set

across a total of 10 separate rounds of genomic PCR analysis. This gave a

transformation frequency, calculated as number of independent confirmed transgenic

plants produced per one hundred treated cotyledons, of 0.13%. These 10 plants,

designated 15-1.3, 15-2.2, 16-4.1, 16-5.1, 16-5.2, 16-7.1, 16-8.5, 16-8.6, 16-9.1 and 16-10.1, were selected for further testing and advancement to soil. A further 5 plants were selected as non-transformed control plants based on not testing positive for any primer pair, these plants were designated 15-4.2, 15-5.7, 16-3.2, 16-5.6 and 16-11.1.

Variation in plant growth habit was observed between some clones, indicating the possibility of chimeric tissues, and also the possibility of somaclonal variation was apparent in some lines. For example, clone 16-8.6 was observed to have a dwarfed growth habit when compared to other clones of line 16-8. For this reason these plants were selected for future testing alongside clones of the same line that displayed a normal growth habit. This was performed because any somaclonal variation capable of yielding an altered growth habit would result from altered gene expression, and this could yield plants that behaved in a novel manner compared to plants of the same line with a normal growth habit, despite their apparent genetic similarities.

Table 3.1: Screening by PCR of all surviving regenerated clover plants. Only positive band sizes are shown.

Primer set used	Plants							
	15-1.2	15-1.3	15-2.1	15-2.2	15-4.2*	15-4.3	15-5.3	15-5.4
SSUp								
SSUp		600						
SSUp::NCED								
SSUp								
SSUp::NCED								
NptII		400	400					
SSUp::NCED		300						300
SSUp::NCED				300				
NptII		400		400				
SSUp::NCED		300		300				
Total		5	1	3				1

Primer set used	Plants							
	15-5.6	15-5.7*	16-3.2*	16-4.1	16-5.1	16-5.2	16-5.5	16-5.6*
SSUp						600		
SSUp								
SSUp::NCED	300							
SSUp								
SSUp::NCED								
NptII			400		400	400		
SSUp::NCED				300	300			
SSUp::NCED				400		300		
NptII								
SSUp::NCED					300	300		
Total	1		1	2	3	4		

Primer set used	Plants							
	16-6.1	16-7.1	16-7.2	16-8.1	16-8.2	16-8.3	16-8.4	16-8.5
SSUp		600						
SSUp								600
SSUp::NCED	300	300						
SSUp								
SSUp::NCED								
NptII		400	400					400
SSUp::NCED								
SSUp::NCED								
NptII		400						
SSUp::NCED		300						
Total	1	5	1					2

* Denotes plants used as non-transformed controls

Table 3.1 continued: Screening by PCR of all surviving regenerated clover plants. Only positive band sizes are shown.

Primer set used	Plants 16-8.6	16-9.1	16-10.1	16-11.1*	18-1.1	18-1.2	18-1.3	18-1.4
SSUp								
SSUp			600					
SSUp::NCED								
SSUp								
SSUp::NCED	300		300					
NptII	400	400						
SSUp::NCED								
SSUp::NCED	300							300
NptII	400	400				400		
SSUp::NCED	300	300			300			300
Total	5	3	2		1	1		2

Primer set used	Plants 18-1.5	18-1.6	18-1.7	18-1.8	18-1.9	18-1.10	18-1.11
SSUp							
SSUp							
SSUp::NCED							
SSUp							
SSUp::NCED							
NptII							
SSUp::NCED							
SSUp::NCED							
NptII	400	400	400	400			400
SSUp::NCED	300						
Total	2	1	1	1			1

* Denotes plants used as non-transformed controls

3.1.4 Establishment on soil

Due to the use of the *SSU* promoter to drive expression of the transgene construct, plants had to be removed from typical CR0 media that contained sucrose before transgene expression could be tested because sucrose in the media could inhibit the *SSU* promoter-mediated transcription (Van Oosten & Besford. 1994). Prior to the transfer to soil, all available lines of positive transformants (Section 3.1.3) and non-transformants (15-4.2, 15-5.7, 16-3.2, 16-5.6, 16-11.1 [asterisked in Table 3.1]), along with some wild type controls were cloned to provide sufficient copies for multiple establishment attempts. The wild-type controls underwent the same tissue culture as all putative transgenic lines but were not exposed to *Agrobacterium.*

Of the 44 clones transferred to soil (29 cloned positive transformants, 10 cloned non-transformants, 5 wild type controls), a total of seven survived to grow into mature plants, but none of these were clones of the 10 positively transformed plants. All other plants died during hardening off or after succumbing to fungal infections. Of the seven surviving plants, three were wild type control lines, and were given the identifiers of N2, N6 and N7. The remaining four plants were originally exposed to *Agrobacterium* but were shown to have failed to integrate the transgene during the genomic PCR analysis and were designated as non-transformed [15-4.2, 15-5.7, 16-5.6 and 16-11.1 (Table 3.1)]. These could be used as negative controls for the transformant plants that went through tissue culture and so were named N15, N16, N17 and N22.

When transformed plants failed to establish on soil, other methods of generating tissues raised without media containing sucrose were attempted. The use of CR0 media containing no sucrose and supplemented with mannitol to maintain the same osmotic potential was trialed to grow plants but failed due to poor establishment and growth rates, coupled with persistent contamination of the growth media (data not shown).

Nine clones (15-1.3, 16-4.1, 16-5.1, 16-5.2, 16-8.5, 16-8.6, 16-10.1, 18-1.2 and 18-1.4) were also sent to AgResearch Grasslands, Palmerston North for establishment in soil using their well-established protocols. Of these, seven transgenic lines were established on soil via peat plugs and were designated M1 (16-8.5), M2 (18-1.2), M3 (16-8.6), M4 (16-4.1), M5 (15-1.3), M6 (16-10.1) and M7 (16-5.2).

3.1.5 Confirmation of expression

Confirmation of expression of transgenic clover lines was carried out on plants M1 to M7 by RT-PCR analysis of RNA extracted from tissue harvested at two different times; a morning sample (10:00am) and an afternoon sample (2:00pm). This was performed in order to detect any differential expression related to day-time light quality that might have been associated with the transcriptional activity driven by the *SSU* promoter. No lines tested positive consistently for transgene expression. A band of the expected size (150 bp) was observed in line M3 in the pm sample (arrowed, Fig.3.2). This band was never observed in the am sample, and only approximately 50% of the time in the pm sample when the test was repeated.
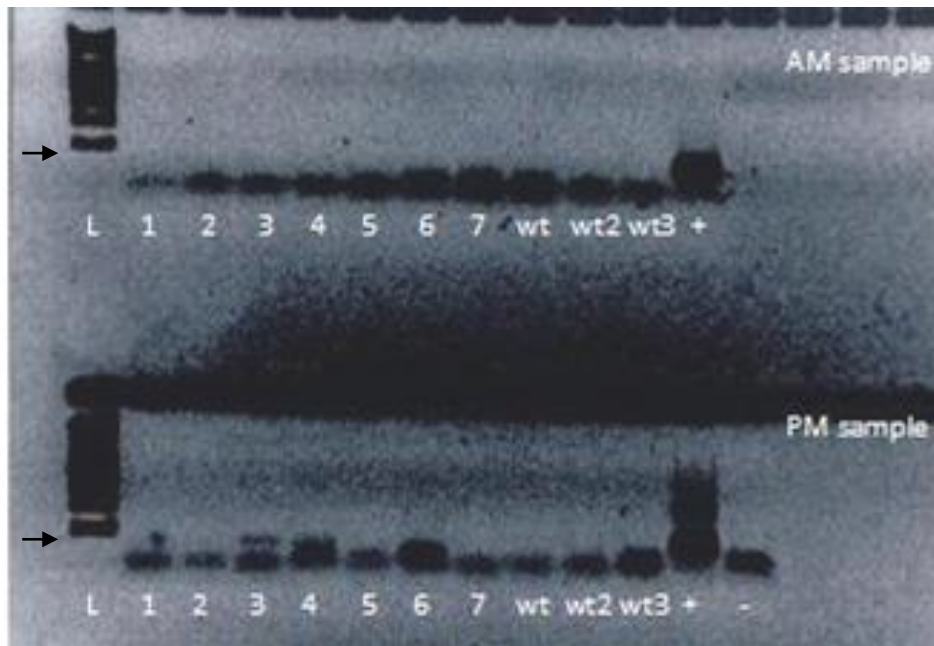
Figure 3.2: *SlNCED1* expression in leaf tissue isolated in the morning (10:00am) or afternoon (2:00pm), as indicated, as determined by RT-PCR. Lanes 1 – 7 represent M1-M7, respectively, with three regenerated wild type controls, as indicated. L represents the molecular mass marker. A distinct positive band was observed in the afternoon sample of line M3. Lines M4 and M6 displayed band signals approximately 50bp smaller than the expected band size. The expected band size is indicated by an arrow.

At this point the use of white clover was stopped as there was only a single successfully expressing transformant that did not appear to have high levels of expression. This would not allow any statistically significant data to be collected, as analysis of a single plant would not allow for the separation of the effect of the introduced *SlNCED1* gene in improving water use efficiency from the genotype into which it was introduced. Ideally, multiple positive transformants would need to have been identified and it would then have been possible to compare these with regenerated non-transformants and wild type controls to separate the effect of the introduced *SlNCED1* gene from the heterogenous genetic background of the Huia cultivar.

It was thus decided that research would shift to a 'proof of concept' focus using tobacco (*Nicotiana tabacum* L.).

3.2 Production and analysis of the transformed *Nicotiana tabacum* lines expressing *SlNCED1*

3.2.1 *Agrobacterium* mediated transformation and tissue culture

Fourteen independent lines of tobacco were initially regenerated from shoots that grew independently from separate pieces of callus using tissue culture. However, this was reduced to eight lines due to poor growth and death of some lines due to fungal contamination on the growth media.

3.2.2 Confirmation of insertion of the *SSUp::SlNCED1* transgene

Confirmation of transformation was performed using genomic PCR. However, the fact that both tomato and tobacco are in the same family (Solanaceae) and thus are predicted to share a high amount of genetic similarity in coding sequences, only the *SSUp::SlNCED1* transcriptional fusion primers were used for screening.

From two batches of leaf cutting, a total of 17 plants from seven lines were available for

use. All lines that survived tissue culture consistently tested positive for transgene

insertion, with the expected 300 bp PCR product present when compared to the wild

type control, and no anomalous band sizes were observed (Table 3.2).

Table 3.2: Positive genomic PCR band results for tobacco (batch) 1 and tobacco (batch) 2.

Tobacco 1	Line.-clone						
Primer	1.-1	1.-2	1.-3	1.-4	2.-1	2.-2	2.-3
SSUp::NCED	+	-	-	+	+	-	-
SSUp::NCED	-	-	-	+	+	-	-
SSUp::NCED	+	-	-	+	+	-	-
Renamed	*NT9			*NT12			

Tobacco 2	Line.-clone									
Primer	1.-1	1.-2	2.-1	2.-1	2.-2	3.-1	7.-1	7.-2	8.-1	12.-1
SSUp::NCED	+	-	-	+	-	-	+	+	-	+
SSUp::NCED	-	-	-	+	-	-	+	+	-	+
SSUp::NCED	-	-	-	+	-	-	+	+	-	+
Renamed				*NT2			*NT5	*NT7		*NT8

* Denotes those lines that survived the transition to soil and were renamed, as indicated.

- Indicates where no bands were observed.

3.2.3 Establishment on soil

A total of twelve plants were transferred to soil. Half of the plants died due to fungal infections during the hardening off period. Six plants survived and managed to establish successfully on soil. These plants were named NT2, NT5, NT7, NT8, NT9 and NT12 (Table 3.2).

3.2.4 Confirmation of expression

To examine the copy number of each line and to verify the number of lines available, Southern blot analysis was carried out (Fig. 3.3). This was performed because some plants originated from the same plate and potentially were collected from tissue descended from the same transformation event. The analysis showed that there were four genetically distinct lines. Of the plants established in soil, NT2, NT5, NT8 and NT9 are genetically distinct from one another. NT7 appears to be derived from the same line as NT5, while NT12 appears to be derived from the same line as NT9, as indicated in Table 3.2.

Confirmation of expression was performed by sqRT-PCR on RNA extracted from healthy leaf tissue collected at 10:00 am to coincide with the expected peak of transgene expression, although the peak expression time of the *SSU* promoter was not investigated directly. The resulting sq RT-PCR product was used to gauge the relative levels of expression of the transgene construct in respect to a constitutively expressed 'house-keeping' gene, *NtEFα1* (Figures 3.4a and b).
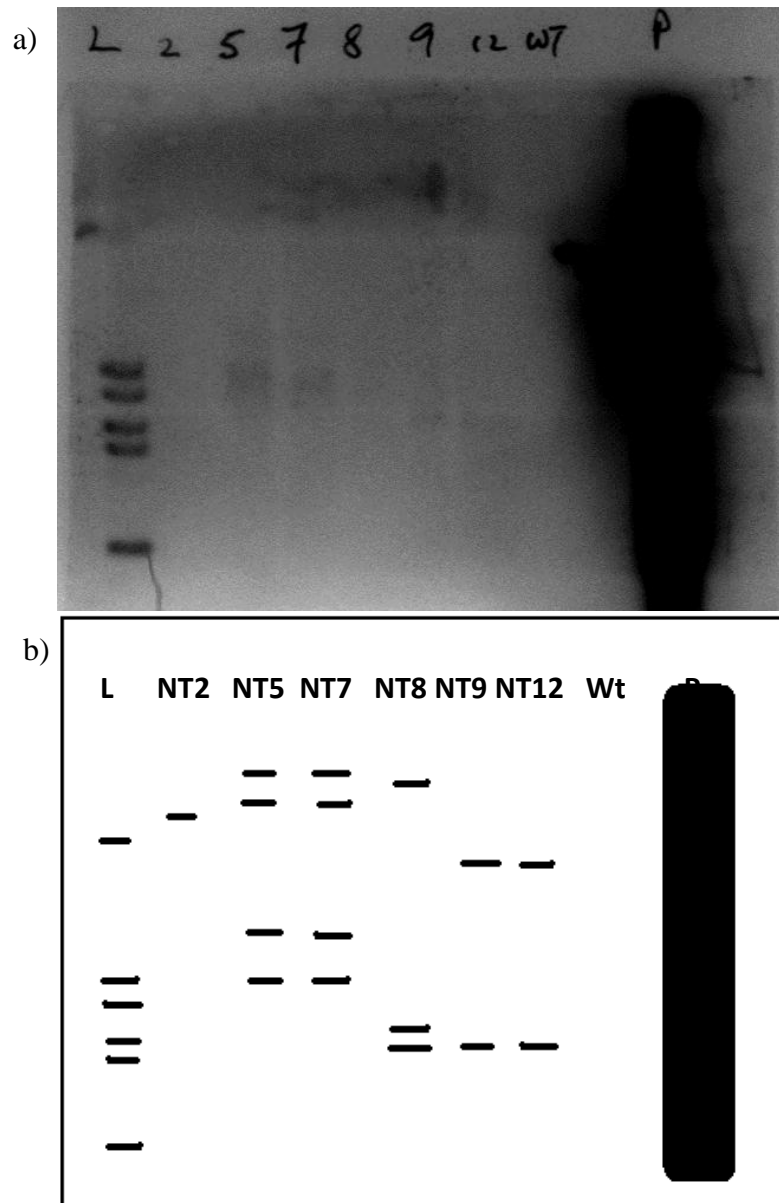
Figure 3.3: a) Southern blot analysis of transgenic T$_0$ tobacco lines, as indicated, established on soil against a wild type (wt) control and a positive control (plasmid containing the *SSUp::SlNCED1* construct). b) Schematic of Southern blot analysis shown in a) displaying all observable bands.

All lines initially tested positive for transgene expression compared to the wild type control, with varying levels of expression inferred due to the intensity of the apparent bands (Figure 3.4a), as a more intense band is expected to be the result of higher levels of transgene mRNA. When the band strength was standardised against $NtEF1\alpha$ (Figure 3.4a) a graphical representation was possible (Figure 3.4b) that allows for ranking of the plants from highest to lowest expressor and gives the following ranking: NT7, NT8, NT5, NT9, NT2, and NT12.
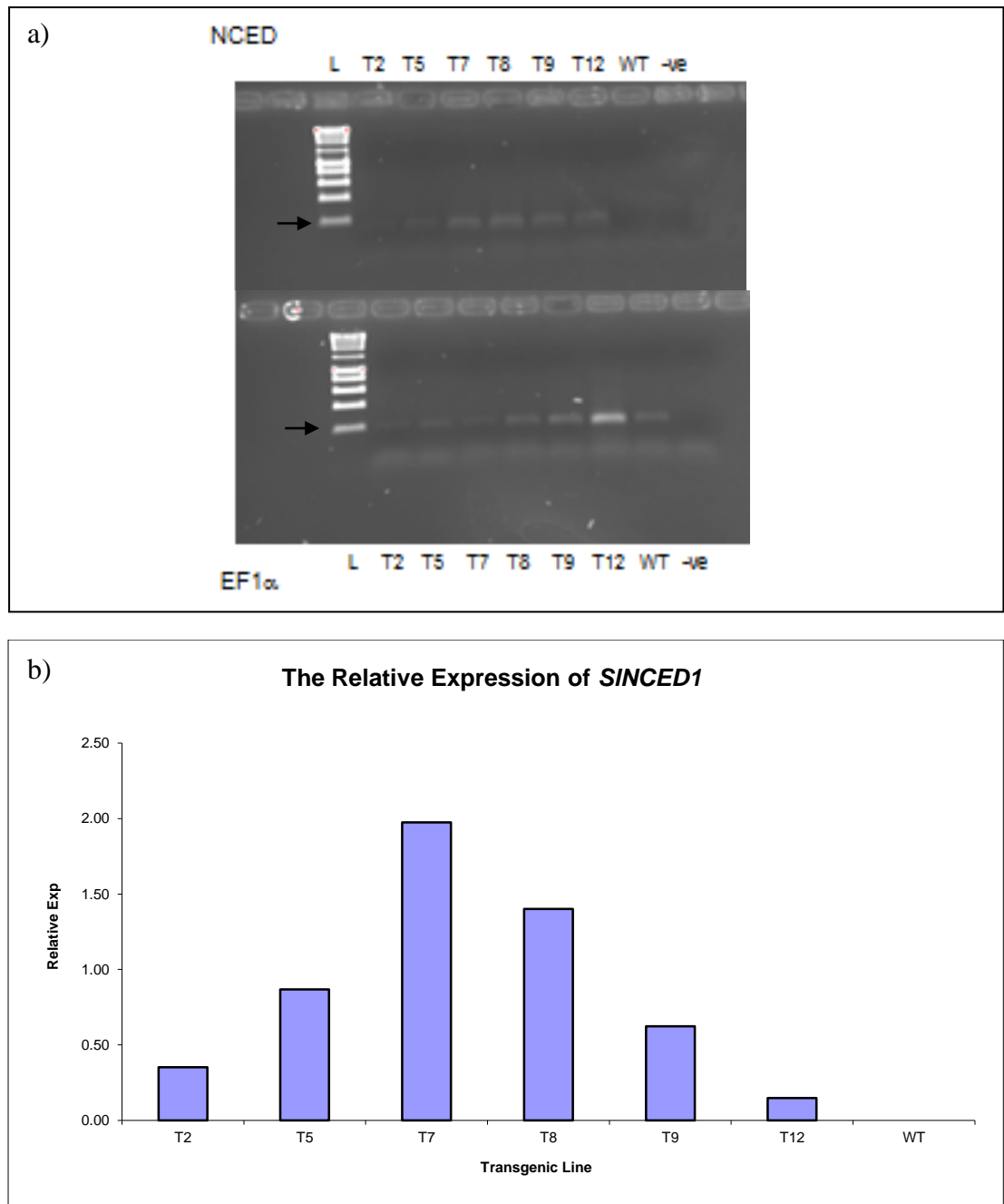
Figure 3.4a, b: a) sqRT-PCR results for *SlNCED1* gene expression in the T$_0$ transgenic tobacco lines, as indicated, when compared to the housekeeping gene *NtEF1α*. The expected band size is indicated by an arrow. b) The relative expression of the inserted *SlNCED* gene construct when compared to the housekeeping gene *NtEF1α*

3.3 Phenotypic analysis of tobacco lines transformed with *SSUp::SlNCED1*

3.3.1 Phenotypic analysis of primary (T_0) transformants

The six surviving primary transformants on soil appeared phenotypically normal,

although detailed growth comparisons with wild-type were not specifically undertaken.

