Evaluation of drought tolerance in genetically modified *Lolium perenne* L. and *Arabidopsis thaliana*

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Ubiquitin like proteins (UBLs) are known to have different functions including a potential role in drought tolerance of perennial ryegrass (*Lolium perenne* L.). UBLs are related in tertiary structure to ubiquitin (UB) and they both serve as regulators of many cellular processes. In previous work over-expression of one UBL, the Lolium perenne L. *Ubl5* (*LpUbl5*) homologue in perennial ryegrass resulted in plants with enhanced drought tolerance. Other studies in the nematode *Caenorhabditis elegans* have associated *Ubl5* with a mitochondrial unfolded protein response (UPRmt), and studies in yeast and human cells suggest a role in alternative splicing for *Ubl5*.

This PhD study aimed to characterize the *LpUbl5* gene in the context of its potential role in drought tolerance using *LpUbl5*-overexpressing perennial ryegrass. The study extended to include *Arabidopsis thaliana* transformed using *LpUbl5* to support the findings. Subsequently subcellular localization of *Lp*UBL5 protein was investigated to identify its potential role in alternative splicing. The study also screened different perennial ryegrass accessions from diverse backgrounds to identify germplasm with potential tolerance under drought stress.

The results rejected any role of the *LpUbl5* gene in drought tolerance of perennial ryegrass and *A. thaliana.* A potential seed lethal phenotype was observed in *Ubl5* mutants of *A. thaliana*. The study found co-localization of the *Lp*UBL5 protein into the cytoplasm and nucleus of *Nicotiana benthamiana* (tobacco) leaves and *Allium cepa* L. (onion) cells. This supports its potential role in alternative splicing. Importantly, the study also identified a germplasm accession of *Lolium perenne* L. from Norway with increased drought tolerance in a controlled environment.

The results generated from the *LpUbl5* overexpressing perennial ryegrass lines emphasize the need to integrate critical evaluation of plant lines in their developmental stages especially in out crossing species. Lack of physiological evidence rejected the potential role of *LpUbl5* in drought tolerance. However, the localization of *Lp*UBL5 indicates its functional significance as UBL5 homologues from vertebrates, yeasts and plants and exhibits high levels of protein sequence conservation. It is conceivable that UBL5 homologues possess similar functions across species. The indications of a potential seed-lethal phenotype of mutants is consistent with findings in humans, yeast and *C.elegans*.

Future research is recommended to explore and confirm the potential seed lethal phenotype of *Ubl5* mutation in plants using *Arabidopsis*. This may identify the phenotype characters of *Ubl5* in plants. Since cultivar development is an integral part of pasture development, the genetic diversity of Norwegian accession provides a candidate to develop perennial ryegrass plants with enhanced drought tolerance.

Keywords: Ubiquitin (Ubs), Ubiquitin-like proteins (UBLs), Ubiquitin like protein 5 (UBL5), Homologous to ubiquitin 1 (HUB1), *Arabidopsis thaliana*, alternative splicing, drought tolerance, Sub-cellular localization, germplasm accessions, mutants.

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Dedication

This PhD is lovingly dedicated to my husband, Tennis Thomas and my daughter, Riya Mary Tennis. Truly thankful for their support, encouragement and constant love.

Table of Contents

List of Tables

List of Figures

Chapter 1

Introduction

Perennial ryegrass (*Lolium Perenne L.)* is a self in-compatible diploid (2n=2x=14) out crossing species (Cornish *et al.*, 1979). Plant breeders have also developed tetraploids (2n = 4x = 28) to improve forage quality and produce larger plants with increased water content (Charlton and Stewart, 1999). Perennial ryegrass belongs to the family *Poaceae* which is native to Europe, Asia, and northern Africa (Yu *et al.*, 2013b). Perennial ryegrass is the most widely used pasture species in New Zealand and temperate regions of the world. It was introduced to New Zealand by British migrants before 1820 along with other pasture species. By 1912, New Zealand was capable of producing sufficient seed to support its pasture system (Stewart, 2006) and it is now a major exporter of perennial ryegrass and other herbage seeds.

The main attributes of perennial ryegrass are its high growth rate under fertile conditions, ease of establishment, ease of management, ability to tolerate treading and hard grazing and its ability to grow in conjunction with other pasture species. However, perennial ryegrass fails to thrive under hot dry summer conditions due to its shallow root system (Hunt and Easton, 1989; Charlton and Stewart, 1999; Liu and Jiang, 2010). Drought is the one of the major environmental stresses in agriculture worldwide and is expected to intensify in future (Jewell *et al.*, 2010; Prudhomme *et al.*, 2014). Drought is a major abiotic stress that constrains the yield and quality of most-popular cool season perennial ryegrasses (Mir *et al.*, 2012; Jonavičienė *et al.*, 2014). Researchers have taken four different approaches to improve the ability of plants to adapt to drought. These are conventional breeding, marker assisted breeding, transgenic and genome editing approaches (Ashraf, 2010; Shi *et al.*, 2017).

This research focuses on a transgenic approach to improve the drought tolerance of perennial ryegrass. Forage Scientists at ViaLactia Biosciences (NZ) Ltd under the aegis of Pastoral Genomics have identified *Homologous to Ubiquitin 1* (*Hub1*) also known as *Ubiquitin Like protein 5* (*Ubl5*) as a potential candidate gene for drought tolerance through serial analysis of gene expression (SAGE)(Sathish *et al.*, 2007). The scientists used a cis-genic approach by genetically modifying the ryegrass plants using the *Ubl5* gene taken from ryegrass (*LpUbl5*) plants. The cultivar used as a parent line for transformation purposes was 'Grasslands Impact', a late flowering diploid certified as a NZ hybrid (Charlton and Stewart, 1999).

Research was also initiated at the Department of Crop Science, North Carolina State University, Raleigh, USA, where amenity ryegrass was transformed with a construct capable of overexpressing

LpUbl5 to screen for drought tolerance. A recent study indicated that over-expression of *LpUbl5* improves drought tolerance in perennial ryegrass (Patel *et al.*, 2015).

The research reported in this PhD was designed to identify the potential molecular mechanism through which these transgenic ryegrass plants exhibited improved drought tolerance. The study was conducted at Lincoln University, NZ using genetically modified seeds supplied by ViaLactia Biosciences in association with Pastoral Genomics, NZ. This project aims to evaluate the physiological basis of any drought tolerance in genetically modified *Lolium perenne L*., and a range of germplasm accessions. The hypothesis underlying this aim is that the genetically modified plants that over-express *LpUbl5* would tolerate, adapt and overcome the imposed drought through physiological, metabolomics or genetic changes that occur during the growth period. Screening a wide range of germplasm was included to see if this process could identify a naturally occurring accession with drought tolerance that could be used as a potential candidate for plant breeders. It would then combine the best features to generate a drought tolerant ryegrass through breeding programs. Based on these concepts the following research objectives were tested.

1.1.1 Research Objectives

I. Evaluate the expression level of *LpUbl5* in wild type and transgenic plants under drought and recovery periods and critically examine the presence or absence and level of *LpUbl5* transcripts at each stage. Critically evaluate the physiological response of *LpUbl5* when over-expressed in comparison with non-modified control.

II. Modify *Arabidopsis thaliana* using *LpUbl5* and study the physiological response of *Arabidopsis* under drought and establish the expression level of *AtUbl5* under drought. Identify *Arabidopsis Ubl5* mutants and study the phenotypic characters of *Ubl5* mutant plants.

III. Examine the localization of *LpUbl5* in *Nicotiana benthamiana and Allium cepa* L.

IV. Evaluate the physiological performance of accessions under imposed drought cycles and study the expression level of *LpUbl5* in these non-modified perennial ryegrass lines in the context of drought.

1.1.2 Design of Chapters

This thesis is written in seven chapters that outline progress through the research program. How they fit together is illustrated in Chapter 1. Chapter 2 provides a Literature Review which contains the history of ryegrass research in NZ, current knowledge about perennial ryegrass, drought related studies, and a sequential analysis of research findings about *Hub1/Ubl5* from published research to the start of the present study. Chapter 3 explains the findings from Research Objective l. It outlines

the physiology response of modified perennial ryegrass to drought and determines *LpUbl5* transcript level under drought. The chapter also describes a failure of this research and important implications which necessitated a change in direction of this study. This specifically indicates the need for Objective ll. Chapter 4 explains the method of genetic transformation of *Arabidopsis* using *LpUbl5,* experiments to screen the plants for drought tolerance, efforts to generate *Arabidopsis Ubl5* mutants and the corresponding results. Chapter 5 portraits the sub-cellular localization procedures of *LpUbl5* in *Nicotiana benthamiana* leaves and *Allium cepa* L. and the results indicating the potential role of *LpUbl5* in plants. Chapter 6 describes methods used for screening 10 germplasm accessions for drought tolerance and results obtained which were validated via a glass house experiment. The final Chapter 7, integrates all the results from the four experimental chapters to discuss limitations of the transgenic approach and propose a potential candidate ecotype for drought tolerance to be incorporated in New Zealand plant breeding programmes.

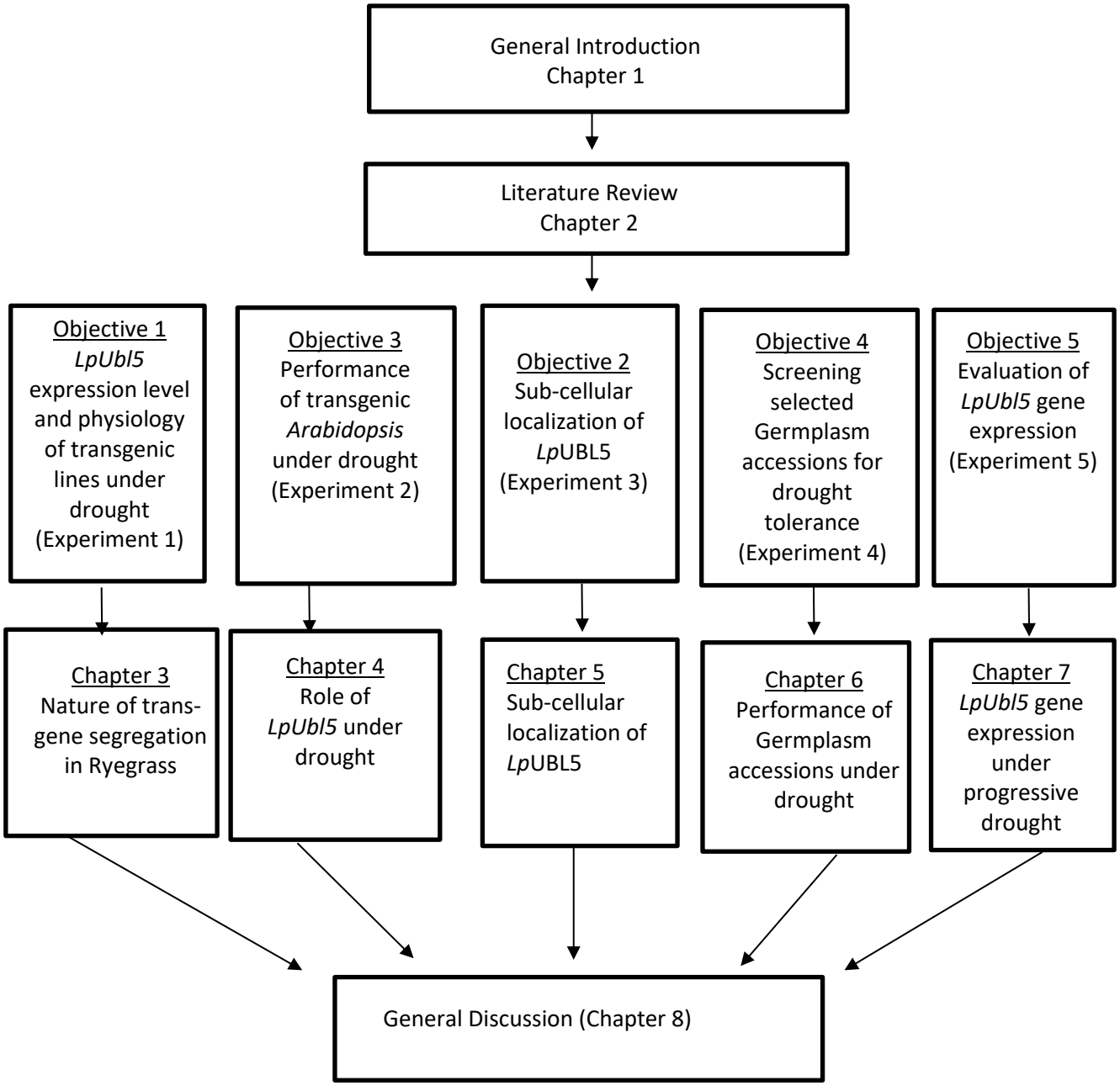


Figure 1-1 Thesis structure

Chapter 2

Literature Review

2.1 Drought

Drought is an important environmental stress, of international interest. The Ministry of Primary Industries (MPI) definition of drought is "When the lack of rainfall has economic, environmental and social impacts on farming business and families and the wider community". The agronomic concept of drought is defined as "The state of the climate system that creates abnormally dry weather, prolonged enough for the lack of rainfall to cause serious hydrological imbalances" (MPI, 2013). Drought related research becomes a prime priority in dryland farming systems when summer moisture stress affects pasture production (Barker *et al.*, 1985). The yield of plants reduces significantly under abiotic stresses, and drought is the major abiotic stress prominent and widespread across the world (Akpinar *et al.*, 2013). Drought has been one of the major factors constraining crop yield and the main focus of several studies in recent years (Chaves and Oliveira, 2004; Marulanda *et al.*, 2007; Atkin and Macherel, 2009; Munoz-Mayor *et al.*, 2012).

2.2 Drought resistance

Drought resistance is defined as "The ability to oppose drought successfully and to prevent the effects of, and be proof against, water deficit" (Lawlor, 2013). The definition states that a true drought resistant plant is one that is unaffected or which can withstand water deficit. Every drought related study considers drought resistance as a quantitative trait expressed relative to a control value. Four different modes of drought resistance are drought escape, drought avoidance, drought tolerance, and drought recovery or survival (Lawlor, 2013; Zhou *et al.*, 2016). Drought escape, is a rapid development in accordance with water availability that is sufficient for potential production mainly in annual plants (Lawlor, 2013). Rapid phenological development and developmental plasticity are the two important mechanisms by which plants escape drought. The former involves rapid plant growth and reduction in seed production before soil water becomes deficient. The latter involves reduction in plant growth with very few flowers and seeds under water depletion and shows remarkable growth and seed production under favourable conditions (Basu *et al.*, 2016). Drought avoidance is prevention of dehydration or maintenance of water status by restriction of water loss by reducing leaf area (LA) and stomatal conductance (g_s) (Lawlor, 2013; Kooyers, 2015) , increased root length to exploit the available water in the soil (Huang and Fry, 1998) and storage of water in the plants tissues (Malinowski and Belesky, 2000; Shao *et al.*, 2009). Drought survival is the ability of the plants to minimize gas exchange by reduced leaf growth to retain vital cellular functions and protect the meristematic tissues to recover

promptly to water availability and restoration of cellular activities with limited damage (Volaire *et al.*, 2009). Drought tolerance is the ability of the plants to tolerate a reduction in tissue water potential via adaptive traits such as increasing protoplasmic resistance and maintenance of cell turgor (Basu *et al.*, 2016). According to Lawlor (2013), drought tolerance is "the ability of a plant to sustain or bear drought without suffering and it often refers to metabolic mechanisms that maintain growth and production unimpaired by water deficit. This form of drought resistance is referred to as a "holy grail" of current plant science and genetic modification technology". Temperature elevation and lack of water availability have motivated scientists to improve plant water use and drought tolerance (Park *et al.*, 2015). Conventional breeding, marker assisted breeding and a genome editing approach are the three different methods to generate drought tolerance in plants (Ashraf, 2010).

2.2.1 Drought: signal perception

Drought leads to osmotic stress, which affects cell division, elongation and arrests growth. Plant growth is determined by cell division which is the principal element of the meristem activity, and cell cycle arrest occurs under osmotic stress in plants (Figure 2-1). Water availability is one of the external stimuli that influences cell expansion mainly determined by cell wall extensibility and cellular osmotic potential (Bartels and Sunkar, 2005). Our knowledge about an 'osmosensor' by which plants sense the osmotic challenges is limited (Zhu, 2002; Bartels and Sunkar, 2005; Haswell and Verslues, 2015). Osmosensing in plants cells are explained using three general models. First model by activation of Histidine kinase osmotic imbalance across the plasma membrane which initiates a signal transduction pathway. Second model, is opening of mechanosensitive channels in response of plasma membrane tension which facilitate the flow of osmolytes across the membrane. Third model is monitoring the cell wall integrity by sensing the movement or disruption of cell wall components potentially by receptor like kinases (Haswell and Verslues, 2015).

2.2.2 Drought: signal tranduction

Unlike osmosensing, signal transduction has been explored to a greater extent (Bartels and Sunkar, 2005; Haswell and Verslues, 2015) and the major pathways include mitogen activated protein kinase (MAPK) cascades, sucrose non-fermenting-1- like kinase (SNF-1-Like Kinases) signalling, phosphatases signalling, and phospholipid signalling (Bartels and Sunkar, 2005). MAPK cascades are highly conserved signal transduction modules in plants, animals and yeast (Jonak *et al.*, 1996). The cascade contains three consecutive protein kinases such as MAPK kinase kinases (MAPKKKs), MAPK kinases (MAPKKs) and MAPKs (Shen *et al.*, 2012; Cai *et al.*, 2014). The dual phosphorylation of conserved threonine and tyrosine residues of MAPKKs or MAPKKKs activates MAPK and MAPKKs activates themselves by MAPKKKS by the phosphorylation of conserved serine and/or threonine residues (Nakagami *et al.*, 2005; Wang *et al.*, 2016a).The phosphorylated MAPK activate the expression of stress-resistance

related genes or transcriptional regulation of target genes which stimulate a stress response (Sinha *et al.*, 2011; Shen *et al.*, 2012). Sucrose non-fermenting 1 (SNF1)-related protein kinase2 (SnRK2) is classified into three groups based on the activation status by Abscisic acid (ABA). Groups of kinases that are not activated by ABA are group 1, group 2 is not activated or weakly activated and group 3 are kinases that are strongly activated by ABA (Saruhashi *et al.*, 2015). ABA dependent SnRK2s pathways occur by direct phosphorylation of downstream targets and transcription factors which contribute to the expression of various stress response genes (Kulik *et al.*, 2011). Phosphatases are ubiquitous enzymes which are grouped based on their substrate specificity. The groups are serine/threonine specific protein phosphatase (PPP), tyrosine specific protein phosphatases (PTPs) and dual-specificity protein Tyr phosphatase (DsPTP) classes (Boudsocq and Laurière, 2005). Several subfamilies of PPP such as PP1, PP2A, PP4, PP5, PP6, PP7, PP2Cs have been identified in plants in which the role of PP2A in plant stress responses has been studied to a greater extent when compared with other subfamilies in this group (País *et al.*, 2009). Phospholipid signalling is hypothesised to initiate with cleavage of phospholipids by phospholipases and the four classes of phospholipases are phospholipase C (PLC), phospholipase D (PLD), and phospholipase A1 and A2 (PL A1 and PL A2) based on their site of cleavage. Inositol 1, 4, 5-triphosphate (IP3), diacylglycerol (DAG) and phosphatidic acid (PA) are the major phospholipid-derived signalling molecules (Bartels and Sunkar, 2005; Xue *et al.*, 2009; Ruelland *et al.*, 2015). Lipid signalling cascade involves the expression of gene clusters and thereby supports plants stress adaptation processes (Hou *et al.*, 2016).

2.2.3 Drought: Calcium signalling

Calcium signalling in response to osmotic stress is another well documented area of research. Calcium is designated as a second messenger which couples extracellular stimuli to intracellular responses (Bartels and Sunkar, 2005). Temporal and spatial changes in cytoplasmic calcium (Ca^{2+}) are evoked by various signals involved in abiotic, biotic stress and developments. The nature of these inducing stimulus defines the intensity, duration, amplitude and frequency of the cytoplasmic calcium levels and these cytoplasmic calcium elevations are called calcium signatures (Zheng *et al.*, 2016). The elevation of calcium levels (Ca^{2+}) in the cytoplasm occur in response to stimulus by glutamate receptor-like channels (GLRs), cyclic nucleotide-gated channels (CNGCs), annexin transporters, two-pore Ca^{2+} channels (TPCs) (Peiter *et al.*, 2005; Kudla *et al.*, 2010; Steinhorst and Kudla, 2013). The calcium signatures are then detected and transduced by different calcium binding proteins such as calmodulin (CaM), calcium sensing receptor (CaSR) calreticulin (CRT) calcineurin B (CBLS) and calcium dependent protein kinase (CDPK). These calcium binding proteins translate calcium signatures into specific cellular responses, such as altered phosphorylation and target gene expression (Wang *et al.*, 2016b; Zheng *et al.*, 2016).

2.2.4 Drought: Transcriptional regulation

Stress-responsive transcription is generated by different sets of cis-acting elements and trans- acting elements and different transcription factors are involved in the regulation of stress responsive gene expression (Yamaguchi-Shinozaki and Shinozaki, 2005). The plant hormone ABA, regulates the expression of many drought responsive genes and it regulates the expression of most of the target genes through an ABA-responsive element (ABRE) binding protein/ABRE binding factor (AREB/ABF). Dehydration-responsive element binding protein (DREB), NAM, ATAF and CUC (NAC transcription factor) regulate drought responsive genes in an ABA-independent manner. Other transcription factors involved in the drought response are MYB/MYC, WRKY, and nuclear factor-Y (NF-Y) transcription factors (Li *et al.*, 2008; Shao *et al.*, 2015; Singh and Laxmi, 2015). Two ABA-dependent pathways have been identified based on the presence of cis-elements in the promoters of ABA- inducible genes. The first pathway mediates gene expression through an ABA-responsive element (ABARE) and b-ZIP transcription factor and the second pathway through MYB and MYC elements and transcription factors (Bartels and Sunkar, 2005). ABARE is an 8 bP long cis- element, ACGTGG/TC and induces ABA-responsive gene expression in the proximity of other ABRE copies or other cis-acting elements known as coupling elements (Singh and Laxmi, 2015). Dehydration-responsive element-binding proteins (DREB) belong to APETALA 2/ethylene-responsive element binding factor (AP/ERF) and two major subclasses are DREB1 and DREB2 (Mizoi *et al.*, 2012). DREB has a conserved 9-bp direct repeat TACCGACAT and the core motif is underlined (Jaglo *et al.*, 2001; Bartels and Sunkar, 2005). DREB1 transcription factors are involved in cold stress response while DREB2 are responsive in drought stress through pathways mediating via regulation of 14 direct target genes, such as LEA class proteins, RD29A (Sakuma *et al.*, 2006; Budak *et al.*, 2013). Apart from these, NAC transcription factors regulate drought stress responses via ABA-dependent and ABA-independent pathways, an example of cross talk between these major pathways (Budak *et al.*, 2013).

2.2.5 Drought: Compatible solutes and protective proteins

Drought leads to accumulation of compatible solutes and protective proteins which enables the plants to tolerate, avoid or survive. Compatible solutes such as sugars, sugar alcohols, proline and protective proteins such as Late embryogenesis abundant (LEA) protein, aquaporin and heat shock protein (Budak *et al.*, 2013). Compatible solute accumulates at high concentration in the cytoplasm without affecting the cellular structure and metabolism (Bowlus and Somero, 1982; Mohammadkhani and Heidari, 2008). They provide protection to plants from stress by contributing osmotic adjustment, reactive oxygen species (ROS) detoxification, and protection of cellular structures and adaptive value in metabolic pathways (Hayat *et al.*, 2012). Compatible solutes are categorized as hydroxy compounds such as sugars, sugar alcohols and nitrogen-containing compounds such as proline and other amino acids (Mohammadkhani and Heidari, 2008). Correlation between osmotic stress tolerance and

accumulation of sugar content such as sucrose, hexose, and sugar alcohols in different plant species indicates the important role of these compounds. These sugars stabilize membrane structure by protecting specific macromolecules, or protect cells by forming cytoplasmic glasses during desiccation or prevention of membrane fusion by interacting with polar head groups of phospholipids in membranes (Bartels and Sunkar, 2005).

Since the first report of proline accumulation in perennial ryegrass under wilting (Kemble and Macpherson, 1954), different studies have reported proline under different environmental stresses. This also includes more than 100 fold increase under drought (Verslues and Sharma, 2010). Proline accumulation and root traits have been proposed as a method in selection of drought tolerance (Mwenye *et al.*, 2016). In plants proline is synthesised mainly from glutamate by the pyrroline-5-carboxylate synthetase (P5CS) enzyme and P5C reductase (Szabados and Savouré, 2010). Proline acts as an osmolyte to mitigate water stress, balancing turgor pressure and cryoprotectants. Proline acts as a chaperone, by enhancing and stabilizing redox enzymes and proteins and also acts as a reactive oxygen species (ROS) scavenger and as a singlet oxygen quencher (Szabados and Savouré, 2010; Liang *et al.*, 2013).

LEA proteins 10-30 kDa are formed during the late period of seed development which consists of seven groups (group 1-7) based on their amino acid sequence and mRNA homology (Hong-Bo *et al.*, 2005; Amara *et al.*, 2014). LEA proteins reduce the protein aggregation in the dehydrated cytoplasm by decreasing the interaction between partially denatured polypeptides. These proteins acts as membrane protector and also use their charged amino acid residues to sequester ions and decrease the concentration of intracellular components under dehydration. LEA proteins reduce the rate of water loss by acting as hydration buffers under drought to allow sufficient water activity for proteins to retain their function (Amara *et al.*, 2014; Fang and Xiong, 2015).

Heat shock proteins (Hsps) range from 200-800 kDa, and are classified into five different classes based on their molecular size (Reddy *et al.*, 2014; Wang *et al.*, 2014). HSP functions as molecular chaperons which prevents mis-folding and thermal aggregation of substrate proteins and facilitates the subsequent reactivation (Bartels and Sunkar, 2005; Al-Whaibi, 2011). Aquaporin superfamily (26–34 kDa) have four major groups and is reported to have a central role in plant water relations (Tyerman *et al.*, 2002). The aquaporin has an important role in the water balance and water use efficiency mediated through opening and closure of cellular gates (Li *et al.*, 2015a). It is also known to be the most abundant transmembrane transporter of urea, glycerol, metalloids, reactive oxygen species (ROS), CO_2, NH_3 and water. To-date a number of aquaporin encoding genes have been identified in different species ranging from 31 in maize to 66 in soya bean. Aquaporins main roles include plant cell

osmoregulation, root hydraulic conductivity (Lpr), leaf hydraulic conductivity, transpiration and cell elongation as a potential plant water regulator (Afzal *et al.*, 2016).

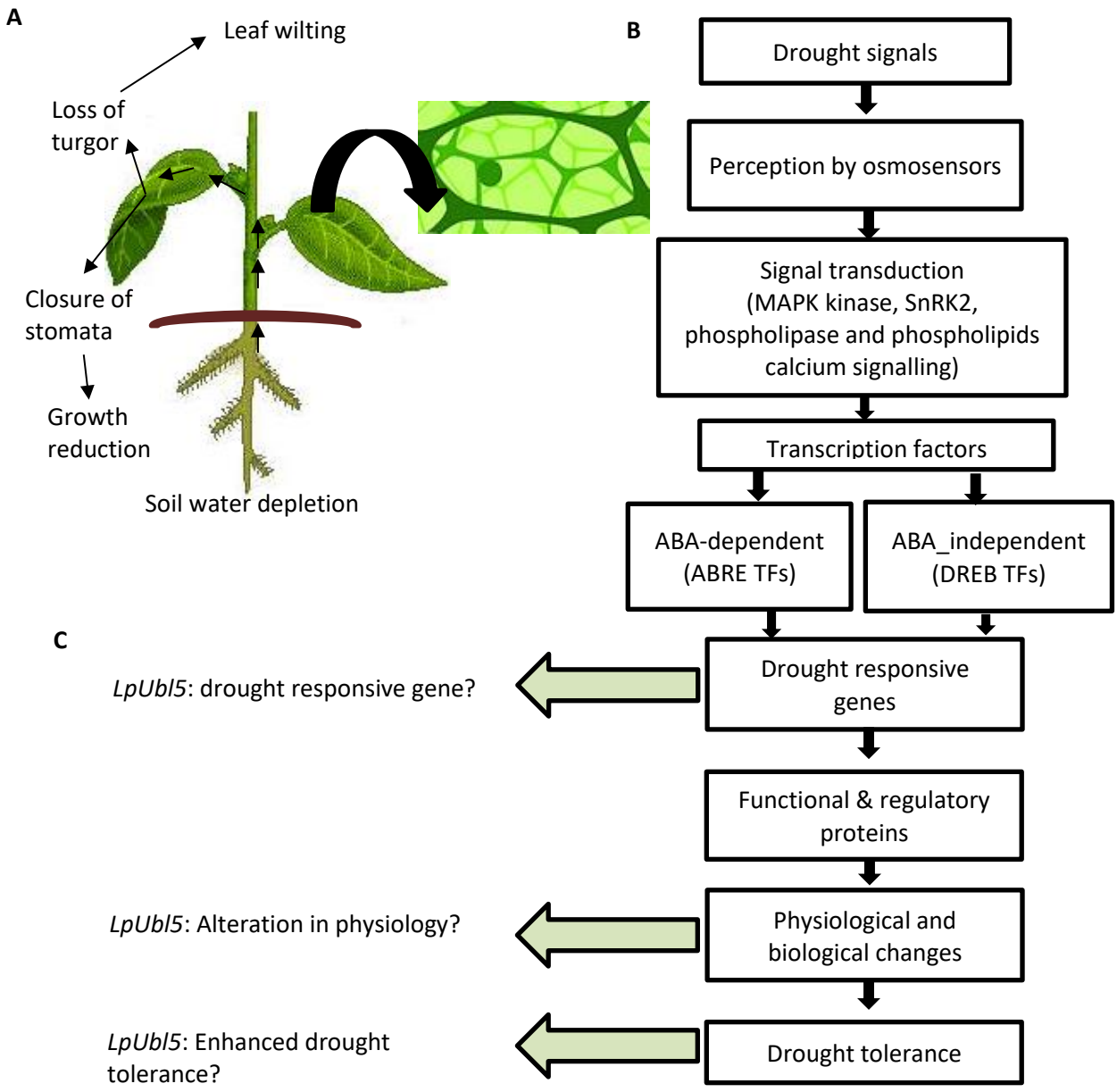


Figure 2-1 Schematic representation of plants responses under water deficit conditions. A. Highlights important causes of growth reduction in plants under drought stress. B. Outlines important molecular mechanisms which regulate stress-responsive gene expression in plants under abiotic stress C. highlights important research questions raised in this study. The basic diagram was adapted from dos Reis *et al.* (2016).

2.2.6 Drought: plants mechanisms to avoid, tolerate or resist

Plants incorporate different mechanisms of drought resistance to fight drought or water deficit conditions. Plants incorporate modulation of root architecture, leaf characters, photosynthesis, osmotic adjustment (OA), antioxidants, and phytohormones to avoid, tolerate or resist the water deficit conditions experienced (Fang and Xiong, 2015). Root architecture comprises important traits,

volume, length and thickness of roots, root penetration, and the root/shoot ratio which contributes to cope with water deficits. Plants have been shown to increase root growth to absorb water from deep soil in the early stages of drought stress (Hu and Xiong, 2014). Root growth increases under early stages of drought (Fang and Xiong, 2015). A significant increase in the surface area of roots when compared with root length was observed in wheat under moderate water deficit (Saidi *et al.*, 2010) and root number was increased in perennial ryegrass a month after the onset of drought (Wedderburn *et al.*, 2010). An extensive root system helps plants to maintain greater water potential and increase the duration of transpiration under water deficit conditions to support growth and development (Fang and Xiong, 2015). Under water deficit, partitioning of the photo assimilates is altered which results in a reduction in shoot growth but maintenance of root growth (Waseem *et al.*, 2011).

Leaf responses to water deficit conditions are mainly leaf wilting, a reduction in leaf area, leaf abscission and shedding to reduce the water loss by transpiration (Waseem *et al.*, 2011). The phenotypic leaf traits include plants with smaller leaves observed in plants adapted to dry environments and hairy leaves to reduce the leaf temperature and transpiration which supports plants under drought stress (Farooq *et al.*, 2009). Under drought or water deficit conditions, morphological and physiological responses of leaves are crucial to reduce the water loss and increase water use efficiency (WUE). Leaf wilting is a mechanism by which plants reduce transpiration rate. It is a passive movement by which plants prevent excess water loss under water deficit and also plants maintain their leaf blades parallel to the solar radiation by rolling which can regulate the intercepted solar radiation (Ludlow and Björkman, 1984; Fang and Xiong, 2015). Leaf rolling is a common plant response to water depletion which is activated by turgor pressure and is considered an adaptive trait (Hsiao *et al.*, 1984; Oosterhuis *et al.*, 1985). Plants under water deficit can maintain leaves vertical to the solar radiation to reduce the amount of radiation received which results in a reduction in water loss (Stevenson and Shaw, 1971; Meyer and Walker, 1981). In ryegrass, leaf senescence rate (LSR) and leaf elongation rate (LER) was reduced under water deficit and LSR was higher than LER which indicates a different adaptation strategy of plants (He *et al.*, 2017a). Plants increase water absorbability and transportation using xeromorphic structures such as small leaf cells with thick cell walls, more epidermal trichomes, numerous small stomata, dense cuticle epidermis and palisade tissue, more developed vascular bundle sheaths and increased lipid accumulation or thick wax. These help to reduce transpiration, reduce leaf temperature, protect from light irradiation which effectively avoids damage from exposure to water deficit by reduction of water loss and enhancing the water holding ability (Zhu *et al.*, 2011; Fang and Xiong, 2015).

Reduction in stomatal conductance is a systemic response under water deficit (García-Mata and Lamattina, 2001). Since Heath (1950) observed reduction in CO_2 concentration induce stomatal opening, research in stomatal behaviour became important to crop production, biodiversity responses

and particularly in the field of water deficit (Haworth *et al.*, 2011). Stomatal movement balances the CO_2 uptake and water transpiration and is therefore an important response to variation in the environmental conditions. Stomata are the pores on a leaf surface and the area of a stomatal pore is determined by turgor changes in the guard cells (Arve *et al.*, 2011) Broadly they have two morphological types which are dumb-bell-shaped stomata found in grasses and kidney shaped stomata in other species (Hetherington and Woodward, 2003). Rapid changes in guard cell turgor and stomatal conductance, in response to internal and environmental signals is mediated by their complex signal transduction networks and modified metabolic pathways (Vavasseur and Raghavendra, 2005). Several mechanisms and signalling pathways lead to stomatal closure under adverse environmental conditions and can be divided into hydro passive and active stomatal closure. A rapid reduction in the cell water content resulting in reduction in osmotic pressure and lose of turgor pressure causes passive stomatal closure as guard cells fail to maintain their shape. Active stomatal closure is caused by signalling pathways activated by ABA and elevated CO_2 levels (Arve *et al.*, 2011). ABA synthesised in roots under water deficit act as chemical information for the closure of stomata (Zhu *et al.*, 2011). K^+ is one of the major ions which alter the osmotic potential of the guard cells and also results in stomatal movement (Hosy *et al.*, 2003).

Photosynthesis is a key factor in plant production which is the most fundamental and intricate physiological process in all green plants and is regulated mainly by stomata for CO_2/water exchange and photosynthetic activity in mesophyll cells. Two main photosynthetic pathways are C_3 plants species that grow well in cool and wet conditions usually with low light exposure and C_4 plants under hot, dry conditions usually with high light intensity (Ashraf and Harris, 2013). Stomata of the C_3 plants opens during the day for CO_2 absorption and fixation and remain closed during night whereas the C_4 plants have evolved a metabolic pump which concentrates CO_2 in the bundle sheath cells. The CO_2 fixation process in C4 plants occurs in bundle sheath cells and mesophyll cells separately and this contributes to increase WUE when compared with C_3 plants. However photosynthetic metabolism of C_3 and C_4 plants are similarly sensitive to water deficit. A third category is crassulacean acid metabolism (CAM) plants which are best adapted to arid environments and their stomata operate at night, concentrate CO_2 into C_4 acids which then re-fixed to carbohydrates during the day time with closed stomata. This contributes higher WUE and some plants behave as facultative CAM species which can switch their photosynthetic pathway from C_3 to CAM under water deficit conditions (Chaves *et al.*, 2003; Ashraf and Harris, 2013). Five sub-process of photosynthetic metabolism based on the inhibition sites under drought are ribulose 1, 5-biphosphate regeneration capacity, ATP synthesis, leaf photochemistry, ribulose 1, 5-biphosphate carboxylase/oxidase activity (Rubisco) and permanent photoinhibition (Flexas and Medrano, 2002). Under drought, stomatal closure limits the CO_2 diffusion from the atmosphere to suboptimal cavities followed by mesophyll conductance which limits the CO_2 diffusion

from the sub-stomatal cavities to the chloroplast stroma and finally resulting in decreased photosynthetic enzyme activity, ribulose-1,5-bisphophate (RuBP) regeneration capacity and triose-phosphate utilization (Zhou *et al.*, 2015).

Osmotic adjustment is the term used for osmoregulation by which turgor potential is maintained by accumulation of organic and inorganic substances in cytochylema to reduce the osmotic potential and improve water retention in the cells under drought stress and hence continue plants growth. Accumulation of organic substances protect the enzymes and plasma membranes by regulating the plasma osmotic potential whereas the inorganic ions regulate the osmotic potential of the vacuole (Morgan, 1984; Fang and Xiong, 2015). Carbohydrates, amino acids, proline and mineral ions are solutes that accumulate in the temperate Poaceae/ Gramineae and accumulation of proline has been linked with drought resistance (Thomas, 1990). Osmotic adjustment under water deficit conditions benefits processes such as cell elongation and stomatal opening during the day (Johnson *et al.*, 1984). Several studies have been conducted in various species including ryegrass to understand the differences in osmotic adjustment and growth and using various genotype to study plant responses to drought (Johnson *et al.*, 1984; Grumet *et al.*, 1987; Thomas, 1987; Thomas and Evans, 1989; Thomas, 1990, 1991; Jongdee *et al.*, 2002). Many of these studies have linked osmotic adjustment and increased yield under drought whereas no effect on yield (Johnson *et al.*, 1984) or reduced yield (Grumet *et al.*, 1987) were also reported. A recent review of osmotic adjustment and yield under drought stress in different genotypes of 12 crops reported osmotic adjustment as a prime adaptive trait supporting the yield under water deficit conditions (Blum, 2016). These above mentioned features briefly summarise common plant responses to drought. Plant responses to drought will be investigated to understand the effect of over-expression of *LpUbl5* in perennial ryegrass and *Arabidopsis*

2.3 An overview of drought related research in ryegrass

In New Zealand perennial ryegrass research was initiated in 1920 by A H Cockyane and Bruce Levy at the Department of Agriculture in the Biology section at Palmerston North (Hunt and Easton, 1989). A plant research station at Palmerston North was then established in 1928 (Matthew *et al.*, 2012). This enabled development of strains with superior traits through pasture grass testing. A seed certification scheme was inaugurated by J W Hadfield in 1929 (Hunt and Easton, 1989). Currently over 30 cultivars of perennial ryegrass and long-rotation hybrid ryegrass with varying morphological types, endophytes and date of reproductive maturity are available in New Zealand (Charlton and Stewart, 2000). Nevertheless drought tolerance is the limiting factor for growth and development of this cool-season perennial grass (Yu *et al.*, 2013b). Thus researchers have focused on this issue since the 1960s. There are a number of studies from 1960 to date that have investigated drought tolerance through

different experimental regimes. Barker *et al.* (1985) emphasised the importance of drought related research due to an increase in the frequency of droughts since 1968.

Persistence of ryegrass in warmer regions of New Zealand has become a concern (Matthew *et al.*, 2012). A review of literature indicates the volume of research carried out to identify the best practice to maintain ryegrass under drought/water deficit conditions and quest for drought tolerance mechanism, identify the desired breeding traits and identify the germplasm resources for desired traits to employ in breeding for drought tolerance. Soil water extraction studies were available from 1967 in ryegrass swards indicating that ryegrass extracts water from 0.91 metre and below under drought conditions (Garwood and Williams, 1967b). Another study by the same authors analysed nutrient uptake and water use efficiency and indicated that water depletion under dry weather constrains nutrient uptake, limiting ryegrass re-growth (Garwood and Williams, 1967a). Studies were also carried out to identify 'water savers and water spenders' in relation to epidermal ridging in ryegrass which showed deep ridged ryegrass as drought tolerant as an importance criteria when breeding for water deficit (Wilson, 1975). Differences between field grown ryegrass swards and simulated boxes (soil filled boxes) to identify the features of droughted ryegrass leaves upon gradual exposure and sudden exposure to water deficit conditions was also focused (Jones *et al.*, 1980). Research suggests spring management to ensure better root growth to exploit available soil moisture and controlled grazing to increase tiller density (Korte and Chu, 1983). Recovery growth after drought indicated increased leaf extension in plants exposed to drought when compared with well watered plants but leaf appearance rate showed little difference (Norris and Thomas, 1982). Apart from this, plant water relations, sugar partitioning upon re-watering after drought, competences of ryegrass and white clover pastures under drought have been studied to a great extent (Thomas, 1987; Thomas and Evans, 1989; Thomas, 1990, 1991).

Research on root growth patterns under drought and well watered conditions reported an increase in root count under drought and reduced death rate of roots in the deeper soil profile (Wedderburn *et al.*, 2010). Studies also indicate that selection of faster recovery after drought exposure as a promising trait for breeding drought tolerant ryegrass (Westermeier and Hartmann, 2016). The effect of plant growth regulators and application of gamma aminobutyric acid (GABA) in drought responses of ryegrass were also investigated (Jiang and Fry, 1998; Krishnan *et al.*, 2013). Different perennial ryegrass accessions were screened to identify candidate genes with drought tolerance (Liu and Jiang, 2010; Yu *et al.*, 2013b), variation in drought tolerance traits using phenotypic drought traits and amplified fragment length polymorphism (AFLP) data (Jonavičienė *et al.*, 2014). Understanding and relating phenotypic responses under drought to genetic diversity (Cui *et al.*, 2015) have also contributed to drought research in ryegrass. Efforts were also taken to understand the physiological and genetic mechanisms underlying drought, recovery and re-growth following drought by quantitative trait loci

(QTL) (Guthridge *et al.*, 2001; Hatier *et al.*, 2013; Hatier *et al.*, 2014) and DNA methylation of ryegrass under drought (Tang *et al.*, 2014). Ryegrass genes regulated under PEG induced water stress using transcriptional and metabolomics approach have also been reported (Foito *et al.*, 2009).

The symbiotic relationship of endophyte and perennial ryegrass were studied extensively in the context of drought tolerance. Endophyte infection and ryegrass persistence under drought in different genotypes (Hesse *et al.*, 2003), evaluation of ryegrass productivity with and without endophyte infection under drought (He *et al.*, 2017b), drought tolerance of ryegrass cultivars with and without endophyte (He *et al.*, 2017a) and effect of endophyte symbiosis in Mediterranean ryegrass accessions has occurred (Kane, 2011). In addition, analysis of the impact of endophyte symbiosis on the ability of ryegrass to recover after drought (Cheplick, 2004) was also studied. Perennial ryegrass has been also studied in comparison with other pasture species under drought or water deficit conditions to identify a species which thrive under water deficit environment (Johnson and Bassett, 1991; Karsten and MacAdam, 2001; Hofmann and Isselstein, 2004; Wang and Bughrara, 2008; Turner *et al.*, 2012).

A transgenic approach to drought tolerance was also reported in perennial ryegrass but this is limited. Perennial ryegrass modified with CBF3/DREB1A showed enhanced drought tolerance by reduced electrolyte leakage malondialdehyde and peroxidase activity under decreased soil moisture when compared with control plants (Han *et al.*, 2008). Zhang and Zheng (2008) reported enhanced drought tolerance in perennial ryegrass when Nicotianamine synthase (NAS) gene was overexpressed. Similarly, overexpression of homologous to ubiquitin-like *LpHub1* (*LpUbl5)* conferred enhanced drought tolerance in perennial ryegrass and this was carried out using the primary transgenic lines (Patel *et al.*, 2015). The current study is also aimed to evaluate the performance of *LpUbl5* overexpressing perennial ryegrass, using the T_3 generation of transgenic plants under imposed drought cycles. This will be further discussed in Chapter 3.1.

2.4 Ubiquitin

Ubiquitin was discovered in 1974 and is an 8.5 kDa protein which was purified from the bovine thymus. Ubiquitin is recognized as a molecule which labels intracellular proteins for degradation by a multi-enzymatic complex. The important function of the Ubiquitin_Proteasome pathway is the degradation of misfolded, damaged or non-functional proteins by a multi-enzymatic complex. Due to the variation in binding of ubiquitin to its substrates, ubiquitin could influence protein function without degradation and thus be involved in the regulation of a number of cell signalling pathways. To date, a large number of ubiquitin-like molecules have been identified. These ubiquitin like molecules have divided into two main categories which are Ubiquitin Domain Proteins (UPDs) and Ubiquitin-Like Proteins (ULMs) (Herrmann *et al.*, 2007).

2.4.1 Ubiquitin Domain Proteins (UPDs)

UPDs have a sequence domain which is similar to ubiquitin. UPDs do not conjugate to proteins but carry out adaptor functions by non-covalent binding (Herrmann *et al.*, 2007). UDPs function as chaperone co-factors, ubiquitin–protein ligases and take part in de-ubiquitination processes (Hartmann-Petersen and Gordon, 2004).

2.4.2 Ubiquitin-Like Proteins (ULMs)

ULMs have less sequence homology in common with ubiquitin but this group exhibits a similar tertiary structure, ubiquitin fold. The carboxyl group of glycine residue at the C-terminal of ULMs attaches to the lysine residue of its substrates through isopeptide bond formation. Therefore, ULMs are described as proteins that conjugate to its targets and function in an “ubiquitin-like” manner. To date, a dozen ULMs have been identified. Table 2-1 lists the ULMs, sequence homology of each ULM to Ubiquitin and its proposed function (Herrmann *et al.*, 2007).

Table 2-1 Ubiquitin like proteins/modifiers (ULMs), their sequence homology to ubiquitin and their proposed functions (Herrmann *et al.*, 2007).

Ubiquitin-Like Modifier	**Ubiquitin Sequence Homology (%)**	**Functions**
ISG15 (UCRP) (2 ubiquitins)	29, 27	Positive regulator of IFN-related immune response, potentially involved in cell growth and differentiation
FUB1 (MNSFβ)	37	Negative regulator of leukocyte activation and proliferation
NEDD8 (Rub1)	58	Positive regulator of ubiquitin E3s; directs to proteasomal degradation
FAT10 (2 ubiquitins)	29, 36	Cell cycle checkpoint for spindle assembly, directs to proteasomal degradation
SUMO-1 (SMT3C, GMP1, UBL1)	18	Control of protein stability, function, and localization, antagonist to ubiquitin, overlap with SUMO-2/-3
SUMO-2 (SMT3B); SUMO-3 (SMT3A)	16	Transcription regulation, cell cycle progression
Apg 8	10	Autophagy, cytoplasm-to-vacuole targeting
Apg 12	17	Autophagy, cytoplasm-to-vacuole targeting
Urm1	12	Potential role in oxidative stress response
UBL5 (Hub1)	25	Pre-mRNA splicing, appetite regulation
Ufm1	16	Potential role in endoplasmic stress response

UBL5/HUB1 has been studied in yeast, *C.elegans*, humans, Israeli sand rat, mouse and plants (Friedman *et al.*, 2001; Dittmar *et al.*, 2002; Brailoiu *et al.*, 2003; Lüders *et al.*, 2003; McNally *et al.*, 2003; Yashiroda and Tanaka, 2004; Benedetti *et al.*, 2006; Mishra *et al.*, 2011; Ammon *et al.*, 2014; Oka *et al.*, 2014; Oka *et al.*, 2015; Patel *et al.*, 2015; Feng *et al.*, 2016).

Gene nomenclature: Ubiquitin like protein 5

Ubiquitin like protein 5 (*Ubl5*) was termed differently by different reports. It is useful to have an overview about the gene name reported so far to facilitate the further reading. *Ubl5* was identified in human adult iris cDNA by Friedman *et al.* (2001) and named as *Ubiquitin-like 5 gene* (*Ubl5*). Whereas, identification of this gene in Israeli sand rat (*Psammomys obesus*) by Collier *et al.* (2000) termed as *Beacon*. Studies of this gene in yeast referred to it as *Homologous to Ubiquitin 1* (*Hub1*) and all the reports available in yeast followed the term *Hub1* (Dittmar *et al.*, 2002; Lüders *et al.*, 2003; Wilkinson *et al.*, 2004; Mishra *et al.*, 2011). Hub1 homologue in humans was termed as both Hub1 (Ammon *et al.*, 2014) and Ubl5 (Oka *et al.*, 2014; Oka *et al.*, 2015). In perennial ryegrass, the gene was named as *Lolium perenne* L. *Hub1* (*LpHUB1*) (Patel *et al.*, 2015) and in wheat, the gene was termed as *ubiquitin-like protein 5* (*TaULP5*)(Feng *et al.*, 2016). This present study involves *Lolium perenne* L and *Arabidopsis thaliana* to further study the role of *Ubl5* in enhanced drought tolerance. This study has decided to term this gene as *LpUbl5* in ryegrass and *AtUbl5* in Arabidopsis to keep it consistent. The name *Hub1* was not adopted as *Arabidopsis* has *histone mono-ubiquitination 1 (Hub1)* under the same name and The *Arabidopsis* Information Resource (TAIR) termed At3g45180 and At5g42300 gene as *Ubl5* which were homologues of *Ubl5*.

2.4.3 Ubiquitin-Like Protein 5 (UBL5) /Homologous to Ubiquitin 1 (Hub1)

UBL5 is unique due to the presence of a double tyrosine residue instead of a double glycine residue in ubiquitin and other ubiquitin like proteins (Figure 2-2). The double glycine residue in ubiquitin is protruding beyond the final β pleated sheet with which ubiquitin like proteins conjugates to their target proteins. Whereas, the double tyrosine residue in UBL5 is part of its final β pleated sheet structure (Herrmann *et al.*, 2007). Therefore, UBL5 does not conjugate to its target proteins and is therefore considered to be involved in different functions.

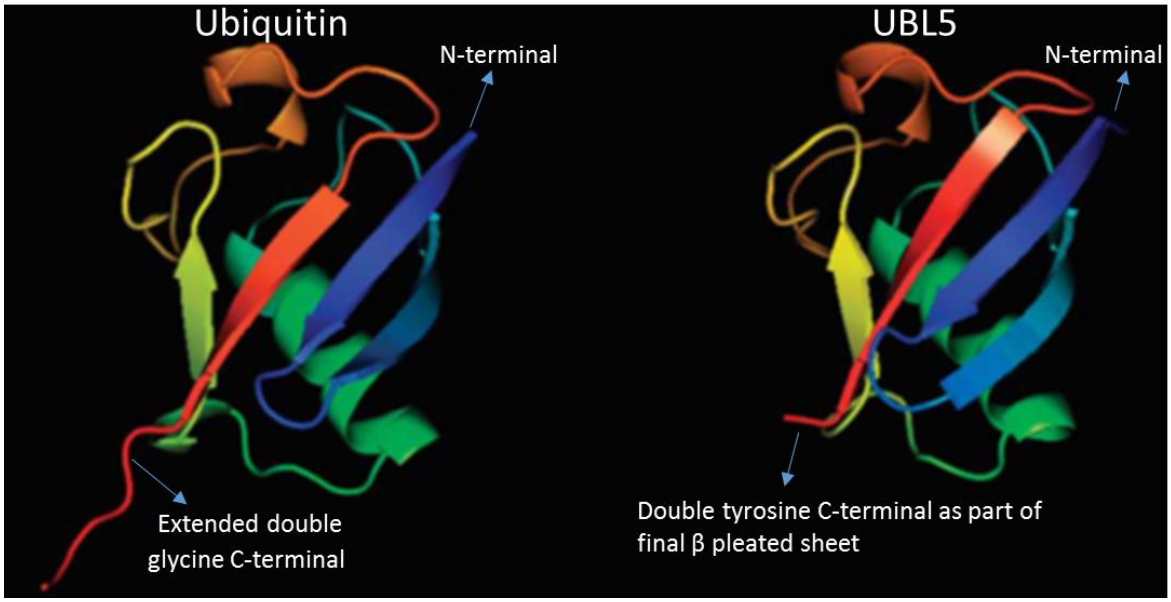


Figure 2-2 Structural comparison of Ubiquitin and UBL5 labelled with N-terminus and C-terminus, figure adapted from (van den Ent and Löwe, 2005).

2.4.4 The proposed functions of UBL5/HUB1

The *Ubl5* gene was reported in different species and their proposed functions are reviewed to understand the depth of research in this category (Figure 2-3). Reports indicate that the *Ubl5* gene was identified in the hypothalamus of the obese *Psammomys obesus* and named as *Beacon* and found to be associated with increased food intake and body weight gain after Intracerebroventricular (ICV) administration of *Beacon* (Collier *et al.,* 2000) . Subsequently identification of *Ubl5* gene in human adult iris cDNA and the cytoplasmic localization of recombinant UBL5 protein in COS-7 cells was also reported by Friedman *et al.* (2001). Further study in *Psammomys obesus* showed ICV administration of *Beacon* and body weight gain was due to increased food intake and fat deposits in the major adipose depots which indicated *Beacon* as an orexigenic peptide which promotes the body fat accumulation (Walder *et al.*, 2001). Kantham *et al.* (2003) reported the interaction between beacon and CLK 1, 2 and 4 (Subfamily of kinases) using yeast two hybrid system. This supported its potential role in energy metabolism either as a modulator of key regulatory molecules or to regulate the gene expression pattern of specific genes (Kantham *et al.*, 2003). Further, McNally *et al.* (2003) confirmed the interaction between UBL5 and CLK4, which was suggested to involve the phosphorylation non-snRNP splicing proteins rich in serine and arginine and differential splicing.

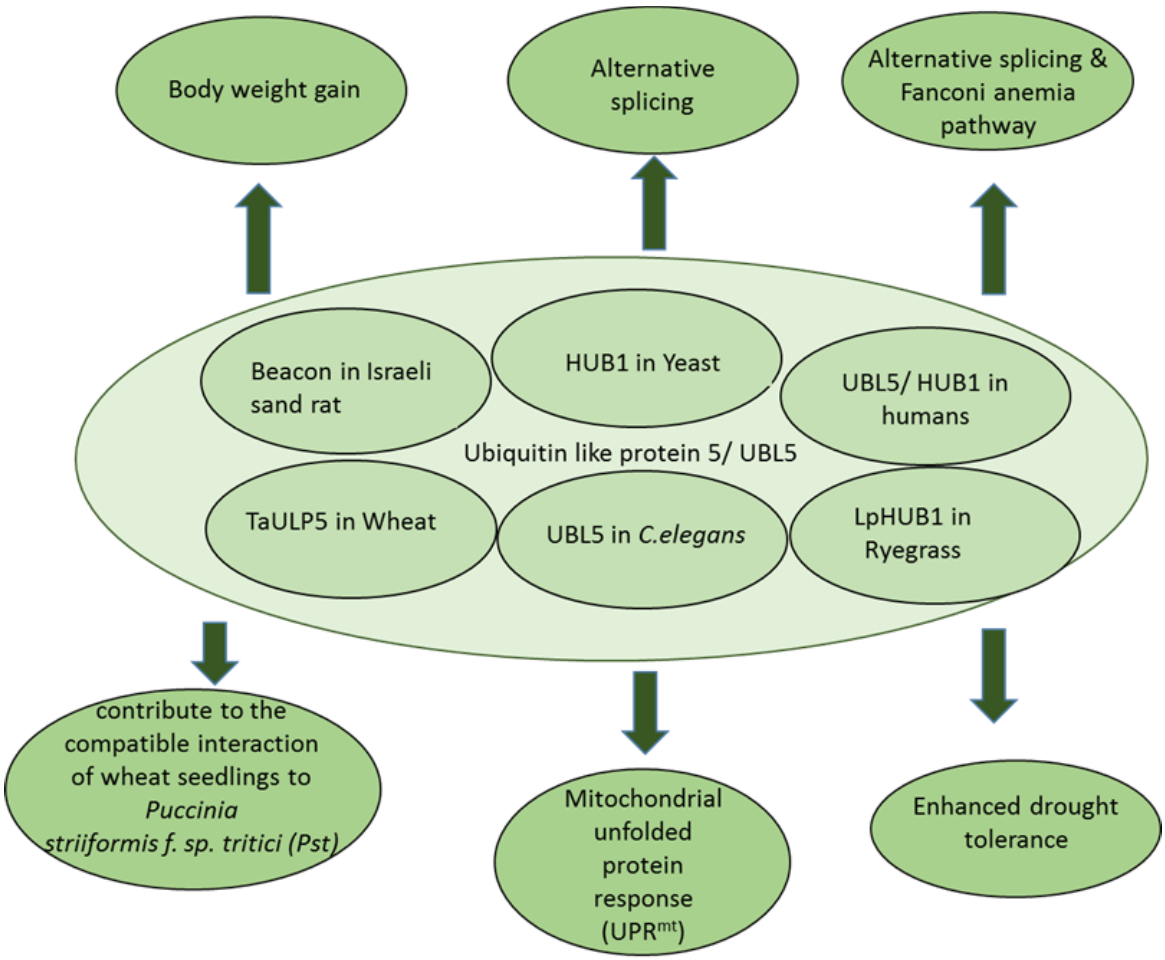


Figure 2-3 Summarized illustration of UBL5 in different species and their proposed functions reported in corresponding species

Deletion of the non-conserved Lysine residue of HUB1 protein resulted in the conjugate formation of Hub1-YDL223c/ HBT1 (Hub1 target) and Hub1-Sph1 in *Saccharomyces cerevisiae* (budding yeast). Further, the study of Hub1 mutants indicated a morphological defect in the mating projections, reduced mating efficiency and identified a mis-localization of its conjugate proteins in Hub1 mutants. This indicated that Hub1 acts as a covalent modifier and is important for polarization during mating in budding yeast (Dittmar *et al.*, 2002). In contrast to this, a possible non-covalent interaction of HUB1 with target proteins was suggested based on the formation of SDS resistant complexes (Lüders *et al.*, 2003). Further, HUB1 in *Schizosaccharomyces pombe* (fission yeast) was shown to interact with Snu66, which is a component of the U4/U6.U5 tri-snRNP splicing complex using yeast two hybrid system. Loss of HUB1 activity reduced the splicing efficiency which caused cell cycle defects probably due to the defective splicing of key cell cycle factors, which blocked the cell cycle progression (Wilkinson *et al.*, 2004). This finding was supported by the report of co-purification of UBL5 (Human homologue of HUB1) with 45S activated spliceosome (B*) complex (Makarov *et al.*, 2002; Makarova *et al.*, 2004). In addition, Yashiroda and Tanaka (2004) reported that HUB1 is essential for the mitotic growth in *S.pombe* using mutants and the conserved di-tyrosine is not essential for HUB1 function. Further the

non-covalent interaction of HUB1 to Snu66 through a conserved element named, Hub1-interaction domain (HIND) does not affect general splicing, but mediates non-canonical 5' splice site usage was also reported (Mishra *et al.*, 2011).

Biochemical and functional studies by siRNA-mediated depletion in human cells strengthened the role of UBL5 in alternative splicing. The study confirmed the formation of a salt bridge between D22 of UBL5 and R127 of HIND of Snu66 and an Alanine replacement of D22, D22A failed to co-isolate endogenous hSnu66 (Ammon *et al.*, 2014). However a deletion of D22 of UBL5 did not affect UBL5 binding to SART1 (Snu66 known as SART1 in mammalian cells)(Oka *et al.*, 2014). UBL5 depletion led to defects in various forms of alternative and constitutive splicing such as exon skipping and higher levels of intron retention (Ammon *et al.*, 2014; Oka *et al.*, 2014). Absence of sequence similarity in the UBL5 sensitive splice sites and intron sequences indicates that UBL5 may function as a splicing qualifying factor for splicing events. Further, the lethal effect of prolonged UBL5 siRNA treatment showed chromosome mis-segregation and loss of nuclear integrity which resulted in segmented nuclei and consequently apoptosis (Ammon *et al.*, 2014). Oka *et al.* (2014) reported the premature loss of sister chromatid cohesion as a result of UBL5 knockdown. This was due to the reduction in Sororin expression as a result of increased intron retention due to the knockdown of UBL5 or SART1 (Oka *et al.*, 2014). Further evidence supporting its role in splicing machinery was reported by Allende-Vega *et al.* (2013). Knockdown of UBL5 and Pre-mRNA processing factor (Prp8) resulted in the activation of tumour-suppressor p53 this in turn result in a p53-dependent G1 cell cycle arrest (Allende-Vega *et al.*, 2013).

Apart from this, a direct interaction of UBL5 with a non-spliceosomal protein FANC1, was observed and explained its role in the Fanconi anemia pathway for DNA interstrand crosslink repair. This was unrelated to pre-mRNA splicing function of UBL5 (Oka *et al.*, 2015). Another role which was unrelated to alternative splicing was the role of UBL5 in mitochondrial unfolded protein response (UPR^{mt}), identified in *Caenorhabditis elegans. Ubl5* (RNAi) inactivation lead to reduced expression of UPR^{mt} reporter genes, *hsp-6* and *hsp-60* and compromised mitochondrial protein folding (Benedetti *et al.*, 2006). A UPR^{mt} signal in cytosol triggered UBL5/DVE-1 complex formation, which re-localized to the nucleus along with a bZip transcription factor. The complex, UBL5/DVE1, activates the expression of hsp6 and hsp60 and also activates the expression of UBL5 (Haynes *et al.*, 2007; Haynes and Ron, 2010). The downstream nuclear role of Ubl5 in UPRmt was further supported by its elevated level in the nucleus of stressed *C.elegans* indicated by the nuclear to cytosolic ratio of GFP fluorescence (Benedetti *et al.*, 2006).

A functional role of UBL5 has been reported in plants but to a lesser extent. UBL5 was identified in APR wheat cultivar 'Xingzi 9104 (XZ)' when infected with *Puccinia striiformis* f. sp. *tritici* (*Pst*) pathogen and named it as *TaULP5 (Ubiquitin Like Protein 5)*. Resistance of 'XZ' seedlings to *Pst* was improved when

TaULP5 (UBL5) was knocked down which indicates the contribution of TaULP5 in the compatible interaction of wheat seedlings to the *Pst* pathogen (Feng *et al.*, 2016). Finally overexpression of *Lolium perenne* gene coding for Ubiquitin like protein 5 (LpHUB1) or LpUBL5 has shown enhanced drought tolerance in ryegrass. The identification of *LpUbl5* gene in perennial ryegrass and further studies involved in this area are discussed below. (Patel *et al.*, 2015).

2.4.5 Identification and over-expression of *LpUbl5*

Seasonal changes in ryegrass gene expression in samples collected from active paddocks during different seasons were analysed using SAGE™ (Serial Analysis of Gene Expression) based ryegrass transcriptome program (Sathish *et al.*, 2007). This SAGE based gene expression analysis facilitated by a proprietary GeneThresher® database (methyl filtered sequence mapping) (Bajaj *et al.*, 2007) resulted in identification of *LpUbl5*. *LpUbl5* gene expression was increased about three fold under water deficit when compared with before stress, rehydration and summer heat condition. This indicated that *LpUbl5* may have a potential role in drought response in ryegrass. *LpUbl5* was over-expressed in 'pasture type' perennial ryegrass 'Grasslands Impact' NZ Agriseeds, Christchurch, NZ using vector pLpHUB1 in which a Double CaMV35S (D35S) promoter driving *LpUbl5* and another D35S driving the expression of marker gene, *hptII* for hygromycin resistance. 'Turf type' perennial ryegrass 'Monterey II' (MT) was transformed using pTTS44 with 35S promoter driving the *LpUbl5* gene and another 35S promoter driving the *bar* gene (bialaphos resistance) encodes phosphinothricin acetyl transferase (PAT) (Patel *et al.*, 2015). Embryogenic calli derived from meristematic regions of the vegetative tillers were transformed using high throughput *Agrobacterium*-mediated genetic transformation (Bajaj *et al.*, 2006). Patel *et al.* (2015) reported higher shoot biomass accumulation, increased relative water content, reduced water potential and increased chlorophyll content under stress when compared with controls. A two to three fold increase in the *LpUbl5* transcript level was observed under drought when compared with the transcript level before imposing drought stress in vector only control and transgenic lines. This has been promoted as evidence for its involvement in enhanced drought tolerance (Patel *et al.*, 2015). However, their study did not identify a physiological response as a result of the over-expression of *LpUbl5* to support the mechanistic role of LpUbl5 in plants under drought stress conditions. Therefore, evaluation of physiological response under imposed drought help to investigate its hypothesised role in drought response. This led to the present evaluation of T_3 generation of 'pasture type' perennial ryegrass to further evaluate and understand the physiological functions of *Ubl5* in ryegrass as highlighted in Figure 2-1. The research reported in this thesis is focused on the physiology measurements and transcript abundance of *LpUbl5* in ryegrass under imposed drought cycles in controlled environment. Further, efforts were taken to evaluate the function of *Ubl5* in *Arabidopsis,* as a model plant under drought, and also incorporated into *Nicotiana Benthamiana* and *Allium Cepa* L. to investigate the subcellular localization of LpUBL5.

2.5 Arabidopsis as a model plant

Arabidopsis thaliana (family Brassicaceae) has been used in experimental research for about half a century (Page and Grossniklaus, 2002). *Arabidopsis* was the third multicellular organism, and first plant to be sequenced, known to have the smallest plant genome (125 Mb) (Initiative, 2000; Bevan and Walsh, 2005). *A. thaliana* was designated as a model plant in 1943 due to its small plant size, rapid generation within 5-6 weeks under optimum conditions, ability to grow in soil or defined media under controlled conditions, ability to generate up to 10,000 seeds per plant via self-pollination and easy maintenance (Page and Grossniklaus, 2002). Apart from these key features, efficient *Agrobacterium* mediated transformation of *Arabidopsis* allowed researchers to express or over express the gene of interest and analyse the effect of newly introduced genes within short time periods due to its short life cycle. These are also highly efficient methods to generate insertional mutants such as *agrobacterial* plasmid T-DNA transformation and transposon based mutants. Subsequently, high-throughput ways of sequencing simplified the laborious stage in reverse genetics. The availability of these mutant seed stocks of any gene of interest from publically available resources has simplified gene function studies. Researchers are able to study gene function in *Arabidopsis* mutant lines which helps to identify the functions of an orthologous gene of interest in other plants species (Provart *et al.*, 2016).

Arabidopsis was incorporated in drought tolerance studies either by genetically modifying the plant using the gene of interest or by screening the mutant lines of specific genes. Over expression of genes such as, ABRE binding factors 3 and 4 (*ABF3/4*) (Kang *et al.*, 2002), *AtMYC2/AtMYB2* (Abe *et al.*, 2003), *CBF4* (Haake *et al.*, 2002), *SRK2C* (Umezawa *et al.*, 2004), *HDG11* (Yu *et al.*, 2008), *XERICO* (Ko *et al.*, 2006), *MYB15* (Ding *et al.*, 2009), *ADC2* (Alcázar *et al.*, 2010), DREB1A and DREB2A (Liu *et al.*, 1998) have successfully demonstrated enhanced drought tolerance in *Arabidopsis*. Incorporation of mutant lines to study drought sensitivity using genes such as *max2* (*MAX2* gene) (Bu *et al.*, 2014), study of SIZ1 using siz1-3 mutants and a complementation line C-siz1-3 (Catala *et al.*, 2007) and ABO3 using *abo3* mutant line and Line 4 (ABO3 overexpressing in *abo3* mutant line) (Ren *et al.*, 2010) are examples of utilizing mutant lines to query gene function. Incorporation of both mutants and overexpression of the gene of interest, to identify drought responsive genes, *CAMTA 1* (Pandey *et al.*, 2013), and YUC6 (Cha *et al.*, 2015) , *WRKY57* (Jiang *et al.*, 2012) are also a method of choice.

2.5.1 *Arabidopsis thaliana Ubl5* gene (*AtUbl5*)

Plant response to drought or water deficit can be measured from the whole plant to the molecular level. Technologies such as micro arrays help to understand expression patterns of different genes simultaneously and allows us to understand the types and quantities of RNA in a cell in response to drought (Bray, 2004). A review of literature suggests several studies have been published to date to identify the molecular response of *Arabidopsis* under different abiotic stresses such as drought, heat, salt and UV radiation (Seki *et al.*, 2001; Kreps *et al.*, 2002; Seki *et al.*, 2002; Oono *et al.*, 2003; Kilian *et*

al., 2007; Huang *et al.*, 2008). The gene of interest of this study, *Ubl5* (At5g42300) was looked up in published literature to identify any involvement of *Ubl5* in response to drought and ABA. At5g42300 was identified as drought induced, ABA and PBI425 (ABA analogue) induced gene out of 1310 other genes. The comparison of data was between results obtained from water withdrawn and rehydrated aerial parts of the plants (Huang *et al.*, 2008). Whereas, a former study by the same authors listed At5g42300 as a gene induced by PBI425 at 24 hours along with 1501 genes whereas the same study did not list this gene under ABA (20 µM) induced either at 6 or 24 h (Huang *et al.*, 2007). This variation was explained as PBI425 in high concentration and long persistence contributing to the expression of more ABA-responsive genes and thereby increasing drought tolerance (Huang *et al.*, 2008). However, a meta-analysis using the data generated by Huang *et al.* (2008) did not list At5g42300 in differentially expressed gene (DEGs) under drought and re-watering (Xu and Wu, 2013). Apart from this, a challenge to these above results was found in study by Wang *et al.* (2011) where 30 µM ABA treated plants were used for analysis. At5g42300 was detected in both leaves and guard cells of the plants but they did not list At5g42300 as an ABA-responsive gene (Wang *et al.*, 2011). Li *et al.* (2006) studied ABA-responsive (10 µM) genes in *Arabidopsis* and did not list At5g42300 as an ABA-responsive gene.

Furthermore, 2678 Arabidopsis genes were listed as genes which showed a 2 fold change in response to drought in 3 week old seedling where the drought was initiated by transferring plants to 200 mM mannitol in hydroponic medium. These did not include At5g42300 (Kreps *et al.*, 2002). Similarly, studies which queried genes under drought using either soil grown of hydroponic medium grown plants did not list At5g42300 as a drought induced gene (Seki *et al.*, 2002; Oono *et al.*, 2003; Kawaguchi *et al.*, 2004; Kilian *et al.*, 2007). In contrast, another group of studies have reported both At5g42300 and At3g45180 as drought responsive genes. At3g45180 was upregulated by drought after 5 days, and continued to be upregulated at 7 and 9 days of water withdrawal in shoots. Whereas, At5g42300 was reported to be upregulated only by 9 days after water withdrawal, when compared with 0 days. This study used 17 day old soil grown plants subjected to water withdrawal up to 9 days and samples were collected on 0, 1, 3, 5,7 and 9 days. At3g45180 and at5g42300 were not up-regulated in root (Rasheed *et al.*, 2016). Two week old media grown plants subjected to dehydration by leaving the harvested plants on parafilm have reported At3g45180 (1 fold) and At5g42300 (2.9 fold) changes under drought, when compared as droughted versus wild type well-watered (Nishiyama *et al.*, 2013). In other study, plants were grown in pellets and moderate (30% of the field capacity) or progressive drought (complete water withdrawn) drought were applied to 35 day old plants. At5g42300 was upregulated only by progressive drought (until symptoms of wilting was observed) and not by ABA treatments or moderate drought (Harb *et al.*, 2010). In a separate study using soil grown 3 week old plants, At3g45180 (-1.5) was down regulated and At5g42300 (1.4 fold) was upregulated in wild type droughted plants versus wild type well watered plants (Van Ha *et al.*, 2014). These studies differ in

terms of their plant growth medium, application of drought/water deficit conditions, time of exposure of plants to stress, type of samples employed and their controls to which they compare the data to identify the fold change. Collectively results are inconclusive so *Ubl5* (At3545180 or At5g42300) cannot yet be designated as a drought responsive gene.

2.6 *Nicotiana benthamiana* as a model plant

Nicotiana benthamiana (*N. benthamiana*) is a unique allotetraploid species, indigenous to Australia. The plant was discovered and collected from Australia on the "N.W. Coast" by Benjamin Bynoe in 1980s. The plant genome is estimated to be 3 Gb (gigabases) and consists of 19 chromosomes. *N. benthamiana* is a herbaceous plant belonging to the *Solanaceace* family. *N. benthamiana* is a close relative of economically important species such as Tomato (*Solanum lycopersicum)* and potato (*Solanum tuberosum*) (Goodin *et al.*, 2008; Bombarely *et al.*, 2012). *N. benthamiana* was accepted as a model organism by the plant virology community due to its unique and general characteristic of susceptibility to virus. Later, the popularity of *N. benthamiana* within the plant biology research community was increased due to factors such as its ability to express the gene of interest (foreign gene) from a plant virus vector, Virus Induced Gene Silencing (VIGS) and efficient transient expression of the protein of interest through *Agrobacterium* mediated infiltration method (Goodin *et al.*, 2008). Firstly, the amenability of genetic transformation in *N. benthamiana* enables researchers to track viral movement within living cells, gating of plasmodesmata and movement of macromolecules between the cells and allowed the definition of the proteins targeted to them (Chapman *et al.*, 1992; Cruz *et al.*, 1996; Escobar *et al.*, 2003; Howard *et al.*, 2004; Lucas, 2006). Secondly, identification of VIGS has made *N. benthamiana* a powerful reverse-genetics system which allows systemic down-regulation of practically any gene-of-interest in plants (Kumagai *et al.*, 1995; Goodin *et al.*, 2008). The method has reduced genetic redundancy and also been able to target individual or multiple members of a gene family (Burch-Smith *et al.*, 2004) *N*. *benthamiana* is widely used in this area of research (Senthil-Kumar *et al.*, 2007; Liu and Page, 2008; Hosseini Tafreshi *et al.*, 2012).

Finally, agroinfiltration has made *N.bethamiana* a popular research model which serves as a platform to express a protein of interest, often in fusion with an autofluorescent protein (Goodin *et al.*, 2008). *N. benthamiana* is used as a platform due to its attributes such as easy management, high level expression of protein in the leaves through agroinfiltration. The process of agroinfiltration of *N. benthamiana* leaves is simple, time saving, does not require costly equipment and the whole process completes within a week (Liu *et al.*, 2010). The transient expression of the protein of interest (LpUBL5) with Carboxy terminal fusion of green fluorescent protein via *Agrobacterium* mediated infiltration of *N. benthamiana* was employed in this study.

2.7 *Allium cepa* L. as a model plant

Allium cepa L (onion) belongs to family *Liliaceae* which is close relative to *Allium sativum* L. (garlic), *Allium porrum* (leek) and *Allium schoenoprasum* L. (chives) (Eady, 1995). Onion epidermal peels have been used as a platform and model system to use in microscopic studies because it occurs naturally as a monolayer in large size and is transparent (Scott *et al.*, 1999). The onion epidermis cells have been useful in the study of various cellular structures such as Plasma membrane, plasmodesmata (Oparka *et al.*, 1994) endoplasmic reticulum (ER) (Allen and Brown, 1988), nucleus (Kinkema *et al.*, 2000), callose deposits (Amstel, 1996), the cytoskeleton (Allen *et al.*, 1993; Timmers *et al.*, 2002), and Hechtian threads (Pont-Lezica *et al.*, 1993) which are network-like structures and fine strands in the periplasmic space (Lang *et al.*, 2014). Onion epidermis cells have also proven to be useful in studying various cell processes such as cytoplasmic streaming (Quader and Schnepf, 1986; Ueda *et al.*, 2010), endocytosis (Oparka *et al.*, 1990), mitosis (Runthala and Bhattacharya, 1991), characterization of mechano- sensory channels (Ding *et al.*, 1993), secretion (Miesenböck *et al.*, 1998), plasmolysis and effects of osmotic stress on the plasma membrane (Mansour, 1995). Onion epidermis is also used as a platform to study plant pathogen interactions using a GFP reporter (Sornkom *et al.*, 2015).

The transient expression in onion epidermis via particle bombardment serves as an ideal tool for studying subcellular localization of organelles and organellar dynamics in the living cells (Scott A1 *et al.*, 1999). The direct DNA delivery into the onion epidermal cells by particle bombardment provides a clean and easy method to transiently express the protein of interest in onion epidermal cells (Eady, 1995). Biolistic bombardment of onion epidermal cells using a GFP tagged with the protein of interest was used in this study to identify subcellular localization of LpUBL5

2.8 Green fluorescent protein as a reporter gene

A green fluorescent protein (GFP) was discovered from the Pacific North West jellyfish *Aequorea Victoria* (Ormo *et al.*, 1996). The discovery and application of GFP as a powerful reporter molecule for monitoring gene expression, protein localization and non-destructive method of protein–protein interaction studies in living cells has revolutionised molecular science (Cinelli *et al.*, 2000; Philips *et al.*, 2017). Wild-type GFP contains a single chain of 238 aa with a molecular weight of 27-30 KDa. The protein is stable, proteolysis-resistant and has two absorption maxima at about 395 and 475 nm. The chromophore of GFP is formed by post-translational modification within the protein by cyclisation and oxidation of the three amino acids, Ser^{65} (or Thr^{65}), Tyr^{66}- Gly^{67} (Gerdes and Kaether, 1996; Ormo *et al.*, 1996). The fluorescence is stable, not dependent on species, allows non-invasive monitoring techniques such as macroscopic imaging, fluorescence microscopy and flow cytometry and moreover, the GFP fluorescence does not require any additional cofactors, gene products or substrates (Zhang *et*

al., 1996). Wild type GFP has two absorption maxima, one is a major peak at wavelengths of 395 nm and a minor at 475 nm (Gerdes and Kaether, 1996).

Genetic engineering of the wild type GFP has resulted in several mutants that alter the fluorescence properties of wild type GFP and resulted in single excitation peak, 6 fold stronger fluorescence and four fold faster maturation (Heim *et al.*, 1994; Cubitt *et al.*, 1995; Heim *et al.*, 1995; Cormack *et al.*, 1996). A stronger fluorescence and a faster maturation was obtained by Ser^{65}- Thr (S65T) GFP but the fluorescence intensity was temperature dependent. Further a substitution of Ser^{147} to Pro (S147P GFP) provided 6-fold brighter fluorescence when compared with a single S65T GFP mutation (Kimata *et al.*, 1997). To date, there are a number of GFP mutants available such as EGFP, sGFP, sGFP(S65T) which enhances the fluorescence, (Heim *et al.*, 1994; Chiu *et al.*, 1996; Haas *et al.*, 1996; Kimata *et al.*, 1997). Two mutations (F64L and S65T) in the EGFP contribute to improved properties such as greater folding efficiency (higher proportion of correctly folded protein increases the fluorescence), a single excitation peak at 490 nm and a codon optimized for expression in mammalian hosts. This is the most widely used GFP mutant (Cinelli *et al.*, 2000; Arpino *et al.*, 2012) This PhD study incorporates EGFP for the sub-cellular localization of the protein of interest (LpUBL5) using GATEWAY vectors suitable for *Agrobacterium*-mediated plant transformation (Karimi *et al.*, 2002; Karimi *et al.*, 2007).

2.9 Research questions

The literature review has highlighted the various aspects of plant response in the context of drought (Figure 2-1), briefly outlined the research outcomes in ryegrass drought studies and most importantly, reviewed various proposed function of *Ubl5* in different species. The informations and resources available have facilitated the design and execution of different experimental procedures to investigate the role of *LpUbl5* in drought tolerance. A number of research questions were formed to identify functions of *LpUbl5* in plants.

- Does over-expression of *LpUbl5* occur in ryegrass and does it provide enhanced drought tolerance in the T_3 generation of transgenic ryegrass lines? Has the generation of T_3 lines in a self-incompatible species been proved successful?

- Does the over-expression of *LpUbl5* in Arabidopsis confer enhanced drought tolerance, given the highly conserved nature of *Ubl5* across species? Do the *AtUbl5* mutants expedite phenotypic characterization?

- Does subcellular localization of *Lp*UBL5 exhibit its potential involvement in alternative splicing?

- Does the ryegrass germplasm have any potential candidates to incorporate in breeding for drought tolerant trait? Does the performance of selected germplasm accession under drought

correlate with the *LpUbl5* transcript level? Does the *LpUbl5* transcript level increase with increased severity of drought?

- Does the *LpUbl5* transcript level increase under water withheld treatment in primary transgenic ryegrass lines and cultivar 'Impact'?

These research questions have been used to formulate the objectives outlined in Chapter 1.1.2 and the hypothesis provided at the beginning of each experimental chapter.

Chapter 3

Physiological evaluation and transcript levels of *LpUbl5* in *Lolium perenne* L. under different stages of drought and recovery periods

3.1 Introduction

Perennial ryegrass is the most widely used grass in New Zealand but its summer performance is limited by relatively poor drought tolerance. Previous research in the species by Patel *et al.* (2015) has shown that overexpression of *LpUbl5*, under a double 35S promoter, enhanced drought tolerance (Literature review 2.4.5). The species is a self-incompatible diploid (2n = 2x = 14), species (Sathish *et al.*, 2007) with cultivars consisting of a population of genotypes. The research of Patel *et al.* (2015) was focused on primary transgenic T_0 plants from a single genotype of 'Grasslands Impact'. As a follow-up to their research, this PhD study has used T_3 generation of plants of 2 primary transgenic events, 8AC1 and 8AC2 of the same single transformed genotype that was subsequently backcrossed to a number of plants of the parent cultivar 'Grasslands Impact'. The 8AC1 T_3 perennial ryegrass populations used in this study resulted from a seed increase of T_2 plants putatively homozygous for the transgene for over-expression of *LpUbl5*. The 8AC2 T_3 perennial ryegrass were generated from T_2 heterozygous plants and resulted in generation of T_3 plants segregating for the transgene. To study the enhanced drought tolerance in physiological context, a null hypothesis was formed;

"Drought tolerance in perennial ryegrass is not different between control plants and those with over-expression of *LpUbl5*".

This research was carried out on the T_3 plants when sufficient seeds had been generated to examine plant physiology through sequential measurements of dry matter, plants scores and leaf extension, leaf area, and solute potential. The *LpUbl5* transcript abundance was studied by qRT-PCR on samples from drought cycles which included a period of well watered recovery and progressive drought. This chapter describes the experimental design, materials and methods, physiology results and transcript level of *LpUbl5.*

3.2 Experiment Design

The experiment was carried out in the Biotron growth room facility at Lincoln University. The experiment assessed the effect of periodically imposed drought on four perennial ryegrass lines in rhizotrons. The perennial ryegrass lines in this experiment were one parent line as control, and three genetically modified cis-genic lines that are negative or positive for an additional copy of a gene that

may confer drought-tolerance, including a homozygous line. The experimental plan included eight replicates of each line across two rhizotrons.

