Understanding phenotypic and epigenetic drought responses in *Trifolium repens* (white clover)

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Abstract

Trifolium repens (white clover) is an allopolyploid species belonging to the clover genus (*Trifolium*). White clover is very important in New Zealand agriculture as feed for livestock. Yet this species is facing challenges due to drought, an abiotic stress that has impacted New Zealand's economy in the past and is predicted to have increased effects in the future due to climate change. Therefore, analysing plant phenotypic and epigenetic responses to drought is an important area of research because it will broaden understanding of drought resistance/tolerance and mechanisms that plants use to survive drought stress.

An epigenetic mechanism of interest is DNA methylation, which has a role in gene expression in response to drought. There is also evidence that DNA methylation is involved in plant memory and 'priming' which assists in subsequent stress responses. However, this response has not been explored in depth in white clover. In other species there has been evidence of transgenerational inheritance of DNA methylation marks associated with stress. Therefore, research into stress-related DNA methylation in white clover is important as it may be able to be incorporated into the plant breeding process in the future to breed more drought-tolerant varieties.

In this study, a drought trial with two subsequent drought stress periods was run on white clover varieties and related species. A range of phenotypes were measured throughout the drought trial, including relative water content and leaf area. There was evidence that these phenotypes were significantly impacted by drought stress. The relative water content measurements revealed significant differences in drought response depending on whether plants were experiencing their first or second drought, suggesting that some aspect of plant stress memory was involved.

Subsequently, DNA methylation analysis was run on a subset of the white clover plants involved in the drought trial. It was found that DNA methylation played a role in the plant

drought response and that there were differences in methylation patterns between plants that were experiencing their first and second drought exposures. Further investigation into genomic regions with DNA methylation profiles retained after drought stress revealed examples of stress-related genes. This supported the hypothesis that areas of differential methylation had a role in the stress response.

This study revealed insights into white clover's drought response and provides a starting point for further research into how these findings can be incorporated into plant breeding/generating more drought tolerant varieties in the future.

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Abbreviations

3D	3-dimensional
5-mC	5-methylcytosine
°C	degrees Celsius
µmol	micro mole
A	leaf area
ABA	abscisic acid
BLUE	best linear unbiased estimator
BS	bisulphite sequencing
bp	base pair
CAM	crassulacean acid metabolism
CO_2	carbon dioxide
cv	cultivar
DMC	differentially methylated cytosine
DNA	deoxyribonucleic acid
DW	dry weight
EM-seq	enzymatic methyl-seq
FW	fresh weight
g_s	stomatal conductance
H_2O	water
kb	kilobase
LMM	linear mixed model
LSD	least significant difference
mol	mole
N/ha/year	kg nitrogen per hectare per year

NZ	New Zealand
PCR	Polymerase chain reaction
ppm	parts per million
RdRM	RNA-directed DNA methylation
RFO	raffinose family of oligosaccharides
RNA	ribonucleic acid
ROS	reactive oxygen species
RRBS	reduced representation bisulphite sequencing
RT-qPCR	reverse transcription-quantitative polymerase chain reaction
RWC	relative water content
SAM	S-adenosyl-L-methionine
SDDL5	S10 double-droughted leaf at time point 5
SDDL7	S10 double-droughted leaf at time point 7
SSDL5	S10 single-droughted leaf at time point 5
SSDL7	S10 single-droughted leaf at time point 7
SWL5	S10 watered leaf at time point 5
SWL7	S10 watered leaf at time point 7
TE	transposable element
TP	time point
TW	turgid weight
UV	ultraviolet
V	volt
WC	white clover
WGBS	whole genome bisulphite sequencing

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1 Introduction

1.1 White clover

1.1.1 Taxonomy, morphology, and growth

Trifolium repens, white clover (WC) is a member of the Fabaceae family of flowering plants. The clover genus (*Trifolium*) is named for its trifoliate (three leaflets) leaf composition. These leaflets are usually elliptical, egg-shaped or heart shaped with miniature serrations on the edges (Thomas, 1987).