There was no noticeable dwarfing, or chlorosis as observed in previous studies using

tomato (Thompson, *et al*. 2000; Thompson, *et al*. 2008). In these studies, constitutive

overexpression of NCED resulted in leaf margin chlorosis, overguttation and leaf

flooding due to the plants no longer being able to lose sufficient amounts of water to

keep pace with water uptake from the roots.

3.3.2 Germination trials

All six primary transformants (NT2, NT5, NT7, NT8, NT9, and NT12) were allowed to

flower, self-pollinate, and set seed. This seed (T_1 generation) was collected and sown on

media without hormones to determine if any delays in germination could be observed in

any of the seeds harvested from the T_0 transgenic lines.

3.3.2.1 Germination on MS media

When the rate of germination on MS media (designated the control media) of seeds

from the T_0 lines is compared with wild type there is a significant delay of the onset of

germination observed in all lines of the transformants (Figure 3.5). The progeny of the

mid-range expressor NT5 showed the highest germination rate after a month followed

by those of the low expressing line NT2, while the progeny of the lowest expressing

line, NT12, showed the lowest germination rate. In addition to the delay in germination

of all lines, there were also a significant number of seedlings which displayed an

aberrant growth behaviour. The degree of this unusual growth was variable but appeared to display the common feature of arrested growth of the apical meristem. It should be noted that even though NT5 and NT7 have been postulated to have originated from the same callus (on the basis of the Southern analysis; Fig. 3.3), their progeny show a significant degree of difference in terms of their germination responses, particularly compared to the other paired group of NT9 and NT12, the progeny of which show a very similar germination response to each other.

The disparity between putatively genetically identical lines NT5 and NT7 is further illustrated by the time taken to reach 25% and 50% germination (Table 3.3). Line NT5 was able to reach 25% germination by day 17 of sowing, six days ahead of NT7. The difference in germination rate only increased after this point with NT5 reaching 50% germination (on day 20) three days after reaching 25% germination, while NT7 achieved 50% germination (on day 31) eight days after reaching 25% germination (Table 3.3).

Figure 3.5: Mean germination rate for progeny of T_0 transgenic tobacco (T_1 lines), as indicated in relation to a wild type control. Standard deviations are shown for each time point. Three containers, each containing 30 seeds from each parent were sown on MS media containing no hormones or antibiotics. The putative genetically identical T_0 lines NT5, NT7 and NT9, NT12 are boxed.

Table 3.3: Comparative germination rates for progeny of T_0 transgenic tobacco (T_1 lines), as indicated, in relation to a wild type control (WT)showing days to 25% and 50% germination[1].

	WT	NT2	NT5	NT7	NT8	NT9	NT12
Days to 25% germ.	1	17	17	23	16	-	-
Days to 50% germ.	3	24	20	31	-	-	-

[1]Data from Fig. 3.5

3.3.2.2 Germination on gibberellic acid-containing media

To examine the effect of added GA_3 on germination percentage, 90 seeds from each transgenic line were sown on two media types. The first contained 1 mg/L GA_3 (2.89×10^{-6} mol/L) dissolved in absolute ethanol (designated as the GA_3 media) and the second contained 1 mL/L ethanol only (designated as the non-GA_3 media).

When the rate of germination of seed derived from the transgenic lines was compared against wild type there was a very significant delay in the onset of germination (Fig. 3.6a). This delay was longer than that observed for seed on MS media, with delays of up to ten days in order to reach 50% germination observed on GA_3 and non-GA_3 media compared to MS media (compare Table 3.4 with Table 3.3). An increased delay in germination was also observed in wild type seed when compared with germination on MS media (compare Figure 3.5 with 3.6a,b). This observed delay was more prominent on non- GA_3 media, with the overall rate of germination lagging behind that of the GA_3 containing media (Figure 3.6a,b and Table 3.4). However, after this initial delay in germination, the general trend was similar across all media types in that seed derived from plant NT5 had the highest germination rate, after the wild type control, followed by seed derived from NT2. Seed originating from NT7 and NT8 consistently clustered at a final germination rate between 30% and 50%. Seed originating from NT9 and NT12 consistently clustered between 5% and 15% for their final germination rate.
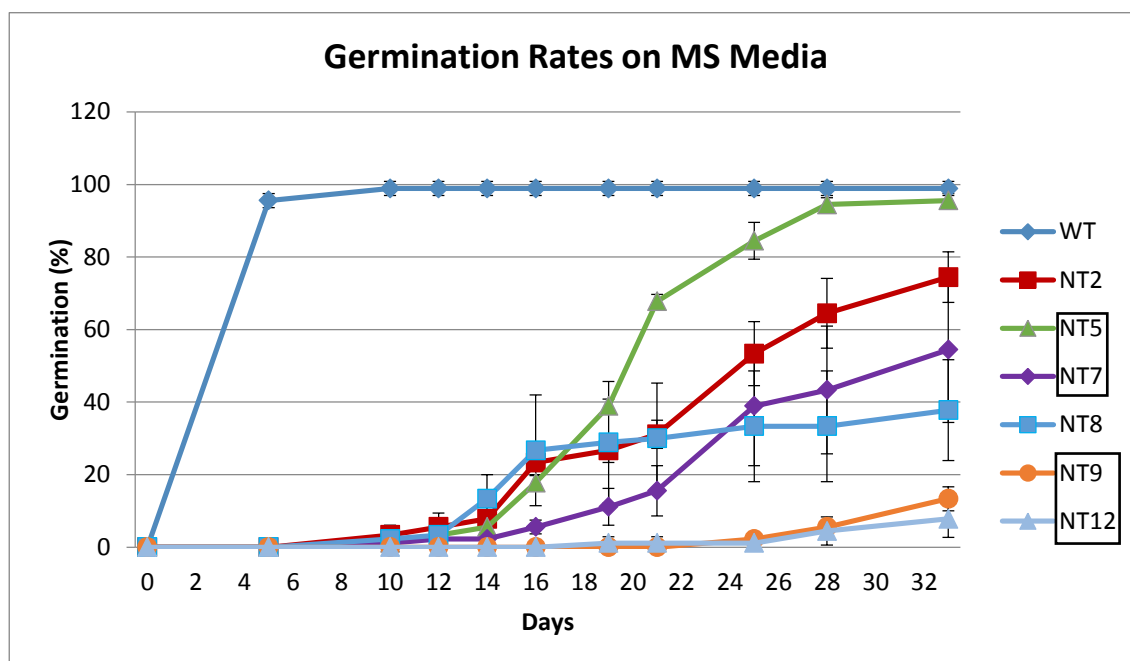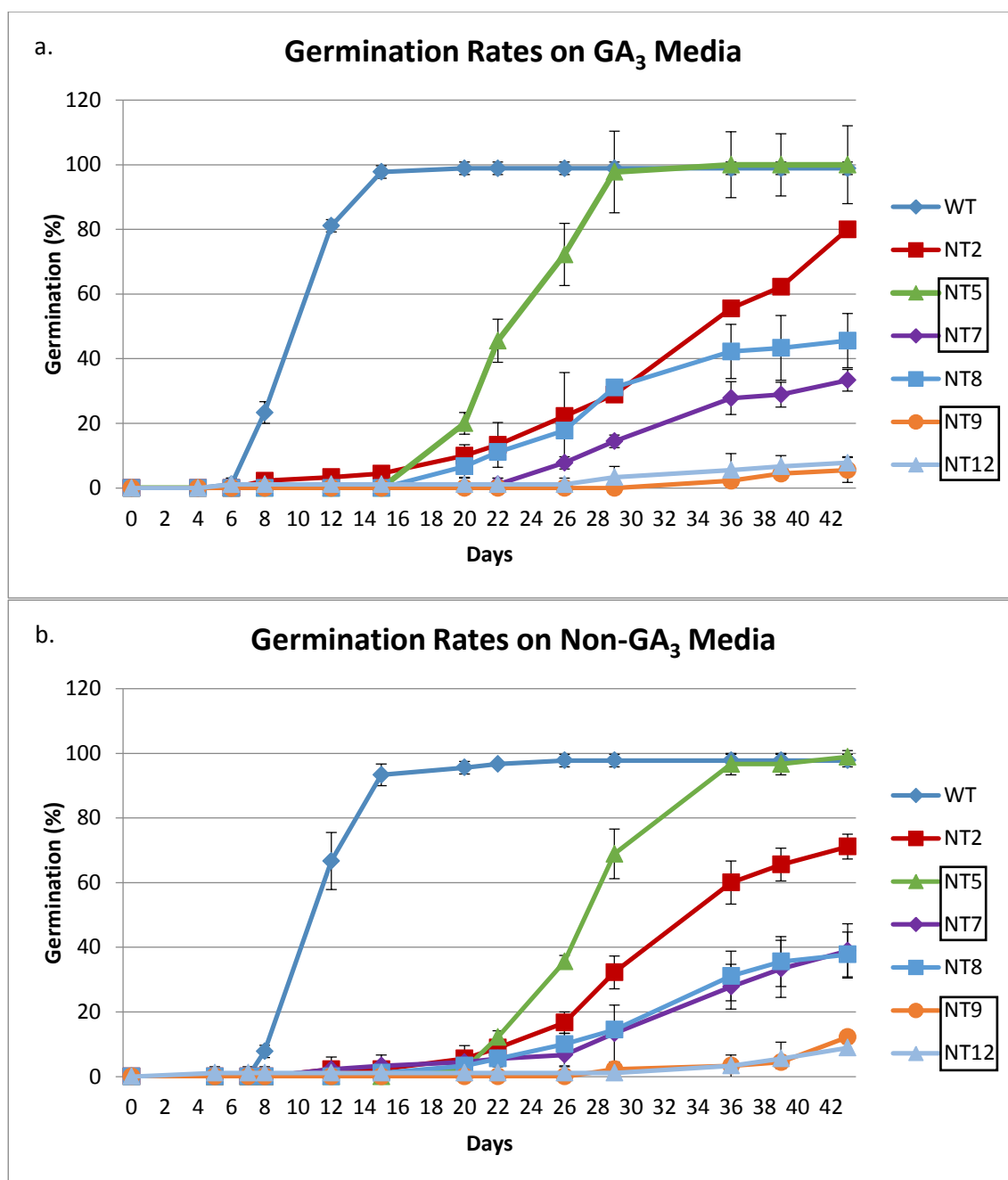
Figure 3.6a, b: Mean germination rate for progeny of T_0 transgenic tobacco (T_1), as indicated, in relation to a wild type control. Standard deviations are shown for each time point a) gibberellic acid media containing 1 mL L^{-1} ethanol) and, b) non-GA$_3$ media containing 1 mL L^{-1} ethanol. The putatively genetically identical T_0 lines NT5, NT7 and NT9, NT12 are boxed.

Table 3.4: Summary of germination rates for progeny of T_0 transgenic tobacco (T_1 lines), as indicated, in relation to a wild type control (WT) with a total of 90 seeds germinated on GA_3 media and non-GA_3. Failure to reach 25% or 50% germination is marked by a dash[1].

GA_3 media	WT	NT2	NT5	NT7	NT8	NT9	NT12
Days to 25% germ.	8	27	20	35	27	-	-
Days to 50% germ.	10	34	23	-	-	-	-
non-GA_3 media	WT	NT2	NT5	NT7	NT8	NT9	NT12
Days to 25% germ.	9	28	24	35	33	-	-
Days to 50% germ.	11	34	27	-	-	-	-

[1]Data is from Fig. 3.6

Germination on media containing GA_3 reduced the time to reach 25% germination by 2.4 days on average across all lines that managed to reach 25% germination when compared with media that contained ethanol alone. There was an increase in the time taken to reach 25% germination of 8.6 days on average on GA_3 media compared to MS media (compare Table 3.4 with Table 3.3), with a further increase to 11 days on non-GA_3 media. This may be attributed to the potentially poisoning effect of ethanol delaying germination. Line NT5 displayed the most resistance to ethanol delayed germination with the lowest increase in time to reach 25% germination, with three days on GA_3 media and seven days on non-GA_3 media.

3.3.3 Description of aberrant seedlings

3.3.3.1 Aberration on MS media

After examination of a total of 350 T_1 seedlings from all transgenic lines (167 of which displayed abnormal growth), the morphology of the aberrant seedlings was grouped into three types.

   Mildly aberrant plants with normal cotyledons and in most cases, the presence of shoots, that then failed to develop beyond this point, were termed "Type 1 aberrants" (Fig. 3.7b and c; wild type shown as Fig. 3.7a). However, the most commonly observed aberrant growth pattern was an elongated hypocotyl shoot apparently lacking in cotyledons accompanied by a failure to shed the seed coat. These plants were termed "Type 2 aberrants" (Fig. 3.7d). It was later observed, using a dissecting microscope, that these plants did in fact have undeveloped cotyledons which had failed to expand and cause shedding of the seed coat. This served to hide the cotyledons from all but histological investigation or direct removal of the seed coat. A third class of aberrant was also observed that displayed the typical under-developed cotyledon aberrant growth

habit plus a swollen hypocotyl and a section of enlarged, callus-like tissue on the lower hypocotyl just above the roots (Figures 3.7e,f). Often these plants also displayed abnormal root growth, such as swollen, callus-like protrusions on the roots, combined with stunted growth and chlorophyll bleaching. These were termed "Type 3 aberrants" (Fig. 3.7e,f).
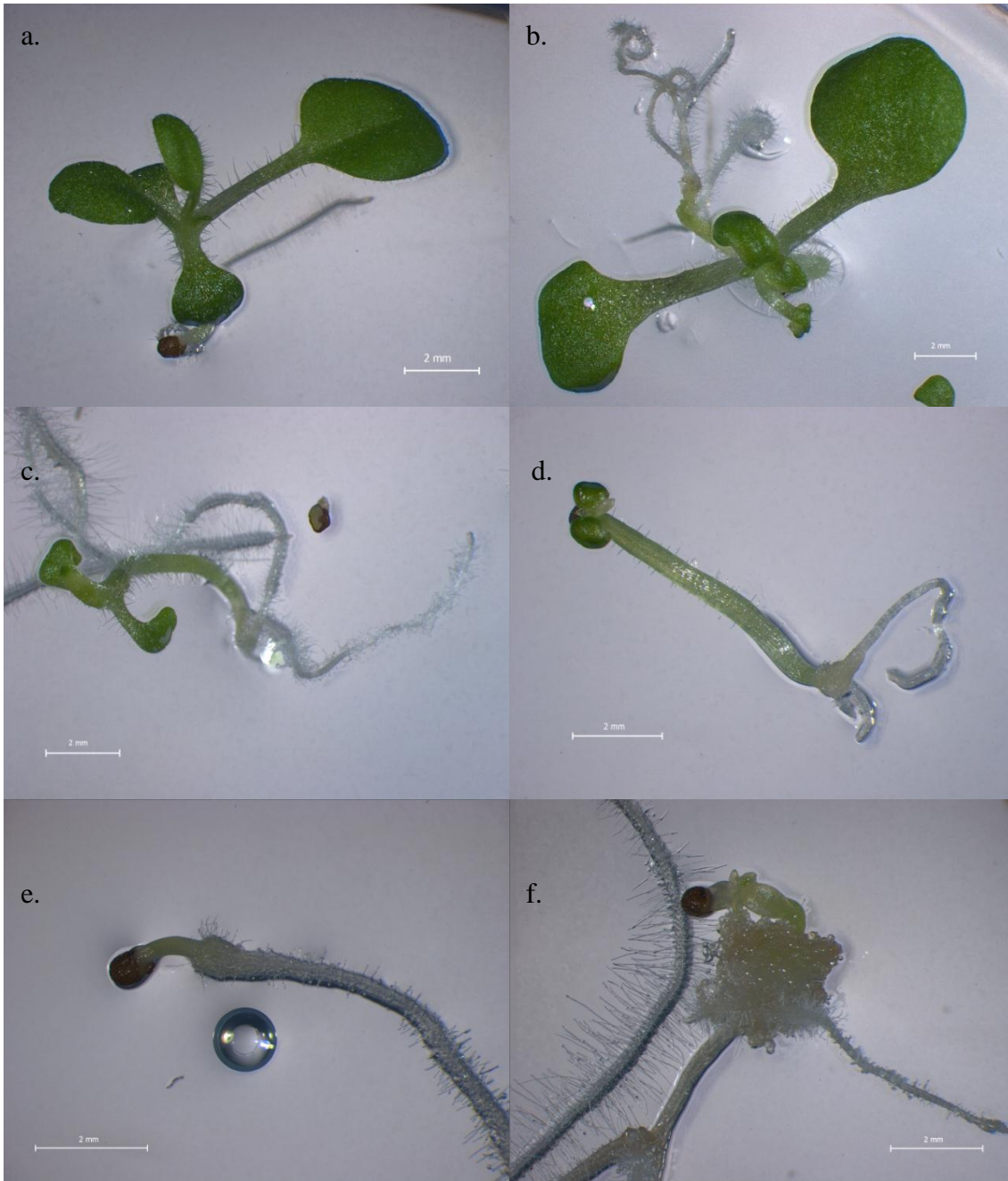
Figure 3.7: Examples of the three types of aberrant growth behaviour observed in T$_1$ seedlings a) wild type control; b) Mild Type 1 aberrant; c) Type 1 aberrant; d) Type 2 aberrant; e) Type 3 aberrant; f) Severe Type 3 aberrant.

The rate of aberrant growth and the severity of the abnormality varied with each line (Fig. 3.8). Line NT5 had the highest overall aberration rate at 97.7% of total germination, while NT2 had a 0% aberration rate, similar to the wild type control (Figure 3.8). When the total number of aberrants for each line was compared, the lines ranked from highest to lowest as: NT5, NT7, NT12, NT9, NT8, NT2 and then wild type. However, this did not take into account the severity of aberration present in some lines. Thus when each type of aberrant was weighted so that type three was given more emphasis than type two, and type two was given more emphasis than type one, i.e. [(proportion as type 1 x 1) + (proportion as type 2 x 2) + (proportion as type 3 x 3)], then a ranking of NT12, NT8, NT7, NT9, NT5, NT2 was obtained. It is interesting to note that this ranking closely resembles the ranking for germination rate when it is listed from lowest to highest, i.e. NT12, NT9, NT8, NT7, NT2, NT5, and wild type. Only the positions of NT2 and NT9 prevent a perfect match.

3.3.3.2 Aberration on gibberellic acid-containing media

The aberration rate for both GA_3 containing and non-GA_3 media was similar to that observed on MS media. Seed derived from NT2 had the lowest aberration count, although there were now individual plants observed that were aberrant in their growth habit when germinated on either GA_3 containing media or non-GA_3 media (Fig. 3.9), in contrast with no aberrant plants observed in NT2 derived seed germinated on MS media (Fig. 3.8). Seed harvested from NT5 had the highest proportion of aberrant germination, and once again contained the largest proportion of type 1 aberrants. NT7 seed contained the largest fraction of type 2 aberrants. Seed harvested from NT8 had the second highest proportion of type 3 aberrants and of these, this line consistently generated the most severe forms of type 3 aberrants based on visual scoring. NT8 seed also contained the

Figure 3.8: Aberration rate and type of aberration for progeny of each tobacco T_0 line, as indicated, compared to a wild type control when sown on MS media. The term uncountable refers to any freshly germinated seedlings that were unable to be assessed for aberration.