3.2.1 Seedling establishment

The source of genetically modified seeds was ViaLactia Biosciences through the Pastoral Genomics Consortium. The transgenic lines carried an expression cassette that consists of a double-enhancer version of *CaMV 35S* promoter, which drives the two genes of interest. These genes from a small open reading frame 135 (ORF135) of 464 bp long from perennial ryegrass which codes for the *LpUbl5* gene and the *hptII* selectable marker. Both are under the control of the *CaMV 35S* 3' UTR. The transgenic lines were 8AC1, 8AC2- and 8AC2+. 8AC1 was a homozygous transgenic line which carried an additional copy of *LpUbl5* (Figure 3-1). The T_0 transgenic lines from Patel *et al.* (2015) were not available to include as a positive control

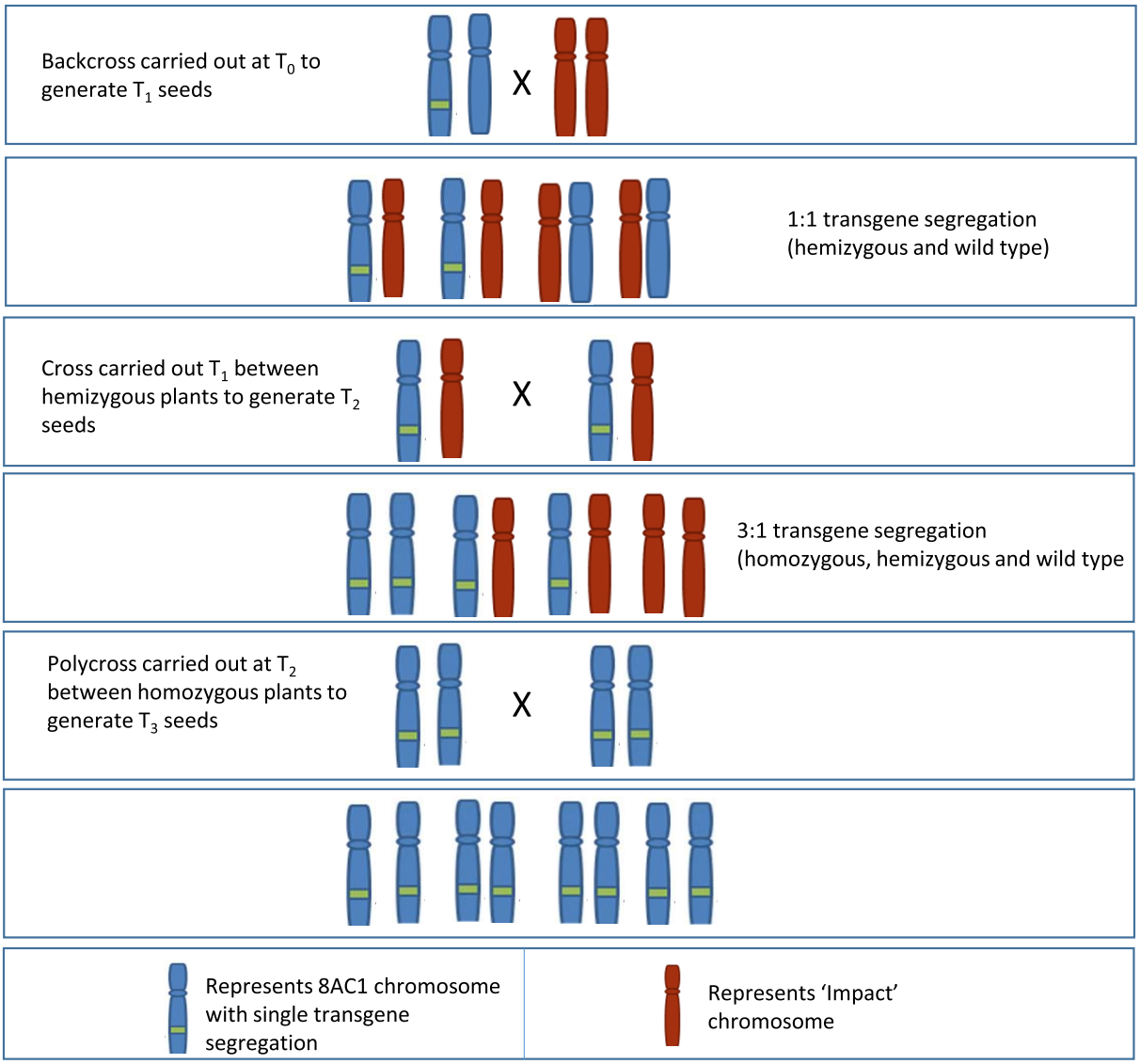


Figure 3-1 Schematic representation of transgene segregation for 8AC1 during the crossing. The chromosome images were adapted from (Passricha *et al.*, 2016).

8AC2+ also had an additional copy of the transgene and the 8AC2- did not have an additional copy of the transgene. 8AC2+ and 8AC2- were the progeny of a heterozygous line for the transgene. The Control line was cultivar 'Grasslandz impact' (will be referred as 'Impact' throughout) which was the parent line used to generate the transgenic lines (Table 3-1). A single seed was sown into each cell plug of cell trays and these trays were watered and germinated in containment in the Biotron. After emergence, the seedlings were watered regularly to ensure that they were not drought stressed at any point in the seedling stage. Hoagland's solution (Appendix B.12) was added once every two weeks or depending on the appearance of seedlings to ensure they were never nutrient deficient.

Table 3-1 Lines and cultivars used in the Experiment 1 carried out in Biotron, Lincoln University

Line & clutivars	Transgenic status
8AC1	Positive
8AC2+	Positive
8AC2-	Negative
Impact	Wild type

3.2.2 Determination of genetic status of seedlings

Seedlings were grown until they had produced 3 to 4 tillers. The youngest-leaf of these well-established seedlings was then sent to Vialactia Ltd to confirm its genetic status, i.e. GM +ve, or GM –ve. The transplanting of seedlings from cell tray to rhizotrons was carried out based on the genetic-screen results provided by Kerry Templeton, Research Associate, ViaLactia Ltd.

3.2.3 Growth Room

The experiment occurred in a 5.0 X 2.4 m Conviron BDW120 growth room equipped with metal halide (Model MS400W/HOR, Venture) and incandescent bulbs (100W, Philips). Lights are mounted above a clear Perspex barrier, and a downward airflow distribution system maintains the ambient (350-400 ppm) CO_2 conditions. The room underneath the growth room was air-conditioned which has Rhizotrons (107 cm length X 80 cm width) that holds the soil. The growth media (soil) was filled in Rhizotrons in horizon "A" (24 cm) and "B" (24 cm) with a layer of sand (2 cm) below horizon "B".

3.2.4 Growth Media for Rhizotrons

A Templeton silt loam soil (Cox, 1978) was used for the experiment after sieving out stones and large pieces of plant material and processing the soil using a soil shredder. The top soil "A" horizon was amended with sand in a 4:1 ratio (soil: sand by volume) and subsoil "B" horizon at a 5:2 ratio. Each

horizon was recreated within four rhizotrons to reflect the bulk density equivalent to that found in the field. The resulting rhizotrons were placed in the controlled environment and the soil profiles stabilized by watering the rhizotrons (1 L water per day).

3.2.5 Growth Room Condition

The environmental conditions within the Conviron during seedling establishment and sward establishment were set to a constant 15°C air temperature, 8°C soil temperature, 16/8 h (day/night) photoperiod and the relative humidity was maintained at 70% constant. A 30 min ramped twilight either side of 15 hours was applied to adjust to a total of 16 h "daylight".

The light intensity at plant level was 463 $\mu mol.m^{-2}.s^{-1}$ measured with a LI-COR® Radiation Sensor (LI-250A Light Meter, Turfschipper 114, 2292 JB Wateringen, Netherlands).

3.2.6 Time Domain Reflectometry Installation

Soil moisture was measured using Time Domain Reflectometry (TDR, 0-0.2 m, Trace systems, soil moisture equipment, Trace systems, Model 6050X1, Soil Moisture Equipment, Santa Barbara, California, USA). Two 0.2 m long TDR rods were inserted on 14/6/2013 in the rhizotrons during their establishment phase to measure the soil moisture content over the course of the experiment (Figure 3-2).

The TDR was set to zero with the TDR rod holder before each measurement. The holder was cleaned before each measurement as leaf or soil residuals within the TDR holders may alter results. The soil moisture measurement was carried out once in two days during the watering cycles and twice a week to estimate the availability of water in the soil during the imposed drought cycles. TDR rods were moved within each rhizotron between each regrowth cycles. The movement of TDR rods were impossible as the drought progressed and reduced water content of the soil. The experiment had an establishment phase and six drought cycles in total. Each drought cycle is explained separately below.

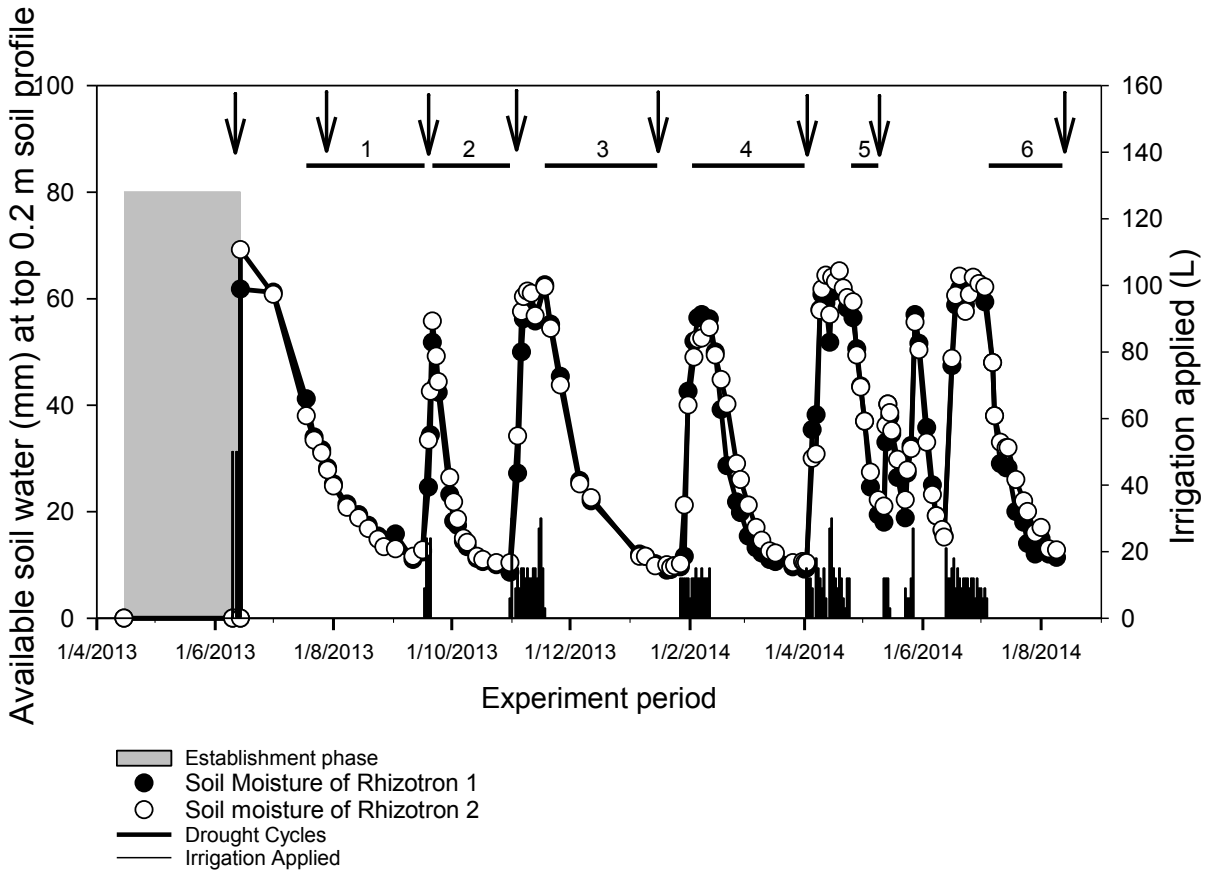


Figure 3-2 Soil moisture of the rhizotron profile, with the establishment phase, highlighted water application rate ((L) bars), drought cycles shown as horizontal lines marked above the graph. Arrows represent harvest dates, at the end of the drought cycle.

Micro-sward Establishment

The transplantation of seedlings was based on the genetic analysis report which categorised the different perennial ryegrass plants for their transgenic status (Figure 3-3). The rhizotrons were divided into 32 individual plots designed for each treatment (Appendix E.1). The plants were established on 15/04/2013 in Rhizotrons 1 and 2 and the soil profile was watered to field capacity by 10/06/2013. Field capacity was identified as irrigating the rhizotrons with 3 Litres of water at 2 or 3 hour intervals until the drainage of water through the bottom of rhizotrons was detected. This process usually lasted for 20 to 30 days based on the dryness of the soil profile. At the end of the establishment phase the plants were trimmed about 2 cm above ground level to commence the first drought cycle.

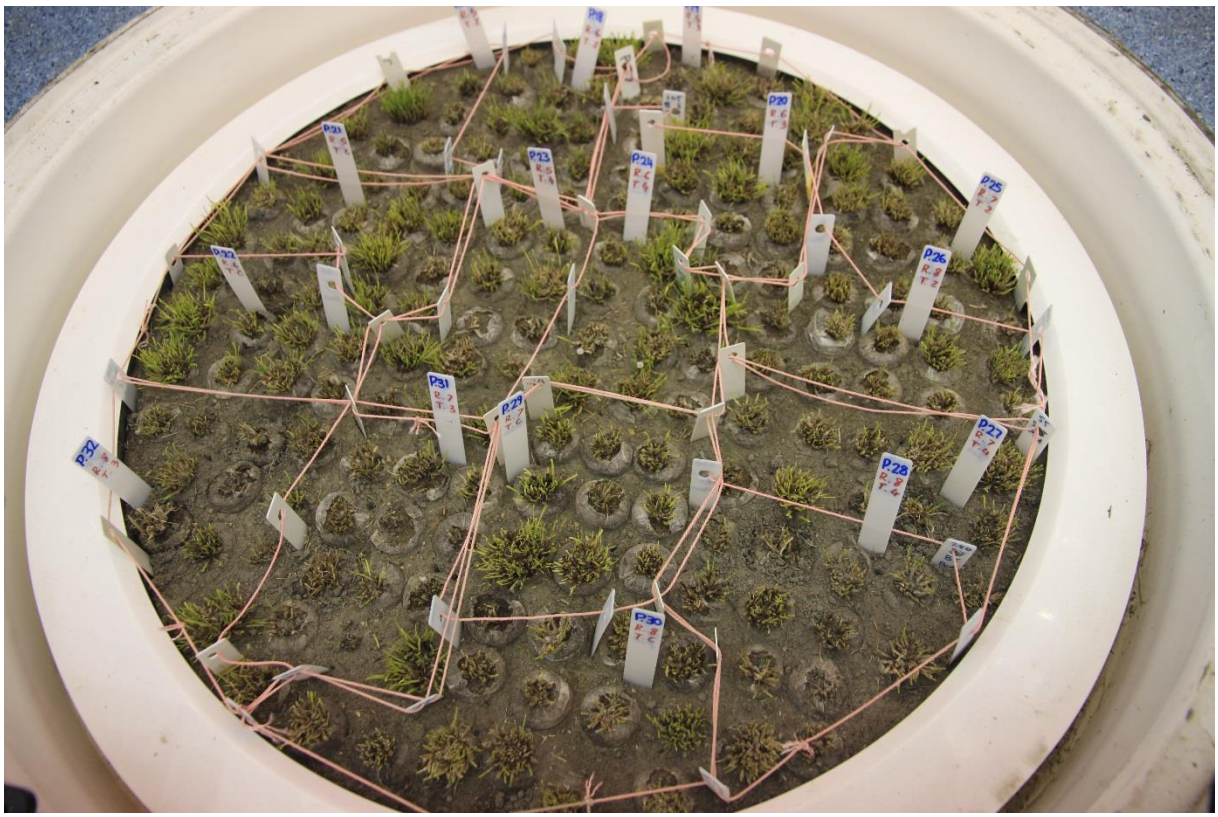


Figure 3-3 Photo taken after the plants have been trimmed at the end of establishment phase of the Rhizotrons showing the separation of different lines in micro-swards using strings.

3.2.7 Drought cycles

Drought Cycle 1

The first drought cycle was started by ceasing irrigation of the soil on 18/7/2013. The cycle was completed after 61 days on 19/09/2013. To impose the first drought cycle, the growth chamber conditions were increased to 20^{o}C constant air temperature, 15^{o}C constant soil temperature, 70% relative humidity and day length was 16/8 h (D/N).

Drought Cycle 2

After Cycle 1 the profile was re-watered to 25% soil moisture and drought Cycle 2 was initiated on 21/09/2013 and completed after 40 days on 31/10/2013. To impose the second and all subsequent drought cycles, soil temperature was increased to 25^{o}C and the air temperature was 15^{o}-25^{o}C (D/N) with the humidity set to 0%.

Drought Cycle 3

After the harvest from Cycle 2 the soil profile was not irrigated for a further 13 days. This allowed the plants to remain at 4% soil moisture which exposed plants to a simulated drought after defoliation analogues to grazing in field conditions followed by a dry summer. Drought Cycle 3 was initiated on

18/11/2013 after watering the soil profile to field capacity. The cycle was continued for 58 days and competed on 15/01/2014.

Drought Cycle 4

The fourth drought cycle was initiated on 2/2/2014 after watering the soil profile to field capacity. Drought Cycle 4 was completed on 1/04/2014 after 58 days of dry down.

Drought Cycle 5

Drought Cycle 5 was initiated on 25/04/2014 after irrigating the soil profile to field capacity and the cycle was suspended due to appearance of insects (thrips) on the plants and in the rhizotrons. The plants were harvested on 9/5/2014 after 14 days of dry down and the rhizotrons were sprayed using "Attack" insecticide, in 1ml /L of water. (Active ingredients is 25 g/litre permethrin + 475 g/L pirimiphos-methyl).

Drought Cycle 6

The final cycle was initiated on 5/7/2014 after irrigating the soil profile to a field capacity. Cycle 6 was completed on 12/08/2014 after 38 days of dry down.

3.3 Physiological Measurements

If the transformed plants conferred drought tolerance, then an understanding of its physiological basis is important. Therefore the following measurements taken based on their simplicity, repeatability and sensitivity to water stress.

3.3.1 Dry matter

Dry matter production is a robust evaluation of performance of different lines under experimental conditions (Robson, 1973; Thomas, 1991; Wilkins, 1995). It is also important to assess the capability of plants to maintain yield which is the key agronomic requirement during experimental conditions (Hatier *et al.*, 2014). Plants from each plot were harvested at the end of each drought cycle and labelled with plot number and number of live plants harvested per plot. The shoot dry matter was analysed per live plant because all the physiology measurements carried out were based on individual plants. In addition, the area of mini-swards was created to contain an equal number of plants to provide similar level of competition to all the plants but the area of individual mini-swards was not measured. The dry matter was measured before imposing a drought and at the end of each imposed drought cycle to evaluate the productivity of each of the treatment groups. The harvested plants were dried to constant weight after each dry down cycle and total dry matter from each treatment was calculated.

3.3.2 Plant scores (Grading)

Visual appearance of plants during each recovery and imposed drought cycle was used as a non-destructive measure to record the performance of plants from lines over time as the drought stress increased. The scoring also helped to identify the number of dead plants from within each line and the time when death occurred within the drought cycles. Initially it was planned to only score plants during drought periods. However during Drought Cycle 2, a decision was made to score the plants during recovery and at the beginning of drought to access the performance of each plant at each stage of growth. The scale was based on the best performing plants at each stage of a dry down cycle so changed between measurement dates.

During imposed drought, the plants were graded based on the following Table 3-2(Derrick Moot, 2011, Unpublished data).

Table 3-2 Table explains the definition of plant score which used for the research

SCORE	DEFINITION
0	Dead
1	One or few live tillers
2	More live tillers but growth only 25-50% of best performing plants
3	plant achieving 50% of best performing plant
4	75% of best performing plant
5	Best performing plant

3.3.3 Leaf Extension

The leaf extension rate contributes to canopy development which influences light interception and consequently plant growth rates. The leaf extension rate is also sensitive to temperature and availability of water (Baker and Younger, 1987). Therefore, leaf extension rate was used as an indicator of moisture stress throughout the growing cycles. Two tillers of two different plants of each plot were labelled using a metal string attached to a wooden stake to mark plants (Figure 3-4) for repeated measure of the leaf extension rate (mm/tiller/day). The tillers were labelled with their corresponding plot number and the first plant was “a” and the second plant in the same plot was labelled as “b”. This enabled two measurements from each plot and provided 16 measurements from each line. The mean of the two measurements was used in the analysis.

The measurements were taken using a ruler until the appearance of the ligule which indicated that leaf was fully expanded. Then the measurements were shifted to the next newly emerging leaf on the

same tiller. This measurement was carried out weekly. The tiller markings were removed during each harvest and new tillers were marked again for the next re-growth period at the appearance of new leaves.

Figure 3-4 Marked tiller in one plant to measure leaf extension of ryegrass plant growing in the rhizotrons at Lincoln University on 13/4/2014 during Drought cycle 5.

3.3.4 Leaf Area (LA)

Leaf area is a component of canopy expansion that affects plant dry matter. Maintenance of leaf area is one method of increasing the capture of light which in turn results in dry matter (Dalirie *et al.*, 2010). Leaf area is also highly sensitive to drought conditions and as a result the expansion and development of the transpiration surface is reduced (Canavar *et al.*, 2014). It is important to establish a clear understanding of the mechanisms of different lines to survive or cope with drought stress. The leaves collected for relative water content (RWC Section 3.3.5) were also used to measure leaf area. The harvest of leaves for different measurements coupled with senescence meant there were fewer leaves towards the end of each drought cycle for all physiological measurements. Therefore length and width of leaves harvested for RWC were measured each time and recorded to quantify the leaf area. The length of the leaves was recorded after the leaf harvest and width was measured after the saturation of leaves in the water overnight. To calculate LA, a fully expanded leaf from each plant was excised from the plant and the laminar area was scanned using an Area Meter AM 300 (12 Spurling Works,

Pindar Road, Hoddesdon, Herts EN110DB, England). The values obtained from the leaf scanner were used to determine the relationship between leaf length and width to estimate leaf area.

3.3.5 Relative Water Content (RWC)

RWC of the leaves is used to measure plant water status (Shepherd, 1977). RWC is one of the main plant water status measurements and is an important trait which indicates the leaf water content (Saura-Mas and Lloret, 2007). A wider evaluation of plant water status is important in imposed drought studies because it is important to monitor the plant water status of each treatment under different soil moisture conditions. The RWC measurements were taken during drought and recovery cycles. During the last two drought cycles, RWC was measured every week to evaluate the effect of progressing drought on plants. Two fully expanded leaves from each group were collected and the fresh weight recorded. Each leaf was placed in a labelled petri-dish (Figure 3-5).

Figure 3-5 Ryegrass leaves were placed into the petri-dish containing water after measuring the fresh weight (FWt).The saturated leaves in the water were used to measure saturated weight (SWt) after overnight incubation at $4^{o}C$.

Approximately 10 petri-dishes were stacked together and wrapped with foil to prevent the evaporation of water from the petri-dishes. Then these stacked petri-dishes were refrigerated overnight to slow down the metabolism. After incubation, the leaves were reweighed after pat drying (Figure 3-6). Then the leaves were placed in small paper bags, labelled and bags of leaves were oven dried at $66^{0}C$ until constant weight to determine their dry weight.

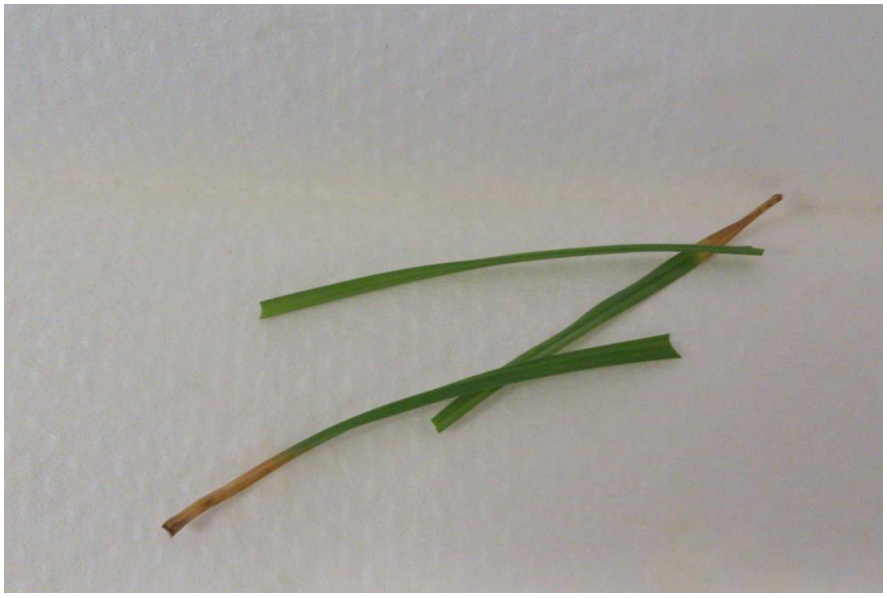


Figure 3-6 The ryegrass leaves placed on tissue paper after saturation in water. This leaves were pat dried, placed in paper cover and dried until constant weight at 60°C to measure the dry weight (DWt).

RWC was calculated using the Equation 3-1.

$$RWC = [(FWt-DWt) / (SWt-DWt)] \times 100$$

Where FWt is leaf fresh weight (g), DWt is leaf dry weight (g), and SWt is the saturated weight (g) (Shepherd, 1977).

3.3.6 Solute Potential

Under drought conditions, leaf osmotic potential decreases due to increased solute concentrations (Callister *et al.*, 2008). Grasses could acclimate to drought by accumulating solutes in the tissues in response to water deficit by osmotic adjustment (Qian and Fry, 1997). Solute potential was used to assess plants water status. The method helps to determine the osmotic adjustment of the plant. To do this 1.7 mL microfuge tubes were prepared by placing a metal mesh in their bottom. The leaves were placed on top of the metal mesh, so that the leaf sap could be collected at the bottom of the tube for measurements at the end. Leaf samples from plots were harvested in a pre-arranged 1.7 mL microfuge tube and labelled with plot number. These microfuge tubes with leaf samples were snap chilled using liquid nitrogen and stored at -80°C until processing.

Leaf samples were processed by centrifuging the microfuge tubes at 10000 RPM for 5 minutes before the leaves and metal mesh were removed. The leaf sap collected at the bottom of the microfuge tube which was then analysed using an osmometer to measure the osmolality of the leaf sap. The osmometer was calibrated each day prior to analysing the samples. The leaf sap was used to measure

the osmolality from which the solute potential was calculated Vaan't Hoff relation was used to calculate osmotic potential (Ψπ) from osmolality (mol.kg^{-1}) using Equation 3-2.

Equation 3-2 $\Psi\pi=-RT_{cj}$

Where RT = -0.002437 m^3 MPa.mol^{-1} at 20^{0}C (Assuming that we are working at this temperature), and *cj* is the total solute concentration (∑jcj) or osmolality (mol.kg^{-1}) (Blum, 1989).

3.4 Transcript abundance analysis of *LpUbl5*

3.4.1 Reference gene selection

Kozera and Rapacz (2013) explain the importance of careful selection of reference genes and this is important to validate the gene of interest (*LpUbl5*) under different experimental conditions to obtain a reliable gene expression result. Lee *et al.* (2010) validated the six perennial ryegrass reference genes from the 442 samples collected from field grown and lab grown perennial ryegrass. *eEF1A*(s) (eukaryotic elongation factor 1 alpha) and *eIF4A* (eukaryotic translation initiation factor 4 alpha) were chosen to validate the current study to use as reference genes in the qualitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays. The reference gene primers were used from the study of Lee *et al.* (2010). The selected reference genes were validated in the current experimental samples and analysed using GeNorm applet before incorporating the reference genes into expression level analysis of *LpUbl5*(Vandesompele *et al.,* 2002).

3.4.2 Sample collection

Sample collection was carried out during recovery until the end of each drought cycle at different soil moisture levels. A sample of 100 mg of leaf tissue was collected in Eppendorf tubes. The tubes were labelled with a unique plant ID and plot number indicating the treatments. The samples were snap frozen using liquid nitrogen and stored at -80^{o}C until processing

3.4.3 *LpUbl5* Primer Design

The perennial ryegrass *LpUbl5* complete coding sequence with the EST sequence (GenBank: GR521568.1) Figure 3-7 was used to design primers using (NCBI) Primer-BLAST (National Centre for Biotechnology Information). A primer (Table 3-3) was designed to study the *LpUbl5* transcript level in different samples collected during the course of the experiment using qRT-PCR.

```
>ELPLPE1032A02-g1RSP_20001211

        1 cagaagaaaagagaagagctagggtttggtcgagaggaggcctggcggccgatcgagcga

       61 agatgatcgaggtggtgctcaacgaccgtctggggaagaaggtgcgcgtcaagtgcaacg
            ***
      121 aggacgacaccatcggcgacctcaagaagctggtggcggcgcagaccgggaccaggcccg

      181 agaagatccgcatccagaagtggtacaccatctacaaggaccacatcaccctcggcgact
                                          >>>>>>>>>>>>>>>>>>>>
      241 acgagatccacgacggaatgggactcgagctctactacaactagcccattcaatctcccc
                                                    ***
      301 agccatgttggtatgcatccccctagccatccctagatgatgtctttggttgtgttccag
          <<<<<<<<<<<<<<<<<<<
      361 tcccagtgtggtcagagttcatgtgtgagctaaaaaaagctactagtattatgtaagtac

      421 tgcatgacccatcatgactgttgacagcttgaactttgtgtcctattgtcaccccgtgaa
                           ***************
      481 tgtaatgcgctgttactctcaaggctataaataa
```

Figure 3-7 EST sequence of the *LpUbl5* gene. SAGE tag region is indicated by underline beneath the region. Stop codon and start codon of *LpUbl5* coding region are represented using an asterisk. The forward and reverse primers are underlined with > and <.

3.4.4 Preparation of Standard

Standards are included in the qRT-PCR to evaluate the efficiency of reaction and as a positive control. Total RNA was isolation from the perennial ryegrass leaf samples and cDNA was synthesised using the total RNA as described in Section 3.4.6. To prepare the standards, the reference gene primers and *LpUbl5* primers were used to amplify the reference gene and *LpUbl5* from the cDNA by using end point PCR. All the primers used in this study were ReadyMade primers by Integrated DNA Technologies, Singapore. The end point PCR products were run on a 3.5% gel. Axygen™ Axyprep™ DNA Gel Extraction Kit (Axygen, Ray lab, NZ). This was used to clean the amplified products separated on the gel based on the molecular weight following the manufacturer's protocol. The purified product was run on the gel to confirm the size of the product and quantity using Qubit® flourometer (Life Technologies, NZ) quantification.

Table 3-3 Primers used to amplify *Lolium perenne* L. reference genes and *LpUbl5* to carry out TOPO® TA Cloning® to develop standards to be used in qRT-PCR assay.

Name	Sequences	Amplicon
eEF1A (s)_FWD	CCGTTTTGTCGAGTTTGG T	
eEF1A (s)_RVS	AGCAACTGTAACCGAACATAGC	113 bp
*eIF4A*_FWD	AACTCAACTTGAAGTGTTGGAGTG	
*eIF4A*_RVS	AGATCTGGTCCTGGAAAGAATATG	168 bp
*LpUbl5*_FWD	TACAAGGACCACATCACCCT	
*LpUbl5*_RVS	GGATGCATACCAACATGGCT	108 bp

These amplified products were cloned using TOPO® TA Cloning® kit (ThermoFisher Scientific, Auckland, NZ) into the pCR™2.1-TOPO® vector (ThermoFisher Scientific, Auckland, NZ). The transformation (Appendix B.4) was carried in 3:1 ratio of PCR product: plasmid using Equation 3-3.

Equation 3-3.

$$\frac{(\text{Ng plasmid} * \mu\text{l plasmid used})\ \text{Kbp insert size} * (\text{insert} : \text{plasmid})}{\text{Kbp plasmid size}} = \text{ng insert to add}$$

The competent DH5α™ *E.coli* cells were transformed by a heat shock method (Appendix B.6) using the pCR™2.1-TOPO® vectors carrying the PCR products of reference genes or *Ubl5* according to Sambrook and Russell (2001). The Laurie Bertani (LB) media with kanamycin (50 µg/mL) as a selective agent along with X-gal (5-Bromo-4-Chloro-3 Indolyl-Beta-D-thiogalactopyranoside) for blue white colony screening. A mix of 40 µl of X−Gal stock solution (20 mg/ml stock solution) at room temperature and 4 µl of a 200 mg/ml solution of IPTG (isopropyl-β−D−Thiogalactoside) solution was spread evenly over the entire surface of the plates. The transformed *Ecoli* cells were plated on to these and incubated at 37°C overnight (Sambrook and Russell, 2001). The white colonies were screened by colony PCR using the specific primers for each insert. The positive colonies were grown overnight in 5 mL of Luria Broth Base with 50 mg/mL kanamycin. The cells were harvested and the *E.coli* plasmid mini-prep (Appendix B.7) procedure was followed to isolate plasmids from the cells. The isolated plasmids were cleaned using Axygen™ Axyprep™ PCR Clean-up Kit (Axygen, Ray lab, NZ) following the manufacturer's protocol. The cleaned plasmids were sequenced by Sanger sequencing to confirm the insert (Sanger *et al.*, 1977) by the Department of Bio-Protection Lincoln University, New Zealand (Appendix B.11).The sequence results were analysed using Geneious 7.1.7 (http://www.geneious.com/, (Kearse *et al.*, 2012).

3.4.5 Restriction digestion of Plasmids

Restriction digestion was carried out to linearize the plasmids to be used as the template in the qRT-

PCR assay. The restriction enzyme BamHI was selected to linearize the TOPO-TA plasmids at 3889 bp; as the selected enzyme digests the plasmid at a single site. The restriction digestions (Appendix B.10) were carried out by following manufacturers protocol (New England BioLabs *Inc.*). The digested plasmids were run on the agarose gel and the Axygen™ Axyprep™ DNA Gel Extraction Kit (Axygen, Ray lab, NZ) was used to carry out gel purification of the digested plasmid. The purified plasmids were quantified using Qubit® flourometer (Life Technologies) and also run on 1% agarose gel for further confirmation. The 1 ng of linearized and gel purified plasmids were serially diluted as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} to be use as standards in the qRT-PCR assay. All the standard curve generated throughout this study is shown in Appendix C.

3.4.6 RNA isolation from the leaf tissue of *Lolium perenne* L.

To synthesize cDNA for the qRT-PCR assay, RNA was isolated from the leaf samples. The samples were selected from the final drought cycle ranging through 30%, 15%, 10%, 8%, 7%, and 5% soil moisture. RNA was isolated from the ground leaf tissue using Spectrum™ plant Total RNA kit (Sigma-Aldrich, Auckland, New Zealand). The RNA was treated with TURBO DNA-free™Kit Ambion ® TURBO DNA-free™ DNase (ThermoFisher, Auckland, NZ) to completely remove the DNA. RNA was quantified using Nano-drop (Thermo Scientific, Auckland, NZ). A Qubit® flourometer was used to quantify RNA using Qubit® RNA buffer and dye (life technologies, NZ) after calibration using the supplied standards.

Quality analysis of isolated RNA

Quality analysis of RNA was important to ensure the integrity of isolated RNA before proceeding to cDNA synthesis. The RNA integrity was analysed by running denaturing formaldehyde agarose gel (Appendix B.13) and confirmed that the RNA was intact. The general PCR (Appendix A.1) using primers of reference gene, *eEF1A*(s) was used to confirm the complete removal of genomic DNA in the isolated total RNA. The primers used to carry out general PCR are given in Table 3-4.

Table 3-4 The primers used for running general (Appendix A.1) PCR on RNA samples to identify genomic contamination in total RNA isolated from leaf samples.

Name	Sequences	Amplicon
*eIF4A*_FWD	AACTCAACTTGAAGTGTTGGAGTG	
*eIF4A*_RVS	AGATCTGGTCCTGGAAAGAATATG	168 bp

Genomic DNA isolated from perennial ryegrass was used as a template as a positive control and molecular grade water was used as a template in the negative control. The PCR products were run on 3.5% agarose gel to visualise the result along with Hyperladder V (Bioline, London, UK).

cDNA Synthesis

The template used for the qRT-PCR was cDNA synthesised using reverse transcription reaction of RNA samples. There were three biological replicates from each isolated RNA from each treatment Control, 8AC2+ and 8AC2- (Section 3.2.1). The selection of replicates was based on the quantity of RNA and also the samples chosen for the RNA sequencing experiment. 300 ng of total RNA based on the Qubit® flourometer (life technologies, NZ) quantification was used for synthesising cDNA for downstream processing. cDNA was synthesised using PrimeScript RT Reagent Kit (Perfect Real Time) from TAKARA, Clonetech following manufacturer's instruction. Then the cDNA was diluted 20 fold for further procedures. Efficiency of cDNA was analysed using general PCR with *eIF4A* gene using the primers listed in Table 3-5. Genomic DNA was isolated from perennial ryegrass and used as a template as the positive control and molecular grade water was used as the template for the negative control. The protocol in Appendix A.3 was followed to carry out the reverse transcriptase reaction to synthesis cDNA from the total RNA.

Table 3-5 Primers used for general PCR (Appendix A.1) for analysing the quality of cDNA synthesised from total RNA

Name	Sequences	Amplicon
eIF4A_FWD	AACTCAACTTGAAGTGTTGGAGTG	
eIF4A_RVS	AGATCTGGTCCTGGAAAGAATATG	168 bp

3.4.6..1 Determination of PCR efficiency

Standard preparations were explained in Section 3.4.5. Plasmids were linearized using specific restriction enzymes, (New England BioLabs®Inc) following manufacturer's instructions. Linearized plasmids containing the cloned gene fragment were used to create a seven point standard curve of 10 fold dilutions. The concentrations of 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} were used as templates for the qRT-PCR to determine the qRT-PCR efficiency. Table 2.1 lists the primer pairs used for each assay. Sterile water of molecular biology grade (UltraPure™ Distilled Water, Life Technologies, NZ) was used instead of serially diluted plasmids in a non-template control (NTC) to rule out any contamination in the assay. The standards and NTC were carried out in triplicates. The master mix containing qRT-PCR reagents and primers and serially diluted plasmids were pipetted into 2x48 well plates using an epMotion 5070 liquid handling robot (Eppendorf, Germany) to minimize any pipetting error. The qRT-PCR assay followed manufacturer's instructions as given in Appendix A.4.

3.4.6..2 qRT-PCR assay

A qRT-PCR assay was carried out to investigate the *LpUbl5* transcript level in each tissue sample. qRT-PCR was carried out on cDNA synthesised from Control (Impact), 8AC2+ and 8AC2- using TaKaRa SYBR® Premix Ex Taq™ II following manufactures instructions. The primers given in Table 2.1 were used for

the qRT-PCR assay to determine expression level of the reference gene and *LpUbl5*. The absolute quantification assays were run on the Illumina Eco™ Real Time PCR Systems (Illumina, Victoria, AU). This run a qRT-PCR reaction in 2x48 well plates (34.5cm x 31cm) across selected samples in triplicates. The master mix containing qRT-PCR reagents and primers and diluted cDNA (20 fold) were pipetted into 2x48 well plates using an Eppendorf epMotion 5070 liquid handling robot to minimize any pipetting error. A plate control sample (cDNA from Impact) was included with each plate using *eEF1A*-(s) primers to normalize any plate variation. A standard curve of 10 fold dilution of linearized plasmid (10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8}) was also run along with each assay as a positive control, to ensure PCR efficiency and quantify the Cq into gene copy number. A non-template control using molecular grade water (Life Technologies, NZ) instead of cDNA was used to rule out any contamination in the master mix. All the test samples, plate control and NTC were run in triplicate. The qRT-PCR reaction was carried as given in Appendix A.4.

Raw data obtained from the qRT-PCR assay were entered into Ecostudy 4.0 software (Illumina, NZ) for plate normalization. The highest Cq standard deviation accepted was 0.25 across a technical replicate. The plate normalized data were then exported to Excel (Microsoft, WA, USA) and the Cq obtained was converted into nanogram per microliter (ng/µL) using the equation derived from the standard curve of reference gene and *LpUbl5*. The amount was converted into copy number using the following formula (Equation 3-4) adapted from Integrated DNA Technologies Inc. (https://sg.idtdna.com/site)

Equation 3-4

$$\text{Number of copies (molecules)} = \frac{X\ (ng) * 6.0221 \times 10^{23}\ \text{molecules/mole}}{(N * 660\ \text{g/mole}) * 1 \times 10^{9}\ \text{ng/g}}$$

where X = amount of amplicon (ng), N = length of dsDNA amplicon and average mass of 1 bp dsDNA used = 660 g/mole. Conversion formula was adapted from Integrated DNA Technologies Inc.

3.5 Transgene evaluation in the transgenic plants

The results obtained from qRT-PCR and the physiology data were ambiguous (Section 3.7). This lead to the conclusion that there was a need to re-confirm the genotyping of the 8AC1, 8AC2+ and 8AC2- plants in this experiment. In particular, it was decided to carry out a thorough investigation of the transgene status of the plants supplied via ViaLactia Biosciences for the physiology experiment. They indicated that research to date had confirmed the transgenic status based on the presence or absence of an *hptII* gene which was the selective marker used for the transformation purposes. This process was followed due to difficulty in designing an efficient primer set to amplify the transgene in the plants (Sathish Puthigae, Pastoral Genomics Pers. Comm.). It was decided that the transgene status needed

to be confirmed based on the presence or absence of *LpUbl5* to critically evaluate the progress and future directions of the research programme. Therefore, new primers were designed in and around the *CaMV* double *35S* promoter (double *CaMV 35S/*D *35S*) sequence and *LpUbl5* sequence (Figure 3-8). The whole process followed the general end point PCR protocol explained in Appendix A.1 using genomic DNA as the template unless otherwise stated.

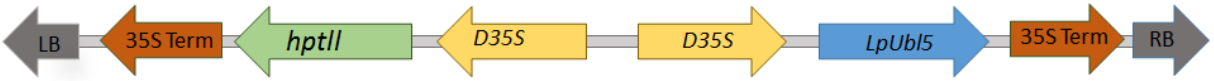


Figure 3-8 Schematic representation of T-DNA used for the transformation of *Lolium perenne* L. containing CAMV double 35S promoter driving *hptII gene* and *LpUbl5* gene.

3.5.1 Genomic DNA isolation

Genomic DNA was isolated from the leaf samples to be used as the template in the end point PCR procedure to identify the transgene status. Leaf samples from plants used for qRT-PCR were used for genomic DNA isolation to confirm the transgene status of samples used for the expression level study. Genomic DNA was isolated using NucleoSpin® Plant II, MACHEREY-NAGEL GmbH & Co. KG following the manufactures protocol. Genomic DNA was isolated from 100 mg of ground leaf tissue. The genomic DNA was quantified using Nano-drop and then a Qubit® flourometer (Life Technologies, NZ) was used to quantify DNA using Qubit dsDNA BR Assay Kit (Life Technologies, NZ) after calibration using the supplied standards. The isolated genomic DNA was used in the PCR procedures to define the transgene status of the plants. The samples used for initial PCR procedures are given in Table 3-6. Along with these samples, a positive control (binary vector) and an NTC were used in the reaction to validate the PCR efficiency.

Table 3-6 Information regarding the plants and corresponding groups of plants used for pilot trial end point PCR to define transgene status of the plants.

Plot ID	Plant ID	Group
5	59	8AC2+
13	125	8AC2+
24	64	8AC2+
27	113	8AC2+
10	97	8AC2-
19	23	8AC2-
20	35	8AC2-
32	148	8AC2-
32 _Expt 2	91	Norwegian (Germplasm Accession described in Chapter 6)

3.5.1..1 Sequence analysis and Primer design

The *CaMV 35S* promoter sequence was repeated twice in the sequence to be used as double *CaMV 35S* promoter for the transgene and the *hptII* gene. Therefore, the vector contained two double *CaMV 35S* promoters, one double *CaMV 35S* promoter driving *LpUbl5* gene, and another double *CaMV 35S* promoter driving the *hptII* gene. This made the primer design difficult. As an initial step, a commonly used *CaMV 35S* forward primer (5' GACGTTCCAACCACGTCTTCAAAGCAA 3') was matched against the vector sequence which showed that the primer sequence matched with multiple sequence in the T-DNA. Then efforts were made to search for a specific sequence which does not repeat in the vector. This lead to identification of a primer pair which is shown in Table 3-7. The primers used in this whole process were designed using the software Geneious® 7.1.7 (© 2005-2014 Biomatters Ltd NZ, Website: www.geneious.com) (Kearse *et al.*, 2012).

Table 3-7 Primers used for general PCR (Appendix A.1) for amplifying the transgene present in the ryegrass plants listed in the Table 3-6 to detect a partial T-DNA segment stretching from *D35S* promoter to *35S* terminator.

Name	Forward	Amplicon
*35S*_End_FWD	GCTAGGGTTTGGTCGAGAGGAGGCCGGCG	
*35S*_Term_REV	TATCGAATTCCTGCAGGGCG	713 bp

This primer pair was used to carry out general PCR (Appendix A.1) using the genomic DNA as the template. This combination was used to amplify the whole length of the *LpUbl5* gene insert until the end of the *35S* terminator sequence.

Table 3-8 Different combinations of primers used for carrying out end point PCR as genomic DNA as a template.

Combinations	Forward	Reverse	Amplicon
1	GCTAGGGTTTGGTCGAGAGGAGGCCGGCG (*35S*_END_FWD)	TATCGAATTCCTGCAGGGCG (*35S*_Term RVS)	713 bp
2	GCTAGGGTTTGGTCGAGAGGAGGCCGGCG (*35S*_END_FWD)	CCAACATGGCTGGGGAGATT (*LpUbl5*_Mid_RVS)	297 bp
3	GCTAGGGTTTGGTCGAGAGGAGGCCGGCG (*35S*_END_FWD)	CCGGGTGACAATAGGACACA (*LpUbl5*_End_RVS)	462 bp
4	AAC TCA ACT TGA AGT GTT GGA GTG (*elF4A*_FWD)	AGA TCT GGT CCT GGA AAG AAT ATG (*elF4A*_RVS)	168 bp

The first three combinations were used to identify the presence of the transgene using the same forward primer with two different combinations of reverse primer. The first combination was the same primer pair as shown in Table 3-7. This combination was repeated to confirm the results obtained. *LpUbl5*_Mid_RVS (Table 3-8) was generated to amplify the *LpUbl5* sequence which would include the complete coding sequence. *LpUbl5*_End_RVS (Table 3-8) was used to amplify the complete *LpUbl5*

transgene insert which was cloned into the binary vector. A third combination using *elF4A*_FWD and RVS (Table 3-8) was used to check the efficiency of genomic DNA and the PCR reagents as a quality control. This validates the quality of the genomic DNA used as the template in the end point PCR reactions. A decision was taken to carry out a stab PCR and sequence the PCR product to identify the amplified sequence.

A fresh set of genomic DNA was isolated from a new set of samples including samples from the cultivar "Grasslandz Impact'. Quantification of genomic DNA was carried out as explained in Section 3.5.1. 'Impact' was included in the samples because it is the parent line used to generate the transgenic lines under investigation. A general end point PCR using the primers of *elF4A*_FWD and RVS was used to evaluate the efficiency of genomic DNA. Primer combination 1 in Table 3-8 was used to carry out the end point PCR. The obtained PCR product was run on 1% agarose gel to identify the PCR product. Samples from plots 5 (8AC2+), 10 and 20 (8AC2-) had multiple bands indicating that these reactions contained multiple PCR products generated by the same primer pair. Efforts were taken to carry out band stab PCR (Bjourson and Cooper, 1992) from all PCR products obtained from the PCR reaction. To carry out band stab PCR, a fine needle (0.50X25 mm, TERUMO® Needle) was used to stab the PCR product on the agarose gel and the needle tip was mixed with the end point PCR reaction mix (Appendix A.1) which was then used for the PCR reaction. The PCR product was run on a 1% agarose gel then cleaned using an Axygen™ Axyprep™ DNA Gel Extraction Kit (Axygen, Ray lab, NZ) following manufacture's protocol. A total of 11 PCR products were cleaned and sent for sequencing (Bio-Protection Research Centre, Lincoln University, NZ). Sequencing was repeated using different primers.

A fresh start with new genomic DNA as template

A fresh set of genomic DNA was isolated including 8AC1 along with the samples described in Table 3-6 to avoid using any degraded samples through multiple thawing, freezing and handling issues. Quantification of genomic DNA was carried out as explained in Section 3.5. The samples included 8AC1 to identify the transgenic status of 8AC1, which was also one of the transgenic lines characterised to carry an additional copy of *LpUbl5*. This sample set included all the lines used in Experiment 1 (21 samples) in the biotron which survived till Drought Cycle 6. An end point PCR using *elF4A*_FWD and RVS primers was carried out to confirm their efficiency. Two primer combinations (Table 3-9) were used to identify the presence of the *hptII* gene and *LpUbl5* transgene in the samples. Hygromycin was the selective agent used in the binary vector used for the selection purpose in the transformation procedure. A total of 21 samples were used in which 'Impact' was used in the PCR reactions as a control for both the *LpUbl5* transgene detection and *hptII* gene. Binary vector was used as the positive control and molecular grade water (Life Technologies, NZ) was used as the negative control for both combinations.

Table 3-9 Primer combinations used for end point PCR reactions to evaluate the transgene status of ryegrass plants by detecting the components of T-DNA.

Combinations	Forward	Reverse	Amplicon
1	GCTAGGGTTTGGTCGAGAGGAGGCCGGCG (*35S*_END_FWD)	TATCGAATTCCTGCAGGGCG (*35S*_Term RVS)	713 bp
2	CCGCAAGGAATCGGTCAATA (Hygro _FWD)	CCCAAGCTGCATCATCGAAA (Hygro_RVS)	435 Bp

Troubleshooting using genomic DNA from Impact as template

Effort was taken to investigate different combinations of primer pairs in and around the complete coding sequence of *LpUbl5.* This was carried out to confirm the presence of the *LpUbl5* sequence in the genomic DNA sample from a non-transgenic plant, 'Impact'. This attempt included eight different combinations of primer pairs (Table 3-10).

Table 3-10 Different combinations of primer pair designed and used for end point PCR to detect the presence of *LpUbl5* in genomic DNA isolated from 'Impact'.

Combinations	Forward	Reverse	Amplicon
1	GAAGAGCTAGGGTTTGGTCG (*LpUbl5*_Full_FWD)	CCGGGTGACAATAGGACACA (*LpUbl5*_Full_RVS)	467 bp
2	ATGATCGAGGTGGTGCTCAAC (*LpUbl5*_ORF_FWD)	CTAGTTGTAGTAGAGCTCGAGTCC (*LpUbl5*_ORF_RVS)	222 bp
3	CCATCGGCGACCTCAAGAAG (*LpUbl5*_Int_FWD)	CGAGGGTGATGTGGTCCTTG (*LpUbl5*_Int_RVS)	105 bp
4	CCATCGGCGACCTCAAGAAG (*LpUbl5*_Int_FWD)	CTAGTTGTAGTAGAGCTCGAGTCC (*LpUbl5*_ORF_RVS)	155 bp
5	ATGATCGAGGTGGTGCTCAAC (*LpUbl5*_ORF_FWD)	CGAGGGTGATGTGGTCCTTG (*LpUbl5*_Int_RVS)	172 bp
6	GAAGAGCTAGGGTTTGGTCG (*LpUbl5*_Full_FWD)	CTAGTTGTAGTAGAGCTCGAGTCC (*LpUbl5*_ORF_RVS)	235 bp
7	ATGATCGAGGTGGTGCTCAAC (*LpUbl5*_ORF_FWD)	CCGGGTGACAATAGGACACA (*LpUbl5*_Full_RVS)	414 bp
8	TACAAGGACCACATCACCCT (*LpUbl5*_qRT_FWD)	GGATGCATACCAACATGGCT (*LpUbl5*_qRT_RVS)	108 bp

Eight different end point PCR reactions were carried out using the genomic DNA isolated from 'Impact' as the template. The binary vector was used as a positive control and molecular grade water as a negative control. The PCR products were run on the 3% agarose gel to visualise the results.

Screening primary transgenic ryegrass plants

Primary transgenic plants were selected to evaluate primer pair combinations (Table 3-11) as the T_3 lines used in the Experiment 1 were generated from these primary transgenic plants. The primary transgenic lines were 8AC1, 8AC2, 7AE5 and 7AE15. 8AC1 and 8AC2 were the primary transgenic lines

from which T_3 lines used in Experiment 1. 7AE5 and 7AE15 are perennial ryegrass carrying a T-DNA containing a dehydrin promoter driving an AN1-A20 type zinc finger transcription factor. 8AC1, 8AC2, 7AE5 and 7AE15 have *hptII* gene as a selection marker.

Table 3-11 Primer combinations used for screening primary transgenic lines to detect different T-DNA components.

Combinations	Forward	Reverse	Amplicon
1	GCTAGGGTTTGGTCGAGAGGAGGCCGGCG (*35S*_END_FWD)	TATCGAATTCCTGCAGGGCG (*35S*_Term RVS)	713 bp
2	CCGCAAGGAATCGGTCAATA (Hygro _FWD)	CCCAAGCTGCATCATCGAAA (Hygro_RVS)	435 Bp

These were selected because they had not undergone any crossing or transgene segregation which could possibly lead to transgene deletion, duplication and rearrangements. cDNA synthesised from RNA isolated from 'Impact' was used as the positive control in primer pair (Table 3-11) combination 1 and a binary vector and molecular grade water (Life Technologies, NZ) were used as negative controls. A binary vector was used as the positive control for primer pair combinations 2 and 3 and cDNA and molecular grade water were used as negative controls.

Designing new primer combinations

Three different primers were designed using sequences from the double 35S promoter. A primer was picked from the beginning of the *D35S* promoter sequence with the addition of 12 bp at the 5' end from the vector. A second forward primer was completely selected from the double *CaMV 35S* promoter sequence. A third forward primer was selected from the end of the double *CaMV 35S promoter*. These three primers made a total of 12 different primer combinations with previous reverse primers (Table 3-10) and the combinations (Table 3-12). As an initial step, the binary vector was used as the template to study the efficiency of primer pairs with the inclusion of molecular grade water (Life Technologies, NZ) as the negative control in all reactions.

Table 3-12 Primer pair combinations use to evaluate the efficiency of primer pairs using binary vector as template.

Combinations	Forward	Reverse	Amplicon
1	CACTCTCGTCTACTCCAAGAAT (*D35S*_FWD_1)	CCGGGTGACAATAGGACACA (*LpUbl5*_Full_RVS)	1241 bp
2	CACTCTCGTCTACTCCAAGAAT (*D35S*_FWD_1)	CTAGTTGTAGTAGAGCTCGAGTCC (*LpUbl5*_ORF_RVS)	1049 bp
3	CACTCTCGTCTACTCCAAGAAT (*D35S*_FWD_1)	CGAGGGTGATGTGGTCCTTG (*LpUbl5*_Int_RVS)	999 bp
4	CACTCTCGTCTACTCCAAGAAT (*D35S*_FWD_1)	GGATGCATACCAACATGGCT (*LpUbl5*_qRT_RVS)	1085 bp
5	GTCTCAGAAGACCAAAGGGC (*D35S*_FWD_2)	CCGGGTGACAATAGGACACA (*LpUbl5*_Full_RVS)	1207 bp
6	GTCTCAGAAGACCAAAGGGC (*D35S*_FWD_2)	CTAGTTGTAGTAGAGCTCGAGTCC (*LpUbl5*_ORF_RVS)	1015 bp
7	GTCTCAGAAGACCAAAGGGC (*D35S*_FWD_2)	CGAGGGTGATGTGGTCCTTG (*LpUbl5*_Int_RVS)	965 bp
8	GTCTCAGAAGACCAAAGGGC (*D35S*_FWD_2)	GGATGCATACCAACATGGCT (*LpUbl5*_qRT_RVS)	1051 bp
9	AGGACACGCTGAAATCACCA (*D35S*_FWD_3)	CCGGGTGACAATAGGACACA (*LpUbl5*_Full_RVS)	520 bp
10	AGGACACGCTGAAATCACCA (*D35S*_FWD_3)	CTAGTTGTAGTAGAGCTCGAGTCC (*LpUbl5*_ORF_RVS)	328 bp
11	AGGACACGCTGAAATCACCA (*D35S*_FWD_3)	CGAGGGTGATGTGGTCCTTG (*LpUbl5*_Int_RVS)	278 bp
12	AGGACACGCTGAAATCACCA (*D35S*_FWD_3)	GGATGCATACCAACATGGCT (*LpUbl5*_qRT_RVS)	364 bp

3.5.1..1 Finalizing the efficient primer pair combinations

A decision was taken based on the results generated from these methods to use primer pair combination 9 (Table 3-12) for the evaluation of genomic DNA isolated from selected samples. This approach would confirm the presence of the complete *LpUbl5* insert in the plants. The samples selected from primary transgenics were 8AC1, 8AC2, 7AE5, 7AE15. 8AC1 and 8AC2 were the primary transgenic lines of the T_3 transgenic lines (Section 3.2.1) used in this research. Samples selected from Experiment 1 in the Biotron were 'Impact', 8AC2-, 8AC2+ and 8AC1. Controls used for the reaction were the binary vector as a positive control, and ryegrass cDNA and molecular grade water as negative control. Further, it was important to identify the presence of *LpUbl5* full open reading frame in the same set of samples used above to confirm the presence of ORF in the plants while the complete *LpUbl5* insert was absent. This was carried out using the primer pair combination 10 (Table 3-12). This step also aimed to compare the efficiency of both the primer pair combinations.

Table 3-13 Primers used for screening plants for the presence of intact T-DNA in the transformed plants.

Combinations	Forward	Reverse	Amplicon
1	AGGACACGCTGAAATCACCA (*D35S*_FWD_3)	CCGGGTGACAATAGGACACA (*LpUbl5*_Full_RVS)	520 bp
2	CCGCAAGGAATCGGTCAATA (Hygro_FWD)	CCCAAGCTGCATCATCGAAA (Hygro_RVS)	435 bp

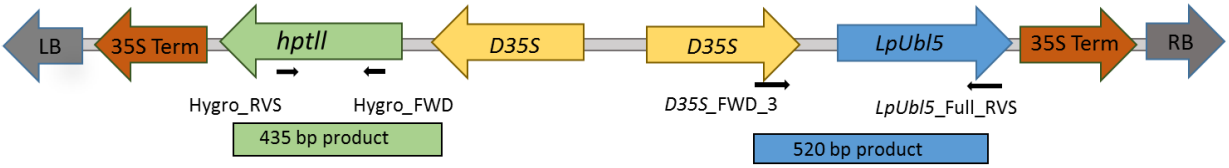


Figure 3-9 Schematic representation of primer pairs used for the identification of plants for the presence of *hptII* gene and *LpUbl5* transgenes.

The whole process helped to identify a unique set of primers (Figure 3-9) which could amplify the transgene in the transformed plant thus confirming the presence or absence of the transgene in the transformed plants. The primer pair combination 1 in Table 3-13 detects the presence of the *LpUbl5* sequence and combination 2 identifies the presence of *hptII*. Screening other plants with *LpUbl5* and *hptII* primer pairs, helps to understand the presence of the complete T-DNA insert in the transgenic plants or any truncation in the T-DNA which occurred over generations. This process allows to identify and categorise them based on the presence or absence of *LpUbl5* and the *hptII* gene. The same samples were screened for presence of the *hptII* gene to evaluate whether the T-DNA was intact within the plant system. Two separate endpoint PCRs were carried out by following the general PCR protocol explained in Appendix A.1.

3.6 Statistic analysis

3.6.1 Shoot biomass production

Dry matter production was initially analysed by GenStat Version GenStat Release 16.1, (Copyright 2013, VSN International Ltd.) using a One-way Analysis of Variance (in a Randomized Blocks). Further dry matter yield (dry matter/ plant) was analysed by REML (Restricted Maximum Likelihood). The method was chosen to analyse data due to the fact that the data generated were from repeated measurements from the same plants over the course of the research. The data were analysed by repeated measurements, data in one variate. In this analysis the fixed models were Harvest date and Line and Random model was termed a 'multfact'. The individual selection of these three factors generated an error report by Genstat. Therefore these were converted to multfact to overcome the error. The data used for analysis were log10 transformed due to the high number of zeros in the results due to plant deaths. The data were transformed using Equation 3-5 and the result was back transformed using Equation 3-6.

Equation 3-5 $z=\log10(y+3/8)$

Equation 3-6 $y=10z - 3/8$

The mean values given are back transformed data obtained from the predicted means by REML, repeated measurement analysis.

All the other physiology measurements were analysed using One-way Analysis of Variance (in a Randomized Blocks) and a post hoc analysis using Fisher's protected LSD test.

3.7 Results

3.7.1 Biomass

3.7.1..1 Shoot biomass accumulation

Total accumulated yield ranged (P<0.05) from 2.3 g/plant (8AC1) to 6.7 g/plant from the Control (Figure 3-10). The yield of 8AC2+ (5.2± 1.01 g/plant) and 8AC2- was (4.8 ± 1.01 g/plant) was not different to either the Control or 8AC1 yields.

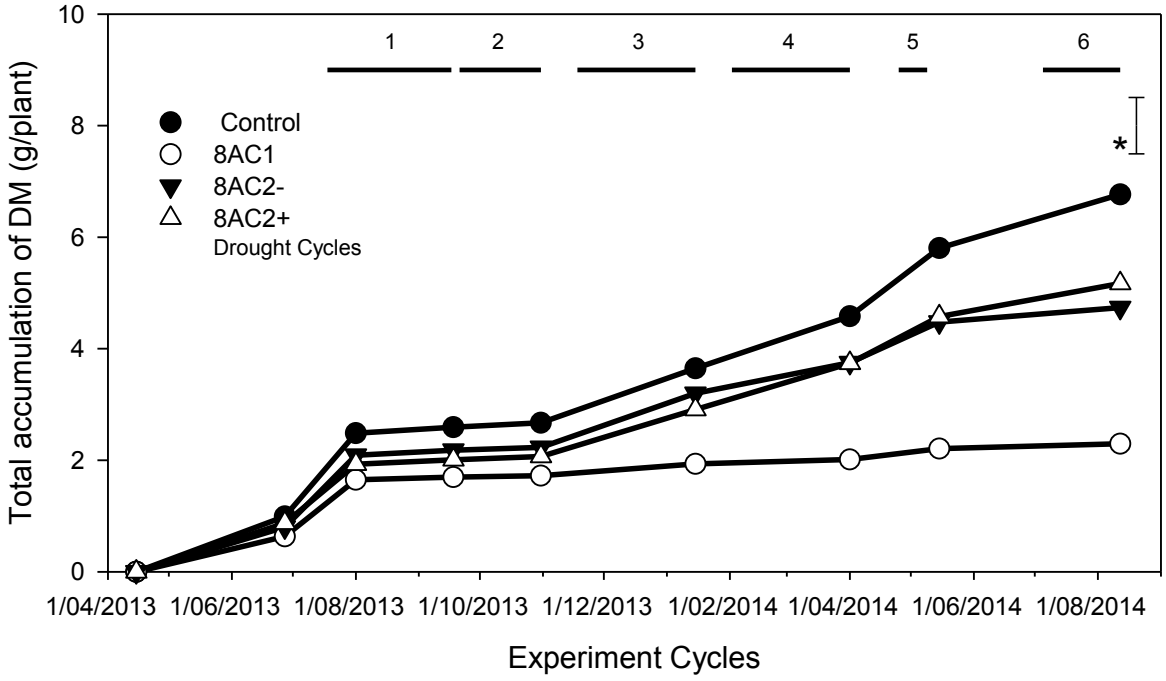


Figure 3-10 Shoot biomass accumulation over time from four lines of perennial ryegrass from eight harvests dates (27/6/2013, 1/08/2013, 18/09/2013, 31/10/2013, 15/01/2014, 1/4/2014, 15/05/2014 and 12/08/2014), Error bar is SEM for the total dry matter accumulation from One-way ANOVA in randomized blocks. Asterisks shown where significant difference was observed.

Shoot biomass accumulation during the establishment phase and Drought Cycle 1 did not show any difference (P=0.11) across the four lines. This suggests that they had all established to a similar level and responded similarly to the initial drought cycle. However, from the end of Drought Cycle 3 to the end of Drought Cycle 5 there was a trend (P=0.09, P=0.07, P=0.12) where 8AC1 showed no increase in shoot biomass accumulation compared with the Control, 8AC2- and 8AC2+. Control had the highest (p<0.05) shoot biomass (0.9 ± 0.21 g/plant) at the end of Drought Cycle 6 whereas 8AC2+ (0.6 ± 0.21 g/plant) was not different to Control and 8AC2- (0.25 ± 0.21 g/plant) and 8AC1 (0.08 ± 0.21 g/plant) were not different to 8AC2+.

3.7.1..2 Shoot biomass generated at each harvest

The second harvest was carried out at the beginning of the Drought Cycle 1. As expected after the establishment phase under well watered conditions shoot biomass was high compared with the subsequent regrowth periods because each successive drought cycle restricted plant growth. The dry matter production showed an interaction (P=0.028) between harvest dates and line. The results of back-transformed mean dry matter are shown in Table 3-14.

Table 3-14 Mean of shoot dry matter (g/plant) data of Control, 8AC1, 8AC2+ and 8AC2- from eight harvest dates generated from the individual harvests (g/plant).

	27-Jun-13		1-Aug-13		18-Sep-13		31-Oct-13	
Line	Mean dry matter (g/plant)	$log_{10}(x)$	Mean dry matter (g/plant)	$log_{10}(x)$	Mean dry matter (g/plant)	$log_{10}(x)$	Mean dry matter (g/plant)	$log_{10}(x)$
8AC1	0.652	0.011	0.980	0.132	0.044	-0.378	0.024	-0.400
8AC2+	0.890	0.102	0.955	0.124	0.073	-0.349	0.055	-0.366
8AC2-	0.812	0.074	1.238	0.208	0.087	-0.336	0.047	-0.374
Control	1.016	0.143	1.419	0.254	0.104	-0.320	0.074	-0.348
E.s.e $log_{10}(x)$		0.092		0.066		0.066		0.066
	15-Jan-14		1-Apr-14		15-May-14		12-Aug-14	
8AC1	0.201	-0.240	0.060	-0.361	0.131	-0.296	0.068	-0.354
8AC2+	0.568	-0.025	0.436	-0.091	0.492	-0.062	0.356	-0.136
8AC2-	0.734	0.045	0.427	-0.096	0.577	-0.021	0.170	-0.264
Control	0.926	0.114	0.758	0.054	1.043	0.152	0.858	0.091
E.s.e $log_{10}(x)$		0.066		0.066		0.066		0.066

Note: REML analysis of transformed data (Equation 3-5) using data in one variate, and the back-transformed mean dry matter values using Equation 3-6.

3.7.2 Plant survival

The evaluation of plant survival was initiated after the establishment phase, during each harvest. There was a decline in the number of plants after each harvest from each treatment (Figure 3-11). All plants from control, 8AC1, 8AC2- and 8AC2+ were established and survived during the establishment phase. At the end of Drought Cycle 1, 8AC2- had the lowest plant survival (86.9 ± 2.0%) ($P<0.01$) than the 96.3 ± 2.0% for the Control, 8AC1 and 8AC2+. At the end of Drought Cycles 4, 5 and 6, the highest ($P<0.01$) plant survival was from Control (52 ± 6.3%) compared with 8AC2+ (23.3 ± 6.3%), 8AC2- (20 ± 6.3%), 8AC1 (16 ± 6.3%).

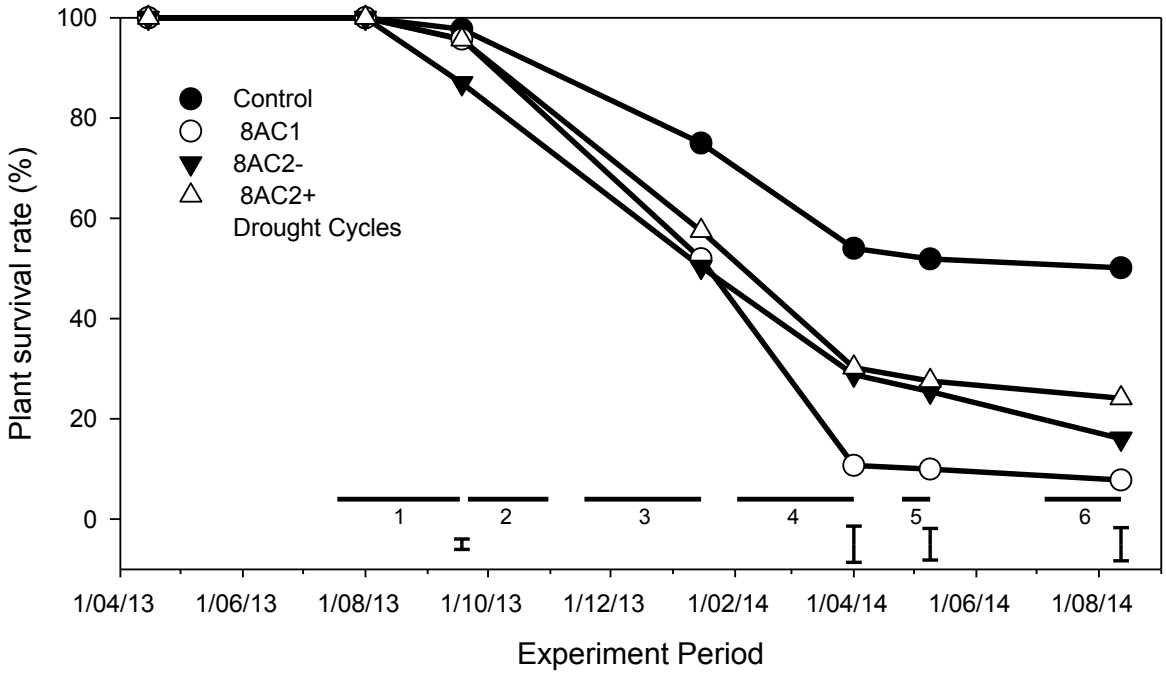


Figure 3-11 Percentage of plant survival throughout Experiment 1 after each imposed drought cycle. Error bar is SEM for the plant survival and is shown where differences ($P<0.05$) were detected**.**

3.7.3 Plant Score

At the end of Drought Cycle 1, the control had the highest plant score of 2.7 (P<0.01) compared with 8AC1, 8AC2- and 8AC2+ ((2.0 ± 0.12) Figure 3-12). This pattern continued during Drought Cycle 2 for two consecutive scorings of 2.8 and 2.7 (P<0.001). During Drought Cycle 3, scoring occurred on three occasions. At recovery values ranged from 0.8 (8AC1) to 1.9 (P<0.01) for the Control. The grades of 8AC2+ and 8AC2- was 1.3 ± 0.18 which were intermediate. The second scoring under Drought Cycle 3 exhibited the same pattern. However, a third grading under the drought conditions showed the Control had the highest score of 2 (P<0.001) which was higher than 8AC2+, 8AC2- and 8AC1 (1 ± 0.17). The Control also had the highest score of 1.5 to 1.8 (P<0.001) through Cycles 4, 5 and 6. 8AC1 had the lowest (P<0.001) score 0.11 to 0.15 and 8AC2+ and 8AC2- were similar.

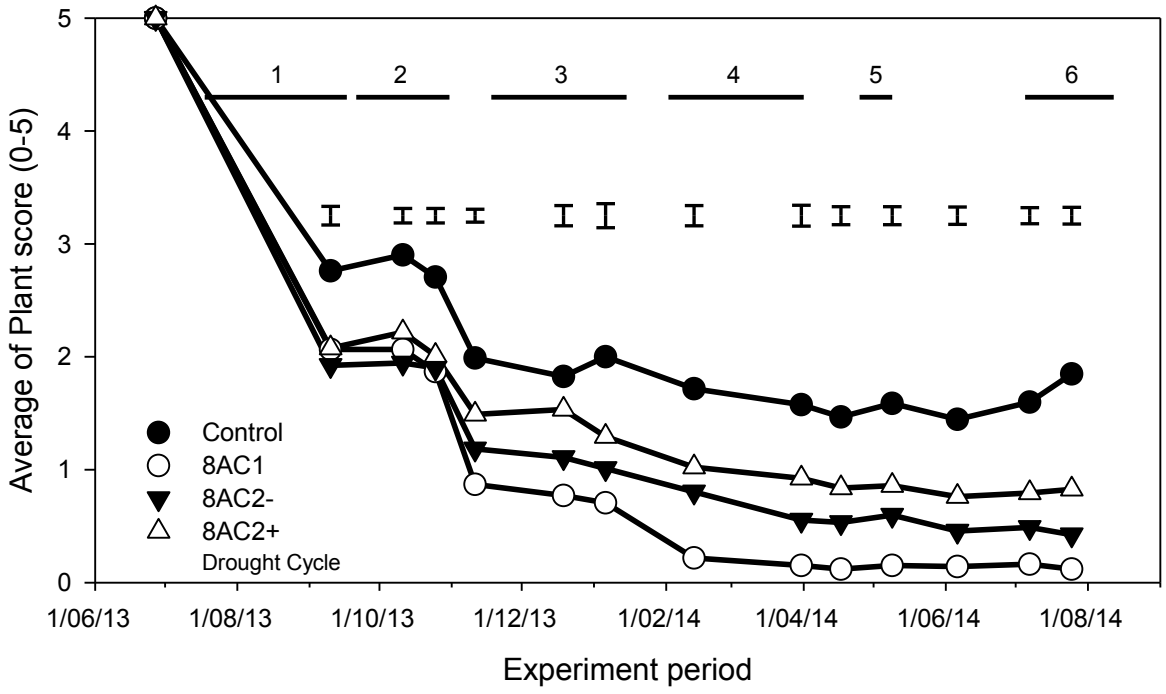


Figure 3-12 Plant score obtained throughout the Experiment 1 period. Error bar is SEM for the plant score shown when differences (P<0.05) were detected.

3.7.4 Total leaf extension (mm/rotation)

Leaf extension result was measured across six dry down cycles in total. The total leaf extension did not show any difference (P=0.10) among the lines until the Drought Cycle 3 (Figure 3-13). At this point the Control and 8AC2- had mean leaf extension of 365 ± 28 mm and this was longer (P<0.05) than 8AC1 (234 mm). 8AC2+ (313 mm) was not different to all other lines. At the end of Drought Cycle 4, the Control, 8AC2- and 8AC2+ had longer (P<0.01) total leaf length of 490 ± 63.7 mm compared with 8AC1 (234 mm). During Drought Cycle 5, the Control had the longest (P<0.05) leaf extension of 568 mm. 8AC2+ and 8AC2- showed no difference leaf extension at 351 ± 82.8 mm and 8AC2+ and 8AC2- were not different to the Control. 8AC1 had the shortest leaf extension of 142 mm. In the final (6th) drought cycle, the Control had a leaf extension of 446 mm which was longer (P<0.01) than 8AC2+, 8AC2- and 8AC1 which averaged 211 ± 55.6 mm.

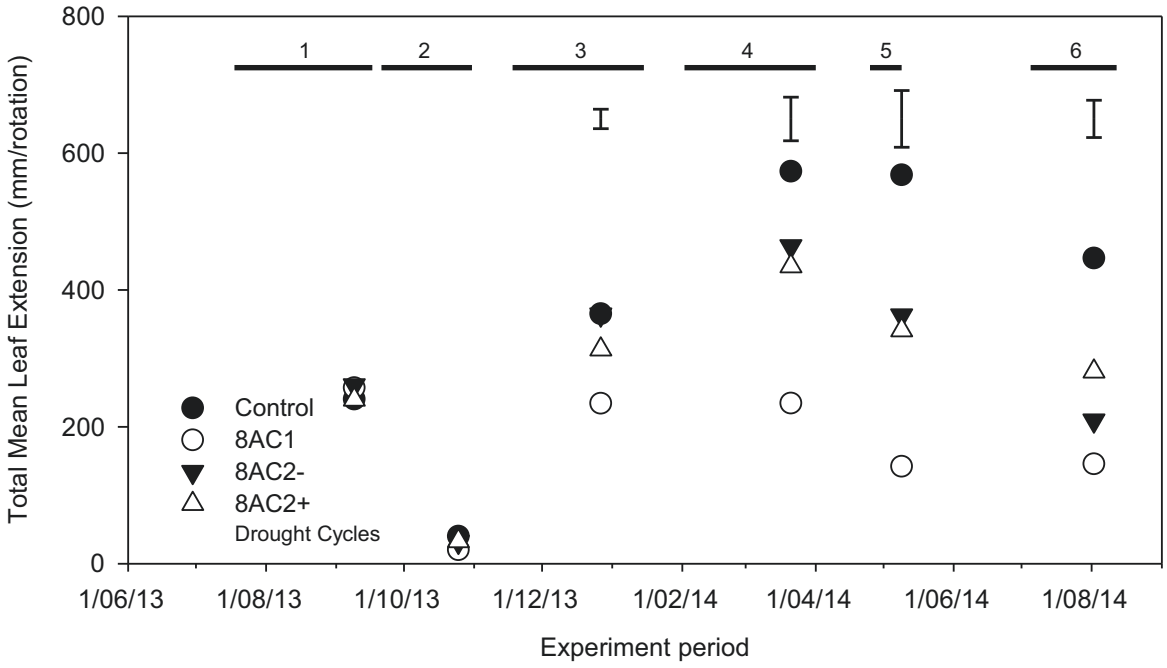


Figure 3-13 Total leaf extension mm/rotation from each imposed drought cycle. Error bar is SEM for the total leaf extension shown when difference (P<0.05) were detected

3.7.5 Leaf extension rate (mm/day)

As expected the leaf extension rate per day (mm) showed the same pattern as total leaf extension during the first two imposed Drought Cycles (Figure 3-14). Differences started to be observed during Drought Cycle 3 when (P<0.01) Control and 8AC2- (7.5 ± 0.59 mm/day) were different to 8AC1 (4.8 mm/day) but 8AC2+ (6.5 mm/day) was not different to the Control, 8AC2- or 8AC1. During the Drought Cycle 4, Control, 8AC2+ and 8AC2- (7.6 ± 0.99 mm/day) were longer (P<0.01) than 8AC1 (3.6 mm/day). The Drought Cycle 5 exhibited a similar result to Drought Cycle 3. In the final drought Cycle, Control had a faster (P<0.01) leaf extension rate (5.6 mm/day) where 8AC2+, 8AC2- and 8AC1 had 2.6 ± 0.70 mm.

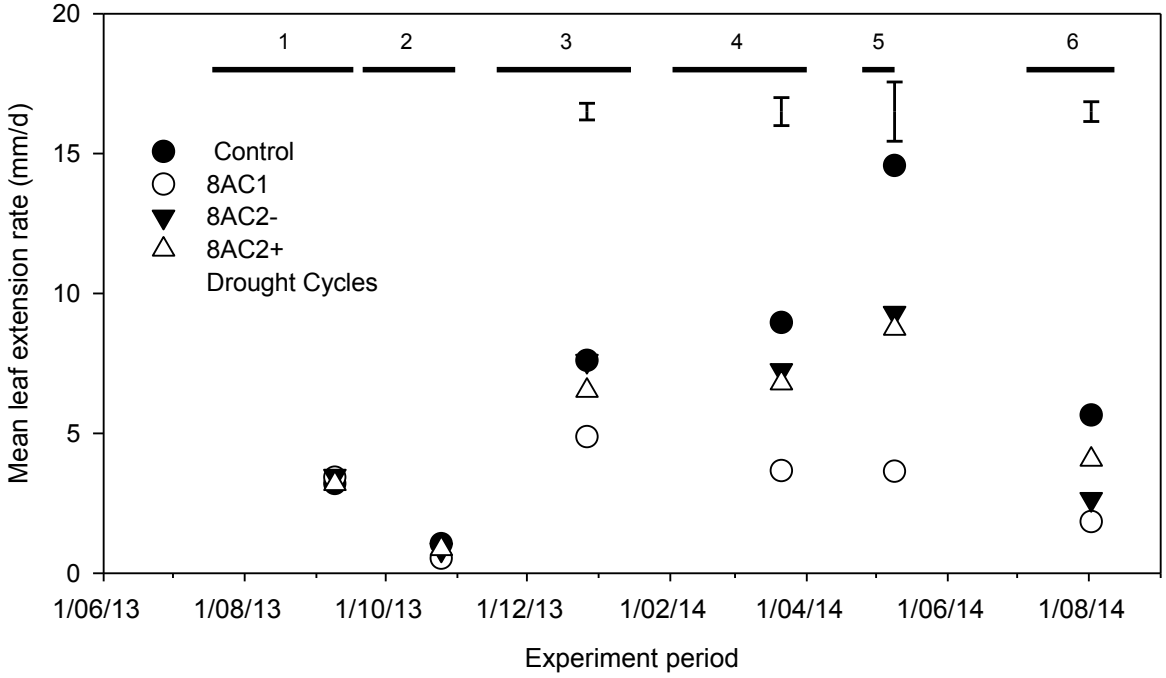


Figure 3-14 Leaf extension rate mm/day during each imposed drought. Error bar is SEM for the leaf extension rate and is shown when differences (P<0.05) were detected.

3.7.6 Leaf area (mm^2)

The leaf area per leaf was measured only once during the first drought cycle and showed no differences P=0.43) among lines (Figure 3-15). During Drought Cycle 2, control (240 mm^2) was larger (P<0.05) than 8AC2+, 8AC2- and 8AC1 (132 ± 26.9 mm^2) at the beginning of drought. During the middle of the Drought Cycle 3, Control, 8AC2+ and 8AC2- (522 ± 43.8 mm^2) were larger (P<0.01) than 8AC1 (346 mm^2) but this did not continue during the drought. However this result was repeated during Drought Cycle 4. During the final Drought Cycle, leaf area was measured five times in which Control was larger (P<0.01) than 8AC2+, 8AC2- and 8AC1 at three times of measurements but not at the end of the drought where Control and 8AC2+ had 191 ± 44 mm^2 leaf area and 8AC1 had the lowest (27.2 mm^2) leaf area.

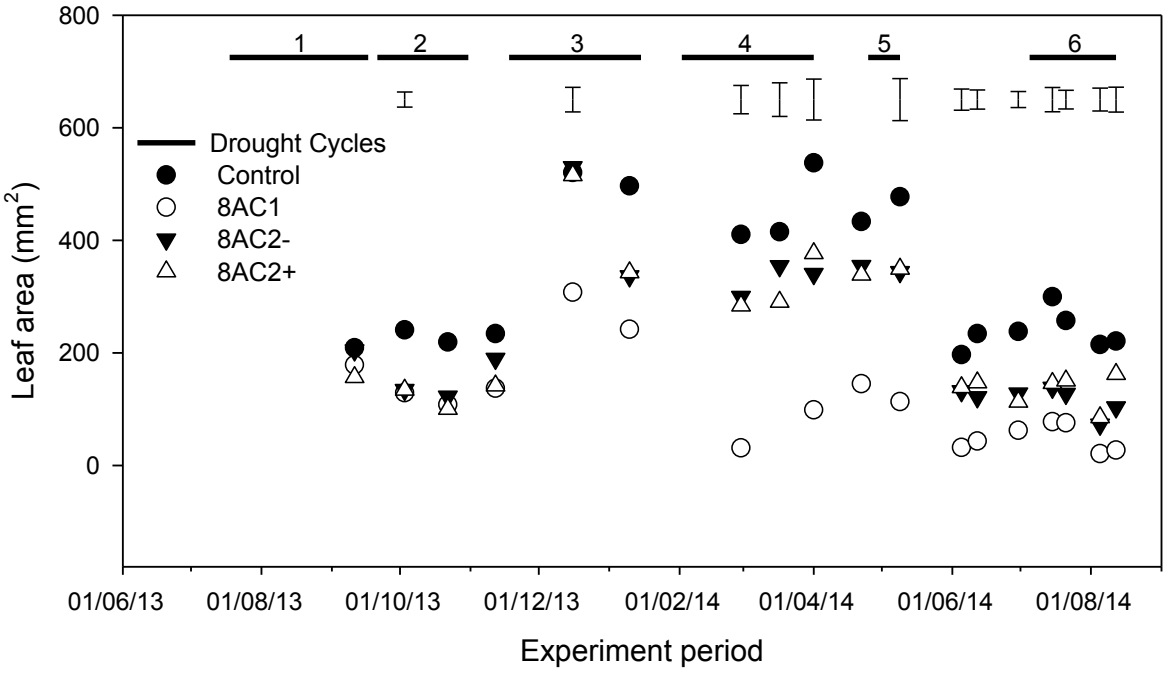


Figure 3-15 Leaf area (mm^2) during each imposed drought cycle. Error bar is SEM for the leaf area and is shown when differences (P<0.05) were detected.

3.7.7 Relative water content (RWC)

The relative water content was not different (P=0.4) during Drought Cycle 1. During Drought Cycle 2, the control, 8AC2+ and 8AC2- had higher (P<0.05) RWC than 8AC1 during recovery and drought stress (Figure 3-16). There was no difference in RWC during Drought Cycle 3 but Drought Cycles 4 and 5 showed the same pattern of difference (P<0.05) to Drought Cycle 2. During the final drought cycle, five consecutive RWC measurements were taken in which the lines did not show any difference except at the fifth measurement. At the fifth measurement at the end of the imposed drought period, the Control and 8AC2+ had a RWC of 24 ± 5.18% which was higher (P<0.05), than 8AC1 (4%) and 8AC2- (15%) was intermediate.

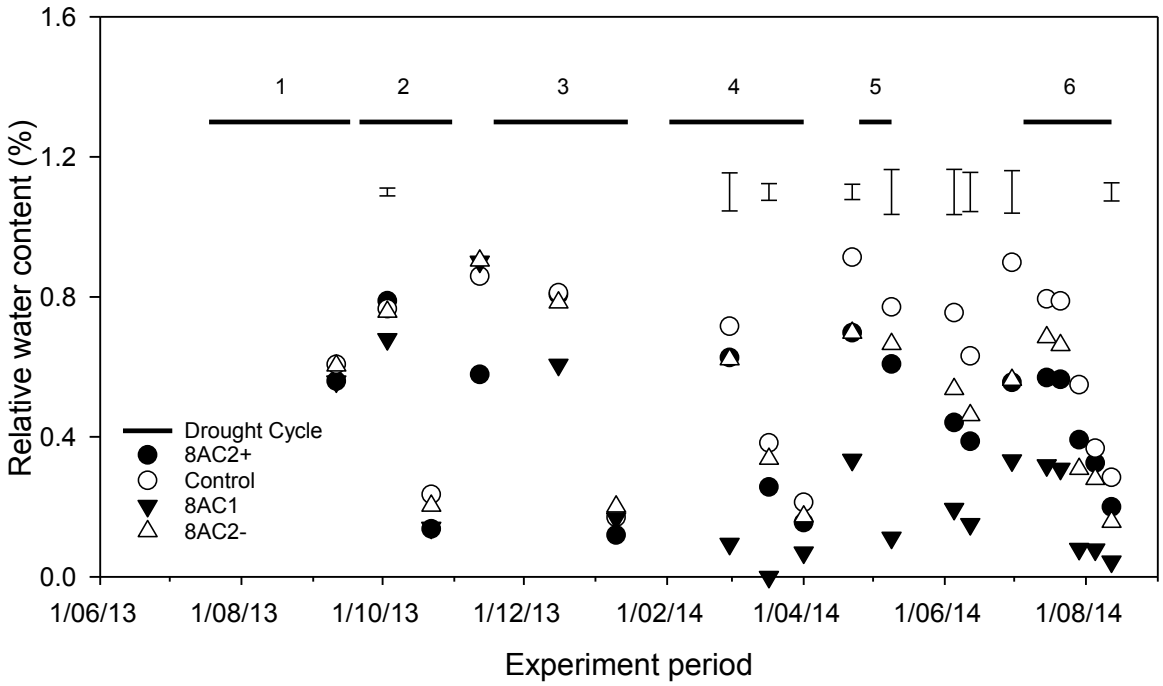


Figure 3-16 Relative water content (%) during each imposed drought cycle. Error bar is SEM for the leaf area and is shown when differences (P<0.05) were detected.

3.7.8 Solute Potential (MPa)

The solute potential of the Control, 8AC1, 8AC2+ and 8AC2- did not show any difference (P=0.45) at the end of the Drought Cycle 1 (Figure 3-17). At the end of Drought Cycle 2, the solute potential of Control and 8AC2+ were -4.41 ± 0.58 MPa which was not different (P=0.23) to either 8AC2- or 8AC1. Under Drought Cycle 3, solute potential was measured once which did not show any difference (P=0.09) among the lines. However under Drought Cycle 4, solute potential was measured twice in which the Control, 8AC2+ and 8AC2- averaged -1.3 ± 0.24 MPa and -2.8 ± 0.52 MPa which were lower (P<0.01) than 8AC1 at 0 MPa. In the final Drought Cycle, the first two measurements under recovery to progressive drought did not show any difference (P<0.5) in solute potential across the lines. However, during the final measurement at the end of the imposed drought, the 8AC1 and 8AC2+ had higher (P<0.05) solute potential of -0.61 ± -0.524 MPa compared with the Control (-2.59 MPa), 8AC2- (-1.38 MPa) intermediate.

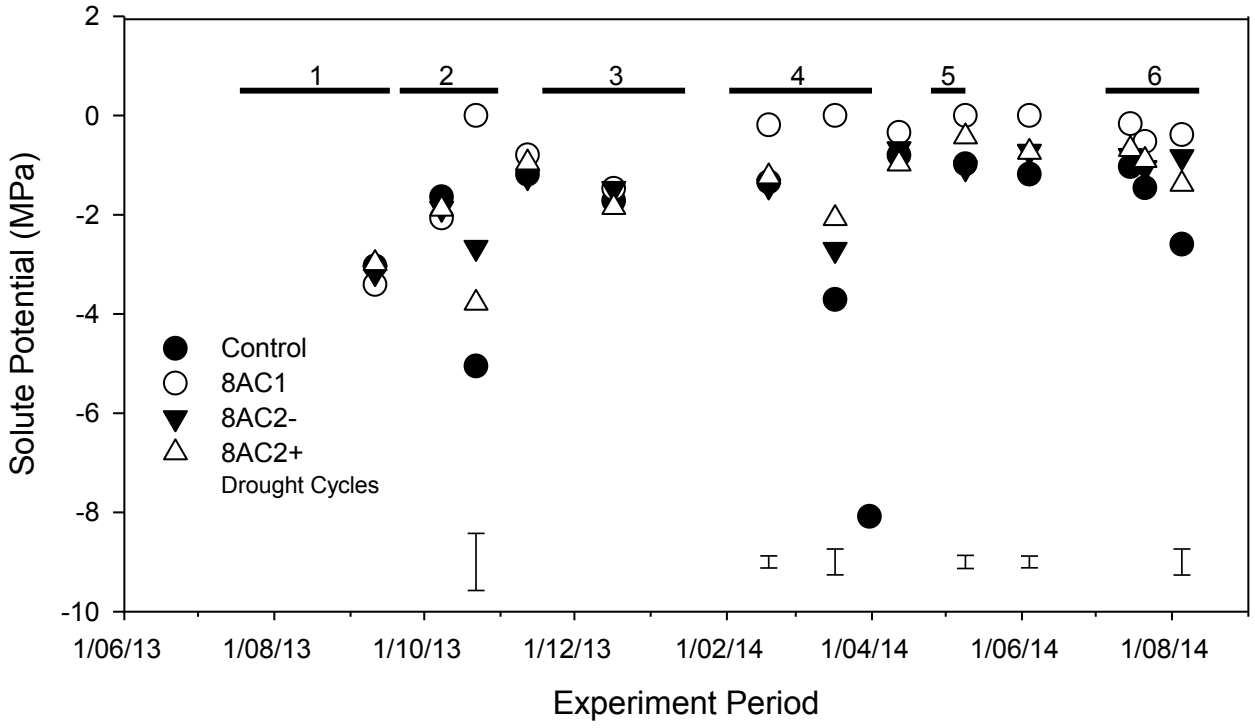


Figure 3-17 Osmotic potential (MPa) during each imposed drought cycle. Error bar is SEM for the solute potential and is shown where differences (P<0.05) were detected.

3.7.9 *LpUbl5* expression level analysis

Quality Analysis of isolated RNA

Quality analysis of RNA was carried out by formaldehyde agarose gel and endpoint PCR using RNA as a template to identify the presence of genomic contamination (Section 3.4.6). The corresponding representative results are given below. The RNA isolated from each sample was run on denaturing formaldehyde agarose gel to confirm the integrity of RNA. The intact bands show the RNA isolated was intact and the ribosomal RNA bands 28S (first band from the top) and 18S (second band from the top) are visible in Figure 3-18.

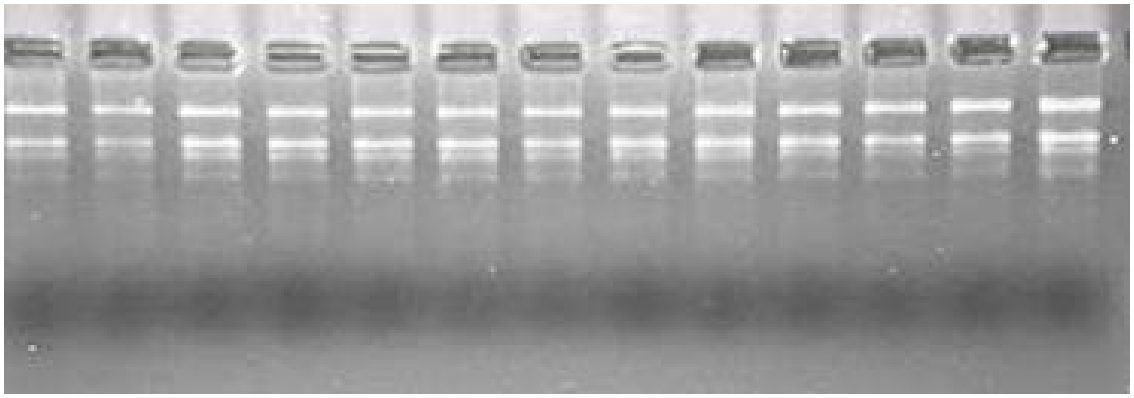


Figure 3-18 The representative formaldehyde agarose gel, showing the integrity of total RNA isolated from the *Lolium perenne* L. An equal amount of RNA was added to each well.

Evaluation of presence of genomic DNA in RNA samples by End point PCR

The result of endpoint PCR using RNA as the template is in Figure 3-19. This is a representative image of all gel images obtained from the procedure. The sample numbers are given above each well in the gel where no amplification is observed. The positive control shows an amplification where TOPO/TA: *elF4A* was used as a template to evaluate the efficiency of the PCR reaction. The negative control shows the absence of any amplification where NTC was used as a template. The negative control and positive controls give confidence in the results.

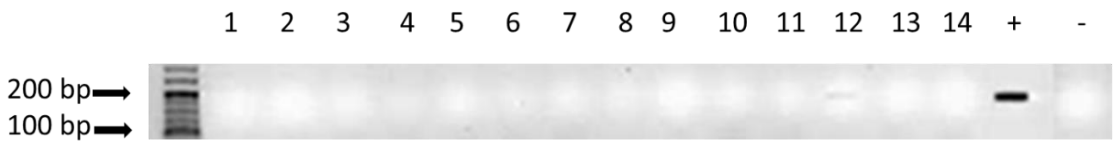


Figure 3-19 3.5% agarose gel showing the PCR product of total RNA sample along with positive control (TOPO/TA: elF4A) and negative control (molecular grade water). Sample labels are provided above the gel. The HyperLadder™ 25 bp (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

The result of end point PCR using total RNA as a template shows that the RNA does not have any genomic DNA contamination. The absence of a band in sample 1-14 indicates that there is no genomic contamination in these samples. A positive control (genomic DNA as template) and a negative control (PCR grade water) are shown. It is important to confirm the absence of genomic DNA contamination to obtain an accurate transcript copy number.

Quality analysis of synthesised cDNA

Quality analysis of cDNA was carried out using End point PCR to confirm the efficiency of the RT reaction. The gel image in Figure 3-20 shows the amplification product of *elF4A.*

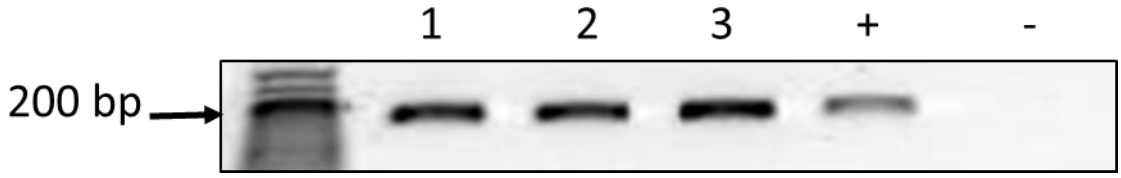


Figure 3-20 Validation of quality of synthesised cDNA as template along with positive control (TOPO/TA: elF4A) and negative control (molecular grade water). The HyperLadder^{TM} 25 bp (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as a DNA ladder.

The quality check on RNA and synthesised cDNA gave sufficient confidence to progress to qRT-PCR using the cDNA synthesised and these quality evaluations have confirmed the efficiency of procedures carried out from RNA isolation until cDNA synthesis.

Results of qRT-PCR

The results in Figure 3-21 were ambiguous. The results show wide variation within each treatment. This has led to a rethinking of the status of plants and meant an evaluation of the transgene in the plants used for the qRT-PCR was necessary. 8AC1 plants did not have three biological replicates and therefore did not include in the transcript level analysis.

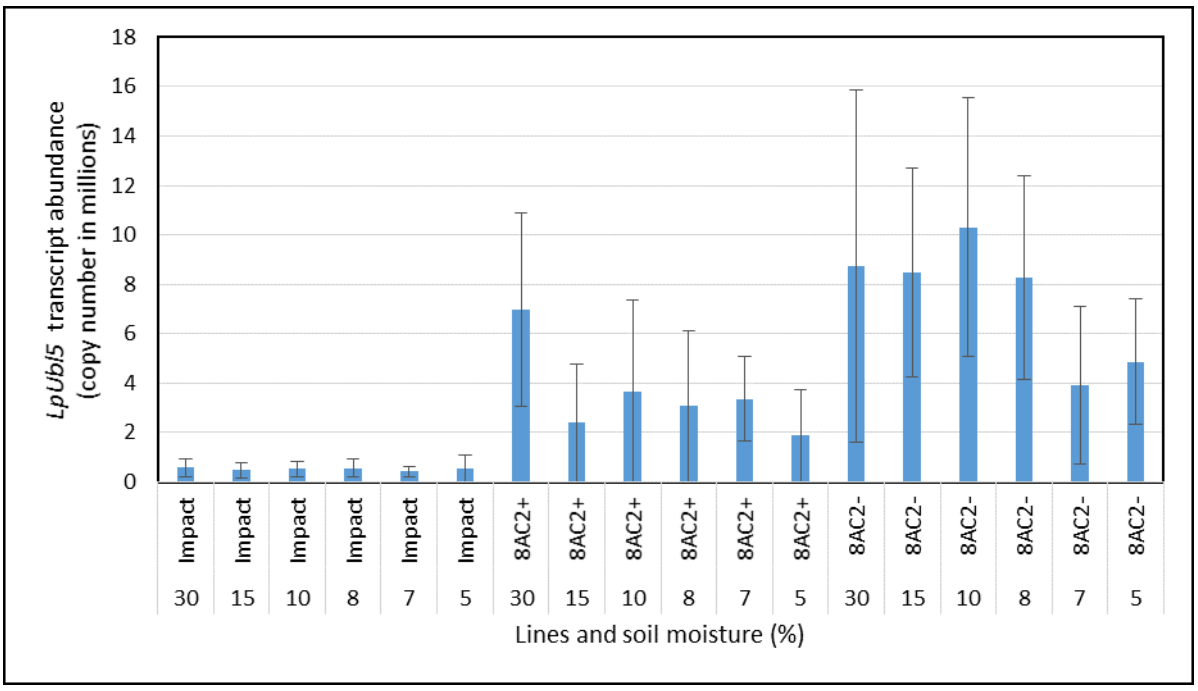


Figure 3-21 Copy number of *LpUbl5* gene in the analysed samples. The samples were collected under Drought Cycle 6 during progressive drought. The available soil moisture (%) at the time of sample collection. Error bar shown is the standard deviation observed between the biological replicates.

3.8 Transgene Evaluation

3.8.1 Primary evaluation of transgene

Figure 3-22 shows the amplification product of *35S*_End_FWD and *35S*_Term_RVS. The results show multiple products in the test samples regardless of plants categorised as 8AC2+ or 8AC2-. The negative control and genomic DNA from the Norwegian line (Chapter 6, Section 6.1.2) did not show any amplification. The positive control was a binary vector which showed a thick band indicating strong amplification.

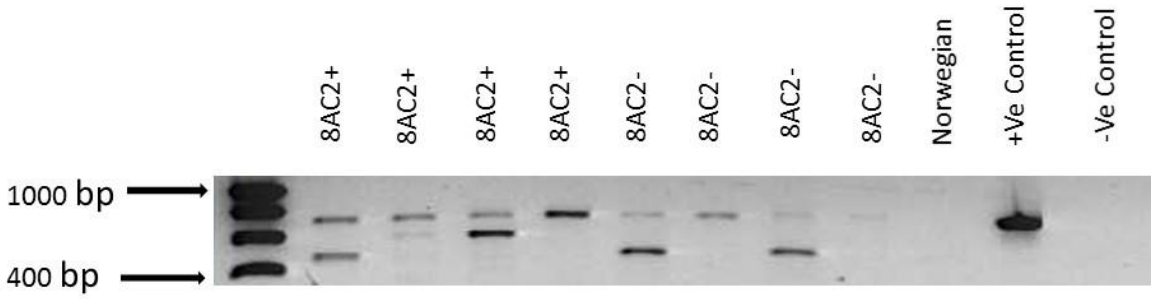


Figure 3-22 Gel images of PCR product obtained using the *35S*_End_FWD and *35S*_Term_RVS (Gel 1), *35S*_End_FWD using the genomic DNA as the template and loaded onto 1% gel. The HyperLadder™ 1 kb (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used a as DNA ladder.

This result (Figure 3-22) did not provide a satisfactory result for 8AC2+ and 8AC2-. It indicated that this primer pair had multiple binding sites in the genomic DNA of ryegrass. Therefore a decision was taken to include more primer pairs along with the combination shown in Figure 3-22, to obtain an understanding of the transgene (*LpUbl5*) insert status.

The result shown in gel image Figure 3-23 shows three gel products. Gel 1 is the repetition of Figure 3-22 to confirm the result. Primer pair combinations shown in Gel 2 and Gel 3 aimed to understand the length of the *LpUbl5* insert within the plants. The results showed that two of the 8AC2+ plants and two of the 8AC2- plants possessed partial *LpUbl5* transgene insert. Whereas Gel 3 showed that only two of the 8AC2- plants possessed a complete transgene insert.

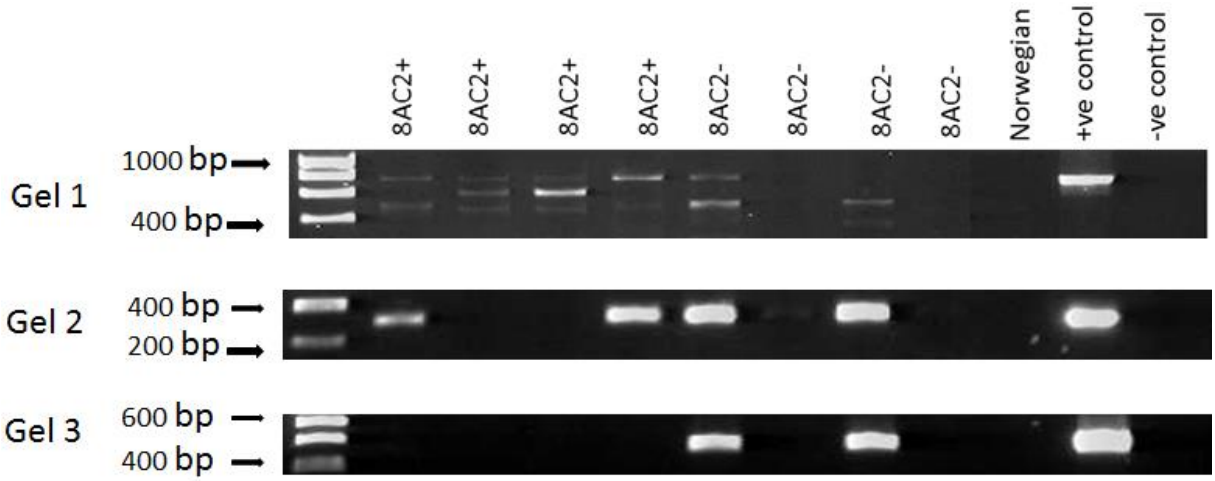


Figure 3-23 Gel images of PCR product obtained using the *35S*_End_FWD and *35S*_Term_RVS (Gel 1), *35S*_End_FWD and *LpUbl5*_Mid_RVS (Gel 2) and *35S*_End_FWD and *LpUbl5*_End_RVS (Gel 3) using the genomic DNA as the template and loaded onto 1% gel. The HyperLadder™ 1 kb (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

Though two of the 8AC2- plants had a correlation in results in Gel 2 and Gel 3, the result of 8AC2- in Gel 1 did show an uncertainty due to multiple amplification products. All of the 8AC2+ plants did have multiple amplification products. It was important to understand the reason behind the multiple products obtained in Gel 1. Therefore the gel stab PCR method (Figure 3-24) was employed. The cleaned gel products were sequenced. The sequence results were not satisfactory even when repeated using different primers and did not yield a quality result. Therefore the amplified gene sequence identification was impossible.

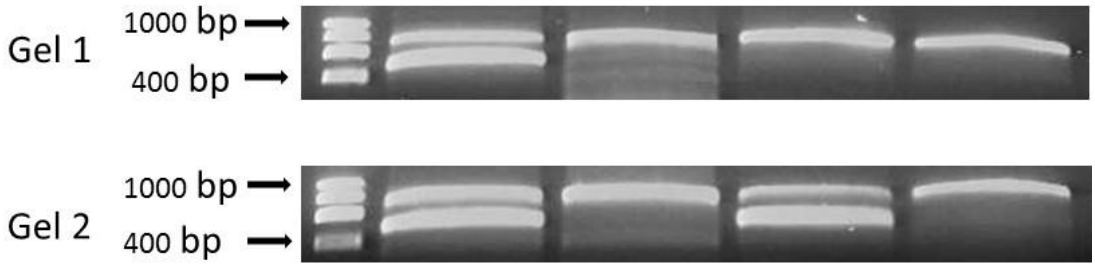


Figure 3-24 Representative gel images using gel stab of PCR products as templates. The primers used here were *35S*_End_FWD and *35S*_Term_RVS (Gel 1). The product was loaded onto 1% gel. The HyperLadderTM 1 kb (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

3.8.2 Newly isolated genomic DNA as template

The result (Figure 3-24) did not yield a quality result. Therefore fresh genomic DNA was isolated and used as a template to avoid any chances of contaminations. Three different primer combinations were used for three end point PCR reactions. Gel 1 (Figure 3-25) shows the PCR product obtained using primers for *eIF4A,* a housekeeping gene used for the gene expression study in Section 3.4.1. This result shows that the freshly isolated genomic DNA was not degraded.

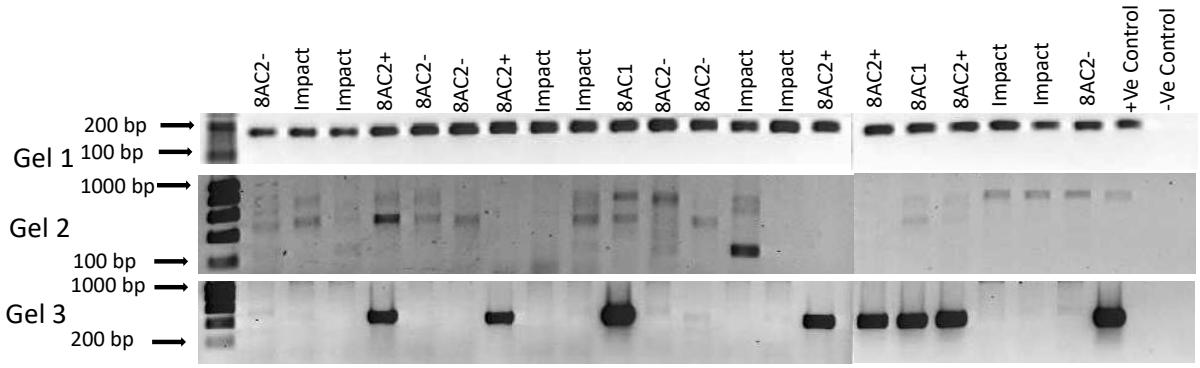


Figure 3-25 Gel images of PCR product obtained using the *eIF4A*_FWD and *eIF4A*_RVS (Gel 1), *35S*_End_FWD and *35S*_Term_RVS (Gel 2), Hygro_FWD and Hygro_RVS (Gel 3) using the freshly isolated genomic DNA as the template and loaded onto 3.5% gel agarose (Gel 1) and 1% agarose gel (Gel 2 & Gel 3). The HyperLadder™ V (Gel 1) HyperLadder™ 1 kb (DNA ladder) (Gel 2&3) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder

The results from Gel 2 (Figure 3-25) shows multiple amplification products in the Gel. There is strong amplification products of *hptII* gene using the primers Hygro_FWD and Hygro_RVS. Gel 3 shows that all the 8AC2+ and 8AC1 plants did possess the *hptII* gene. Figure 3-25 also shows that the Control PCR reaction (Gel 1) and the detection of the *hptII* gene (Gel 3) provided a strong indication that genomic DNA and the PCR reaction mix were effective. Whereas Gel 2 (Figure 3-25) shows that the amplification of "*LpUbl5*" is uncertain.

3.8.3 Trouble shooting *LpUbl5* amplification

Trouble shooting was carried out using eight primer pairs in and around the *LpUbl5* sequence. The results from the first six combinations are shown in Figure 3-26. All the primer pair combinations except *LpUbl5*_Full_FWD and RVS in Figure 3-26 (Gel 1), which were designed to amplify the complete *LpUbl5* insert in the binary vector, were used for the transformation purpose. The primer pairs designed to amplify the *LpUbl5* open reading frame (Figure 3-25, Gel 2) successfully amplified the sequence from the genomic DNA obtained from 'Impact'. Gel 3 shows the amplification products of four different primer pair combinations which efficiently amplified partial or complete sequence of *LpUbl5.* The positive control (binary vector) and negative control (molecular grade water) were efficient in all the reactions indicating efficiency of the end point PCR and primers and also the absence of any contamination.

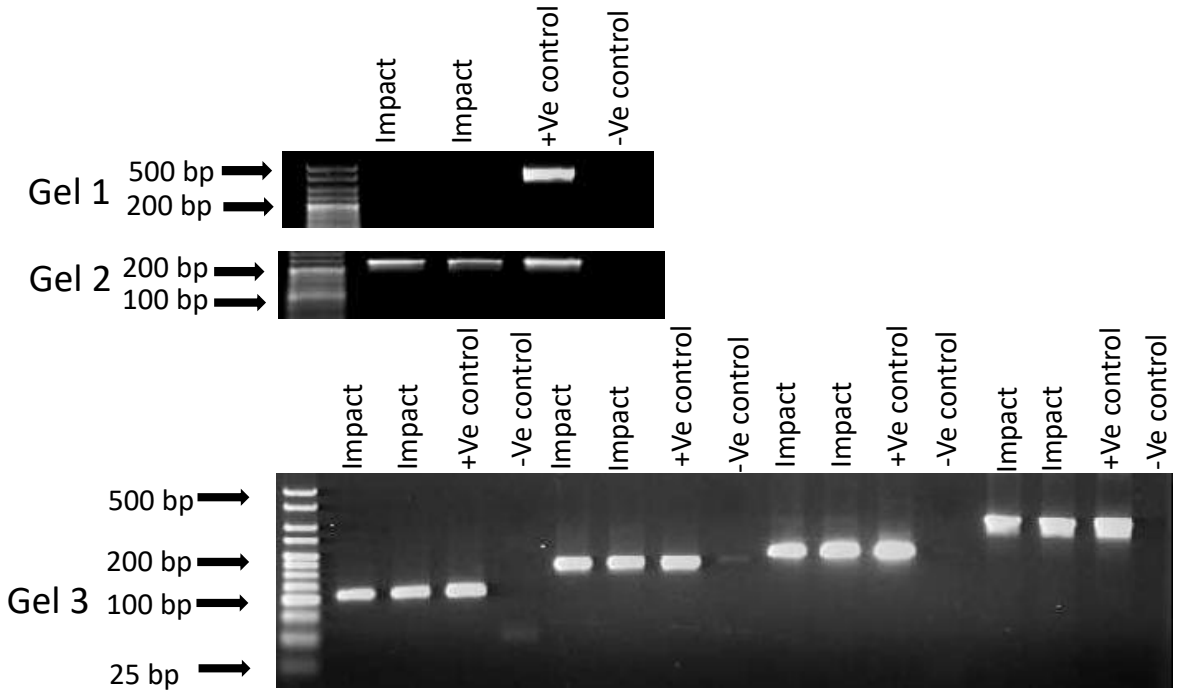


Figure 3-26 Gel images of end point PCR products using six primer pairs using genomic DNA from Impact as template which is repeated twice in all the reactions along with positive (binary vector) and negative control (molecular grade water). The end point PCR products are from primer pair *LpUbl5*_Full_FWD and *LpUbl5*_Full_RVS (Gel 1), *LpUbl5*_ORF_FWD and *LpUbl5*_ORF_RVS (Gel 2), *LpUbl5*_Int_FWD and *LpUbl5*_Int_RVS, *LpUbl5*_Int_FWD and *LpUbl5*_ORF_RVS, *LpUbl5*_ORF_FWD and *LpUbl5*_Int_RVS and *LpUbl5*_Full_FWD and *LpUbl5*_ORF_RVS (Gel 3). The product was loaded onto 3 % agarose gel. The HyperLadder™ V (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

3.8.4 Screening primary transgenics ryegrass

The Primary transgenic plants were screened using primer pair combinations Hygro_FWD and Hygro_RVS and *35S*_End_FWD and *35S*_Term_RVS. The *35S*_End_FWD and *35S*_Term_RVS did not show any amplification. The result of Hygro_FWD and Hygro_RVS is shown in Figure 3-27. This indicates that the primer pair combination of *35S*_End_FWD and *35S*_Term_RVS was not able to amplify the transgene insert in the plants.

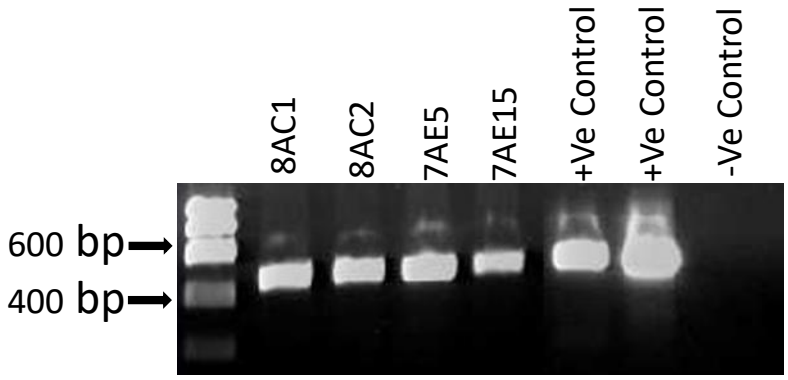


Figure 3-27 Gel images of end point PCR products using primer pair combination Hygro_FWD and Hygro_RVS genomic DNA from primary transgenic plants. The product was loaded onto 3 % agarose gel. The HyperLadder™ V (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

3.8.5 Study of new primer pair combinations

The primer pair efficiency was first validated by endpoint PCR using a binary vector (+Ve control) as the template and molecular grade water as negative control. Figure 3-28 has Gel 1, Gel 2 and Gel 3 in which three forward primers were coupled with four different reverse primers. Gel 1 shows the result of *D35S*_FWD_1 coupled with four different *LPUbl5* reverse primers which were *LpUbl5*_Full_RVS, *LpUbl5*_ORF_RVS, *LpUbl5*_Int_RVS and *LpUbl5*_qRT_RVS in order. Gel 2 shows the result of primer pair combinations of *D35S*_FWD_2 with the LpUbl5 reverse primers. Gel 3 had *D35S*_FWD_3 coupled with *LpUbl5* reverse primers.

Figure 3-28 shows that the primer pair in Gel 3, which is *D35S*_FWD_3 with LpUbl5 reverse primers, efficiently amplified the *LpUbl5* transgene insert when compared with primer pair combinations used in Gel 1 and Gel 2. This confirms that the *D35S*_FWD_3 was an efficient forward primer as the reverse primers used in Gel 1, Gel 2 and Gel 3 were the same. The primer pair combination of *D35S*_FWD_3 with *LpUbl5*_Full_RVS was chosen to screen the samples of the representatve plants. This primer pair was designed to amplify the complete *LpUbl5* transgene insert in the plants.

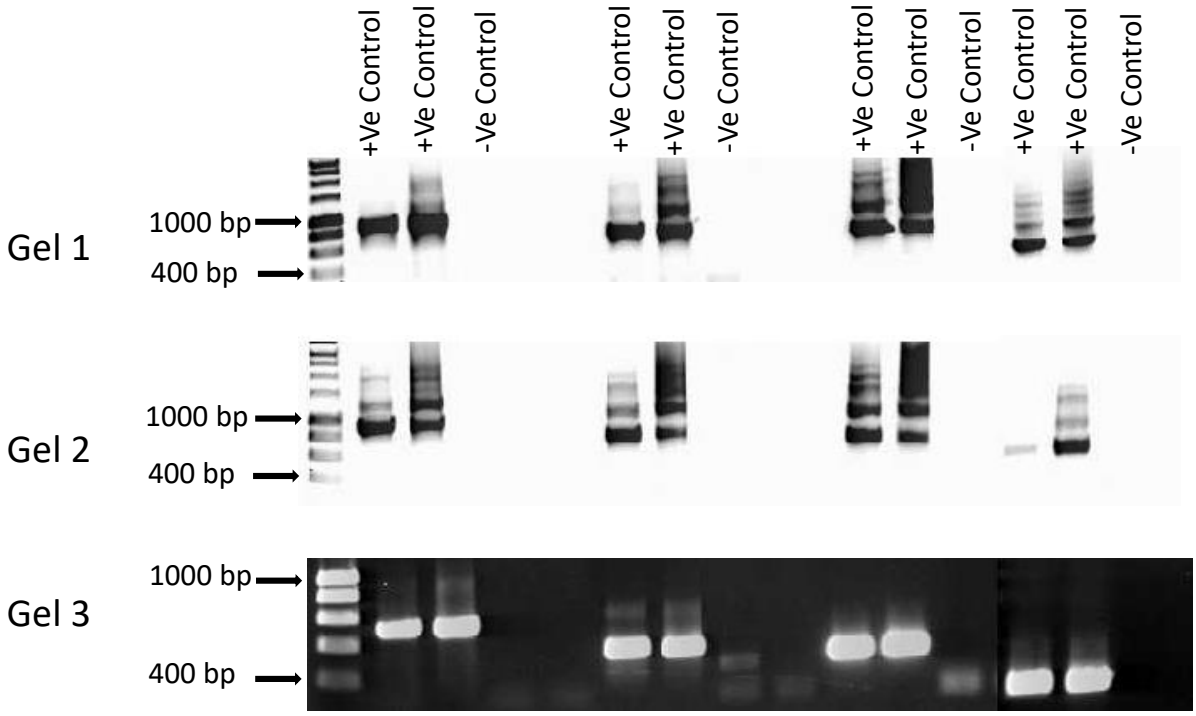


Figure 3-28 Gel images of end point PCR carried out using binary vector as using 12 different primer pairs given in Table 3-12. The product was loaded onto 1 % agarose gel. The HyperLadder™ 1 (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used a as DNA ladder.

3.8.6 Validation of selected primer pair on the samples

Figure 3-29 shows that the 8AC1 plants and 8AC2 plants both carry the *LpUbl5* transgene. 7AE5 and 7AE15 were used as negative controls as these lines carried a different transgene. The transformation of 7AE5 and 7AE15 was carried out using a binary vector with dehydrin promoter driving an AN1-A20 type zinc finger transcription factor. The sample from 'Impact' could also be considered as a negative control because it does not have a transgene. The results from plants 8AC2-and 8AC2+ did not behave as expected. 8AC2- plants were originally categorised as 8AC2- by them not having a transgene due to the absence of the *hptII* gene. Whereas 8AC2+ plants were considered to have *LpUbl5* transgene due to the presence of *hptII*. Figure 3-29 shows *LpUbl5* transgene amplification in 8AC2- and amplification of the transgene was not observed in 8AC2+. Positive controls were binary vector and negative controls were molecular grade water were acceptable, indicating the validity of the reaction.

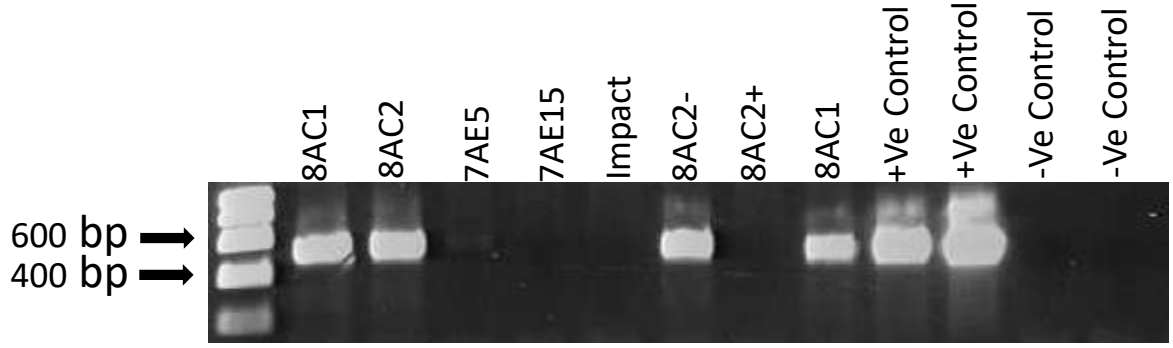


Figure 3-29 Gel images of end point PCR carried out using *D35S*_FWD_3 and *LpUbl5*_Full_RVS using genomic DNA from primary transgenics and Impact, 8AC1, 8AC2+ and 8AC2-. The product was loaded onto 1 % agarose gel. The HyperLadder™ 1 (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

3.8.7 Application of selected primer pair to screen plants

Gel 1 and Gel2 (Figure 3-30) show the result of end point PCR products using *D35S*_FWD_3 and *LpUbl5*_Full_RVS and Hygro_FWD and Hygro_RVS respectively. Gel 1 shows a 520 bp amplification product which indicates the presence of the *LpUbl5* transgene. Gel 2 shows a 435 bp amplification product indicating the presence of the *hptII* gene. The result comparison of Gel 1 and Gel 2 shows that, out of three 8AC2+ plants a single plant possessed *LpUbl5* transgene and *hptII* gene. Whereas, the other two plants only possess the *hptII* gene. Its presence led to their categorisation as 8AC2+. The result of 8AC2- plants in Gel 1 shows that two plants do carry the *LpUbl5* transgene even in the absence of a *hptII* gene (Gel 2). This shows that only one plant was a true transgenic plant carrying the *LpUbl5* transgene and *hptII* gene in total of these six 8AC2+ and 8AC2- plants. The result for 'Impact' did not show any amplification indicating that there was no non-specific amplification occurring in the reaction. This also confirmed that there was no mixing up of plants while establishing the experimental plots. The 8AC1 plants show amplification in Gel 1 and Gel 2 (Figure 3-30) indicating the presence of both the *LpUbl5* transgene and *hptII* gene. The positive and negative controls were efficient and provides confidence in the reliability of these results. The end point PCR was repeated to confirm the result.

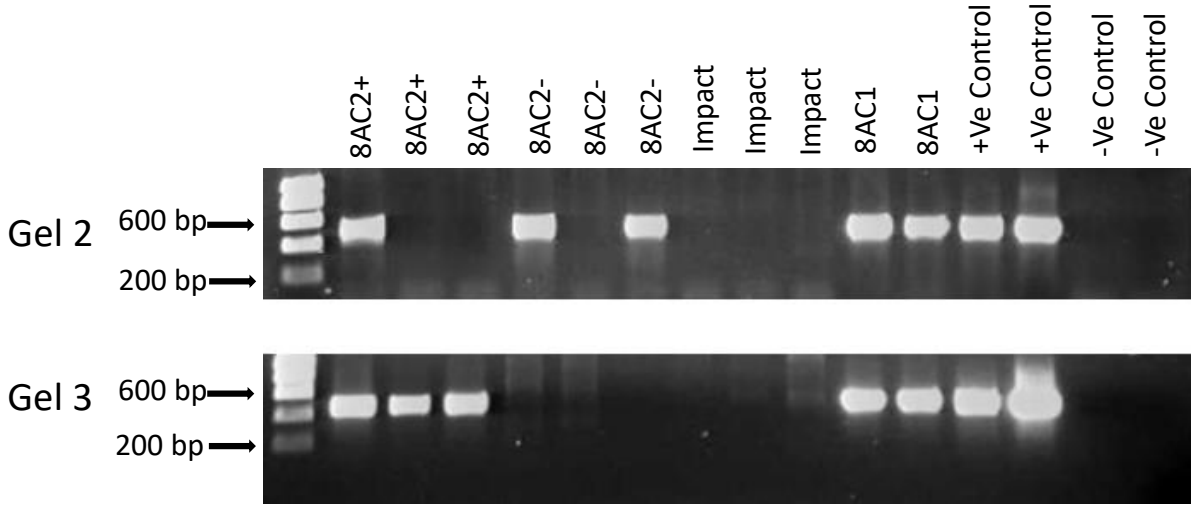


Figure 3-30 Gel 1 shows the presence and absence of transgene in genomic DNA of the plants amplified by end point PCR. Gel 2 shows the presence and absence of *hptll* gene in the genomic DNA of the selected plants, amplified by end point PCR. The HyperLadder™ 1 kb (DNA ladder) from Bioline (Total Lab Systems Ltd, NZ) was used as a DNA ladder.

Figure 3-30 confirmed the transgene status of the plants and thus clarified the reason for the ambiguous nature of the results obtained from the physiology study and *LpUbl5* expression level data. This could categorise the 8AC2 plants into four groups such as 8AC2 with complete T-DNA, 8AC2 with *hptll* gene, 8AC2 with *LpUbl5* transgene 8AC2 without T-DNA insertion which could be labelled as 8AC2- plants (Table 3-15).

Table 3-15 Plants were given a new labelling based on the results obtained from the transgene evaluation study (Figure 3-30)

New labelling	**Conclusions made from the results obtained from current transgene evaluation study (**Figure 3-30**)**
8AC2+(+)	Plants originally categorised as 8AC2+ and were positive for *LpUbl5* transgene and *hptll*
8AC2+(-)	Plants originally categorised as 8AC2+ and were negative for *LpUbl5* transgene but positive for *hptll* gene
8AC2-(+)	Plants originally categorised as 8AC2- and were positive for *LpUbl5* transgene but negative for *hptll* gene
8AC2-(-)	Plants originally categorised as 8AC2- and were negative for *LpUbl5* transgene and *hptll* gene

This result allowed the rearrangement of the expression level data based on the presence and absence of the transgene and *hptII* gene in each sample used.

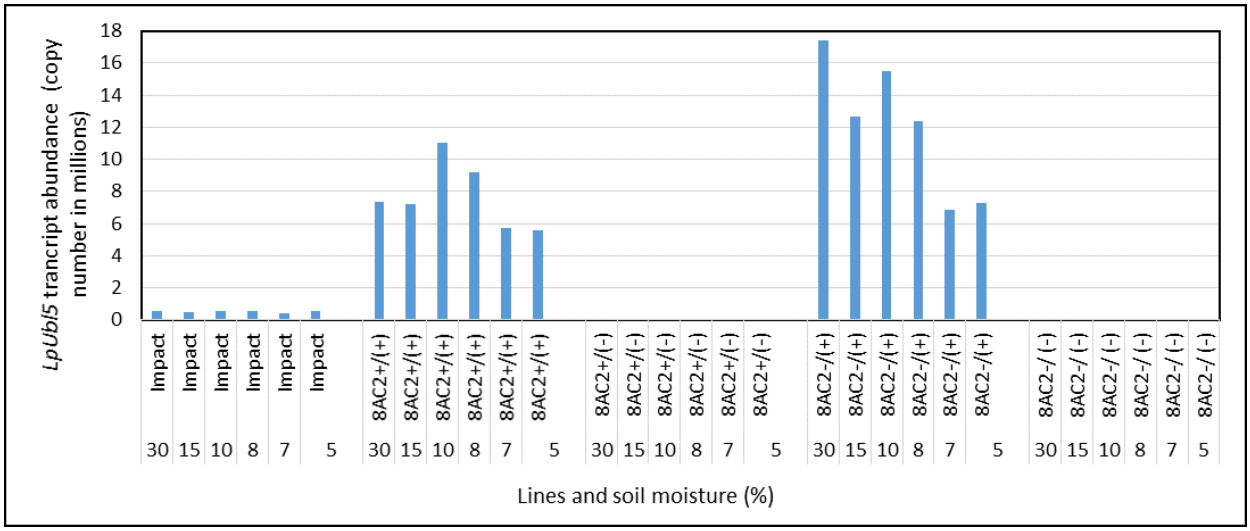


Figure 3-31 Copy number of LpUbl5 gene during the 6th drought cycle. The data were categorised based on the result obtained from Section 3.6.10 Transgene evaluation and the plants from 8AC2+ and 8AC2- were separated based on the presence or absence of the transgene. The available soil moisture (%) at the time of sample collection is shown in the graph.

Figure 3-31 shows that the *LpUbl5* transcript level in the plants, grouped based on the presence or absence of both the transgene and hygromycin gene. This results supports the ambiguous nature of the physiology results obtained, from shoot dry matter to solute potential. Shoot dry matter was taken from all the plants in each plot. This was a sum of transgene positive and negative plants in terms of 8AC2 category. In terms of other physiology results such as leaf extension, RWC, grading, plants survival rate and solute potential, the measurements were obtained from any plants in the group. The measurements were taken randomly and the individual transgene status of each plant was assumed based on the initial hygromycin screening. The sample for transcript level study was collected based on the plants ID from Drought Cycle 3 which enabled me to re-sort the data after obtaining the transgene evaluation data. However, in the case of 'Impact' and 8AC1 the results are considered as the real result obtained as their transgene status was not mis-interpreted. The mixed culture of the transgene status compromised the physiology results obtained. Their further analysis to draw a conclusion or reach a verdict regarding the main objective of this research was therefore invalid.

3.9 Discussion

The dry matter yield data were ambiguous. They did not show any difference in accumulation between 8AC2+ and (containing an additional copy of *LpUbl5*) and 8AC2- (without the additional copy of transgene). Furthermore, plant survival rates (Figure 3-11) show 8AC1, 8AC2+ and 8AC2- were not different, which indicates that plants did not benefit from having an additional copy of *LpUbl5* under consecutive drought cycles. Plant grading (Figure 3-12) showed that the Control (Impact) had higher grades when compared with 8AC1 with 8AC2+ and 8AC2- were intermediate. This indicates that the original 8AC2+ lines, expected to have an additional copy of the transgene, did not exhibit a difference in grading when compared with 8AC2- without the presence of the *LpUbl5* transgene. The nature of the results was the same for each measurement made during the experiment (Figure 3-10 and Figure 3-17). On the basis of these measurements the null hypothesis was accepted. In short there was no evidence that LpUbl5 conferred any drought tolerance on perennial ryegrass. Given these unexpected physiology results (Section 3.7) and the ambiguous nature of *LpUbl5* transcript data with huge variation between the 8AC2+ and 8AC2- plants, several questions needs to be answered. Specifically,

1. Was the experiment set up incorrectly in terms of labelling of plants while transplanting from germination trays to rhizotron?
2. Were the plants mixed up while transplanting?
3. Could a transgene be silencing either at the transcriptional or post transcriptional level over three generations?
4. Was the method chosen for the transgene determination compromised? These fundamental questions then led to a detailed investigation of the transgene status of the T_3 plants. These fundamental questions then led to a detailed investigation of the transgene status of the T_3 plants.

Background

The transgene status of plants was evaluated at Vialactia Biosciences (NZ) Ltd, at the beginning of the project. The experiment was set up based on their results to determine the presence or absence of the *hptII* gene. The plants that possessed the *hptII* gene were categorized as transgene positive and the plants without *hptII* gene were categorised as transgene negative. This result predicted that the presence of the *hptII* gene indicated an insertion of T-DNA into the plants genome, which in turn was expected to carry the transgene. This was a compromised choice due to the difficulty in deriving a suitable primer pair which could efficiently amplify the transgene in the genomic DNA isolated. The primer designing was difficult due to the presence of the double *Cauliflower mosaic virus* (*CaMV) 35S* promoter. This compromised the transgene evaluation by using the primer pair for *hptII* gene by PCR amplification and categorizing the plants into 8AC2+ and 8AC2- solely based on the presence of the *hptII* gene (Personnel Com. Sathish Puthigae, Vialactia).

Troubleshooting

However, upon obtaining ambiguous physiology results and *LpUbl5* expression level data, it was decided to re-evaluate the transgene status of the plants used in the experiment. This commenced with primer design in and around the transgene. Section 3.5 explains the procedure used to identify a suitable and efficient primer pair to detect the presence of T-DNA in plants. As noted, the primer design was difficult due to the presence of the *D35S CaMV* promoter repeating twice in the T-DNA.

Several primer pairs were evaluated and these listed in Table 3-13 were identified and confirmed as an efficient primer pair. This gave a clean PCR amplification on the vector as a positive control and genomic DNA isolated from the plants. This assay was used to evaluate the plants and was used to reveal the transgenic status of the plants. Figure 3-30 shows the transgenic status of plants based on the presence or absence of the *LpUbl5* transgene and hygromycin gene. This result showed that there was truncation of T-DNA which led to the integration of *LpUbl5* and *hptII* gene independently in some of the transgenic plants. These results showed that there was no error caused by labelling of plants during experiment set up and a mix up while transplanting. The re-evaluation of *LpUbl5* transcript data shown in Figure 3-31, with the revised transgene status of the plants, could disprove the transcriptional transgene silencing (Flachowsky *et al.*, 2008). The *LpUbl5* transcript level Figure 3-31 correlates with the transgene status of the plants in Figure 3-30. This results answered the question 4 mentioned above, that the method chosen for transgene determination was fatally compromised and discards first three questions.

Reviewing the generation of T_3 Plants

This study was conducted using the T3 generations of transgenic lines of 8AC2 and 8AC1. Ryegrass is an outcrossing (Copeland and Hardin, 1970) wind pollinated species. Cornish *et al.* (1979) reported a gametophytic, S and Z locus incompatibility system acting at the stigmatic surface which was first elucidated by Lundqvist (1954) in rye (*Secale cereale* L.). Because ryegrass is an obligate outcross species, the transgenic lines used in this research were generated by backcrossing genetically modified lines (8AC1 and 8AC2) with a number of genotypes of the cultivar 'Impact' (Accession number_ A10745). The crossing and generation of the T_3 generation of transgenic plants were carried out by Kim Richardson at AgResearch, Palmerston North, NZ.

The T_0 plants were crossed with a number of genotypes of 'Impact' to generate T_1 seeds. The single copy insertion of the transgene was confirmed by Southern blot analysis using *hptII* coding sequence which showed the transgene was hemizygous at T_0 (Patel *et al.*, 2015). This yielded a 1:1 segregation pattern of hemizygous transgenic plants and wild type. T_2 seeds were generated by crossing the hemizygous transgenic plants which yielded a 3:1 transgene segregation. This resulted in a transgene segregation pattern of 1:2:1 for homozygous: hemizygous: wild type as expected. The identification of homozygous plants were determined by qPCR. These plants were intercrossed to form the T3 generation which had 100% homozygous plants. This Mendelian segregation pattern was observed in

the case of the 8AC1 line where they had a transgene segregation pattern of 1:1 in the T1 generation, 3:1 in the T2 and 100% in the T3 generation (Figure 3-1).

In contrast, the line 8AC2 had a non- Mendelian transgene segregation which was 1:3 in the T_1 and 1:1 in the T_2 and T_3 generation. The backcross carried out between 8AC2 with the parent cultivar, 'Impact' to generate T1 and T2 generations resulted in plants with non-Mendelian transgene inheritance. Therefore a polycross between the T_2 plants did not yield 100% transgene segregation in T_3 lines. This was explained as, "transgenics don't always follow an obvious Mendelian segregation pattern and is not unusual. These transgenic lines are often discarded" (Personnel Com. Kim Richardson, AgResearch).

It is possible that the use of *hptII* coding sequence near left border of T-DNA for southern blot hybridization was not conclusive. This method does not detect any truncation in T-DNA and therefore resulted in non-Mendelian transgene segregation pattern. The reason for non-Mendelian segregation could also be either an unstable transmission or poor expression of the transgene (Yin *et al.*, 2003). Figure 3-30 shows that the problem of transgene segregation was not limited to non-Mendelian pattern or an unexpected Mendelian pattern of transgene segregation but also to T-DNA instability. The results of 8AC2 (Figure 3-30) shows there were four groups of plants being those plants carrying complete T-DNA, *LpUbl5* (only transgene), *hptII* (only selective agent,) and finally a group without the T-DNA. This could be due to either the nature of the recipient genome or nature of the transgene and it could be also due to the interaction between the recipient genome and introduced transgene (Yin *et al.*, 2003).

Reviewing the structure of T-DNA

When we examine the nature of the transgene, an assessment of the plants transformation vector employed here is a critical factor which needs to be analysed (Figure 3-8). The transformation of ryegrass was carried out using the pLpHUB1 plant transformation vector. The vector contained a double *CAMV 35S* promoter driving the transgene and another double *CAMV 35S* promoter driving the *hptII* gene (Bajaj *et al.*, 2006). The *CAMV 35S* promoter is a major promoter of the *Cauliflower Mosaic virus* which infects *Brassicaceae* or crucifers (Fang *et al.*, 1989). The promoter was identified as a model plant nuclear promoter system and is a constitutive promoter (Covey *et al.*, 1981; Hohn and Richards, 1982; Odell *et al.*, 1985). To date various combinations of hybrid promoters have been generated from the *CAMV 35S* promoter to improve gene expression (Ho *et al.*, 1999; Podevin and Du Jardin, 2012). The double *CAMV 35S* is generated by the duplication of the -343 to -90 fragment of the 35S promoter sequence and is one of the hybrid promoters which showed a 10 fold increase in the gene expression (Kay *et al.*, 1987). The use of a double *35S* promoter employed here in the pLpHUB1 plant transformation vector was aimed to maximise the *LpUbl5* expression in the transformed plants (Puthigae *et al.*, 2015).

To date a large number of transgenic approaches have used the *CAMV 35S* promoter as the promoter of choice in the T-DNA driving the gene of interest or gene of selection purpose or both (Spangenberg *et al.*, 1995; Hisano *et al.*, 2004; Wu *et al.*, 2005). Approximately 80% of genetic modifications have used the *CAMV 35S* promoter as their T-DNA component (Hull *et al.*, 2000). CAMV 35S promoter has also been shown to affect the activity of adjacent tissue or organ-specific gene promoters or convert them to constitutive promoters (Yoo *et al.*, 2005; Zheng *et al.*, 2007).

Rearrangements of DNA during transformation have been reported (de Groot *et al.*, 1994; Register III *et al.*, 1994) in which case a transgene malfunction can be identified at an early stage. Use of multiple *CAMV 35S* promoters in the same plasmid caused plasmid rearrangements. The 3' end of the 35S promoter is identified as highly recombinogenic, and the use of the 35S promoter increases the chances of plasmid rearrangements following plant transformation (Kohli *et al.*, 1999). In that case, the use of multiple and inverted repeats of the 35S promoter (Figure 3-8) increases the chance of T-DNA rearrangements when compared with a single 35S promoter. Apart from these, the sequence repeats and use of these repeats in inverse orientation, increases the risk of gene silencing (Kooter *et al.*, 1999).

Furthermore, the sequences have potentially been identified as a *CAMV35S* hotspot for recombination events (Kohli *et al.*, 1999). The sequence repeat of "ATATAAG" is repeated four times in the T-DNA region two in either direction. This "ATATAAG" is a purine rich sequence which was identified to be involved in cross over events (Machida *et al.*, 1986). This could be a potential cause of the recombination of T-DNA observed in the 8AC2 lines. The junction between the CAMV 35S promoter and transgene also increases the instability of the plasmid (Ho *et al.*, 1999). Kohli *et al.* (1999) reports the chances of intermolecular recombination is high for constructs containing the 35S promoter.

Conclusion

In this study, the presence of T-DNA in the plants was screened by tracking the presence of the associated *hptII* gene, which in turn failed to identify the plasmid rearrangements. The break in the T-DNA could have been identified at initial stages of the project if the selection of transformants were carried out using a screening procedure involving two steps. This would select seedlings with hygromycin resistance and the amplification of *LpUbl5* transgene from the genomic DNA of hygromycin resistant seedlings by an end point PCR. A better comprehensive method will be carrying out a southern using *LpUbl5* coding sequence which would confirm the presence of transgene and copy number insertion. Instead, the compromised method of choosing only the *hptII* gene in transformed plants to determine the presence of complete T-DNA, failed to detect the rearrangements or T-DNA breakage separating the *hptII* gene and *LpUbl5* gene. TaqMan probes for the *hptII* gene were used for qPCR to study the segregation pattern by AgResearch Ltd during the development of the lines 8AC1 and 8AC2 due to the design of the T-DNA which left them with no choice other than the *hptII* gene (Personnel comm. Kim Richardson, AgResearch Ltd). However, a confirmation using southern

analysis using T-DNA sequence containing *LpUbl5* coding sequence could identify any break in the T-DNA.

Adaptation of a compromised method for transgene determination has caused a compromise in the whole project as highlighted by the lack of the difference in of physiology data. The plants used for the physiology measurements were from a mixed population of 8AC2+ and 8AC2- plants. The physiology measurements and sample collections were carried out till the end of the drought stress experiment in good faith that 8AC2+ plants carried the *LpUbl5* transgene. The collected data could not be analysed because the data were collected from a mixed population of plants which could not be separated based on the result generated by the newly identified transgene status using the new set of primer pairs. This necessarily caused the abandonment of the physiology data obtained through the course of drought stress experiment.

The research question asked (Chapter 2.9) were, does over-expression of *LpUbl5* in occur in ryegrass and does it provide enhanced drought tolerance in the T_3 generation of transgenic ryegrass lines? Has the generation of T3 lines in a self-incompatible species been proved successful? To answer these questions, the research project was designed and co-ordinated to deliver a thorough understanding of the *LpUbl5* gene in ryegrass, with the aim to establish its role in plants via detailed physiology, gene expression and transcriptomics data. This PhD was initially designed to characterise the *LpUbl5* gene in *Lolium perenne* L. using the physiology data, *LpUbl5* expression level analysis and RNA sequencing data obtained from the experiment set up in Rhizotrons 1 and 2 in the Biotron. The compromised transgene status generated at the beginning of the project by Vialactia compromised the project. However, a comparison of homozygous transgenic line, 8AC1 with 'Impact' shows that the transgenic lines did not perform equally well which suggests that *LpUbl5* is not involved in drought tolerance. The null hypotheses was "Drought tolerance in perennial ryegrass is not different between control plants and those with over-expression of LpUbl5". The study accepted the null hypotheses because of the superior performance of Impact over 8AC2 and 8AC1 plants. However, this compromised transgenic status and compromised results in turn meant the PhD needed to be reframed to confirm the findings. The reframed project has used *Arabidopsis thaliana* as a model plant system to understand the role of *LpUbl5* in enhanced drought tolerance in plants.

Chapter 4

Over-expression of *LpUbl5* in *Arabidopsis thaliana* and performance of transformed lines under drought stress

4.1 Introduction

Arabidopsis is a small flowering plant used as a model in plant science research (Koornneef and Meinke, 2010). It is convenient to generate *A. thaliana* transgenic plants, the whole genome sequence is available, it has molecular genetic markers and there are collections of sequence-indexed DNA-insertion mutants (Hayashi and Nishimura, 2006). Gene functional studies often use *Arabidopsis* as a plant of choice (Dubouzet *et al.*, 2003; Xiong and Fei, 2006). Understanding plant response and biological mechanisms to drought in *Arabidopsis* and transferring this information to crop plants or desired species is a method of engineering for stress tolerance (Zhang *et al.*, 2004). A review of the current literature that focuses on the use of Arabidopsis as a model for studying plant responses to drought have shown that withdrawal of water for 14 days is sufficient to highlight drought tolerance. (Umezawa *et al.*, 2004; Yu *et al.*, 2008; Yoo *et al.*, 2010; Ding *et al.*, 2015) that focuses on the use of *Arabidopsis* as a model for studying plant responses to drought have shown that withdrawal of water for 14 days is sufficient to highlight drought tolerance. Therefore 14 days of water withdrawn experiments were incorporated in this research to identify the potential involvement of *LpUbl5* in drought tolerance.

The null hypothesis was "Drought tolerance in *Arabidopsis* is not affected by over-expression of *LpUbl5*"

This chapter describes the generation of transgenic plants, exposure of these to imposed drought stress and evaluation of the plant performance under drought. *LpUbl5* Transcript abundance under *CaMV 35S* promoter in transgenic lines was determined to understand the variation in transcript level in each lines. *AtUbl5* transcript abundance in wild type *Arabidopsis* under progressive drought was studied to analyse any change in transcript level as the drought progress.

LpUbl5 was amplified from cDNA by general PCR and cloned into an entry vector. *Escherichia coli*, DH5α competent cells were transformed using the entry clone. Then an expression vector was generated by LP Clonase reaction with the entry vector. This expression vector was used to transform DH5α competent cells. The isolated plasmids were used to transform *Agrobacterium* GV3101 strain which was used to generate transgenic *A. thaliana* plants by a floral dip transformation method. The T_0 seeds were collected from primary transgenic lines and planted out to generate T_1 seeds. The collected T_1

seeds were screened to identify resistant plants from which a determination of individual lines that had segregation ratios of 3:1 indicating that these lines had segregated at a single locus (Bandupriya and Dunwell, 2012). Then the collected T_2 seeds were screened for 100% germination and the generated homozygous T_3 seeds. This provides stable integration of a transgene. The T_3 plants were used to carry out experiments to study the potential role of *LpUbl5* in plants.

4.2 Materials and methods

4.2.1 Cloning *LpUbl5* in pENTR/D-TOPO to generate entry clone

Prior to cloning, *LpUbl5* was amplified from ryegrass leaves by KAPA HiFi Hotstart PCR protocol (Appendix: A.2) using the primers given in Table 4-1.

Table 4-1 The primers used for amplification of *LpUbl5* for cloning *LpUbl5* into an entry clone.

Primer	Sequences	Amplicon
*LpUbl5*_FWD	**CACC**ATGATCGAGGTGGTGCTCAAC	
LpUbl5+TAA_RVS	CTAGTTGTAGTAGAGCTCGAGTCC	226 bp

The highlighted "**CACC**" in *LpUbl5*_FWD primer facilitates directional cloning by pENTR™/D-TOPO® Cloning.

PCR amplified *LpUbl5* was run on a 3.5% gel to confirm the PCR result. The correct amplification band on the gel was sliced out and purified using Axyprep DNA Gel Extraction kit (Axygen, Ray lab, NZ) following the manufacturer's protocol. The cleaned product was quantified (µg/ml) using Qubit® flourometer (Life Technologies, NZ) quantification following manufacturer's instruction. This amplified product (*LpUbl5*) was cloned into pENTR/D-TOPO vector (Invitrogen). The cloning was carried in a 3:1 ratio of PCR product: plasmid (*LpUb5*: pENTR/D-TOPO) using the following formula.

$$\frac{(\text{Ng plasmid} * \mu\text{l plasmid used})\ \text{Kbp insert size} * (\text{insert} : \text{plasmid})}{\text{Kbp plasmid size}} = \text{ng insert to add}$$

Equation 4-1 Transformation was carried in a 3:1 ratio of PCR product: plasmid using the following formula

Ng plasmid = Ng/µl Plasmid (entry clone)

Kbp insert = Kilo base pair insert (*LpUbl5*)

The whole procedure was carried out by following the manufacturer's instruction (Appendix B.5)

4.2.2 Transformation of *Escherichia coli* DH5α competent cells

E.coli (DH5α cells) was transformed with the entry vector according to Sambrook and Russell (2001). One tube of *E.coli* chemically competent DH5α cells (See Appendix B.2) from -80°C was thawed on ice

to be used for transformation. Two µl of cloned TOPO Cloning reaction form above step was added to the thawed DH5α cells and incubated on ice for 30 minutes. After incubation, this reaction mix was heat shocked at 42°C for 30 seconds and placed back on ice immediately. Two hundred and fifty microliters of SOC medium (Appendix B.2) was then added to the reaction mix. Then the tube containing reaction mix was placed horizontally in the incubator at 37°C at 250 RPM for one hour. 50 and 100 µL of the cells were plated onto LB plates containing of Kanamycin (50 µg/mL) (Appendix B.2) and incubated at 37°C overnight.

4.2.3 Screening for positive colonies

To identify E. coli cells that contained the desired *LpUbl5*-pTOPO construct, a general PCR protocol (Appendix A.1) was carried out. The colonies grown after overnight incubation were screened using colony PCR by following a general PCR protocol (Appendix A.2) by using bacterial colonies as templates. The primers used were M13 F and *LpUbl5* reverse (Table 4-2)

Single transformed colonies were inoculated in 3 mL of LB broth (Appendix B.2) containing kanamycin (50 µg/mL). These were then incubated at 37^{o}C in the rotary shaker at 250 rpm for 16-18 hours. The bacterial cells were pelleted by centrifugation and a mini-plasmid extraction was carried out using alkaline lysis method (See Appendix B.7) (Sambrook and Russell, 2001). Purified pENTR/TOPO-D carrying the *LpUbl5* gene, pENTR: *LpUbl5* plasmid were sequenced (Department of Bio-Protection, Lincoln University, New Zealand) using the primers in Table 4-2. The sequence results were analysed using Geneious 7.1.7, http://www.geneious.com/ (Kearse *et al.*, 2012).

Table 4-2 The primers used for colony PCR to screen transformed E.coli (DH5α cells)

Primer	Sequences	Amplicon
*M13*_FWD	GTAAAACGACGGCCAG	
*LpUbl5*_RVS	CTAGTTGTAGTAGAGCTCGAGTCC	374 bp

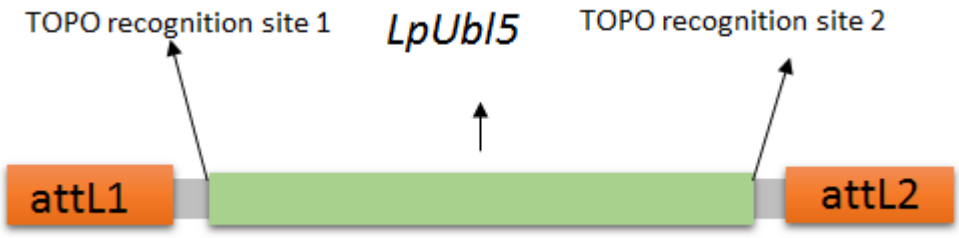


Figure 4-1 Schematic representation of pENTR/D_TOPO:*LpUbl5* which was used for LR Clonase reaction to carry out a recombination reaction to make the expression vector to carry our floral dip *A. thaliana* transformation.

4.2.4 LR Clonase recombination reaction using Gateway Cloning technology

An LR Clonase recombination reaction of Gateway Technology (Invitrogen Inc., Life Technologies, NZ) was carried out to generate the expression clone using the LR Clonase reaction mix with an entry clone and a destination vector (Appendix B.8). The destination vector used was pARTB_GW_egfpER (a kind gift from Fernand Kennel, Plant and Food Research Institute, Canterbury, New Zealand) and the vector possessed *CaMV 35S* promoter to drive transgene transcription and the phosphinothricin-N-acetyltransferase resistance gene to confer resistance to the herbicide Glufosinate 200, (AGPRO, NZ) a non-selective herbicide which is commercially known as BASTA, as a selection marker.

The LR Clonase reaction mix was prepared as listed in Table 4-3. Then 4 µl of LR Clonase™ enzyme mix were added and vortexed briefly to mix well.

Table 4-3 LR Clonase Reaction mix for generating the expression clone.

Reagent	Quantity	Final Concentration
Entry clone	100-300 ng	1-10 µl
Destination vector	150 ng	2 µl
5X LR Clonase™ reaction buffer	5X	4 µl
TE Buffer	pH 8.0	up to 16 µl

The reagent mix from Table 4-3 was incubated at 25^{o}C for 1 hour and the reaction was stopped by adding 2 µl of 2 µg of proteinase K solution and incubated for 10 minutes at 37^{o}C. Then the *E.coli* DH5 α cells were transformed by following the protocol outlined in Appendix B.6 and was plated on the LB agar media containing spectinomycin (50 µg/mL) as the antibiotic selection agent. A general PCR (See Appendix A.2) was carried out to identify the positive colonies (*pARTB_GW_egfpER::LpUbl5*) using the primers in Table 4-4.

Table 4-4 Primers used for colony PCR to identify *pARTB_GW_egfpER::LpUbl5* harbouring cells

Primer	Sequence	Amplicon
*pART_35S*_FWD	GACGTTCCAACCACGTCTTCAAAGCAA	
*LpUbl5*_RVS	CTAGTTGTAGTAGAGCTCGAGTCC	459 bp

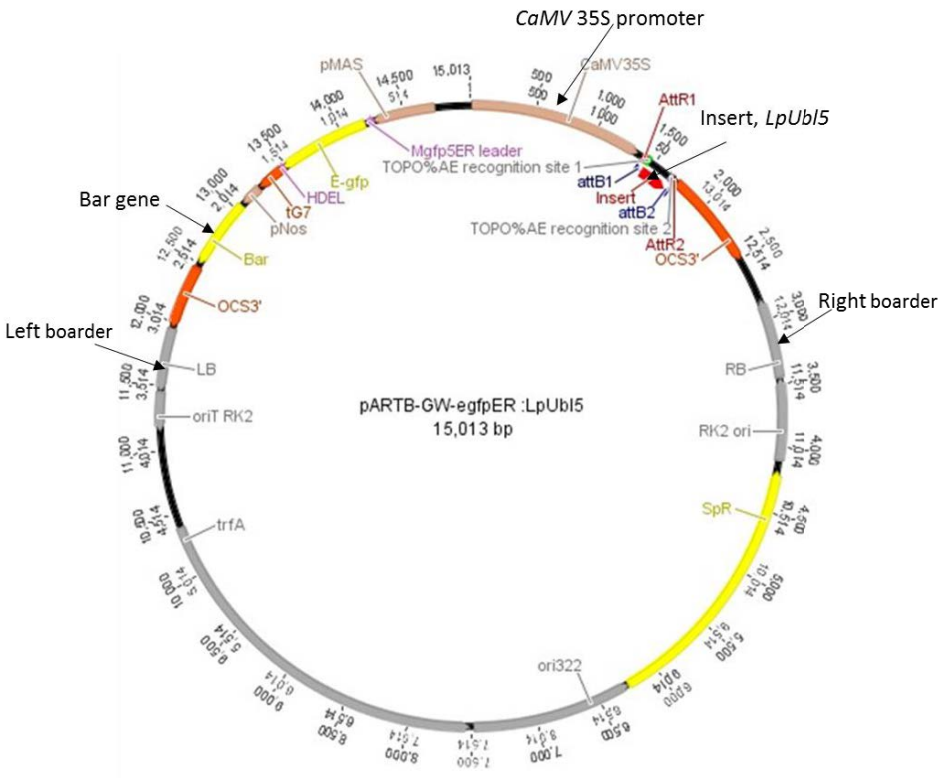


Figure 4-2 Schematic representation of expression vector *pARTB_GW_egfpER::LpUbl5*

The positive colonies identified through colony pCR were inoculated into 3 mL LB medium with 3 µL of spectinomycin (50 µg/mL) and incubated at 37°C shaking at 250 RPM overnight. Then the *E.coli* plasmid mini-prep (Appendix B.7) was carried out to isolate plasmids. The isolated plasmids were cleaned using an Axyprep PCR Clean-up Kit (Axygen, Ray lab, NZ) following manufacturer's protocol. The cleaned plasmid was quantified using Qubit flourometer (Life Technologies) quantification. An end point PCR was carried out to confirm the insert sequence and orientation of insert using the primers used for colony PCR (Table 4-4). The plasmids were sequenced using *pART_35S_FWD* primer by the Department of Bio-Protection, Lincoln University, New Zealand. The sequence results were analysed using Geneious 7.1.7 (http://www.geneious.com/, (Kearse *et al.*, 2012).

4.2.5 Transformation of *Agrobacterium Tumefaciens* (GV3101 strain)

Agrobacterium tumefaciens (GV3101 strain) was transformed using pARTB_GW_egfpER::*LpUbl5* to carry out *A. thaliana* transformation. The *pARTB_GW_egfpER::LpUbl5* was used to transform *A. tumefaciens* by electroporation as defined in Appendix B.9 (Sambrook and Russell, 2001). The reaction was plated onto LB medium containing Spectinomycin (50 µg/mL) as a selective agent and the plates were incubated at 28°C in the dark for two days. Colony PCR was carried out using the forward and reverse primers given in Table 4-4 to confirm the transformed colonies containing *pARTB_GW_egfpER::LpUbl5*. The templates for colony PCR was prepared by were diluting single

bacterial colonies in 20 µl of deionised water and incubated at 99^{o}C for 10 minutes. Aliquot of 5 µl of this cell suspension was used as a template in colony PCR to ensure the lysis of cells to expose the plasmids. The result was confirmed by loading the PCR products onto 1% agarose gel to confirm the insert, based on the size (bp).

4.2.6 Preparation of *Arabidopsis* plants for floral dip transformation

To transform *A. thaliana* plants using *pARTB_GW_egfpER::LpUbl5* carrying *Agrobacterium Tumefaciens* (GV3101 strain), *A. thaliana* wild type plants were sown. Stratification of wild type *A. thaliana* (Columbia) seeds was achieved by spreading seeds on wet filter paper placed in a petri-dish followed by incubation at 4^{o}C for 2 days. The seed sowing soil mix was prepared with a mixture of peat and sterilized pumice in a 3:1 ratio and an additional fertiliser of Osmocote exact mini (i.e. NPK (2 g/L), dolomite (4 g/L) and hydraflo (1 g/L)). This soil mix was used for all the experiments in this chapter. The soil was filled in small black pots (cm x 7 cm x 8 cm LWH) in which the top of each pot was covered using Cheese cloth (spotlight support group, NZ) to help to support the floral dip transformation method. Approximately 5-6 seeds were sown on the surface of moistened cheese cloth, carefully placing the individual seeds at the gap between the threads on the cloth. The pots were maintained in the plant growth room at 21^{o}C at 16h day/8h night regime under 55–65% humidity and light intensity was approximately 100 µmol.m^{-2}.s^{-1} using cool white fluorescent tubes. To achieve higher number of floral buds, inflorescences bolts were removed to promote growth of secondary inflorescences by enhancing synchronised emergence of multiple secondary bolts. The plants were grown until the appearance of a large number of flower heads which was achieved by within 4-8 days after clipping. The transformation procedure was then carried out using *Agrobacterium* mediated stable transformation with *pARTB_GW_egfpER::LpUbl5* by the floral dip transformation method (Bent, 2006; Weigel and Glazebrook, 2006; Zhang *et al.*, 2006). The watering was stopped two days prior to transformation to allow the soil to dry slightly.

4.2.7 Floral dip *Arabidopsis* transfromation

In order to prepare the *Agrobacterium* containing transformation solution, a single colony of *Agrobacterium* containing the binary vector was first (Section 4.2.5) inoculated in 3 ml LB medium containing spectinomycin (50 mg/ml) as a selective agent. This was incubated at 28^{o}C at 250 RPM for two days in the dark. An aliquot of 200 µl of 3 ml *Agrobacterium* culture was inoculated into 100 ml of LB medium with spectinomycin (50 mg/ml) incubated in the same conditions. The bacterial cells were pelleted using a Beckman centrifuge at 5000 g for 5 minutes when the OD_{600} reached 0.8-1. The pellet was re-suspended in 50 ml of infiltration medium (Appendix B.1 for media preparation) containing 2 ml/L of Acetosyringone (100 µm). Then the medium was incubated at 28^{o}C for a minimum of 3 hours. After the 3 hour incubation period the inoculated infiltration media (50 ml) was diluted to 1 L with

fresh Infiltration medium 500 µl PULSE (0.05%) (800g/L organomodified polydimethyl siloxane, Nufarm, NZ) was added to the 1 L of cell culture prior to dip transformation.

The cell culture was transferred into a wide mouthed beaker to allow easy dipping of flower heads into the medium. The inflorescence shoots were dipped completely into the medium and held for approximately 30 seconds. After dipping, the pots were laid flat on the tray and covered with a plastic lid for 24 hours to maintain the humidity and to prevent contamination of soil with *Agrobacterium*. The lid was removed after 24 hours and the pots were kept upright (Weigel and Glazebrook, 2006). Then the plants were watered to prevent any drought conditions until seed collection from primary transgenic plants. Seeds were collected from the brown matured siliques and labelled as T_0 seeds.

Figure 4-3 Floral dip transformation procedure by dipping the floral head into the infiltration medium

4.2.8 Screening of T_0 seeds of *A.thaliana* transformed by floral dip method

A tray (37 X 23 X 6 cm WLH) was filled with soil and the T_0 seeds (Section 4.2.7) were sown evenly on the soil surface. The seeds were stratified by incubating the tray containing seeds at 4^{o}C for 24 hours. Then the tray was kept in the growth room (Section 4.2.6). The seeds sown on the trays were germinated in closed trays to maintain humidity until the majority of the seedlings developed two fully

spread leaves. Then 1.7% (v/v) commercial BASTA herbicide containing 200 g/L Glufosinate-ammonium (AGPRO 200, AGPRO NZ Ltd, Auckland, NZ) was sprayed onto the plants as a selective agent and the tray was kept in the safety hood for 10 minutes. The tray was then moved back to the plant growth room and kept closed to maintain moisture. The seedlings were monitored daily to identify the transformed plants as green and healthy looking compared with pale yellow, herbicide affected un-transformed plants. The un-transformed plants died within a week.

The green transformed plants were re-planted into a propagation tray containing 50 round cells per sheet (5.9 cm Depth X 4.7 cm Top Diameter X 1.13 cm bottom Diameter). The trays were placed inside the plant growth room at 20^{o}C and 16h day/8h night regime. ARACON tube and ARACON base (BetaTech bvba, Belgium) were used to isolate each plants to prevent cross fertilization of flowers and release of transgenic seeds. The plants were maintained well-watered for 3 - 4 weeks until the first of the siliques formed began to yellow. Then the seeds were collected and labelled as T_1 -seeds stored for generating T_1-plants.

4.2.9 Screening of T1 & T2 seeds

Approximately, 100 T_1 seeds were transferred into 1.7mL microcentrifuge tube (Axygen, New York, USA) and seeds were sterilized by vapour phase sterilization method as described by Clough and Bent (2000) . The microcentrifuge tube containing the seeds were placed in the desiccator jar along with 100 mL commercially available bleach (Cyclone, premium Bleach, Contents; sodium hypochlorite: 50 g/L, available chlorine: 4.0% W/V). A 5 mL aliquot of concentrated hydrochloric acid (HCl, 36%) was added gently into the 100 mL bleach to generate chlorine gas to facilitate the seed sterilization. The desiccator was sealed using parafilm to avoid the escape of chlorine gas. The sterilization process was continued for four hours after which the parafilm was removed. This allowed the release of fumes from the desiccator and microcentrifuge tube. The vapour sterilised seeds from 17 plants were sprayed on to ½ MS medium with 10 mg/ml of Basta (200 g/L Glufosinate-ammonium, AGPRO 200, AGPRO NZ Ltd, Auckland, NZ) (see Appendix B.1 for media composition) and stratified at 4^{o}C for 48 hours where then allowed to germinate at 18-20^{o}C at 16h day/8h night regime until herbicide resistant versus sensitive plants could be discriminated. Each plate was then scored to determine the ratio of sensitive to resistant plants and subsequently 8 plants from those lines that possessed a 3:1 ratio were transplanted onto soil, grown and seed collected from each individual plant and labelled as T_2- seeds.

The T_2-seeds were sterilized and sprinkled onto ½ MS medium with BASTA (10 mg/mL BASTA) to identify the lines with 100% germination and survival indicating a homozygous line transformed with *LpUbl5* gene. Eleven lines displayed a 100% survival rate indicating the homozygous nature of the integrated transgene in these plants. These lines were planted out in the soil and maintained in the plant growth room until seed collection. The plants were screened by following the end point PCR

method (Appendix A.2) using the primer combinations from Table 4-2 to confirm the presence of the transgene in the selected lines. The seeds were labelled T_3-seeds and were used as the seed source for this research. The 11 lines selected were named as T3/1, T3/12, T3/15, T3/19, T3/22, T3/27, T3/31, T3/32, T3/35, T3/41, T3/43 based on their seed of origin.

4.2.10 *LpUbl5* transcript abundance in transfromed *Arabidopsis* lines

An understanding of the *LpUbl5* transgene expression was important to study the level of *LpUbl5* expression in each line obtained via transformation. *LpUbl5* gene expression would also aid in selection of different lines to be used in experiments. A subset of seeds from T_3-seeds were spread on wet filter paper placed in a petri-dish and was incubated at 4^{o}C for 24 hours to allow stratification of seeds. Approximately 4-5 T_3-seeds were sown on top of soil (soil mix from Section 2.2.6) filled in small black pots (7 cm X 7 cm X 8 cm LWH). The plants were grown in a plant growth room at 21^{o}C in a 16h day/8h night regime. The leaves were harvested when the plants were four weeks old for RNA isolation.

RNA isolation and cDNA synthesis

A sample of 100 mg of leaf tissue was collected from each line. RNA was isolated from the ground leaf tissue using a Spectrum™ plant Total RNA kit (Sigma-Aldrich, Auckland, New Zealand). The RNA was treated with TURBO DNA-free™ Kit Ambion ® TURBO DNA-free™ DNase treatment (ThermoFisher, Auckland, NZ) to completely remove the DNA. Then the total RNA quality was assessed using a DeNovix DS-11 spectrophotometer (DeNovix Inc, Wilmington, USA). A Qubit® flourometer was used to quantify RNA using Qubit® RNA buffer and dye (Life Technologies Ltd, NZ) after calibration using the supplied standards. Total RNA (300 ng) was run on a denaturing formaldehyde gel to check the RNA integrity (Appendix B.13). To check for DNA contamination one microlitre of total RNA extraction was used as template for a general end point PCR (Appendix A.1) was carried out using *AtEF1α* primers. cDNA isolated from *Arabidopsis* leaves was used as positive control and sterile water of molecular biology grade (UltraPure™ Distilled Water, Life Technologies, NZ) was used as negative control. Total RNA that produced amplification products were treated with TURBO DNA-free™ Kit ThermoFisher, Auckland, NZ) according to the manufacturer's protocol and re-tested. cDNA was synthesised using 300 ng of total RNA using PrimeScript RT Reagent Kit (Perfect Real Time) from TAKARA, Clonetech following manufacturer's instruction (Appendix A.3). The method described in this section was consistantly followed to isolate RNA and cDNA synthesis for this chapter.

Selection of reference genes

Reference genes used for normalising the LpUbl5 gene expression were *F-BOX* (*AtF-Box*, accession: At5g15710) and *elongation factor 1-alpha* (*AtEF1α*, accession: At5g60390) genes (Lilly et al., 2010). TOPO/TA cloned reference genes were kindly gifted by Walftor Dumin (Dumin, 2015).

Table 4-5 Primers used for the amplification *AtF-Box*, AtEF1α and *LpUbl5* primer used in the initial analysis of the LpUbl5 gene expression using qRT-PCR in transformed *A.thaliana* plants.

Name	Sequence	Amplicon
AtF-Box-qPCR_FWD	GGCTGAGAGGTTCGAGTGTT	
AtF-Box-qPCR_RVS	GGCTGTTGCATGACTGAAGA	108 bp
AtEF1α-qPCR_FWD	TGAGCACGCTCTTCTTGCTTTCA	
AtEF1α-qPCR_RVS	GGTGGTGGCATCCATCTTGTTACA	76 bp
*LpUbl5*_Int_FWD	CCATCGGCGACCTCAAGAAG	
*LpUbl5*_Int_RVS	CGAGGGTGATGTGGTCCTTG	105 bp

Plasmid mini-prep was carried out by following a protocol (Appendix B.6). The isolated plasmids were linearized, cleaned and quantified by following the protocol (Appendix B.9). Then, the 1 ng of linearized and gel purified plasmids were serially diluted as 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7}, and 10^{-8} to be use as standards in the qRT-PCR assay. The primers shown in Table 4-5 were used for qRT-PCR assay to query *AtF_Box*, *AtEF1α* and *LpUbl5* in transformed *Arabidopsis*. The qRT-PCR results were analysed as described in Section 3.4.6.

4.2.11 Initial screening of transformed *Arabidopsis* lines for drought tolerance

An initial screening of transformed *LpUbl5* overexpressing *A. thaliana* was designed to understand the nature of the response of transformed plants to drought in comparison with the wild type *A. thaliana*. A subset of seeds from the 11 lines (Section 4.2.9) obtained through the BASTA selection process was stratified and used for this experiment. An experiment layout is given in the Table 4-6.