Table 3.5: Numerical summary of aberration rate for seed collected from each tobacco T_0 line, as indicated, in relation to a wild type control when sown on MS media. Absolute numbers of aberrant seedlings and percentage of total germination are both shown (data from Fig. 3.8).

MS media	Wt		NT2		NT5		NT7		NT8		NT9		NT12	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%
#	89		67		87		50		34		14		9	
normal	89	100	67	100	-	0	3	6	10	29.4	-	0	-	0
Type 1 Aberrant	-	0	-	0	48	55.2	11	22	1	2.9	1	7.1	-	0
Type 2 Aberrant	-	0	-	0	34	39.1	27	54	6	17.6	7	50	3	33.3
Type 3 Aberrant	-	0	-	0	3	3.4	5	10	15	44.1	2	14.3	4	44.4
uncountable	-	0	-	0	2	2.3	4	8	2	5.9	4	28.6	2	22.2

second highest fraction of seedlings with a normal growth habit, after NT2. Seed obtained from plants NT9 and NT12 each had very high proportions of aberrant growth, though due to the extremely low germination rates of both of these lines any results based upon them are unreliable (Fig. 3.5).

With the differences in media there were some variations in the proportions of growth habit. However, overall the severity of aberration was reduced in most lines on media containing GA_3 (Fig. 3.9a) and there was a greater proportion of plants observed growing with a wild type growth habit on GA_3 enhanced media when compared with MS media (compare Table 3.6 with Table 3.5). The fraction of uncountable plants was considerably increased on non-GA_3 media (Fig. 3.9b) compared to all other media types.
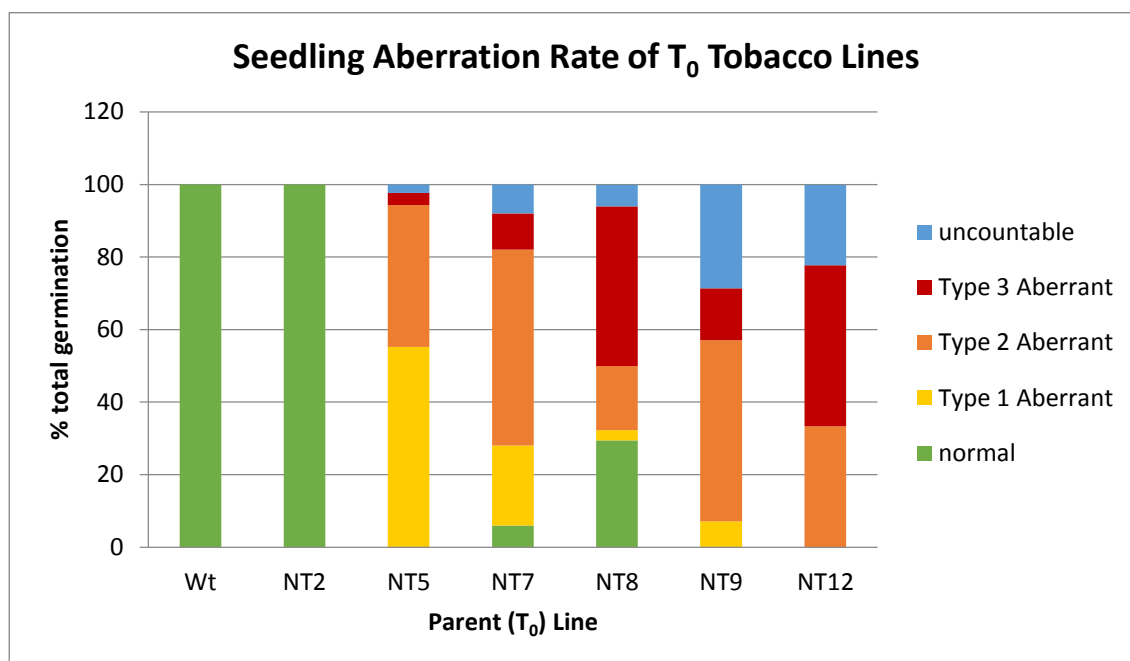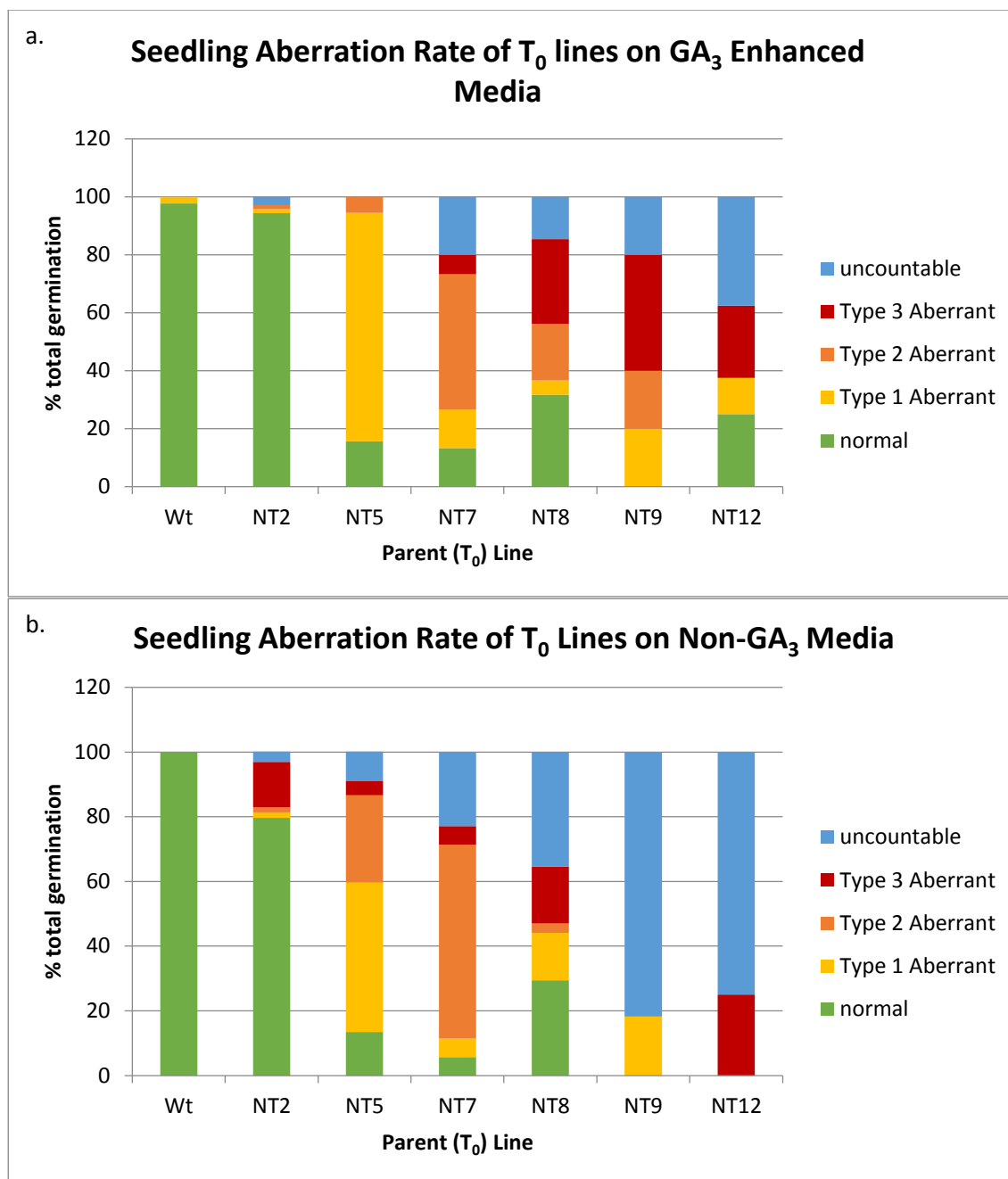
Figure 3.9a, b: Aberration rate and type of aberration for progeny of each tobacco T$_0$ line, as indicated, compared to a wild type control germinated on; a) gibberellic acid enhanced media (containing 1 mL L^{-1} ethanol) and, b) media containing 1 mL L^{-1} ethanol. The term uncountable refers to any freshly germinated seedlings that were unable to be assessed for aberration.

Table 3.6: Numerical summary of aberration rate for seed collected from tobacco T_0 lines in relation to a wild type control when sown on GA_3 media and non-GA_3 media respectively. Absolute numbers of aberrant seedlings and percentage of total germination are both shown (data from Fig. 3.9).

GA_3. media	Wt		NT2		NT5		NT7		NT8		NT9		NT12	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%
#	89		72		90		30		41		5		8	
normal	87	97.8	68	94.4	14	15.6	4	13.3	13	31.7	-	0	2	25
Type 1 Aberrant	2	2.2	1	1.4	71	78.9	4	13.3	2	4.9	1	20	1	12.5
Type 2 Aberrant	-	0	1	1.4	5	5.6	14	46.7	8	19.5	1	20	-	0
Type 3 Aberrant	-	0	-	0	-	0	2	6.7	12	29.3	2	40	2	25
uncountable	-	0	2	2.8	-	0	6	20	6	14.6	1	20	3	37.5

non-GA_3. media	Wt		NT2		NT5		NT7		NT8		NT9		NT12	
	#	%	#	%	#	%	#	%	#	%	#	%	#	%
#	88		64		89		35		34		11		8	
normal	88	100	51	79.7	12	13.5	2	5.7	10	29.4	-	0	-	0
Type 1 Aberrant	-	0	1	1.6	41	46.1	2	5.7	5	14.7	2	18.2	-	0
Type 2 Aberrant	-	0	1	1.6	24	27	21	60	1	2.9	-	0	-	0
Type 3 Aberrant	-	0	9	14.1	4	4.5	2	5.7	6	17.6	-	0	2	25
uncountable	-	0	2	3.1	8	9	8	22.9	12	35.3	9	81.8	6	75

3.3.4 Preliminary observations of the anatomy of aberrant seedlings

Sections (10 µm) of types 1 and 2 aberrants as well as phenotypically normal seedlings, were examined (Fig. 3.10). Sections were selected for clarity and for structural characteristics. A comparison between the wild type growth habit sections and the type 1 aberrants shows the cellular organisation of the stem tissue cells appears to be disorganised in the aberrants. Development of leaves in type 1 aberrants appears perturbed, as does the development of vasculature tissue (Fig. 3.10a). Type 2 aberrants appear to have no functional apical meristem and resemble newly germinated seedlings in their morphological characteristics (Fig. 3.10b).
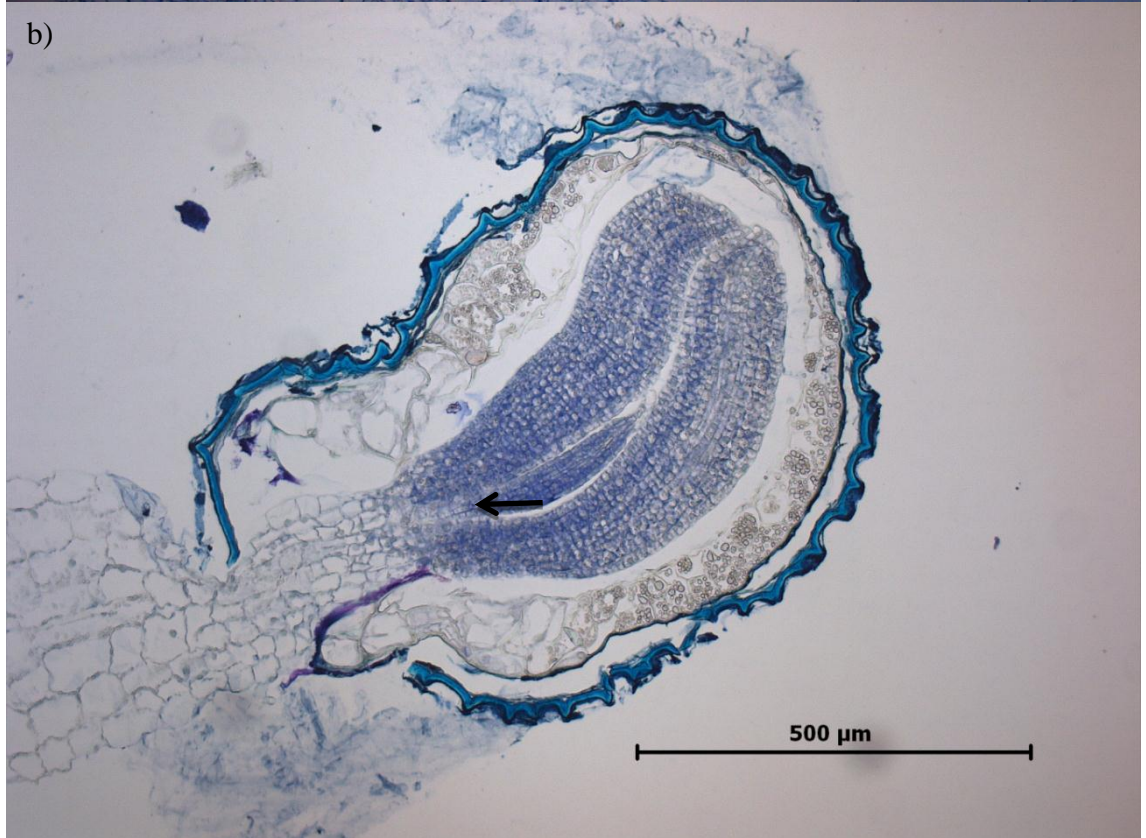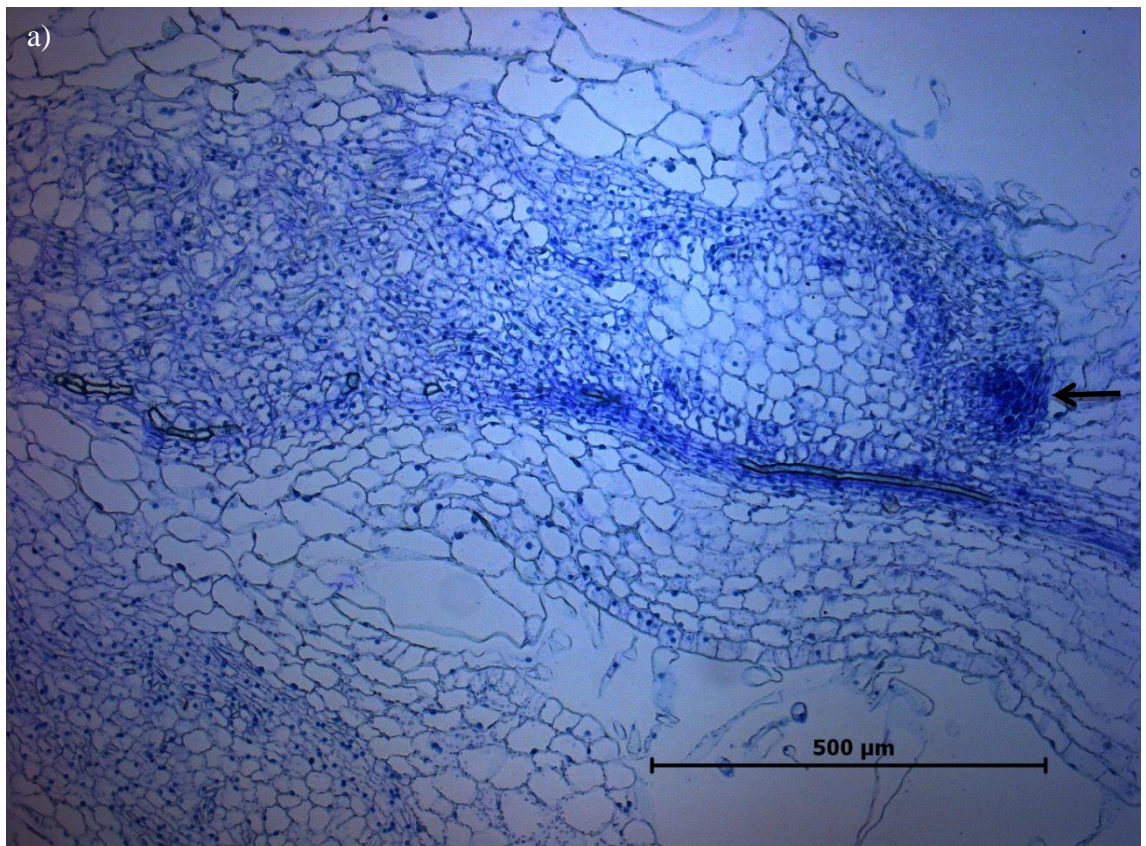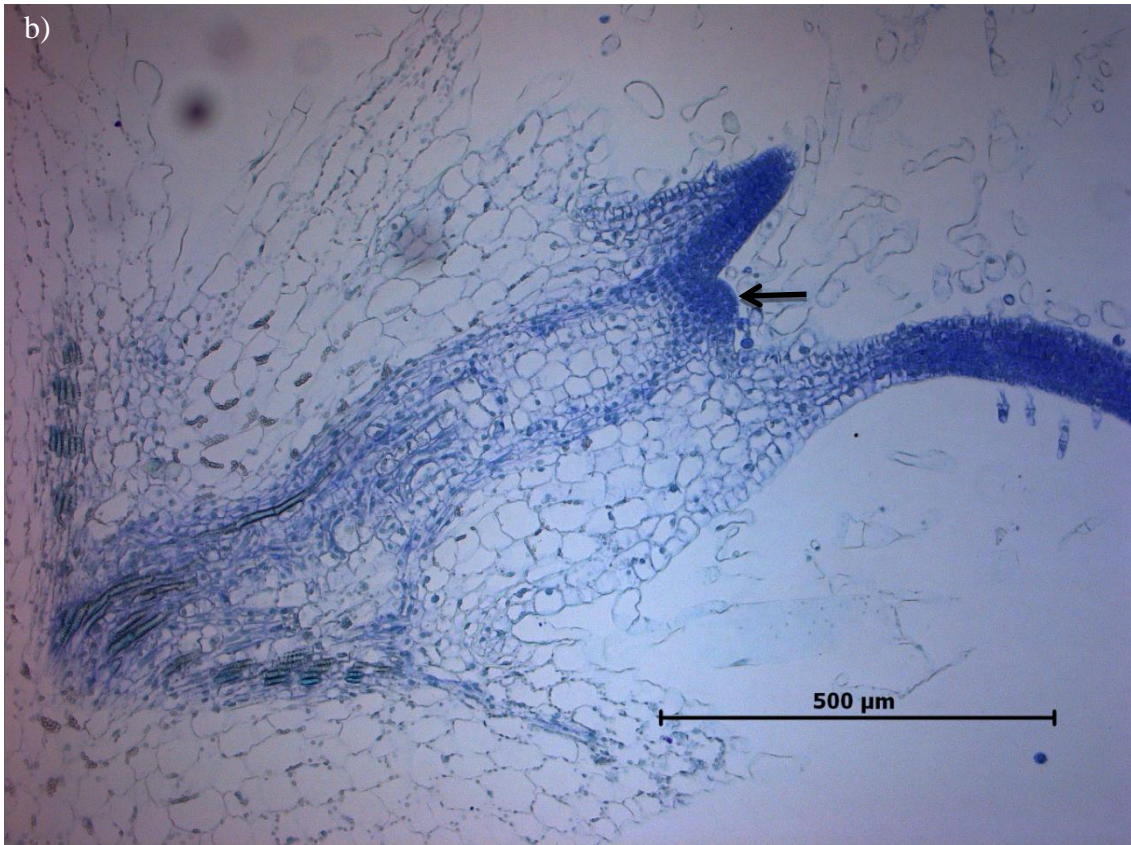
Figure 3.10 a, b, c: Sections (10 µm) of seedlings from a) type 1 aberrants; b) type 2 aberrants and; c) a wild type growth habit seedling prepared and stained using toluene blue stain. The location of the apical meristem is indicated with an arrow.