Table 4-6 Representative design of experiment layout used for the initial screening of transformed lines for drought tolerance. A 60 cell propagation tray was divided into two parts for including two transformed lines along with alternate arrangements of wild type *A. thaliana* plants**.**

Line 1					Line 12				
1	2	3	4	5	6	7	8	9	10
WT	GM	WT	GM	WT	GM	WT	GM	WT	GM
GM	WT	GM	WT	GM	WT	GM	WT	GM	WT
WT	GM	WT	GM	WT	GM	WT	GM	WT	GM
GM	WT	GM	WT	GM	WT	GM	WT	GM	WT
WT	GM	WT	GM	WT	GM	WT	GM	WT	GM
GM	WT	GM	WT	GM	WT	GM	WT	GM	WT

Half of the trays contained genetically modified (GM) *A.thaliana* carrying the *LpUbl5* gene under a *CAMV35S* promoter with an alternate arrangement of wild type (WT) plants. Approximately, 4-5 stratified seeds were sown on top of the soil in each cell of the propagation tray. The tray was kept in

the plant growth room at 21^{o}C at a 16h day/8h night regime at 20^{o}C and trays were covered to maintain the humidity and moisture. After germination, upon obtaining four leaves, the extra seedlings were removed to leave a single seedling in each cell of the trays. The cover of the trays was removed and the trays were well watered until five weeks. A representative photograph of the experiment is shown in Figure 4-4

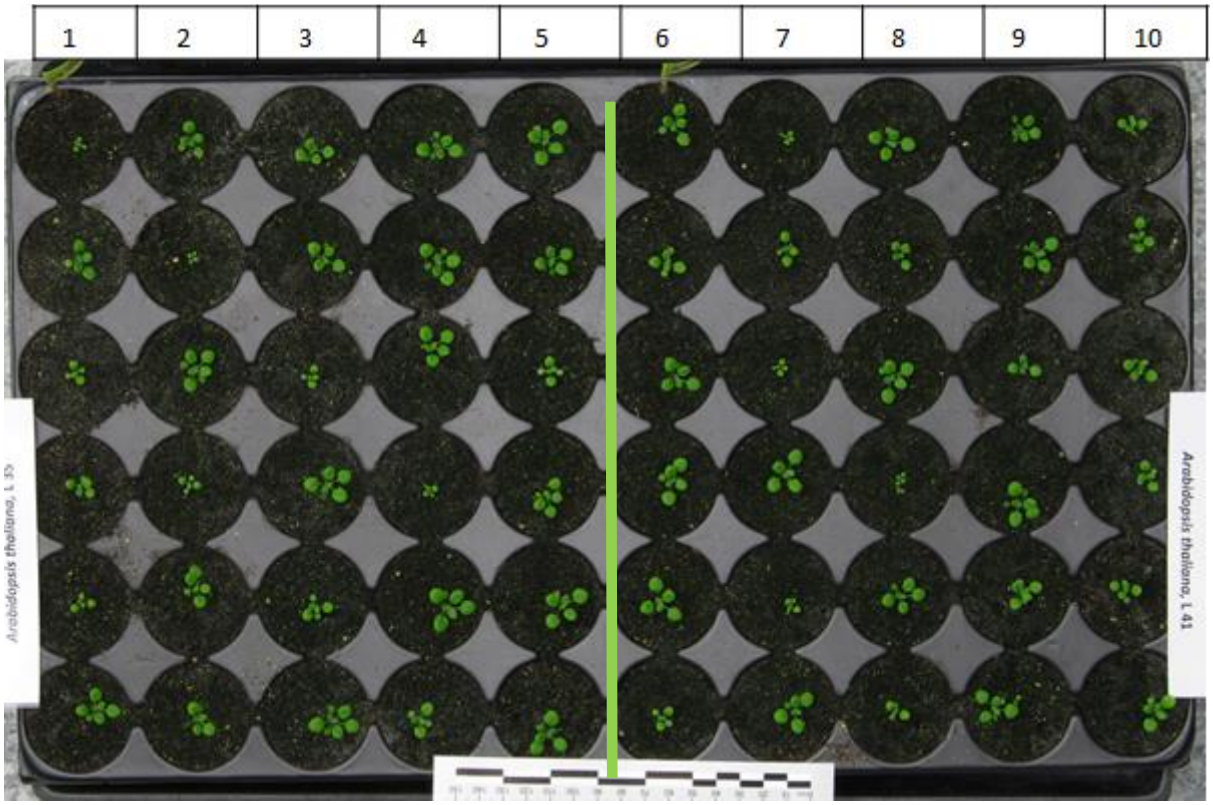


Figure 4-4 Representative photograph of a 60 cell propagation tray carrying two lines of transformed *A. thaliana* plants over-expressing *LpUbl5* gene along with alternate arrangements of wild type *A*. *thaliana* plants as shown in Figure 4.3. The green line at the centre shows the division of one tray to two, column 1-5 holding *A. thaliana* Line 35 and column 6-10 holding *A. thaliana* Line 41.

Trays were imaged every day to identify any change in morphology under well watered conditions and after water withdrawal in comparison with its nearest spaced wild type *Arabidopsis.* Water was withdrawn for 15 days to understand any morphological difference between wild type *Arabidopsis* and transformed *Arabidopsis* through visual evaluation of individual plants as the soil dried. Photographs were taken every day to record the changes that occurred.

Measurements

The plants used for screening lines for drought tolerance were also used for measurement of leaf length and number of leaves per plants. The total leaf length was measured using a ruler and the number of leaves per plant was counted prior to imposing drought stress. Twelve plants from each

modified line in a tray and plants from the nearest spaced wild type plants were used for these measurements.

4.2.12 Analysis of transpirational water loss

The experiments were carried out to analyse the rate of water loss from transformed *Arabidopsis* plants and wild type *Arabidopsis*. The lines used were T3/1, T3/35, T3/41 and T3/43 and wild type, arranged in completely randomized design and these lines were selected based on the *LpUbl5* transcript abundance (Section 4.3.2). The stratified seeds were sown into each cell in the trays. The plants were grown in the plant growth room at 20^{o}C at 16h day/8h night regime until harvest. The plants were watered well until four weeks.

Figure 4-5 Representative photograph of 60 cell propagation tray carrying four lines of transformed *A. thaliana* plants over-expressing *LpUbl5* gene wild type *A. thaliana*. The plants were arranged in completely randomized design.

After four weeks of growth, plants were detached from the roots and the water loss was recorded. The weight of the plants was weighed soon after the shoot detachment and then these detached shoots were left on the lab bench to measure the water loss over time. The shoots were measured every 20 minutes for the next 3 hours and 20 minutes. Ten plants from each line and wild type were used for the study. This study was carried out twice (Yu *et al.*, 2008).

4.2.13 Analysis of drought tolerence and shoot biomass

The plants for this study was T3/1, T3/35, T3/41 and T3/43 and wild type and were arranged in a split plot design. The plants were grown in FTTS tubes of 50 X 120 mm deep and these tubes were arranged

in Native Tube Tray, Egmont, Christchurch, NZ. The study included two treatments of five lines in four replicates including the wild type. Each pot had a single seedling. The plants were grown until three weeks under regular watering and water was withdrawn for two weeks from the pots assigned for drought. Photographs were taken and shoot biomass (See methods Section 3.3.1) was measured at the end of the study.

Figure 4-6 Representative photograph of 50 native tube tray holding different lines of transformed *A. thaliana* plants over-expressing *LpUbl5* gene wild type *A. thaliana* plants. Each tray had eight plants from each line used for the experiment.

4.2.14 Measurements of relative water content and solute potential

Relative water content and osmotic potential were measured (see methods in section 3.3.5 and 3.3.6) for T_3/L1, T_3/L35, T_3/L41, T_3/L43 and wild type to understand the response of plants to imposed drought stress. Plants were grown in pots as shown in Figure 4-6. The drought stress was imposed on three week old plants for two weeks. Before measurements were taken and plants re-watered. The measurements were repeated on recovered plants to understand the difference in plant water status and chlorophyll content.

4.2.15 Analysing number of leaves generated

The number of leaves was counted to understand the effect of overexpression of *LpUbl5* in *Arabidopsis* plants on development. This measurement provides a rapid quantitative assessment of any morphological difference observed between the transformed lines and wild type *Arabidopsis*.

4.2.16 Analysing the effect of osmotic stress

The selected lines of transformed *Arabidopsis* were used for analysing the effect of osmotic stress using MS medium containing increasing concentrations of mannitol. Mannitol concentrations were 100, 200, 250 and 300 mM. The plants were grown for 18 days and root elongation was recorded every day. The transformed lines T3/41 and T3/43 were used along with wild type *Arabidopsis* to study the effect of osmotic stress due to the increasing concentrations of mannitol in the MS medium (Appendix B.2). The shoot dry matter was measured at the end of the experiment to understand the effect of osmotic stress on the yield. The experiment was carried out twice to quantify the response. A trial was carried out without replication to understand the difference observed by transplanting one week old seedlings into MS medium compared with directly sowing seeds to MS medium and MS medium containing different concentrations of mannitol.

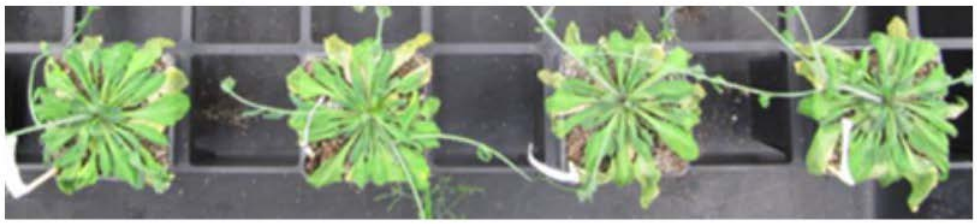
4.2.17 Drought stress experiment to study *Ubl5* transcript level in *A thaliana . thaliana*

An experiment was set up to understand the level of *Ubl5* expression in *Arabidopsis* prior to drought stress, under imposed drought stress and under recovery. This experiment included four lines of *A. thaliana* over-expressing *LpUbl5* and wild type *A.thaliana*. The *LpUbl5* over-expressing lines included in this study were T3/1,T3/35,T3/41 and T3/43. The experiment included 200 FTTS tubes of 50 X 120 mm deep and this tubes were arranged in Native Tube Trays, from Egmont, Christchurch, NZ.

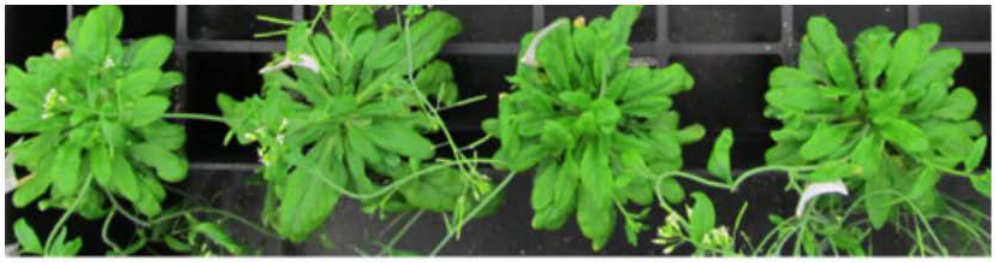
Figure 4-7 Representative photograph of 25 native tube tray holding lines of transformed *A.thaliana* plants over-expressing *LpUbl5* gene wild type *A.thaliana* plants. Each tray carried five plants from each line used for the experiment. Experiment included native tube trays to hold 200 plants.

The seeds were sown after stratification at 4^{o}C for two days. Approximately four seeds were sown in each tube which were thinned down to one seedling per tube after germination. The trays were kept in the plant growth room at 20^{o}C at 16h day/8h night regime and trays were covered to maintain the humidity and moisture until the appearance of true leaves. The plants were watered until five weeks. Leaf samples were collected from each line for the next two weeks at five day intervals for the *LpUbl5* gene expression study. Sample collection was carried out for the next three weeks and a final sample collection was carried out after three days recovery of drought plants. Pots were weighed to record the water loss from the pots.

Well watered pots_20 days drought stress



Water withdrawn pots_ 20 days drought stress



Well watered pots at recovery stage

Water withdrawn pots at recovery stage

Figure 4-8 Representative photograph of *A.thaliana* plants growing in well watered (WW) pots and water withdrawn drought (DR) pots for gene expression analysis. The photos were taken at the end of drought stress and after the recovery.

Analysing endogenous *AtUbl5* transcript abundance under progressive water withdrawal

Arabidopsis thaliana Ubl5 (AtUbl5) gene expression analysis under progressive drought used SAND family protein (At2g28390) and PDF2 (AT1G13320) as reference genes (Czechowski *et al.*, 2005). The SAND, PDF2 and AtUbl5 (AT5G42300) and the gene of interest were amplified using cDNA of wild type *A.thaliana* and cloned into TOPO/TA vector to be used as standards in the qRT-PCR procedure (Section 3.4.4).

Table 4-7 Primers used for the amplification of reference genes and *AtUbl5* (AT5G42300) gene to clone into TOPO/TA vector to use as standards.

Name	Sequences	Amplicon
*SAND*_FWD	AACTCTATGCAGCATTTGATCCACT	
*SAND*_RVS	TGATTGCATATCTTTATCGCCATC	61 bp
*PDF2*_FWD	TAACGTGGCCAAAATGATGC	
*PDF2*_RVS	GTTCTCCACAACCGCTTGGT	61bp
AT5G42300_FWD	TGGTGGTTATAGAAGAAGAATCGT	
AT5G42300_RVS	ACACATAAAATCACGTACCAGAGGA	276 bp

LpUbl5 was amplified using the endpoint PCR method (Appendix A.2) and the gel purified (Section 3.4.4). PCR product was cloned into TOPO® TA Cloning® vector to be used as standards for qRT-PCR assay. The efficiency of the qRT-PCR assay was analysed using the method given in Section 3.4.6..1and qRT-PCR was carried out as described in Section 3.4.6..2.

The primers used for the normalisation of *AtUbl5* gene expression in wild type *A.thaliana* were *SAND* and *PDF2* primers given in Table 4-7 and the *AtUbl5* gene expression was obtained using the primers shown in Table 4-8. The qRT-PCR and the data processing was carried out as described in Section 3.4.6.

Table 4-8 Primer sequences used to analyse the endogenous *Ubl5* gene expression in *A.thaliana* using qRT-PCR

Name	**Sequence**	**Amplicon**
*AtUbl5*_FWD	GAACACGAGCCGAGAAGATCA	
*AtUbl5*_RVS	CGGCATGGGTCTTGAGCT	103 bp

4.2.18 Ubl5 gene in *Arabidopsis* (*AtUbl5*)

An understanding of Ubl5 gene in *A.thaliana* was important to understand the background, importance and the nature of the host plant used for transformation. This information was also crucial to understand the potential role of *Ubl5* in plants. *A.thaliana AtUbl5* gene information was acquired from "The Arabidopsis Information Resource" (TAIR). Search for UBL5 gene in the TAIR resulted in gene information from two loci with two different gene models. The genes were At3g45180 (Ubiquitin like superfamily protein) and At5g42300 (ubiquitin like protein 5 _Ubl5). Both genes contained the Ubiquitin domain (IPR000626) and are grouped as a Ubiquitin supergroup (InterPro: IPR019955). At3g45180 had the 222 bp full length genomic DNA, which encodes the complete coding sequence for the UBL5 protein. In contrast, AT5G42300 had a 1598 bp genomic DNA sequence. The gene model for At5g42300 is explained in Table 4-14. The genomic DNA of At5g42300 was also used to analyse the UBL5 protein coding gene sequence using Emboss six pack (Li *et al.*, 2015b).

Analysing the sequence identity

ClustalW alignment of nucleotide sequence and amino acid sequence for At5g42300, At5g45180 from TAIR and ryegrass UBL5 (GenBank: AIZ94609.1) protein sequence from the National Centre for Biotechnology Information (NCBI) database was carried out using Geneious 7.1.7, http://www.geneious.com/, (Kearse *et al.*, 2012).

Amplification of Ubl5 gene *Arabidopsis*

A.thaliana Ubl5 (*AtUbl5*) gene was amplified by end point PCR to detect the presence of At3g45180 and At5g42300. Two primers were designed using Geneious 7.1.7 http://www.geneious.com/, (Kearse *et al.*, 2012) (Table 4-9) to amplify both the genes from genomic DNA (AT3G_coding_FWD and RVS & AT5G_coding_FWD and RVS) and two different primer pairs (AT3G45180_FWD and RVS & AT5G45180_FWD and RVS) were designed to amplify from cDNA of *Arabidopsis*. The template genomic DNA and cDNA was isolated from leaves of the plants at the stage of flower production (stage 6).

Table 4-9 Primers designed by Geneious 7.1.7 to amplify At5g42300 and At3g45180 to amplify both the genes in the *Arabidopsis*.

Name	Sequence	Amplicon
AT3G_Coding_FWD	TGGTGGTTATAGAAGAAGAATCGT	
AT3G_Coding_RVS	ACACATAAAATCACGTACCAGAGGA	276 bp
AT5G_Coding_FWD	AGAAAATTGGGGGCTAGGGT	
AT5G_Coding_RVS	TCACATACCACAAAAACCGAAAGA	334 bp
AT3G45180_FWD	GGAACCCGACCCGAAAAGATTC	
AT3G45180_RVS	GTAGAGCTCGAGACCCATGC	108 bp
AT5G42300_FWD	GAACACGAGCCGAGAAGATCA	
AT5G42300_RVS	AGCTCAAGACCCATGCCG	103 bp

4.2.19 *A.thaliana Ubl5* mutants

The aim of this experiment was to generate a homozygous mutant for Ubl5 (At3g45180 and At5g42300). Mutant information was gathered from TAIR and one mutant line from At3g45180 and two lines from At5g42300 were ordered from the Arabidopsis Biological Resource Centre (ABRC). The mutant line of At5g45180 was SALK_126377 using vector pROK2, At5g42300 was mutant line CS829912 (SAIL_682_E03) using vector pDAP101 and SALK_067245 using pROK2. These were ordered to identify a homozygous mutant for each gene (At3g45180 and At5g42300) and generate a homozygous *AtUbl5* mutant line by cross pollinating two homozygous mutant lines of each gene.

The seeds were sown in soil mix and the leaf samples were collected to isolate genomic DNA to identify the homozygous mutant lines.

Primer design for mutant screen

Primers were generated using T-DNA Primer Design at the Salk Institute Genomic Analysis Laboratory (SIGnAL) based on the schematic representation (Figure 4-9) of T-DNA insertion adapted from http://signal.salk.edu/tdnaprimers.2.html)

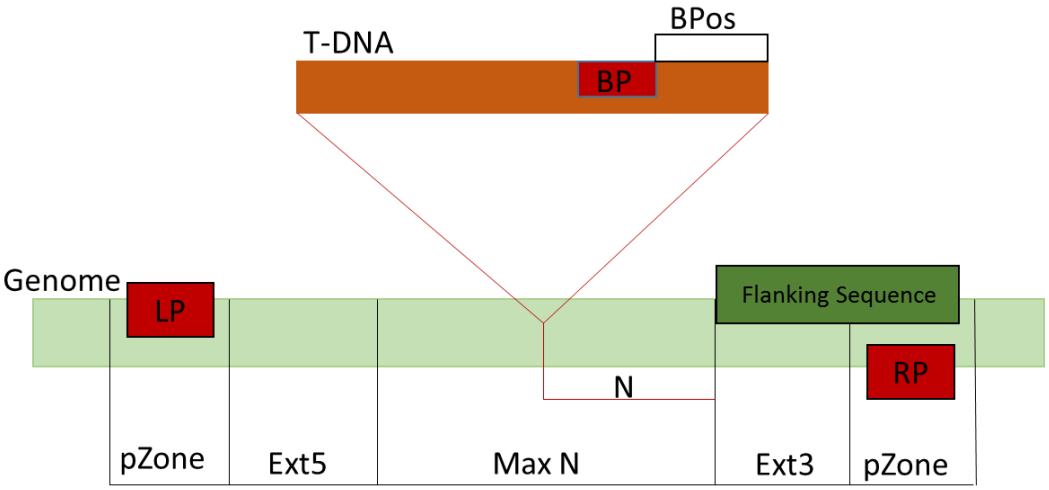


Figure 4-9 Schematic representation of T-DNA inserts in the genome and the primer design was carried out based on this scheme to identify the homozygous, heterozygous and wild type plants in the mutant seeds. Difference between actual insertion site and the flanking sequence position (N= 0-300 bases), Maximum difference between the actual insertion and the sequence (MaxN = 300 bases), Regions selected to design primers (pZone) reserved regions for not picking primers (Ext5 and Ext3), Left and Right genomic primers (LP and RP) T-DNA border (BP) and the distance between the T-DNA border primer to the insertion site.

Table 4-10 Amplification pattern of wild type (WT), heterozygous (HZ) and homozygous (HM) lines of T-DNA insertion mutants (N= ~0-300 Bp)

Amplicon	WT	HZ	HM
900 bp	Positive	Positive	
410 +N bp		Positive	Positive

The wild type plants will be generating one amplification product with approximately 900 bp indicating the absence of T-DNA insertions. Whereas the heterozygous lines will have two amplification products indicating the presence of a wild type allele and a mutant allele. A homozygous plant will result in one amplification product of 400 +N bp indicating the insertion of T-DNA at both alleles and thus homozygous for *Ubl5* gene (Table 4-10).

Table 4-11 Primer pairs used to screen the mutant plants to identify homozygous mutant lines for At3g45180 and At5g42300.

Name	Sequence	Amplicon
SALK_126377_LP	TAACGACATGCCTTATTTGGC	
SALK_126377_RP	TATCACGGACAATTCCTTTCG	1186Bp
SALK_126377_LBb1.3	ATTTTGCCGATTTCGGAAC	
SALK_126377_RP	TATCACGGACAATTCCTTTCG	556-859 Bp
SAILseq_682_LP	CCCCCTTGTCTCTATTGGAAC	
SAILseq_682_RP	GTATATCTCGCAGATGCAGCC	1122 Bp
SAILseq_682_LP_LB1	GCCTTTTCAGAAATGGATAAATAGCCTTGCTTCC	
SAIL_682_RP	GTATATCTCGCAGATGCAGCC	440-740 Bp
SALK_067245_LP	CGTAAATTGGTGGCATATTTATTG	
SALK_067245_RP	ACAACAACATCTCAATTCCGC	1209 Bp

Wild type plants from the SALK lines (SALK_126377 and SALK_0675245) were identified using corresponding LP and RP primer (Table 4-11) pairs whereas the LBb1.3 was used along with RP to identify the mutation status. Wild type plants from SAIL (CS829912) used corresponding LP and RP primers (Table 4-11). A combination of LB1 with RP was used to identify the mutant status (http://signal.salk.edu/tdnaprimers.2.html).

An end point PCR was carried out using the genomic DNA from mutant plants as a template to identify the mutational status of the plants. This resulted in categorizing the plants into wild type, heterozygous and homozygous.

Investigation embryo lethal effect in *Ubl5* mutation

The seeds generated from heterozygous *Ubl5* mutant plants were screened to study the potential effect of *Ubl5* mutation. The siliques were peeled under Olympus SZX16 stereo microscope (Olympus, Tokyo, Japan) and images acquisition was carried out using an Olympus DP71 digital camera (Olympus, Tokyo, Japan) to observe any variation in seed filling (Tsugeki *et al.*, 1996).

4.3 Results

4.3.1 Molecular charactarization of transgenic *Arabidopsis* lines containing the *LpUbl5* transgene

In order to determine the impact of over-expression of the *LpUbl5* gene in *Arabidopsis*, a number of transgenic lines containing the *LpUbl5* transgene driven by the strong CaMV-35S promoter were generated and analysed using end point PCR to understand the transgene abundance.

An end point PCR on genomic DNA isolated from transgenic lines confirmed the presence of the T-DNA insert using the specific primers pairs (Table 4-4) in the 11 selected lines of transformed Arabidopsis. Screening T_3 lines for the presence of the transgene was carried out using *pART_35S*_FWD and *LpUbl5*_RVS primers. The gel in Figure 4-10 shows the end point PCR result of 11 transformed lines with the expression vector as a positive control, which shows similar amplification products which confirms the transformation. Wild type (WT) and molecular grade water were used as negative controls to validate the reaction. This confirms that the lines selected based on 1:2:1 Mendelian segregation ratio did possess the transgene, *LpUbl5*.

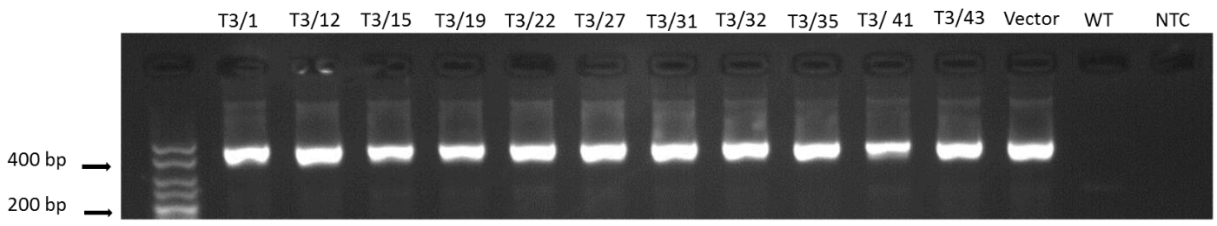


Figure 4-10 Gel images of end point PCR product obtained using the using *pART_35S*_FWD and *LpUbl5*_RVS primers showing 459 bp product. The HyperLadder™ V from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

All the lines were used for initial drought tolerance screening whereas only lines T3/1, T3/35, T3/41 and T3/43 were used for other physiological analyses of drought tolerance.

4.3.2 *LpUbl5* transcript abundance in modified *Arabidopsis*

The 11 lines of transformed *Arabidopsis* lines confirmed different transcript level of *LpUbl5* gene. The *LpUbl5* transgene expression was analysed for all of the 11 transformed lines and the result is given in modified *A.thaliana* lines (Figure 4-11).

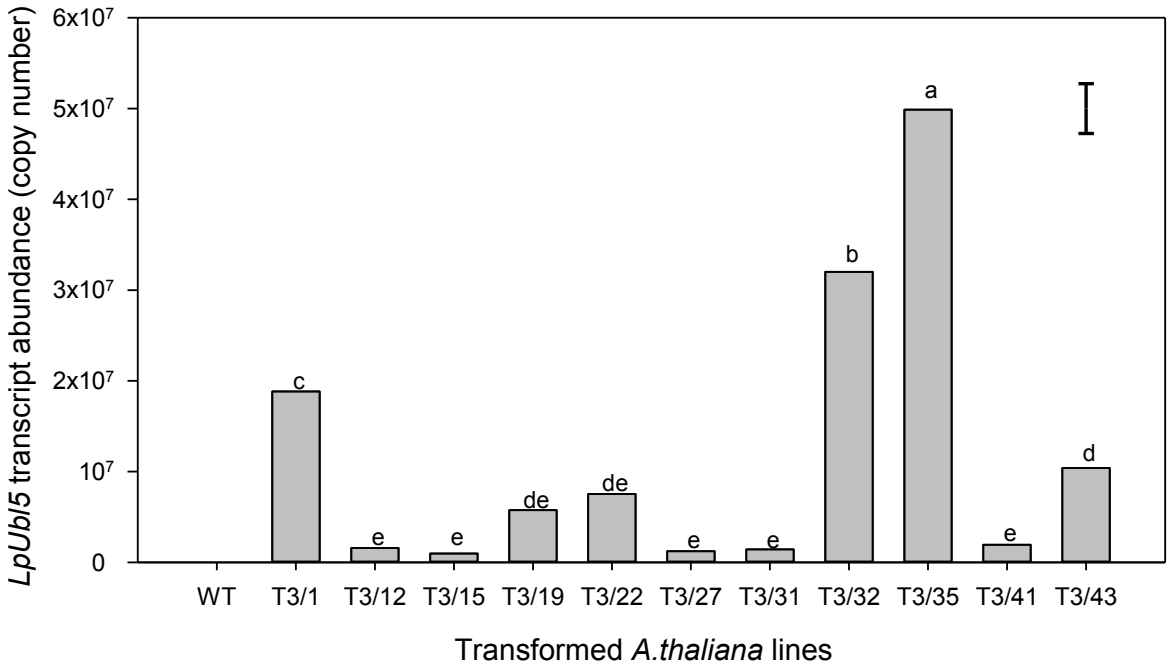


Figure 4-11 *LpUbl5* transcript abundance in 11 transformed *A.thaliana* lines and the standard errors of means are shown as error bars. Fischer's Protected Least Significant Differences (LSD), bars with letters in common are not significantly different α = 0.05 level.

The transgene expression level was analysed using One-way anova. A post hoc analysis using Fisher's protected least significant difference test showed that the transgene, *LpUbl5* expression was different ($P<0.001$) among the lines. Line T3/35 had the highest (49881219 copies) transgene expression level. The lines T3/12, T3/15, T3/27, T3/31, T3/41 has the lowest (977107 copies) transgene expression whereas T3/19, T3/22 and T3/43 had similar levels of gene expression.

To include a range of *LpUbl5* transcript abundance, T3/35, T3/1, T3/43 and T3/41 are selected to carry out all the experiments except, visual screening of drought tolerance and analysis of number of leaves and length where total 11 lines were used.

4.3.3 Visual screening of 11 lines for drought tolerence

Visual screening and photographs were obtained to evaluate 11 transformed lines of *A.thaliana* to identify any differences in performance under drought (Figure 4-12). There was no clear evidence of a link between *LpUbl5* transgene expression and enhanced tolerance to drought.

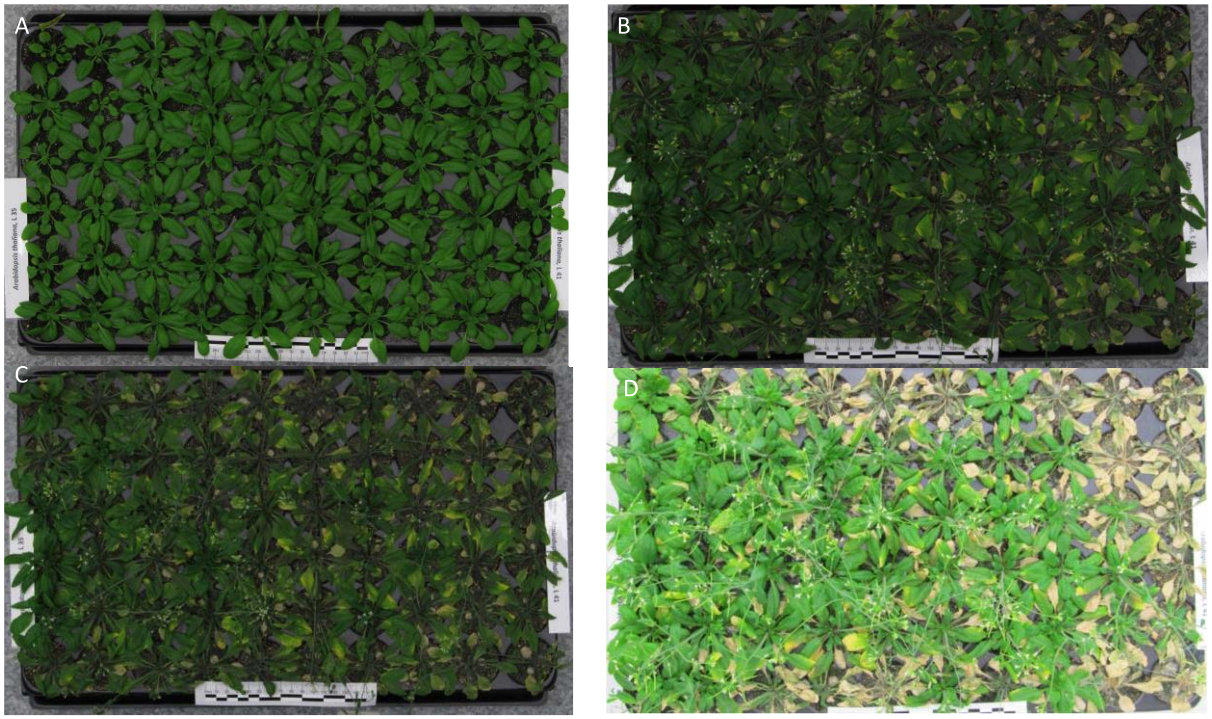


Figure 4-12 Shows the photographs of *A.thaliana* Lines T3/35 and T3/41 taken at different phases of a drought stress experiment to study the performance of lines under drought in comparison with wild type plants. Image A shows 5 week old plants before imposing water withdrawal. Image B shows the plants after 10 days of water withdrawal and image C shows the state of the plants after 14 days of water withdrawal (Yu *et al.,* 2008). Image D shows the recovery of plants after three days after re-watering.

The experiment screened 11 lines of transformed *A.thaliana* in comparison with the nearest spaced wild type (Figure 4-12). The screening could not identify any visual differences between the wild type and transformed lines after 14 days of water withdrawal.

4.3.4 Number of leaves and leaf length

The analysis indicated a higher number of leaves and increased leaf length in transformed *Arabidopsis* lines but this did not correlate with the *LpUbl5* transcript abundance (Figure 4-11). Linear regression showed no correlations between number of leaves and transcript abundance (P=0.26; r= 0.375) or length and transcript abundance (P =0.98; r = 0.009). The number of leaves per plants was counted (Section 4.2.11) when the plants were five weeks old. The results of paired T-test are shown in Table

4-12. Line T3/15 (P<0.05), T3/19 (P<0.05), T3/35 (P<0.001), T3/41 (P<0.05) and T3/43(P<0.05) had higher number of leaves in comparison with wild type plants.

Table 4-12 Number of leaves per plants analysed using T-test from 11 transformed *A.thaliana* lines when compared with wild type.

Lines	Difference of means	SED	p
T3/1	1.7	0.858	0.052
T3/12	1.1	0.0807	0.193
T3/15	3.8	1.678	*0.041*
T3/19	3.2	1.306	*0.024*
T3/22	1.3	0.713	0.075
T3/27	1.5	0.768	0.064
T3/31	1.1	0.807	0.193
T3/32	-0.6	0.838	0.437
T3/35	3.0	0.0704	*< 0.001*
T3/41	2.8	0.716	*0.002*
T3/43	3.2	1.242	*0.025*

Difference of means between each line and nearest spaces wild type, SED: standard error of difference, P: probability (under null hypothesis of equal variance) and n=24

Table 4-13 Leaf length (mm) analysed using paired T-tests from 11 transformed *A.thaliana* lines when compared with wild type.

Lines	Difference of means	SED	p
T3/1	35.2	25.3	0.179
T3/12	28.9	21.2	0.187
T3/15	74.8	29.2	*0.022*
T3/19	96.6	28.42	*0.003*
T3/22	29.8	30.97	0.346
T3/27	41.2	29.45	0.176
T3/31	80.2	30.5	*0.015*
T3/32	-32.8	30.5	0.29
T3/35	91.0	26.71	*0.003*
T3/41	99.5	19.92	*< 0.001*
T3/43	106	26.79	*< 0.001*

Difference of means between each line and nearest spaces wild type, SED: standard error of difference, P: probability (under null hypothesis of equal variance) and n=24

The result of paired T-test in Table 4-13 shows that lines T3/15 (P<0.05), T3/19 (P<0.05), T3/31 (P<0.05), T3/35 (P<0.05), T3/41 (P<0.001), and T3/43 (P<0.001) had increased leaf length when compared with wild type plants.

4.3.5 Comparison of Water loss rate

Water loss rate was low in lines T3/41 and T3/43 in comparison with other tested lines and wild type.

Non-linear regression analysis fitted a standard curve (Figure 4-13) of water loss over time. The resultant Equation 4-2 A + B*(R**X) was used to calculate the response variate using estimated parameters.

Equation 4-2 Fitted curve: A + B*(RX)**

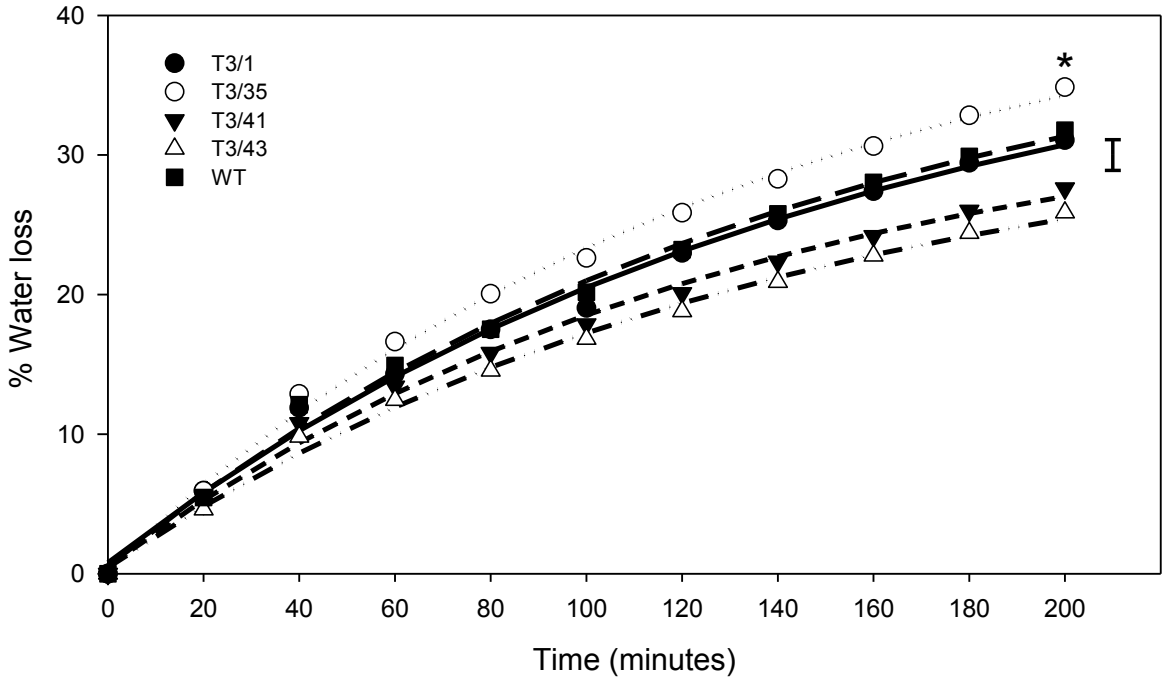


Figure 4-13 Water loss of T3/1, T3/35, T3/41 and T3/43 wild type over time from a detached rosette. Asterisks is shown to indicate the difference. The error bar shows the standard error of mean for the lines.

A One-way Anova of water loss at the final measurement point (200 mins) showed T3/41 (27.6±2.19) and T3/43 (25.9±2.19) had the lowest water loss and T3/35 (34.8 ± 2.19) had the highest with the wild type (31.8±2.19) and T3/1 (31.8±2.19) intermediate ($P<0.05$).

4.3.6 Shoot Biomass Yield

The biomass yield did not show any difference between transformed lines and wild type *Arabidopsis*. The biomass yield was obtained after imposing 14 days of water withdrawal from three week old plants and the experiment included a well-watered control (Figure 4-14).

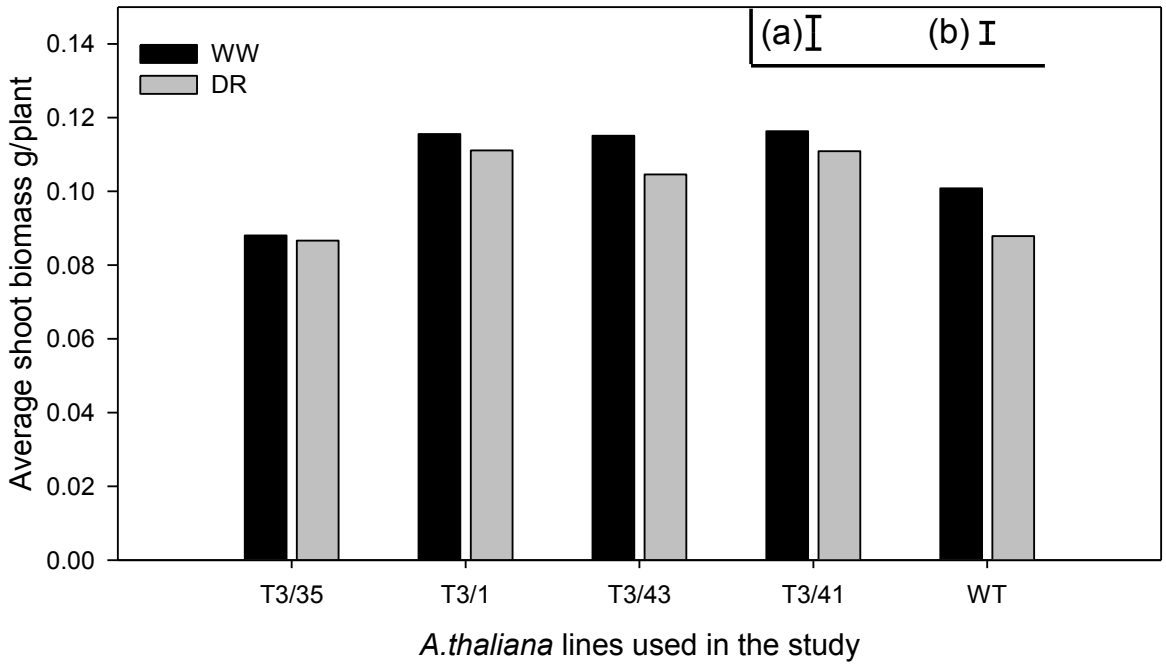


Figure 4-14 Shoot biomass yield of transformed *A.thaliana* lines (T3/1, T3/35, T3/41, T3/43 and WT) after 14 days of water withdrawal. This includes results from well watered (WW) controls and water withdrawn drought (DR) pots. Error bar shown are standard error of mean for the lines (a) and treatments (b).

The shoot biomass (Figure 4-14) was analysed using a Two-way Anova in GenStat (VSN International (2015) with lines and water treatments. There were no differences in shoot biomass between the lines (P= 0.12), water treatments (P= 0.38) or their interaction (P=0.99).

4.3.7 Number of leaves per plant

Average number of leaves per plant was similar in transformed lines and wild type *Arabidopsis*. The number of leaves was also counted on the plants collected for shoot biomass yield (Figure 4-15).

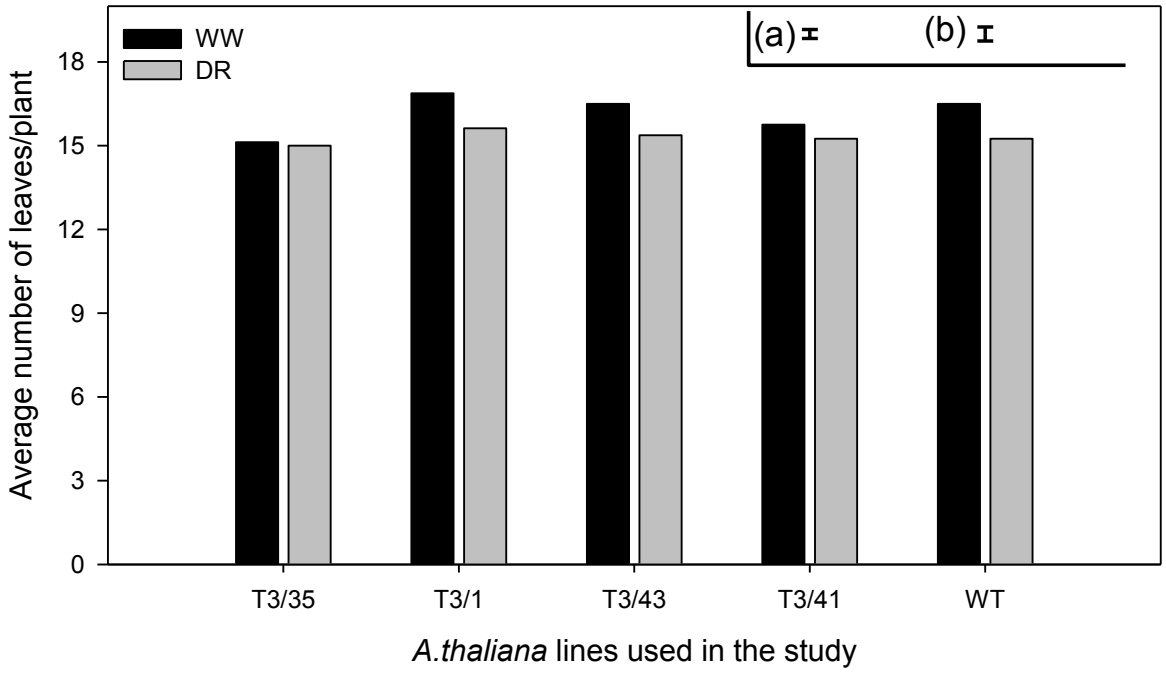


Figure 4-15 Leaves per plants of transformed *A.thaliana* lines (T3/1, T3/35, T3/41, T3/43 and WT after 14 days of water withdrawal. This results shown from well watered (WW) controls and water withdrawn drought (DR) pots. Error bar shown are standard error of mean for the lines (a) and treatments (b).

The number of leaves per plant (Figure 4-15) was analysed by Two-way Anova with lines and water treatments. There was no difference (P= 0.51) among lines and water treatments (P= 0.06) and no interaction (P= 0.90), with all plants having approximately 15 leaves.

4.3.8 Relative water content (RWC)

The RWC (Figure 4-16) was analysed using Two-way Anova and showed no difference (P=0.42) among lines but, as expected, an effect (P <0.001) of water stress. A One-way Anova was carried out with each line using water treatment as factor. Differences (P< 0.05) were only observed in wild type plants between well watered (0.83 ± 0.009%) and water withdrawn drought (0.89± 0.009%) plants. No interaction (P=0.59) was observed under recovery between lines and water treatments.

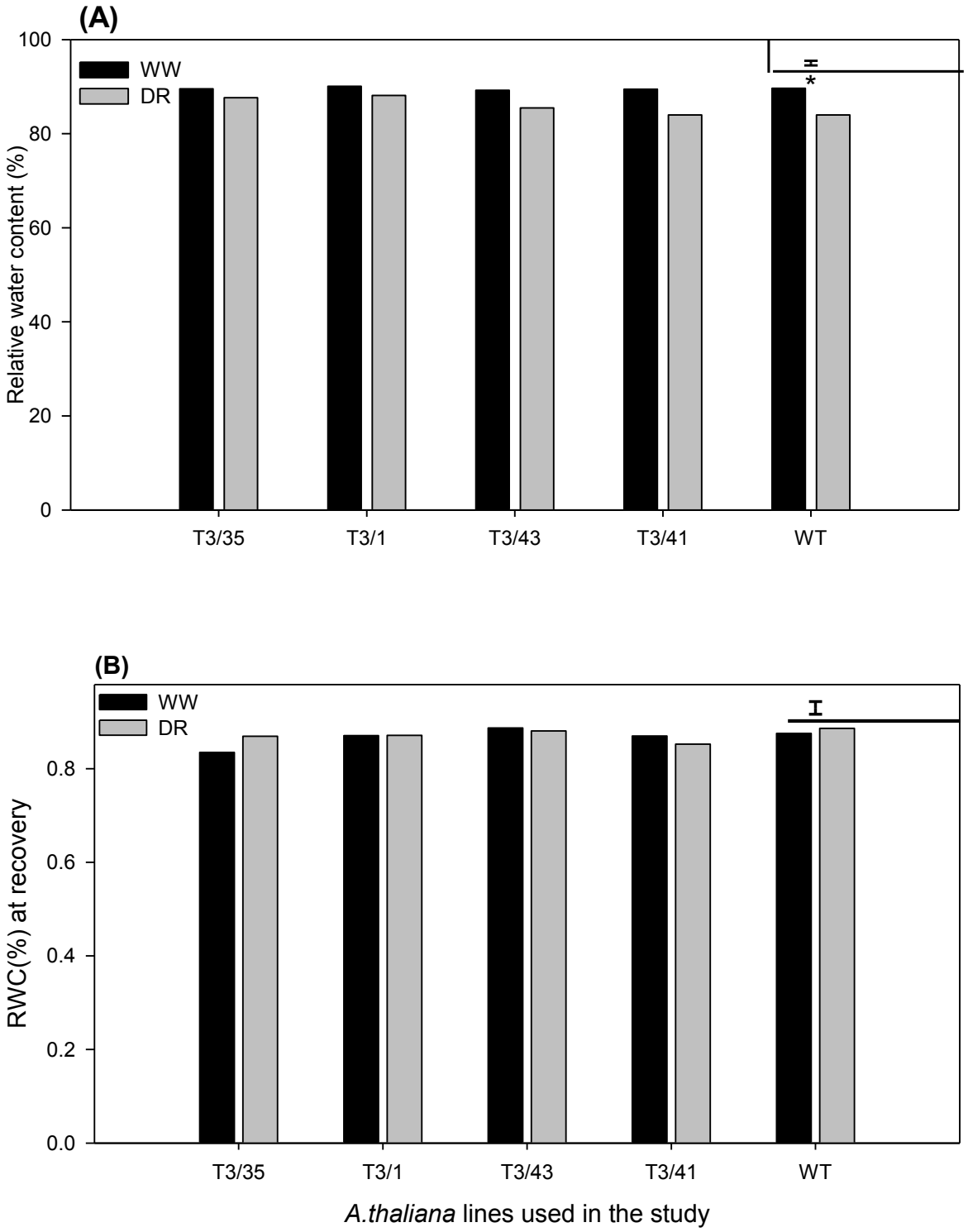


Figure 4-16 Relative water content (RWC) of the transformed *A.thaliana* lines (T3/1, T3/35, T3/41, T3/43 and WT after 14 days of imposed drought stress (A) and recovery (B) for well watered (WW) and water withdrawn (DR) treatments. Error bars shown are standard error of mean for the treatments. Asterisks shown where difference (P< 0.05) observed.

4.3.9 Solute potential (MPa)

The pattern of result indicated by RWC (Figure 4-16) was not followed by solute potential (Figure 4-17) measurements. Solute potential (Figure 4-17) was analysed using Two-way Anova with no difference

(P=0.82) observed among lines but a difference (P< 0.05) between water treatments, with an osmotic potential of -1.3 ± 0.03 MPa and under drought -1.1 ± 0.03 MPa. In contrast, there was no difference among lines (P= 0.38) or water treatments (P = 0.45) at recovery and observed no interaction (P=0.87) between line and treatments.

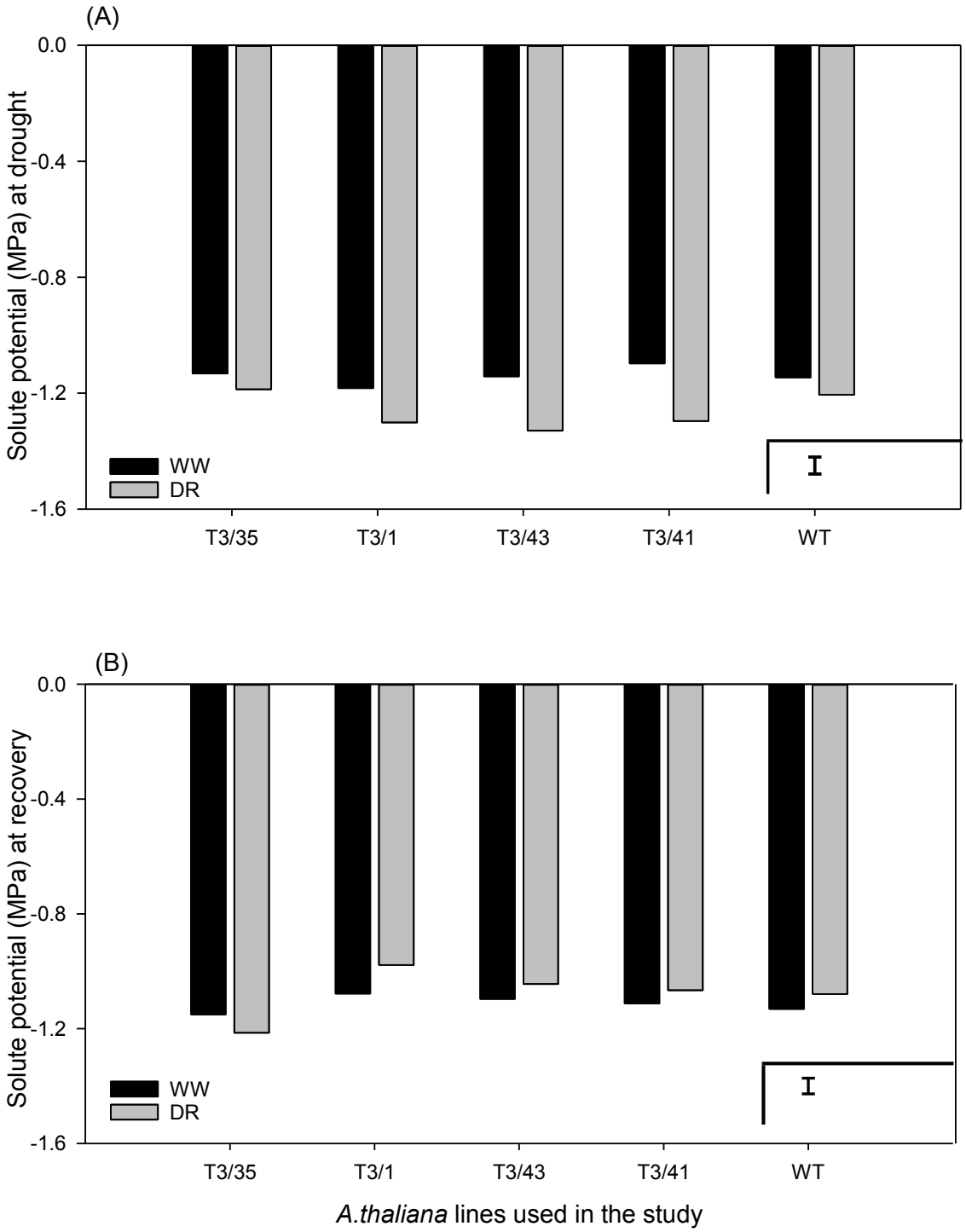


Figure 4-17 Solute potential of the transformed *A.thaliana* lines (T3/1, T3/35, T3/41, T3/43 and WT) after 14 days of imposed drought stress (A) and recovery (B) for well watered (WW) controls and water withdrawn drought (DR) treatments. Error bar shown are standard error of mean for the treatments.

4.3.10 Effect of osmotic stress on root length and shoot biomass

Line T3/41 and T3/43 were selected initially for osmotic stress experiment with an intention to test more lines based on the experimental outcome of these lines.

Effect of osmotic stress on root length

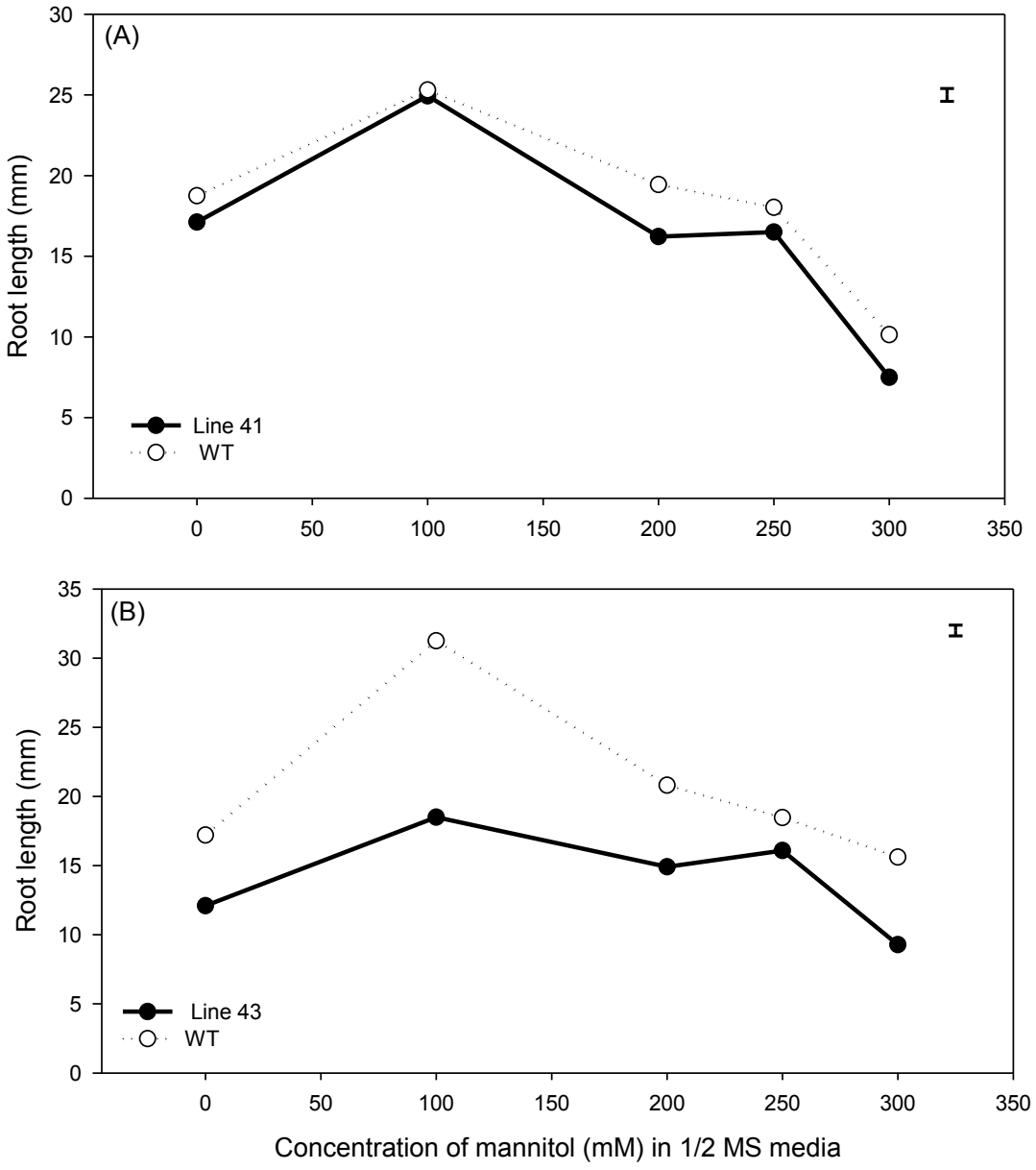


Figure 4-18 Root length of transformed line (A) T3/41 and wild type *A.thaliana* (WT) , (B) T3/43 and wild type *A.thaliana* (WT) grown with increasing concentrations of mannitol in ½ MS medium. Error bar shown is the standard error of the treatments.

An addition of mild quantities of (100 mM) mannitol under seed germination showed a potential to increase the root length of *A.thaliana* seedlings. Lines T3/41 and T3/43 were used to analyse the effect of osmotic stress by using wild type *A.thaliana* as the control. The measurements obtained were root length (Figure 4-18) and shoot biomass (Figure 4-19) and the data were analysed using Two-way Anova.

Root length (25.18 ± 1.26) was increased (P <.001) for both T3/41 and WT with 100 mM mannitol containing media and then decreased to be lowest (8.8 ± 1.26) at 300 mM mannitol. Analysis showed no difference (P=0.09) between T3/41 and wild type.

Analysis using Line T3/43 and wild type, the longest (P<0.001) root length was observed in 100 mM (24.8 ± 1.23) mannitol and the shortest root length (12.4 ± 1.23) was observed in 300 mM. A difference was observed between lines and an interaction (P<0.05) between lines and mannitol treatments.

Effect of Osmotic stress on shoot biomass yield

The yield of *Arabidopsis* shoot biomass decreased as mannitol concentration increased in ½ MS media (Figure 4-19).

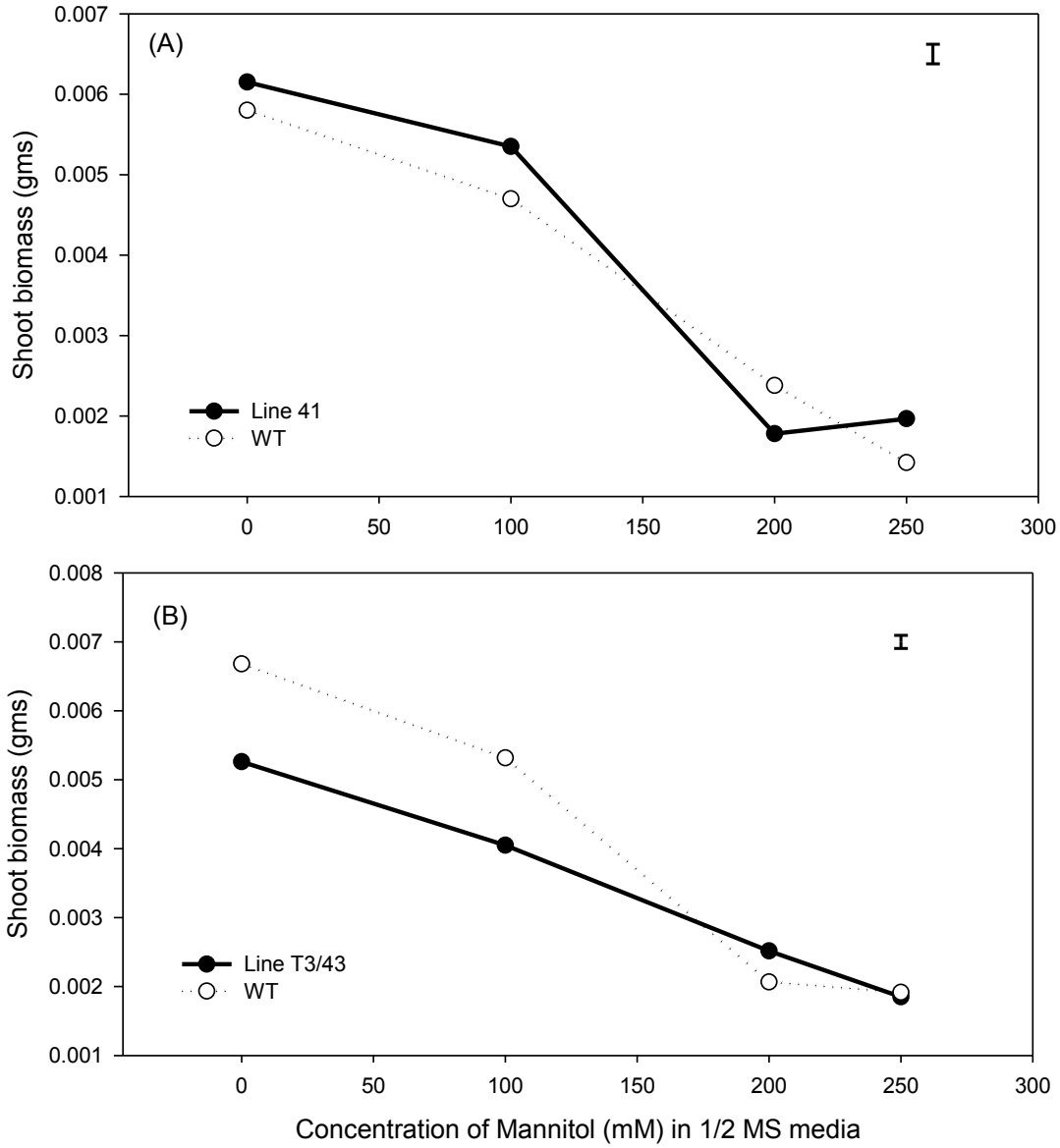


Figure 4-19 Shoot biomass of transformed line (A) T3/41 and wild type *A. thaliana* (WT) , (B) T3/43 and wild type *A. thaliana* (WT) grown with increasing concentrations of mannitol in ½ MS medium. Error bar shown is the standard error of the treatments.

The Figure 4-19 shows the effect of osmotic stress on shoot biomass yield from Line T3/41 and T3/43 in comparison with wild type *A.thaliana*. Shoot biomass decreased (P <0.001) with increasing concentrations of mannitol in ½ MS medium.

There was a difference (P <0.001) in shoot biomass for line T3/41 and the wild type in different concentrations of mannitol but no difference (P= 0.41) among lines and was no interaction (P= 0.50). Whereas the analysis of line T3/43 and wild type showed a difference (P <0.001) in different concentrations of mannitol and an interaction between line and treatment (P < 0.05). Wild type plants increased (P <0.001) shoot biomass when compared with T3/43 whereas there was no difference (P= 0.41) between wild type and T3/41.

Based on these results, a decision was taken not to extend the experiment to more lines but restrict to T3/41 and T3/43.

Photographic record of status of plants on day 18

Figure 4-20 to Figure 4-23 show a photographic record of in root length in seedlings germinated on ½ MS media containing different concentrations of mannitol compared with one week old seedlings transplanted on to ½ MS media containing different concentrations of mannitol.

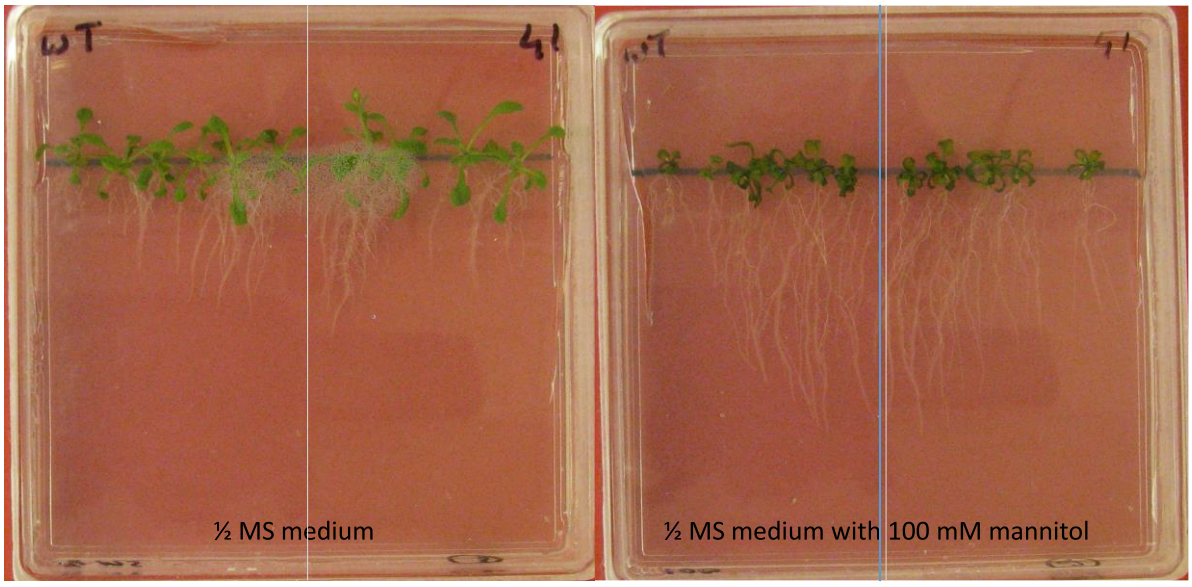


Figure 4-20 Wild type (WT) *A. thaliana* and transformed line T3/41 (41) are grown in ½ MS medium and ½ MS medium containing 100 mM mannitol exhibiting the variation in root length and shoot growth. Each plate contains 6 seedlings from wild type (first half of the Petri-dish) and T3/41 (the second half of the petri-dish).

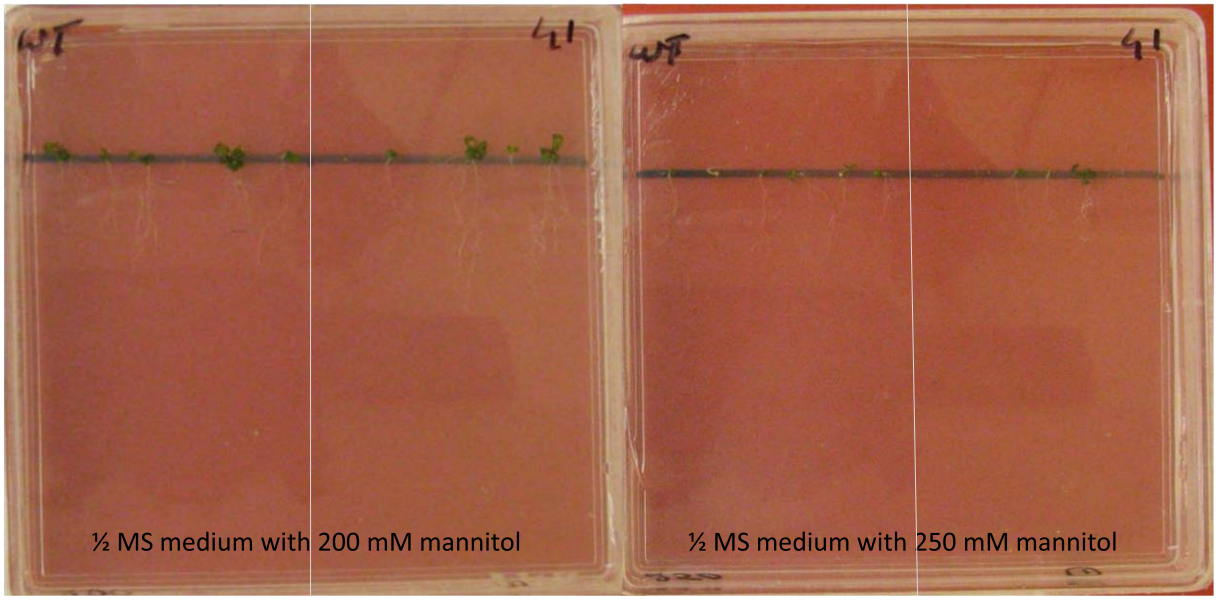


Figure 4-21 Wild type (WT) *A. thaliana* and transformed line T3/41 (41) are grown in ½ MS medium containing 200 mM mannitol and ½ MS medium containing 250 mM mannitol exhibiting the variation in root length and shoot growth. Each plate contains 6 seedlings from wild type (first half of the Petri-dish) and T3/41 (second half of the petri-dish).

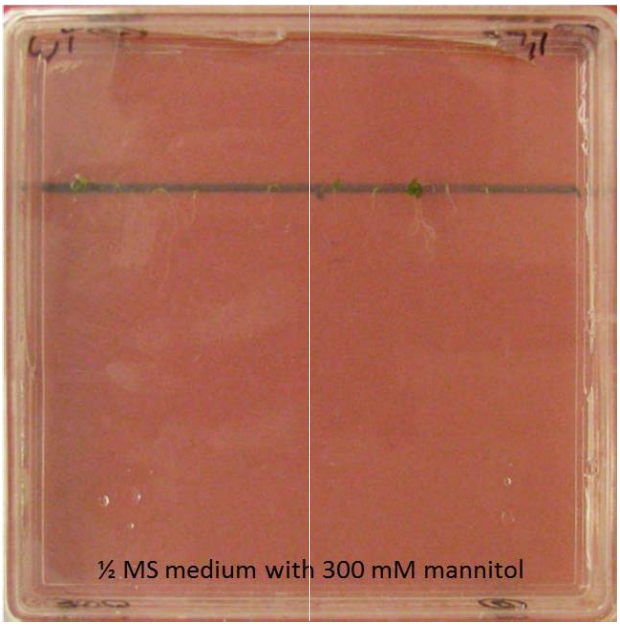


Figure 4-22 Wild type (WT) *A. thaliana* and transformed line T3/41 (41) are grown in ½ MS medium containing 300 mM mannitol exhibiting the root length and shoot growth. The plate contains 6 seedlings from wild type (first half of the Petri-dish) and T3/41 (second half of the petri-dish).

Figure 4-23 Wild type (WT) *A. thaliana* and transformed line T3/41 (41) are grown in ½ MS medium, ½ MS medium containing 100 mM mannitol and ½ MS medium containing 200 mM mannitol exhibiting the variation in root length and shoot growth. Each plate contains 7 seedlings from wild type and T3/41. One week old seedlings were transplanted from ½ MS medium to different test mediums.

The photos obtained on day 18 (Figure 4-20 to Figure 4-22) show the differences in shoot and root growth in different concentrations of mannitol in comparison with ½ MS medium without mannitol addition. There was a difference in root growth when compared with Figure 4-23 when one week old seedlings were transplanted to ½ MS medium and ½ MS medium that contained different concentrations of mannitol.

Figure 4-23 was obtained from the trial experiment and the experiment was not replicated and therefore an analysis is not carried out. These photos are presented here to demonstrate the difference in root length of seeds sown directly on to the ½ MS medium that contained 100 mM mannitol (Figure 4-20) compared with seedlings transplanted from ½ MS medium on to ½ MS medium with 100 mM mannitol (Figure 4-23).

4.3.11 Pot weight and *AtUbl5* transcript abundance under progressive drought

AtUbl5 transcript abundance under progressive drought was analysed in wild type *Arabidopsis* to understand any variation in the level of transcripts as drought progress. *AtUbl5* transcript level (B_ Figure 4-24) was analysed from control unstressed plants over time and from stressed plants under progressive drought. The results showed no difference ($P \geq 0.15$) between well watered and stressed plants under progressive drought.

Similar levels of transcript level were observed in leaf samples obtained from well watered pots and water withdrawn pots (Figure 4-24).

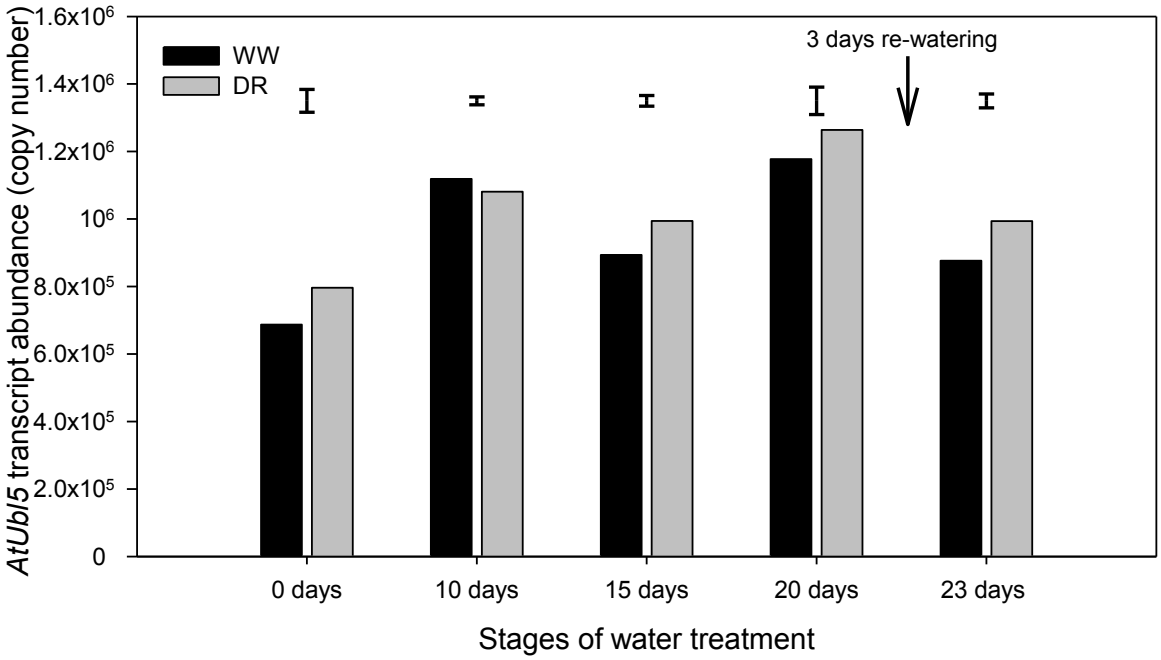


Figure 4-24 The endogenous *AtUbl5* gene expression in wild type *Arabidopsis* under progressive drought and the error bar shown are the SEM of treatments.

4.3.12 *Ubl5* genes in *Arabidospsis*

Ubl5 Information from TAIR

Search for UBL5 gene in the TAIR resulted in gene information from two loci with two different gene models. The genes were AT3G45180 (Ubiquitin like superfamily protein) and AT5G42300 (ubiquitin like protein 5 _Ubl5). Both genes contained the Ubiquitin domain (IPR000626) and grouped as a Ubiquitin supergroup (InterPro: IPR019955). AT3G45180 appears to possess a single exon of 222 bp length whose putative nucleotide sequence matched the ryegrass *LpUbl5* coding sequence (GenBank: KJ782026.1) frame at 82%. The AT5G42300 gene (Table 4-14) appears to possess a single intron at position 321-1308 in the gene model. This genes coding region at position 99 – 320 bp also corresponds to ryegrass *LpUbl5* model at 82%.

Table 4-14 Summary of AT5G42300 protein coding gene model obtained from TAIR

Type	Coding
ORF (Open reading frame)	99-320
5' utr	1-98
Coding region	99-320
exon	1-320
intron	321-1308
exon	1309-1598
3' utr	1309-1598

The genomic DNA of AT5G42300 was used to analyse the UBL5 protein coding gene sequence using Emboss six pack ((Li *et al.*, 2015b)) which generated a similar result for the protein coding gene sequence, which starts open reading frame at 99 bp and ends at 320 bp. This showed that AT5G42300 had no introns within the coding region.

Analysing the sequence identity

Nucleotide sequence alignment was performed using ClustalW alignment in Geneious 7.1.7. The results are given as a percentage indicating the percentage bases which are identical when compared with other sequences.

Table 4-15 Distance results of AT5G42300, AT3G45180 and LpUbl5 obtained by Nucleotide sequence alignment result carried out using ClustalW alignment in Geneious 7.1.7

Gene Name	At5G42300	AT3G45180	*LpUbl5*
AT5G42300		83.78%	81.98%
AT3g45180	83.78%		82.88%
LpUbl5	81.98%	82.88%	

A ClustalW alignment was performed using the putative protein sequence in Geneious 7.1.7 for At5G42300, AT5G45180 from TAIR and ryegrass UBL5 (GenBank: AIZ94609.1) protein sequence from the National Centre for Biotechnology Information (NCBI) database. Protein alignment results are given in Table 4-15 as alignment view (Figure 4-25). The putative amino acid sequences showed 94.52% sequence identity indicating the percentage of residues which are identical.

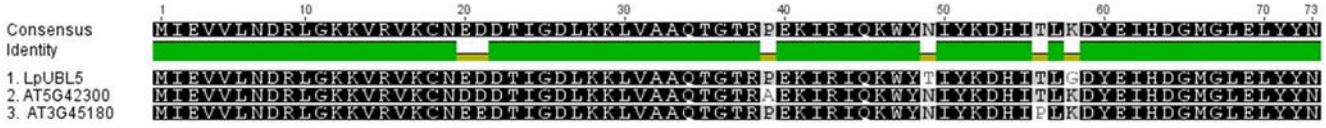


Figure 4-25 Protein sequence alignment results obtained by ClustalW alignment using protein sequence of AT5G42300, AT3G45180 and LpUBL5 in Geneious 7.1.7

These alignments confirmed that UBL5 was highly conserved across species and thus its potential role is expected to be similar across these species.

Amplification of AtUbl5 from genomic DNA and cDNA

An end point result using the specific primers (Table 4-9) showed that only *Arabidopsis Ubl5* gene AT5G42300 was detected in genomic DNA and cDNA, not At3G45180. Gel 1 and Gel 2 show the end point PCR results using primers for AT3G45180 and AT5G42300. The first part of Gel 1 shows the end point PCR result using AT3G_Coding_FWD and AT3G_Coding_RVS which shows amplification (product 276 bp) in the genomic DNA but no amplification product observed in cDNA samples. The second part

of Gel 1 contains the amplification (product 334 bp) of AT5G_Coding_FWD and AT5G_coding_RVS which shows the amplification product of similar size in the genomic DNA as well as in cDNA. This also indicates that there were no introns within the coding region and is an important indication of the size of the coding region. The information obtained from TAIR (Table 4-14) shows that 98-320 as the coding region and 321- 1308 as intron. However the endpoint PCR result using AT5G42300_coding FWD and RVS shows that the product is 334 bp where reverse primer is nested at 422 – 442 which is given as an intron (321- 1308) by TAIR.

The first part of Gel 2 shows the amplification product of At3G45180_FWD and AT3G45180_RVS. The primers were designed from within the open reading frame of AT3G45180 and showed amplification (product 108 bp) in gDNA and was absent in cDNA which confirms that the gene was not transcribed. The primer pair was specific to AT3G45180 and that did not amplify the AT5G42300. The second part of Gel 2 shows the amplification (product 103 bp) of AT5G42300 in genomic DNA as well as in the cDNA. This confirms that *Arabidopsis* expressed only AT5G42300 but not AT3G45180. Therefore qRT-PCR was carried out only for AT5G42300.

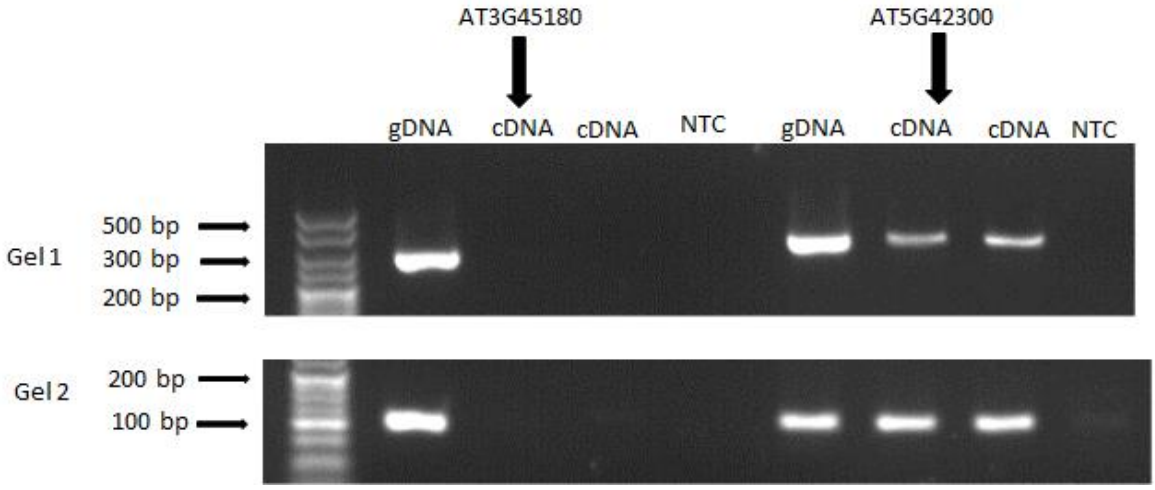


Figure 4-26 Gel images of end point PCR products obtained using the AT3G_Coding_FWD and AT3G_coding _RVS, AT5G_Coding_FWD and AT5G_coding _RVS (Gel 1) and AT3G45180_FWD and AT3G45180_RVS, AT5G42300_FWD and AT5G42300_RVS (Gel 2) using genomic DNA and cDNA as template. No template control (NTC) was included in all reactions. The HyperLadder™ V from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

4.3.13 AtUbl5 mutant screening

Identification of *Ubl5* homozygous mutant of AT5G42300 was not successful by screening seeds (SAIL_682_E03). Whereas identification of homozygous mutants in AT3G45180 (SALK_067245) was successful. The *AtUbl5* mutant screening was carried out using end point PCR using primer pairs shown in (Table 4-11). The lines screened were SALK_126377 (AT3G45180), SALK_067245 and SAIL_682_E03

(AT5G42300). A total of 20 plants from each line were used for screening and the representative images are shown here.

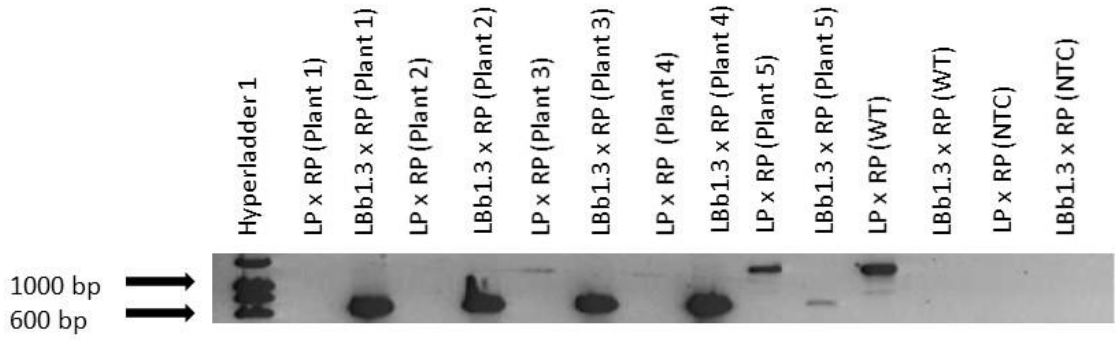


Figure 4-27 Gel images of end point PCR product obtained using the specific LP and RP primers and LBb1.3 and RP primer using genomic DNA from line SALK_126377 as template. Wild type (WT) and no template control (NTC) were included in all reactions as controls. The HyperLadder™ 1 from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder**.**

Figure 4-27 shows no amplification using LP and RP primers in plants 1, 2, 3, and 4 but a strong positive amplification product from plant 1, 2, 3 and 4 using LBb1.3 and RP primer pair. This indicates the insertion of T-DNA in both the alleles of AT3G45180 and thus homozygous mutation for AT3G45180. Plant 5 and wild type did not show a strong amplification using the LBb1.3 and RP primers pair whereas they showed amplification using LP and RP primer pair. This indicated that plant 5 did not have a TDNA insertion at AT3G45180 and thus resulted as wild type. No template control (NTC) showed any amplification products, which indicates no contamination.

A similar reaction was carried out using genomic DNA from SALK-067245 using the specific primers designed for SALK-067245 lines. The reactions failed and a troubleshooting was carried out such as quality of genomic DNA, running end point PCR using housekeeping gene (F-Box shown in Table 4-5). The genomic DNA was found to be of good quality and the end point PCR using *AtF-Box*_qPCR_FWD and reverse primers showed specific amplification product (Results not shown). Therefore the use of this line was suspended.

The SAIL_682_E03 was also analysed using specific LP and RP primers along with LB1 and RP primers (Table 4-11) to identify AT5G42300 mutation status.

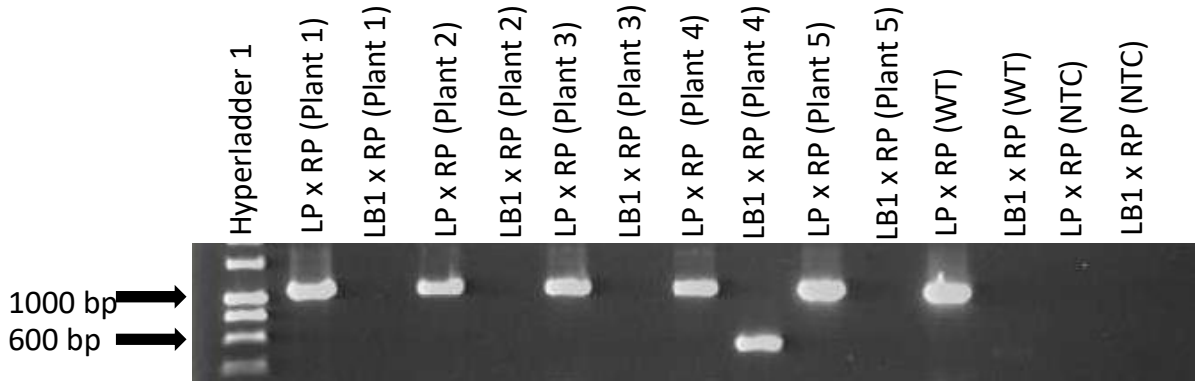


Figure 4-28 Gel images of end point PCR product obtained using the specific LP and RP primers and LB1 and RP primer using genomic DNA from line SAIL_682_E03 as template. Wild type (WT) and no template control (NTC) was included in all reactions as controls. The HyperLadder™ 1 from Bioline (Total Lab Systems Ltd, NZ) was used as DNA ladder.

Figure 4-28 shows amplification from samples of plants 1, 2, 3, 4, 5 and wild type using LP and RP primer pair combinations. Plant 4 also shows amplification using primer combination of LB1 and RP. This result (Table 4-11) indicates that plants 1, 2, 3, and 5 are wild type and plant 4 had T-DNA insertion in one of the alleles of AT5G42300 indicating a heterozygous status. No homozygous mutants for AT5G42300 were identified by screening 20 plants. This led to screening of siliques as a preliminary study to identify any potential embryo lethal phenotype of the *AtUbl5* mutation.

4.3.14 Imaging of siliques

A preliminary analysis showed aborted seed development in the siliques of plants from SAIL_682_E03 seeds lines indicating a potential seed abortion phenotype by mutation of AT5G42300. Figure 4-29 photo A shows discoloration of seeds and photos B and C show a complete absence of seeds. Photo D shows a silique from wild type plants which is completely filled with seeds. Considering this result in conjunction with end point PCR, the result of SAIL_682_E03 (Figure 4 12) indicates a potential embryo lethal phenotype of the AT5G42300 mutation. However, these results are considered only as an indication of potential seed abortion phenotype of AT5G42300 mutation and further studies are required for confirmation. An attempt to generate an AtUbl5 homozygous mutant (AT3G45180 & AT5G42300) by cross pollinating homozygous mutants of AT3G45180 and AT5G42300 failed to obtain a homozygous mutant in AT5G42300. Therefore generating *AtUbl5* mutant and incorporating the generated mutants to study the phenotype of *AtUbl5* by different experiments was concluded at this point

Figure 4-29 Photos of *A. thaliana* siliques collected from SAIL_682_E03 (A,B and C) and wild type (D) obtained using Olympus SZX16 stereo microscope (Olympus, Tokyo, Japan) with Olympus DP71 digital camera (Olympus, Tokyo, Japan) at Biotron, Lincoln University.

4.4 Discussion

This discussion is in two parts. The first part, "Outcome of drought study" explains the results of the drought study using *Arabidopsis* lines transformed with *LpUbl5* driven by *CaMV* 35S promoter in comparison with wild type. The second part "Information of *AtUbl5* in *Arabidopsis*", explains findings of the *AtUbl5* gene.

Outcome of drought study

A successful generation of pARTB_GW:*LpUbl5* vector, transformation and development of T_3 generation of transformed *Arabidopsis* lines carrying pARTB_GW:*LpUbl5* vector was achieved. *LpUbl5* gene (222 bp) was successfully amplified using specific primer pairs (Table 4-1) which were then cloned into an entry clone to generate a binary vector. The binary vector carrying complete open reading frame (ORF) of the *LpUbl5* gene (222 bp) was used for the *Arabidopsis* transformation procedures via the *Agrobacterium* mediated floral dip transformation method (Weigel and Glazebrook, 2006). End point PCR using specific primer pairs (Table 4-4) was employed to confirm the presence of T-DNA in the selected 11 transformed *Arabidopsis* plants lines (Figure 4-10). The amplification of 222 bp *LpUbl5* gene for the development of pARTB_GW:*LpUbl5* vector was important as the binary vector, pCORF135

plant transformation vector used for the ryegrass transformation (Chapter 3) carried a 464 bp sequence which include 222 bp *LpUbl5* gene. Therefore, pARTB_GW:*LpUbl5* vector carried only the 222 bp ORF without the interference of any untranslated region in the T-DNA.

The selection of transformed lines for physiological screening of drought tolerance was based on the level of *LpUbl5* transcript abundance to include a range of transcript abundance to correlate the plants performance with the transcript abundance. The *LpUbl5* transcript abundance in transformed *A.thaliana* in Figure 4-11 shows the level of transcript abundance in each line under the *CAMV 35S* promoter. The results showed different levels of *LpUbl5* transcript abundance in each line. The lines selected for further analysis based on the transcript abundance were T3/1, T3/35, T3/41 and T3/43. The selection process aimed to include different levels of *LpUbl5* transcripts to see if these were related to the performance of plants under drought. Among the selected lines T3/35 had highest *LpUbl5* transcript abundance level and the lowest transcript abundance was exhibited by T3/41. This was aimed to relate to physiological performance of each line and quantify the plant performance under drought in comparison with transgene (*LpUbl5*) transcript abundance.

Initial screening of drought tolerance was carried out including 11 lines of transformed *A. thaliana* regardless of the transcript abundance. It showed no difference in performance under drought but indicated an increase in the number of leaves and leaf length per plants. Specifically each of the 11 transformed lines was screened in comparison with its nearest spaced wild type plant. The screening did not show any variation of performance under drought between transformed *A.thaliana* lines and wild type (Figure 4-12). These experimental plants indicated that lines, T3/15, T3/19, T3/35, T3/41 and T3/43 had differences in number of leaves and lines T3/15 T3/19, T3/31, T3/35, T3/41, and T3/43 had differences in leaf length when compared with nearest spaced wild type. However, this result should be treated with caution because the seeds used to grow the wild type plants were older compared with seeds of the transformed lines. This could also be due to difference in germination rate which unfortunately were not recorded.

To investigate this result a repetition of number of leaves per plant in a newly established experiment was not show an increase in number of leaves in transformed lines of *Arabidopsis* when compared with wild type plants generated from new set of seeds. A second experiment was set up with selected lines (T3/1, T3/35, T3/41 and T3/43. These lines did not show an increased number of leaves when compared with wild type plants (Figure 4-15). This suggests that difference observed from the initial experiment (Table 4-12 and Table 4-13) was probably due to the difference in seed age and difference in seed germination.

Data generated from biomass yield of *LpUbl5* transformed *Arabidopsis* lines in comparison with wild type *Arabidopsis* did not support the potential role of *LpUbl5* in drought tolerance. The biomass results

shown in Figure 4-14 showed no difference in biomass yield under drought and well watered conditions between transformed lines and wild type Arabidopsis. This shows that over-expression of the *LpUbl5* gene did not confer a high biomass yield under drought or well watered conditions. Consistently, the results from leaf number (Figure 4-15) also indicated a similar result with line and treatment.

The RWC (Figure 4-16) in wild type *Arabidopsis* was different between well watered and water withdrawn wild type *Arabidopsis* and the osmotic potential (Figure 4-17) were not different. Whereas both the RWC and osmotic potential was not different between water treatments in over expression lines of *LpUbl5* gene. This indicate a minor contribution to increased RWC in leaves under water withdrawn conditions for the modified lines but the magnitude of change was minor. Given this finding was not supported by other results, such as biomass yield, performance under osmotic stress, rate of water loss and over all morphological appearance under water withdrawn conditions, its practical importance appears limited.

An enhancement of root growth was observed when *Arabidopsis* seeds germinated in 100 mM mannitol. The root length (Figure 4-18) and shoot biomass (Figure 4-19) indicate that the over expression of *LpUbl5* in line T3/41 and T3/43 did not improve root length or shoot biomass under osmotic stress conditions when compared with wild type. The results of root length (Figure 4-18) indicated a different pattern of results where 100 mM mannitol containing MS medium produced longer roots when compared with roots in MS medium without mannitol addition. Studies usually replant the one week old or 5 day old seedlings into different concentrations of mannitol containing MS medium to measure root length responses (Xiong *et al.*, 2006; Pandey *et al.*, 2013). Whereas, in this study, the seeds were sown directly into different concentrations of mannitol to avoid any damage and stress caused by replanting. This resulted in the longest roots in 100 mM mannitol compared with MS medium without addition of mannitol. Lines T3/41 and T3/43 showed similar results in root length elongation when compared with the wild type. The shoot biomass (Figure 4-19) showed similar results to previous reports (Pandey *et al.*, 2013) with a decreased shoot biomass in increasing mannitol concentrations. A comparison of transplanted one week old seedlings and seeds sown was used to cross check the pattern of root length (Figure 4-23). This produced similar pattern of root growth as in Pandey *et al.* (2013) and Xiong *et al.* (2006). The shoot biomass and the photographic representation (Figure 4-23) of transplanted seedlings into ½ MS media containing 100 mM mannitol also confirmed that these media preparation procedures were correct.

Wild type plants and transformed lines performed equally under water withdrawn conditions. Drought studies often analyse drought tolerance by visual inspection of plant performance under drought and recovery (Nelson *et al.*, 2007; Yu *et al.*, 2008; Yoo *et al.*, 2010). The current study also incorporated

visual evaluation of 11 lines with nearest spaced wild type plants. Transformed lines did not exhibit any obvious tolerance to drought in daily visual examinations when compared with wild type plants under drought, or show evidence of greater recovery response (Figure 4-12). Survival rate of transformed lines and wild type were not recorded because of the lack of visual evidence for the difference between the lines. The visual evaluation of the morphological appearance of wild type and transformed *Arabidopsis* under water withdrawn conditions indicated that these lines were not different with increased expression of *LpUbl5*.

The rate of transpiration water loss was not different in transformed and wild type *Arabidopsis* plants. The rate of water loss (Figure 4-13) shows that increased levels of *LpUbl5* did not reduce transpiration water loss when compared with wild type plants. A water loss experiment is one of the main methods used to understand a reduction in transpiration water loss which is an important factor that contributes to drought tolerance. It can also be used as an indication of reduced stomatal density (Yu *et al.*, 2008; Jiang *et al.*, 2012; Yu *et al.*, 2013a). Diffusion of CO_2 through stomatal openings also results in evaporation and water loss. This water loss triggers root water uptake which fails in case of de-rooted plants. This in turn leads to a reduction in stomatal density or partial closure of stomata resulting in limited CO_2 uptake as the soil water is limiting (Gollan *et al.*, 1985; Socias *et al.*, 1997; Des Marais *et al.*, 2014). The lack of measured differences between wild type and transformed plants further indicate that *LpUbl5* has not conferred drought tolerance on *Arabidopsis*.

Implications of plant water status is an important factor determining the drought performance of plants. Drought tolerance studies and water loss experiments often include osmotic stress experiments using *A.thaliana* as model plants for drought tolerance (Liu *et al.*, 1998; Umezawa *et al.*, 2004; Ko *et al.*, 2006; Xiong *et al.*, 2006; Catala *et al.*, 2007; Yu *et al.*, 2008; Ren *et al.*, 2010; Jiang *et al.*, 2012; Bu *et al.*, 2014; Cha *et al.*, 2015). Plant water relationship studies such as RWC and solute potential were analysed as an indication of water status and a reliable measurement to understand the water status of the plants tissue (Schonfeld *et al.*, 1988; Yoo *et al.*, 2010). This informs the value of these experiments in the identification process of genes involved in any drought tolerance mechanism. This study incorporated drought tolerance screening, a water loss experiment, RWC measurement, osmotic potential and osmotic stress experiment using different concentrations of mannitol. This was used to identify any potential involvement of *LpUbl5* under water deficit conditions to improve the water status of the plants when expressed under a constitutive promoter when compared with well watered controls (Yoo *et al.*, 2010). These failed to expose any potential role of *LpUbl5* gene in drought tolerance mechanism. Ten to 14 days of drought stress is the method integrated in most drought studies to differentiate drought tolerant plants via physiological measurements (Taji *et al.*, 2002; Ko *et al.*, 2006; Peng *et al.*, 2007; Jiang *et al.*, 2012; Pandey *et al.*, 2013; Cha *et al.*, 2015). This study imposed complete water withdrawal for 14 days which did not show visual signs of pronounced wilting in water

withdrawn plants in comparison with well watered plants. However, RWC in Figure 4-16 showed a difference (P <0.001) between treatments which indicate that plants were under drought. This also confirms the methods involved were sufficient to identify any potential involvement or enhancement of *LpUbl5* in drought tolerance mechanism.

The *AtUbl5* transcript abundance in *Arabidopsis* was not affected under progressive drought by imposed water withdrawal. *AtUbl5* transcript abundance was carried out under progressive drought where water was withdrawn and the control plants under regular watering regime. The results (Figure 4-24) showed only a difference (P <0.001) in pot weight (Appendix C.11), whereas the transcript abundance level was not correlated with a reduction in pot weight indicating the severity of drought stress by water withdrawal. This study has incorporated samples from well watered control plants for the equal comparison of the transcript abundance of water withdrawn plants. This allowed a comparison of transcript abundance data of plants under drought stress with well watered plants in which they showed no difference (P≥0.15) at each of three time points under progressive drought stress. Gene expression level shown in ryegrass after eight days of stress showed a two to three fold increase in gene *LpUbl5* gene expression under drought when compared with samples collected before stress. This increase in gene expression after stress compared with before stress was interpreted as an indication of a physiological role of *LpUbl5* under drought stress. Authors did not include a well-watered control to compare the gene expression of drought stressed plants (Patel *et al.*, 2015). Literature indicates the importance of well watered controls or unstressed control samples for comparison of gene expression studied (Uno *et al.*, 2000; Catala *et al.*, 2007). The comparison of transcript abundance data and the pot weight (Figure 4-24) indicating the water withdrawal status with well watered control plants gives confidence in accepting these results.

Information of *AtUbl5* in *Arabidopsis*

The background study shown that *Arabidopsis* has two *Ubl5 (AtUbl5)* genes. Initially, the "Arabidopsis Information Resource" (TAIR) was browsed to find *AtUbl5* gene information. Two *AtUbl5* genes were identified as present on Chromosome 3 (AT3G45180) and Chromosome 5 (AT5G42300). The AtUBL5 protein sequences were retrieved from TAIR. A ClustalW alignment with LpUBL5, ryegrass protein sequence, from the NCBI database showed a 94.5% sequence similarity (Table 4-15) and is evolutionarily highly conserved across species (Wilkinson *et al.*, 2004; Ammon *et al.*, 2014).

The results (Figure 4-26) showed that a single *AtUbl5* gene is expressed in *Arabidopsis* and a homozygous mutation of this gene leads to a potential embryo lethal phenotype. Specific primer pairs were designed to amplify the two *AtUbl5* genes (AT3G45180 and AT5G42300) in *Arabidopsis*. The result observed (Figure 4-26) was consistent with the statement by Vierstra (2012), At5g42300 was strongly expressed and At3g45180 is called as a pseudogene. However, a pseudogene listing by Yang

et al. (2011) did not include At3g45180 as pseudogene along with 1939 classified pseudogenes in *Arabidopsis*. Further, RNA expression using high resolution genome tilling arrays have detected both At3g45180 and At5g42300 (Stolc *et al.*, 2005). Consistently, drought stress related studies also listed At3g45180 in their results indicating evidence of the expression of At3g45180 in Arabidopsis (Nishiyama *et al.*, 2013; Van Ha *et al.*, 2014; Rasheed *et al.*, 2016). The results of these studies (Nishiyama *et al.*, 2013; Van Ha *et al.*, 2014) showed that mean of the normalized intensity (Log2) was negative values (-7.5 and -8.2) indicating low level expression compared with a control. However, this transcriptional activity can hardly considered as criteria for gene function and exclude At3g45180 from a pseudogene categorisation. Further, the efforts to assign non-functionality to pseudogenes is considered impractical (Podlaha and Zhang, 2010). Johnson *et al.* (2005) have categorised the expressed pseudogenes as dark matter transcription which still needs to be explained. Whole genome array of *Arabidopsis* have detected expression of approximately 20% of the 1332 annotated non-expressed genes and they have listed both At3g45180 and At5g42300 in the mRNA sample (Yamada *et al.*, 2003). It is possible that At3g45180 is a pseudogene and the mutation does not impart any observable phenotype or the effect is too subtle to observe (Podlaha and Zhang, 2010). Collectively, At3g45180 can be potentially a pseudogene which is transcribed at low level and undetectable by an end point PCR method.

Subsequently, mutant lines were also identified and ordered from the Arabidopsis Biological Resource Center (ABRC) to generate double mutants of *AtUbl5* genes (AT3G45180 and AT5G42300). The study of Arabidopsis *Ubl5* genes *AtUbl5* (AT3G45280 and AT5G42300) showed (Figure 4-26) AT3G45180 was not detected whereas AT5G42300 was detected. Consistently, the result from screening of mutants (Figure 4-27and Figure 4-28) showed homozygous mutation in At3g45180 whereas the At5g42300 did not produce homozygous mutation. In support of this idea, analysis of siliques from SAIL_682_E03 (Figure 4-29) showed an absence of seed filling which can be due to a potential seed abortion phenotype. It is also possible that the homozygous mutation of At3g45180 did not impart a lethal effect due to the high level of expression of At5g42300 complimented the mutation of At3g45180. Collectively, these results (Figure 4-26, Figure 4-27, Figure 4-28 and Figure 4-29) indicate that AT5G42300 serves an important role which is vital to the plant and needs to be explored.

Ubl5 mutants have been studied in humans using RNA interference (RNAi) and identified *Ubl5* as an essential gene for viability of human cell lines. Apoptosis was caused by the segmented nuclei due to chromosome mis-segregation and loss nuclear integrity in Ubl5 SiRNA treated cells after 72 hours (Ammon *et al.*, 2014). Whereas in *S.cerevisiae Ubl5* was identified as not essential for viability (Mishra *et al.*, 2011). Studies in *S.pombe*, *Ubl5* identified as essential for mitotic growth and the loss of *Ubl5* function showed defective cell cycle progression (Wilkinson *et al.*, 2004; Yashiroda and Tanaka, 2004). *Ubl5* inactivation via RNAi has been shown to be associated with decreased expression of UPR^{mt}

reporter genes, such as *hsp*-6 and *hsp*-60 in *C.elegans* (Benedetti *et al.*, 2006). These studies show that the inactivation of *Ubl5* plays a vital role in each species and therefore suggest *Ubl5* mutations can lead to lethal phenotypes in *A.thaliana*. This assumption is supported by the nature of the Ubl5 protein which is highly conserved across species (Wilkinson *et al.*, 2004) which suggests that mutation or loss function of *Ubl5* has serious effects on each species, including *Arabidopsis*.

Conclusion

Previous overexpression of *LpUbl5* gene suggested a potential involvement in drought tolerance (Patel *et al.*, 2015) but attempts in ryegrass to validate the gene function did not provide clear evidence for this role (Chapter 3). Given the highly identical protein sequence (94.52%) Table 4-15 and Figure 4-25) of ryegrass and *Arabidopsis*, we would expect the *LpUbl5* gene to act similarly in *Arabidopsis*. However, the experiments designed and performed in *Arabidopsis* did not exhibit any potential drought tolerance and accepted the null hypothesis "drought tolerance in Arabidopsis is not affected by overexpression of *LpUbl5*". Collectively, the potential role of *LpUbl5* in drought tolerance was not supported by the results generated using the *A.thaliana* transformed using *LpUbl5.* This research has identified *AtUbl5* is serving a potentially vital role in plants, as indicated by the results from mutant screening. Although this initial hypothesis needs further study to identify the potential embryo lethal phenotype and the potential vital role of *Ubl5* in plants, the collective results do not support any role of *Ubl5* in drought tolerance.

Chapter 5

Sub-cellular localization of *Lolium perenne* L. Ubiquitin like protein 5

5.1 Introduction

The HUB1/UBL5 homologues in yeast and humans is involved in alternative splicing of mRNA (Mishra *et al.*, 2011; Ammon *et al.*, 2014). HUB1/UBL5 homologues exhibit a high sequence homology across species including in perennial ryegrass. Patel *et al.* (2015) suggested transcriptional responses in perennial ryegrass indicated a role for the *Lpubl5* in drought stress response. However, whether the drought stress response is due to alternative splicing of mRNA is unknown. To participate in alternative spicing, *Lp*UBL5 needs to localise in the nucleus. The analytical results obtained from the prediction tools for protein localization in plants were inconsistent and therefore we designed a biological experiment to determine sub-cellular localization of *Lp*UBL5. The ideal method for localization of protein was immunofluorescence which is highly specific in cells and tissues (Vitha and Osteryoung, 2011). However, immunofluorescence was not chosen due to the lack of resources and unavailability of a specific antibody to *Lp*UBL5. Therefore, our preferred method for sub-cellular localization was *Agrobacterium*-mediated transient transformation of *Nicotiana benthamiana* leaves or biolistic bombardment of onion epidermal cells with gold particles coated with gene expression cassettes or plasmids (Collings *et al.*, 2003; Kokkirala *et al.*, 2010).

The null hypothesis was "*Lp*UBL5 is not localized to the nucleus"- rejecting the potential involvement of *Lp*UBL5 in alternative splicing.

The final binary Vector *pB7_FWG2:LpUbl5* was constructed using the *LpUbl5* amplified from cDNA, cloned into an entry vector. Then *pB7_FWG2:LpUbl5* expression vector was generated using the entry clone destination vector via LR Clonase reaction. Resultant LR reaction mix was used to transform *E.coli.* The plasmids were isolated and used to transform *Agrobacterium* by electroporation to transiently transform *N. benthamiana* leaves. The isolated plasmids were also coated onto gold particles for biolistic bombardment of *Allium cepa* L (onion) epidermal cells. Confocal microscopy was used to determine the sub-cellular localization in *N. benthamiana* leaves and onion epidermal cells. This chapter describes the sub-cellular localization procedure and results.

5.2 Materials and methods

5.3 Sub-cellular localization of *Lp*UBL5

Transient transformation of *Nicotiana benthamiana* leaves and onion epidermal cells was acquired using the green fluorescent protein (GFP) fused to the carboxy terminus of the target proteins. This is

a powerful tool used to monitor gene function, protein-protein interaction and protein localization (Cinelli *et al.*, 2000). GFP is a naturally fluorescent protein in the jellyfish *Aequorea Victoria* and GFP is able to fold into the fluorescent structure without the need for any unique factor from jellyfish and can therefore be used in different cell types (Patterson *et al.*, 1997). GFP could be seen within the intact tissue which is highly desirable in the case of plant tissue due to its impermeability to different stains and dyes (Hanson and Köhler, 2001).

Gateway expression vector pB7_FWG2 (Karimi *et al.*, 2002) carrying *CaMV 35S* as a gene promoter and containing a C-terminal GFP fusion was used to express *LpUbl5* and study the subcellular localization of *Lp*UBL5 protein in *N. benthamiana* leaves. The method followed is described in detail in the following sections.

5.3.1 Cloning LpUBL5 in pENTR™/D-TOPO®

To clone *LpUbl5* into an entry vector, *LpUbl5* from *Lolium perenne* L was amplified by KAPA HiFi Hotstart PCR protocol (Appendix: A.2) using the primers given in Table 5-1.

Table 5-1 The primers used for amplification of *LpUbl5* without the end codon for cloning into entry clone to generate a plasmid carrying *LpUbl5*

Name	Sequences	Amplicon
*LpUbl5*_FWD	5' CACCATGATCGAGGTGGTGCTCAAC 3'	
LpUbl5+TAA_ RVS	5' CTAGTTGTAGTAGAGCTCGAGTCC 3'	226 bp

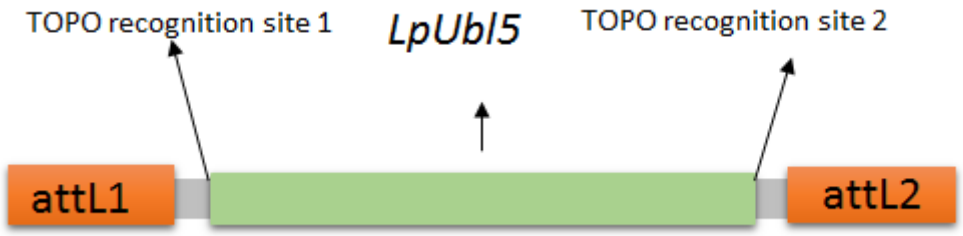


Figure 5-1 The schematic representation of pENTR/D_TOPO:*LpUbl5* explained in Section 5.3.1. *LpUbl5* was cloned without the end codon. The forward primer that designed with the addition of CACC to the 5' end and along with a reverse primer was used to carry out amplification of *LpUbl5* for directional cloning.

5.3.2 Transformation of DH5α cells

E.coli DH5α competent cells were transformed as explained in Section 4.2.2

5.3.3 Screening for positive colonies

The colonies were screened as explained in Section 4.2.3. The primers used for endpoint PCR were *M13*-FWD and *LpUbl5-TAA* reverse given in Table 5-2.

Table 5-2 The primers used for carrying out colony PCR of *E.coli* colonies to identify the transformed colonies to isolate plasmids possessing *LpUbl5* from corresponding colonies carrying *LpUbl5* gene.

Primer	Sequences	Amplicon
M13-FWD	5' GTAAAACGACGGCCAG 3'	
LpHUB1-TAA Reverse	5' GTTGTAGTAGAGCTCGAGTCC 3'	371 bp

The screened colonies were also inoculated into 3 mL LB medium (Appendix B.2) containing 3 µL of Kanamycin (50 µg/mL) as the selective agent and incubated overnight at 37^{o}C at 250 RPM.

5.3.4 Cloning *LpUbl5* into Expression vector using Gateway® Cloning Technology

The expression vector *pB7_ FWG2* which has a C-terminal GFP was used to clone the *LpUbl5* to carry out the subcellular localization study as explained in Section 4.2.4. Colony PCR (Appendix A.2) was carried out to identify transformed colonies using the *pB7_FGW2* forward and *pB7_FWG2* reverse primers given in Table 5-3.

Table 5-3 The primers used for colony PCR of transformed E.coli cells using *pB7FGW2::LpUbl5* binary vector

Name	Sequences	Amplicon
pB7_WG2_FWD	5' CGCCGGACACGCTGAACTTG 3'	
pB7_WG2_RVS	5' TCCAACCACGTCTTCAAAGCAA 3'	634 bp

Identification and confirmation of positive colonies were carried out as explained in Section 4.2.4

5.3.5 Transformation of *Agrobacterium tumefaciens*

The *pB7_FWG2:LpUbl5* was used to transform *A. tumefaciens* by the electroporation method (Appendix B.9). The resultant reaction was plated on to LB medium containing spectinomycin (50 µg/mL) as a selective agent and the plates were incubated at 28°C for two days. Colony PCR was carried out using the *pB7_FWG2* forward and reverse primers described in Table 5-3, to amplify the positive colonies and the result was confirmed by loading the PCR products on to 1% agarose gel.

Figure 5-2 The schematic representation of T-DNA, *pB7FWG2:LpUbl5* generated in Section 1.2.6 used for transient transformation of *N. benthamiana* plant tissue to determine the sub-cellular localization of *Lp*UBL5.

5.3.6 Preparation of the positive control

A positive control was chosen to validate the efficiency of transient transformation. This control was also included as a positive control for the cytoplasmic targeted protein. The binary expression vector *pB:35S-3gfp* was provided by Darrell Lizamore (Lizamore, 2013). This vector contains three GFP open reading frame driven by *35S CAMV* promoter inserted into the *pART27* multiple cloning site by Gateway cloning via *pENTR1A*. The three GFP construct was expected to be localized in the cytoplasm and included as a cytoplasmic targeted protein.

5.3.7 Tobacco transformation protocol

To carry out the transient transformation of tobacco plant, single colony *of A. tumefaciens* was suspended in 3 mL LB medium containing 50 µg/mL spectinomycin and incubated at 28^{o}C, at 250 RPM for two days. After two days, 50 mL LB medium containing the same concentration of spectinomycin was used in the inoculum culture was inoculated with 100 µL of the two days old culture. The culture was then incubated in the same conditions for four to five hours to reach log phase or exponential phase of bacterial growth (OD_{600} = 0.8-1.0).

Then the cells were harvested and re-suspended in re-suspension (Appendix B.2) medium to a density of OD_{600} = 0.2. The re-suspended culture was incubated at 28^{o}C, at 250 RPM for a minimum of 3 hours. The culture was then gently infiltrated at the bottom/ventral part) of *N. benthamiana* leaves with an approximate size of 3 X 1.5 cm for best results (Kokkirala *et al.*, 2010).

5.4 Transient transformation of onion epidermal cells

It was important to validate the result obtained from the dicot *N. benthamiana* leaves in a monocot system perennial ryegrass is a monocot plant. Onion epidermal cells are used as a model system to study the cellular structures. They are ideal for transient gene expression to study the subcellular localization of GFP tagged proteins. (Scott A1 *et al.*, 1999). Biolistic transformation of onion epidermis has become the preferred method for determining the subcellular localization of protein in monocot background. Biolistic rather than Agrobacterium is used because of the poor transformation efficiencies delivered by agrobacterium based methods in monocots (Eady, 1995). This adds

confidence to our results being relevant to perennial ryegrass. Biolistic bombardment of onion epidermal cells was carried out using the same binary vector that used for tobacco transformation purpose, *pB7_FWG2:LpUbl5*. Control vectors used were *pCAMBIA1304GUS::GFP*, *PBI121:: mgfp4-ER* to transform the onion epidermal cells (Hollender and Liu, 2010). The transformation of onion epidermal cells and confocal microscopy was carried out at Biomolecular Interaction Centre, University of Canterbury, NZ under the supervision Dr. David Collings.

5.4.1 Selected plasmids

Different plasmids were included as controls along with *pB7_FWG2::LpUbl5* to validate the results. Control plasmids were *GFP-GUS (pCAMBIA1304)*, mgfp4-ER and a nuclear targeted protein (Dr John Gardiner, Sydney University). *pCAMBIA1304GUS::gfp* was a 96 kDa fusion protein. *pCAMBIA1304GUS::gfp* localises in cytoplasm (Lechner *et al.*, 2012). *mgfp4-ER* which comprises an N terminal signal peptide derived from an *Arabidopsis* vacuolar basic chitinase and C terminal HDEL sequence cloned into *pBI121* vector (*pBI121: mgfp4-ER*) (Haseloff *et al.*, 1997). The constructs used as controls (*pCAMBIA1304GUS::gfp* and *pBI121: mgfp4-ER*) were gifted by Dr. David Collings, Biomolecular interaction centre, Canterbury University. A nuclear targeted protein was also included as a control which as donated by Dr John Gardiner, Sydney University, Australia to Dr. David Collings. This nuclear targeted protein was received from Dr. David Collings with gratitude and used as control in this study.

5.4.2 Preparation of gold particles

Gold particles were prepared ready for coating with plasmids to be used in the transformation procedure. 50 mg of gold particles were weighed and placed in a microfuge tube. The gold particles was re-suspended in 1 mL of sterile distilled water by vortexing the microfuge tube for 20 seconds. Then the microfuge tube was centrifuged at 13000 RPM for 1 min and the supernatant was discarded. The gold particles were then re-suspend the gold particles in 1 ml of 100% ethanol by vortexing for 20 seconds. Then the tubes were centrifuged at 13000 rpm for 1 min and the supernatant was discarded. The washing step using ethanol was repeated twice. Then the gold particles were re-suspended in sterile distilled water by vortexing for a further 20 seconds. Finally, 25 µl of gold particles were aliquoted into screw cap tubes and the particles were re-suspended after each aliquot to make sure an even number of particles were transferred into each tube. The aliquots were stored at -20^{o}C until use.

5.4.3 Coating plasmid DNA into gold particles

Plasmids were coated onto gold particles for the biolistic bombardment. Required number of tubes with gold particles was removed from -20^{o}C for each construct. Then the particles were re-suspended

by vortexing for 20 sec and 5 µl of plasmid DNA at a concentration of 200-250 ng/µl was added. The gold particles and plasmid DNA were mixed by pipetting up and down and continued mixing by vortexing for 20 seconds. 25 µl of 2.5M $CaCl_2$ was added to the tubes and mixed by pipetting and vortexing. 10 µl of 0.1 M spermidine was added to the tubes and mixed as above. Then the tubes were vortexed for 3 min to ensure complete mixing. Tubes were briefly centrifuged to pellet the particles and the supernatant was discarded. The pellets were re-suspended in 180 µl of 100% ethanol each. Then particles were spun down and re-suspended in 100 µl of 100% ethanol which were subsequently used for onion epidermal cell transformation (Collings *et al.*, 2002).

5.4.4 Preparation of onion epidermal cells for transformation

Red or white onion can be chosen for transformation and red onion was used here. The outer layers of onion were peeled off. Then the whole onion was cut into four equal halves. Then the different layers of onion were separated and approximately 2-3 cm wide onion slices which looked healthy were chosen and placed onto petri-dishes with moistened tissues and used for transformation.

5.4.5 Transient transformation by particle bombardment

Biolistic® PDS-1000/He Particle Delivery System, (model PDS 1000, Bio-Rad Laboratories Pty Ltd, Auckland, NZ) with 1100psi rupture disc was set up prior to the transformation procedure. This set up was used inside a biosafety cabinet for transformation to ensure sterile condition. The particle bombardment system was connected to a vacuum pump and helium cylinder. Then the helium cylinder was turned on until the upper scale reading reached to 60 psi. A 10 µl of gold particles coated (Section 1.3.2) with plasmid DNA in 100% ethanol was added to a 13 mm Swinnex filter holders (Millipore, USA). The filter was dried briefly for 20 seconds at room temperature and attached to the upper port inside the chamber.

A petri-dish of 90 mm diameter with a moistened tissue paper was placed on the middle shelf of the chamber which is 10 cm beneath the nozzle of Swinnex filter holders. The sliced red onion was placed on the petri-dish at the middle shelf facing the curving side facing the Swinnex filter and the chamber was closed. The onion epidermis was bombarded at 350-400 kPa of helium gas and under 500 mm hg of vacuum. This can be repeated to ensure complete removal of gold particles from the Swinnex filter (Collings *et al.*, 2002).

The transformed onion tissue was placed onto a petri-dish. The onion epidermal cells were placed onto the moistened tissue paper and covered with moistened tissue paper. Then the petri-dishes were incubated at room temperature overnight. Then each construct was used to transform the onion epidermal cells with new Swinnex filters to avoid cross contamination. The epidermal peeling was carried out after screening the whole onion slices using a Stereo Research Microscope (Olympus Model

SZX16). Then the peeled epidermal peels were mounted onto glass slides using water and imaged using a confocal microscope.

5.5 Confocal microscopy

Confocal microscopy was the method of choice to visualise the subcellular localization of LpUBL5 and was carried out at Biomolecular Interaction Centre (BIC) at the University of Canterbury. The transient transformation of *N. benthamiana* leaves were carried out at Lincoln University. Transformed leaves from tobacco plants were excised from the tobacco plants. The excised leaves were placed into the petri-dishes prepared with a wet filter paper. The petri-dishes were closed to maintain the moisture for transfer. The Labelled petri-dishes were transferred to University of Canterbury with the transfer permit CL6626 under the applicable HSNO approval number GMD100510 from the Lincoln University plant growth room RFH026.

Transient transformation of onion epidermal cells were carried out at University of Canterbury. The onion epidermal peels were prepared and confocal imaging was carried out.

The leaves were screened for the presence of GFP using a Leica MZ10F stereo fluorescence microscope (SP5 system; Leica, Wetzlar, Germany). Then the leaves were sectioned and mounted on a glass slide using water. Leaves were examined under the confocal microscope (SP2 system; Leica, Wetzlar, Germany) and Images obtained (Collings *et al.*, 2003). GFP localization in tobacco leaf cells and onion epidermal cells was carried out using an argon 488 nm laser excitation with complimentary GFP emission band of 500-550 nm filter. The Images were taken with 40 X objective lens with four fold line averaging and time series Images were taken to evaluate the streaming of cells. The observation of streaming within the cells is the evidence of activities of living cells which indicates that the cells were live and dynamic (Haseloff, 1998).

5.6 Results

5.6.1 Result from transient transformation of N*. benthamiana*

5.6.1..1 pB7_FWG2:LpUbl5

Transiently transformed *N. benthamiana* leaves using *pB7_FWG2:LpUbl5* were imaged using confocal microscopy. Figure 5-3 shows the confocal image of *N. benthamiana* leaves using green fluorescence in Panel A, chlorophyll in Panel B and white light in panel C. The chlorophyll image indicates that the cells are alive while obtaining the images whereas the white light image indicates the integrity of cell structures.

Panel A shows the green fluorescence image of *Lp*UBL5 in both cytoplasm and nucleus of *N. benthamiana* leaves. Viability of cells was determined by judging the cytosolic content streaming as described above.

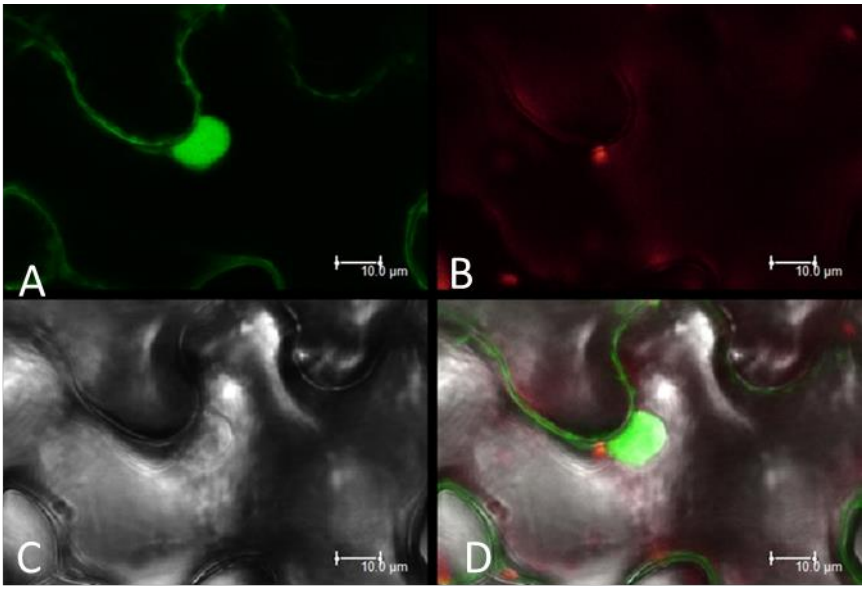


Figure 5-3 Confocal images of *N. benthamiana* leaves showing cytoplasmic and nuclear localization of *Lp*UBL5 protein (Panel A) chlorophyll fluorescence (Panel B), white light image (Panel C), and merged image of Panel A, Panel B and Panel C (Panel D).

5.6.1..2 Cytoplasmic protein

The Image from *N. benthamiana* leaves transiently transformed using *pB:35S-3gfp* was imaged as a cytoplasmic targeted protein and validation of transformation procedure. Panel A in Figure 5-5 shows the cytoplasmic localization of *pB:35S-3gfp.*The chlorophyll and white light Images of the leaf were also obtained. The streaming of cells was monitored to evaluate that cells were live and dynamic to make sure the viability of cells while imaging (Images not shown).

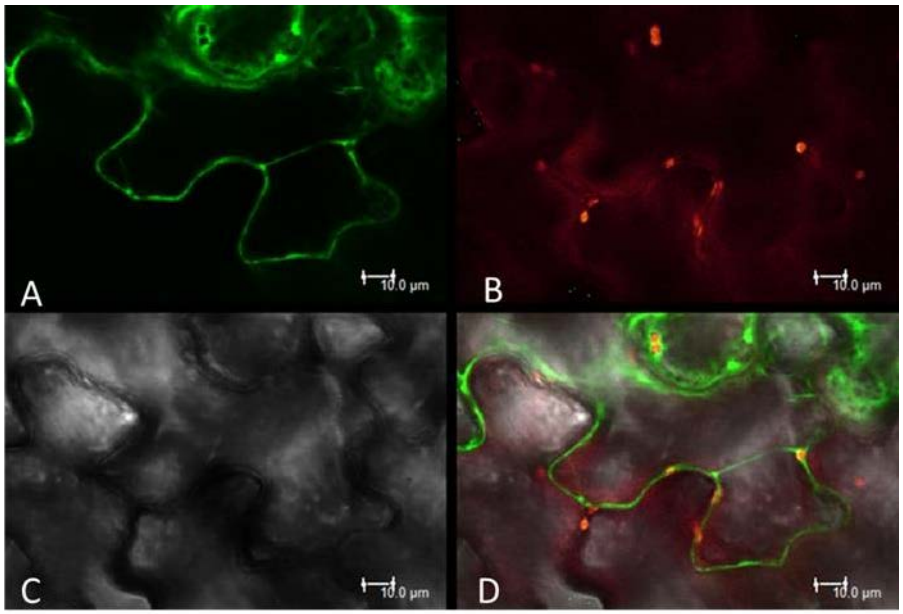


Figure 5-4 Confocal images of *N. benthamiana* leaves showing cytoplasmic localization of *pB:35S-3gfp* (Panel A) chlorophyll fluorescence (Panel B), white light image (Panel C), and merged image of Panel A, Panel B and Panel C (Panel D).

5.6.1..3 Wild type N. benthamiana

The image of wild type *N. benthamiana* leaves was used as a control as wild type tobacco leaves do not possess any fluorescence. Figure 5-5 shows the confocal image of wild type *N. benthamiana* leaves using green Panel, chlorophyll and white light images. The chlorophyll image (Panel B) indicates that the cells were alive and the white light image in (Panel C) indicates integrity of the cellular structures. The green Panel A does not show any green fluorescence which show the absence of green fluorescence in wild type *N. benthamiana* leaves. The merged image in the Panel D helps to visualize the different Panels in one image.

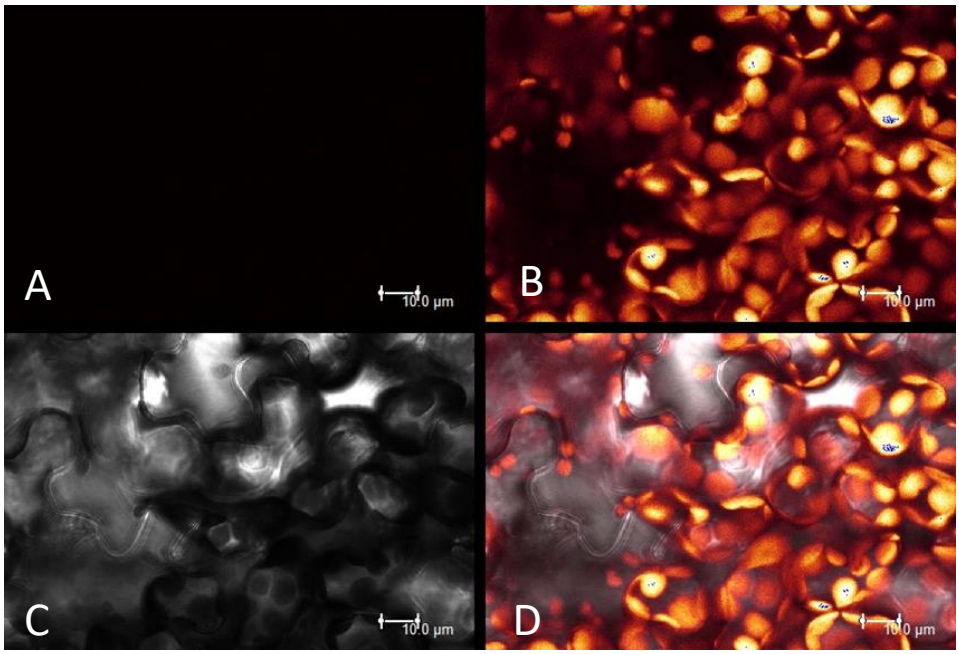


Figure 5-5 Confocal Images of wild type *N. benthamiana* leaves showing no GFP (Panel A) chlorophyll fluorescence (Panel B), the white light Image (Panel C), and the merged image of Panel A, Panel B and Panel C (Panel D).

5.6.2 Transient transformation of onion epidermal cells

5.6.2..1 pB7FWG2:LpUbl5

The transient transformation of onion epidermal cells using *pB_7FWG2:LpUbl5* shows the sub-cellular localization of *Lp*UBL5 (Figure 5-6). The Panel A shows the *Lp*UBL5 green fluorescent protein in both cytoplasm and nucleus. The Panel B shows same cell structures under the white light where GFP cannot be detected. The Panel C shows that the GFP is present in both cytoplasm and nucleus of the transformed cells. Time series images were obtained to ascertain streaming in the cells, indicating cells were live and dynamic (Images not shown).

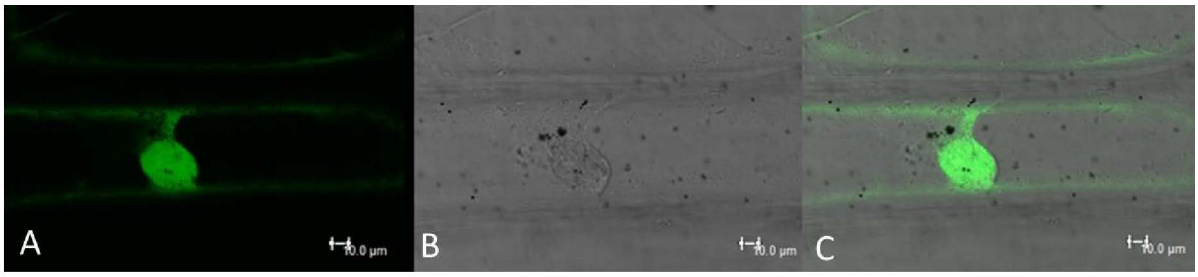


Figure 5-6 Confocal image of in onion epidermal cells *pB7_FWG2:LpUbl5* in both cytoplasm and nucleus (Panel A), white light Image (Panel B) and merged image of Panel A, Panel B (Panel C).

5.6.2..2 Cytoplasmic targeted protein

The onion epidermal cells were transformed with the cytoplasmic targeted protein, GUS::GFP construct. The confocal image shows the cytoplasmic localized *pCAMBIA1304GUS::gfp* (Lechner *et al.*, 2012) protein in cytoplasm in Panel A and the white light image and merged images show that the GFP is excluded from the nucleus of onion epidermal cells (Figure 5-7). The Panel C shows the merged image of complete nucleus under the white light image and the GFP image from Panel A which highlights the nucleus without the presence of any GFP. The streaming of the cells was evaluated using time series images. The results show that a protein without a nuclear localization signal tagged with GFP does not localize to the nucleus.

Figure 5-7 Confocal image of cytoplasmic localization of *pCAMBIA1304GUS::gfp* in onion epidermal cells (Panel A), white light image (Panel B) and merged image of Panel A and Panel B (Panel C).

5.6.2..3 Nuclear targeted protein

Nuclear targeted protein was used to transiently transform the onion epidermal cells (Figure 5-8). Panel A shows the nuclear localization of green fluorescent protein and Panels B and C shows the white light and merged images respectively. The images indicate that the GFP is confined onto the nucleus of the cell. This indicates that when a protein possesses a nuclear localization signal and is tagged with GFP it completely localizes to the nucleus of the cell and no GFP would be observed in the cytoplasm of the cell.

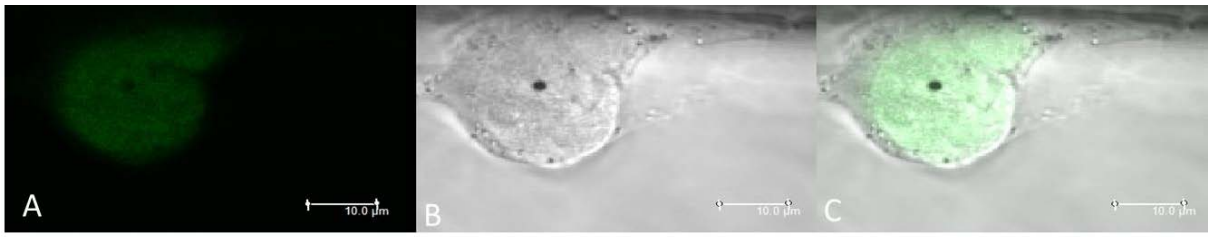


Figure 5-8 Confocal image of nuclear localization of nuclear targeted protein attached to GFP in onion epidermal cells (Panel A), white light image (Panel B) and merged image of Panel A and Panel B (Panel C).

5.6.2..4 Endoplasmic reticulum targeted protein

PBI121:: mgfp4-ER (Haseloff *et al.*, 1997)was used to transiently transform the onion epidermal cells as another positive control in this study (Figure 5-9). The Panel A shows GFP endoplasmic reticulum and Panel B and C are the white light and merged images respectively. The GFP tagged protein contains a HDEL sequence, an endoplasmic reticulum calcium binding protein which retains in the ER using a carboxy terminal His-Asp-Glu-Leu motif(Weis *et al.*, 1994). The Panel C shows that the protein is confined to the ER which shows the other cell structures without GFP. The cells were streaming highly which indicates that the cells were live and dynamic while obtaining the images (data not shown).

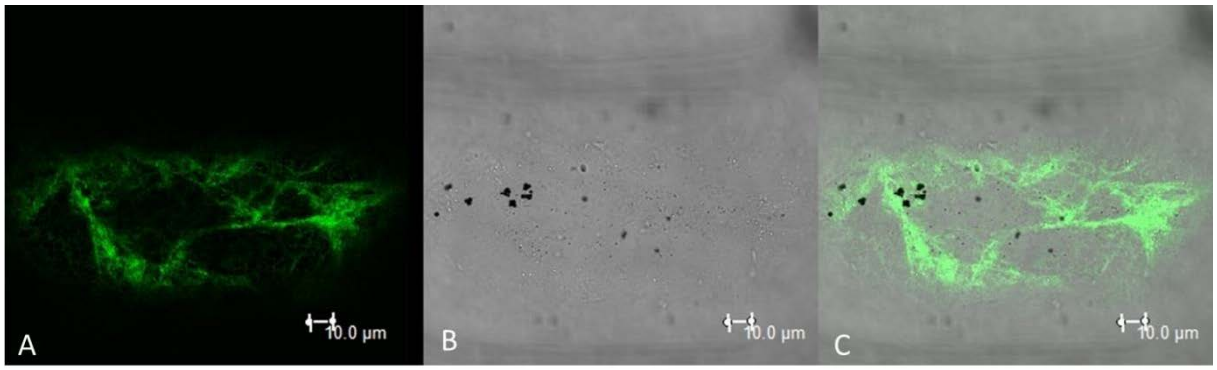


Figure 5-9 Confocal image of endoplasmic reticulum targeted *PBI121:: mgfp4-ER* the endoplasmic reticulum of onion epidermal cells (Panel A), white light image (Panel B) and merged image of Panel A and Panel B (Panel C).

5.6.3 Discussion

The methods used to study the sub-cellular localization of *Lp*UBL5 in *N. benthamiana* (tobacco) and *Allium cepa* L. (onion) expanded the understanding of *Lp*UBL5 expression pattern in both dicots and monocots.

In order to study the function of *Lolium perenne* L. *Ubl5*, *LpUbl5* was overexpressed in *A. thaliana* (Chapter 1) and in *Lolium perenne* L. (Chapter 3). The results obtained were not sufficient to support the role of *Lp*UBL5 in drought tolerance. However, functional studies often require to understand cellular environment of protein in question and UBL5 has been shown to be involved in alternative splicing in yeast and humans (Mishra *et al.*, 2011; Oka *et al.*, 2014) and mitochondrial unfolded protein response (UPRmt) in *Caenorhabditis elegans* (Haynes and Ron, 2010). As UBL5 homologous from vertebrates, yeasts and plants exhibits high levels of protein sequence conservation and it is conceivable that UBL5 homologues possess similar functions across species. Therefore a biological experiment to identify the localization pattern of *Lp*UBL5 was essential.

The use of online prediction tools for subcellular localization resulted in generation of contradicting result from each prediction tools when used for *Lp*UBL5 protein sequence. The results obtained from some of the online prediction tools for *Lp*UBL5 protein are given in Appendix D. The results from four online prediction tools does not indicate the presence of nuclear localization signals in the *Lp*UBL5 sequence. However, the results from the ESLpred, Appendix D.5 predicted *Lp*UBL5 as a nuclear localised protein and the cNLS mapper result score, (Appendix D.4) suggested chance of compartmentalisation of *Lp*UBL5 in both cytoplasm and nucleus.

The transient expression of *Lp*UBL5 in tobacco plants (Figure 5-3 in Panel A) shows the GFP expressing *Lp*UBL5 is compartmentalised both in the cytoplasm and nucleus. The positive control which was a 3GFP fused together showed a cytoplasmic distribution (Figure 5-4 in Panel A) and this shows that the protein end up in the cytoplasm when a specific localization signal is absent in the protein. The complete absence of GFP (Panel A Figure 5-5) in wild type tobacco leaves is a proof of absence of any GFP in the wild type background. This clearly shows that the GFP obtained in Figure 5-3 in Panel A is due the transient expression of *Lp*UBL5 fused to GFP which is localized both in the cytoplasm and nucleus due to the signals it possesses. The positive control, *pRT27::3gfp* also validates the efficiency of transformation procedure. This results obtained is consistent with the hypotheses of potential role of UBL5 in alternative splicing (Mishra *et al.*, 2011; Oka *et al.*, 2015). In order to take part in alternative splicing, *Lp*UBL5 needs to be localised in the nucleus. The experiment has proven that the *Lp*UBL5 is localised in the nucleus which supports the hypothesis of potential role of *Lp*UBL5 in alternative splicing. However, the role of *Lp*UBL5 in alternative splicing needs to be further studied.

This research extended further to identify the subcellular localization of *Lp*UBL5 in a monocot (onion) by transient expression to rule out any change in pattern obtained from transient expression in tobacco plants. This has allowed to include a number of controls such as cytoplasmic, nuclear and endoplasmic reticulum targeted proteins to compare the results of *Lp*UBL5 localization.

The Figure 5-6 shows the subcellular localization of *Lp*UBL5 in both the cytoplasm and nucleus of the onion epidermal cells as observed in tobacco leaves (Figure 5-3). The Figure 5-7 shows onion epidermal cells expressing the GFP protein (cloned with GUS), which is a cytoplasmic protein (Lechner *et al.*, 2012). The Image shows a clear compartmentalization of protein to the cytoplasm by excluding the nucleus. The vector carried a GUS protein which is 603 aa protein (weighing 68.47 kilo Daltons) fused to GFP of 238 aa protein of 27 kDa. Then the Figure 5-8, Panel A shows the nuclear localization of protein which is targeted specifically to the nucleus of the onion epidermal cells. This shows that the presence of NLS helps the proteins to localize to the nucleus and remains within the nucleus. The Figure 5-9, Panel A shows the localization of GFP protein in the endoplasmic reticulum of onion epidermal cells. The *PBI121:: mgfp4-ER* which comprises an N terminal signal peptide derived from an *Arabidopsis* vacuolar basic chitinase and C terminal HDEL sequence cloned into *pBI121* vector used for transient transformation of onion epidermal cells (Haseloff *et al.*, 1997). This Image clearly shows the GFP tagged protein is spread in the ER of the onion epidermal cells.

The three controls *pCAMBIA1304GUS::GFP, PBI121:: mgfp4-ER* and nuclear targeted protein used in the study (Figure 5-7, Figure 5-8, Figure 5-9) aimed to validate the result obtained by *Lp*UBL5. Endoplasmic reticulum (ER) serves as an entry point from where the proteins are targeted to different cellular compartment based on the signal each protein possess (Gomord *et al.*, 1999; Crofts *et al.*, 2004) . Therefore, the results from the control gene constructs used for transformation of onion epidermal cells indicate that GFP tagged protein localizes to the different cell compartment through ER, based on the signal peptide which each protein possess. This confirms the cytoplasmic and nuclear localization of *Lp*UBL5 (Figure 5-6 Panel A). This result is consistent with the result obtained by transient transformation of *N. benthamiana* leaves. This result also confirms that *Lp*UBL5 do have a similar subcellular compartmentalization in both dicot and monocot backgrounds.

These results indicate that the *Lp*UBL5 either do have a signal peptide which aid its localization to the nucleus, or the protein is passively diffusing to the nucleus due to its size (>40 kDa) (Dingwall and Laskey, 1986; Jans, 1995; Schlenstedt, 1996; Cole and Hammell, 1998; Nardozzi *et al.*, 2010). However, the amino acid sequence evaluation indicated that the UBL5 protein possess a consensus sequence similar to a importin α-dependent nuclear localization signal (LGKKVRVK) (Kosugi *et al.*, 2009a). This would exclude the argument of passive diffusion of UBL5 to the nucleus. The UBL5 protein contains 73 aa and the potential NLS is present at ^{10}LGKKVRVK^{17}. The potential NLS is very similar to class 5 NLS

which functions only in plant cells (Kosugi *et al.*, 2009a). In addition, the UBL5 protein possess a Tyrosine and Threonine residues (^{59}TIY^{60}) similar to extra cellular signal-regulated kinase (ERK5) and a TEY activation motif (Kondoh *et al.*, 2006; Nardozzi *et al.*, 2010) in which the phosphorylation of tyrosine and threonine residues expose the bipartite NLs in the C-terminal of ERK5 and hence promotes nuclear accumulation (Kondoh *et al.*, 2006). Similarly, the potential class 5 NLS present at the C-terminus of UBL5 protein might be exported to the nucleus when tyrosine and threonine residues (TIY) of the UBL5 protein are phosphorylated. However, further studies are required to identify the relation between the phosphorylation and potential monopartite class 5 NLS in the *Lp*UBL5 protein.

The nuclear localization of *Lp*UBL5 is consistent with the potential role of *Lp*UBL5 in alternative splicing as in humans(Ammon *et al.*, 2014; Oka *et al.*, 2014) and in yeast (Wilkinson *et al.*, 2004; Yashiroda and Tanaka, 2004; Mishra *et al.*, 2011). UBL5/HUB1 homologues in yeast bind to spliceosomal protein, Snu66 through a Hub1 interaction domain (HIND) and they observed the localization of yeast HUB1 homologues in both cytoplasm and nucleus. (Mishra *et al.*, 2011).

This finding is also consistent with the potential role of *Lp*UBl5 gene in the mitochondrial unfolded protein response (UPR^{mt}) as shown in *Caenorhabditis elegans (*Benedetti *et al., 2006;* Broadley and Hartl*, 2008;* Haynes and Ron*, 2010;* Haynes *et al., 2010).* The subcellular localization pattern of *Lp*UBL5 was also consistent with the observation of Benedetti *et al.* (2006). UBL5 homologues in *C.elegans* is involved in the Mitochondrial Unfolded Protein Response (UPR^{mt}) in which protein forms a transcriptional complex with homeobox transcription factor DVE-1 in the nucleus which then upregulate the mitochondrial molecular chaperon genes expression as well as UBl5 gene expression. Broadley and Hartl (2008) explains, that the UBL5 homologue is trafficked back to the cytoplasm where it continues to amplify the UPR^{mt} signal. This phenomenon would explain the cytoplasmic localization of *Lp*UBL5 observed in the current study. The inactivation of UBL5 expression resulted in a compromised protein folding environment in the mitochondria due to the relative deficiency of chaperone gene expression. This indicates that the involvement of UBL5 is in the afferent limb of the UPR^{mt} process (Benedetti *et al.*, 2006) where UBL5 forms a transcriptional complex with DVE-1 which upregulates the mitochondrial chaperone gene expression and thus restores protein homeostasis which is considered as the efferent limb of the entire process. Benedetti *et al.* (2006) have studied the subcellular localization of UBL5 in *C.elegans* using UBL5 tagged by GFP at the C-terminus. They observed cytoplasmic and nuclear localization and a high proportion of UBL5::GFP was localized in the nucleus in the mitochondrially stressed animals. This supports the current finding of nuclear localization of *Lp*HUB1 in the potential role of *Lp*UBL5 in UPR^{mt}.

The LpUBL5 was localized both in cytoplasm and nucleus of both *N. benthamiana* leaves (Figure 5-3) and onion epidermal cells (Figure 5-6). This cytoplasmic localization could be either an artefact due to

GFP tagging of *Lp*UBL5, a relatively small (73 aa) protein, or lack of endogenous protein partners which aid its localization to the nucleus. A recent study using *Ta*ULP5 (UBL5) in the APR wheat cultivar 'Xingzi 9104' (XZ) reported that *Ta*ULP5 is likely to be localized in cytoplasm with a percentage of 76%. The method employed used GFP tagged *Ta*ULP5 and the platform used was *Arabidopsis* mesophyll protoplasts to predict the localization. However, the result obtained from this PhD study was consistent with results obtained in *Schizosaccharomyces pombe* (Yashiroda and Tanaka, 2004) and humans (Sveda *et al.*, 2013; Ammon *et al.*, 2014) and *C.elegans* (Benedetti *et al.*, 2006). Yashiroda and Tanaka (2004) in *S. pombe* and Benedetti *et al.* (2006) in *C.elegans* used GFP tagged UBL5 to carry out localization study. They reported that the UBL5 localized in both nucleus and cytoplasm which is consistent with the result obtained in the current study. Further, Ammon *et al.* (2014) and Sveda *et al.* (2013) reported similar result using immunofluorescence method by using UBL5 specific antibody. Taken together, we can speculate that the cytoplasmic localization could be potentially a real observation. Further studies are required by developing *Lp*UBL5 specific antibody to carry out immunofluorescence (Ammon *et al.*, 2014) and identification of its potential protein partners (DVE-1 or Snu66 via HIND) using the yeast two hybrid assay (Mishra *et al.*, 2011) to establish UBL5 function.

This research has transiently expressed GFP tagged *Lp*UBL5 in tobacco (*N. benthamiana)* and in onion (*Allium cepa* L.) with a variety of controls such as cytoplasmic (Figure 5-7), nuclear (Figure 5-8) and endoplasmic (Figure 5-9) targeted proteins. As such the multiple results obtained by transient expression of GFP tagged *Lp*UBL5 can be accepted with a high degree of confidence. These results obtained are consistent with the hypothesis that *Lp*UBL5 may have a potential role in alternative splicing or UPR^{mt} or both processes. The observation of cytoplasmic and nuclear localization of *Lp*UBL5 is consistent with the demonstrated role of *Ubl5* homologous in yeast (Mishra *et al.*, 2011), humans (Ammon *et al.*, 2014) and *C. elegans* (Benedetti et al., 2006). The sub-cellular localization of *Lp*UBL5 in both cytoplasm and nucleus rejected the null hypothesis "*Lp*Ubl5 is not localized to the nucleus"- rejecting the potential involvement of *Lp*UBL5 in alternative splicing. The subcellular localization of *Lp*UBL5 supports the potential role of UBL5 in alternative splicing and UPR_{mt}. However, this finding can only be considered as an indication of the function of *Lp*UBL5 in plants and further research is essential to fully understand the function of UBL5 in plants.

Chapter 6

Physiological evaluation of germplasm accessions in response to imposed drought and expression level study of *LpUbl5* under various stages of drought

6.1 Introduction

Chapters 3 and 4 described a transgenic approach to identify any contribution of *LpUbl5* to confer enhanced drought tolerance when the gene was over-expressed. In this chapter, the aim was to determine if the same methodology can be used to select drought tolerance from ecotypes held in the Margot Forde Germplasm Centre. This is the national gene bank for grassland species and contains plant material gathered from a wide range of geographic locations. Identification of accessions with drought tolerant phenotypes could potentially provide a non-modified genotype with potential for further development in a conventional breeding programme. New Zealand ryegrass breeding programmes produced 'Grasslands Manawa', the world's first hybrid ryegrass using perennial ryegrass and Italian ryegrass in 1943 (Lee et al., 2001; Stewart, 2006). Since then a wide range of cultivars has been released in New Zealand including 'Grasslands Ruanui' in 1955, 'Grasslands Ariki' in 1965 and 'Grasslands Marsden' in 1980. The cultivars introduced include some imported from UK and Belgium but predominantly they have incorporated genetic material from Northwest Spain, Mediterranean Spain and Italy (Stewart, 2006). Germplasm that originated from Western Europe has also been used in New Zealand. Introgression of wild type ecotypes with superior morphological and physiological performance under drought could be a potential candidates for drought tolerance traits. Further, analysis investigated whether enhanced drought tolerance was associated with *LpUbl5* transcript abundance. This approach was adopted because field testing of genetically modified material in New Zealand is legislatively difficult. The study also assessed the endophyte symbiosis of perennial ryegrass, to ensure endophyte infection had not confounded drought tolerance results (Hesse *et al.*, 2003; Hahn *et al.*, 2008).

The null hypotheses are: "Drought tolerance of the germplasm accessions is not different and not associated with *LpUbl5* transcript abundance."

This chapter describes Experiment 4 which used 10 germplasm accessions plus 8AC1 (the homozygous T_3 transgenic line from Experiment 1, Section 3.2.1) and 'Impact'.

6.1.1 Germplasm Accessions

The 10 germplasm accessions from the Margot Forde Forage Germplasm Centre, New Zealand (Table 6-1) had different centres of origin. These were selected specifically to evaluate accessions from a range of climatic and environmental conditions. Design of the experiment is given in Appendix E.3 and E.4.

Table 6-1 Information of germplasm accessions obtained from Margot Forde Forage Germplasm Centre, AgResearch, NZ.

Accession	Known As	Called as
A 6889	Ecotype collection from Otago/Southland, New Zealand	'Otago/Southland'
A 6932	Forde & Easton, SW Europe Collection Forde & Easton 79.01	Portugal'
A 7798	French Ecotypes ex Charmet 376	'French'
A 14499	Increase of 1999 Turkish collection	'Turkey'
A 14542	Increase of SW Europe Collection Forde & Easton	'Italy'
A 15323	PI 231586 Algeria increase	'Algeria'
A 15334	PI 198958 Cyprus increase	'Cyprus'
A 15369	PI 598909 Tunisia increase	'Tunisia'
A 17183	Increase of PI 577269 (A16719) Norway	'Norway'
A 17187	Increase of PI 538976 (A16717) Russian Federation	'Russia'

6.1.2 Background information of Germplasm Accessions

Information about the background and origin of each accession was obtained from the National Plant Germplasm System : USDA and Margot Forde Forage Germplasm Centre Custom enquiry (AgResearch; USDA).

A6889

A6889 is an original germplasm, collected from "Wendon, Southland" on 1/4/1991.

A6932

A6932 was collected from Vila Pouca, Tras os Montes, North Portugal on 1/04/1991. This germplasm was collected by Forde & Easton, in the SW Europe collection, Forde & Easton 79.01. This is an original germplasm and the seeds used were harvested from Gore, NZ on 1/1/1998.

A7798

The accession was collected from France and is known as French Ecotypes ex Charmet 376. A7798 is recorded as an exchange germplasm. The seeds used were harvested in Palmerston North on 1/07/1992.

A14499

Accession A14499 was collected from North of Tabarka at the Turkish fort (Borj Massaoud) in Tunisia on 24/06/1994. NPGS received the accession on 19/8/1994 and PI was assigned by 1997. The collection habitat was a roadway between sea & road and there was no grazing, with a slope of 6-10%. The climate is seasonally dry and the land was at sea level. The accession is known as increase of 1999 Turkish collection and is an original germplasm. The seeds used were harvested from Palmerston North on 1/03/2006.

A14542

This accession was collected from Campo Reggino, Umbria, Central Italy. The habitat of collection was a gateway of field & rough grazing land. This accession is known as increase of SW Europe Collection Forde & Easton 131.01 and is an original germplasm. The seeds used were harvested at Palmerston North on 1/1/2006.

A15323

Accession A15323 was collected in Algeria and maintained by the Western regional PI station. PI 231596 was received by the National Plant Germplasm System (NPGS) on 13/3/1956. Original plant inventory data shows the accession was from Berrouaghia, Algeria and the serial number given was 3228 with the date of entry 31/12/1956. The accession has been used in various studies including Hulke *et al.* (2007). The accession is known as PI 231586 Algeria increase and this accession is an exchange germplasm. The seeds used were harvested at Palmerston North on 26/1/2007 and observed to have poor heading.

A15334

This accession was collected from Limassol, Cyprus and donated to NPGS on 10/01/1952. PI was assigned in 1952. The accession was used in three different studies by Casler (1995), Eline van Zijll de Jong *et al.* (2008) and Hulke *et al.* (2007). The screening of endophyte by Hulke *et al.* (2007) showed 2% of endophyte incidence out 52 tested plants. The accession is known as PI 198958 a Cyprus increase and is an exchange germplasm. The seeds were harvested at Palmerston North on 26/1/2007 and observed to have poor heading.

A15369

A15369 was collected near Skalba, 5 km west of Menzer Temine in Tunisia on 21/06/1994. The site of collection was dominated by tall grasses in a moist floodplain (Cheplick, 2007). NPGS received the accession on 19/8/1994 and the PI was assigned in 1997. The accession was studied by Cheplick (2007) and Hulke *et al.* (2007). This accession is called PI 598909, Tunisia increase and is an exchange

germplasm. The seeds used were harvested at Palmerston North on 12/2/2007 and observed to have poor heading.

A17183

This accession was collected in Bryne, Rogaland state, Norway and maintained by Western regional PI station. It was donated to NPGS in Wales, United Kingdom by the Welsh Plant breeding station on 03/09/1991. The PI was assigned in 1994. This material was used by Hulke *et al.* (2007) for winterhardiness and A17183 was ranked 5.5 in 2005 and 4.8 in 2006 by mean performance in tiller survival whereas the best performing elite variety, Citation Fore had a rank of 3.2 and 2.7 score. The highest tiller survival was observed in accession (PI 502412) from Soviet Union and the score was 7.2. Spring growth was ranked 15.4 in 2005 and 15.2 in 2006 and the Citation Fore had 10.6 and 9.6 in subsequent years. The highest score for spring growth was 22 in 2005 and 23 2006 by accessions from Romania and Greece respectively. The spreading vigour ranking by mean performance for P1 577269 was 19.6 but the Citation Fore had 16.7. The highest vigour ranking was 30.5 by accession from Belgium. These results indicate the potential winterhardiness of Norway accession (A17183). This accession is an exchange germplasm and is an increase of PI577269 (A16719) Norway. The seeds used were harvested at Palmerston North on 17/1/2011.

A17187

The accession is originally from Leningrad, Russian Federation. The location of collection was an Experimental farm, Cherga, Russian Federation on 14/08/1988. The accession was developed by the Institute of Cytology and Genetics on 05/02/1990 and donated to NPGS on 05/02/1990 in Utah, United States. The accession is known as Increase of PI 538976, and is an exchange germplasm. The seeds used were harvested at Palmerston North on 13/1/2011.

6.1.3 Seedling establishment

The seeds from these 10 germplasm accessions plus 'Impact' and the 8AC1 line from Experiment 1 were sown into individual cells as single seeds in trays for germination. Trays were maintained in the same growth room where the experiment was conducted under the same conditions used for Experiment 1 (section 3.2.1). The seedlings were irrigated periodically to avoid any drought stress at the seedling stage. Additions of Hoagland's solution and insecticide applications to control thrips were carried out as required.

6.1.4 Media

The Templeton silt loam soil (Cox, 1978) was used as for Experiment 1 (Section 31).

6.1.5 Growth room

The experiment were carried out in the same Biotron at the same time as Experiment 1 (Section 3.2.3).

6.1.6 Growth room conditions

The environmental conditions within the Conviron, controlled environment chamber during seedling establishment and sward establishment were set to a constant 20^{o}C air temperature, 15°C soil temperature, 16/8 hr day night photoperiod and a constant relative humidity of 70%. A 30 min ramped twilight on either side of 15 hours was given to adjust a total of 16 hours day light.

During the first imposed drought cycle, the soil temperature was increased to 25^{o}C and the air temperature was 15^{o}-25^{o}C and the humidity was decreased to 0%. The controlled environment conditions were maintained for the second imposed drought cycle to evaluate the performance of these perennial ryegrass lines.

6.1.7 Microsward establishment

Experiment 4 included two rhizotrons 3 & 4 and each rhizotron included 24 plots. Different accessions were arranged in randomized blocks. The rhizotrons accommodated 2 X 185 plants, including "Fill" plants, which provided four replicates across the two rhizotrons. The seedlings were trimmed to uniform height before transplanting. TDR rods measuring 20 cm were inserted to measure the soil moisture using Time Domain Reflectometry (TDR, 0-0.2 m, Trace systems, Model 6050X1, Santa Barbara, California, USA). The soil moisture profile, drought cycle and harvest dates are shown in Figure 6-1.

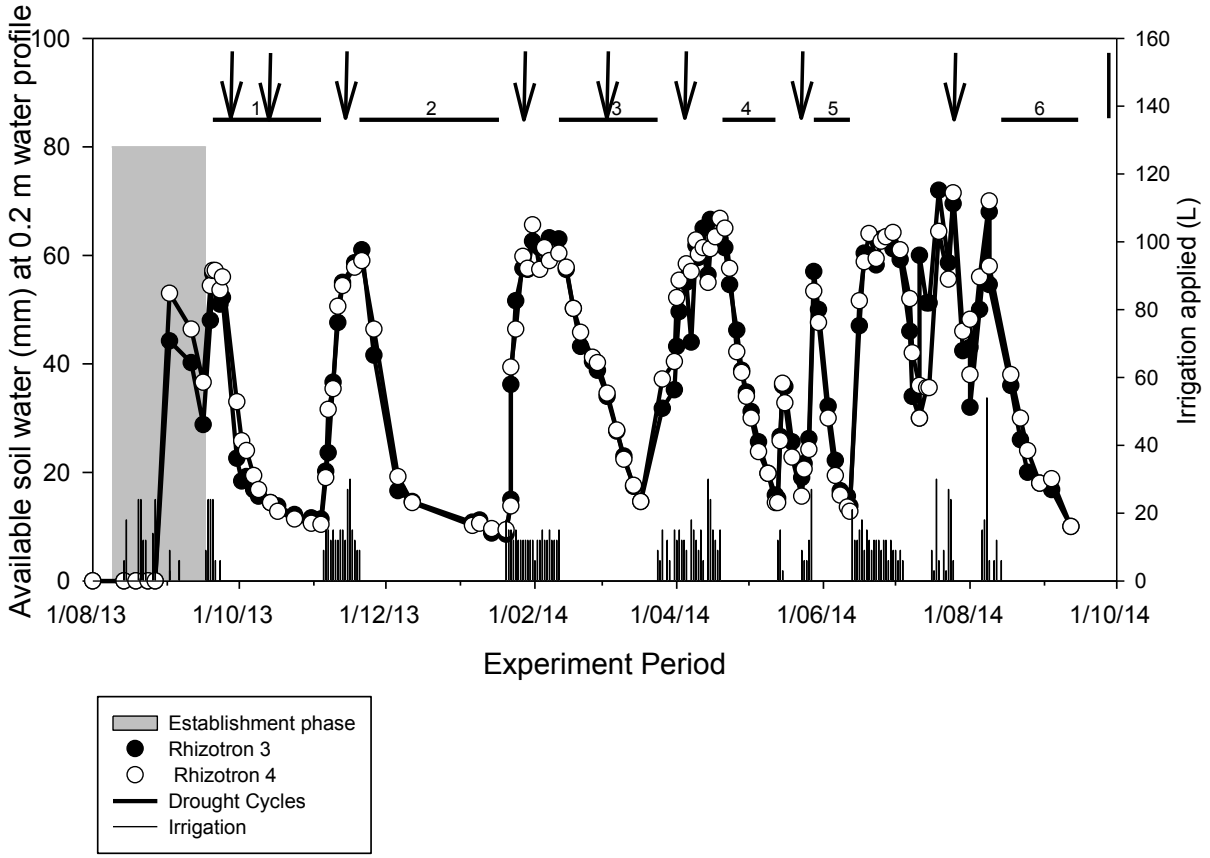


Figure 6-1 Soil moisture of the rhizotron profile during the establishment phase of Experiment 4, with highlighted water application rate (L, bars), drought cycles shown as horizontal lines marked above the graph. Arrows represent harvest dates during and at the end of each drought cycle. ↓ indicates harvest dates.

6.1.8 Drought cycles

The accessions were established on 1/8/2013 in mini swards that contained six plants in each replicate. The accessions were replicated four times across two rhizotrons. The rhizotrons were watered continuously during their establishment phase to ensure vigorous seedling growth (Figure 6-2).

Figure 6-2 Photo taken after the establishment phase of Experiment 4. The image shows the well-established plants in Rhizotron 3. The photo was taken on 12/09/2013 at Lincoln University.

Drought Cycle 1

The first harvest was carried out on 18/9/2013 and Drought Cycle 1 was initiated on 20/09/2013. During the first drought cycle the plants were harvested on 3/10/2013. The plants were not re-watered post-harvest and the re-growth was slow (Figure 6-3). A final harvest was conducted at the end of drought on 4/11/2013 at ~5% soil moisture (Figure 6-3).

Figure 6-3 Photograph of Experiment 4 taken on 14/10/2013 after the mid-harvest during Drought Cycle 1 which shows a slow growth of the plants and the soil moisture was ~7%.

Drought Cycle 2

Drought cycle 2 was initiated on 20/11/2013 after re-watering the rhizotrons to field capacity (30%) over 15 days. The re-growth lasted 58 days until harvest on 17/01/2014.

Drought Cycle 3

Following the harvest on 17/1/2014 rhizotrons were left without watering for five days at ~4% soil moisture. Then the rhizotrons were re-watered to field capacity over a 20 day period. Drought Cycle 3 was initiated on 11/2/2014 and ended on 28/3/2014 with a final harvest.

Drought Cycle 4

The rhizotrons were re-watered to field capacity before initiating drought on 20/04/2014 (Figure 6-4). Drought Cycle 4 was suspended on 12/05/2014 as the plants were infected by thrips (Figure 6-5). The plants were treated as explained in Experiment 1 (Section 3.2.7)

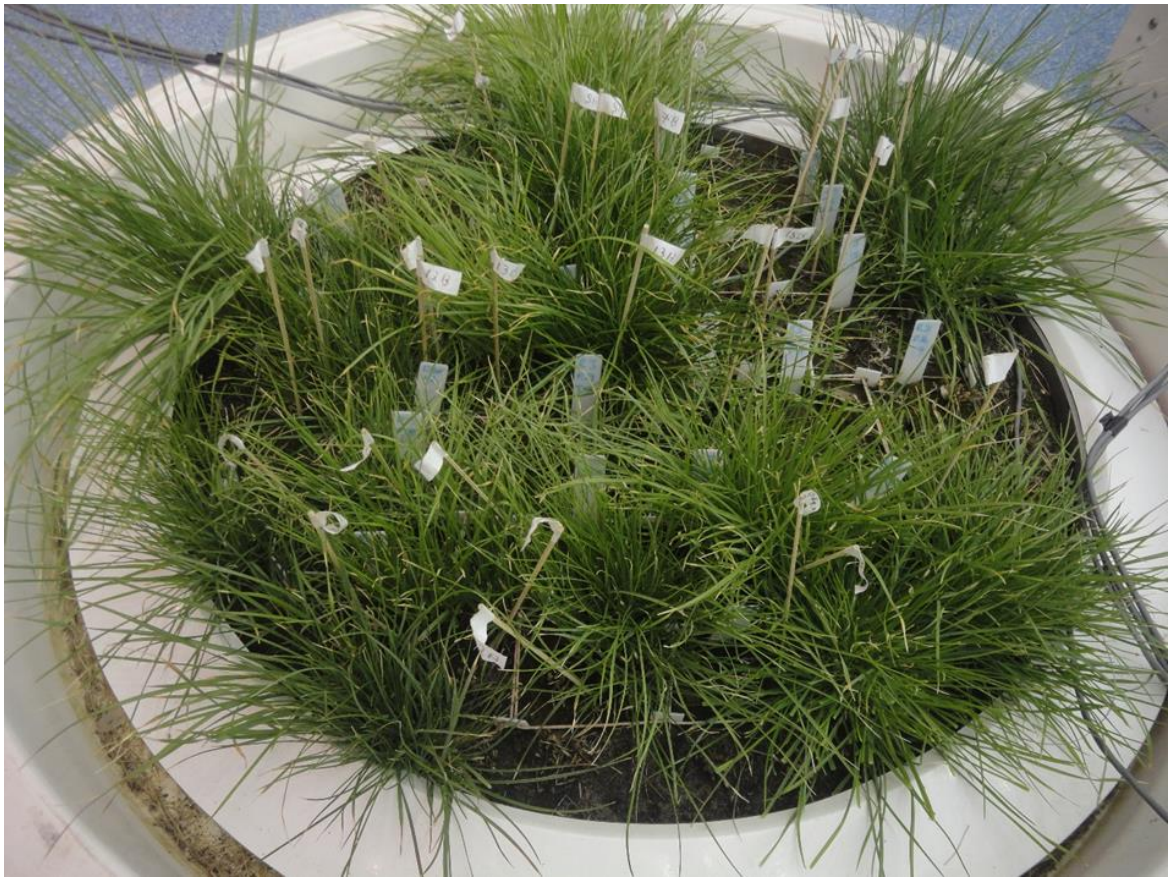


Figure 6-4 Photograph taken on 20/4/2014 before initiating Drought Cycle 4. The photo was taken when the soil moisture was 30%.

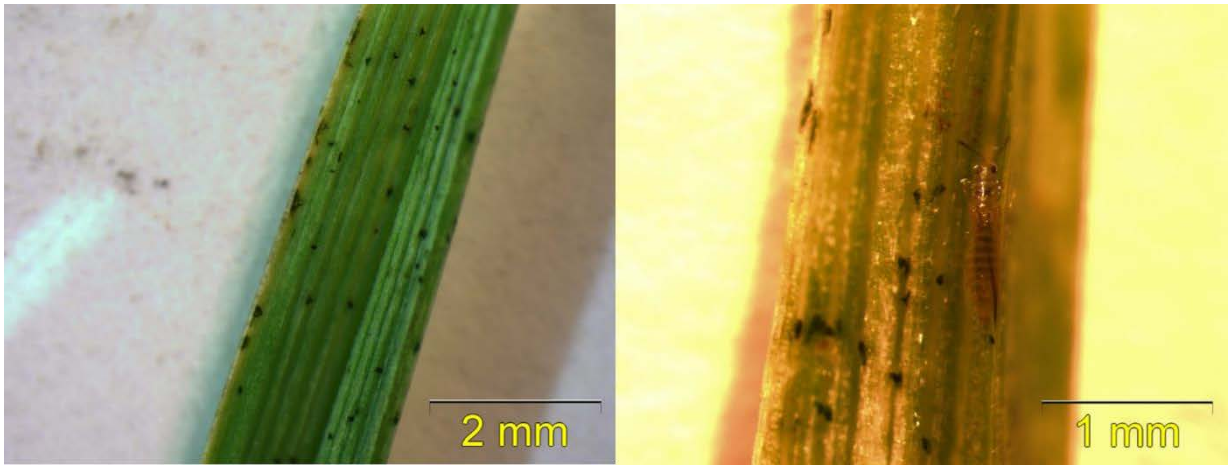


Figure 6-5 Photograph of infected leaves and the thrips which infected the leaves.