3.3.5 Expression of *SlNCED1* in aberrant seedlings

RNA was also extracted from bulked tissue samples made of aberrant T_1 progeny derived from T_0 lines NT5, NT7 and NT8, as well as bulked tissue from non-aberrant progeny derived from T_0 line NT2. Semi-quantitative PCR was performed on the RNA extracted from these tissues, using the housekeeping gene *NtEF1α* as a reference gene and the relative expression of *SlNCED1* in each line calculated as a ratio of the accumulation of *SlNCED1* against the accumulation of *NtEF1α* for each RNA sample. When the relative expression of *SlNCED1* was plotted for NT5 type 1 aberrants, NT7 type 2 aberrants, NT8 type 2 aberrants and NT2 plants with a wild type growth habit, there was lower levels of relative expression (under 1.0) observed in all samples with little difference observed between the type 2 aberrants and plants growing with a wild type growth habit (Figure 3.11). Type 1 aberrants had the lowest level of expression relative to *NtEF1α*, and this was lower than that observed for bulked seedlings with a wild type growth habit. It should be noted that all seedlings prior to collection were maintained on MS media containing sucrose levels that would inhibit the activity of the *SSU* promoter, and at light levels that may further reduce promoter activity. Because of these results, it cannot be stated with any certainty that post-germination expression of *SlNCED1* was responsible for the aberrant germination and growth of seedlings observed in all lines. Rather, expression of the transgene in the T_0 lines during embryogenesis and seed development may have contributed to the aberrant growth habits.
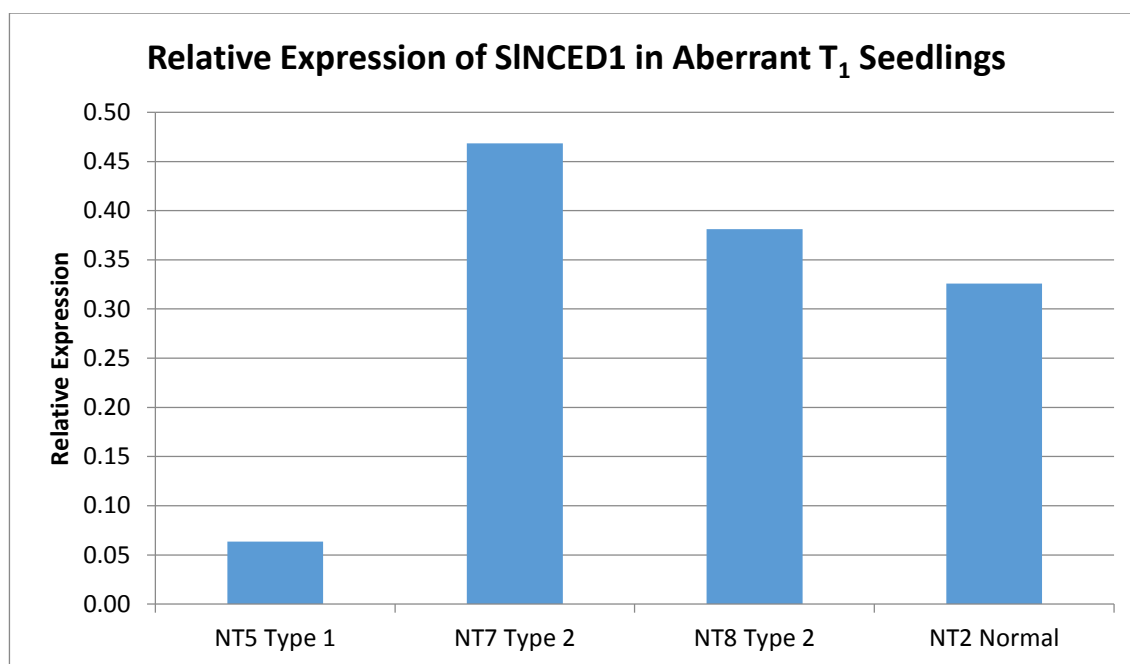
Figure 3.11: Relative expression of the *SlNCED1* gene in bulked T$_1$ aberrant seedlings, as indicated, compared to phenotypically normal T$_1$ seedlings obtained from line NT2, as indicated. Relative expression is determined against the constitutive expression of *NtEFα1*.

3.3.6 Evaluation of water use efficiency of *SlNCED1* transformed tobacco

3.3.6.1 Trial one

Seedlings germinated from each T_0 line that appeared to be phenotypically normal (5 derived from each of the parent plants NT2, NT7, NT8, NT9, NT12 and wild type) were established on soil with one plant per pot (3x3x5cm capacity). Each pot was weighed at field capacity (FC; defined here as the amount of water required to cause liquid water to leak from the base of the pot), before being deprived of water. At 24 hour intervals, each pot was rewatered to FC and the amount of water required to do so recorded (FCW; the weight of water required to reach field capacity). Growth of the seedling was also recorded by measuring each plant across the widest point (leaf tip to opposite leaf tip) as well as the plant height (soil to apex).

When plant water use was plotted against mean changes in plant width, or plant height, differences in apparent water use efficiency were observed (Fig. 3.12a,b). A plant with an apparent high water use efficiency is defined as one in which a minimal amount of water is expended in order to achieve a higher growth rate. Plants that exhibited this characteristic were termed 'efficient', while a plant that expended a large amount of water for a low growth rate was determined to have a low water use efficiency, and these plants were termed as 'poor'. Of those in the trial, four plants were identified which can be considered efficient users of available water (T2-5, T8-3, T9-1, and T12-1), nine plants as average users of water (C-3, T2-3, T7-2, T7-4, T7-5, T8-4, T9-3, T9-5, and T12-3), seven plants which can be considered poor users of the available water (C-2, C-4, T2-1, T7-1, T8-1, T9-2, and T12-5), and ten plants that fall between the two extremes (Figure 3.12a). When plant water use was plotted against change in plant height, six plants were identified which can be considered 'efficient' users of available water (C-5, T2-5, T7-3, T8-3, T9-4, and T12-1), five plants as

'average' users of water (T2-3, T7-4, T7-5, T9-3, and T9-5), six plants as 'poor' users of the available water (C-2, T7-1, T8-1, T8-4, T12-4, and T12-5), and thirteen plants that fall between the two extremes (Figure 3.12b).

Plotting plant water use against plant width generated a trend line with an R^2 value of 0.654 (Fig. 3.12a), compared with an R^2 value of 0.343 (Fig. 3.12b) when water use was plotted against plant height. When these efficient, average and poor water use efficient plants were plotted on the plant width against water use graph (Fig. 3.12a), plants designated as efficient cluster above the trend line at the lower end of water use, those selected as average clustered on the trend line and while those designated as poor clustered below or near to the upper end of the trend line. The efficient, average and poor water use efficient plants were as plotted on the plant height against water use graph (Fig. 3.12b). Again, plants selected as efficient were observed to spread out above the trend line, those designated as average clustered on the trend line and those designated as poor formed a loose group at the upper end of the trend line, but consistently near or below it.

As this was a trial to determine the best methods for establishing, maintaining, and measuring seedlings, and the small number of plants used would have hindered statistical analysis, no investigation of *NCED1* expression was carried out. However, this first trial did serve as a preliminary experiment to determine how best to assess differences in water use efficiency, and it was of interest to note that three of the genotypes identified as efficient using plant width (T2-5, T8-3, T12-1) were also deemed efficient when plant height was used as a parameter.
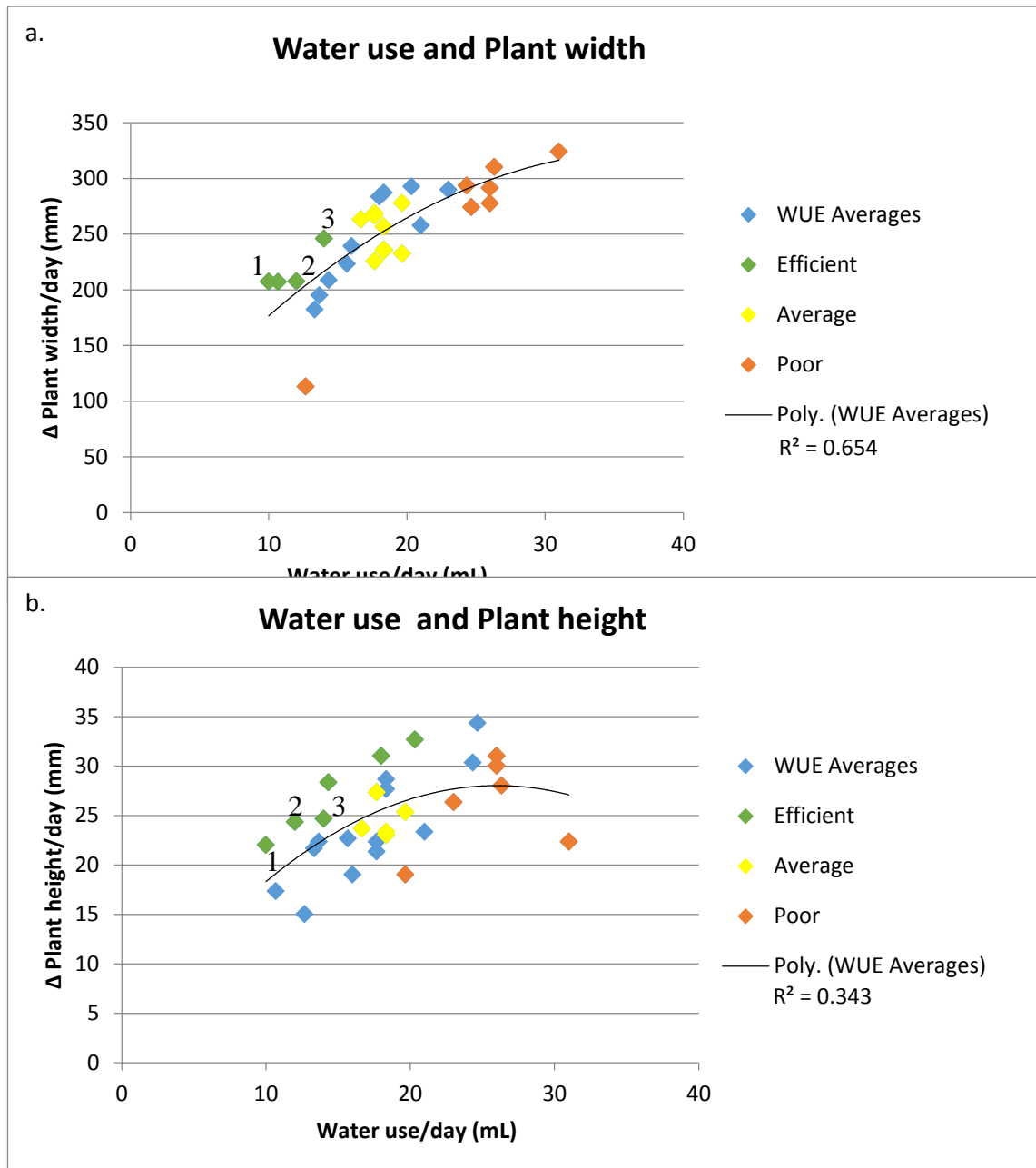
Figure 3.12a, b: Mean changes in plant width (a) and plant height (b) against water use per day for transgenic T_1 seedlings of tobacco. Plants that ranked as 'efficient' using both methods have been marked and designated as 1) T2-5, 2) T8-3, and 3) T12-1.

3.3.6.2 Trial two

Phenotypically normal seedlings germinated from each line (21 derived from the parent plant NT5, 16 derived from NT8, 10 derived from NT12 and 7 wild type plants) were each maintained on soil within a 50 mL falcon tube with a pin-hole in the base to allow for water drainage. Each plant was trimmed to remove all but four of the most recently emerged leaves longer than 10 mm. Each tube was watered to FC and weighed. At 24 hour intervals, each tube was rewatered to FCW and the amount of water required to do so recorded. At the same time, all leaves on all plants were measured for leaf length along the midvein and leaf width at the widest point. As an initial comparison, mean daily leaf growth (mm^2) was plotted against the average daily water use (mL) (Appendix Fig. A5.3.1). To do this, the mean average growth of length and width of each leaf per plant were independently multiplied before being added together to generate the total average growth.

Next, total leaf growth for all four days of experimentation (mm^2) was plotted against the total amount of water used during the experimental period (mL) (Appendix Fig. A5.3.2). In order to generate this, the growth of leaf length and width for all four days of experimentation were added independently before the length was multiplied by width for each individual leaf. At this point the values for each leaf were added together to generate the final value for total leaf growth.

Total mean daily leaf growth (mm^2/day) for all four leaves for all four days of experimentation was plotted against the average daily water use (mL/day) (Appendix Fig. A5.3.3). In order to generate this value, the average growth of leaf length and width were independently added across all four leaves before the value for length was multiplied by the value for width. This was done to eliminate any negative growth values that may have been increased unrealistically by multiplication of the values.

Finally the average daily leaf growth of the second emerged leaf (mm^2/day) was plotted against the average daily water use (mL) (Fig. 3.13). This was selected for further use as the determinant on whether a plant was deemed 'efficient', 'average' or 'poor' in terms of its water use due to its simplicity. The method of its construction used the least number of variables, and thus limited the number of confounding principles that would increase the amount of error.

Plants that were separate from the centralised cluster were considered to be of 'efficient' or 'poor' water use status. Those plants that were the greatest distance above the trend line were marked as efficient (green; 7 genotypes), and were clustered into two groups designated as groups A and B. Group A contains the efficient plants WC-3 and W5-22, while group B contains the efficient plants W8-9, W5-15, W5-18, W5-3 and W5-19. Those plants that were the greatest distance below the trend line, but above negative relative growth, were marked as poor (orange; 5 genotypes) users of water. Plants that clustered closest to the trend line were selected as average users of water (Figure 3.13). All plants selected as efficient, average or poor users of water were then used in a transpiration assay.
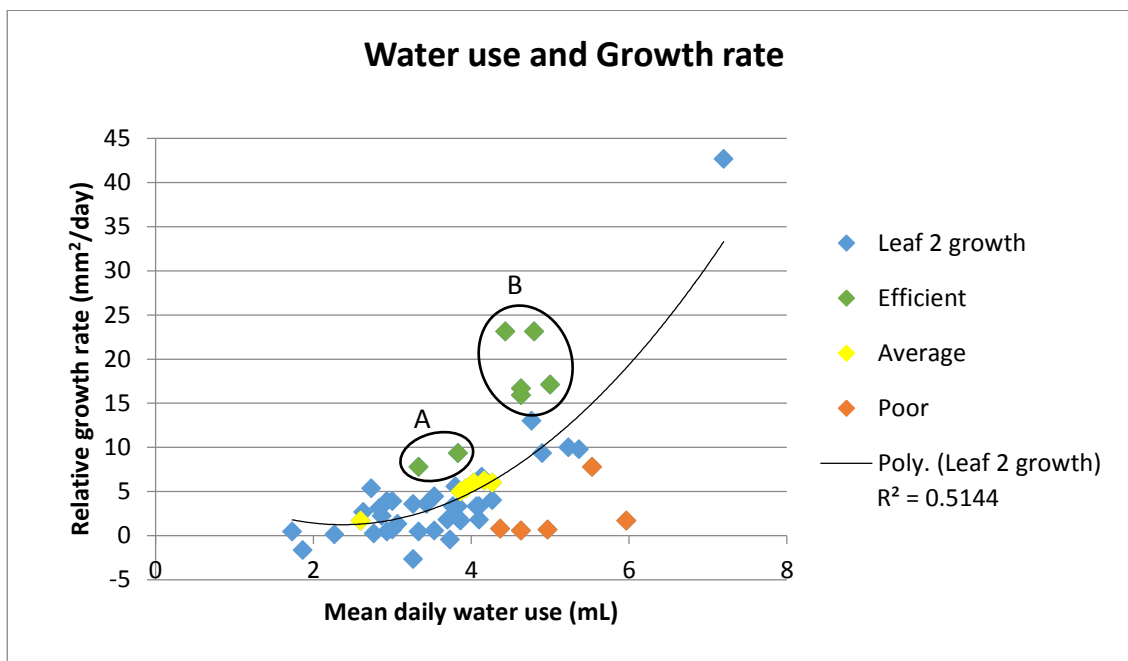
Figure 3.13: Change in the rate of extension of the second emerged leaf against mean daily water use of transgenic T_1 lines.

3.3.6.3 Transpiration assay

The relative transpiration rate (RTR), expressed as g H_2O loss/min/g leaf tissue, was plotted against plant genotype and analysed for efficient, average or poor water use (Fig. 3.14). No clear clustering of classes of water use efficiency (determined in Fig. 3.13) with transpiration rate could be observed.

This may have been caused by a light intensity that was insufficient to drive maximum transpiration as a trade-off was necessary due to the heat generated by the lamps used. The plants from the efficient A group displayed the highest transpiration rates of either efficient group despite being the group with the lowest mean daily water use (refer Fig. 3.13).

However, when the transpiration rate of each genotype was plotted against the relative growth rate (mm^2/day) observed in the water use efficiency trial (Section 3.6.2) it was observed that plants previously designated as efficient (Fig. 3.13) had a higher growth rate for the same transpiration rate and that this growth rate was consistently higher than plants from both the average and poor water use groups (Fig. 3.15). Once again, the efficient plants clustered into two groups comprised of the same plants as observed previously in Figure 3.13.
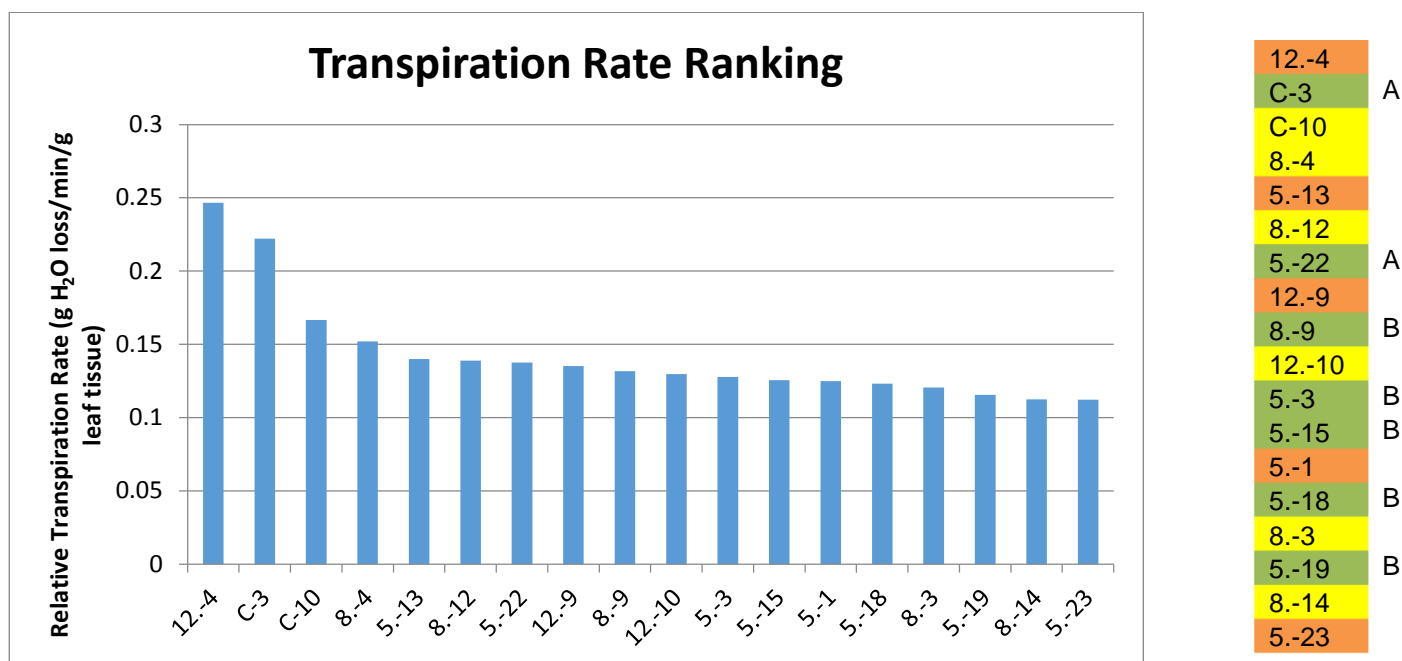
Figure 3.14: Relative transpiration rate of all T_1 transgenic plants tested stacked from highest to lowest, with what class each plant is derived from. The proposed efficiency designation that each of the plants clustered into is also displayed (right hand panel; data from Fig. 3.12; green shading = efficient; yellow shading = average; orange shading = poor). Plants in Group A and B (section 3.3.6.2) are indicated.
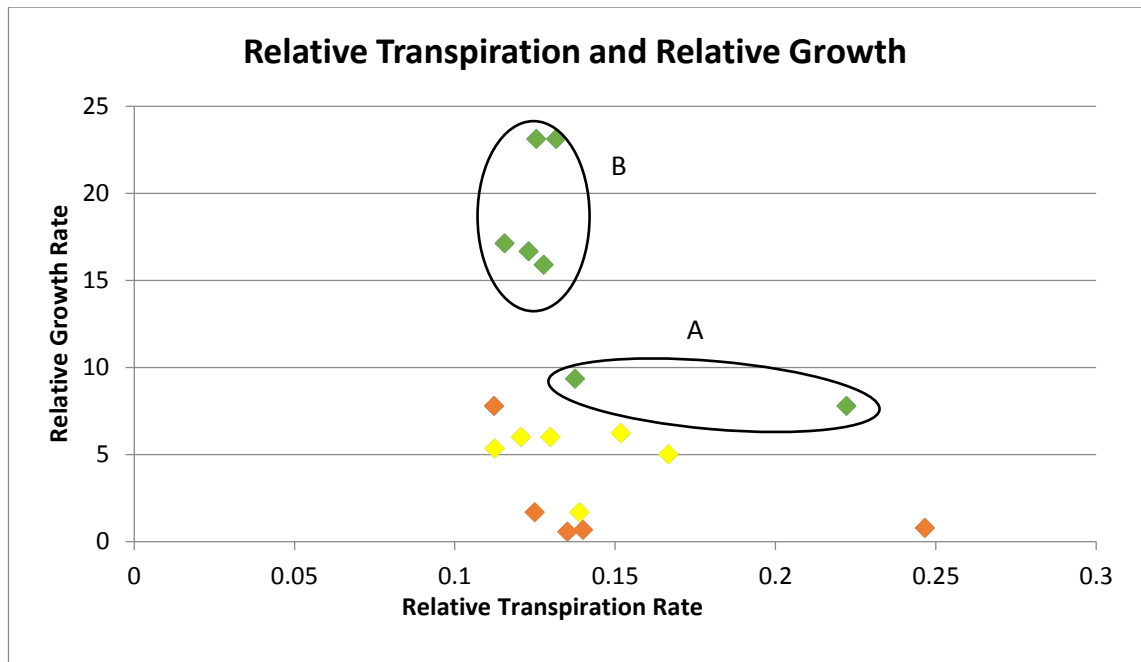
Figure 3.15: Relative transpiration compared with relative growth for the T_1 transgenic plants classified as efficient, average or poor users of available water. Efficient plant cluster groups A and B are shown.

3.3.7 Expression of *SlNCED1* in T_1 genotypes

RNA was extracted from a total of nine T_1 plants identified during the second water use

efficiency trial; three each from the water use efficient, average, and poor groups

[Efficient: 5-15, 5-18, 8-9 (Group B); Average: 8-3, 8-4, 12-10; Poor: 5-1, 5-13, 12-9;

Fig. 3.13]. Semi-quantitative PCR was performed on the RNA extracted from these

tissues, using the housekeeping gene *NtEF1α* as a reference gene and the relative

expression of *SlNCED1* in each line calculated as a ratio of the accumulation of

SlNCED1 against the accumulation of *NtEF1α* for each RNA sample.

When the relative expression of *SlNCED1* was plotted against individual plants that

had previously been determined to be efficient, average and poor in their use of water,

no relationship was observed between expression of the gene and water use efficiency

(Figure 3.16). No expression was observed for any of the plants designated as average

users of water, the plants designated as poor users of water were the highest, third

highest, and lowest expressers of the transgene, while those plants designated as

efficient had the second highest expressor, the second lowest expressor, and one

genotype that did not express the transgene. From this, it can be determined that

expression of the transgene was not responsible for the classification of plants into these

categories, and assuming that the construct was functioning correctly, the increased

level of ABA failed to increase the water use efficiency of expressing plants above what

could be expected by natural variation (although the absolute levels of ABA were not

determined).
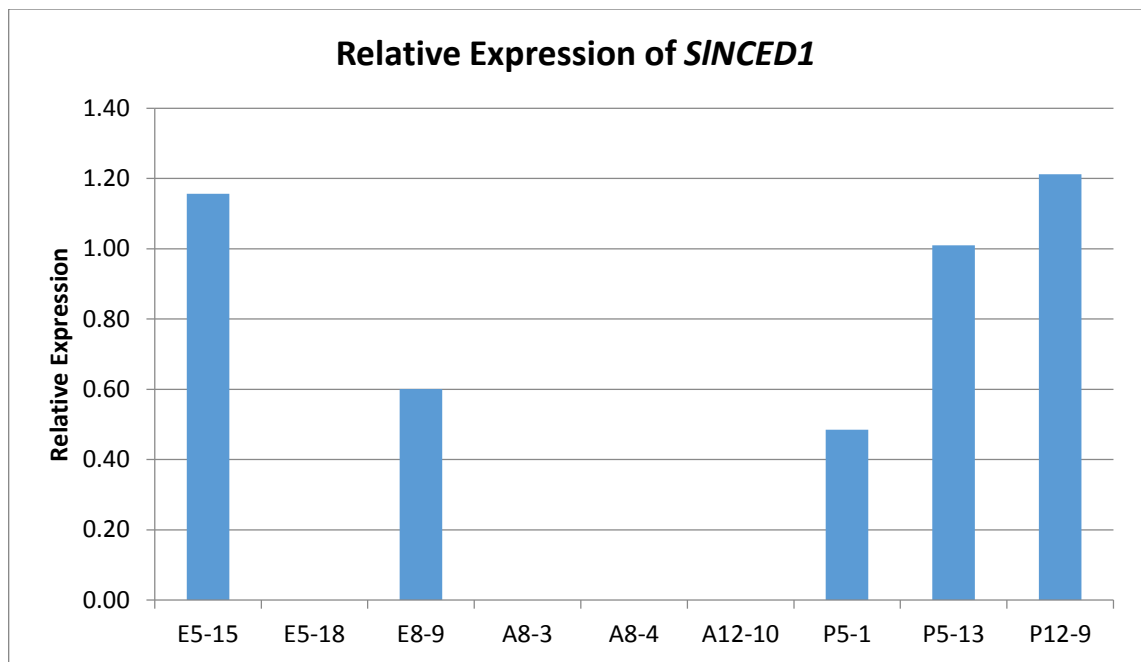
Figure 3.16: Relative expression of *SlNCED1* in T$_1$ transgenic plants deemed efficient (E series), average (A series), and poor (P series) users of water (data from Fig. 3.13).

In terms of this analysis (see sections 3.3.6 and 3.3.7) it cannot be stated whether any variation observed throughout the study was the result of the transgene or simply natural variation. In order to exclude natural variation, more wild type plants would need to be studied to find the limits of the natural variation under test conditions and establish a natural mean. This would then be compared against the variation and mean observed in the transgenic plants to determine its effectiveness in improving WUE under non-stressed conditions.

This was always the intended goal of this thesis but, unfortunately time restraints forced a limit to the size and number of studies that could be performed. It should be noted that follow up work is currently being carried out by other individuals and some findings from this follow up work will be discussed.

4.   Discussion

The purpose of this thesis was to assess the use of the transgene construct *pART-rbcS3Cp::SlNCED1* (here designated *SSUp::SlNCED1*) developed by Tung *et al.* (2008) to drive light dependent upregulation of ABA biosynthesis in the agronomically important crop white clover (*Trifolium repens*) with the aim of increasing the water use efficiency of the transformed plants. White clover was chosen as the target species because it exhibits a relatively low water use efficiency (Hart, 1987) and continued expansion of agronomic practices to areas of limited water availability requires greater conservation of water.

4.1 White clover (*Trifolium repens* L.)

4.1.1 *Agrobacterium*-mediated transformation of white clover

The low clover regeneration rate of 0.29% achieved in this thesis was lower than the expected published rate of between 85 and 95% for white clover (Ding *et al*. 2003). The transformation frequency of 0.13% was also lower than the expected 0.3 to 6% expected for white clover (Ding *et al*. 2003). Possible reasons for this are the high seasonality effect observed during tissue culture, photo-bleaching of high expressing tissues, possible lethality as a result of high transgene expression, or a combination of all the above.

The observed seasonality of tissue regeneration was a large contributing factor for the low regeneration rate, as all attempts to regenerate plants outside of the spring time window resulted in the death of all tissues in a matter of weeks. Despite being grown and handled under climate controlled conditions these plants were obviously still responding to some form of timing cue, be it external or internally mediated by an as yet

undescribed molecular clock that functions to elicit annual rhythms in an epigenetic manner in tissues not previously entrained by a timing cue. A seasonally dependent response of tissue culture is not a novel concept; indeed much anecdotal evidence points towards a general trend for more successful regeneration rates in spring. However, actual publications on this subject appear infrequently in literature (Carman *et al*. 1987; Doctrinal *et al*. 1989; Mythili & Pious, 1995; Sharma *et al*. 2005) and often are not the result of a rigorous study on the matter but rather are published using data collected during a separate study. In the most recent of these studies, Sharma *et al.* (2005) identified a rise in the rate of regeneration from wheat callus that corresponded with the advent of spring. This regeneration also occurred under conditions that should have precluded the developing plants ability to sense seasonal change, similar to the observation in this study.

How these seasonal responses are coordinated in white clover is still unknown. The possibility that the plants were being entrained by an external cue, however unlikely, must be considered. During the periods when the tissues were being checked for growth or transferred to fresh media, it is possible that the small amount of exposure to differing light quality or changes in temperature, while being kept in a growth chamber, was able to entrain the regenerating plants and allow them to determine an 'optimal time for growth'. Another notion to be considered is that devoid of external environmental cues, the regenerating plants may be sensing plant derived volatiles originating from free growing plants in the external environment, and are thus sensing the entraining environmental cues by proxy. It is well established that plants are capable of purposefully synthesising and releasing volatile compounds (Baldwin *et al*. 2006), although there is no evidence of this form of sensing and entraining having ever been studied to date.

The prospect that high expression of the *SSUp::SlNCED1* transgene in tissues may have resulted in photo-bleaching of the photosynthetic tissues is possible. The photo-bleaching would have resulted from the transgene product acting as a net sink for carotenoids (Tung *et al*. 2008), diverting them from chlorophyll biosynthesis and causing the plant to slowly bleach out. This, in and of itself, would not have resulted in the death of the plants as they were being maintained on media that would support their growth without the plant having to photosynthesise. However, because all early selection was based on kanamycin resistance, these high expressing plants would have appeared sickly in comparison to lower expressers and would be subsequently selected against leading to the net loss of any high expressing genotypes early on in tissue culture. However, the chances of this happening however are perhaps lower as the RUBISCO small subunit promoter (SSUp) used here is inhibited at high sucrose concentrations (Van Oosten & Besford, 1994), and therefore should have been rendered ineffective on the sustaining media.

The possibility that high transgene expression could result in lethality is relatively mild as high expression of abscisic acid is more likely to result in the dormancy or arrest of the apical meristem (Zhang *et al*. 2010) rather than resulting in an unregulated state that leads to cell death. It is possible that some insertions were made in important genomic regions or genes that would result in the death of the host cell should they be incapacitated, but the likelihood of this happening in a large proportion of the treated cotyledons is highly unlikely.

The reason for the low regeneration rate is due to more than one single factor. The seasonality of the regeneration of tissue can be used to explain some of the variability but, even when the trials outside of the spring growth time window are ignored the

regeneration rate is still very low (0.55%), and therefore the regeneration of tissue was likely also subject to an unanticipated gene effect.

4.1.2 Initial evaluation of primary white clover transformants

The lack of transgene integration and expression in clover was anticipated as a possible outcome. White clover (*Trifolium repens*), along with many other legumous species, has a history of being a recalcitrant species that is not particularly amenable to genetic manipulation (Ding *et al*. 2003). Advances in tissue culture have allowed some success in increasing the number of plants that can be regenerated without the use of specific genotypes (Voisey *et al*. 1994; Larkin *et al*. 1996; Ding *et al*. 2003), but due to the nature of white clovers' genetic structure it can still react in an unpredictable manner to genetic manipulation. For example, the process of gene silencing is very difficult to achieve in white clover due to the fact that it is an allotetraploid (Ellison *et al*. 2006). Because it effectively has two independent genomes that act cooperatively, the targeted silencing of what is considered to be the dominant active gene may result in the compensatory upregulation of the corresponding recessive gene, effectively nullifying any gene silencing. Other problems arise when genes are inserted from less related (non-leguminous) sources, as species specific regions of the genome can result in an AT-GC mismatch that results in down-regulation of the foreign gene and renders it useless (Iglesias *et al*. 1997; Matzke & Matzke, 1998).

Techniques are available to increase the uptake and expression of foreign genes such as procuring the genes to be inserted from closely related gene sources such as other members of the *Trifolium* genus or from the *Medicago* genus. Should this still not allow gene expression or not be an available option, the removal of the introns of the foreign

gene and replacement with native introns may increase expression levels due to unforeseen position effects (Lumbreras *et al*. 1998).

Unfortunately, even when acceptable levels of expression are achieved the growth habit of white clover lends itself well to removal of expressing tissues. Due to its stoloniferous growth habit, any chimeric tissues that are generated have the potential of producing shoots that have regressed to the wild type. As maintenance of clover on growth media is based on selecting individual stolons for transferral to new media, unless each individual is tested for insertion prior to transfer (a very labour intensive, if practical method) there is the very real possibility that regressive stolons will be selected, and successfully transformed tissues will be discarded unknowingly. It is likely that this is what has occurred during the course of this study and would possibly explain some of the variability in the genomic DNA PCR results where a genotype would test positive for a time before suddenly losing the insertion (eg. Plants 15-5.6 and 16-3.2; Table 3.1). As well, other genotypes apparently lacking any insertion would suddenly test positive as a chimeric stolon was selected from a regressive group (eg. Plants 15-2.2 and 16-4.1; Table 3.1).

The unexpected band sizes obtained in the clover genomic PCRs cannot be explained by any of the usual models such as fragmented insertion of the transgene, multiple native copies of the gene, or contamination of the PCR samples. If any of these were the case more consistent bands would be observed. For example, if there was fragmented insertion of the transgene it would be expected that the number and size of the bands would remain consistent within each genotype. If there were multiple native copies of the NCED gene that were being amplified by the primers used, then there would be bands of a consistent size across most, if not all, samples. Finally if there was large scale contamination of the PCR reaction or the gels, then the bands would be consistent

within each gel that was run. As none of these scenarios are the case, it can be concluded that there must be some other factor involved in producing the anomalous bands. Exactly what was occurring is uncertain. However, the most likely answer is the amplification of sections of the genome that had a weak binding affinity to the primers used, coupled with sporadic contamination of the samples and/or PCR reaction mixture yielded erratic bands of variable sizes.

4.1.3 Establishment of primary clover transformants on soil

Plants were to be established on soil prior to any expression analysis because the *SSU* promoter is suppressed by sucrose (Van Oosten & Besford, 1994) and as such was predicted to be down-regulated, if not inactive on the growth media. Before expression of the transgene could be measured, the plants had to be removed to a system that allowed them to grow without the aid of sucrose.

The initial poor establishment of the regenerated clover lines on soil was due to a combination of factors. These included recurring fungal infections, both on the soil and on the senescing leaves, insect herbivory on the emerging leaves, and poor establishment of the roots caused by withdrawal from the media. However, wild-type control plants were successfully established on soil suggesting that the methodology used for establishment was suitable.

The continued lack of establishment of any of the putative transgenic plants led to the implementation of alternative methods of eliciting gene expression. Thus clones of all putative transgenic lines were sent to Dr. Alicia Scott at AgResearch Grasslands, Palmerston North for establishment on peat plugs, while at the same time several clones were transferred to media that had had the sucrose replaced with mannitol at the same osmotic potential.

The intention of the mannitol media was to provide an extra step whereby the plants could be weaned from their dependence on sucrose and subsequently established on soil. This principle also underpinned the use of peat plugs at AgResearch and which was able to yield lines M1 through M7 when the use of mannitol-enhanced media failed.

4.1.4 Final assessment of primary white clover transformants

Prior to establishment on soil, lines M1 – M7 had consistently tested positive for the transgene *SSUp::SlNCED1* by genomic PCR. Once established on soil, all plants were analysed for transgene expression and only plant M3 was observed to display any transgene expression, and only during a brief circadian window.

There are several possible causes for the lack of expression observed in lines M1, M2, M4, M5, M6 and M7. An un-recognisable gene, high copy number and chimeric tissues are all potential sources of concern. Insertion and expression of a foreign gene can be hindered if the foreign gene is from a source too distantly related for the transcriptional and translational machinery of the host plant to recognise it. In this case, this seems an unlikely outcome as *NCED1* is a highly regulated gene and part of a highly regulated metabolic pathway present in all higher plant species (Hartung, 2010), and so the chances that *SlNCED1* would be un-recognisable for transcription and translation are very low.

High copy number can result in a feedback effect on the inserted gene, resulting in gene silencing (Assaad *et al*. 1993). As Southern analysis was not carried out on lines M1 – M7, it is impossible to say whether this was the cause of the low expression observed in line M3 and the lack of expression observed in all other lines. However, it does seem unlikely that all 7 plants would be subject to high copy numbers, when the occurrence of this phenomenon is random and occurs at a relatively low frequency.

The most likely reason for the failure of lines M1, M2, M4, M5, M6 and M7 to express the transgene is that they no longer contained the transgene. It had already been established that chimeric tissues were present in this study and that the maintenance of lines to ensure genetic purity was difficult at best. Lines that had regressed were indistinguishable from transgenic lines, and were more easily established on soil (section 3.1.4). It is likely that chimeric tissues were unintentionally established on soil and that regressed shoots were preferentially selected for due to their greater potential to successfully establish.

The decision to discontinue using white clover was not made lightly, as clover was the original focus of the thesis. However, a single expressing individual would not be sufficient for determining the efficacy of *SlNCED1* in increasing water use efficiency as the outcrossing nature of white clover results in a heterogeneous population, even within cultivars. This would mean that testing this single positive genotype against a wild type control of the same cultivar (Huia), would not allow any relevant data to be collected on water use efficiency, or plant responses to water deficit.