Drought Cycle 5

The Rhizotrons were watered to 25% soil moisture before imposing drought. The drought was initiated on 28/5/2014. This fifth cycle was also suspended after the rhizotrons were flooded due to the breakdown of the Conviron facility on 16/6/2014. The roof of the Conviron leaked as the refrigeration system failed and Rhizotrons 3 and 4 were saturated with water particularly Rhizotron 4. This caused uneven moisture availability and a delay in initiating the next drought cycle because it took a prolonged period to re-water Rhizotrons 3 and 4 back to a similar soil moisture level. The watering continued at a low rate until 14/08/2014 to maintain both rhizotrons at similar moisture levels.

Drought Cycle 6

The final drought cycle, Drought Cycle 6 was initiated on 14/08/2014. The final drought cycle was completed on 15/9/2014 which ended Experiment 4. The plants at the end of this drought cycle are shown in Figure 6-6.

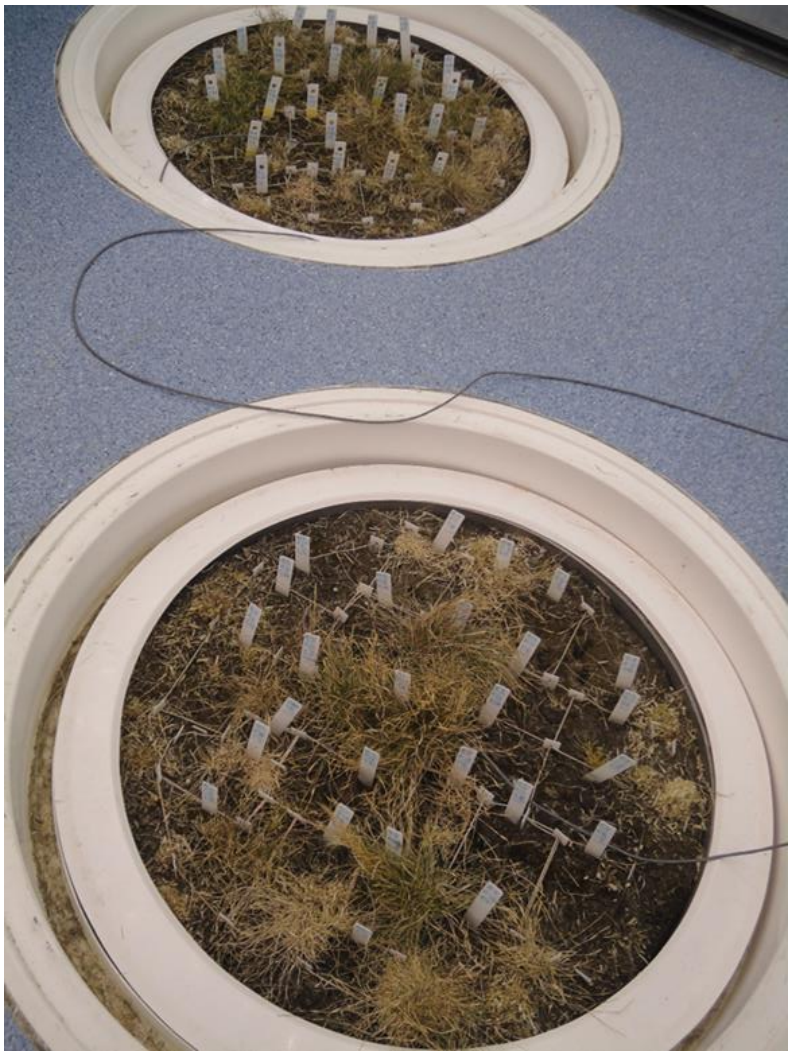


Figure 6-6 Photographs of Rhizotron 3 and 4 taken at the end of the Drought Cycle 6 on 18/09/2014**.**

6.2 Physiology measurements

All the physiology measurements were carried out as described in Chapter 3, Section 3.3

6.2.1 Adjusted solute potential

Plants typically respond to water loss under drought with a reduction in solute potential. This reduction can either be passive (merely by water lost from the cell) or active (via osmotic adjustment). In the latter case, plants actively accumulate compatible solutes to reduce their overall water potential below that of the surrounding solution and thus attract water back into the cell (Girma and Krieg, 1992). In order to understand the active accumulation of compatible solutes, adjusted solute potential was calculated. The adjusted solute potential is the Ψ_{S100} estimated using Equation 6-1.

Equation 6-1 $\Psi s_{100} = \Psi s$ **(RWC-0.1)/ (1-0.1)**

Where Ψs is solute potential and 0.1 refers to the correction for water in the apoplast tissue (Wilson *et al.*, 1979)

6.3 *LpUbl5* transcript abundance

The leaf sample collection, storage, RNA isolation, quality analysis and qRT-PCR and data processing were carried out as described in Chapter 3, Section 3.4 to identify *LpUbl5* transcript abundance before imposing drought stress and under water withdrawn conditions.

6.3.1 Endophye detection

Endophyte detection was carried out to determine the presence of endophyte in the seeds. Due to the lack of sufficient seeds, detection could not be carried out in A6932 (Forde & Easton 79.01) & A15334 (Cyprus). Seeds from the other eight accessions were sown in small pots. The plants were grown up to 3-4 tiller stage. Then the plants were removed from the soil and the main tiller was cut from the base, just above the roots. The necrotic sheaths were carefully removed from the main tiller. The tillers were cut transversely and the cut end was pressed onto a nitrocellulose membrane (NCM) (0.45 mm). This results in a circular moist mark on the NCM. The blotted paper was stored at 4^{o}C until processing. A positive and negative control were also blotted.

The reagents used and the preparation of the reagents for the processing of NCM are given in (Appendix B.14) The blotted paper was immersed in milk protein blocking solution for two hours to block the NCM with no bound protein. After two hours, blocking solution was removed and fresh blocking solution was used to rinse the blotted paper. The blotted paper was immersed in primary antibody and the membrane was shaken for 15 minutes at room temperature. Then the membrane was incubated at 4^{o}C overnight. The excess primary antibody was discarded and the membrane was

rinsed twice using fresh blocking solution. The blotted membrane was immersed in secondary antibody and shaken for 15 minutes at room temperature. After shaking, the membrane was incubated at 4^{o}C for five hours. The excess secondary antibody was discarded and the membrane was washed using fresh blocking solution. The blotted membrane was stained by immersing in chromogen solution (Appendix B.14. 4) and this was shaken for 15 minutes at room temperature. The shaking was stopped when a red colour developed in the positive control. The stained blotting paper was rinsed three times in reverse osmosis (RO) water. The red colour indicated the positive control (Simpson *et al.*, 2012).

6.3.2 Statistical analysis

Dry matter production data were generated from the repeated harvesting of the same plants over the period of Experiment 4 and were analysed by REML (Restricted Maximum Likelihood) in Genstat Version Release 16.1, (Copyright 2013, VSN International Ltd.) to understand the interaction of accessions and harvest date over time. Further, the dry matter production was analysed using REML using residuals form the previous harvest as the covariate to understand the contribution of plant death to dry matter production and the variation as residuals. Subsequently, total accumulated dry matter and dry matter production from individual harvests were analysed by One-way Anova in randomized blocks to understand the performance of each accession individually at each time point. Best subset regression was carried out using Minitab 17 Statistical Software (2010) with the area as the response variate using length and width as predictors to generate leaf area. All the physiology measurements were analysed by One-way Anova. Fisher's protected LSD was used for means separation. As the drought progressed, some accessions died. Accessions with less than three live replicates were removed from the analysis at each time point. The *LpUbl5* transcript abundance was analysed by Two-way Anova in randomised blocks. Relationships between parameters were tested using regression analysis and Pearson correlation coefficients in Minitab 17 Statistical Software (2010).

6.4 Results

6.4.1 Shoot biomass accumulation

The REML analysis of dry matter showed an interaction (P<0.001) between accessions and harvest date. Based on this analysis results from each drought cycle are presented separately (Figure 6-7). Analysis using residuals from the previous harvest as co-variate showed differences in dry matter production from 20/02/2014 (P<0.05) to 15/9/2014 (P<0.001).

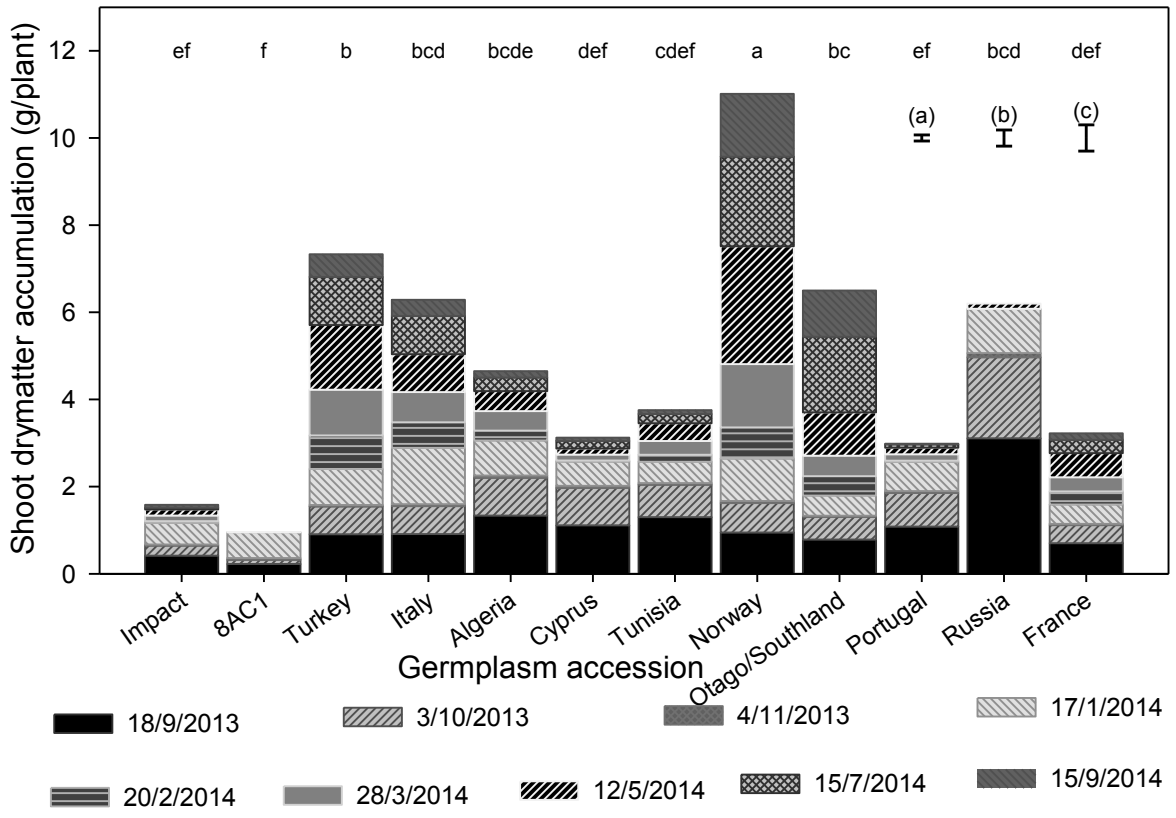


Figure 6-7 Shoot dry matter accumulation over time from 10 different germplasm accessions, Impact (Control) and 8AC1 from 9 harvest dates (3/10/2013, 18/09/2013, 4/11/2013 17/01/2014, 20/02/2014, 28/03/2014, 12/05/2014, 15/07/2014, 15/09/2014). Error bar is LSD for the Accessions (a), Harvest Date (b) and Accessions and Harvest Date (c) by REML analysis of Data in one variate. Bars with letters in common are not significantly different α = 0.05 level.

In addition to the REML, a One-way Anova was used to assess the total dry matter accumulation over the experiment period. The accession A17183, Norway had the highest (P<0.001) shoot dry matter accumulation at the end of six imposed drought cycles (Figure 6-7). Total accumulated shoot dry matter ranged from 0.95 ± 1.10 g/plant (8AC1) to 11 ± 1.10 g/plant ('Norway'). The dry matter accumulation of ('Turkey') was second highest (7.3 ± 1.27 g/plant) and not different to several others. Yield of 'Tunisia' (3.8 ± 1.27 g/plant), 'France' (3.2 ±1.27 g/plant), 'Cyprus' (3.1 ± 1.27 g/plant),

'Portugal' (3 ± 1.27 g/plant) and "Impact' (1.6 ± 1.27 g/plant) were all not different to 8AC1 which had the lowest yield.

The 'Russian Federation' line outgrew all accessions during the establishment phase and Drought Cycle 1 but did not regenerate after the second drought cycle. Its visual appearance of long, wide leaves and rolled emerging leaf suggests it may have been an annual ryegrass.

Shoot dry matter generated at each harvest

The highest shoot dry matter was measured for the 'Norway' but only from Drought Cycle 3 to Drought Cycle 6 (Figure 6-8). Before this, there were no yield differences among accessions, except the initial growth from the 'Russia' line. Therefore the interaction occurred because of the greater biomass produced by the Norwegian line approximately seven months after the start of the experiment.

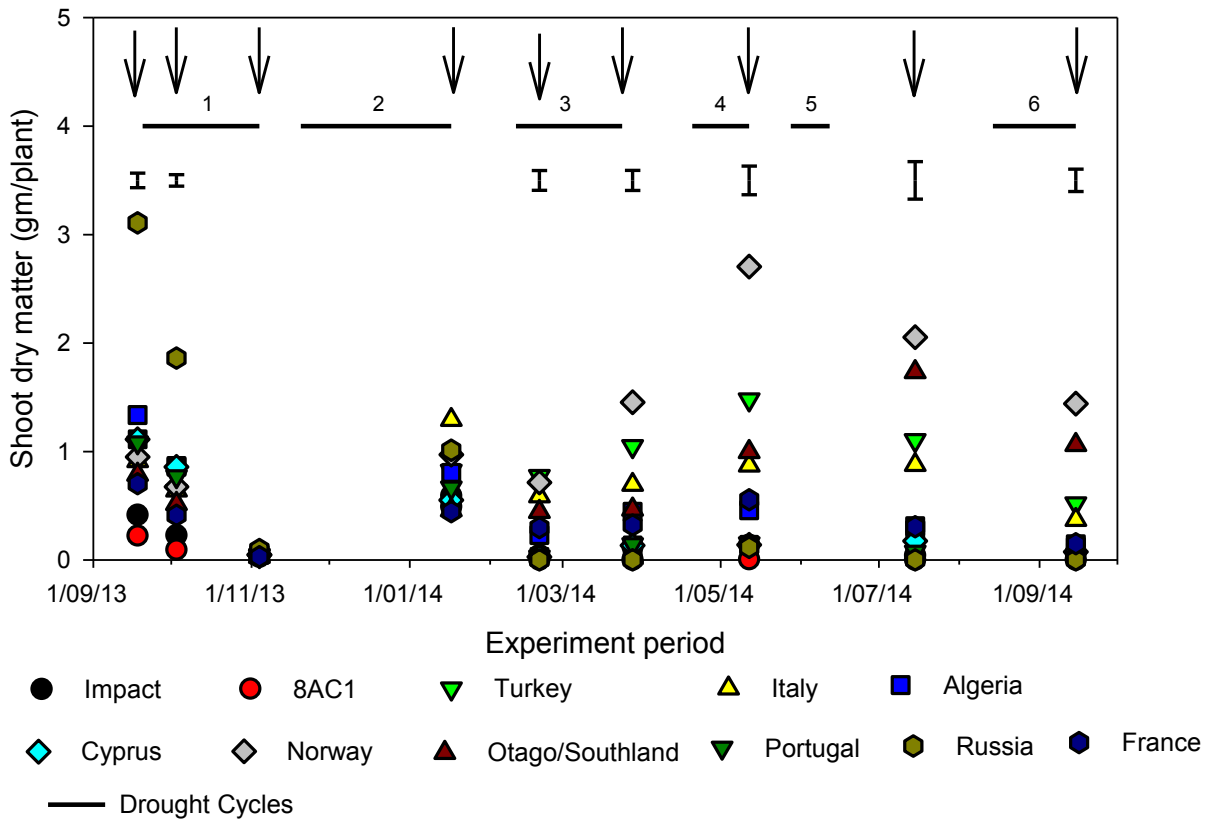


Figure 6-8 Shoot dry mater generated from each harvest from 10 different germplasm accessions, Impact (Control) and 8AC1. Eight harvest dates are (3/10/2013, 18/09/2013, 4/11/2013 17/01/2014, 20/02/2014, 28/03/2014, 12/05/2014, 15/07/2014, 15/09/2014). Error bar is SEM for the shoot dry matter from One-way Anova in randomized blocks and is shown where difference were detected. The ↓ indicates harvest date.

Specifically during the first two harvests (18/09/2013 and 3/10/2013) the highest (P<0.001) shoot dry matter accumulation was in 'Russia' at 3.1 ± 0.13 g/plant and 1.9 ± 0.10 g/plant respectively. The lowest shoot dry matter was in 8AC1 (0.2 ± 0.13 g/plant and 0.09 ± 0.10 g/plant). The third harvest at the end

of Drought Cycle 1 showed minimum shoot dry matter generation as the soil moisture was limited (~10% to ~5%) and there was no difference (P= 0.13) among the accessions. Consistently, the fourth harvest at the end of Drought Cycle 2 also showed no difference (P=0.31) among the accessions. From Drought Cycle 3, 'Norway' had the highest (P<0.001) shoot dry matter and this trend continued to be the same until the end of Drought Cycle 6. In addition, 'Otago/Southland' and 'Turkey' were the other accessions which had high shoot dry matter yields towards the end of the experiment.

6.4.2 Plant Survival

Plant survival (Figure 6-9) after the first two harvests, before imposing drought stress at Drought Cycle 1, was 100% across all accessions. No difference in plant death was observed after the Drought Cycle 1 (P=0.51) and Drought Cycle 2 (P=0.73). Differences were observed during Drought Cycle 3, where the 'Norway' and 'Italy' (95 and 87 ± 13.55%) accessions had high survival rate. After Drought Cycle 3 'Otago/Southland' had highest (P<0.05) plant survival rate (69 ± 10.64%). The lowest survival rate was observed in 8AC1 (4.17 ± 10.64%). Most of the transformed lines were dead by the end of the Drought Cycle 3.

The plant survival rate after Drought Cycle 4 showed differences (P<0.001) in plant survival rate similar to Drought Cycle 3. After Drought Cycles 5 and 6, the highest (P<0.001) plant survival (62.5 ± 8.69%) was also from Otago/Southland. The plant survival rate of the 'Norway' was same from Drought Cycle 3 (37.5 ± 10.64%) to the end of Drought Cycle 6 (37.5 ± 8.95%).

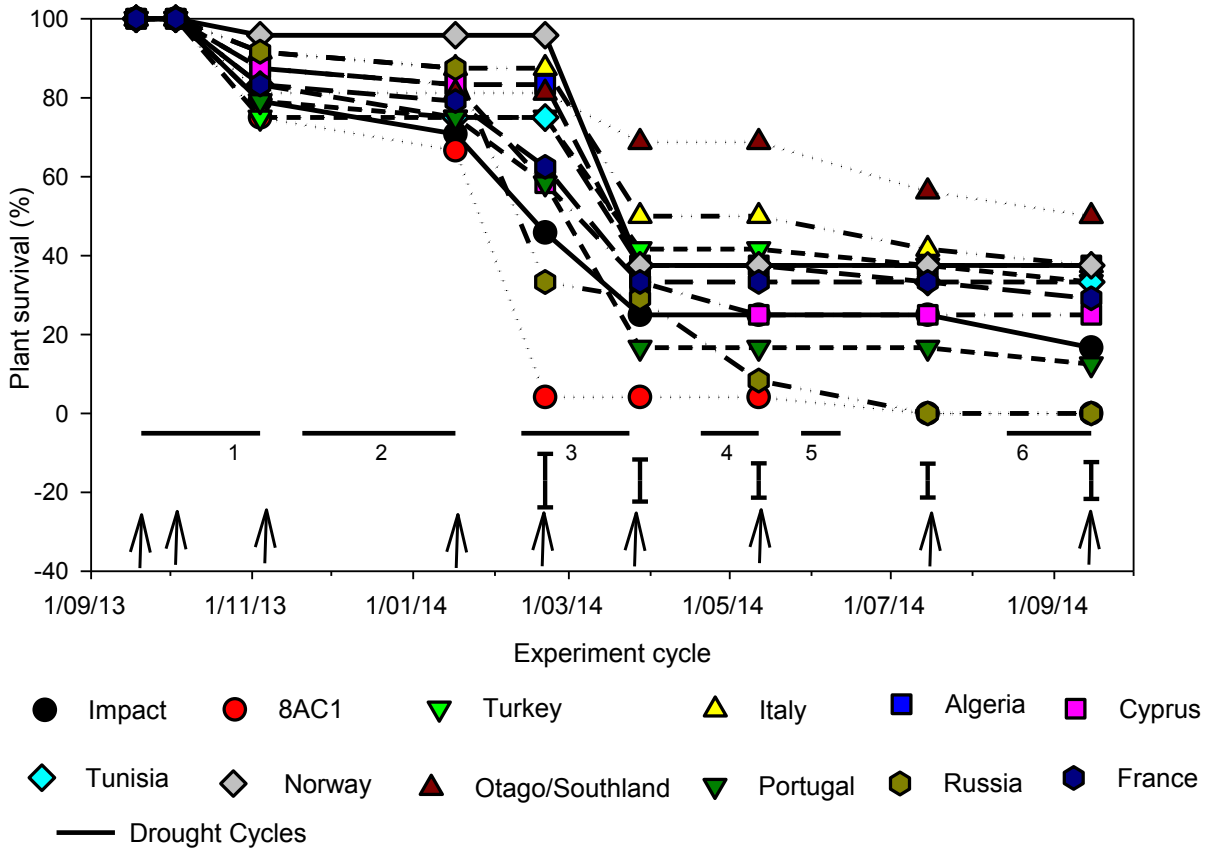


Figure 6-9 Plant survival rate after each imposed drought cycle from Experiment 4. Error bar is SEM for the plant survival from One-way Anova in randomized blocks and is shown where difference were detected. The ↓ indicates harvest date.

6.4.3 Plant score/ Grading

Grading of plants (Figure 6-10) was initiated at the middle of Drought Cycle 2 where each accession was scored as a plot based on the performance and morphological appearance ranging from 1-5 as described in Table 3-2. Initial grading at the middle of Drought Cycle 2 showed 'Norway' had the highest ($P<0.01$) score (4.3 ± 0.42) and 8AC1 had the lowest score (1.3 ± 0.42). The highest ($P<0.001$) scoring during Drought Cycles 3, 4, 5 and 6 was also shown by the 'Norway', 'Otago/Southland', 'Turkey' and 'Italy'. At the end of the Drought Cycle 6, 'Norway', 'Otago/Southland', 'Turkey' and 'Italy' had highest score ($p<0.001$) and 'Tunisia', 'Cyprus', 'Control', 'Portugal', 8AC1 and 'Russia' were the lowest ($P<0.001$) scored accessions (0 to 1 ± 0.49).

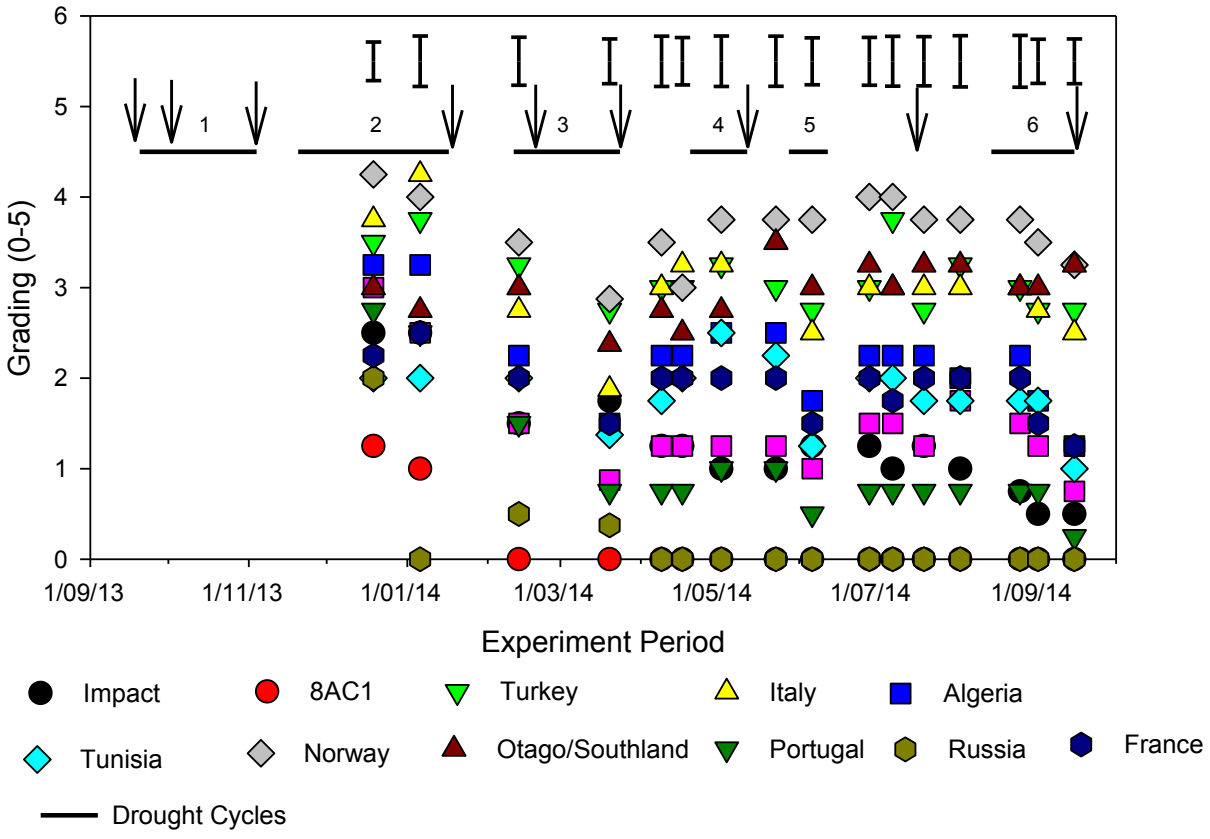


Figure 6-10 Grades obtained throughout the imposed drought cycles of Experiment 4, from 19/12/2013 until 15/09/2014. Error bar is SEM for the plant score from One-way Anova in randomized blocks and is shown where difference were detected. The ↓ indicates harvest date.

6.4.4 Leaf extension (mm/rotation)

Norway had the longest leaf extension during the final two drought cycles (Figure 6-11). Leaf extension was measured from Drought Cycle 2 when a trend (P=0.07) were observed among accessions.

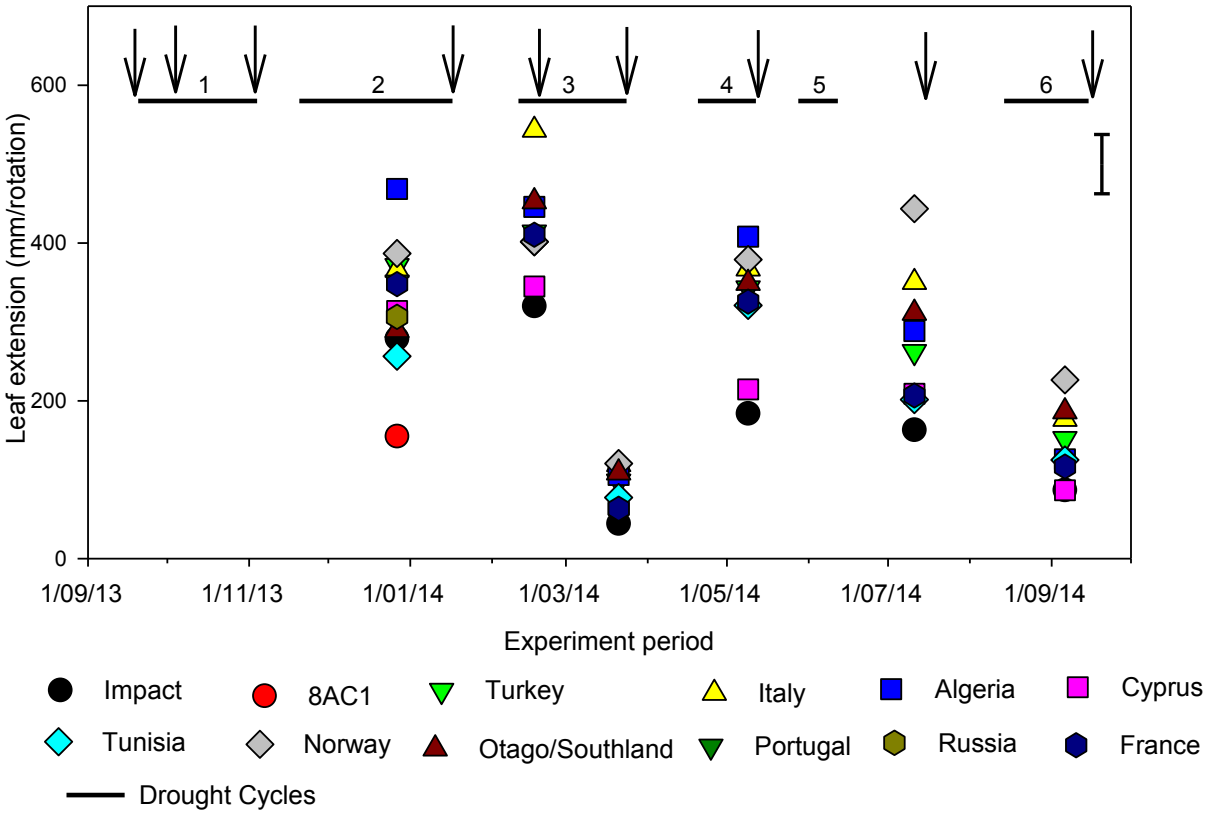


Figure 6-11 Leaf extension (mm/rotation) from each imposed drought cycle during Experiment 4. The ↓ indicates harvest date. The error bar shown is the maximum SEM.

By the end of Drought Cycle 5, 'Norway' had the longest (P=0.08) leaf extension (443 ± 61.5). This pattern through Drought Cycles 6 where Norway had longer (P=0.06) leaf extension (226 ± 31.5) than all other accessions.

6.4.5 Leaf area

Leaf length and width were measured by using a Leaf Area Meter AM300 (12 Spurling Works, Pindar Road, Hoddesdon, Herts EN110DB, England). Best subset regression was carried out using Minitab 17 Statistical Software (2010) with the area as the response variate using length and width as predictors. The highest adjusted R^2 (R^2_{adj}=0.82) was generated using length as a predictor (Figure 6-12). This was not improved when length and width were used as predictors together (R^2_{adj} =0.81). Therefore, the length of the leaf was used to predict the leaf area (mm^2) of the plants (Figure 6-13) using Equation 6-2. The constant (-147) in the Equation 6-2 resulted in negative leaf area when leaf length was less than 46 mm. However, this equation was accepted because adjusted R^2 was dropped (R^2_{adj}=0.75) when intercept was forced through the origin.

Equation 6-2 Area mm^2 = -147+3.2(±0.27)*Length)

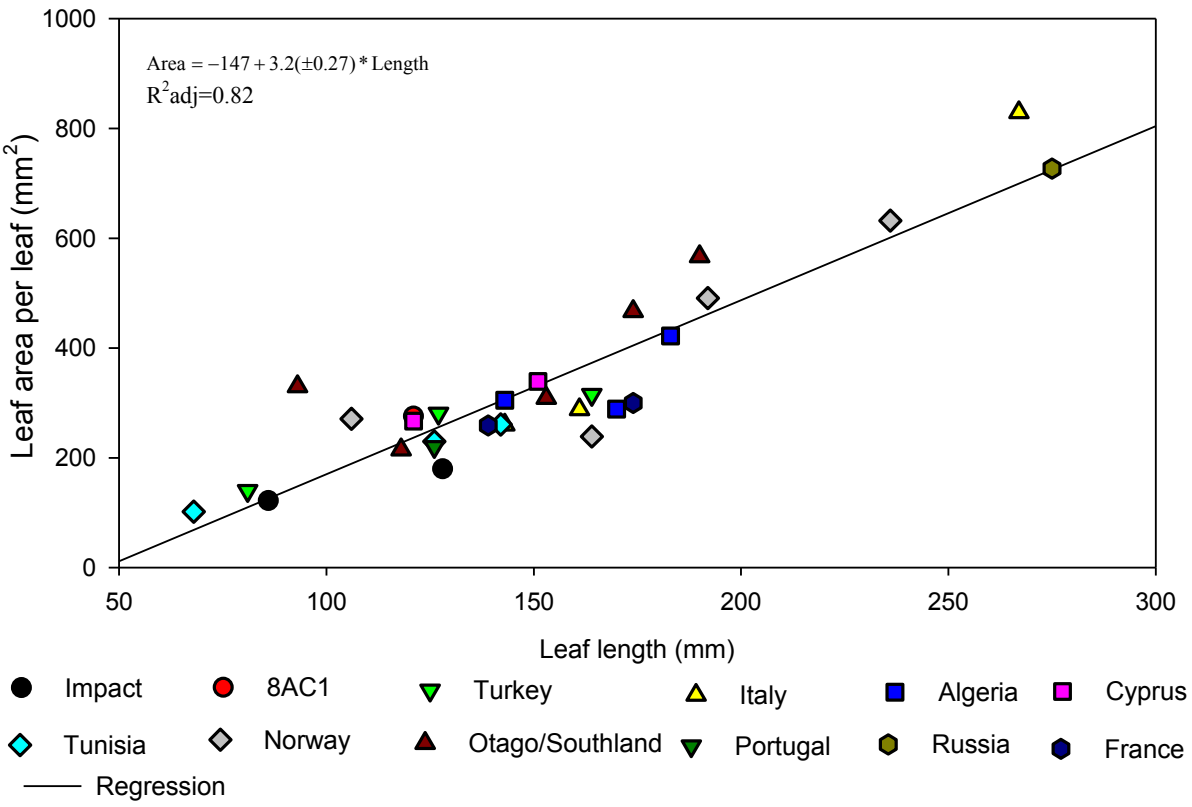


Figure 6-12 Regression coefficients using leaf length to estimate the leaf area of the 10 germplasm accessions, 8AC1 and Impact in Experiment 4.

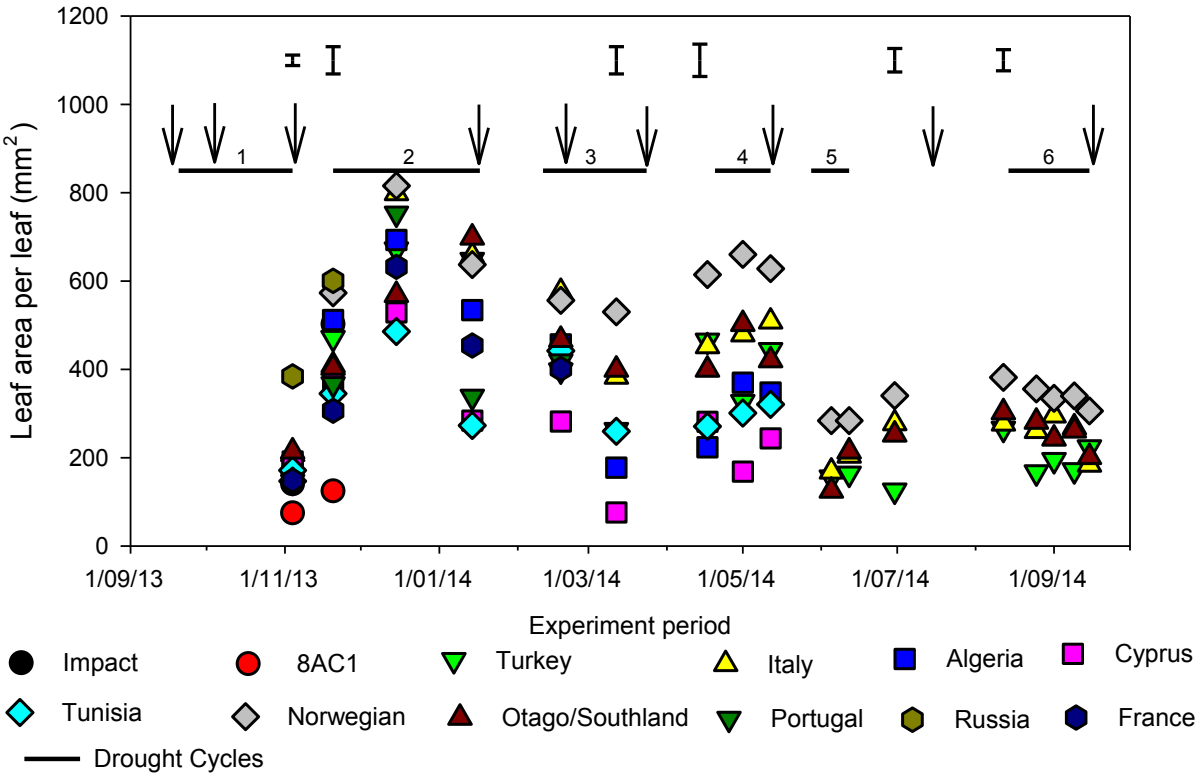


Figure 6-13 Leaf area during each imposed drought cycle during Experiment 4. Error bar is SEM for the leaf area from One-way Anova in randomized blocks and is shown where differences were detected. The ↓ indicates harvest date.

The 'Russian Federation' and 'Norway' had the largest ($P<0.001$) leaf area (530 ± 55.9 mm^2) at the beginning of Drought Cycle 2 and 8AC1 had the lowest (155 ± 55.9 mm^2) leaf area. All other accessions had leaf area that ranged from 438 to 263 ± 55.9 mm^2. This pattern was repeated during the recovery stage after the Drought Cycle 1 (Figure 6-13). 'Norway' had the largest ($P<0.05$) leaf area at the beginning of Drought Cycle 4. In contrast, the 'Tunisia' and 'Cyprus' accessions had the smallest leaf area. The similar trend ($P=0.06$) repeated at the beginning of Drought Cycle 6.

6.4.6 Relative water content (RWC %)

RWC (Figure 6-14) was measured multiple times during each drought cycle except Drought Cycle 1. The 'Turkey' had the highest (P<0.001) RWC (35 ± 1.6%) at the end of Drought Cycle 1 and accessions, 'France', 'Norway' and 'Algeria' were not different to Turkey. 'Portugal' and 'Otago/Southland' had the lowest RWC (18 and 17 ± 1.6%).

At the end of Drought Cycle 3, the accessions that showed the highest (P<0.05) RWC were 'Algeria', 'Norway', 'Turkey', 'Otago/Southland', and 'Italy' and ranged from 55 to 75 ± 10%. The 'Cyprus' had the lowest (P<0.05) RWC (20 ± 10%). At the end of Drought Cycle 6, 'Norway', 'Otago/Southland' and 'Italy' had RWC ranging from 22 to 31 ± 7.2%.

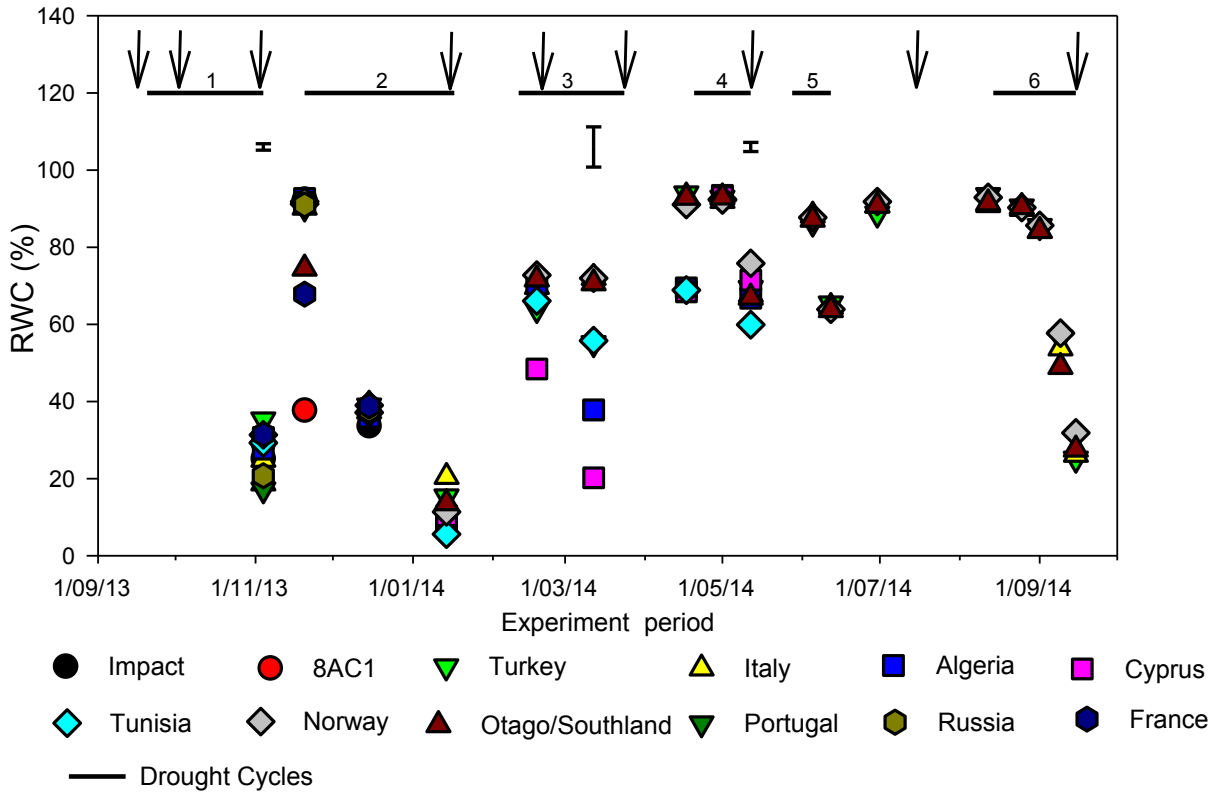


Figure 6-14 Relative water content (%) during each imposed drought cycle in the Experiment 4. Error bar is SEM for the relative water content from One-way Anova in randomized blocks and is shown where differences were detected. The ↓ indicates harvest date.

6.4.7 Solute Potential (MPa)

As for RWC, solute potential was consistently low in the accession from 'Norway' (, 'Otago/Southland' and 'Italy' during Drought Cycles 4 to 6 (Figure 6-15).

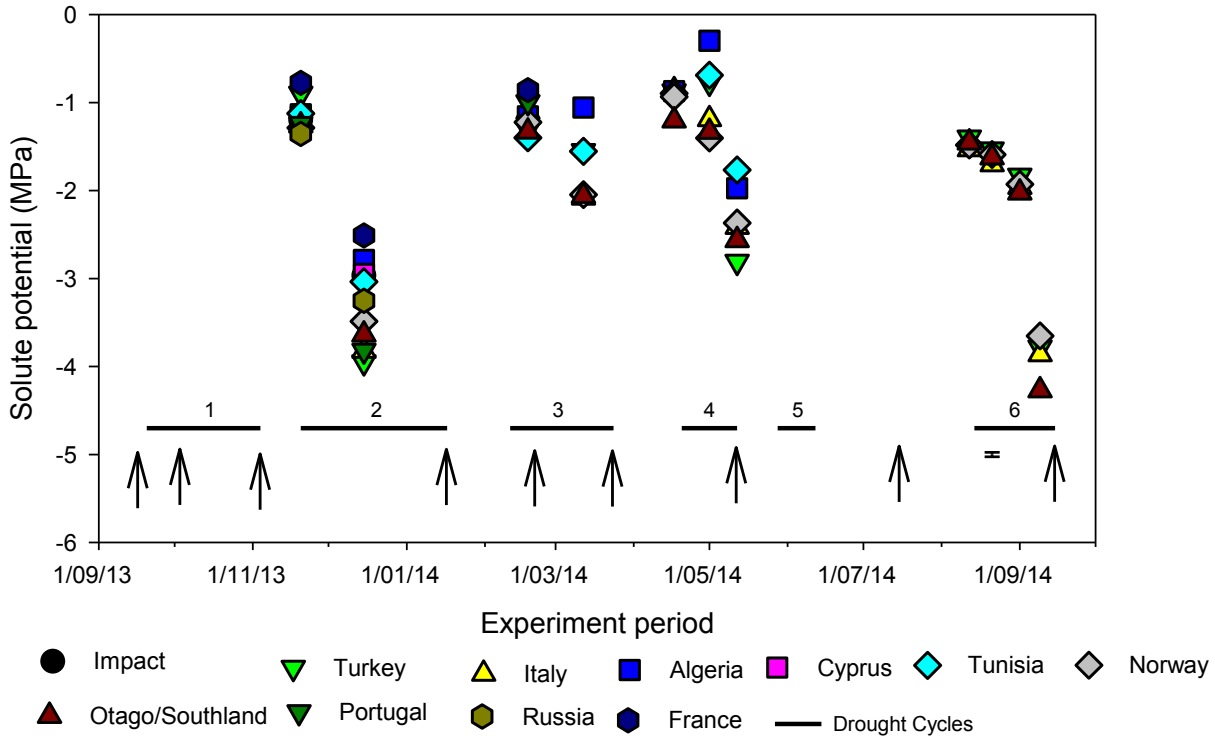


Figure 6-15 Solute potential (MPa) during each imposed drought cycle in Experiment 4. Error bar is SEM for the relative water content from One-way Anova in randomized blocks and is shown where difference were detected. The ↓ indicates harvest dates.

'Norway', 'Italy' and 'Otago Southland' had similar solute potential from Drought Cycles 3 to 6. At the beginning of Drought Cycle 6, Otago/Southland and 'Italy' had the lowest ($P<0.05$) solute potential (-1.6 and -1.1 ± 0.05 MPa). Whereas 'Turkey' and 'Norway' had the highest (-1.6 and -1.5 ± 0.05 MPa). Solute potential of these surviving accession was not different at the end of Drought Cycle 6.

Adjusted solute potential

The adjusted solute potential for the surviving accessions was calculated using relative water content and solute potential from Drought Cycle 6 (Figure 6-16).

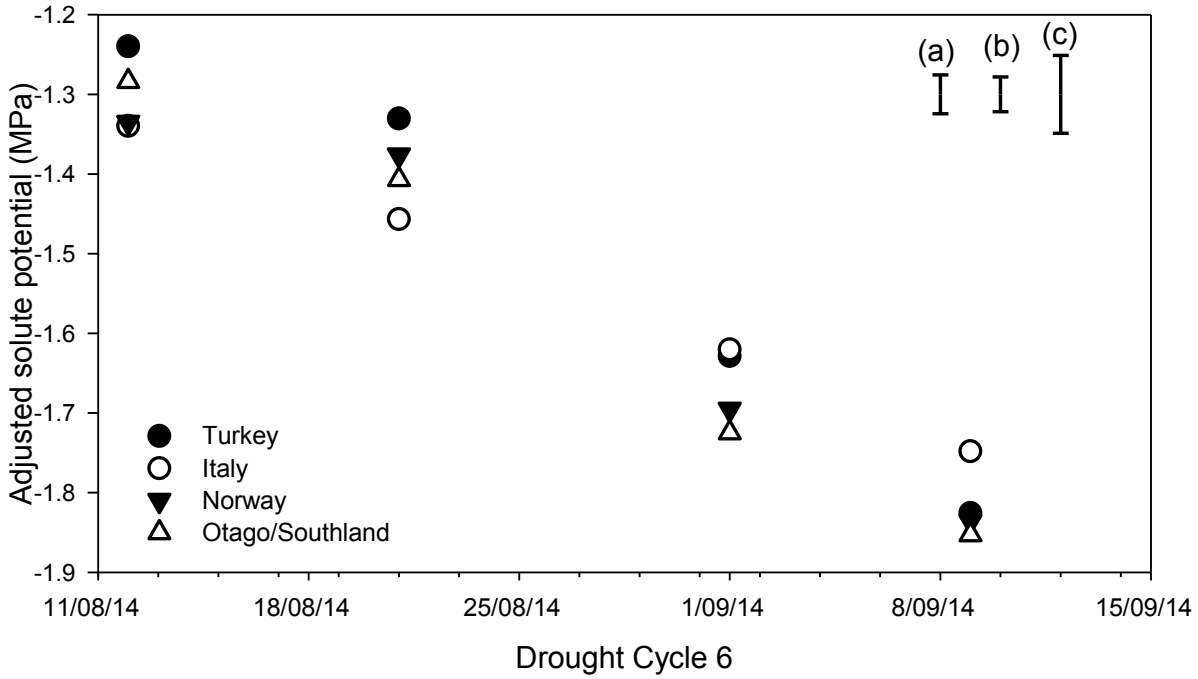


Figure 6-16 Adjusted solute potential from surviving accessions during Drought Cycle 6. Error bar is SEM for date (a) accessions (b) and date and accession (c) for the adjusted solute potential from Two-way Anova in randomized blocks.

The adjusted solute potential calculated in Drought Cycle 6 was different ($P<0.001$) as the drought progressed from 12/8/2014 to 9/9/2014, whereas there was no difference in its response among the accessions ($P=0.35$).

6.4.8 Relationships

There was an association between accumulated shoot dry matter at the end of Drought Cycle 6 and relative water content ($r=0.72$, $P<0.0.5$) after 26 days of moisture stress during Drought Cycle 6 (Figure 6-17). Accumulated shoot biomass after six stress cycles was also correlated to the adjusted solute potential ($r=-0.75$, $P<0.05$) after 26 days of drought at Cycle 6 (Figure 6-18). There was a tentative relationship of solute potential after recovery of Drought Cycle 1 with accumulated dry matter ($r=-0.56$, $P=0.08$) after six stress cycles (Figure 6-19).

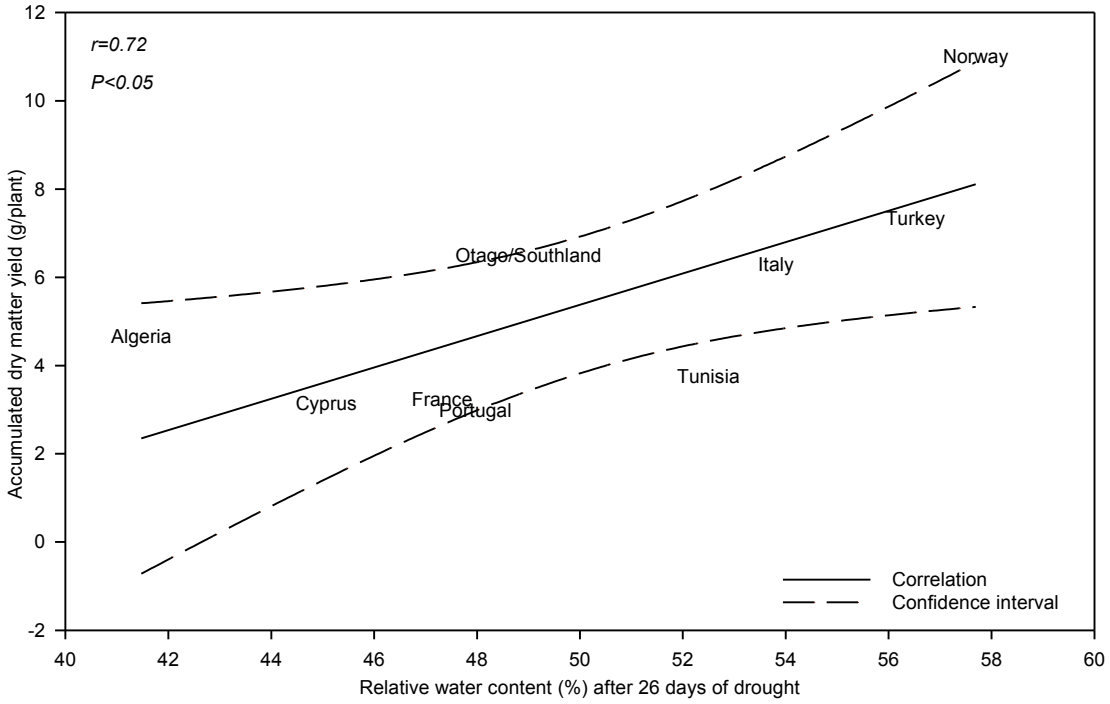


Figure 6-17 Accumulated shoot dry matter at the end of Stress Cycle 6 against relative water content (%) after 26 days of moisture stress in Stress Cycle 6.

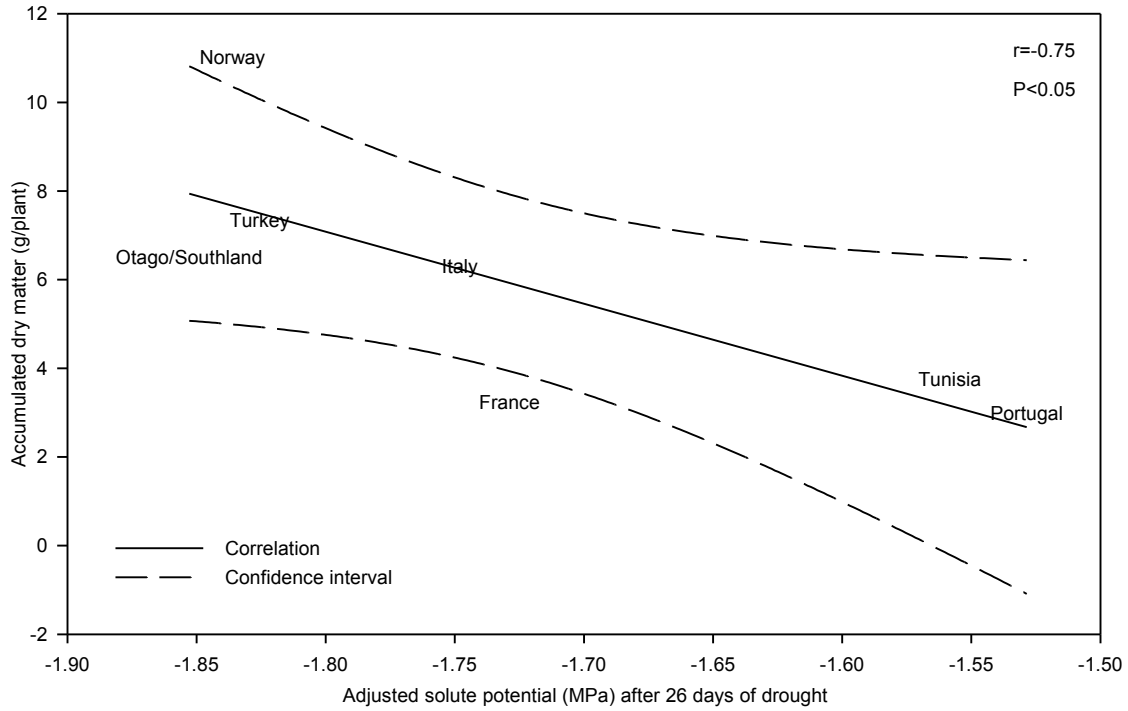


Figure 6-18 Accumulated shoot dry matter at the end of Stress Cycle 6 against adjusted solute potential after 26 days of moisture stress during Stress Cycle 6.

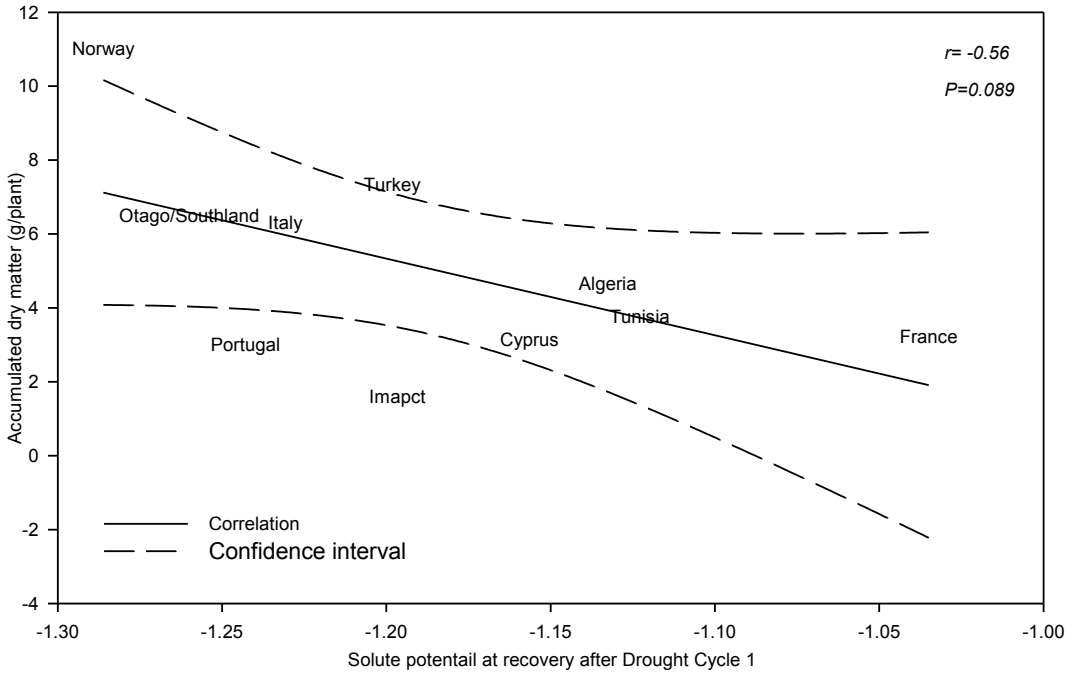


Figure 6-19 Accumulated shoot dry matter at the end of Stress Cycle 6 against solute potential after recovery from Drought Cycle 1.

6.4.9 *LpUbl5* transcript expression

The gene expression profile of different germplasm accessions (Figure 6-20) did not support the plant performance evaluated via physiology measurements.

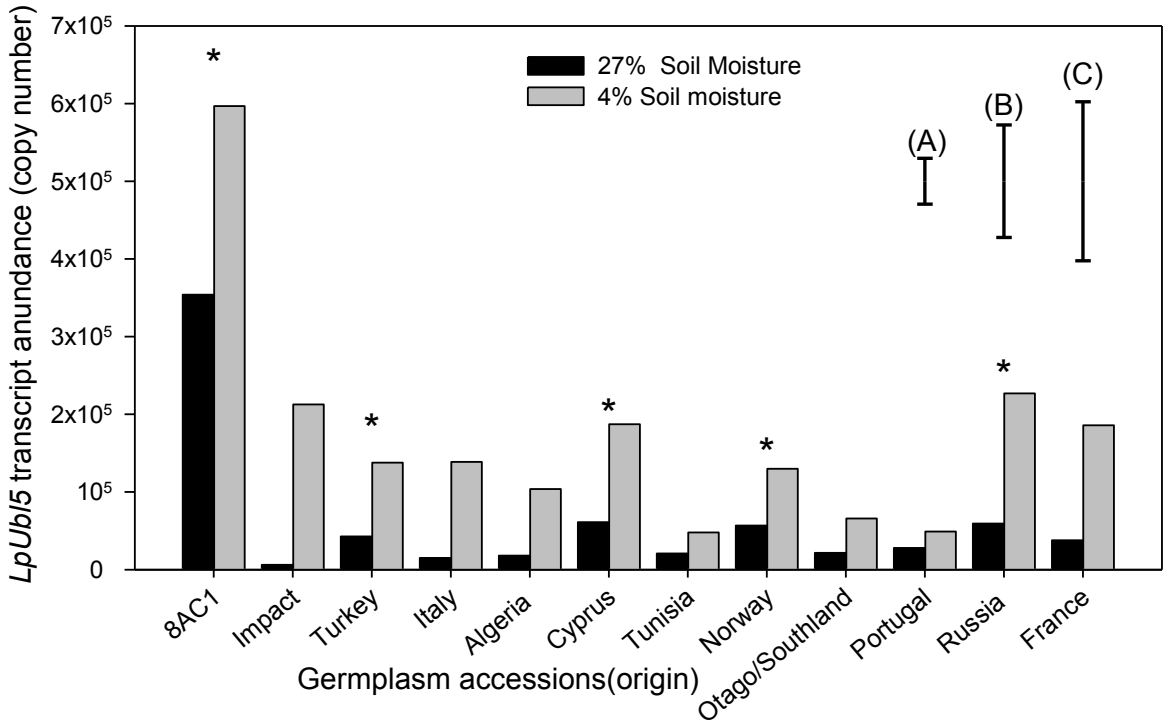


Figure 6-20 *LpUbl5* transcript abundance in 8AC1, Control (Impact) and germplasm accessions in unstressed (27% soil moisture) and under drought (4% soil moisture). The error bar shows the SEM for treatment (A) (soil moisture), Accessions (B) and treatment and accession (C) from Two-way Anova. Asterisks shown where differences (P<0.05) were observed using One-way anova using each accessions.

Two-way Anova showed that *LpUbl5* transcript level was different in accessions (P<0.05) and at 27% and 4% soil moisture (P<0.05) but there was no interaction (P=0.99). The *LpUbl5* transcript was highest in 8AC1 (475282 ± 72388 copy number) but the majority of 8AC1 plants did not survive Drought Cycle 2. Further, One-way Anova was carried out using *LpUbl5* transcript level from each accession before stress and at 4% soil moisture. 8AC1, 'Turkey', 'Norway', and 'French' had increase (P<0.05) in *LpUbl5* transcript level under 4% soil moisture.

6.4.10 Endophyte detection

Only 3 out of 68 seedlings possessed endophyte, which indicates that the drought response of the accession used for Experiment 4 were not confounded by endophyte (Table 6-2).

Table 6-2 Results from endophyte screening from the seedlings generated from the available seeds.

Accessions	Number of seedlings	Positive	Negative	% of positive
A6889	2	1	1	50
A17183	11	0	11	0
A7798	8	2	6	25
A15334	15	0	15	0
A14499	3	0	3	0
A17187	6	0	6	0
A15323	11	0	11	0
A14542	12	0	12	0
Total	68	3	65	

6.4.11 A comparison plants appearance at recovery and end of drought

A photographic representation of plants during their recovery and at the end of the Experiment 4 is used to show the visual differences in recovery between accessions and their appearance after 32 days of water withdrawal. Figure 6-21-A illustrates the rapid recovery of Norway after the 8th harvest and also shows the slow regeneration of accessions such as Tunisia, Cyprus and 'Impact'. Figure 6-21-A also shows that 'Russian Federation' and 8AC1 had no surviving plants at Drought Cycle 6. Figure 6-21-B shows the plant appearance at the end of Drought Cycle 6 (32 days) in which the high yielded accessions are marked in comparison with low yielded 'Impact'. Collectively, Figure 6-21 shows the superior performance of the 'Norway' during this final recovery and also at the end of six consecutive drought cycles with intermittent irrigation experienced over 360 days.

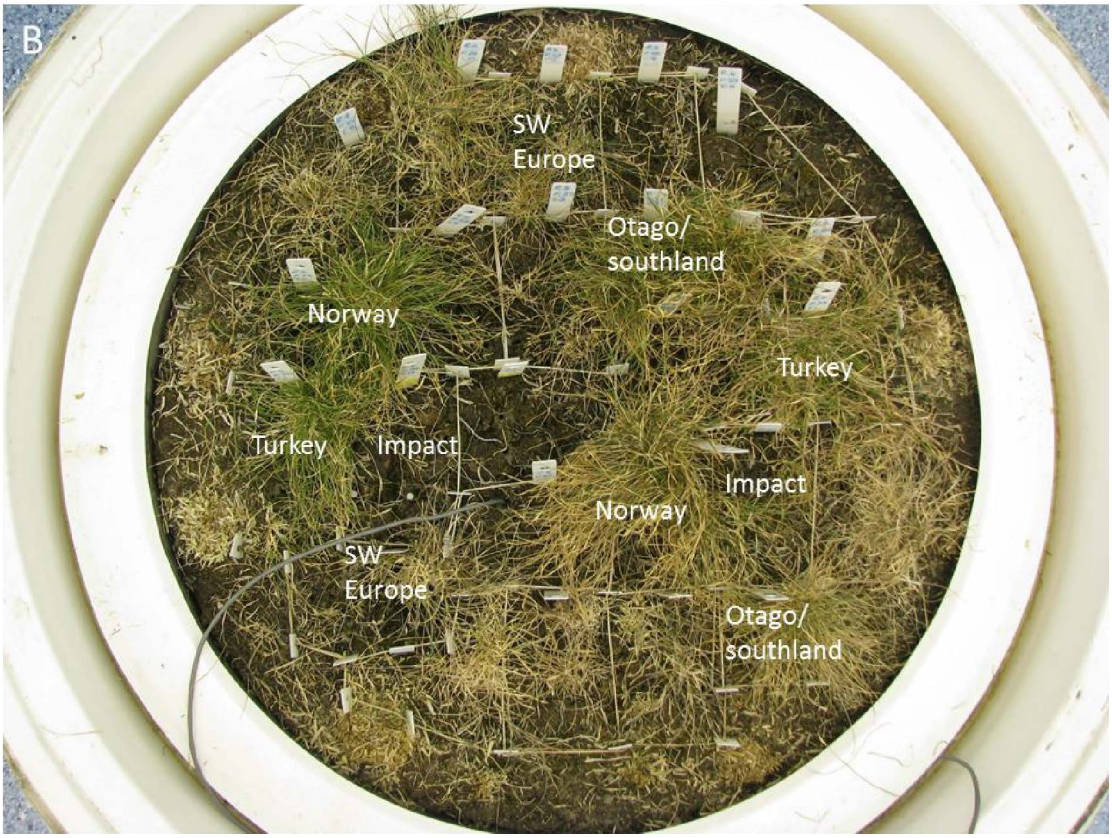


Figure 6-21 Photographs A and B taken during the recovery phase (A) and at the end Drought Cycle 6 (B) on 21/07/2014 and 15/9/2014, respectively. Rhizotron 4 is labelled with some of the high yield accessions and low yield 'Impact' to compare the recovery and response to water deficit. Photo B is taken after the removal of edge plants.

6.5 Discussion

Accessions and cultivars, selected from a wide environmental, climatical and geographical range were successfully established in two rhizotrons (Figure 6-1) which enabled six drought cycles to be imposed to evaluate the performance over 360 days.

Physiology

By the end of Experiment 4, the highest total shoot dry matter accumulated in the accession from 'Norway' (A17183). In contrast, the accession from the 'Russia' had highest shoot dry matter after the establishment phase but it did not re-generate after Drought Cycle 3. Differences in shoot dry matter among the accessions started to appear at the end of Drought Cycle 3 and where reflected by differences in morphology, such as higher leaf extension leaf extension (Figure 6-11) and leaf area in (Figure 6-13) 'Norway'. By the end of Drought Cycle 6 highest shoot dry matter accumulation in 'Norway' was reflected in the other physiological attributes such as plant survival rate (Figure 6-9), plant grades (Figure 6-10), leaf extension (Figure 6-11), leaf area (Figure 6-13).

As expected, the accessions with high shoot dry matter accumulation at the end of Experiment 4 were those with the highest survival rate and plant grades. Plant survival represented the number of regenerated plants but this does not indicate their vigour or morphological status. This was represented by their plant grade (Figure 6-10). 'Norway' had lower survival than the New Zealand accession 'Otago/Southland' at the end of Drought Cycle 6, but its growth was vigorous as indicated by higher leaf extension (Figure 6-11) and leaf area (Figure 6-13) from Drought Cycle 3. The dry matter accumulation of 'Norway' was also characterised by maintenance of higher RWC (Figure 6-14) and accumulation of solutes (Figure 6-15) through each of the drought cycles. Taken together, this suggests morphological and physiological acclimatisation to drought in 'Norway'.

At end of the Drought Cycle 2, RWC was low (Figure 6-14) in all the accessions. These measurements indicate the severity of Drought Cycle 2 at ~4% soil moisture for the 58 day of water withdrawal. The severity of water deficit used in this experiment was high when compared with other studies (Liu *et al.*, 2008; He *et al.*, 2017a; Mohammadi *et al.*, 2017). The severe drought experienced in Drought Cycle 2 and delay in re-watering were designed to mimic a drought after grazing (Section 6.1.8, Drought Cycle 3) and this appeared to contribute to the death of plants and caused a delay in the recovery phase of Drought Cycle 3 in which plant loss was also high. The accession 'Russia', did not survive this drought cycle and its tiller size, rolled emerging leaves and wider leaf blades suggests it was actually *Lolium multiflorum*.

Osmotic adjustment is a drought tolerance strategy in plants, in which active accumulation of compatible solutes decreases the solute potential and therefore promotes water uptake (He *et al.*,

2017a). The solute potential (Figure 6-15) after 30 days water withdrawal was consistent with solute potential observed after 20 to 40 days of water withdrawal in perennial ryegrass (Volaire *et al.*, 1998). At this stage, the leaf water content (Figure 6-14) was only ~40% in most accessions. It is possible that the accessions that survived, preserved their meristems and the integrity of metabolic functions by tolerating decreased leaf water content via accumulation of compatible solutes that acted as osmolytes and osmoprotectants and supported their recovery after 58 days of water withdrawal (Volaire *et al.*, 1998). Active accumulation of compatible solutes in accessions that survived through Drought Cycle 6 was confirmed by the reduced adjusted solute potential Figure 6-16.Consistently, during Drought Cycles 3-6, the accession 'Otago/southland' with highest survival rates (Figure 6-9) showed active accumulation of solutes by which plants protect the cellular components. The accumulation of compatible solutes protects the protein-synthesizing machinery against the damage caused by water withdrawal. This in turn helps to repair the damage more efficiently and rapidly than the rate of damage (Chen and Murata, 2002).

The most obvious difference in terms of dry matter accumulation was observed between 'Norway' and other accessions after Drought Cycle 3, which lasted for 41 days. During this period plants experienced a third severe drought period and were harvested twice in which the Norwegian accession had the highest dry matter accumulation (Figure 6-8). It is possible that dry matter accumulation of 'Norway' (Figure 6-7) benefitted from the short drought cycles that inadvertently occurred in Cycles 4 and 5 (Figure 6-8) which lasted only 22 and 15 days, respectively. However, the availability of water was similar for all the accessions and it is notable that the 'Norway' utilised the availability of water within short periods to generate longer leaves and more vigorous tiller generation. This resulted in its high dry matter accumulation and was consistent with the osmotic adjustment. Tiller survival ranking through winter by (Hulke *et al.*, 2007) showed that 'Norway' had a similar ranking to the top eight superior performed accessions and was included in top 10 accessions which were not different to NK200, the most winterhardy check variety they used.

Dry matter accumulation was related to relative water content (Figure 6-17) and adjusted solute potential (Figure 6-18), illustrating that sustained moisture stress tolerance in perennial ryegrass was effected by osmotic adjustment. Osmotic adjustment can be the prime adaptive trait that supports plant yield under water deficit conditions (Blum, 2016). This is further substantiated by the observation that the moisture stress-resistant perennial ryegrass accessions were able to retain more water in their leaves during water deficit (Figure 6-17) (Figure 10). These relationships suggest that reduced adjusted solute potential could be a potential screening method to identify high yielding ecotypes in the field after experiencing seasonal moisture stress. A further relationship (Figure 6-19) indicated that stress responsiveness after six drought cycles was linked to plant solute potential after Cycle 1. This suggests that solute potential could be used as an early predictor of moisture stress to identify tolerant

phenotypes before plants experience multiple drought cycles. This method could thus be incorporated into germplasm screens for moisture stress tolerance using exposure to a single moisture stress period.

This study did not record physiological measurements in Drought Cycle 1. This means we could not identify any potential mechanism by which the accessions responded to the initial drought experience. However, 'Norway' and 'Turkey' have been examined further in a subsequent glass house study in which dry matter yield, leaf extension, RWC and osmotic potential were analysed during the initial drought exposure (Wilson, 2016). In that study, 'Norway' maintained a high RWC (60%) and the solute potential was unchanged, which indicates the absence of any osmotic adjustment during the initial exposure to water deficit. In the present study, the 'Norway' showed the highest RWC and lowest solute potential from Drought Cycle 3 to 6. It is possible that this accession did not react to its initial exposure to drought cycles as quickly as others tested. Instead, it preserved its leaves and maintained them live to protect cellular components (Section 2.2.6) with its high RWC. Under subsequent drought cycles, the 'Norway' accession displayed a drought tolerance mechanism by increasing osmotic adjustment as indicated by the decreased solute potential to sustain and regenerate quickly and for longer than the other accessions tested.

Crosstalk between stress signalling

The agro-climatic zone of the SW Europe accessions (Italy and Portugal) is a Mediterranean zone. The Mediterranean zone plants experience mild winters, dry and warm summer temperatures and relatively wet winter (Bouma, 2005). In contrast to the Mediterranean zone, almost all of Norway has frozen ground for extended periods of the year and sometimes remains so until late spring, when a complete melt occurs (Tveito *et al.*, 2005). This suggests the 'Norway' experiences extended periods of cold, frost and drought in its native habitat. The cold and frozen winter with extended periods of frost exposure in Norway, means plants experience freeze induced dehydration (Guy, 2003). The mechanisms responsible for this were not part of the current study. However, there are several potential explanations that would support this hypothesis. For example, it is possible that the 'Norway' has integrated genes induced by cold that enabled it to sustain growth during the six drought cycles, and generate high dry matter yields after a 360 day experimental period. Consistently, this line showed the greatest growth under long periods of water withdrawal and comparatively rapid recovery when re-watered (Figure 6-21_A). Cold and drought-stress induce cellular dehydration protection systems such as LEA (Late Embryogenesis Abundant) proteins (Section 2.2.5). These are regulated by common DRE/CRT (dehydration responsive element/C-Repeat element) (Mizoi *et al.*, 2012). The ABA Responsive Element (ABRE) also plays a vital part in the expression of osmotic and cold stress genes. These drought- and/or cold inducible genes have identified a minimum of four independent regulatory systems for gene expression in their promoter. A number of genes are induced by abiotic stress such as drought, cold and salinity which implies a crosstalk between these stress signalling pathways. This

indicates the interaction of different cis-acting elements in their promoter (Yamaguchi-Shinozaki and Shinozaki, 2005). Apart from these, a calcium sensor CBL1 (calcineurin B-like 1 protein) plays a central role in abiotic stress stimuli (drought, cold and salt) and regulates early stress related transcription factors of the CBF/DREB (C-repeat-binding proteins/dehydration-responsive element-binding protein) type (Albrecht *et al.*, 2003). The frost prone Norwegian accession was able to survive in this drought study best, and this could be due to the cross talk between stress signalling pathways, which altered the physiological function to survive and be productive.

Endophyte Symbiosis and Drought tolerance

Analysis of endophyte symbiosis of these accessions ensured results were not confounded (Table 6-2). Only two accessions, 'Otago/Southland' and 'French' showed the presence of endophyte. Endophyte symbiosis in drought tolerance research is also an important area of discussion as there is increasing evidence of endophyte symbiosis and enhanced drought tolerance in plants (Bacon, 1993; Elmi and West, 1995; Hesse *et al.*, 2003; Kannadan and Rudgers, 2008; He *et al.*, 2017a; He *et al.*, 2017b). The presence (50%) of endophyte in the 'Otago/Southland' accession indicates plants may have benefited from the presence of endophyte, but the number of plants analysed was too low (2) to make a conclusive statement. Another high yielding accession 'Turkey' (A14499), had three plants analysed and all three were negative for endophyte. In contrast, a similar glass house study using 'Turkey' (A14499) showed 11 positive stains from 15 replicates (Wilson, 2016). The seeds for this were also from the Margot Forde Forage Germplasm Centre, AgResearch, NZ. This indicates that the low number of seeds used for screening endophyte in the present study may have failed to detect the presence of endophyte in the accessions from 'Turkey'. However, for 'Norway', 11 plants had endophyte screening and the results (Table 6-2) confirmed that the superior performance from 'Norway' under six drought cycles was not due to endophyte infection. This gives sufficient confidence to exclude endophyte symbiosis as a possible drought tolerance mechanism in 'Norway'.

LpUbl5 transcript level and drought tolerance

LpUbl5 transcript expression was increased at 4% soil moisture when compared with unstressed plants (Figure 6-20). The comparison was carried out between samples collected before imposing drought stress and samples collected when the soil moisture was 4%. The lack of an individual well watered control with similar aged plants was a disadvantage in this experiment. However, there was no relationship between *LpUbl5* transcript level and dry matter accumulation. Despite its elevation the results obtained from the *Arabidopsis* (Figure 4-24), primary transgenic lines (Figure 7-8 and Figure 7-9) and 'Impact' (Figure 7-12) do not support the role of *Ubl5* in drought tolerance for perennial ryegrass.

New Zealand breeding system

Experiment 4 indicates, the Northern Europe originated 'Norway', has potential to be a candidate in conventional plant breeding programmes for improved drought tolerance trait. However, Margot Forde germplasm centre holds 23109 accessions (pers. comm. Michelle Williamson., at Margot Forde Germplasm Centre) and this study used a limited number from which the Norwegian accession was identified. Therefore it is possible that accessions with high potential might be available in other germplasm which needs to be studied.

Conclusion

Collectively, 'Norway' (A17183) had the highest dry matter and the highest plant grade from Drought Cycles 3 to 6 which indicate its superior performance of under repeated drought cycles. This finding is consistent with its plant survival rate, leaf extension, leaf area, RWC, osmotic potential. Consistently, the variation in drought tolerance demonstrated by different germplasm accessions was not correlated with *LpUbl5* transcript abundance and therefore the study accepted the null hypothesis. These results suggest 'Norway' as a potential candidate for further investigation in breeding programmes for drought tolerance in perennial ryegrass.

Chapter 7

Analysis of *LpUbl5* gene expression in *Lolium perenne* L. under progressive drought

7.1 Introduction

The expression patterns of genes associated with drought tolerance traits are important to understand the molecular mechanisms that confer its adaptation to drought stress (Zhou *et al.*, 2014). The transcript abundance pattern of *LpUbl5* under progressive drought can be highly informative about the involvement of *LpUbl5* in drought tolerance. Chapter 3 described the physiological performance of perennial ryegrass lines under drought, whereas the transcript abundance data were not sufficiently comprehensive to understand the transcript abundance patterns of *LpUbl5* under progressive drought. The experiments carried out using transformed *Arabidopsis* using *LpUbl5* did not support the potential role of *LpUbl5* in drought tolerance (Chapter 4). Consistently, the transcript abundance patterns of *LpUbl5* in germplasm accessions were not correlated with the physiological performance of plants under imposed drought cycles (Chapter 6). The unstable transgene segregation explained in Chapter 3 led to the re-evaluation of primary transgenic perennial ryegrass lines to confirm the *LpUbl5* transcript abundance pattern under normal growing conditions and under progressive drought. Therefore it was important to understand the *LpUbl5* transcript abundance as this was reported to be increased under drought stress and supressed under water restoration (Patel *et al.*, 2015).

Based on the findings from previous Chapters, the null hypothesis was established that, "Transcript abundance of *LpUbl5* under drought or water withdrawn conditions is not different in perennial ryegrass". This hypothesis was tested using two different drought stress experiments. Experiment 5-A was carried out in a controlled environment growth room and Experiment 5-B was performed in the glass house at Lincoln University, NZ, using the perennial ryegrass cultivar 'Grasslands "Impact"'. Experiment 5-A included primary transgenic lines obtained from Dr. Kim Richardson, AgResearch, Palmerston North, NZ. The primary transgenic lines were taken from long-term culture storage and established through a series of MS media (Appendix B.2) subcultures which were then planted out into soil. The perennial ryegrass seeds for Experiment 5-B were germinated in seed plugs, which were then transplanted into soil to continue with drought stress experimentations.

7.2 Experiment 5-A

Experiment 5-A was carried out using the primary perennial ryegrass transgenic lines developed by ViaLactia Biosciences, Pastoral Genomics Consortium. The lines were maintained by AgResearch, Palmerston North, NZ in long term tissue culture.

7.2.1 Primary transgenic lines of perennial ryegrass

The growth room experiment included four different lines, 8AC1, 8AC2, 7AE5 and 7AE15. 8AC1 and 8AC2 were transformed with *LpUbl5* gene under CaMV 35S promoter. 7AE5 and 7AE15 are perennial ryegrass carrying a T-DNA containing a dehydrin promoter driving an AN1-A20 type zinc finger transcription factor. These lines were included in the experiment to substitute for wild type perennial ryegrass plants as there were no wild type perennial ryegrass plants available which been through the long term tissue culture experienced by 8AC1 and 8AC2. The transgenic status of these plants were analysed as part of presence of transgene evaluation in T_3 lines in Section 3.8.4 and 3.8.6.

7.2.2 Experiment 5-A design

A split plot design was used with the four lines and two water treatments in four replicates. Each replicate had one set of lines randomly assigned to well watered or water withdrawn treatments after an establishment period.

7.2.3 Primary transgenic establishment

The primary transgenic perennial ryegrass lines were received from AgResearch in long term culture media. The perennial ryegrass tillers were sub-cultured into MS media (Appendix B.2) in clear plastic tissue culture tubs (60 mm high x 80 mm diameter, Alto, Auckland, NZ) and were grown in the tissue culture room with 16 hours day/8hours night, at approximate of 100 $\mu mol.m^{-2}.s^{-1}$ light intensity provided by cool white fluorescent tubes, at 20°C temperature. After two weeks, the tillers were sub-cultured into MS media with 0.2-0.5 mgL^{-1} benzyl adenine (Praveena and Veeresham, 2014) and maintained in the media until transplanting to the soil for the drought experiments.

The perennial ryegrass tillers were transplanted from agar to soil. The seed pellets, 40 mm x 7 mm in height, (Jiffy-7 Pellets, Egmont seed company, NZ) were autoclaved and placed into freshly prepared MS media containing benzyl adenine (0.2-0.5 mgL^{-1}) before solidifying the agar in tubs (60 mm high x 80 mm diameter, Alto, Auckland, NZ). The perennial ryegrass tillers were sub-cultured into the seed pellets immersed in the MS media in tissue culture tub. The plants grown in the seed plugs were transplanted into soil filled 1.5 L pots (105 cm bottom diameter, 140 mm tall and 135 mm top diameter) to carry out the water withdrawn experiment.

Pots were filled with 1 kg of soil (mixture of peat and sterilized pumice in a 3:1 ratio and an additional fertiliser of Osmocote exact mini (i.e. NPK (2 g/L), dolomite (4 g/L) and hydraflo (1 g/L)) and the seed plugs with perennial ryegrass tillers were transplanted (Figure 7-1). The perennial ryegrass was grown in a growth room at 16 h day/8h night, 100 µmol.m^{-2}.s^{-1} light intensity, temperature at 20°C and the humidity between 50 -60%.

Figure 7-1 Representative photograph of primary transgenic perennial ryegrass pots with lines 8AC1, 8AC2, 7AE5 and 7AE15 in the growth room RFH026 at Lincoln University on 11/10/2016. The marked perennial ryegrass tillers for leaf extension measurements are labelled.

7.2.4 Drought Cycle 1: Primary transgenic perennial ryegrass lines

Water treatments were imposed to initiate the drought cycle after 56 days of establishment. A shoot biomass harvest was carried out 25 days after the establishment phase on 20/09/2016.

Figure 7-2 Representative photograph of primary transgenic perennial ryegrass pots with lines 8AC1, 8AC2, 7AE5 and 7AE15 in the growth room RFH026 at Lincoln University on 15/11/2016. The plants in the front raw were water withheld treatments and plants in the back raw were well watered plants.

Plants were watered continuously until 20/10/2016 and then the plants were subjected to water stress treatment for 25 days. Water was withheld from the four pots as the drought treatment and four pots were maintained as well watered controls by applying water at regular intervals. Shoot biomass was harvested on 26th day (Figure 7-2) because the plants were infected with insects (thrips). After harvest, the plants were sprayed using "Attack" (Active ingredients is 25 g/L permethrin + 475 g/L pirimiphos-methyl) insecticide in 1 mL /L of water. All pots were re-watered to recover the drought stressed plants. Leaf extension measurements were carried out during the drought cycle as explained in Chapter 3.3.3.

7.2.5 Leaf sample collection for gene expression study

Approximately 100 mg of leaf samples were collected during Drought Cycle 1 to determine *LpUbl5* transcript abundance. Samples were collected before the start of the drought treatment (20/10/2016) and the after 8 and 16 days. Samples after 24 days were not collected, as the plants were infected with thrips. A recovery sample was collected 10 days (25/11/2016) after the shoot biomass harvest on 15/11/2016. The samples were placed in Eppendorf microcentrifuge tubes (Safe-Lock tubes 2 ml, Eppendorf AG, 22331 Hamburg, Germany) and snap frozen in liquid nitrogen and subsequently stored at -80°C until RNA isolation.