Ideally, more positively expressing individuals would have been obtained and this would have allowed for testing against wild type control plants. The aim of these tests would have been to identify the limits of natural variation within the mixed genotype of the cultivar and determine what effects the addition of *SlNCED1* driven by *SSUp* had in increasing the WUE of white clover under non-water stressed conditions.

This would have been achieved by cutting several thousand cotyledons during the apparent spring window for transformation in order to get around the low regenerative percentage and maintaining all cotyledons and potential plantlets on a mild selective media (Kan 50). Genomic PCR would be carried out on all potential stolons with only 'true breeding' stolons being selected for transfer to fresh media in order to reduce the

number of chimeric stolons. The transition to soil would be accomplished in the same manner as performed by Alicia Scott of AgResearch Grasslands, Palmerston North, in that peat plugs would be saturated with decreasing strengths of sustaining media until the plants were successfully weaned to soil.

At this point genomic PCR would be utilised to ensure the purity of the plants, and Southern blot and sqPCR would be used to determine copy number and gene expression respectively. Water use efficiency (WUE) trials would be conducted in a similar manner as those performed using tobacco (section 2.2.3) using T_1 seedlings, generated by hand-crossing T_0 parents, after they had been used for germination trials. A greater number of clover plants would be necessary due to the outcrossing nature of white clover, in order to isolate the effect of *SSUp::SlNCED1* in increasing WUE from natural variation present within white clover.

4.2 Tobacco (*Nicotania tabacum*)

The purpose of this thesis was to assess the use of the transgene construct *SSUp::SlNCED1* to drive light dependent upregulation of ABA biosynthesis with the aim of increasing the water use efficiency in white clover. Tobacco was to be used as facile transformation system in a heterologous genetic background should any issues arise in the clover transformation. Thus as no white clover transformants were recovered, then the bulk of the evaluation of *NCED1* was carried out in tobacco.

4.2.1 Assessment of *SlNCED1* relative expression in T_0 plants

The relative expression study showed that all T_0 lines were expressing the transgene construct at varying levels and could be ranked from highest to lowest expressor as follows; NT7, NT8, NT5, NT9, NT2 and NT12. However, as NT5, NT7 and NT9, NT12 have been postulated to be clones of each other (Fig. 3.3; section 3.2.4), it would be reasonable to assume that their expression levels would be more closely matched than the 2 – 3 fold difference observed, respectively.

The reasons for this difference in expression levels between clones can be explained by the plants experiencing small differences in growing conditions, resulting in slightly adjusted expression and overall protein yield. This would have been amplified by any somaclonal variation between the clones that would have arisen during their separate culture as callus and resulted in different expression patterns between their identical genomes. Finally, shading of some plants was unavoidable within the confines of the glasshouse used for growing the plants, and as the transgene was driven by the light inducible *SSU* promoter, any loss in light intensity would also mean a corresponding loss in transgene expression.

This does confirm, however, that the tomato *SSUp* can drive transgene expression in tobacco. Although diurnal regulation of transgene transcript was not tested as part of this thesis, it would be a topic for future focus.

4.2.2 Relationship between NT5/7 and NT9/12

It was postulated that the tobacco plants NT5, NT7 and NT9, NT12 were clones collected from the same callus (Fig. 3.3; section 3.2.4). At the time of collection these plants were collected from apparently separate calli that must have originally been joined. This is logical as transgene insertion is random, meaning that in order for them

to have identical Southern analyses they must be from a single transformation event and descended from the same progenitor cell.

In future, greater care should be taken to ensure that all collection of callus takes place from well separated leaf discs, and maintained in separate media to ensure purity of all callus collected.

4.2.3 Progeny of transformed tobacco display altered germination rates

The wild type seed achieved higher than 95% germination within 5 days of sowing on media, taking 3 days to reach 50% germination compared with 17-28 days observed in the regenerated tobacco lines that managed to reach 50% germination. This significant delay in germination was unexpected, particularly in comparison to the 2-4 day delay compared to wild type in time taken to reach 50% germination observed by Tung *et al.* (2008) using the same construct in tomato (*Solanum lycopersicum*). The possible reasons for this delay in germination are that the seed may have been collected or stored improperly prior to the trial, or that a stronger ABA hormonal effect than seen previously with this promoter-transgene construct was observed, possibly due to the difference in genetic background.

Germination trials performed since the end of this trial, by other individuals using the same seed stocks (Leung, *pers comm*), have shown that the dormancy effect appears to be increasing over time, leading to greater times before germination begins and time to 25% and 50% germination, as well as lower overall germination rates in progeny from all lines.

When seeds were germinated on media containing gibberellin (GA_3 media) there was a trend towards decreased time to 25% and 50% germination and greater germination compared to media that contained ethanol (non-GA_3) media (section 3.3.2.2). This

implies that, at least in part, the dormancy of the affected seeds can be overcome hormonally by GA_3, a known antagonist of ABA induced dormancy (Mahouachi *et al*. 2005).

Future studies involving the germination of seed from the T_0 lines may benefit from imbibition and germination of the seed on media containing norflurazon. As norflurazon is a known inhibitor of ABA biosynthesis that acts by reducing the pool of available substrate for NCED, it has been shown to increase germination rates and reduce time to germination in seeds where the main barrier to germination is hormonal (Thompson *et al*. 2000; Tung *et al*. 2008).

The use of norflurazon to aid in germination would also demonstrate that it is the production of ABA *de novo* that is preventing germination. Any failure of germination would imply that germination is instead being inhibited by developmental processes. The use of norflurazon as a germinative aid would also give further insight into the potential cause of aberrant growth observed in this study. If the use of norflurazon resulted in a significant shift in the proportion of aberrant seedlings, then it could be assumed that the level of ABA just prior to germination is a determinant factor in the aberrant growth habit of seedlings.

4.2.4 Evaluation of aberrant seedlings in transformed tobacco progeny

The advent of aberrant seedlings was predicted by previous studies (Tung *et al*. 2008), but not to the extent observed in this study. These aberrant seedlings were identified by a significant loss of shoot apical meristem growth and function (see section 3.3.6.2 for a full description). It can be safely assumed that the simple insertion of the transgene construct was not the cause of the aberrant growth behaviour for two reasons; 1) the chance of disruptive insertions of a transgene occurring in four independently derived

lines is highly unlikely, and 2) no defects in shoot apical meristem growth or function were observed in these four lines as they regenerated from callus. The possibility that an unregulated amount of ABA was affecting the seedlings post-germination can also be discounted as the media that they were germinated on (functionally MS media) contained levels of sucrose expected to inhibit the activity of the *SSU* promoter (Van Oosten & Besford, 1994). From this it can deduced that whatever the cause of the aberrant growth was, it did not occur until after the parent plants were established on soil and before the progeny were germinated on media. This is supported by the findings of Tung *et al.* (2008) that seeds derived from a wild type maternal source and germinated on media containing norflurazon, a known inhibitor of ABA biosynthesis, would still display an aberrant phenotype if pollen was obtained from a plant containing the same transgene construct used here. This indicates that these morphological irregularities were the result of hormonal effects independent of the parent plant that occurred prior to the onset of germination.

It is well established that ABA has roles in floral development and seed maturation (Razem *et al.* 2006; Finkelstein *et al.* 1985), and that both of these processes are tightly regulated by a multitude of hormones. In this case it would seem that any excess of ABA created by the transgene was insufficient to inhibit floral initiation (Razem *et al.* 2006). However, as seed maturation is a more tightly regulated process and developmentally more sensitive to hormonal cues, it is possible that the separately regulated levels of ABA were able to effect the growth and development of the developing embryos.

It also bears noting that no hormone acts in isolation. While ABA is generally accepted to be antagonistic to GA, it is also recognised that ABA can also influence the action of other hormones such as cytokinins, auxin, and ethylene (Gazzarrini &

McCourt, 2001; Seo *et al*. 2006; Tanaka *et al*. 2006); all of which have wide reaching

effects on plant growth and development. Perturbation of the hormonal balance at any

point during development can result in disordered growth and even death. When the

disruption occurs during the early stages of development, this can generate greater

changes in the patterns of growth as meristematic activity is not yet functional and the

embryo initially develops according to a very tightly regulated, hormonally driven and

species specific framework. Disruption of this stage of development often results in

death of the embryo, or the development of severely impaired seedlings that display a

variety of abnormalities ranging from disordered growth patterns, to impaired or non-

existent meristematic activity (reviewed by Dodeman *et al*. 1997).

ABA has been identified as being capable of rendering meristems into a quiescent

state, usually during extreme drought (Zhang *et al*. 2010). A high level of ABA will

slow down the development of both shoot and root apical meristems, rendering them

inactive until the ABA signal is relieved.

Sectioning of aberrants was carried out on types 1 and 2, and compared to sections

from phenotypically wild type plants from other T_1 seedlings, to determine the source of

the disturbed growth. Type 2 aberrants were characterised by a failure to progress

beyond the initial stages of germination, whereby the radicle and eventually hypocotyl

emerge from the seed coat (Fig 3.10b). Sections of these aberrants showed little to no

growth of the cotyledons (in preparation for shedding of the testa), and little to no

function of the apical meristem. Type 1 aberrants displayed vascular growth

abnormalities and apparently disorganised cellular organisation (Fig. 3.10a). Type 1

aberrants also displayed a poorly organised apical meristem that bears a resemblance to

apical sections from the Arabidopsis apical-basal deletion mutant *SHOOT-*

MERISTEMLESS (*STM*), or one of the associated mutations that affect the functional integrity of the shoot apical meristem (Barton & Poethig, 1993).

These type of structural abnormalities (Fig. 3.10a,b) are not typical of high-ABA expressing plants. High expression of ABA is known to result in stunting of plants, but mostly due to low photosynthetic gas exchange caused by lowered stomatal permeability (Thompson *et al*. 2000), not due to poor structural organisation. It is possible that the promoter used was the instigator of expression of *SlNCED1* and production of ABA during embryogenesis, a highly sensitive stage to hormonal input in plant growth and development. However, this fails to explain why constitutive super promoters (used by Thompson *et al*. 2000) would fail to generate such a phenotype when an apparently milder promoter is capable of generating such abnormalities. One potential explanation for the lack of such aberrant phenotypes being observed during the use of constitutive promoters may be that such high expression during all stages of germination and embryogenesis results in more severe phenotypic abnormalities and a higher abortion rate of developing embryos, thus obscuring the phenotype seen here.

For further investigation of what is occurring in aberrant seedlings at a cellular level, a greater degree of sectioning would need to be performed. Stains targeting the vasculature would be needed to identify how disordered growth may be affecting overall stem growth, and stains targeting dividing cells would be needed to identify how active the apical meristems are in aberrant plants and whether there is any unregulated growth in other areas of the plant. The use of a confocal microscope would be recommended in order to overcome the limits of light microscopy and give greater resolution at the single cell level.

As the aberrant growth observed here appears to be due to the low growth rate of the shoot apical meristem, a high level of ABA promoting meristem dormancy (Zhang *et al*.

2010) is a possible explanation for the rate of aberrant growth. However, the level of ABA required to stop shoot apical meristem growth would also reduce root apical meristem growth, and would be expected to be occurring on media that should be directly inhibiting the promoter used here to drive ABA biosynthesis in this situation. As such, this scenario of inducing aberrant growth can be discounted.

Based on the proportion of aberrant seedlings across lines it can safely be assumed that the collection and storage of the seed contributed only a minor amount, if at all, to any aberrant growth as seed derived from line NT2 showed a markedly reduced aberration rate (0% on MS media; see section 3.3.6.2). As that seed was collected from NT2 at the same time as all other plants and stored in an identical manner, it is most likely that this is not the main cause. Also of note is that during the assessment of plants for aberrant growth two plants germinated from wild type seed stock also displayed type 1 aberrant growth habits (Fig. 3.9a & Table 3.6), suggesting that natural mutation is also easily capable of yielding this phenotype.

There appears to be a relationship between the severity of aberration and germination rate. When the germination rankings (highest to lowest; NT5, NT2, NT7, NT8, NT9, NT12) are compared with the ranking for severity of aberration (lowest to highest; NT2, NT5, NT9, NT7, NT8, NT12), only the placement of NT2 and NT9 prevent a perfect match. It stands to reason that a greater degree of aberrance will hinder germination, with some aberrants being unable to germinate due to the severity of their malformation, or even some being so severely affected that their aberration was embryo lethal. This would explain why lines with a greater rate of aberrant growth also experienced a reduced germination rate compared to lines with low aberration rates, and to the wild type itself.

In order to test whether this aberrant growth habit is a maternal gene effect or a gene effect active during development, four groups of seed would need to be collected. One group of selfed seed from T_0, one group of selfed seed derived from wild type plants grown under the same conditions as the T_0 plants, one group of seed derived from T_0 plants pollinated by wild type pollen and one group of seed derived from wild type plants pollinated by T_0 pollen.

It would be expected that the selfed seeds derived from T_0 lines would show the expected aberrant growth in a proportion of the seedlings and that selfed seeds derived from wild type plants would show a wild type growth habit.

If the aberrant growth habit is determined by the maternal tissues it would be expected that maternally T_0 seeds pollinated by the wild type would show aberrant phenotypes consistent with the selfed T_0 seed, while the maternally wild type seeds pollinated by T_0 would be wild type in their phenotype. If, however, the aberrant phenotype is determined by an unanticipated gene effect taking place during development of the embryo, then the proportion of aberrant seedlings in seedlings derived from both T_0 and wild type maternal tissues would be expected to be similar.

4.2.5 Evaluation of relative expression in aberrant seedlings

Exploring the relative expression of *SlNCED1* in the three aberrant seedling types compared with phenotypically wild type seedlings was unable to show a correlation between high *SlNCED1* activity and aberration type (Fig. 3.11). The level of expression of the transgene, post-germination, was not able to explain the aberration type. In this test, type 1 aberrants were the lowest expressors, expressing at a rate less than five times that observed in the phenotypically wild type controls that still contained the transgene and at only 0.06 that of the housekeeping gene. The highest expressor was type 2

aberrants bulked from line NT7, however these still only managed to achieve an expression level 0.47 that of the housekeeping gene. These levels of expression are likely to be to low as to be ineffective in generating biologically active levels of ABA. This is likely due to all aberrants being collected directly from sustaining media that would have down-regulated the activity of the *SSU* promoter. This helps to further reinforce idea that active expression during germination and post-germinative growth was not responsible for the aberrant phenotypes observed.

It should be noted however, that due to the unavailability of the required amounts of tissue for samples of each aberration type existing in any one line, samples had to be bulked from lines that contained the required amount of seedlings to facilitate collection of genetic material. This would have removed variation existing between different seedlings within a given line.

4.2.6 Evaluation of water use efficiency in T_0 progeny

T_1 plants were evaluated for water use efficiency by growing them in a water limited environment and assessing their growth rate per unit water, this allowed for the separation of plants into groups labelled 'efficient', 'average' and 'poor' users of water. A selection of these plants (6 efficient, 6 average and 5 poor users of water) were then used for a transpiration assay that showed efficient plants were achieving a higher growth rate for the same loss of water through transpiration (Fig. 3.14).

Plants that were identified to be efficient, average and poor users of water were assessed for their expression of the transgene construct *SSUp::SlNCED1*. With the discovery that all of the tested plants that had been designated as poor users of water contained the transgene, some expressing at relatively high levels, combined with the medium to low expression of the transgene found in the two expressing efficient plants,

it was deduced that in this instance, the introduction of *SlNCED1* was not a contributing factor in determining water use efficiency in these plants. This was possible due to expression of the transgene being unable to overcome natural variation in water use efficiency displayed by these plants.

Further WUE studies carried out using T_1 lines (NT5, NT8 and NT12) (J. Wargent, Massey University, *pers comm*) showed that when each line, and all over-expressing lines were compared against a wild type control there was a trend for greater WUE (Fig. A5.4.1), and decreased photosynthetic rate (Fig. A5.4.2), stomatal conductance (Fig. A5.4.3), internal CO_2 concentration (Fig. A5.4.4) and transpiration rate (Fig. A5.4.5) in all transgenic lines.

There was no significant difference between the photosynthetic rate observed in any of the transgenic lines compared to the wild type control. Line NT5 showed no significant difference to the wild type control in any of the parameters investigated, although there was a general trend for greater WUE and lower photosynthetic rate, stomatal conductance, internal CO_2 concentration and transpiration rate. Lines NT8 and NT12 had a statistically greater WUE than the wild type control and statistically lower stomatal conductance, internal CO_2 concentration and transpiration rate. This is consistent with a higher degree of ABA biosynthesis in these lines. Further, in some analysis conducted in this thesis (germination rate, Fig. 3.5; degree of aberrant seedlings, Fig. 3.8), NT5 appeared to be closest to wild-type.

However, to truly determine the value of *SSUp::SlNCED1* in increasing water use efficiency in tobacco the isolation of true breeding transgenic (homozygous dominant) and regressive (homozygous recessive, lacking in the transgene) plants from each line would be necessary. Isolation of these plants would allow for investigation of the transgene in genetic backgrounds that share a high degree of homology, and single

crosses between the transgenic and recessive plants (creating heterozygous dominant plants) would allow for determination of the value of a single copy of the construct in increasing WUE. Measurement of WUE would be carried out on true breeding transgenic and recessive plants as well as heterozygous crosses with a wild type control to determine the value of the construct in increasing WUE while attempting to isolate natural variation as much as possible. Ideally, performing this examination alongside transpiration rate, stomatal conductance, internal CO_2 concentration, and photosynthetic rate analyses would give an indication of the effectiveness of *SSUp::SlNCED1* in increasing WUE and how the increase in ABA would impact other plant functions.

4.2.7 Accumulation of ABA levels in transgenic plants

This analysis was not performed as part of this thesis due to time constraints. However, it should be carried out as knowledge of the functional accumulation of ABA and its metabolites in the transgenic plants allows for greater understanding of how effective the construct is in upregulating ABA biosynthesis, i.e; whether there is any suppression of the gene product, post-mRNA, or whether there is any upregulation of the catabolic pathways that may lead to a loss of effectiveness of the transgene construct.

The examination of the transgenic plant material was carried out by Dr. Irina Zaharia, University of Saskatoon, Canada (Appendix 5.5). The analysis showed that there was varying levels of free ABA and its metabolites in all transgenic plants. T8 was the highest accumulator, showing levels more than four times the average wild type levels and almost three times the level of the next highest accumulator. For this reason, T8 was excluded as an outlier for some of the statistical analysis. With the exclusion of T8, the transgenic plants showed an average increase of 25% of free ABA compared to the wild type controls (data not shown). When the mean ratio of deactivated ABA (ABA:GE,

PA, DPA, 7'OH-ABA, neo-PA and *t*-ABA) to active ABA for all transgenic plants was compared with the wild type controls, no statistical difference was detected (data not shown). This suggests that although there was an increase in free ABA in the transgenic plants, there was a step-wise increase in ABA catabolism that maintained the ratio of inactive to active ABA.

4.3 Conclusions

The transformation of white clover by *Agrobacterium tumefaciens* using the plasmid vector *pART-SlSSUp::SlNCED1* was successful in generating transformed white clover plants, with some expression of *SlNCED1* being observed. However, as a viable means of generating a new generation of white clover with artificially improved water use efficiency more testing will be required. This will be discussed in further detail in the next section. In determining whether the inclusion of the tomato *NCED1* gene driven by the *RUBISCO* small subunit promoter was able to increase the WUE of the transformed clover plants, a greater number of transformants and more reliable levels of expression would be required to give a proper statistical analysis of any data. The fact that any transformants were obtained and that there were measurable levels of expression in some instances does indicate that the transformation system and the construct used were working successfully, and only further study will reveal whether this is a viable system for artificial improvement of white clover cultivars.

Transformation of tobacco by *Agrobacterium tumefaciens* using the plasmid vector *pART-SlSSUp::SlNCED1* was successful in generating transformed plants that expressed the transgene. The evaluation of transformants for improved WUE was not possible for the primary transformants due to the small population size, and evaluation of T_1 plants was complicated by unforeseen difficulties in obtaining seedlings for

assessment as well as the use of trial WUE methodologies with some limitations. Subsequent assessment revealed that there was a general trend for increased WUE across all lines compared to the wild type control, although for some lines this difference was not at a statistically significant level.

The use of *SSUp::SlNCED1* to generate transgenic plants with an increased water use efficiency is possible however, but more research is required to assess the viability of this construct in species outside of the *Solanum* plant family.

4.4 Future work

Future work using the *SSUp::NCED1* construct with white clover would rely on generating a new generation of transformants. Initially a greater number of cotyledons (up to 60,000) would need to be cut and co-incubated with *Agrobacterium* containing the plasmid vector *pART-SSUp::NCED1*. If at all possible it would be beneficial to investigate the possible variables involved in the seasonality of tissue culture, if for no other reason than to improve future transformational efficiencies.

Stringent care would be taken in the propagation and maintenance of all plant tissues, from maintaining all plants on mid to low strength selection media until obviously dead to reduce the risk of disposing of bleached but otherwise functional transformants, and limit the growth and regression of chimeras. Multiple DNA PCR would be used to assess whether a stolon was transformed, and whether it was worth being maintained as part of a line.

All plants would be established on soil *via* the method used by Dr. Alicia Scott of AgResearch to provide the greatest possible population size for experimentation. This population would be sampled for tissues at varying times (i.e. at 6:00, 9:00, 12:00, 15:00, 18:00 and 21:00) over several days to determine any circadian control of the

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transgene, and therefore the best time for future sampling. A statistically significant number of plants would be used to determine the water use efficiency of the transgenic plants compared to wild type controls using a variety of methods including: growth measured as g/day; g/mL H_2O, transpiration efficiency, and water stress/pre-stress treatments.

Overall, the viability of the *SSUp::NCED1* construct for generating new white clover cultivars with improved WUE would be assessed. Pending the results, a new construct may be suggested using genes from species more closely related to white clover, such as *Medicago spp*. or white clover itself, in order to improve transformational efficiency and/or gene expression.

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5. Appendices

Appendix 5.1: Media formulations

YEB Broth

Beef extract	0.5% (w/v)
Yeast extract	0.1% (w/v)
$MgSO_4 - 7H_2O$	0.05% (w/v)
Sucrose	0.5% (w/v)
Peptone	0.5% (w/v)

Adjust to pH 7.3 with 2M NaOH.

Autoclave at 121°C at 15 psi for 20 min.

YEB Agar

YEB broth mixture	
Bacteriological agar	1% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

CR7 Media

MS powder (including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)

Adjust to pH 5.7 with 2M KOH

Phytoagar	0.8% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

BAP	1 mg/L
NAA	50 µg/L

CR0 Media

MS powder (including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)

Adjust to pH 5.7 with 2M KOH

Phytoagar	0.8% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

CR0 + mannitol Media

MS powder (including vitamins)	0.44% (w/v)
Mannitol	1.6% (w/v)

Adjust to pH 5.7 with 2M KOH

Phytoagar	0.8% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

Nic I Media

MS powder (including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)

Adjust to pH 5.7 with 2M KOH

Phytoagar	0.8% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

BAP	1 mg/L
NAA	100 µg/L

<u>Nic II Media</u>

MS powder (including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)

Adjust to pH 5.7 with 2M KOH

Phytoagar	0.8% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

BAP	1 mg/L
NAA	200 µg/L

<u>Nic III Media</u>

MS powder (including vitamins)	0.44% (w/v)
Sucrose	3% (w/v)

Adjust to pH 5.7 with 2M KOH

Phytoagar	0.8% (w/v)

Autoclave at 121°C at 15 psi for 20 min.

Appendix 5.2: Gene sequences used in this thesis

SlNCED1

ATGGCAACTACTACTTCACATGCCACAAATACATGGATTAAGACTAAGTTGT

CAATGCCATCATCAAAGGAGTTTGGTTTTGCATCAAACTCTATTTCTCTACT

CAAAAATCAACATAATAGGCAAAGTCTCAACATTAATTCCTCTCTTCAAGCT

CCACCTATACTTCATTTTCCTAAACAATCTTCAAATTATCAAACACCAAGA

ATAATACAATTTCACACCCAAAACAAGAAAACAACAACTCCTCTTCTTCTTC

AACTTCCAAGTGGAATTTAGTGCAGAAGCAGCAGCAATGGCTTTAGATGC

TGTAGAAAGTGCTTTAACTAAACATGAACTTGAACACCCTTTGCCGAAAAC

AGCCGACCCACGAGTCCAGATTTCTGGGAATTTTGCTCCGGTACCGGAAAA

TCCAGTCTGTCAATCTCTTCCGGTCACCGGAAAAATACCCAAATGTGTTCAA

GGCGTTTACGTTCGAAACGGAGCTAACCCTCTTTTTGAACCAACCGCCGGAC

ACCATTTCTTCGACGGCGACGGTATGGTTCACGCCGTTCAATTCAAAAATGG

GTCGGCTAGTTACGCTTGCCGTTTCACTGAAACAGAGAGGCTTGTTCAAGAA

AAAGCTTTGGGTCGCCCTGTTTTCCCTAAAGCCATTGGTGAATTACATGGTC

ACTCTGGAATTGCAAGGCTTATGCTGTTTTACGCTCGTGGGCTCTTCGGACT

TGTTGATCACAGTAAAGGAACTGGTGTTGCAAACGCCGGTTTAGTCTATTTC

AATAACCGATTACTTGCTATGTCTGAAGATGATTTGCCTTACCATGTAAAGG

TAACACCCACCGGCGATCTTAAAACAGAGGGTCGATTCGATTTCGACGGCC

AGCTAAAATCCACCATGATAGCTCACCCAAAGCTCGACCCAGTTTCCGGTG

AGCTATTTGCTCTTAGCTACGATGTGATTCAGAAGCCATACCTCAAGTACTT

CAGATTTTCAAAAAATGGGGAAAAATCAAATGATGTTGAAATTCCAGTTGA

AGACCCAACAATGATGCATGATTTCGCAATTACTGAGAACTTCGTCGTCATT

CCTGATCAACAAGTCGTTTTCAAGATGTCTGAAATGATCCGTGGAGGTTCAC

CGGTGGTTTACGACAAGAACAAAGTTTCCCGATTTGGTATTCTGGATAAGTA

CGCGAAAGATGGGTCTGATTTGAAATGGGTTGAAGTACCTGATTGTTTCTGT

TTCCACCTCTGGAATGCTTGGGAAGAAGCAGAAACAGATGAAATCGTTGTA

ATTGGTTCATGTATGACACCACCAGACTCCATTTTCAATGAATGTGATGAAG

GGCTAAAGAGTGTTTTATCCGAAATCCGTCTCAATTTGAAAACAGGGAAAT

CAACAAGAAAATCCATAATCGAAACCCGGATGAACAAGTGAATTTAGAA

GCTGGAATGGTGAACCGAAACAAACTCGGAAGGAAAACAGAGTATGCTTAT

TTGGCTATCGCTGAACCATGGCCAAAAGTTTCTGGTTTTGCAAAAGTAAACC

TGTTCACCGGTGAAGTTGAGAAATTCATTTATGGTGACAACAAATATGGTG

GGGAACCTCTTTTTTTACCAAGAGACCCCAACAGCAAGGAAGAAGACGATG

GTTATATTTTAGCTTTCGTTCACGATGAGAAAGAATGGAAATCAGAACTGCA

AATTGTTAACGCAATGAGTTTGAAGTTGGAGGCAACTGTGAAGCTTCCATC

AAGAGTTCCTTATGGATTTCATGGAACATTCATAAACGCCAATGATTTGGCA

AATCAGGCATGA

Figure A2.1: *SlNCED1* **gene sequence. The highlighted region denotes the sequence that is most widely divergent from other** *NCED1* **sequences.**

NtGAPDH (AJ133422.1)

GCGGCGCGAATTGCCGGCCGCCCCGTTGTATCAAACCCTTCCACCAACTCTG

AATCCACTACACTCTTCTACAAAGCCCTTCAATCACTCAGTCAGATCGGACT

TCGCTCCTCCTCTCATTTCTCAAAAATC<mark>ATGGCCAAGGTTAAGATTGGAATT</mark>

<mark>AACGGATTTGGAAGAATTGGGCGATTAGTGGCCAGGGTTGCTCTCCAAAGA</mark>

<mark>GATGATGTTGAGCTTGTCGCAGTTAACGACCCTTTCATCTCTGTTGAATACA</mark>

<mark>TGACATATATGTTCAAGTATGATAGTGTACACGGCCAGTGGAAGCACCACG</mark>

<mark>AGCTCAAGGTTAAGGATGACAAGACCCTTCTCTTTGGTGAGAAGGCTGTTA</mark>

<mark>CTGTTTTTGGCTTTAGGAACCCAGAGGAGATTCCATGGGGCCAGACTGGAG</mark>

<mark>CAGATTACATTGTGGAGTCGACTGGTGTCTTCACTGACAAGGACAAGGCTG</mark>

<mark>CTGCTCACTTGAAGGGTGGTGCCAAGAAAGTCATCATTTCTGCCCCTAGCAA</mark>

<mark>GGATGCTCCGATGTTTGTTGTCGGTGTCAATGAGAAAGAATACAAGCCCGA</mark>

<mark>GCTCAACATTGTTTCAAATGCTAGCTGTACCACAAATTGCCTTGCTCCCTTG</mark>

<mark>GCCAAGGTTATAAATGACAGATTTGGAATTGTTGAGGGTCTCATGACCACA</mark>

<mark>GTCCACTCCATCACAGCCACACAGAAAACTGTTGATGGGCCATCAGCCAAG</mark>

<mark>GACTGGAGGGGTGGAAGAGCTGCATCATTCAATATTATTCCCAGCAGTACT</mark>

<mark>GGAGCTGCCAAGGCTGTTGGGAAAGTCCTACCAGCATTGAATGGAAAGTTA</mark>

<mark>ACCGGAATGGCTTTCCGAGTCCCAACTGTTGATGTGTCCGTGGTTGACCTCA</mark>

<mark>CAGTGAGGCTGGAGAAGAAGCTACCTACGATGAAATCAAGGCTGCCATTA</mark>

<mark>AGGAGGAATCTGAAGGAAAATTGAAGGGAATTCTAGGTTACACTGAAGATG</mark>

<mark>ATGTGGTGTCCACAGACTTCGTGGGAGACAATAGATCAAGCATATTTGATG</mark>

<mark>CCAAGGCTGGAATTGCTTTGAGCAAGAATTTTGTTAAGCTCGTTTCGTGGTA</mark>

<mark>CGACAATGAATGGGGTTACAGCACACGAGTGGTGGACTTGATTAAGCACAT</mark>

<mark>GGCATCAGTTCAGTAA</mark>AAGTGGCAGTTACTCATTGGTGGCCAAGTGTAGT

GGT<u>GCTGCTGTGAGG</u><u>AGTCTGT</u>TATTTGTTACTAGATAAAGCAGTTTAGAAT

AAAGTACTCACTCGGACTACAAGTGAGCGGCTCTTTGATGTAATTTAGGGAT
ATCTGCACTCTTGGATATGGTTTGAGGTCATCAATTGGGTTGTTGTAGACTT
CCCCACACATTCCGAGACCCAGTCTTTGCAACTCTGGATATGTTTCTTGTTTT
TGTTGTGTTATGAATGTGTAATAAGTTTGG

GAPDH-qPCR -F1: TGC TGC TGT GAG GAG TCT GT

GAPDH-qPCR -R1: GAC TGG GTC TCG GAA TGT GT

Figure A2.2: *NtGAPDH* gene sequence. The highlighted region denotes the open reading frame. The dotted lines denote the primer binding sites used in semi-quantitative RT-PCR, and the primer sequences are also shown (Table 2.2)

NtEF1α (AF120093)

cggcacgagttttaagtccttttttttcaattaacatgggtaaagagaaggttcacatcaa

cattgtggtcattggccacgtcgactctggtaagtcgactaccactggtcacttgatct

accttggtggtattgacaagcgtgtcattgagaggtttgagaaagaagctgctgagatg

cacaagcggtcattcaagtatgcctgggtgcttgacaaactaaaggctgagcgtgaccg

gggtatcactattgatatcgccttgtggaagtttgagaccaccaagtactactgcactg

tgattgatcgtcctggacacagggatttcatcaagaatatgattactggtacctcccgt

gcctgtcgtgtcctgattgttgcctccaccactggctttgcacaagctggtaatctcaa

ggatggacagacccgtgaaagactgcttattgactccaccactggtaacacccttggtg

tcacccaaatgatttgctgctgcaacaagatggatgctaccacccccaagtattccaag

gctaggtacgatgaaatcgtgaaggaggtttcttcctacctcaagaaggtaggatacaa

ccctgacaagatcccctttgtccccatctctggtttggaaggtgacaacatgctcgaaa

gatcaacaaaccttgactggtacaagggcccaacacttcttgatgctcttgaccagatt

aatgagcccaagaggcccacagacaagcctctcaggctcccacttcaggatgtttacaa

gattggtggaattggtactgtccctgttggtcgtgtggaaactggtgtcctcaagcctg

gtatggttgttactttttggtcccactggtctgaccactgaagttggatctgttgagatg

caccacgaagctcttcaggaggcacttcctggtgacaatgttggatttaatgtcaagaa

tgttgcggttaaggatctcaagcgtgggtttgttgcttccaactccggatgtcccagca

atgggtgcttcagctttacctcccaagctatcatcatgaaccattcaggacagattggc

aatggatatgctccagttctggactgccacacctcccatgctgtcagtgcagaaatttt

gaccaatattgccaggcgttctggtcaggagattgcgaaagagcccaggttccttttga

atggttgtgctggttttgttatgatgattcccacccctacccatggttgttgcaaggatc

ctctctgcgtacccaccattggagcgttttgcgttcaggagcatgcgtcaaactgttgc

tgttggtgttatcaagaacgttgtcaagaaggacccaactggtgctatggtcac<u>caagg</u>

<u>ctgctcagaagaagaaatgaacgttgcagttcaattctggtgttactcgtgctatctac</u>

atcaatatagtgtgttttggagacattgttttctctgcttgtggtgttttttgtacggtc

==tttaa==tgtggttcttaggttcttgttttctgctgtg<u>tatgtctggaaccagctctcaga</u>

gctgggtgcttgacaggcggtggcgaggaaaactttgccggctctgctcgtattttctt

ttgaggtaccttggttatccaaggtggctcggaagttttgtttgttatgttgtcttaag

atactgaattccgaacttaaaaaaaa

NtEF1α-qPCR-F1: CAA GGC TGC TCA GAA GAA GA   Tm=62.5

NtEF1α-qPCR-R1: TGA GAG CTG GTT CCA GAC AT   Tm=62.4

PCR product: 180bp

Figure A 2.3: *NtEF1α* **gene sequence. The shaded region denotes the coding region. The underlined regions denote the primer binding sites, and the primer sequences are also shown (Table 2.2).**

Appendix 5.3: Alternative measures of water use efficiency
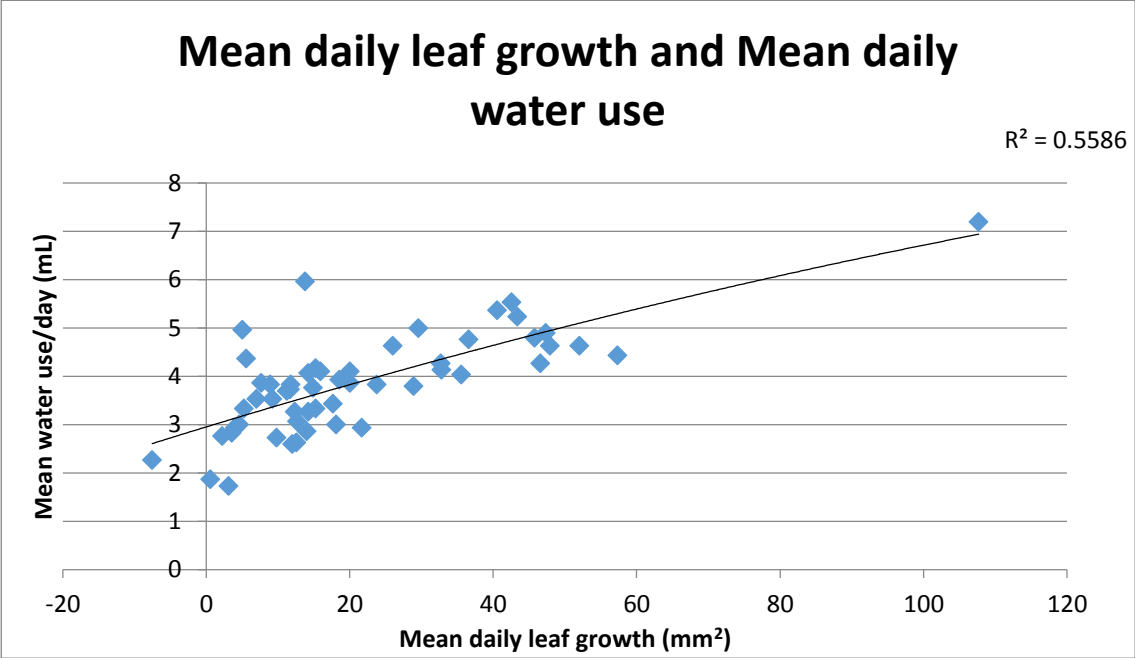
Figure A3.1: Comparison of mean daily leaf growth (mm^2) plotted against average daily water use (mL) generated from data collected during WUE Trial 2 (sections 2.2.3.2 and 3.3.6.2).
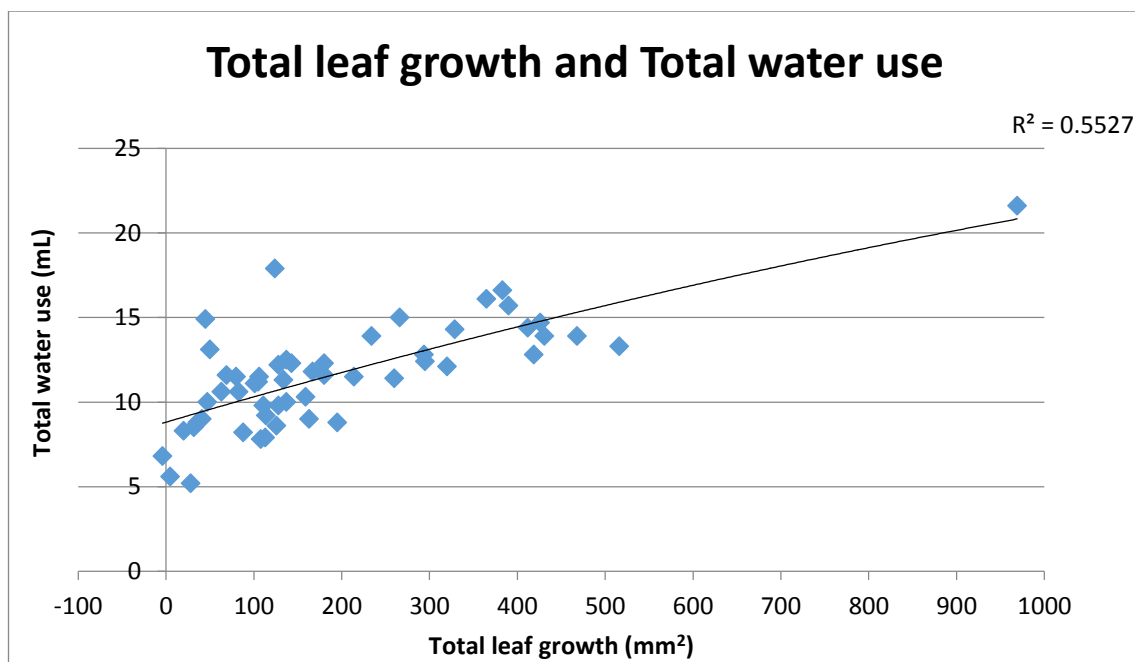
Figure A3.2: Comparison of total leaf growth for all four days of experimentation (mm^2) plotted against total amount of water used (mL) generated from data collected during WUE Trial 2 (sections 2.2.3.2 and 3.3.6.2).
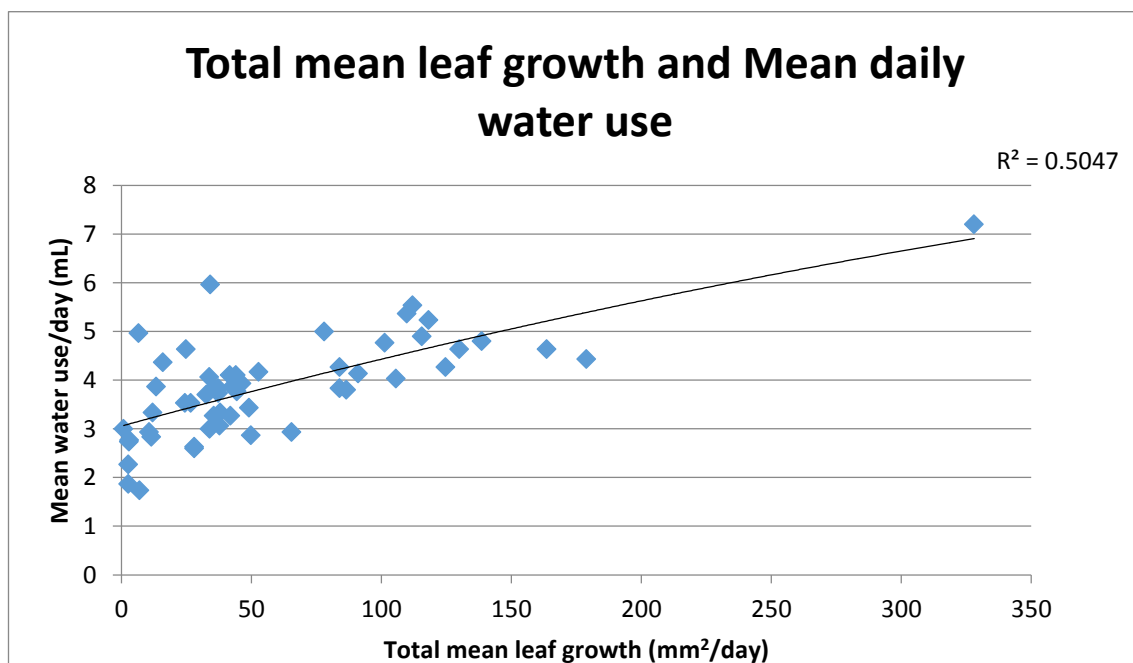