7.3 Experiment 5-B

Experiment 5-B included the ryegrass cultivars 'Impact', 'KAI', 'LP37' and 'M88' as supplied by commercial companies with claims that they perform well under drought conditions. They were grown in the glass house at Lincoln University which is a computer controlled facility for temperature and ventilation. Hot air blowers, fans and vents maintain the temperature between 15°C to 24°C. The experiment had pots in six replicates each containing either a well-watered or water withheld treatment. 'Impact' was the parent line used for the transformation using *LpUbl5*. 'KAI' is a breeder's cultivar which have shown drought tolerance and is a tetraploid perennial ryegrasss (cultivar bred by Cropmark Seeds). LP37 WE and M88 WE were provided by Agriseeds, Christchurch, NZ. LP37 ('Bronsyn') was described as demonstrated heat tolerance in previous experiments conducted by Agriseeds and well proven as an adapted variety in Northern New Zealand. M88 ('Barberia') was described as a winter active, summer dormant perennial categorised as a *Lolium multiflorum* which uses drought avoidance (Information supplied by Agriseeds, NZ).

7.3.1 Media preparation

A soil shredder was used to sieve out stones and large pieces of plant materials from a Templeton silt loam soil (Cox, 1978) extracted from Iversen field, Lincoln University. The soil was prepared by mixing soil with sand in a 4:1 ratio (soil: sand by volume). 10 L pot were filled with the 10 kg of the prepared soil mix and weighed to confirm uniformity. The soil mix was watered until water started to drain out through the bottom of the pots before planting the seedlings.

7.3.2 Experiment 5-B design

Experiment 5-B was designed in randomized blocks. Pots were arranged in six replicates which had two pots of two different treatments either as watered or water withheld (Appendix E.6). Each pot was divided into four quarters and each quarter had three plants of the same cultivar. Pots were weighed prior to any water application and after application and transplantation. The water loss was measured by pot weight throughout the course of the experiment.

7.3.3 Seedlings establishment

The seed plugs were used for germination and establishment of seeds. A seed plug was sown with 4-5 seeds which were thinned after germination to leave a single seedling for replanting. The seedlings were maintained well watered until transplantation (Figure 7-3).

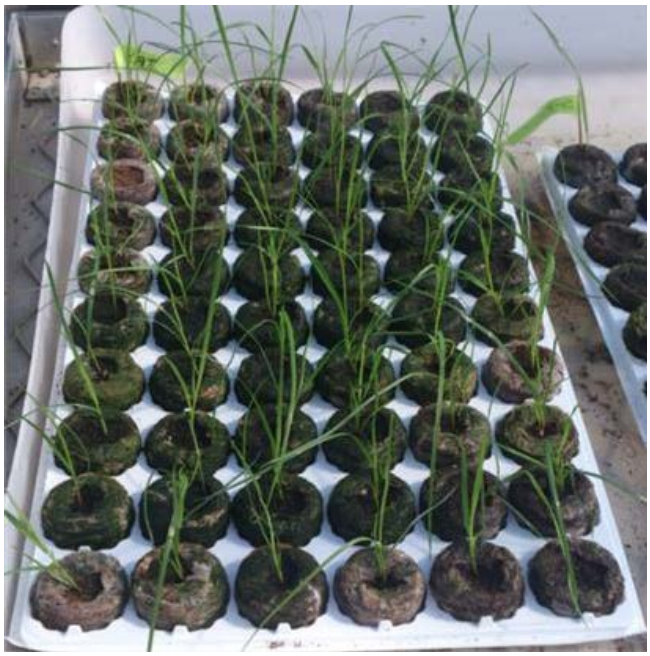


Figure 7-3 The perennial ryegrass seedlings for Experiment 5-B in the seed plug trays in the glass house on 4/12/2015 at Lincoln University.

The seedlings were grown in the same glass house used to carry out the experiment. The seedlings were maintained in the seed plugs for 35 days before transplantation to the pots. The number of tillers were counted before transplanting where each plant had 3-6 tillers. The leaves were trimmed before transplantation and seedlings moved to the pots after opening the seed plug outer cover to help facilitate the extension of roots to the soil in the pots (Figure 7-4).

Figure 7-4 Twelve pots with perennial ryegrass at the establishment period of Experiment 5-B in the glass house on 15/01/2016 at Lincoln University. Each pots contained four cultivars.

7.3.4 Drought Cycle 1: Experiment 5-B

Shoot biomass was harvested after 27 days of an establishment period and then Drought Cycle 1 was initiated. Water was withheld from the designated pots while regular watering continued in the well-watered controls. The water treatment (Figure 7-5) was continued for 20 days during which leaf samples were collected as described previously (Section 7.2.5). Unstressed leaf samples were collected before harvest and drought samples were harvested after 10 days of re-growth and after 18 days of re-growth after first shoot dry matter harvest (15/1/2016). Recovery samples were collected 14 days after the harvest (4/2/2016) at the end of Drought Cycle 1. Leaf extension measurements on marked tillers were carried out at twice weekly to quantify the impact of water treatments (Section 3.3.3).

Figure 7-5 Pots showing the difference in plant growth after the initiation of water treatment between well watered pots and water withdrawn pots in the glass house on 4/2/2016 at Lincoln University.

7.3.5 Transcript expression by qRT-PCR

Leaf samples were collected from Experiments 5-A and 5-B. The lines selected from Experiment 5-A to analyse transcript expression were 8AC1 and 8AC2 with the transgene and endogenous *LpUbl5* and 7AE5 with endogenous *LpUbl5* transcript expression. The perennial ryegrass cultivar used for transcript expression study from Experiment 5-B was 'Impact' to analyse endogenous *LpUbl5* transcript expression. The selected samples were ground and the RNA was isolated from the ground leaf material. RNA isolation, quality analysis, cDNA synthesis and qRT-PCR were carried out as described in Section 3.4.6. The primers used for qRT-PCR are shown in Table 7-1.

Table 7-1 Primers used for qRT-PCR of perennial ryegrass samples collected from growth room and glass house experiment to amplify *LpUbl5* and two reference genes.

Name	Sequence	Amplicon
*LpUbl5*_FWD	ACAAGGACCACATCACCCTCGGC	
*LpUbl5*_RVS	GGACTCGAGCTCTACTACAACTAG	71 bp
eIF4A Forward	AAC TCA ACT TGA AGT GTT GGA GTG	
eIF4A Reverse	AGA TCT GGT CCT GGA AAG AAT ATG	168 bp
eEF1A (s) Forward	CCG TTT TGT CGA GTT TGG T	
eEF1A (s) Reverse	AGC AAC TGT AAC CGA ACA TAG C	113 bp

7.4 Statistical analysis

Data from Experiment 5-A and 5-B were analysed using the Genstat Statistical Package. Shoot dry matter was examined with the REML, procedure with data in one variate. Leaf extension data were analysed using Two-way Anova and the *LpUbl5* transcript level data were analysed using One-way Anova between treatments at each time point.

7.5 Results

7.6 Experiment 5-A

Experiment 5-A was carried out using primary perennial ryegrass transgenic lines, 8AC1, 8AC2, 7AE5 and 7AE15 in the growth room. The pot weight (Appendix C.12) differed by day 8 ($P<0.05$), 16 days ($P<0.001$), 25 days ($P<0.001$) and at recovery ($P<0.05$) indicating the reduction in water content in water withdrawn pots.

7.6.1 Dry matter from Experiment 5-A

Shoot dry matter was obtained on 20/09/2016 after 25 days of establishment and on 15/11/2016 after regrowth and water treatment (56 days). Total dry matter (Figure 7-6) was obtained as repeated measurements from the same pot at each time point from well watered and water withheld treatments. As expected, the treatment had an effect ($P<0.001$) on dry matter with the highest dry matter in well watered plants (5.1 ± 0.27). There was also an effect of date ($P<0.001$) and interaction between date and water treatments ($P<0.001$).

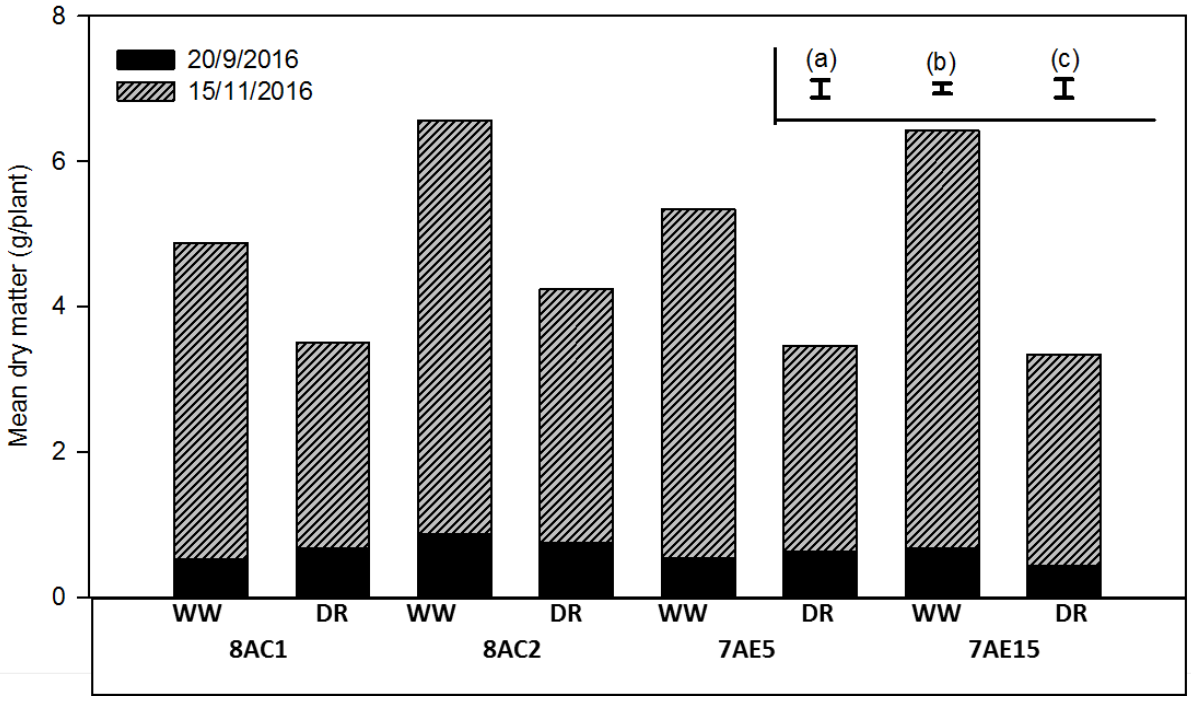


Figure 7-6 Total dry matter accumulation from four primary transgenic lines (8AC1, 8AC2, 7AE5 and 7AE15) after establishment (20/9/2016) and after water treatment (15/11/2016). Water treatments were well watered (WW) and water withheld (DR) treatments. Error bars shown are the SED for treatment (a), dates (b) and treatment and date (c).

7.6.2 Leaf extension from Experiment 5-A

Leaf extension measurement started 21 days after first harvest (20/9/2016). Leaf extension (Figure 7-7) was analysed using Two-way Anova. Leaf extension was different ($P<0.05$) between water treatments by 4/11/2016. This pattern continued through 7/11/2016 ($P<0.001$) and 11/11/2016 ($P<0.001$).

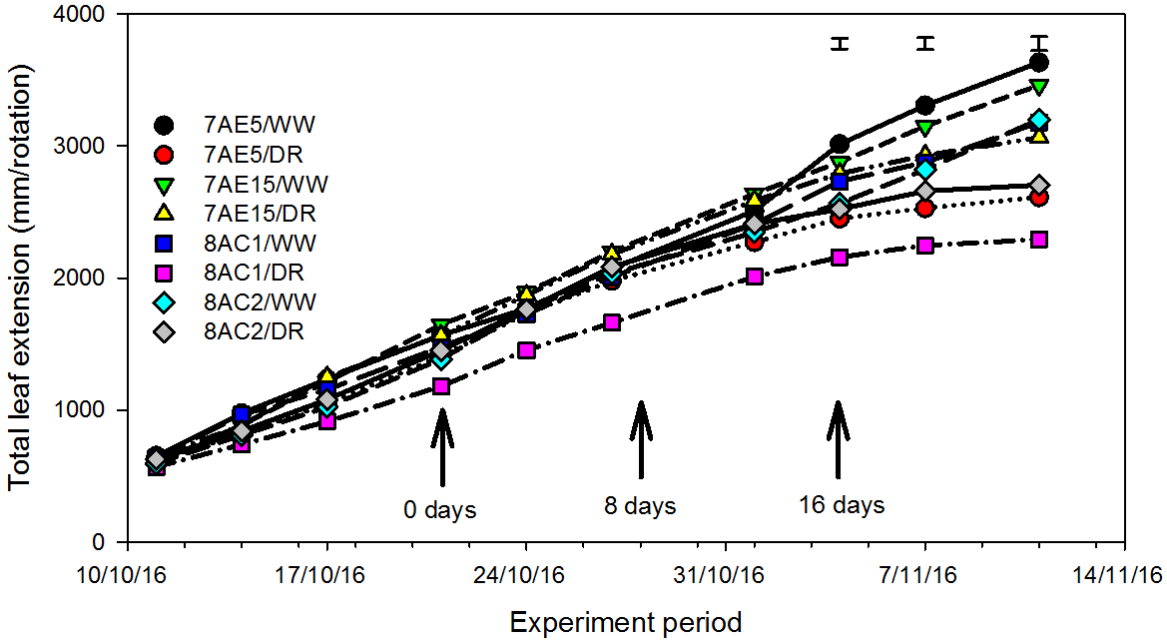


Figure 7-7 Leaf extension during the experiment period from well watered (WW) and water withheld (DR) treatments of primary transgenic lines (8AC1, 8AC2, 7AE5 and 7AE15). Error bar shown were differences observed and is the SEM for the treatments. ↑ indicates sample collection dates for *LpUbl5* transcript level analysis during water treatments.

7.6.3 Transcript abundance from Experiment 5-A

LpUbl5 transcript level (Figure 7-8 and Figure 7-9) and pot weight (Appendix C.12) of each line was analysed using One-way Anova at 0 days, 8 day and 16 days. *LpUbl5* transcript level in 8AC1, 8AC2 and 7AE5 was not different (8AC1; $P\geq0.15$, 8AC2; $P\geq0.47$, 7AE5; $P\geq0.60$) between water treatments at 0 days, 8 days, 16 days and recovery.

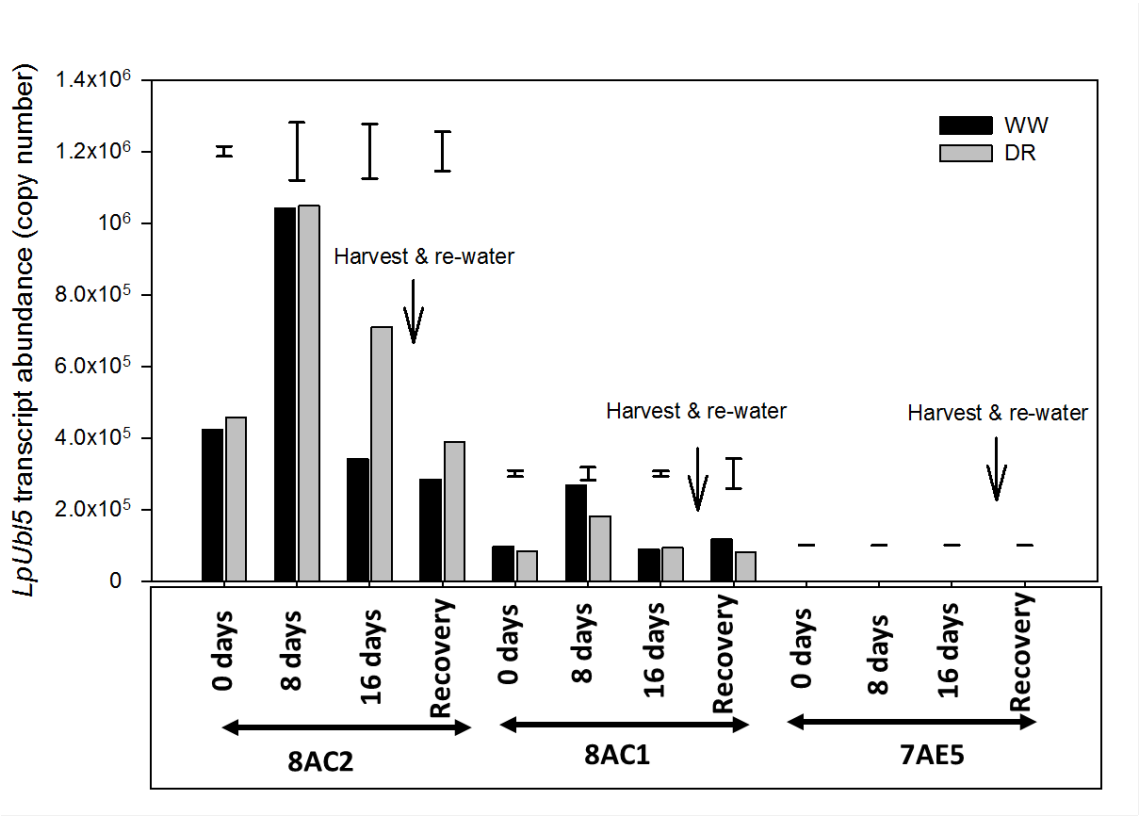


Figure 7-8 *LpUbl5* transcript abundance in 8AC2, 8AC1 and 7AE5 plants for Experiment 5-A in well watered (WW) and water withheld drought (DR) pots. Error bar shown is the SEM for the treatment. Shoot dry matter harvest was carried out on the 26th day of water treatments and re-watered for 10 days to collect recovery sample.

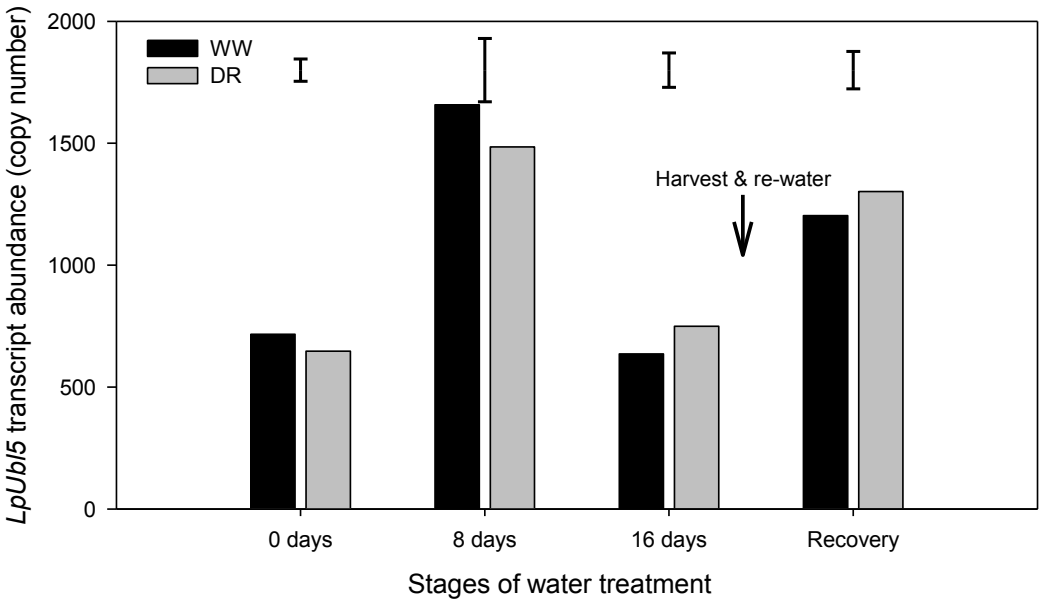


Figure 7-9 *LpUbl5* transcript abundance in 7AE5 plants for Experiment 5-A in well watered (WW) and water withheld drought (DR) pots (Shown separately as the scale is small and not visible in Figure 7-8). Error bar shown is the SEM for the treatment. Shoot dry matter harvest was carried out on the 26th day of water treatment and re-watered for 10 days to collect recovery sample.

7.7 Experiment 5-B

Experiment 5-B was carried out using the ryegrass cultivars 'Impact', KAI, LP37 and M88 in the glass house. The results are explained in the following sections.

7.7.1 Shoot dry matter from Experiment 5-B

Shoot dry matter harvests (Figure 7-10) occurred after the establishment phase on 15/1/2016 (27 days) and at the end of drought treatments on 4/2/2016 (20 days). The data were analysed using REML of data in one variate. There was an effect ($P<0.001$) of date and water treatment ($P<0.01$) and an interaction ($P<0.001$) between date and water treatment.

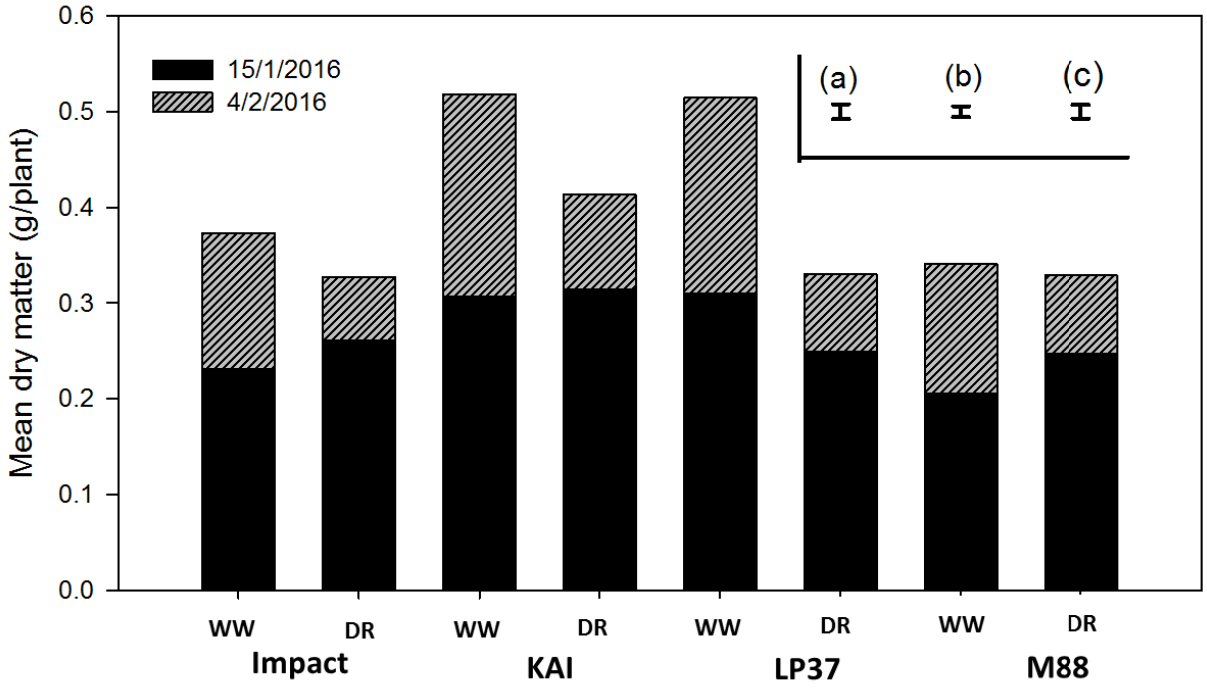


Figure 7-10 Mean shoot dry matter from Impact, KAI, LP37 and M88 plants under well watered (WW) and water withheld drought (DR) conditions in Experiment 5_B. Error bar shown are the SED for the date (a), treatments (b) and date and treatments (c).

Shoot dry matter (0.26 ± 0.015) was highest ($P<0.001$) after the establishment phase (15/1/2016) when compared with dry matter (0.12± 0.015) at the end of water treatment (4/2/2016). Shoot dry matter from well watered and water withheld plants were 0.26 ± 0.011 and 0.27 ± 0.011 at the end of the establishment phase. At the end of water treatments they were 0.17 ± 0.011 and 0.08 ± 0.011, respectively.

7.7.2 Leaf extension from Experiment 5-B

Leaf extension (Figure 7-11) was analysed using Two-way Anova. Leaf extension differed ($P<0.05$) among cultivars on 22/1/2016 and 25/01/2016 with the longest leaf elongation in KAI (289 ± 14.5 and 384 ± 16.6). A difference ($P<0.05$) between water treatments started from 25/01/2016 (9 days of water treatment) and continued through until the 4/2/2016 ($P<0.001$).

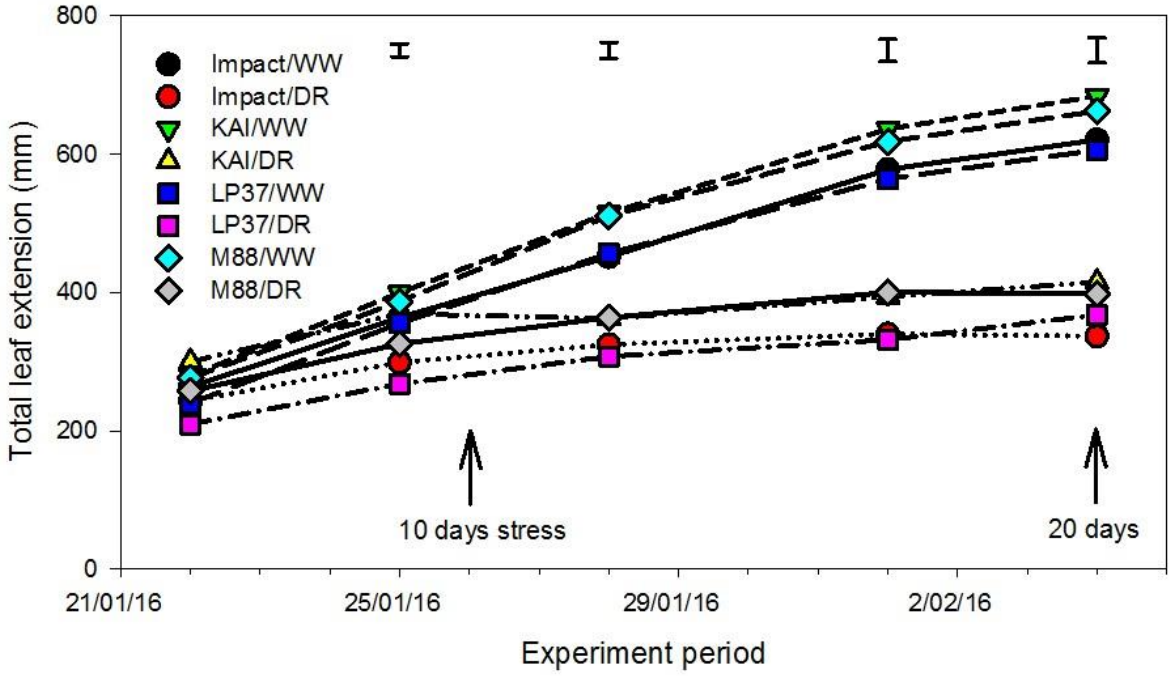


Figure 7-11 Leaf extension period from well watered (WW) and water withheld (DR) treatments of different cultivars (Impact, KAI, LP37 and M88) in Experiment 5_B. Error bar show when differences occurred and is the standard error of the mean. ↑ indicates sample collection dates for *LpUbl5* transcript level analysis.

7.7.3 *LpUbl5* gene expression in Experiment 5-B, Impact

Pot weight (Appendix C.13) and *LpUbl5* transcript abundance (Figure 7-12) in 'Impact' were analysed using One-way Anova in randomised blocks. The pot weight was different ($P<0.001$) after 10 days stress (25/1/2016) and this continued until plant were re-watered ($P<0.05$). *LpUbl5* expression in 'Impact' was not different ($P\geq0.39$) between watered and water withheld treatment on any of the measurement dates nor after recovery

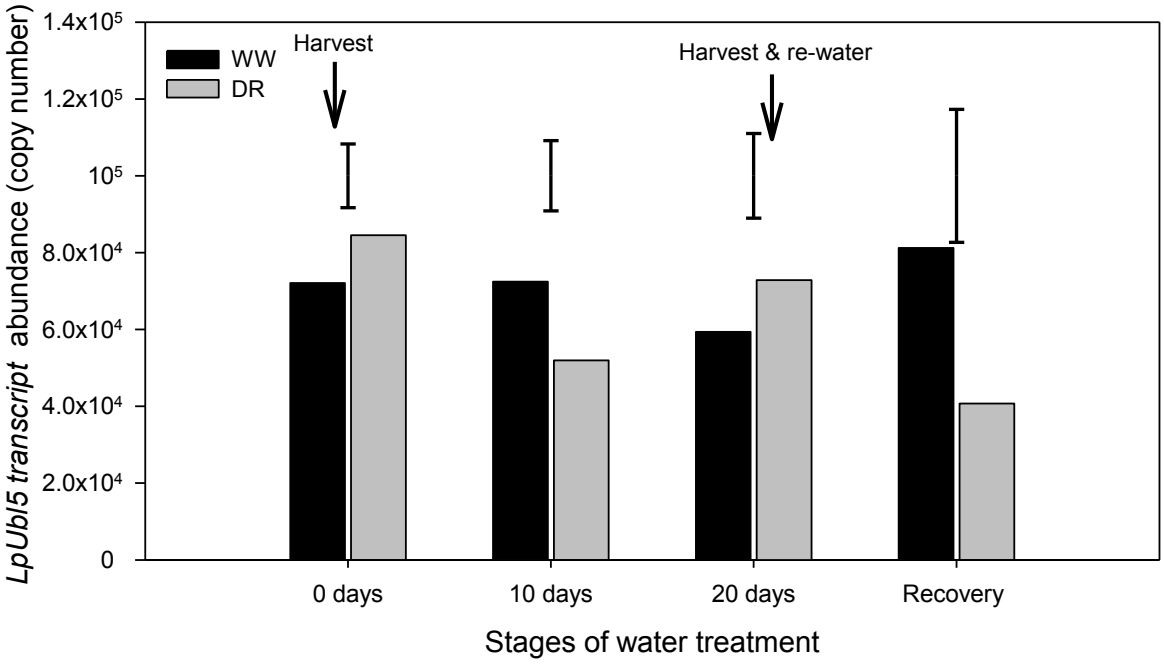


Figure 7-12 *LpUbl5* transcript expression in Impact from well watered (WW) and water withdrawn drought (DR) pots. Error bar shown is the standard error of the treatments. Recovery samples were collected 14 days after harvest and re-water. ↓ shows the harvest before initiating water treatment and after 20 days of water treatment.

7.8 Discussion

Two experiments were set up to study the *LpUbl5* transcript expression of perennial ryegrass under different water conditions: experiment 5-A (Expt 5-A) in a growth room and experiment 5-B (Expt 5-B) in the glass house to more closely mimic field conditions. These were done to measure *LpUbl5* gene expression, and to ensure that *LpUbl5* transcript expression in previous chapters had not been compromised by the controlled environment conditions.

The hypothesis, "Transcript abundance of *LpUbl5* under drought or water withdrawn conditions is not different in perennial ryegrass" was tested by analysing the transcript expression data from Expt 5-A and Expt 5-B and the null hypothesis was accepted. The shoot dry matter harvest (Figure 7-6, Figure 7-10) from both the experiments showed an effect of water treatment. Consistently, leaf extension rate was also lower under water withdrawn conditions. These results confirms that water was limited in the water withheld pots which limited leaf extension, which in turn resulted in reduced dry matter yield. *LpUbl5* transcript expression in 8AC1, 8AC2 and 7AE5 obtained from Expt 5-A (Figure 7-8) and transcript expression in 'Impact' from Expt 5-B (Figure 7-12) did not show any systematic difference between the water treatments. Both 8AC1 and 8AC2 showed the endogenous and transgene *LpUbl5* transcript expression was not different between the water treatments. Similarly, the endogenous *LpUbl5* transcripts expression from 7AE5 was also not different between the water treatments. This indicated that the water stress experienced by plants did not result in increased endogenous or transgenic *LpUbl5* transcript expression.

However, an increase in gene expression was observed after 8 days of which was common in both the treatments and in all three sample types (8AC1, 8AC2 and 7AE5). The samples were collected on 28/10/2016 under standard growth room conditions and no variation was observed. Therefore, the causative factor of the increase in *LpUbl5* transcript expression in both the treatments on day 8 is unknown. 8AC1 and 8AC2 had double *CaMV 35S* promoter driving *LpUbl5* gene and expect to have similar level of *LpUbl5* transcript level due to the constitutive nature of this promoter. The increase observed in 7AE5 which was considered as wild type and thereby representing the endogenous *LpUbl5* transcript abundance. Therefore the effect may not due to the effect of *CaMV 35S* promoter which is known to affect genes thousands of base pairs up-stream or down-stream of the insertion site on the same chromosome and on other chromosomes (Chen *et al.*, 2013). This increase did not sustain till 16 days. Transcript abundance from 24 days of water treatment could not be measured due to Thrips infection and *LpUbl5* transcript abundance on day 24 is not available to identify a periodic variation in gene expression. In addition, these plants were regenerated from long term culture conditions in MS medium without hormones into normal growing condition in MS medium containing hormones to initiate growth. Plants were transferred to soil for water treatments to study the drought response.

The effect of the long term culture on these plants are also unknown. Apart from these, it is possible that this increase can be linked to any biological functions such as leaf appearance which is dependent on temperature, light intensity and defoliation. The leaf appearance rate is 7 days in perennial ryegrass but changes in winter and late spring to 14 days and 5 days respectively (Duble, 2001). Fifteen days interval of leaf appearance was also reported (Vine, 1983). However this present study has neither recorded leaf appearance rate nor evidence to relate the increased *LpUbl5* transcript abundance to any biological functions.

A comparison of present results with previous work on *LpUbl5* in perennial ryegrass is essential when interpreting the *LpUbl5* transcript expression obtained from Expt 5-A and 5-B. Previous published research has also shown that *LpUbl5* was increased 2-3 fold under 8 days of drought stress. However the control to which those data were compared was the samples from well watered conditions before imposing drought stress. The study did not include a well-watered control during the drought stages. Those results led to the hypothesis that overexpression of *LpUbl5* resulted in improved drought tolerance (Patel *et al.*, 2015). An increase in *LpUbl5* transcript expression after 8 days of water treatments was also observed in Experiment 5-A. However, this was not result of the drought treatment as this increase in *LpUbl5* transcript expression also occurred in the well watered controls. In addition, the *LpUbl5* transcript copy number was analysed from the samples collected from the same plants at different stages of water withdrawal. The *LpUbl5* transcript expression data were compared over time between the treatments at each stage. Results indicated that variation occurred in gene copy number at each measurement date but this was not different to well watered control plants. However, an increase in *LpUbl5* transcript abundance in 8AC2 (Figure 7-8) was observed after 16 days. This elevation occurred from one out of three replicate and was not significantly different. This confirms that the *LpUbl5* transcript copy number variation was not associated with the water limiting conditions.

The *Ubl5* homologue in Arabidopsis (*AtUbl5*) expression was reviewed in Chapter 2.5.1 which was not consistent to relate its role in drought tolerance. Similarly, the Ubl5 homologue in APR wheat plants (*TaUlp5*) was reported to increase in wheat seedlings infected with *Puccinia striiformis* f. sp*. tritici* (*Pst*) (Feng *et al.*, 2016). Consistently, the *LpUbl5* endogenous transcript expression from 'Impact' which used as the parent line for generation of transgenic lines, also did not differ in transcript expression between the water treatments at any stage. The sample selections were limited to 'Impact' as this was the parent line used for generation of primary transgenic lines and T_3 generation of same transgenic lines used in the Experiment 1 in Chapter 3. The initial plan was to increase the number of cultivars if the *LpUbl5* transcript expression rejected the null hypothesis to then understand the *LpUbl5* transcript expression in different cultivars. However both experiments supported the null hypothesis

that “Transcript abundance of *LpUbl5* under drought or water withdrawn conditions is not different in perennial ryegrass”.

Collectively, the *LpUbl5* transcript expression was also not associated with increased drought conditions as the progress in the water withheld treatment did not show an increase in *LpUbl5* transcript expression in transgenic and ‘Impact’ (wild type) perennial ryegrass. Future studies are required to understand the functions of *Ubl5* genes in plants but results of this study suggest it is unlikely to have any role in drought tolerance.

Chapter 8

General discussion

8.1 Research Summary

As discussed in Chapter 2, perennial ryegrass is the most commonly sown grass in New Zealand farms. However, it suffers from poor persistence under summer drought conditions. Enhanced drought tolerance has been the focus of research for several years (Section 2.3). The PhD research in this project undertook an independent analysis of transgenic lines which had been transformed and molecularly characterised by ViaLactia Biosciences Ltd. This approach used the *LpUbl5* gene, to define its role in conferring drought tolerance. Initially a physiological evaluation of genetically modified perennial ryegrass was undertaken. The results from the transgenic lines modified using *LpUbl5* driven by a double *CaMV 35S* promoter generated an unexpected result. There was no influence of *LpUbl5* over-expression in tolerance to drought and the data were highly variable. Further investigation showed that the data were generated from a mixed population of plants that indicates not all these plants contained a functional transgene due to a break in the T-DNA (Chapter 3). Therefore, *Arabidopsis*, a model dicot plant with short life cycle, was transformed with *LpUbl5* and generated homozygous progeny plants to unravel its role in conferring drought tolerance. *Arabidopsis* was modified using *LpUbl5* driven by the *CaMV 35S* promoter. Its physiological response was also compared under water withheld conditions and fully watered conditions (Chapter 4). Further, the study confirmed that *Lp*UBL5 was targeted to the same sub-cellular space both in the dicot *Arabidopsis* as in the monocot perennial ryegrass and indicated its potential role in alternative splicing. To take part in alternative splicing, UBL5 needs to be localized within the nucleus to interact with splicing machinery. To ascertain the subcellular localization, transient transformation of the monocot, onion (*Alium cepa* L.), epidermal cells and the dicot, *Nicotiana benthamiana*, with expression vectors carrying a gene construct capable of expressing *Lp*UBL5 fused to a modified Green Fluorescent Protein (GFP) at the carboxy terminal was carried out. This experiment confirmed that LpUBL5 was indeed targeted to identical sub-cellular spaces in both monocot and dicot cells and the nuclear localization of *LpUbl5* indicated its potential role in alternative splicing (Chapter 5). To identify a germplasm accession to incorporate in breeding programs for drought tolerant trait to further study *Ubl5* in drought tolerance, the research programme was extended to screen selected perennial ryegrass germplasm accessions from the Margot Forde germplasm centre (Chapter 6). The study included several hypotheses to address the research questions (Section 2.9). This general discussion examines these research questions and the hypotheses tested using each experiment.

8.1.1 Research question 1

- Does over-expression of *LpUbl5* occur in ryegrass and does it provide enhanced drought tolerance in the T_3 generation of transgenic ryegrass lines? Has the generation of T_3 lines in a self-incompatible species been successful?

In Chapter 3, an independent analysis of T_3 generation of perennial ryegrass transgenic lines was carried out. The transgenic status of the given plants were generated by ViaLactia Biosciences (NZ). Experiment 1 was carried out to identify any potential role of *LpUbl5* in enhanced drought tolerance through measurement of physiological parameters and *LpUbl5* transcript abundance. Analysis of the relationship between the physiology data and transcript abundance data in response to repeated drought were inconclusive. This led to analysis of the *LpUbl5* transgene status in the T_3 ryegrass lines.

Physiological analysis of the supplied transgenic lines was carried out to determine the relationship, if any, between the over-expression of UBL5 and potentially increased tolerance to repeated cycles of drought stress. The data obtained did not support preliminary reports of the impact of UBL5 over-expression in response to repeated drought cycles (Patel *et al.*, 2015). Further analysis of the levels of transgene expression in the lines provided pointed to an issue with the expression of the transgene within the supplied transgenic lines. Careful determination of the presence or absence of the *LpUbl5* transgene revealed surprising structural variation in the transgene locus for lines 8AC2+ and 8AC2-.

Discussions with ViaLactia Biosciences (NZ) (Personnel Com. Sathish Puthigae) revealed that selection of transgenic lines and tracking of the transgenic T-DNA through repeated rounds of backcrossing was achieved through PCR analysis of the hygromycin resistance gene. Southern analysis using *hptII* coding sequence was performed on the T_0 transgenic lines to identify lines containing a single insertional locus (Personnel Com. Sathish Puthigae). This method failed identify any break in the T-DNA which resulted in plants with parts of T-DNA. It is also likely, that at some point in the production of the homozygous lines provided for analysis that either a deletion or recombination event has occurred at a point between the two opposed double *CAMV 35S* promoters. This lead to the loss of the *LpUbl5* transgene while retaining the hygromycin resistance gene. This rare event has gone unnoticed in the production of the seed batches used in this thesis. This highlights the need to monitor the integrity of the T-DNA cassette in transgenic lines.

Discussions with ViaLactia Biosciences (NZ) (Personnel Com. Sathish Puthigae) has revealed that, due to the large numbers of different transgenic experiments being controlled at the same timing, a decision was made to utilise a common target for diagnostic PCR to track transgenic events. This was used in the numerous lines under development at the time. My work has highlighted that this isn't necessarily the safest route to track the key transgene component. Analysis of the primary transgenic

lines indicated that these lines did indeed contain at least a single intact T-DNA. Events that led to an apparent segregation of the *Ubl5* transgene from the hygromycin resistance gene remain unclear.

In transgenic plants, homozygosity is a highly desirable character which confirms the stable integration and inheritance of the transgene (Passricha *et al.*, 2016). The present study used 8AC2 lines which were heterozygous as homozygosity was never achieved (Personnel Com. Sathish Puthigae). This heterozygosity could suggest potential transgene instability which was not identified at earlier generations. *CaMV 35S* promoter is a convenient and most widely used constitutive promoter. However the presence of a recombination hotspot and over use of this promoter (Chen *et al.*, 2013) could be potential reasons for this T-DNA instability. It equally could be that there were intact and incomplete T-DNA insertions at the locus studied by Southern analysis. During the production of the homozygous single copy lines it is likely that these have segregated leaving some plants with an intact T-DNA and some plants with the broken copy. Determination of transgenic plant status using the presence or absence of a selectable marker (*hptII* gene) compromised the experiment.

Genetic transformation is a rapid and powerful tool to alter the plant genome for crop development. Understanding of segregation distortion frequency and its origin, plays a substantial role when breeding transgenic varieties (Passricha *et al.*, 2016). It is also vital to understand the transgene stability in subsequent generations (Armstrong *et al.*, 1995; Jensen *et al.*, 1998; Chareonpornwattana *et al.*, 1999; Demeke *et al.*, 1999; Horvath *et al.*, 2001; Choi *et al.*, 2003; Park *et al.*, 2012). The transgenic line 8AC1 was homozygous for the transgene but did not show enhanced drought tolerance over six consecutive drought cycles. It is possible that transgene insertion in 8AC1 resulted in the disruption of a functional gene leading to a mutant phenotype of the trait controlled by the locus as has been described previously (Bhat and Srinivasan, 2002). Transgenic approaches in self-incompatible and outcrossing ryegrass species have been reported to enhance freezing tolerance (Hisano *et al.*, 2004), salt tolerance (Wu *et al.*, 2005; Cen *et al.*, 2016), drought tolerance (Zhang and Zheng, 2008) or produce hypoallergenic ryegrass (Petrovska *et al.*, 2005), virus resistance (Xu *et al.*, 2001), and high fructan content (Gadegaard *et al.*, 2008) using primary transgenic ryegrass lines. However, no reports have been found with reports of screening subsequent generations of these transgenic lines and nothing has been commercialized (Smith and Spangenberg, 2016). In contrast, 'Roundup' ready (RR) alfalfa (McCaslin and Fitzpatrick, 2000), which is also one of the self-incompatible-outcrossing species, has been produced and commercialised. Given the heterogeneity among the transgenic lines, it is essential to screen a higher number of transgenic lines that could potentially help to understand the role of *LpUbl5* in drought tolerance. However, the use of expression vector, pLpHUB1 with two double *CaMV 35S* promoters which was arranged in inverted orientations could have caused the T-DNA rearrangements in 8AC2 lines. Therefore the study could neither accept nor reject the proffered null hypothesis that,

"Drought tolerance in perennial ryegrass is not different between control plants and those with over-expression of *LpUbl5*."

In conclusion, the generation of homozygous T3 lines in a self-incompatible species was not successful in case of 8AC2. The use of a compromised population in the study could not prove the role of LpUbl5 in drought tolerance. This led to reframing of the project, which then used *Arabidopsis* as a model system to try to define the role of *LpUbl5* in drought tolerance.

8.1.2 Research question 2

- Does the over-expression of *LpUbl5* in *Arabidopsis* confer enhanced drought tolerance, given the highly conserved nature of *Ubl5* across species? Do the *AtUbl5* mutants expedite phenotypic characterization?

Experiment 2 was carried out to answer the research question using *Arabidopsis thaliana* modified using an expression vector *pARTB_GW_egfpER::LpUbl5*. The study generated 11 transgenic lines of which four lines; T3/1, T3/35, T3/41 and T3/43 were used to screen for enhanced drought tolerance in comparison with wild type *Arabidopsis* using different physiological measurements. The results did not suggest any role of *LpUbl5* in enhanced drought tolerance. The study also analysed the performance of T3/41 and T3/43 in different concentrations of mannitol containing MS medium. The increased transcript abundance of *LpUbl5* did not enhance the root growth or the shoot biomass when compared with wild type *Arabidopsis* seedlings. Collectively, the results obtained from Experiment 2 did not support any potential role of *LpUbl5* in enhanced drought tolerance. Therefore the study accepted the null hypothesis. Given the highly conserved nature of *Ubl5* across different species and between ryegrass and *Arabidopsis* (94.52%), over-expression of *Ubl5* was expected to confer enhanced drought tolerance as reported by Patel *et al.* (2015). However, no drought tolerant phenotype was observed in the lines tested using the different physiology measurements. Consistently, transcript levels of *AtUbl5* under progressive drought were analysed and did not show an increase when the water deficit became severe, when compared with well watered control. These results led to acceptance of the null hypothesis;

"Drought tolerance in *Arabidopsis thaliana* is not different between control plants and those with over-expression of *LpUbl5*."

To address the second part of the research question, the study used Ubl5 mutants of *Arabidopsis thaliana*. *AtUbl5* is present in two loci which are At3g45180 and At5g42300. The mutants were obtained from the Arabidopsis Biological Resource Centre (ABRC) and were analysed to identify homozygous mutants for At3g45180 and At5g42300. The study could not isolate a homozygous mutant for At5g42300. Efforts were undertaken to amplify both At3g45180 and At5g42300 from the genomic

DNA and cDNA of Arabidopsis leaves. The study could not amplify At3g45180 which indicated the possibility of this gene being a pseudogene as suggested previously (Vierstra, 2012) . Further evaluation of At5g42300 heterozygous mutant indicated a potential seed lethal phenotype for the homozygous, which needs further study for confirmation. Due to the lack of time and resources, the study of *Ubl5* mutants to unravel the phenotypic characterization of *Ubl5* could not be achieved. Therefore, the second part of the research question was not answered.

The *AtUbl5* gene expression data available from various studies was reviewed in Chapter 2.5.1 and was inconsistent for involvement in drought tolerance. A large number (60-90) of other ubiquitin, ubiquitin like and ubiquitin fold proteins were also upregulated (Nishiyama *et al.*, 2013; Van Ha *et al.*, 2014; Rasheed *et al.*, 2016). Therefore, up-regulation of *AtUbl5* may be in relation to other biological function. As a results, this requires further study to identify the role of *Ubl5* in plants. Identification of the effect of the homozygous mutation of *AtUbl5* (Sparkes *et al.*, 2003) and identification of a lethal phenotype by studying the segregation pattern (Steinebrunner *et al.*, 2011) could identify the potential role of *Ubl5* in plants.

8.1.3 Research question 3

- Does subcellular localization of *Lp*UBL5 exhibit its potential involvement in alternative splicing?

Experiment 3 was carried out by transiently expressing *LpUbl5*:*gfp* fusion under a *CaMV 35S* promoter (*pB7_FWG2:LpUbl5*) in *N. benthamiana* and onion epidermal cells. LpUBL5 was observed both in the cytoplasm and nucleus of *N. benthamiana* leaves and onion epidermal peels. This result indicates a similar sub-cellular localization pattern of *Lp*UBL5 in the dicot (*N. benthamiana*) and monocot (onion) platform. It is also possible that the gene could have a similar function in all plant species. The observed subcellular localization pattern was consistent with the findings in humans, yeast and *C.elegans*. *Ubl5* homologues in yeasts and humans are involved in alternative splicing and UPR_{mt} in *C.elegans*. (Yashiroda and Tanaka, 2004; Benedetti *et al.*, 2006; Sveda *et al.*, 2013; Ammon *et al.*, 2014). The subcellular localization of *Lp*UBL5 supports its potential role in alternative splicing and UPR_{mt}. However, sub-cellular localization needs to be further confirmed using immunofluorescence methods with a specific *Lp*UBL5 antibody to exclude any interference of the C-terminal fusion of 27 kDa GFP protein fusion. The results of Experiment 3 rejected the null hypothesis,

"*Lp*UBL5 is not localized in the nucleus rejecting the potential involvement of *Lp*UBL5 in alternative splicing in plants".

Stadler *et al.* (2013) indicated the need for cross validating the results obtained from fluorescent protein tagged localization results using an immunofluorescence method and vice versa. This study used C-terminal fusion of GFP protein for sub-cellular localization study and a cross validation of the

observed localization using immunofluorescence is recommended. Interaction of UBL5 homologues in yeast and spliceosomal protein Snu66 via Hub1 interaction domain (HIND) was essential for splicing (Mishra *et al.*, 2011) whereas in humans this interaction was not essential for splicing (Ammon *et al.*, 2014). Similarly in *C.elegans*, increased nuclear localization of UBL5 in stressed animals was reported to support its role in UPR_{mt} (Benedetti *et al.*, 2006). It was also hypothesised that formation of DVE1 complex with UBL5 and localization of this complex into the nucleus. This complex then binds to an *Hsp-60* promoter which promotes b-zip transcription factor binding and transcriptional activation in the UPR_{mt} signalling (Haynes and Ron, 2010; Haynes *et al.*, 2010). Similarly, in mammalian cells UBL5 interacts with *SatB2* to mediate UPR_{mt} signalling (Al-Furoukh *et al.*, 2015). The HIND present in Prp38 in plants and the UBL5 interaction with Prp38 could potentially support its role in splicing or its interaction with DVE-1 could be an indication of its role in UPR_{mt} signalling. Given the highly conserved nature of the UBL5 protein across these species (Appendix C.14) it is possible it is involved in similar functions across different species. However, further studies are required to confirm the sub-cellular localization and identify the interacting proteins to elucidate its role in plants.

8.1.4 Research question 4

- Does the ryegrass germplasm have any potential candidates to incorporate in breeding for drought tolerant trait? Does the performance of selected germplasm accession under drought correlate with the *LpUbl5* transcript level? Does the *LpUbl5* transcript level increase with increased severity of drought?

Experiment 4 was carried out using 10 germplasm accessions from a wide geographic origin, 'Grasslands Impact' and T_3 homozygous 8AC1 line. The study subjected the accessions to consecutive drought and recovery cycles to evaluate plant biomass production and survival. Experiment 4 was unique in that plants experienced six consecutive drought cycles for different durations within 360 days. This is prolonged period compared with many studies (Jones *et al.*, 1980; Korte and Chu, 1983; Wang and Bughrara, 2008; He *et al.*, 2017a). The majority of lines were not different until Drought Cycle 3 except form the 'Russian Federation'. The imposition of several drought cycles identified the potential of the Norwegian accession in drought tolerance. The study identified 'Norway' (A17183) as having high dry matter yield after six drought cycles. This indicates enhanced drought tolerance in comparison with other accessions. However, *LpUbl5* expression level under drought tolerance was not related to the performance of plants under drought. In conclusion, the results from Experiment 4 accepted the null hypothesis,

"Drought tolerance of these germplasm accessions is not associated with the *LpUbl5* transcript abundance".

The drought tolerance mechanism shown by 'Norway' could involve a cross talk between cold and drought tolerance pathways (Lit Rev 2.2.4, (Nakashima *et al.*, 2009)). These common signalling components can be identified via forward and reverse genetics along with expression profiling. The specificity and cross talk in these pathways can be determined through biochemical characterisation of signalling complexes (Chinnusamy *et al.*, 2004). According to Austin (1989), determination of successful growth and survival in water-limited conditions should be based on the perception of integrated plant responses to drought. This study measured yield, survival rate, leaf extension, RWC and osmotic potential to understand the effect of imposed drought on the selected accessions. However, further evaluation of stomatal conductance, metabolomics profiling, root elongation and water extraction by individual accessions could potentially deepen the understanding of accessions responses to drought (Johnson and Asay, 1993). The 'Norway' is an ecotype collected from Norway which has frozen ground for long periods during the year. The accession has previously shown similar performance to the most winterhardy check variety NK200 (Hulke *et al.*, 2007) and superior performance under drought when compared with selected accessions in this study. This is possibly due to the ability to decrease metabolic rates, accumulate compatible solutes and repair membrane structure during acclimation. All of these traits would mitigate cell damage in both freeze-thaw and dehydration conditions (Humphreys *et al.*, 1997). This suggests that screening ecotypes from similar cold environments in New Zealand, such as the Mackenzie Basin, may identify ecotypes with enhanced drought tolerance. In conclusion, the Norwegian accession showed a potential for drought tolerant phenotype. Further studies are recommended to deepen the knowledge about the transcriptional pathways and metabolomics status of this accession under prolonged drought.

One of the most important findings of this study is the observation that plant desiccation responses only become apparent after several drought cycles. If the study would have finished after the first cycles, few differences would have been apparent among the various accessions. Thus, drought studies under a limited number of drought cycles can be misleading. The results from this work suggest that analysing accessions under multiple drought cycles for an extended period is required for selection of drought tolerant phenotypes. Further, screening more ecotypes originating form cold environmental conditions could potentially identify other drought tolerant ryegrass phenotypes. One possible explanation for this surprising result could be that the 'Norway' is late flowering and thus invested more carbon into vegetative growth compared to the other accessions. However, in this experiment, flowering was prevented in all accessions by removal of flowering buds. Furthermore, and similar to conditions under grazing in the field, the plant material was removed by cutting at regular intervals, thus bringing them together to a common starting point throughout each experimental cycle.

8.1.5 Research question 5

- Does the *LpUbl5* expression increase under water withheld treatment in primary transgenic ryegrass lines and 'Impact'?

Experiments 5-A and 5-B used primary transgenic ryegrass lines and 'Impact' respectively. Experiment 5-A was used a growth room with 8AC1, 8AC2, 7AE5 and 7AE15. The study did not show an increase in *LpUbl5* transcript abundance in leaf samples under drought when compared with a well-watered control. Experiment 5-B was carried out in a glasshouse and no transgenic lines could be included. The LpUbl5 level in 'Impact' was analysed under water withheld and well watered conditions. The study did not show an increase in *LpUbl5* transcript level in leaf samples from 'Impact'. In short, experiments 5-A and 5-B led to an acceptance of null hypothesis that,

"Transcript abundance of *LpUbl5* under drought or water withdrawn conditions is not different in perennial ryegrass".

Increase in transcript abundance from samples collected from paddocks across different seasons using SAGE database suggested that *LpUbl5* gene may be involved in plant tolerance to drought. Transcript abundance was further studied in samples collected before and 8 days of water withheld period showed increase in *LpUbl5* transcript abundance. Both these instances, the comparison of transcript abundance was with samples collected before stress (Patel *et al.*, 2015). However, Experiment 5 had a well-watered control and the comparison of transcript abundance between water treatments was not different. By accepting the null hypothesis, the study rejects the role of *LpUbl5* in enhanced drought tolerance. The sub-cellular localization reported (General discussion 8.1.3) is an indication of its potential role in plants. Then highly conserved nature of *Lp*UBL5 (Appendix C.14) suggests that the gene would serve a similar function across species. However, *LpUbl5* is associated with a different function in different species (Chapter 2.4.4) and further studies are recommended to identify its role in plants.

The generation of transgenic lines requires careful planning and characterization in each step which may identify any errors at earlier stages. Selection of vectors should also consider efficient screening of transgenic plants at later stages. Use of isogenic plants as controls and clonal propagations to generate swards to account for the genetic variability is also recommended. In case of ryegrass species 70% of genetic variability is observed within the population (Personnel comm. Alan Stewart, PGG Wrightson). Apart from this, generation of transgenic plants by backcrossing also lead inbred suppression in the progeny in obligate outcrossing species. An ideal method to understand the gene function of *LpUbl5* gene, highly conserved across species would be to use model plants *Arabidopsis thaliana*. Phenotypic characterization of *Ubl5* mutants and complementation of *AtUbl5* mutants using

LpUbl5 could confirm the protein function in *Arabidopsis*. Further, understanding of interacting protein partners as indicated in Section 8.2.

8.2 Future prospect

1. Understanding of *LpUbl5* function can be potentially obtained through identification of interacting protein partners (Kantham *et al.*, 2003). We have initiated a study to determine potential interacting protein partners of UBL5 via Matchmaker Gold Yeast Two-Hybrid System (Colnetech, Takara, USA). *LpUbl5* has been cloned into *pGBKT7*, DNA binding domain to be used as bait. Control reactions have been completed and the *pGBKT7:LpUbl5* and the control vectors were grown in *E.coli* DH5 α cells and are stored at -80°C as bead stock. Screening of cDNA libraries from perennial ryegrass could potentially identify the interacting protein partners thus leading to understand the potential role of *LpUbl5*. Further, this study can be extended by screening cDNA libraries from *Arabidopsis* to understand whether *Lp*UBL5 interacts with similar proteins in *Arabidopsis* and thus potentially reveal its conserved function across plant species.

2. The role of *Ubl5* in plants can be potentially identified via phenotypic characterisation of *AtUbl5* mutants in *Arabidopsis*. This study has obtained homozygous mutants of At3g45180 and heterozygous mutants of At5g42300. A segregation analysis using seeds generated from heterozygous mutants of At5g42300 can identify a potential seed abortion or embryo lethal phenotype. Analysis of embryo defects and frequency of aborted seeds per silique in selfed heterozygous plants can extend our knowledge about the effects of the *AtUbl5* mutation in *Arabidopsis* (Sparkes *et al.*, 2003; Michalko *et al.*, 2015).

3. This study has screened 10 germplasm accessions and identified the 'Norway' as a potential drought tolerant accession. Screening a much higher number of germplasm accessions from diverse centres of origin, especially those ecotypes that originate from cold and frozen regions is hypothesised to identify accessions with superior performance under water deficit conditions. This study has generated transcriptomics data from the Norwegian accession under drought and recovery which would deepen our understanding of transcriptional changes in 'Norway'.

Appendix A

PCR protocols

General lab protocols were followed for most of the experiments in this research. The protocols are elaborated here to avoid repetition throughout the theses. References are given in appropriate chapters where the protocols are used.

Polymerase chain reaction (PCR)

Polymerase chain reaction method was developed in 1980 by Kary Mullis which become an indespensable technique in molecular biology. The method is based on repeated thermal cycling in the presence of heat stable DNA Polymerase, nucleotides and DNA oligonucleotides or primers. The variations of this basic PCR is used to employ in different molecular lab works. The different PCR protocol used in this research is described here.

A.1 End point PCR: General PCR

KAPA Taq PCR was used for most of the experiments such as evaluating primer efficiency, genomic DNA contamination in total RNA, efficiency of cDNA and colony PCR. The template come from different sources depending on the aim of procedure such as total RNA, genomic DNA, cDNA (complimentary DNA derived from reverse transcription form mRNA) and plasmids. The reagents used were form KAPA Taq PCR by KAPABIOSYSTEMS and followed manufactures prtotocol as outlined below (Table A.1) and the thermal cycling (Figure A.1). Minor variations were done depending upon the total reaction vloume used for each reactions. The positive and negative controls were included to ensure the efficiency of reaction when carried out.

Table A.1 Genaral PCR Protocol: KAPA Taq PCR

Reagents	Stock Concentrations	Final Concentration
PCR Grade water	N/A	N/A
10X KAPA Taq Buffer	10X	1X
10 mM dNTP Mix	10mM	0.2 mM
Forward Primer	10mM	0.4 mM
Reverse Primer	10mM	0.4 mM
5U/µl of KAPA Taq DNA Polymerase	5U/µl	1 U/µl
Template DNA	Varies	>25-250ng depending upon the type of DNA

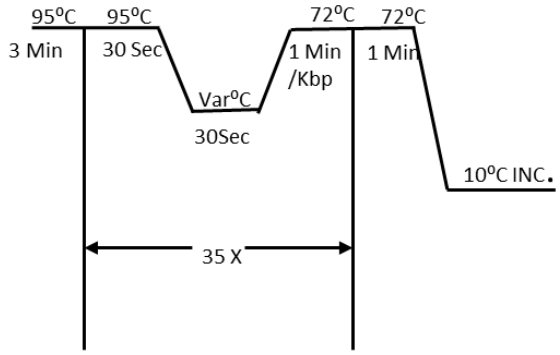


Figure A.1 Visual representation of thermal cycling for general PCR

A.2 Proofreading PCR

The High Fidelity PCR ustilizes a novel DNA polymerase which increases sensitivity, yeild and speed of the whole reaction. The method is employed where high fidelity amplification is required such as generating amplification products for cloning. The reaction would carry out in 3X50 µL to assure enough PCR product to carry out downsteam applications. The reagents were purchased from KAPA HiFi PCR kit (KAPABIOSYSTEMS, NZ) and the followed manufacturers protocol (Table A.2) and thermal cycling (Figure A.2).

Table A. 2 Proof Reading PCR Protocol: KAPA HiFi Hotstart PCR

Reagents	Stock Concentrations	Final Concentration
PCR Grade water	N/A	N/A
5X KAPAHiFi Buffer	5X	1X
KAPA dNTP Mix	10mM	0.3mM
Forward Primer	10µM	0.3µM
Reverse Primer	10µM	0.3µM
1U/µL KAPA HiFi DNA Polymerase	1U/µL	1U
Template	varies	1ng/µL

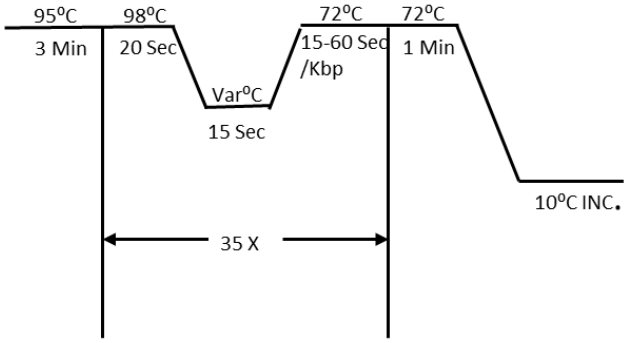


Figure A. 2 Visual representation of thermal cycling used for KAPA HiFi Hotstart PCR

A.3 Reverse transcriptase PCR (RT-PCR)

Reverse Transcription was carried out to synthesis cDNA from the total RNA isolated from the leaf tissue using Sigma Spectrum™ Plant Total RNA kit. Complete removal of genomic DNA was assured by KAPA Taq PCR before proceeding to transcriptase reaction using TaKaRa PrimeScript™ Reagents (Table and Figure A.4).

Table A. 4 RT Reaction protocol

Reagents	Stock Concentrations	Final Concentration
5X PrimeScript Buffer	5X	1X
PrimeScript RT Enzyme Mix	Unknown	0.5 µL
Oligo dT Primers	50 µM	25 pmol
Random 6 mers	100 µM	50 pmol
Total RNA	Varies	300 ng
Sterile Water	N/A	Up to 10 µL

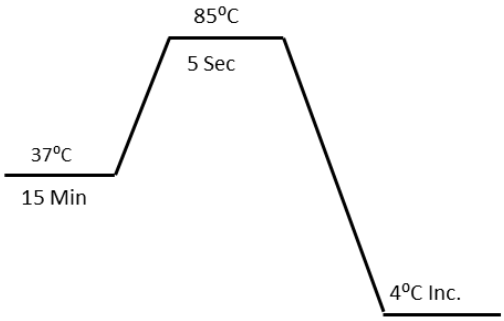


Figure A. 4 Visual representation of thermal cycling for RT-PCR

A.4 Qualitative reverse-transcriptase PCR (qRT-PCR)

The method is used to study the precise amount of gene transcription in the leaf tissue under study. Reverse transcriptase reaction was carried out on the specific amount of total RNA. The cDNA obtained by the reverse transcriptase reaction was diluted to 20 fold and quality was assured as given in the main text. Then qRT-PCR was carried out with TaKaRa SYBR® Premix Ex Taq™ II and the protocol in Table A.5. Eppendorf EP Motion 5070 was used to aliquot cDNA template and master mix into qRT-PCR plates to eliminate manual pipetting errors. The qRT-PCR reactions were done in Eco™ Real-Time PCR System (dnature, NZ) using the thermal cycling in Figure A.5.

Table A. 5 qRT-PCR protocol

Reagents	Stock Concentrations	Final Concentration
2X SYBR ExTaq ll Buffer	2X	1X
Sterile water	N/A	As required
Forward Primer	10 mM	2.5 mM
Reverse Primer	10 mM	2.5 mM
cDNA	~10 ng/µL	~40 ng

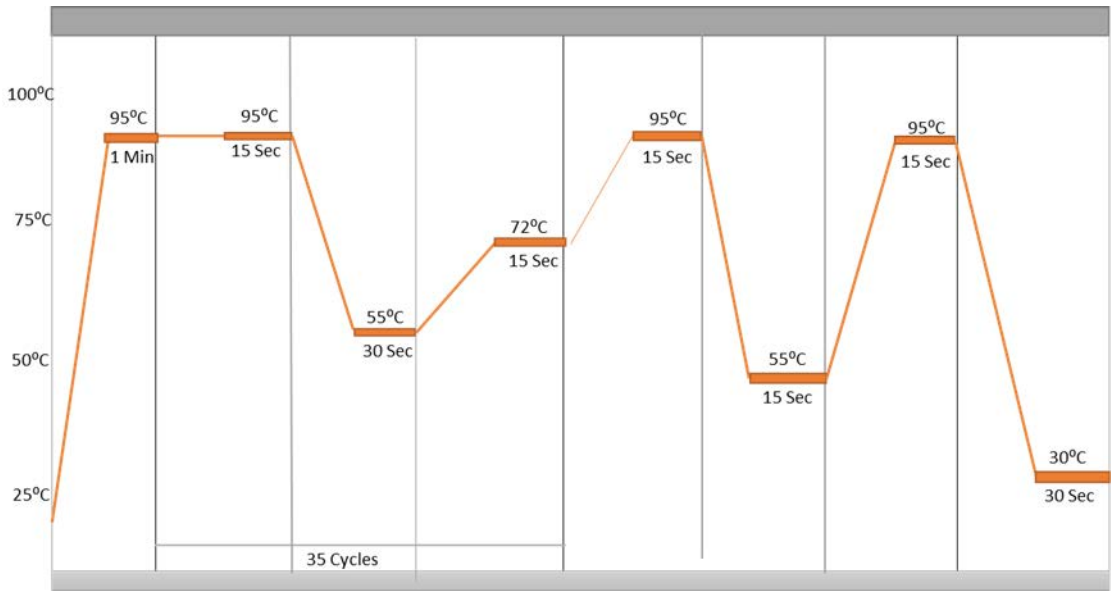


Figure A. 5 Visual representation of thermal cycling used in qRT-PCR

Appendix B

B.1 Cloning and transformation procedures

The *Escherichia Coli (E.coli)* strain of DH5α cells were used for routine cloning purposes in this research. The DH5α competent cells were prepared and the efficiency was analysed once the cells were prepared.

B.2 Media preparations

LB-medium (1 L): (Luria-Bertani (LB) broth)

10 g tryptone

5 g yeast extract

10 g NaCl

Weigh the above given ingredients and dissolve in 900 mL of diH_2O.

Adjust the pH of the medium to 7.5 and bring volume up to 1000 mL.

Autoclave on liquid cycle for 20 min at 15 psi. Allow solution to cool to 55°C, and add antibiotic if needed (50µg / mL of Amp or Kan). Store media at room temperature or +4°C.

LB agar-plates:

Prepare LB medium as above, but add 15 g/L agar before autoclaving. After autoclaving, cool to approx. 55°C, add antibiotic (if needed), and pour into petri-dishes in the laminar flow hood. The plates were left to harden in the laminar flow hood, then invert and store at +4°C in the dark.

SOC Medium (Super optimal broth)

To prepare SOC medium, SOB medium was prepared following the given ingredients below.

5 g Yeast extract

20 g Tryptone

0.584 g NaCl

0.186 g KCl

2.4 g $MgSO_4$

This ingredients were dissolved in 900 mL and adjust the pH to 7.5. The media was then made up to 1000 mL using double de-ionised water. After cooling SOB medium to less than 50°C, 20 ml filter sterilized 20% glucose solution was added.

Infiltration media

30 g sucrose (w/v)

150 µM acetosyringone

0.05% (v/v) of Pulse (Nufarm, NZ)

These ingredients were transferred to 1000 mL flasks and made up the volume to 950 mL double de-ionised water. Infiltration medium was prepared freshly before use.

Re-suspension Medium

4.41 g MS medium with vitamins, (full strength)

30 g sucrose, 3% (w/v)

150 µM acetosyringone

pH was adjusted to 5.8 and made up the volume to 1000 mL with double de-innised water.

MS media

4.41 g Murashige and Skoog medium with vitamins and salts

30 g Sucrose

8 gm Phyto agar

Adjust the pH to 5.7 and make up the media up to 1L.

NB: MS media with benzyl adenine is prepared by adding 0.2 -0.5 mg/L MS media.

½ MS media

½ MS is the ½ strength Murashige and Skoog medium with vitamins and salts (2.2 gm in 1L media).

10 mg/mL BASTA is added to the above as selective agent (based on the requirement).

B.3 Preparation of DH5α competent Cells

The DH5α cells were prepared as described by Inoue *et al.* (1990)

SEM buffer (1L) preparation

3.2 g PIPES (10 mM)

10.88 g $MnCl_2$ (55 mM)

2.20 g $CaCl_2$ (15 mM)

18.64 g KCl (250 mM)

Dissolved everything except $MnCl_2$ in 900 ml of H_2O and adjusted the pH to 6.7 with KOH. Then dissolved the $MnCl_2$ and made up to 1L with H_2O and filter sterilised.

The DH5α cells were streaked on to a plate of LB media from glycerol bead stock from -80^oC. The plate was incubated at 37^oC overnight. The single colony was picked from plate and inoculated into 3 mL LB medium and incubated at 37^oC overnight with shaking at 250 RPM. After overnight incubation the tube was placed on ice to arrest the cell growth. Then two 2 Litre flasks with 200 mL of LB medium was incubated with 1 mL of fresh overnight culture and incubated at 20^oC with vigorous shaking at 200 RPM. Grow the cells until OD_{600} reaches between 0.4-0.8 with an optimum of 0.6. After obtaining the correct OD the cells were chilled on ice for 20 minutes. After the chilling the media was transferred ice cold centrifuge tubes and centrifuge at 5000 RPM for 5 mins at 4^oC. After the centrifugation, the cells were re-suspended in ice cold 20 mL SEM buffer by vortexing. Care was taken to avoid warming up the cells by placing the bottles on ice in between vortex. Complete re-suspension was checked by pipetting up the cells using an ice cold glass pipette to look for any lumps of cells. The re-suspended cells were placed on ice for 10 mins before proceeding to centrifuging at 5000 RPM for 5 mins at 4^oC. Re-suspended the cells in ice 6 mL ice cold buffer by keeping the conditions at 4^oC. Then the contents of two bottles were combined together into one bottle and 920 µL of Dimethyl sulfoxide (DMSO). Then 100 µL of cells were aliquot into ice cold microfuge tubes and snap frozen using liquid nitrogen. The tubes were aseptically transferred at -80^oC freezer.

Evaluating the efficiency of competent cells

The DH5α cells were transformed with 1 µL of pUC19 (10 pg) by gently mixing and incubated the mix on ice for 30 mins. Then the cells were heat-shocked for exactly 30 sec at 42^oC. Then the cells were returned onto ice for 5-10 mins. Then 250 µL of Super Optimal Broth with Catabolite repression (SOC) medium was added to the cells and incubated at 37^oC with shaking at 250 RPM for 1 hour. After the incubation, the cells were serially diluted (1:10, 1:100 and 1:100). 50 µl of each dilution was plated into pre-warmed LB plates and incubated at 37^oC, overnight. The following formula was used to calculate

the efficiency of DH5α competent cells. The efficiency determined was 10^9 which was sufficient to use the cells for routine cloning purposes.

$$\frac{\text{Avg \# of colonies}}{\text{10 pg transformed}} \times \frac{10^6\ \text{pg}}{\mu\text{g}} \times \frac{\text{100+250 SOC µl transformed cells}}{\text{50 µl plated}} = \frac{\text{\# transformants}}{\mu\text{g of plasmid DNA}} \times DF$$

B.4 Cloning protocol using TOPO-TA Vector

The DH5α cells were taken from the -80^{o}C freezer allowed to thaw on ice for 10 mins. The TOPO® TA (Invitrogen) cloning reaction was set up as given in the table by following the manufacturers instruction using 3:1 ratio of PCR product: plasmid using the formula below.

$$\frac{\text{(Ng plasmid * µl plasmid used) Kbp insert size *(insert : plasmid)}}{\text{Kbp plasmid size}} = \text{ng insert to add}$$

Table B1 The reagents used for TOPO® TA cloning

Reagent	Amount
Fresh PCR product	0.5-4 µL
Salt solution	1 µL
Molecular Water	Up to 5 µL
TOPO Vector	1µL
Total Volume	6 µL

The above reaction was gently mixed and incubated at room temperature for 5 mins (Table B.1). 2 µL of the reaction mixture was added to the thawed competent cells and was mixed gently. The reaction mix was placed on ice for 30 mins. Then the cells were heat shocked for 30 Sec at 42^{o}C and placed back on ice immediately for 5-10 mins. Then 350 µL of SOC medium was added to the competent cells and incubated at 37^{o}C for 1 hour. Then the 50 µl and 100 µL of cells were plated onto LB medium with 50 mg/mL of Kanamycin as selective agent. In addition 20 mg/mL X-gal stock (5-Bromo-4-Chloro-3-Indolyl β-D-Galactopyranoside) was used for blue/ white screening method by spreading 100 µL of X-gal stock on to pre-warmed LB agar plates. Colony PCR was carried out to screen the positive colonies.

B.5 Cloning protocol using pENTR™ /D-TOPO® cloning kit

The following reaction was set up in 3:1 molar ration of PCR product : TOPO® vector.

Table B.2 The reagents used for pENTR™ /D-TOPO® cloning.

Reagent	Chemical Transformation
Fresh PCR Product	0.5-4 µL
Salt solution	1 µL
Water	to a final volume of 5 µL
TOPO® vector	1 µL
Total volume	6 µL

The above reaction (Table B.2) were prepared in 1.7 mL microfuge tube and gently mixed and incubated at room temperature for 5minutes.

B.6 Transformation of chemically competent *E.coli*

Chemically competent *E.coli* (DH5α) cells were taken from -80°C whenever required and thawed on ice before transforming. 2 µL of cloning reaction mix was added to the thawed DH5α cells and mixed gently. This reaction mix was incubated on ice for 30 minutes. Then this reaction mix containing the DH5α cells was heat shocked at 42°C for 30 seconds without shaking. Then the tubes were snap chilled on ice and 250 µL of S.O.C medium which had room temperature was added. Then the tubes were placed horizontally and incubated at 37°C at 250 RPM for 1 hour. After 1 hour the 50 and 100 µL of growth was plated onto appropriate pre-warmed (37°C) selective media. The plates were incubated at 37°C overnight (Sambrook and Russell, 2001).

B.7 *E.coli* plasmid mini-prep using alkaline lysis method

The E.coli plasmid mini-prep is carried out as described by (Sambrook and Russell, 2001) Three solutions were required for the E.coli plasmid mini-prep. Solution 2 is prepared freshly every time. The recipe of three solutions are given below.

Alkaline lysis solution 1

Table B.3 The reagents used for preparation of alkaline lysis solution 1

Reagent	Quantity	Final Concentration
Glucose	6.25 mL	50 mM
Tris-Cl (pH 8.0)	6.25 mL	25 mM
EDTA (pH 8.0)	5 mL	10 mM

The solution is made up to 250 mL with sterile dH_2O.

Alkaline solution 2

Table B.4 The reagents used for preparation of alkaline lysis solution 2

Reagent	Quantity	Final Concentration
NaOH	100 µL from 10N	0.2N
SDS	500 µL for 10%	1%

The solution is made up to 5 mL with sterile dH_2O.

Alkaline solution 3

Table B.5 The reagents used for preparation of alkaline lysis solution 3

Reagent	Quantity
5M potassium acetate	60 mL
Glacial acetic acid	11.5 mL

The solution is made up to 100 mL with sterile dH2O and stored at 4^oC.

E.coli plasmid mini-prep protocol

Preparation

- Place alkaline solution and 1 and 3 on ice
- Prepare fresh alkaline solution 2 from stock concentration.
- Aliquot required volume of isopropanol into a small sterile bottle and store at -20^oC
- Prepare 75% ethanol and store at -20^oC.

Single colony of E.coli grown at 37^oC for 15-16 hours with appropriate antibiotic were used plasmid isolation. The culture was transferred into a 1.7 mL microfuge tube and centrifuged at 5000 RPM for 1 minute and the supernatant was discarded. The step was repeated to include the whole 3 mL culture. The cells were suspended in 250 mL of solution 1. After complete re-suspension 250 mL of freshly prepared solution 2 was added and gently mixed by tilting the tubes and placed onto the ice. The addition of solution makes the whole volume of solution into viscous nature. While on ice 350 mL of solution 3 was added to the tubes after 4-5 minutes. Then the tubes were gently mixed by inverting the tubes. This formed white precipitate in the solution.

Then the tubes were centrifuged at maximum speed for 10 minutes to sediment the white precipitate. The clear supernatant was transferred into a fresh microfuge tube. White precipitate was completely removed or repeated centrifugation to remove any carry-over white precipitate. An equal volume of

pre-cooled Isopropanol was added to the tubes, mixed well and incubated at -20^{o}C for better yield. This incubation was an additional precaution to increase the plasmid yield.

After the incubation the tubes were centrifuged at maximum speed for 10 minutes at 4^{o}C. The supernatant was discarded and the pellet was washed with pre-cooled 75% ethanol. The tubes were centrifuged at maximum speed for 2 minutes and the supernatant was removed. Then the tubes were air-dried and re-suspended the pellet in 50 µL of TE buffer containing RNase A (5 µL per mL)

B.8 Gateway® LR recombination reaction protocol

LR recombination reaction performed to between entry clone containing *att*L site and *att*R containing destination vector to obtain an expression vector. The following reaction mix is prepared for the reaction by following manufacturer's protocol (Table B.6).

Table B.6 Gateway® LR recombination reaction protocol

Reagent	Volume
Entry clone	1-10 µL
Destination vector	2 µL
5X LR Clonase Reaction buffer	4 µL
TE Buffer	up to 16 µL
LR Clonase enzyme mix	4 µL

Gently vortex to mix the above reactions (Table B.6) and incubate at 25°C for one hour. Then 2 µL 0f 2 µg/µL proteinase K and incubated the reaction mix at 37°C for 10 minutes. This reaction mix is used to transform DH5α cells.

B.9 Transformation : Electroporation method

Electroporation of *Agrobacterium Tumefaciens* was carried out as described by (Sambrook and Russell, 2001).

Preparations

- Thaw the electro-competent *Agrobacterium Tumefaciens* strain, GV3101 from -80^{o}C freezer.
- Place an electroporation cuvette at -20^{o}C to maintain the cold when added.
- Set up the Gene Pulse (Bio-Rad) apparatus to 25 mF, 2.5 kV voltage and 200 ½ resistance controller.

An approximate of 10-50 ng of binary vector was added into the *Agrobacterium* cells and the cells were incubated on ice for 5 minutes. The cells were added to bottom of the pre-cooled electroporation cuvette. Then the cuvette was placed in the Gene Pulse (Bio-Rad) apparatus chamber slide. The precaution was taken to make sure the notch was facing chamber. The slide was pushed in to the chamber to reach the cuvette between the contacts in the chamber. The button was pushed until the beep sounds to carry out the electroporation (approximately 2 seconds). Then the cuvette was removed and 1 mL of LB medium was added to the cells. This cells were incubated horizontally at 28^{o}C, 150 RPM for minimum 1 hour. This allowed the expression of antibiotic resistance markers. The cells were plated on to LB agar plates containing appropriate antibiotic for selection. The plates were incubated at 28^{o}C for 2 days in the dark. The plates were covered using aluminium foil paper to avoid light. Then colonies were screened using colony PCR to identify the positive colonies for further studies.

B.10 Restriction digestion of Plasmid

Restriction digestion was carried out to linearize the plasmids to be used as the template in the qRT-PCR assay. The restriction enzyme was selected based the availability of restriction digestion sites on the given plasmid to linearize the plasmids, as the selected enzyme digests the plasmid at single site. The restriction digestions were carried out by following manufacturers protocol (New England BioLabs). The digested plasmids were run on the gel and the Axygen™ Axyprep™ DNA Gel Extraction Kit (Axygen, Ray lab, NZ) was used to carry out gel purification of the digested plasmid. The purified plasmids were quantified using Qubit® flourometer (Life Technologies Ltd, NZ) and also run on 1% agarose gel for further confirmation.

B.11 Sanger Sequencing

An approximate of 200 ng of purified plasmid/15-20 ng of purified PCR product along with 5 pmol of corresponding sequencing primer in a final volume of 7.5 µL was used for sequencing reaction. Reactions were carried out using the BigDye Terminator v3.1 kit (Applied Biosystems) according to the manufacturer's recommendations. Subsequently, the amplification products were analysed with separation and detection on an ABI Prism 3130xl Genetic Analyser by capillary electrophoresis (Life Technologies Ltd, NZ).

B.12 Hoagland's solution

Composition of Solution A

280 mg of H_3BO_3, 340 mg of $MnSO_4.H_2O$, 10 mg of $CuSO_4.5H_2O$ (J.T Baker Chemical Co. Phillibsburg, NJ), 22 mg of $ZnSO_4.7H_2O$, 10 mg of (NH4)6- $MO7O_2.4H_2O$. Adjust the volume to 100 ml with deionised water and store at 4°C (All other chemicals from BDH Chemicals Ltd, Poole, England)

Solution B

0.5 ml of concentrated H_2SO_4 (BDH Chemicals Ltd, Poole, England) is made up to 100 ml with deionised water and store at 4°C.

Composition of Solution C

3.36 g of Na_2EDTA (BDH Chemicals Ltd, Poole, England), 2.79 g of $FeSO_4$ (MERCK, Germany) and adjust the volume to approximately 400 ml. The solution is heated to 70°C while stirring until the colour turns yellow brown. After cooling, it is made up to 500 ml with deionized water and stored at 4°C.

Preparation of Hoagland Stock Solution (10x)

4.7 g $Ca(NO3)_2.4H_2O$, 2.6 g of $MgSO_4.7H_2O$ (Sigma Aldrich, USA), 3.3 g of KNO3 (Fisions, England), 0.6 g of $NH_4H_2PO_4$(BDH Chemicals Ltd, Poole, England), 5 ml of Solution A, 0.5 ml of solution B and adjust the volume to 500 ml with deionized water and store at 4°C.

Preparation of Hoagland's Nutrient Solution (1x)

100 ml 10x stock solution and 5 ml solution C and adjust the volume to 1000 ml with deionized water freshly before use (Hoagland and Arnon, 1950).

B.13 Formaldehyde RNA gel electrophoresis

Preparation of solutions

1. **10X MOPS (3-Morpholinopropane-1-sulfonic acid) buffer(500 mL)**

   MOPS: 29.9 g (0.2M) Dissolve MOPS and adjust pH to 7.0

   3M sodium acetate: 3.33 mL (20 mM)

   0.5M EDTA :10 mL (10 mM)

   Adjust pH to 8.0

2. **Formamide loading dye**

   Formamide: 8 mL

   Sterile water: 1.8 mL

   0.5M EDTA (pH 8.0):0.2 mL

   Xylene Cyanol FF: 10 mg

   Bromophenol blue: 10 mg

Kept the stock in -20°C freezer.

3. **Denaturing loading buffer**

   Formaldehyde: 150 µL

   Formamide: 500 µL

   10X MOPS: 500 µL

   Ethidium bromide: 2 µL

4. **Formaldehyde agarose gel (50 mL**)

   DNA/RNA grade water: 0.75 g

   10X MOPS: 5 mL

   Sterile water: 37.5 mL

Add 7.5 mL formaldehyde after melting the agarose and pour the whole content into gel blocking cassette and insert selected comb.

5. **Running buffer**

   10X MOPS buffer was diluted to 1X to use as running buffer. This was used as running buffer for all RNA formaldehyde gel.