Figure A3.3: Comparison of total mean daily leaf growth (mm^2/day) for all four leaves over all days of experimentation plotted against mean daily water use per day (mL/day) generated from data collected during WUE Trial 2 (sections 2.2.3.2 and 3.3.6.2).

Appendix 5.4: Additional WUE testing

Additional testing was carried out on progeny of lines NT5, NT8 and NT12 and compared to a wild type control by Jason Wargent of Massey University, Palmerston North. These tests focussed on analysing water use efficiency, photosynthetic rate, stomatal conductance, internal CO_2 concentration and transpiration rate of each line and all lines as a whole compared to the wild type control.
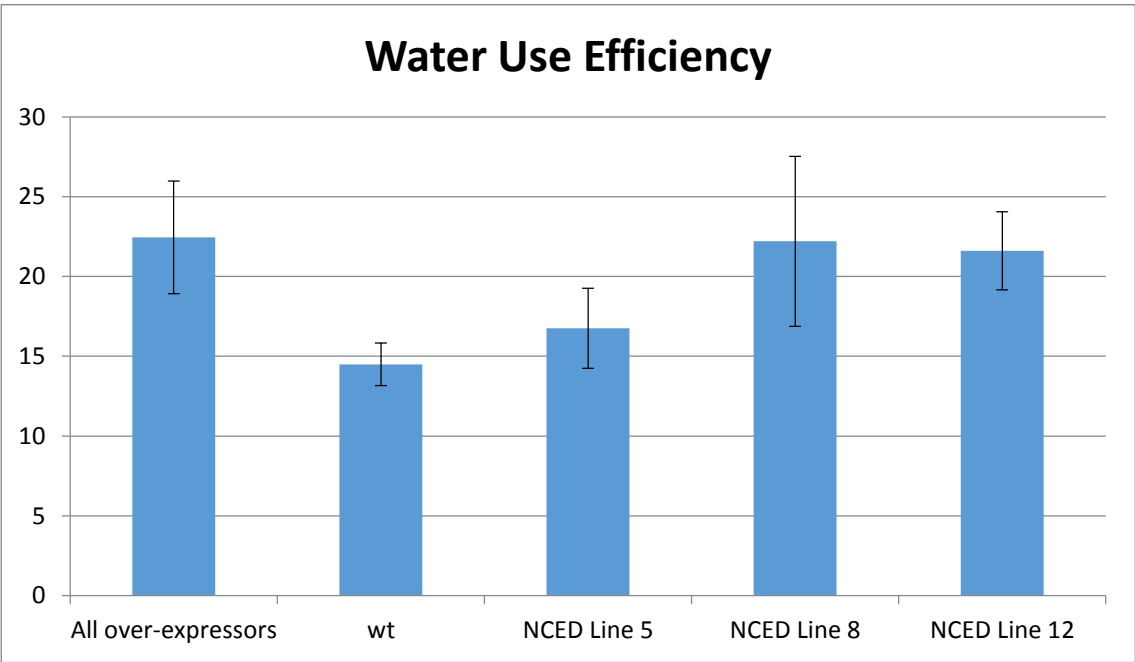

Figure A4.1: Water use efficiency of bulked T_0 line progeny, as indicated, compared with wild type control
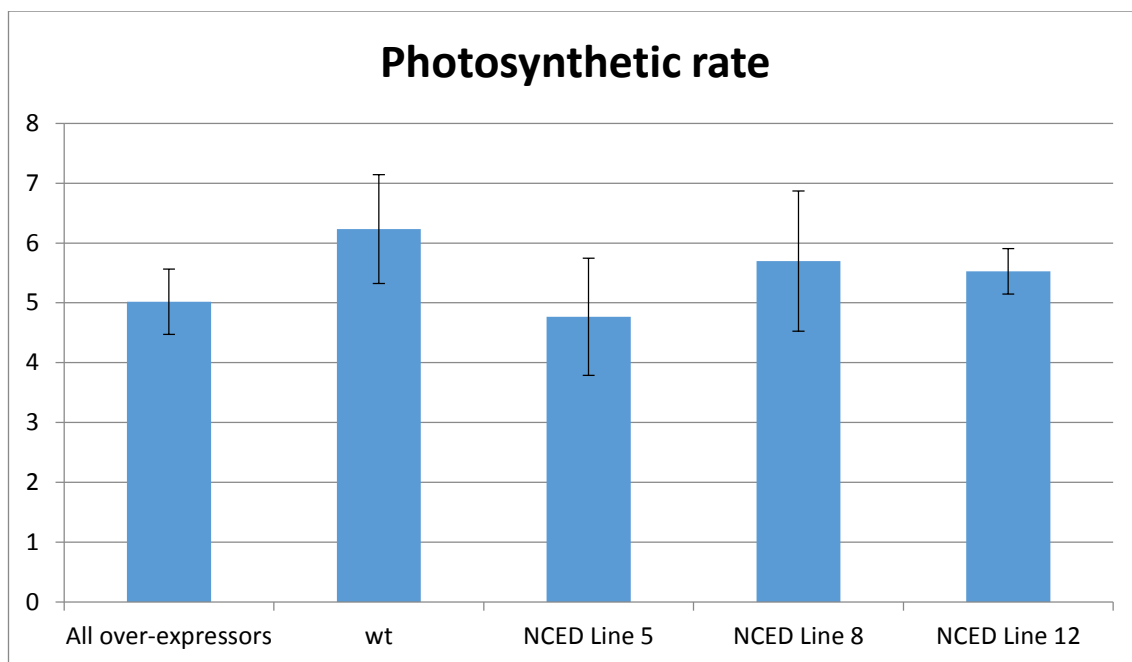
Figure A4.2: Photosynthetic rate of bulked T$_0$ line progeny, as indicated compared with wild type control
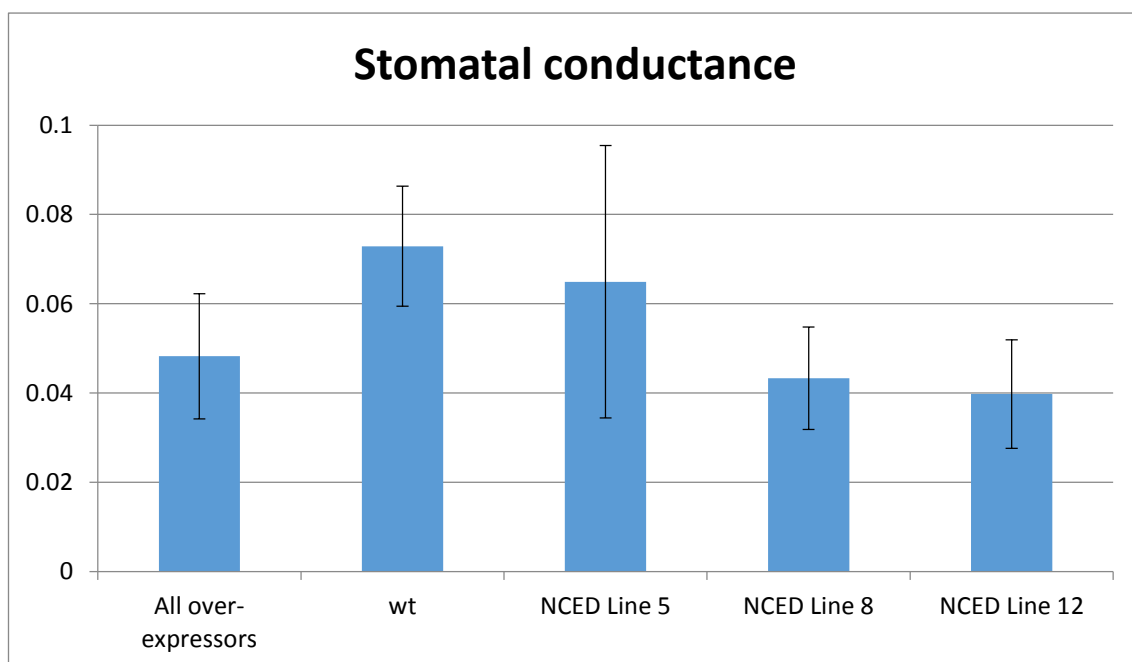

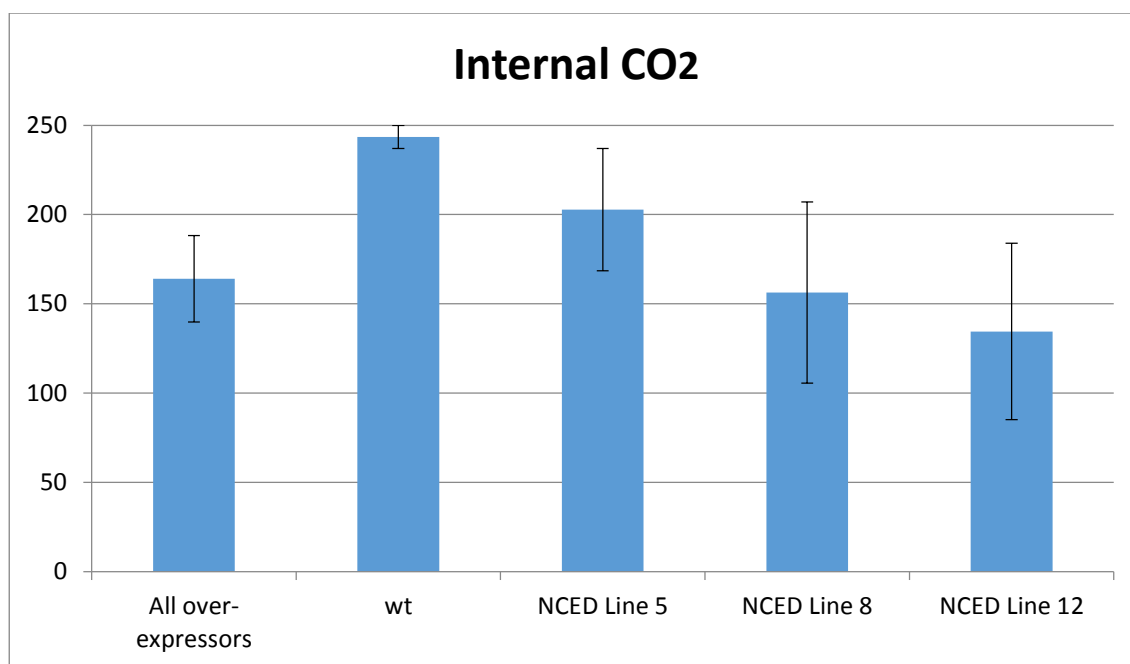**Figure A4.3: Stomatal conductance of bulked T$_0$ line progeny, as indicated, compared with wild type control**

Figure A4.4: Internal CO_2 concentrations of bulked T_0 line progeny, as indicated, compared with wild type control
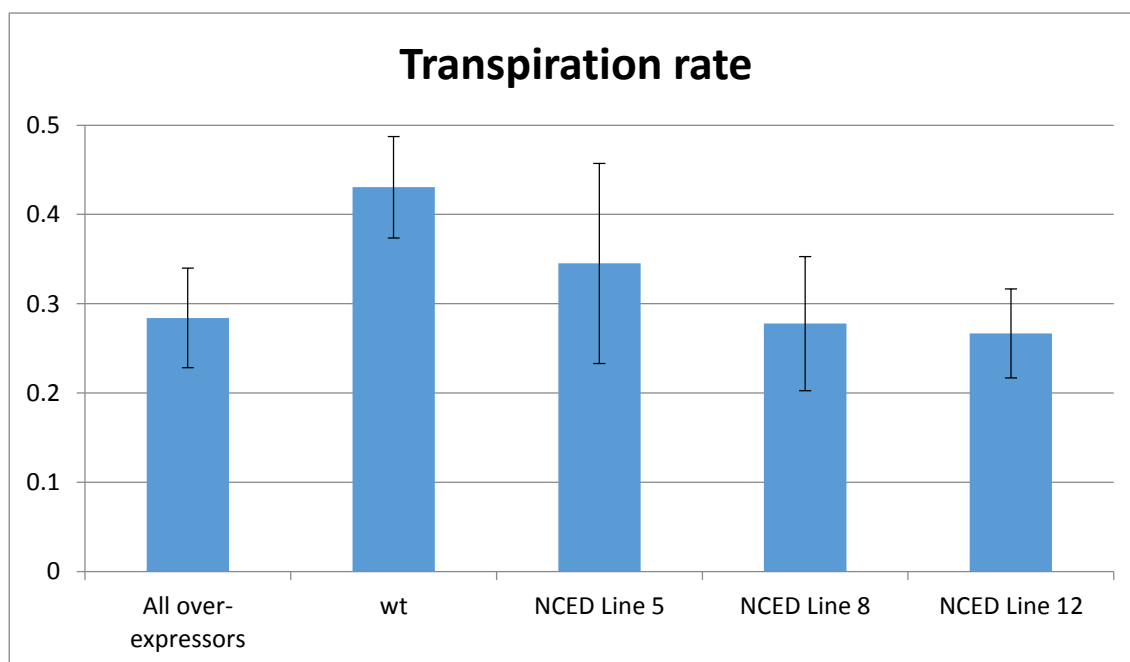

Figure A4.5: Transpiration rate of bulked T_0 line progeny, as indicated, compared with wild type control

Appendix 5.5: Analysis of abscisic acid content

Table A5.1: Analysis ABA and ABA catabolites content in the T_0 tobacco lines used in this thesis

|	ABA	DPA	ABAGE	PA	7'OH-ABA	neo-PA	t-ABA	Total
WT 1 ID:83	673	1789	1889	3920	12	21	9	8313
WT 2 ID:79	1261	3185	3659	3977	23	-	14	12119
WT 3 ID:84	1051	2168	2104	5585	13	-	8	10930
SSUp :: NCED T 2	1228	962	1450	3782	14	21	11	7467
SSUp :: NCED T 5	889	1385	1526	5168	-	39	21	9028
SSUp :: NCED T 7	1399	2191	1806	9695	-	60	9	15159
SSUp :: NCED T 8	4477	9642	15506	24239	21	161	15	54063
SSUp :: NCED T 9	1636	1693	2897	6002	7	37	4	12276
SSUp :: NCED T 12	1147	2338	1740	5962	-	43	52	11282
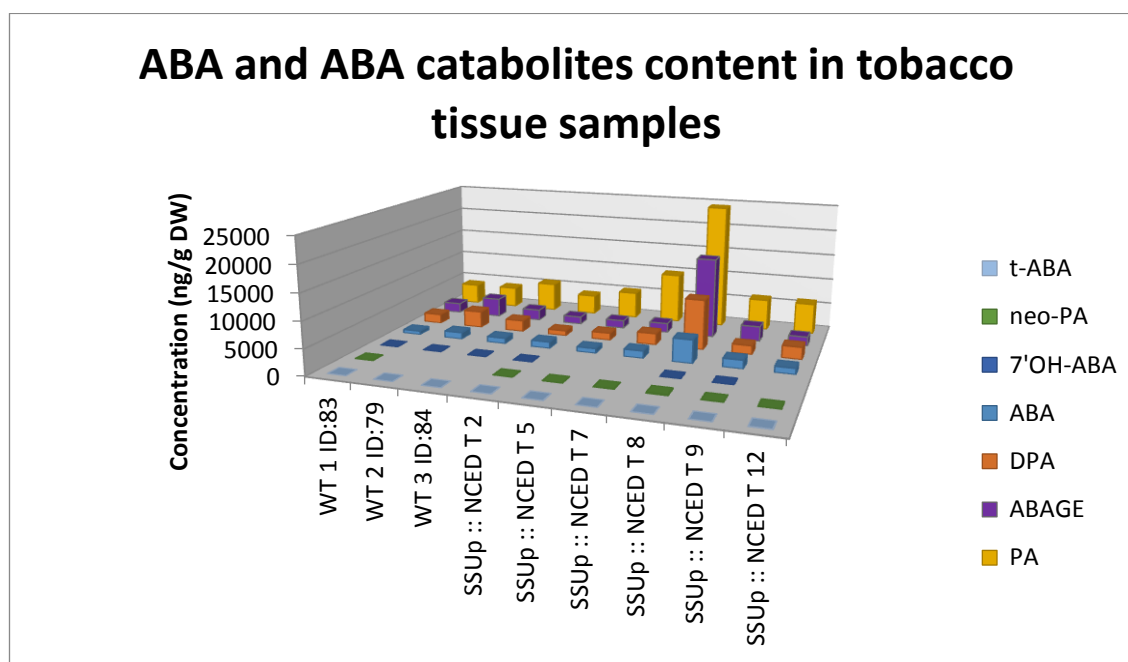
Figure A5.1: ABA and ABA catabolites content in the T_0 tobacco lines used in this thesis