Formaldehyde gel: procedure

The complete electrophoresis apparatus was sprayed and wiped using RNase ZAP (Life Technologies, NZ). The preparation of formaldehyde agarose gel and solidification of agarose gel was done in the fume hood. Then samples were prepared for loading by mixing 5 µL (~400 ng) total RNA sample and 15 µL denaturing loading buffer. The sample and denaturing buffer mix was heated at 65°C by placing it on the heating block for 10 minutes. After incubation place it back on ice to snap chill for at least 10 minutes. Then 1 µL of formamide loading dye was added to each sample while on ice. Then the required running buffer was added to gel tank and place the gel in the gel tank. Then the samples were loaded to the gel and run at 85V for one hour within the fume hood.

B.14 Reagent requirements for immuno-detection of endophyte

1. **Blocking solution**

   Tris(hydroxymethyl) methylamine : 2.42 g

   Non-fat milk powder: 5 g

HCL (1M) 10 ml

Make upto 1 litre and adjust the pH to 7.5

2. **Primary antibody**

Rabbit anti-endophyte produced at Agresearch in conjunction with Massey University.

- 25 µl of primary antibody was added to 25 mL BS (1:1000 dilution)

3. **Secondary antibody**

Goat anti-rabbit IgG-AP, sc-2034, Santa Cruz Biotechnology, USA

- 6.25 µl of secondary antibody was added to 25 mL BS (1:4000 dilution)

4. **Chromogen solutions**

Solution 1:

Fast Red TR (Sigma F -2768) : 20 mg

Tris Buffer (Tris(hydroxymethyl) methyleamine : 24.2 g

RO water : 1 litre

Adjust the pH to 8.2

Solution 2

Naphthol AS-MX phosphate (Sigma N4875)

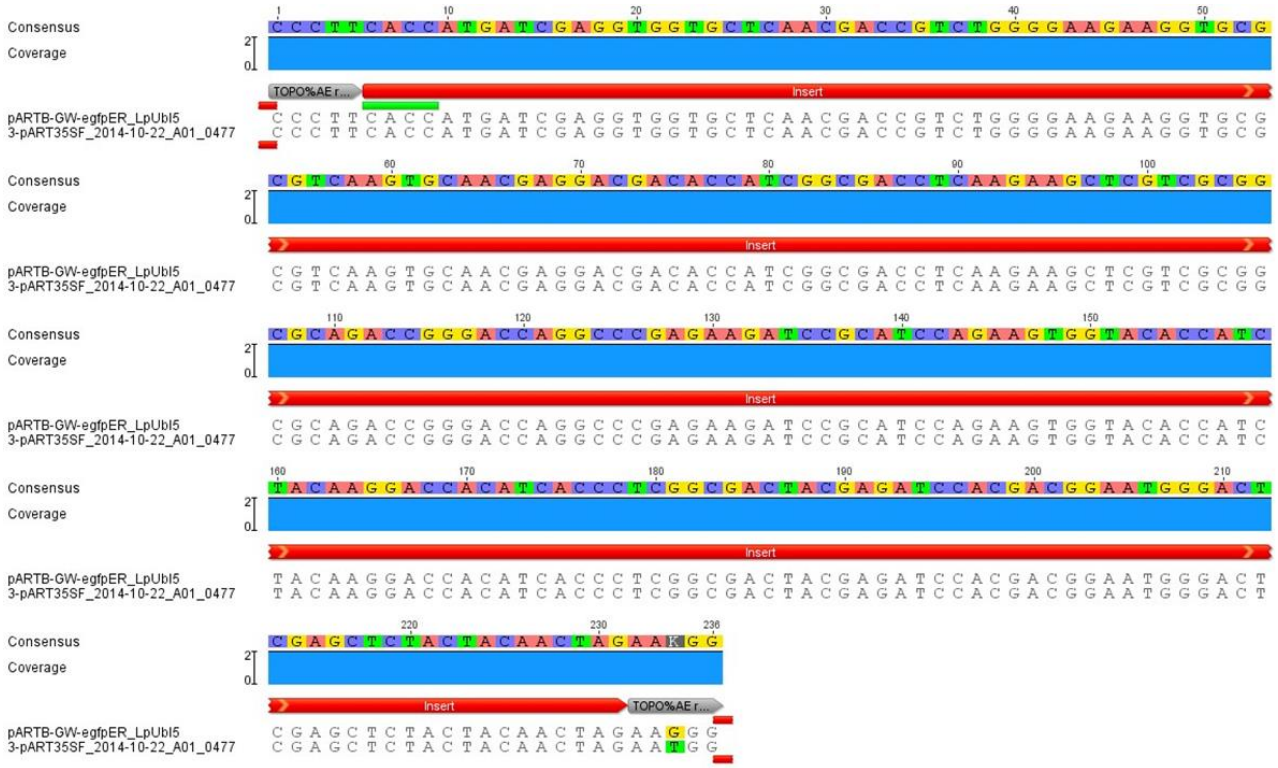
Tris buffer : 10 cm2 of NCM

Solution 1 and 2 is combined and used it to stain NCM.

Appendix C

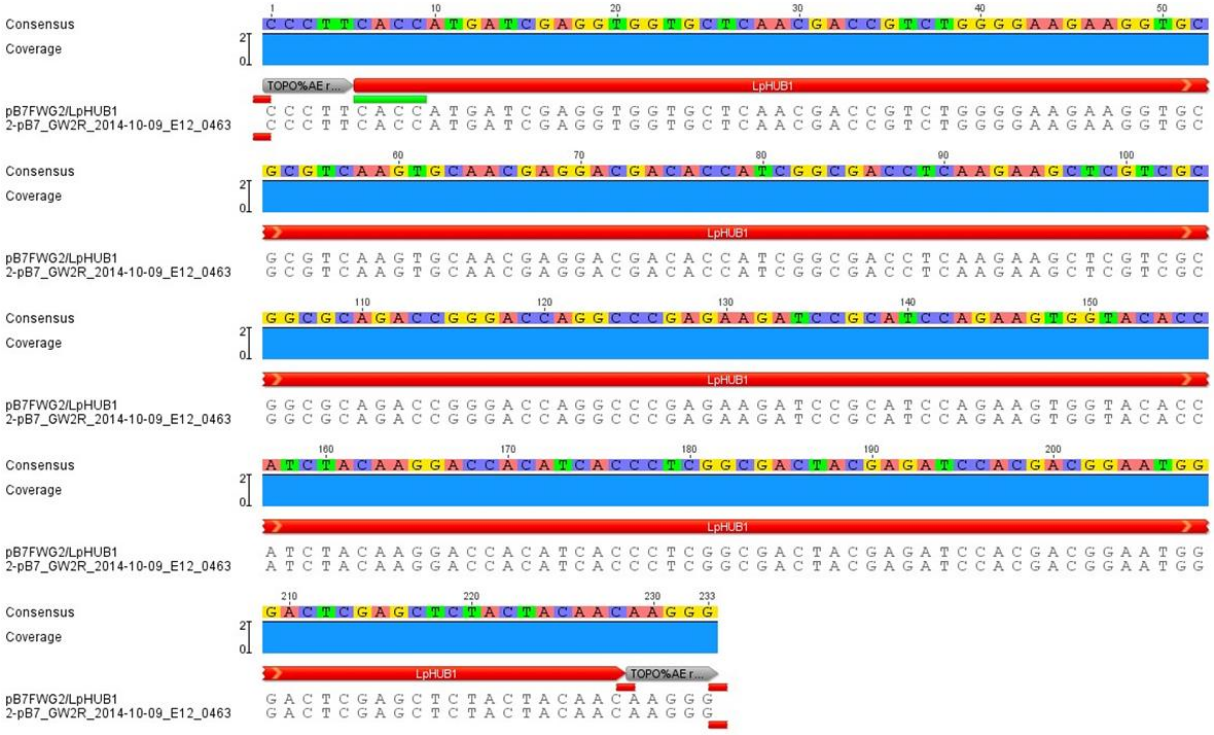
Sequence results and qRT-PCR standard for qRT-PCR

C.1 Sequence results of *pARTB_GW_egfpER::LpUbl5*



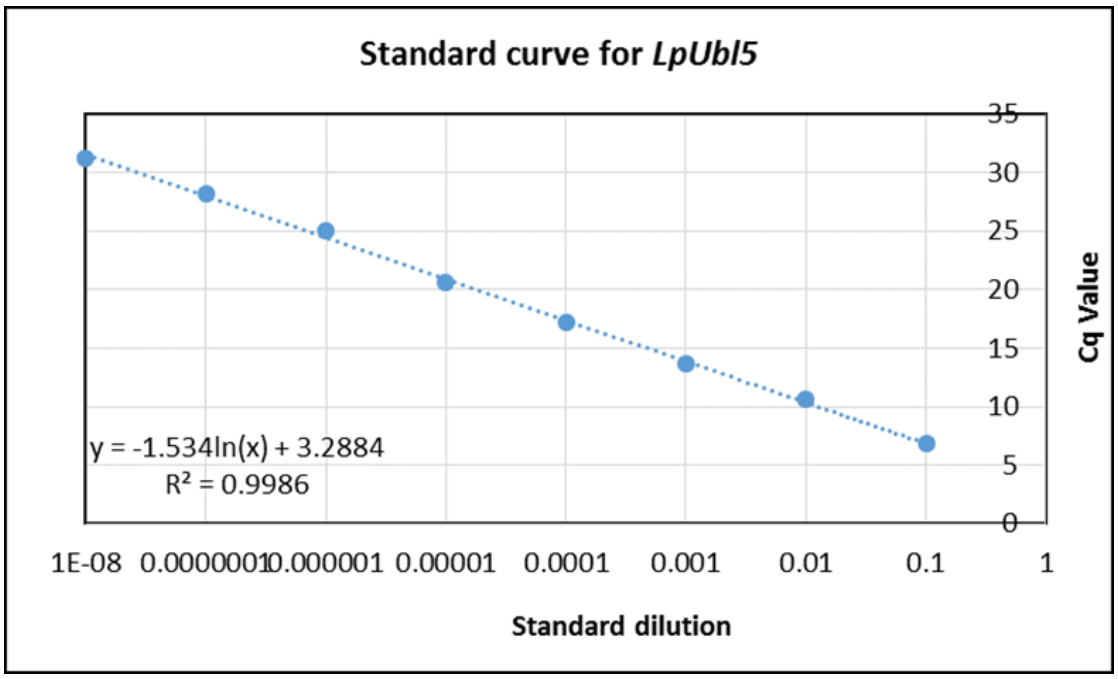
Sequence results of expression vector, *pARTB_GW_egfpER::LpUbl5* shows the sequence coverage of *LpUbl5* gene from the start codon to stop codon used for genetic transformation of *A. thaliana*.

C.2 Sequence results of *pB7F_WG2:LpUbl5*

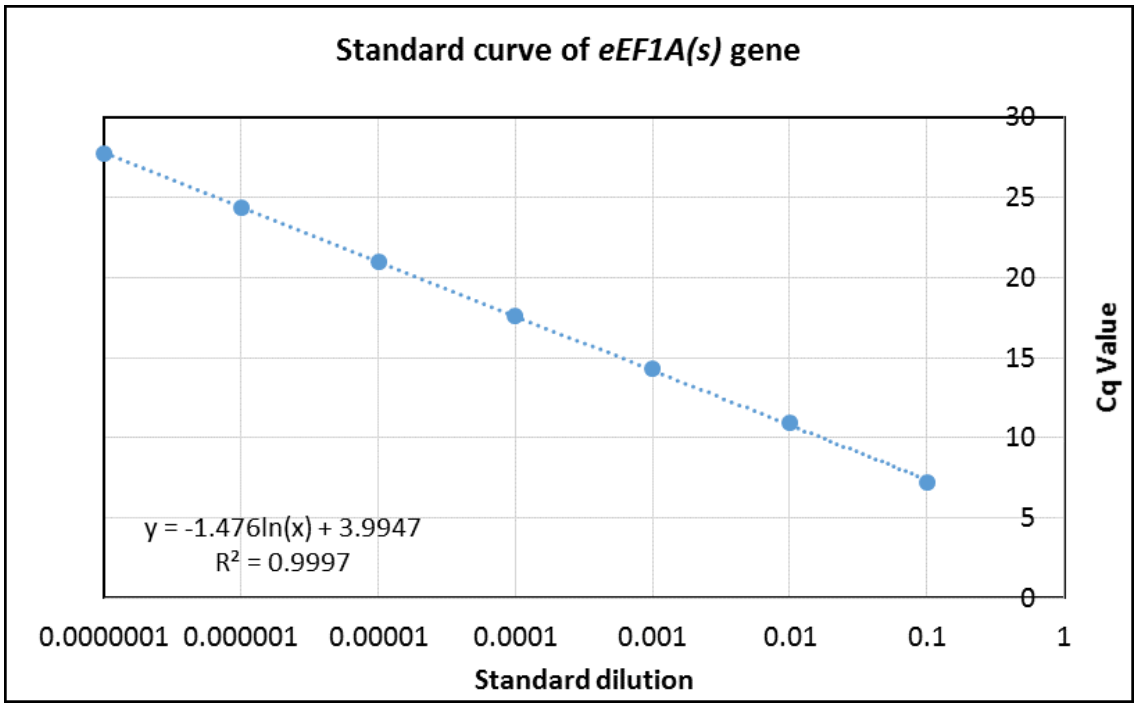


Sequence results of expression vector, *pB7F_WG2:LpUbl5* shows the sequence coverage of *LpUbl5* gene from the start codon and absence of stop codon used for genetic transformation of *N. benthamiana* and onion epidermal cells.

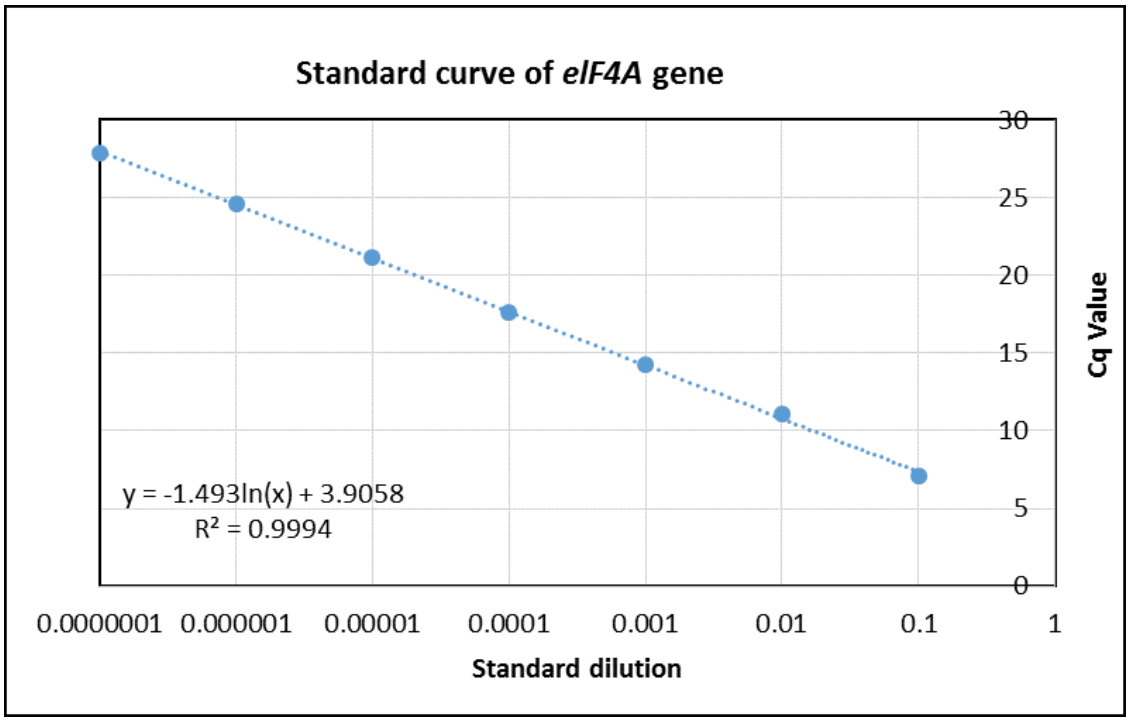
C.3 Standard curve for *LpUbl5* primer pair



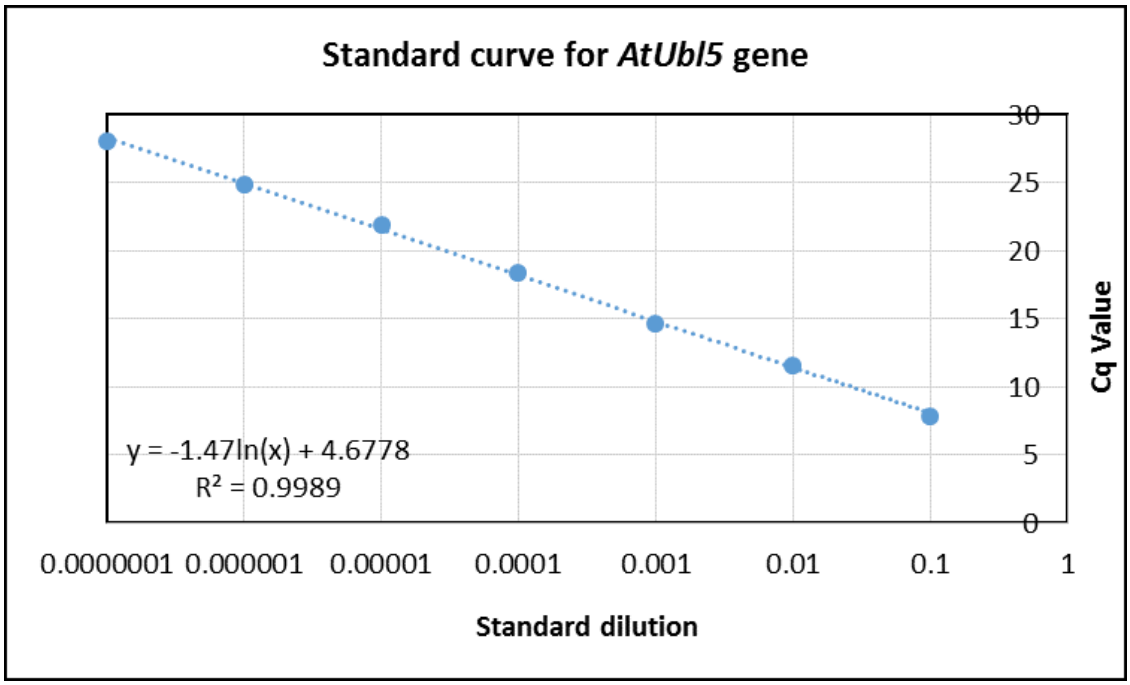
C.4 Standard curve for *eEF1A(s)* primer pair



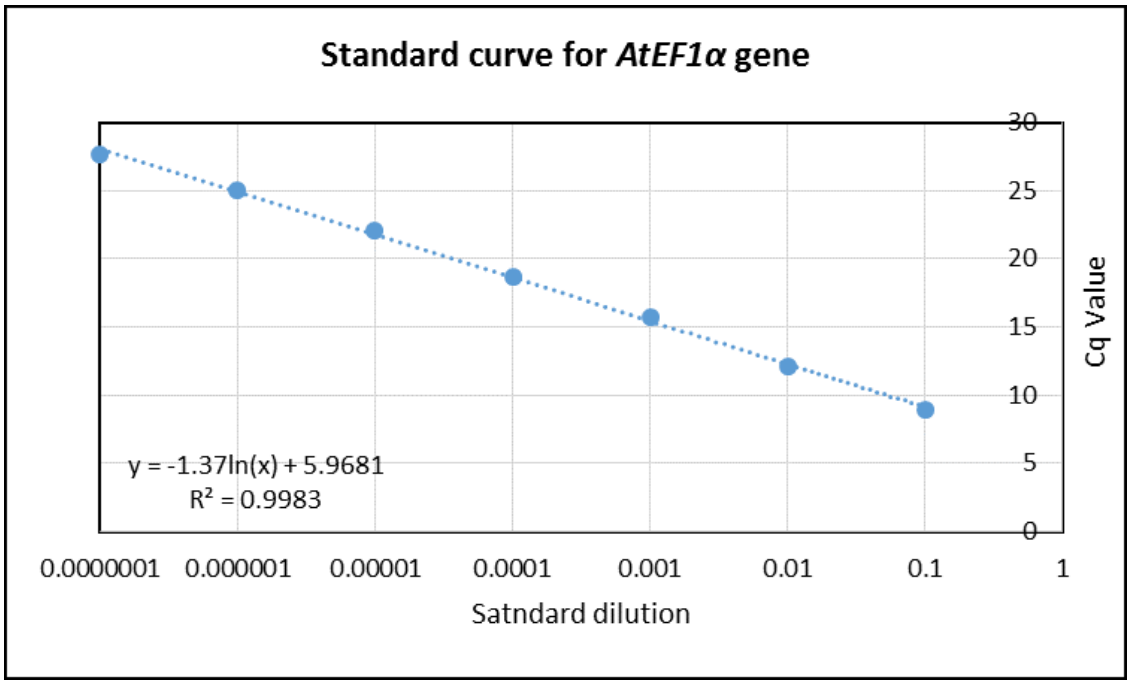
C.5 Standard curve for *elF4A* primer pair



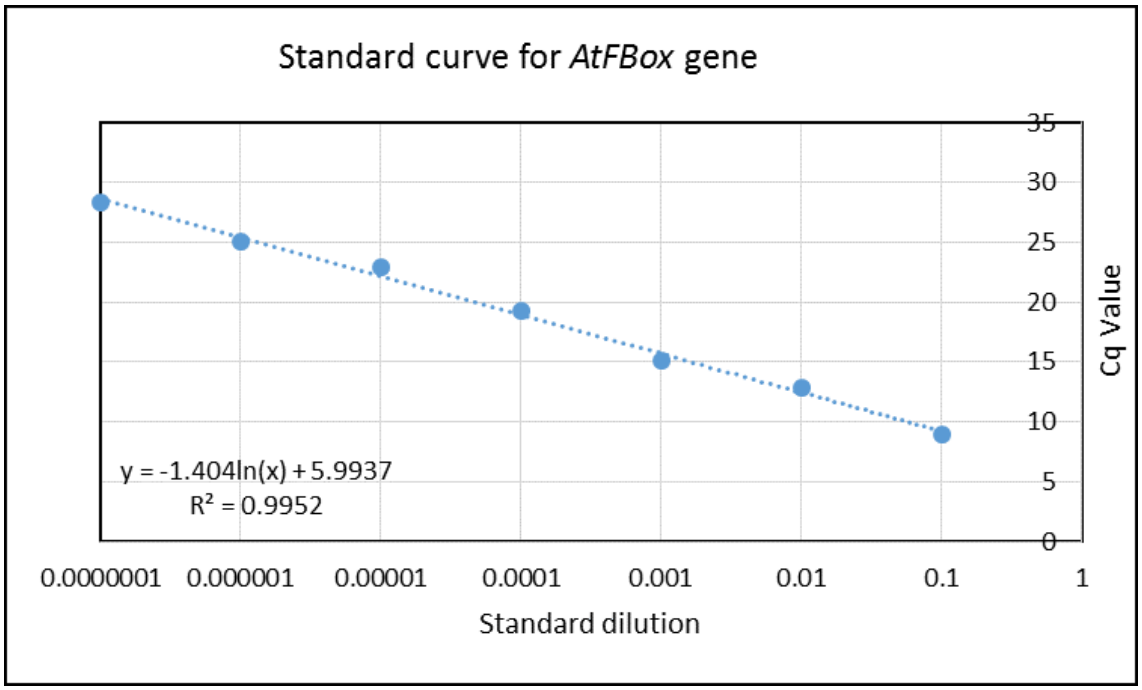
C.6 Standard curve for *AtUbl5* primer pair



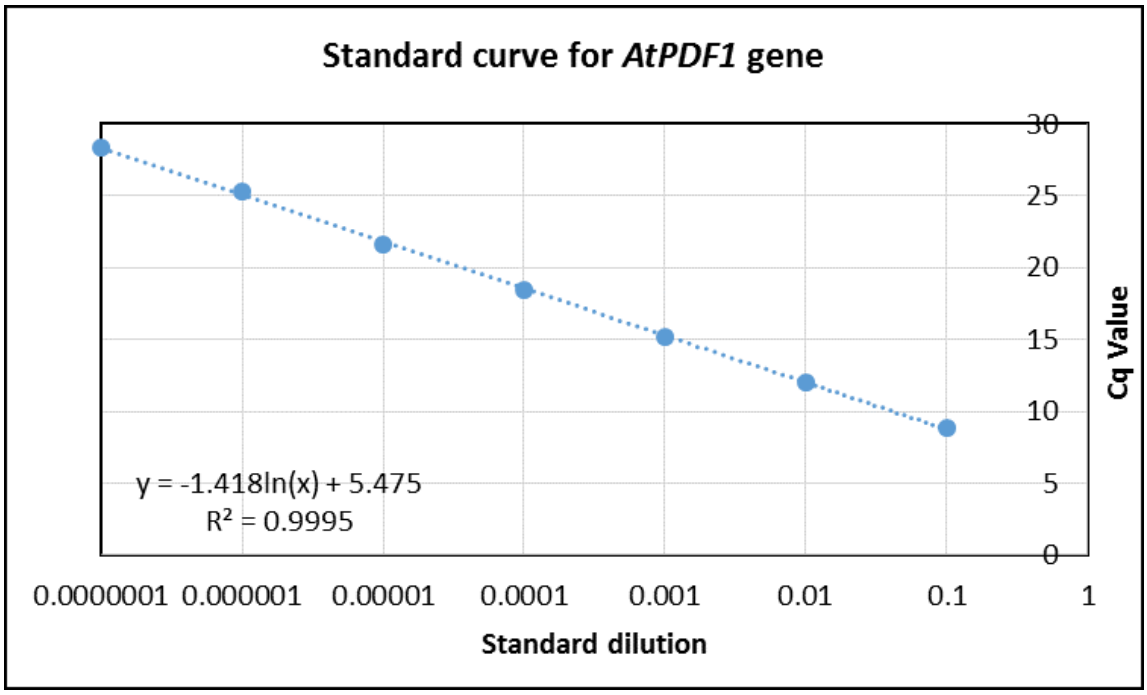
C.7 Standard curve for *AtEF1α* primer pair



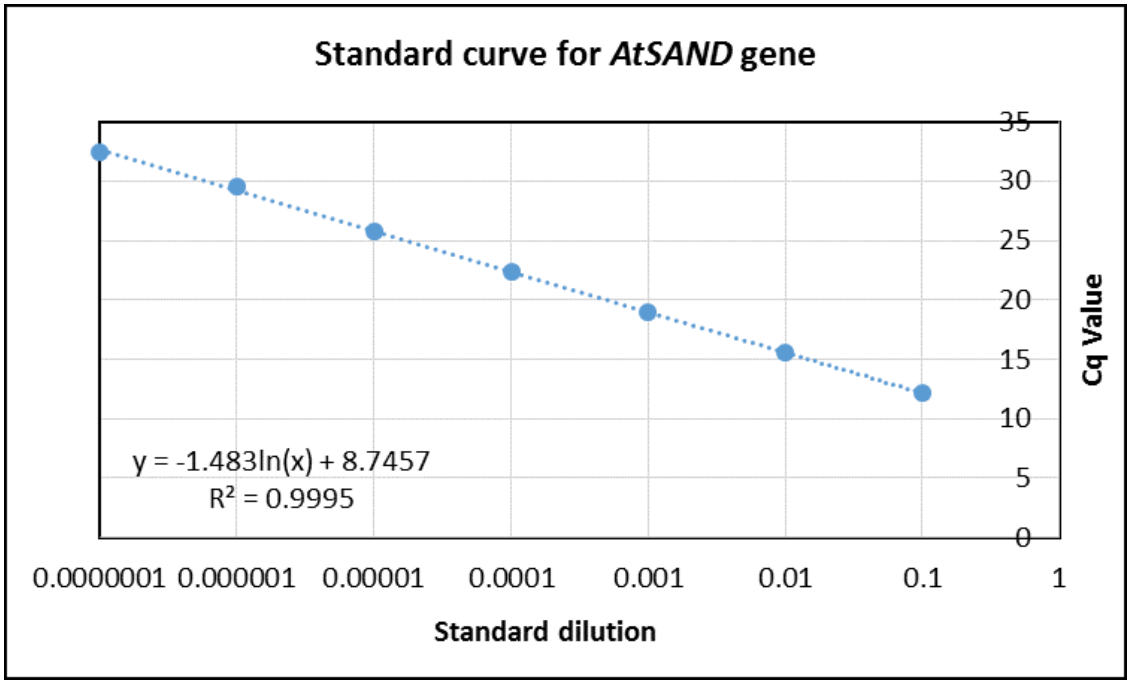
C.8 Standard curve for AtEF1α primer pair



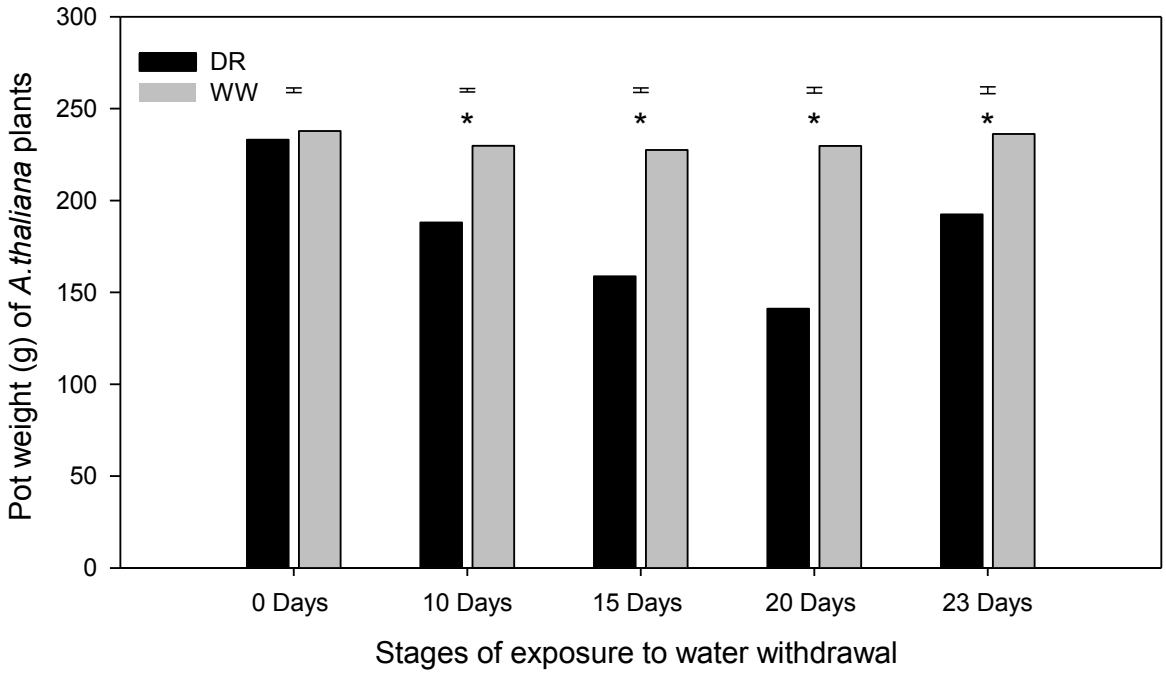
C.9 Standard curve for *AtPDF1* primer pair



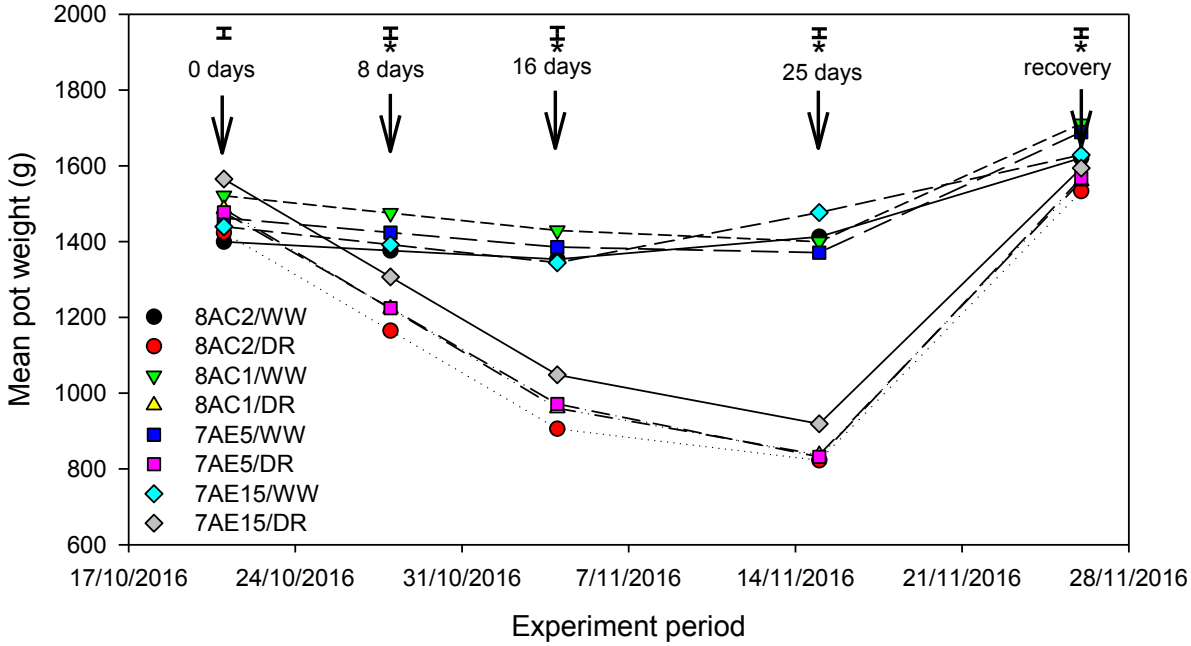
C.10 Standard curve for *AtSAND* primer pair



C.11 Pot weight used to grow *A. thaliana* for *LpUbl5* transcript abundance

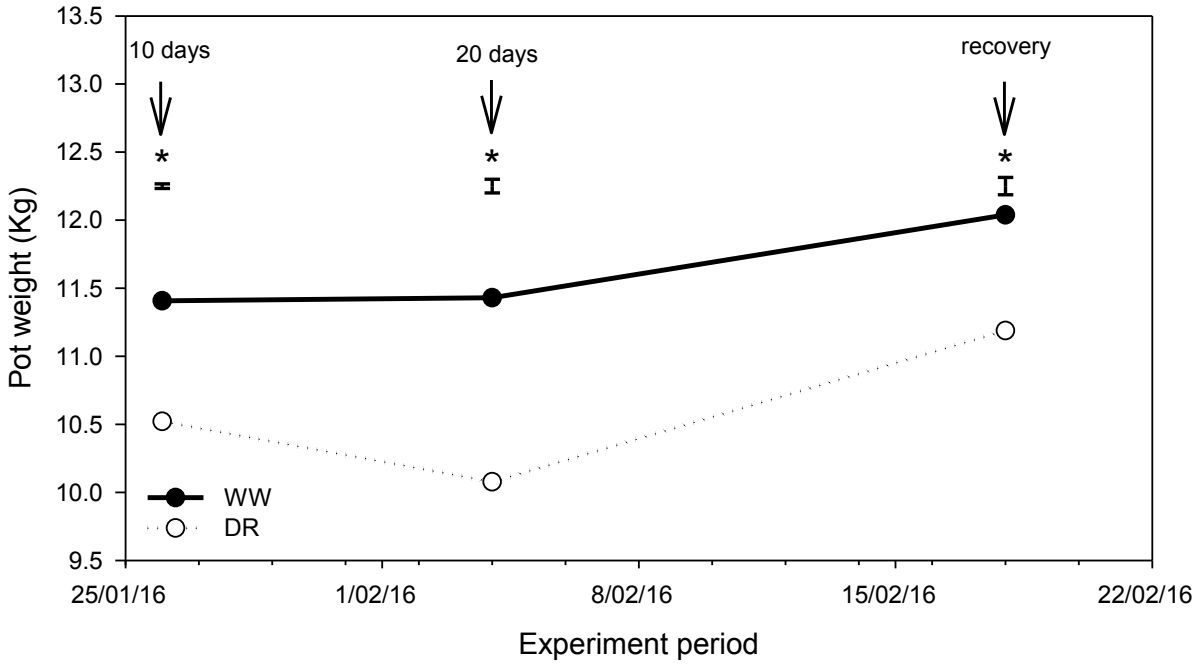


C.12 Pot weight from Experiment 5-A, primary transgenic ryegrass lines



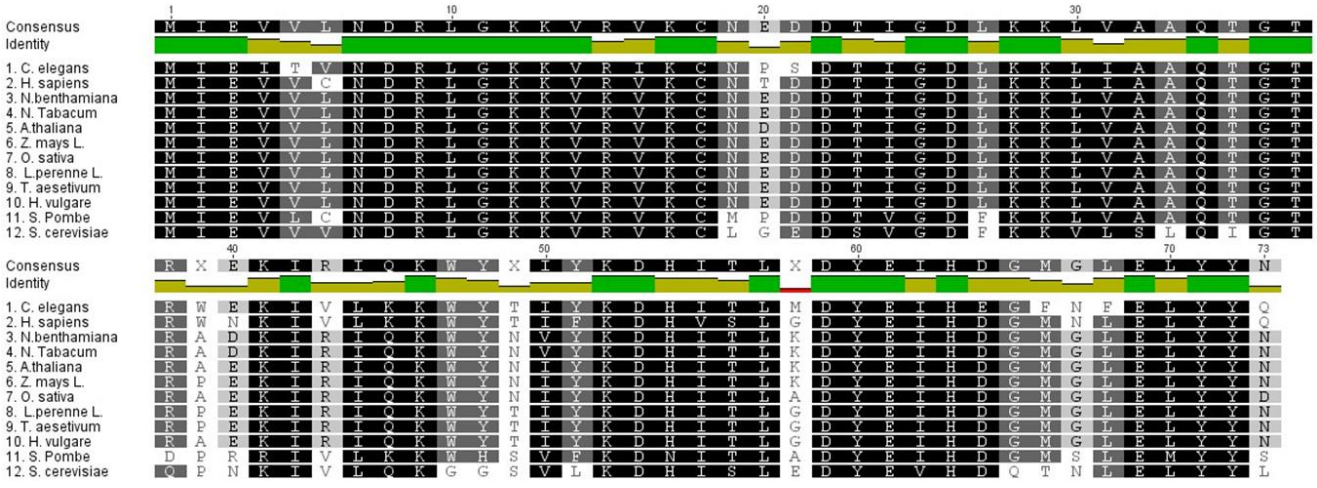
Mean pot weight during Experiment 5-A from well watered (WW) and water withheld drought (DR) pots. Error bar shown is the SEM for the treatment and asterisks where significant differences were detected.

C.13 Pot weight from Experiment 5-B, 'Impact'



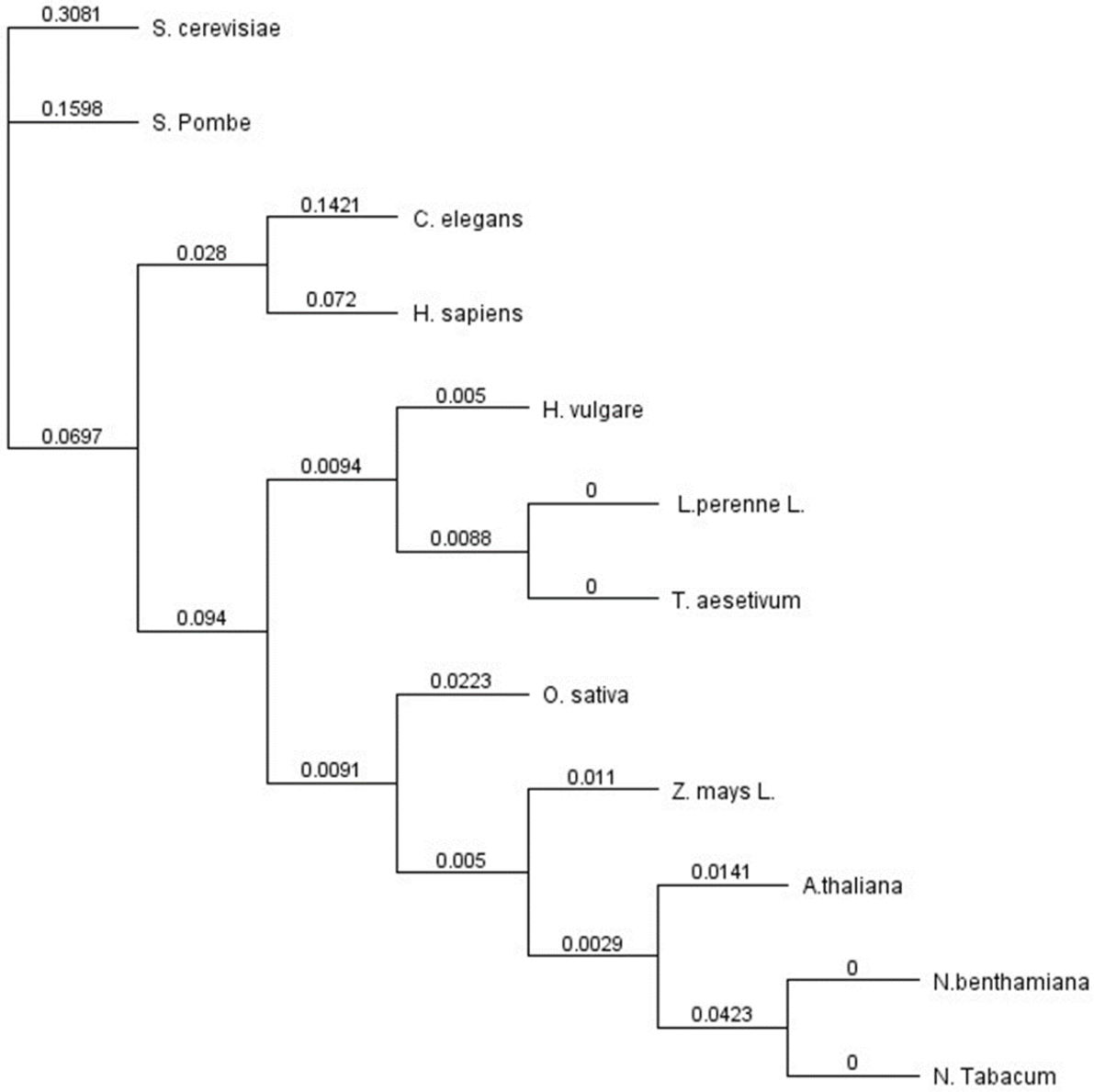
Mean pot weight during Experiment 5-B from well watered (WW) and water withheld drought (DR) pots. Error bar shown is the SEM for the treatment and asterisks where significant differences were detected.

C.14 UBL5 protein sequence alignment



Protein sequence alignment results obtained by ClustalW alignment using UBL5 protein sequence from selected species using Geneious 7.1.7.

C.15 UBL5 protein Phylogenetic tree



An un-rooted protein phylogeny tree by Geneious tree builder using Neighbour joining method of selected proteins and no outgroup was chosen. Distances were obtained from pairwise alignments of all sequence pairs.

Appendix D

Results from online prediction tools for Subcellular Localization of *Lp*UBL5 protein

D.1 Nuc-Ploc Computation Results

Nuc-PLoc:Predicting protein subnuclear localization

| Read Me | Benchmark Data | Citation |

Your input sequence(73aa) is:

>QuerySeq
MIEVVLNDRLGKKVRVKCNEDDTIGDLKKLVAAQTGTRPEKIRIQKWYTIYKDHITLGDY
EIHDGMGLELYYN

----------Predicted Results----------

Protein	Predicted subnuclear location
QuerySeq	**Nucleolus**

Home Page

(Chou, 2005; Shen and Chou, 2005, 2006, 2007), http://www.csbio.sjtu.edu.cn/bioinf/Nuc-PLoc/

D.2 Result generated by NucPred

SBC STOCKHOLM BIOINFORMATICS CENTER

NucPred

The NucPred score for your sequence is 0.02 (see score help below)

```
  1  MIEVVLNDRLGKKVRVKCNEDDTIGDLKKLVAAQTGTRPEKIRIQKWYTI  50
 51  YKDHITLGDYEIHDGMGLELYYN                            73
```

Positively and negatively influencing subsequences are coloured according to the following scale:

(non-nuclear) negative positive (nuclear)

NucPred score threshold	Specificity	Sensitivity
see above	fraction of proteins predicted to be nuclear that actually are nuclear	fraction of true nuclear proteins that are predicted (coverage)
0.10	0.45	0.88
0.20	0.52	0.83
0.30	0.57	0.77
0.40	0.63	0.69
0.50	0.70	0.62
0.60	0.71	0.53
0.70	0.81	0.44
0.80	0.84	0.32
0.90	0.88	0.21
1.00	1.00	0.02

NucPred score threshold for LpHUB1 protein sequence generated is 0.02 which is categorized as fraction of true nuclear protein that are predicted (Brameier *et al.*, 2007) .

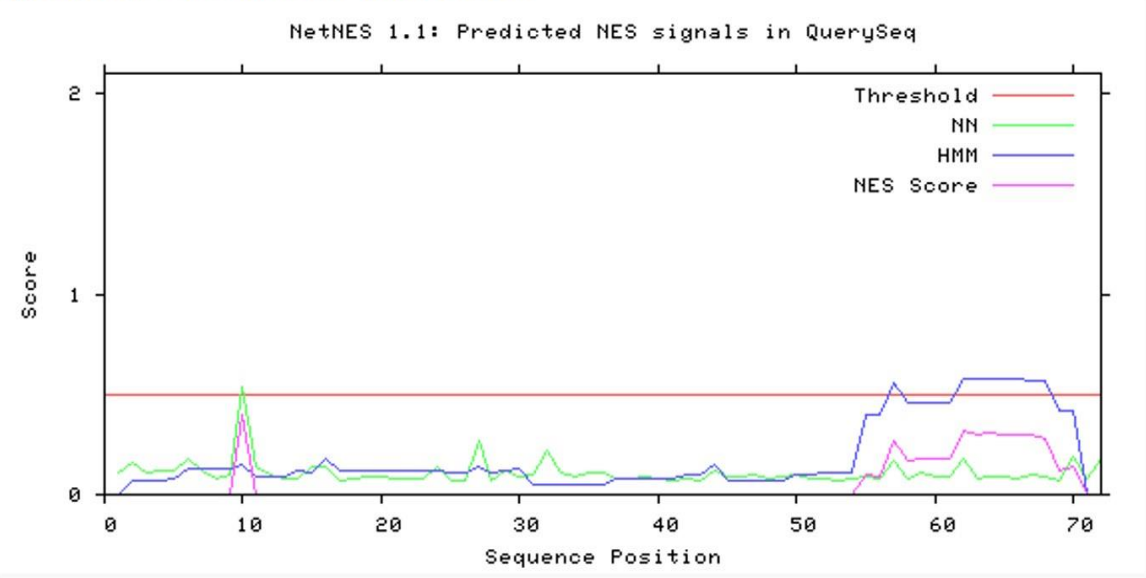
D.3 Result of LpHUB1 protein generated by NetNES Server

CENTERFO RBIOLOGI CALSEQU ENCEANA LYSIS CBS

NetNES 1.1 Server - prediction results

Technical University of Denmark

>QuerySeq - NetNES 1.1 prediction



NES score from the HMM and Artificial Neural Network (ANN) scores obtained for LpHUB1 did not exceed the threshold to identify a nuclear export signal in LpHUB1 protein sequence.
http://www.cbs.dtu.dk/cgi-bin/webface2.fcgi?jobid=5696C0500000583767DC1A5B&wait=20

D.4 Result of LpHUB1 protein generated by cNLS Mapper

cNLS Mapper Result

Predicted NLSs in query sequence	
MIEVVLNDRLGKKVRVKCNEDDTIGDLKKLVAAQTGTRPEKIRIQKWYTI	50
YKDHITLGDYEIHDGMGLELYYN	73

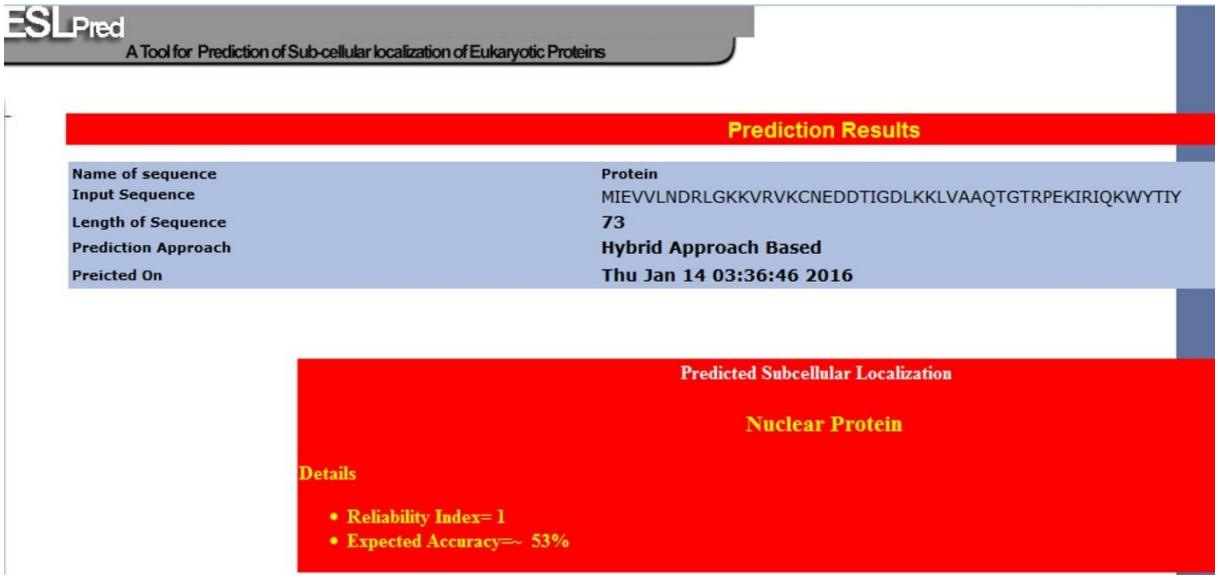
Predicted monopartite NLS		
Pos.	Sequence	Score

Predicted bipartite NLS		
Pos.	Sequence	Score
8	DRLGKKVRVKCNEDDTIGDLKKLVAAQTGTRP	2.2
9	RLGKKVRVKCNEDDTIGDLKKLVAAQTGTRPEKI	2.1

The cNLS Mapper identify putative NLS sequences and score based on the NLS activity which would generate a higher score to indicate stronger NLS activities. A protein with a score of 8, 9, or 10 is localized to the nucleus, whereas a score of 7 or 8 indicate a partial localization in the nucleus, a score of 3, 4, or 5 would localize both in the nucleus and the cytoplasm and that with a score of 1 or 2 localized to the cytoplasm.

The cNLS Mapper result of LpBUB1 protein generated a score of 2.2. and 2.1 and is not a conclusive result. This indicates that protein is predominantly a cytoplasmic protein (Kosugi *et al.*, 2008; Kosugi *et al.*, 2009a; Kosugi *et al.*, 2009b).

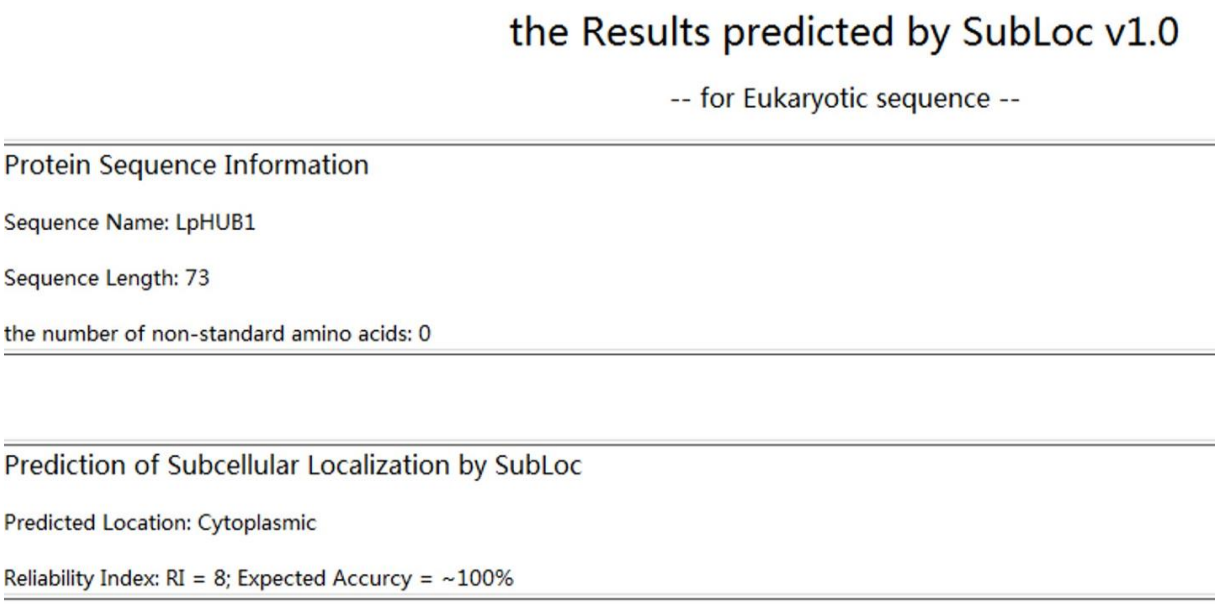
D.5 Result of LpHUB1 protein generated by ESPpred



The results generated has a prediction of nuclear localization of LpHUB1 protein

http://www.imtech.res.in/raghava/eslpred/submit.html

D.6 Result of LpHUB1 protein generated by SubLoc v1.0



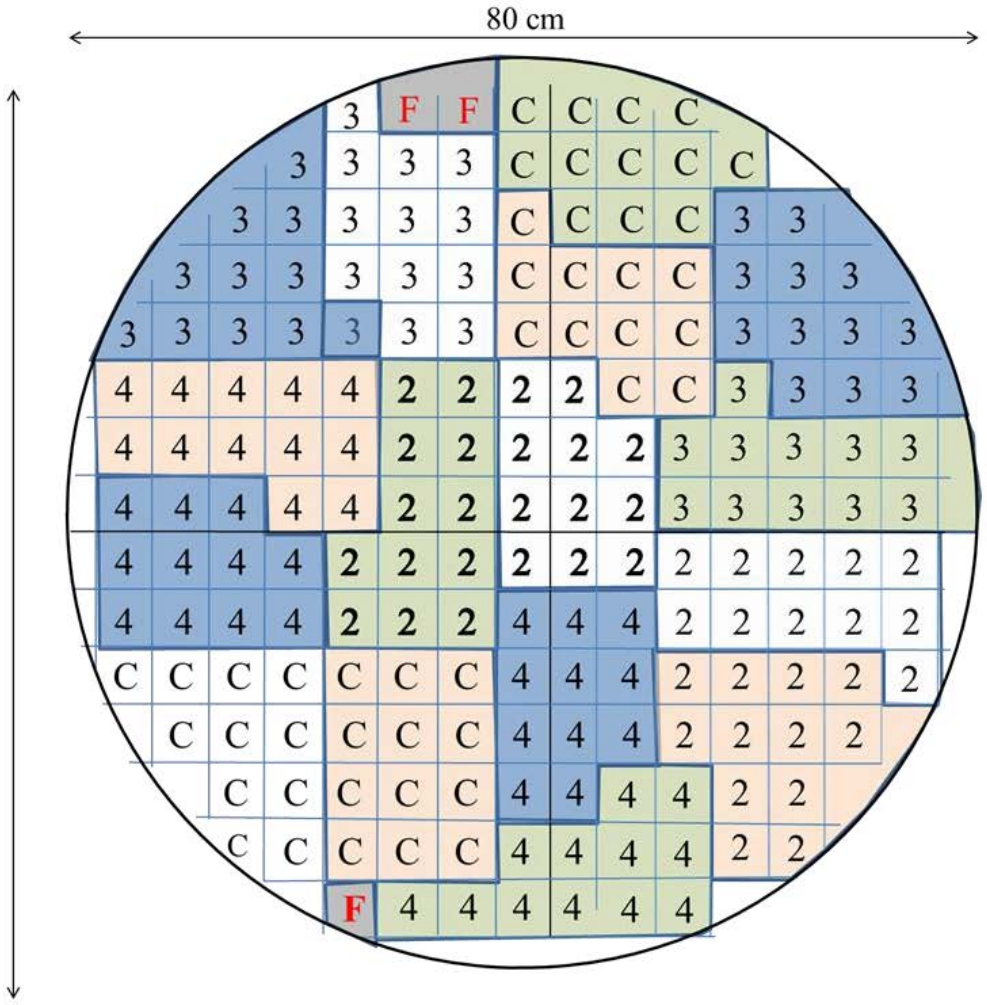
The result generated has a prediction of cytoplasmic localization of LpHUB1 protein.

Appendix E

This section contains experiment design used for experiment 1, and 2 in the Biotron, rhizotrons and glass house.

E.1 Experiment design of Rhizotron 1

Layout of Rhizotron 1 with plot numbers 1 to 16 and treatment replicates 1-4. The red coloured "F" indicates fill plants added as guard rows to exploit the soil volume available to neighbouring plants.

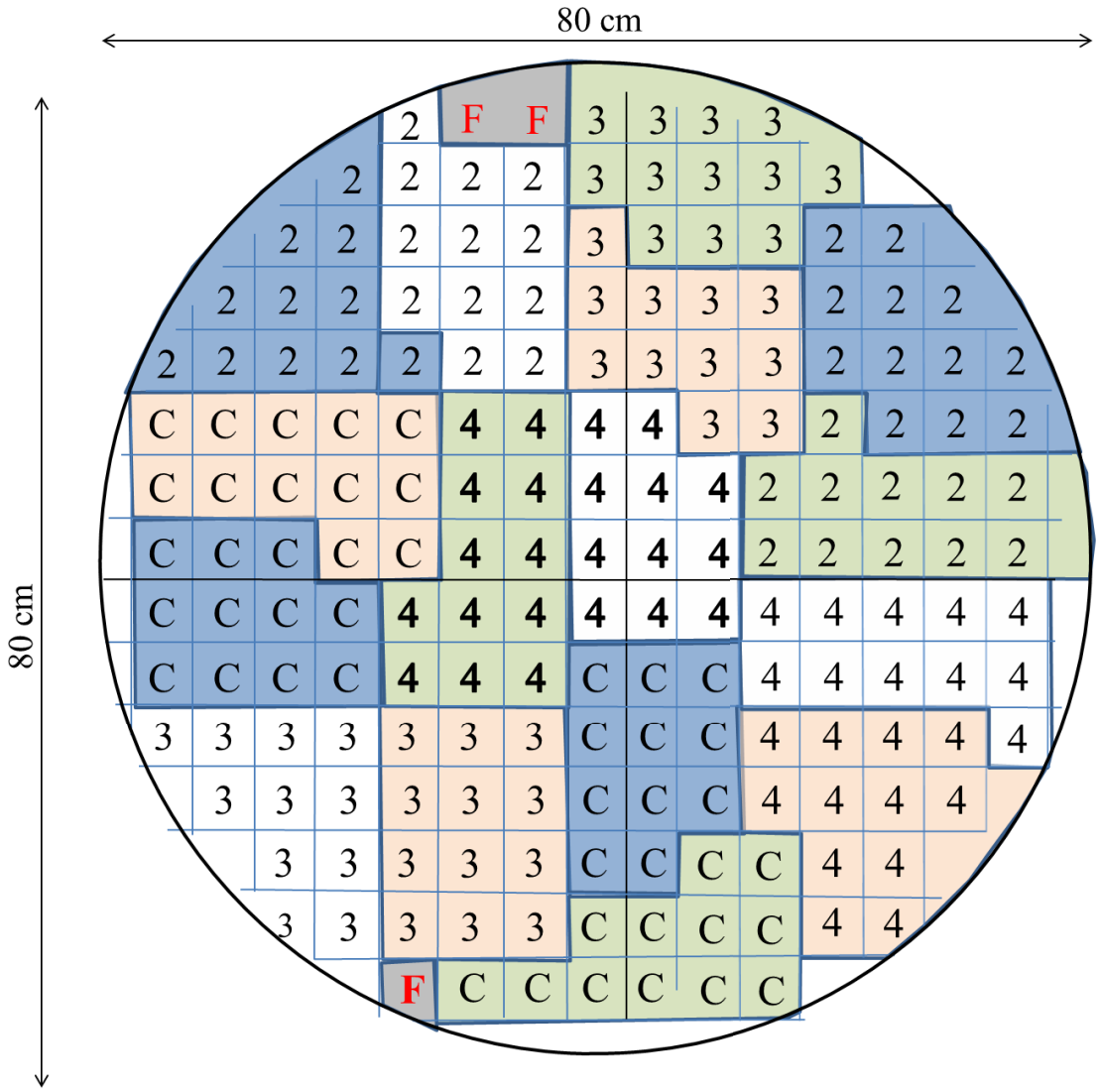


"F"= fill plant (to restrict exploitable soil volume available to neighbouring test plants)

Treatments:
C = Control
2 = 8AC 1
3 = 8AC2 –
4 = 8AC2 +

E.2 Experiment design of Rhizotron 2

Layout of Rhizotron 2 with plot numbers 17 to 32 and treatment replicates 5-8. The red coloured "F" indicates fill plant to exploit the soil volume available to neighbouring plants.

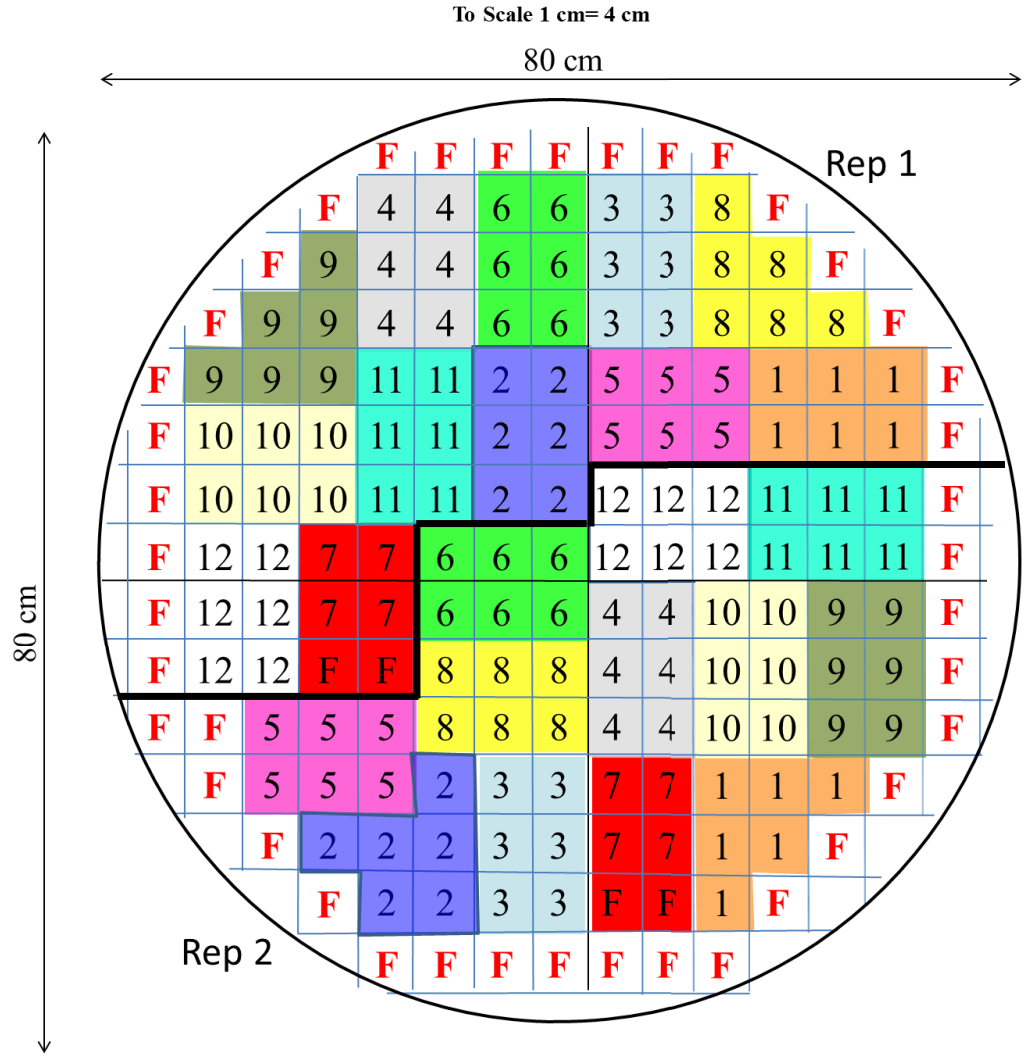


"F"= fill plant (to restrict exploitable soil volume available to neighbouring test plants)

Treatments:
C = Control
2 = 8AC 1
3 = 8AC2 –
4 = 8AC2 +

E.3 Experiment design of Rhizotron 3

Layout of Rhizotron 3 with plot numbers 1to 24 and treatment replicates 1-2. The red coloured "F" indicates fill plant to exploit the soil volume available to neighbouring plants.

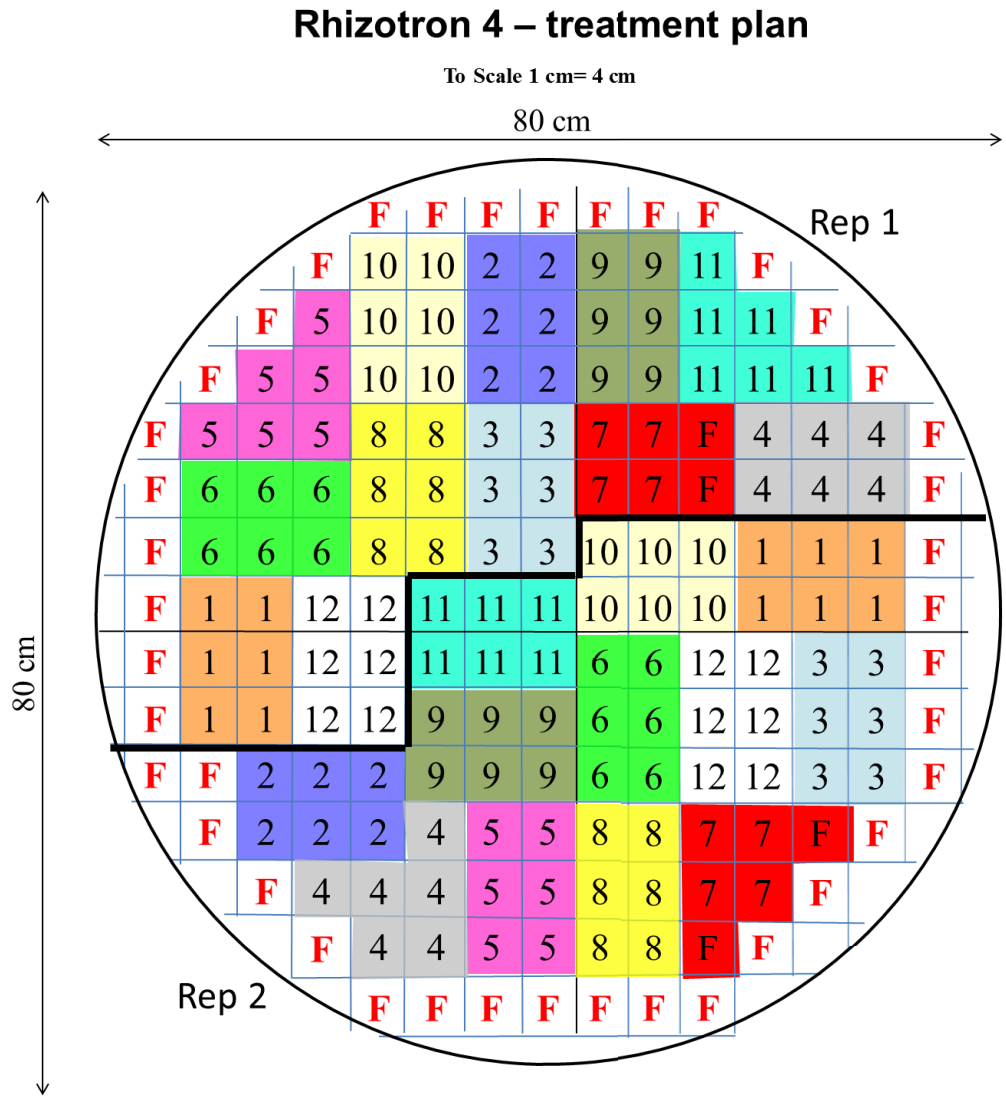


"F"= fill plant (to restrict exploitable soil volume available to neighbouring test plants).
These plants are match the adjacent trtmt group (specified in documentation) except Trts 7,11,12 (see documentation)

Treatments		Treatments	
1	A14499	7	A6889 (2 Reps, 4 plants + 2 fillers)
2	A14542	8	A6932
3	A15323	9	A77187
4	A15334	10	A7798
5	A15369	11	8AC1 (prev. est for Rhizo 1 & 2 Exp)
6	A17183	12	Control (prev. est for Rhizo 1 & 2 Exp)

E.4 Experiment design of Rhizotron 4

Layout of Rhizotron 4 with plot numbers 25 to 48 and treatment replicates 3-4. The red coloured "F" indicates fill plant to exploit the soil volume available to neighbouring plants.



"F"= fill plant (to restrict exploitable soil volume available to neighbouring test plants).
These plants are match the adjacent trtmt group (specified in documentation) except Trts 7,11,12 (see documentation)

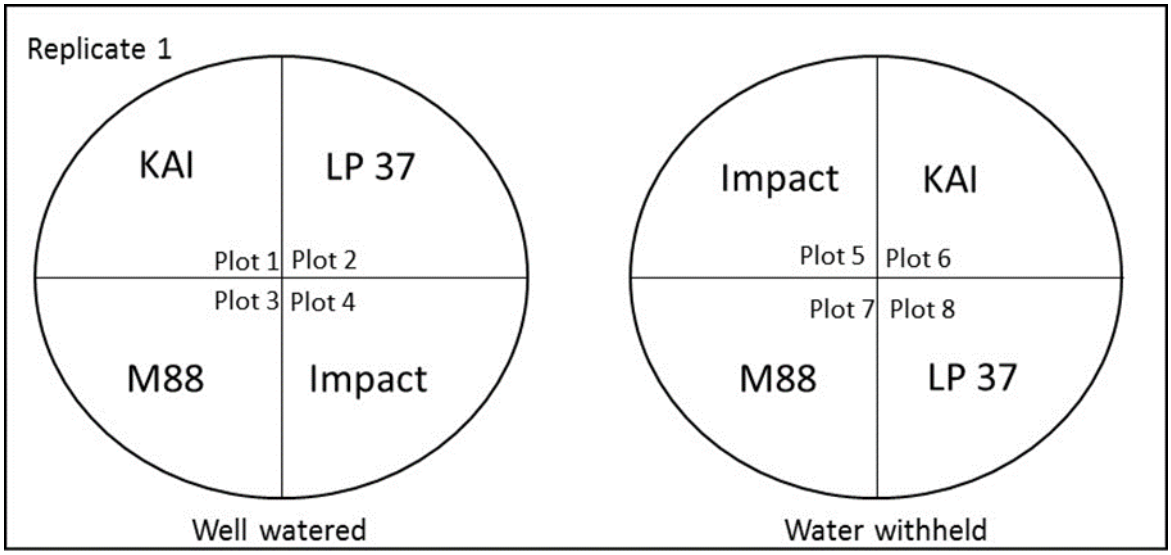
Treatments		Treatments	
1	A14499	7	A6889 (2 Reps, 4 plants + 2 fillers)
2	A14542	8	A6932
3	A15323	9	A77187
4	A15334	10	A7798
5	A15369	11	8AC1 (prev. est for Rhizo 1 & 2 Exp)
6	A17183	12	Control (prev. est for Rhizo 1 & 2 Exp)

E.5 Experiment 5-A design

Well watered	REP 1	7AE5	8AC2	8AC1	7AE15
Water withdrawn	REP 1	8AC1	7AE15	8AC2	7AE5
Well watered	REP 2	7AE15	8AC1	7AE5	8AC2
Water withdrawn	REP 2	8AC2	7AE5	8AC1	7AE15
Water withdrawn	REP 3	7AE5	8AC2	7AE15	8AC1
Well watered	REP 3	8AC1	7AE15	7AE5	8AC2
Water withdrawn	REP 4	7AE15	7AE5	8AC2	8AC1
Well watered	REP 4	8AC2	8AC1	7AE15	7AE5

Design of Experiment used for primary transgenic ryegrass used in Chapter 7.

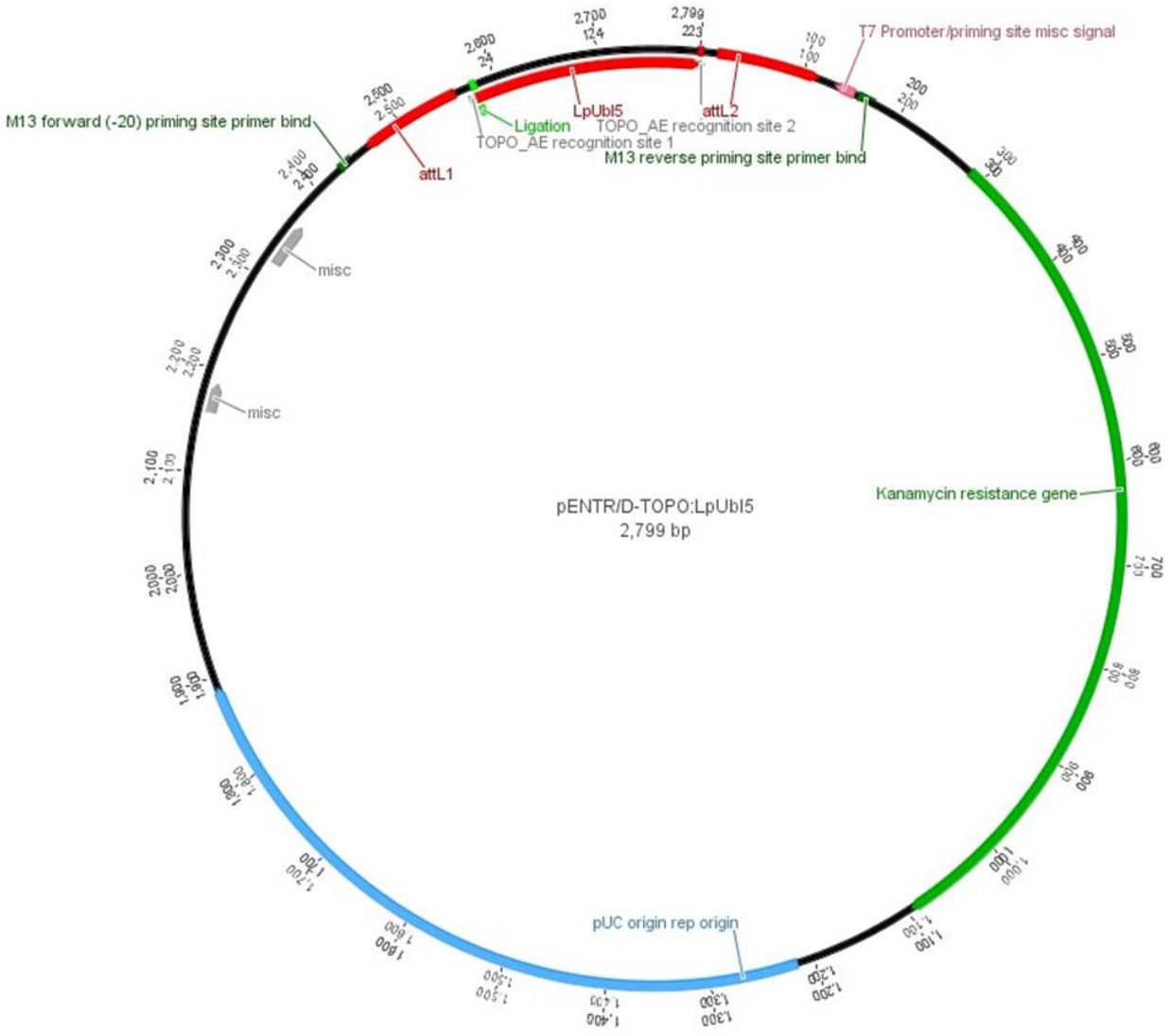
E.6 Experiment 5-B design



Diagrammatic representation of Experiment 5_B, Replicate 1 used for the glass experiment to conduct drought stress experiments.

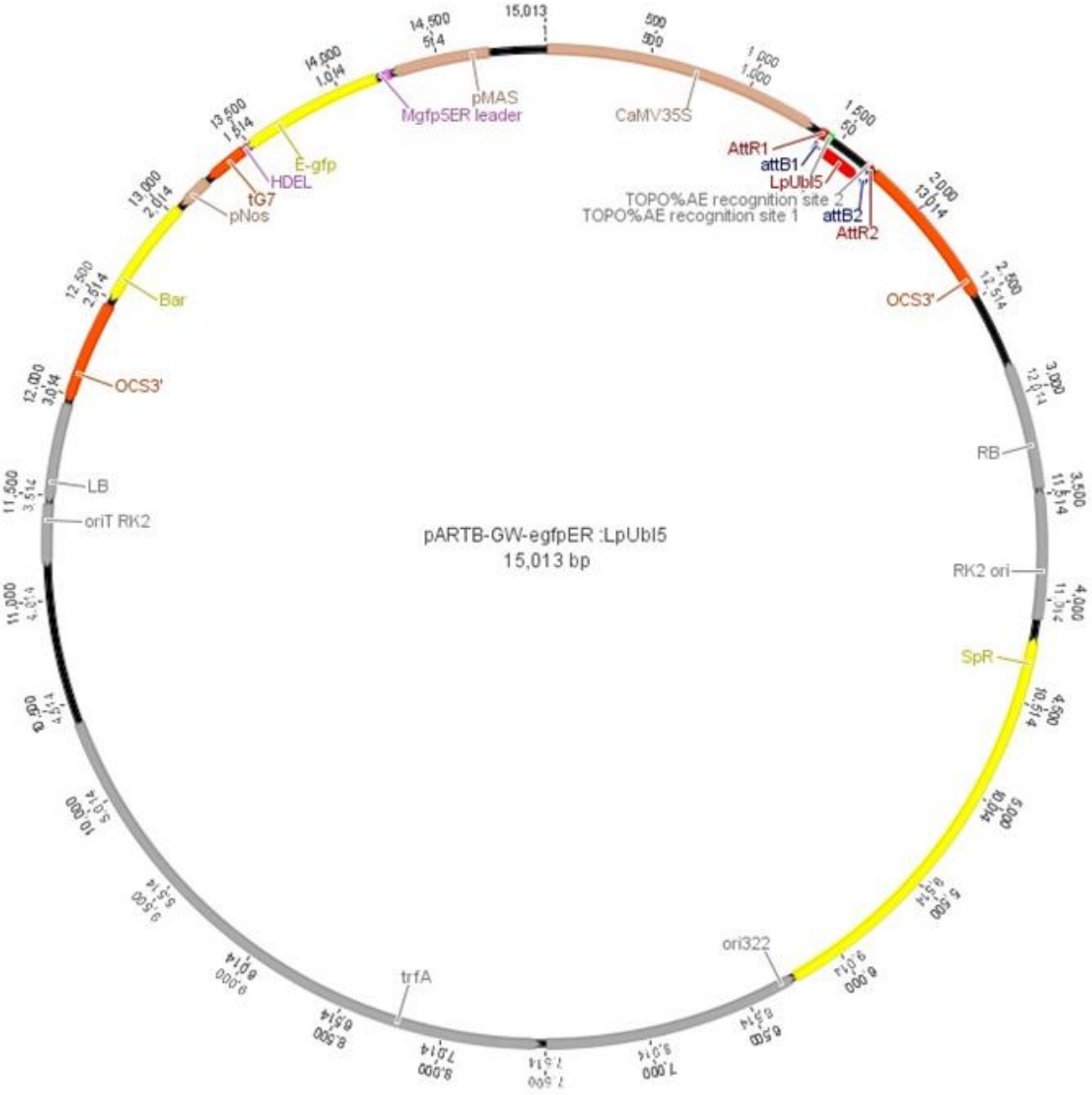
Appendix F

F.1 Schematic representation of pENTR/D-TOPO: LpUbl5



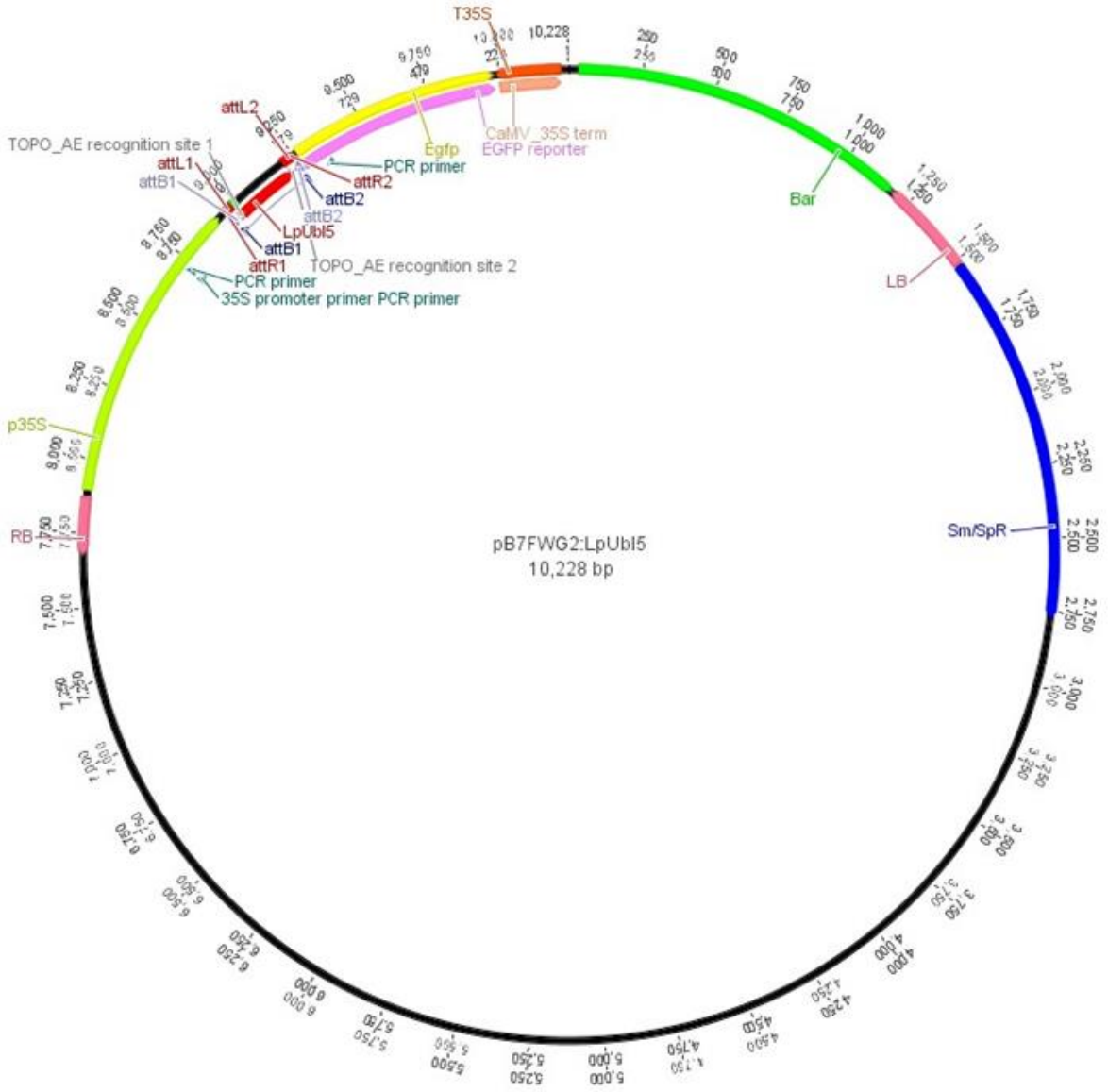
Schematic representation of *pENTR/D-TOPO: LpUbl5* which used to generate expression vector.

F.2 Schematic representation of *pARTB_GW_egfpER: LpUbl5*



Schematic representation of *pARTB_GW_egfpER: LpUbl5* which used as expression vector in *A. thaliana*

F.3 Schematic representation of *pB7F_WG2:LpUbl5*



Schematic representation of *pB7F_WG2: LpUbl5* which used as expression vector in *N. benthamiana* and onion epidermal cells

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