Trifolium species are often prostrate and less than 50 cm tall (Ellison et al., 2006) due to their stoloniferous growth. The stolon is an important structural component of mature WC plants. The stolon is a horizontal, creeping, aboveground stem that is made up of a series of nodes and internodes (Figure 1.1). Each node can have a trifoliate leaf growing from it, as well as two root primordia and an axillary bud (Figure 1.1). The root primordia may form roots if the node contacts moist soil and the axillary buds may grow to form additional stolons (Thomas, 1987).

Figure 1.1. The white clover plant showing basic morphology (Wachendorf et al., 2001)

After about 18 months of growth, the mature WC plant transitions from the initial seedling taproot to stolon growth. After this point, the plant continues as stolon fragments made up of 8-12 nodes supported by nodal rooting and moves horizontally through the sward (Thomas, 1987). Stolon generation is WC's vegetative reproductive system, and seed production and dispersal allow WC to also sexually reproduce (Thomas, 1979). Vegetative reproduction is important for conservation of growth within smaller areas via stolon spread, while sexual reproduction by seed is important for dispersal to new areas.

In the late 1920s, assessment of WC strains and ecotypes began, along with intensive white clover breeding (Brock et al., 1989). WC has been selectively bred to grow in a variety of conditions. In the past, genetic improvements in areas such as herbage yield, forage quality,

pest and disease resistance and nitrogen fixation have been well documented. These core traits are still the target of current breeding goals; however, breeding is expanding to involve adaptation to semi-arid and other marginal environments (Williams et al., 2007).

WC cultivars are varieties produced by selective breeding (Caradus, 1994). More than 250 WC synthetic cultivars and ecotypes have been released worldwide (Caradus, 1986). WC leaf size varies depending on the type/cultivar of WC as well as the environmental conditions in which they are grown. There are four broad groups of WC cultivars based on their leaf size in New Zealand environments (Caradus et al., 1989). There are small-leaved cultivars (leaflets <1 cm in length), intermediate- or medium-leaved cultivars, large-leaved cultivars (leaflets >2 cm in length) and large-leaved Ladino-type cultivars. These leaf-size classes relate to agronomic use.

Stolon density is critical for plant persistence; therefore, this is an important characteristic of different WC cultivars. For maintenance of density, rate of stolon production must be greater than rate of stolon death (Woodfield & Caradus, 1996). Small-leaved cultivars are generally lower-growing and have a greater stolon density, therefore perform better under sheep grazing conditions as they are difficult for animals to completely remove under continuous grazing (Charlton & Stewart, 1999). Large-leaved cultivars were bred for the dairy environment as these cultivars are most productive under rotationally grazed systems. This is because they have a greater yield potential but lower stolon density. Therefore, these cultivars with lower stolon density perform better under rotationally grazed systems. Medium-leaved cultivars perform well under a variety of grazing conditions as they have a good middle ground of stolon density to yield (Woodfield & Caradus, 1996).

1.1.2 White clover's importance in New Zealand

Since white clover's introduction to New Zealand, it has become one of the most important pasture legumes in NZ agriculture as it contributes to ~12% of all livestock nutrient

requirements (NZIER, 2016). Intensification of grazing by ruminant animals has increased the necessity of clover to perform and produce (Clark et al., 2001; Lambert et al., 2004). This further emphasises the importance of developing clover cultivars that are better adapted to the intensive grazing systems of cattle (and other ruminant species) (Woodfield & Caradus, 1996).

Another important capability of WC is its ability to fix atmospheric nitrogen via a symbiotic relationship with *Rhizobium* bacteria (Ledgard & Steele, 1992). This reduces the need for synthetic nitrogen fertilisers, as this nitrogen-fixing symbiotic relationship can produce on average 80-100 kg N/ha/year in grazed permanent clover/grass pastures in temperate regions of the world (Ledgard, 2001). Due to its nitrogen-fixing capability and pasture quality, WC is a significant component of the economy and is the third most economically important plant species in New Zealand where it contributes NZ$2.3 B to gross domestic product, behind perennial ryegrass (NZ$14.6 B) and pine ((NZ$4.5 B) and an order of magnitude greater than the next import crop, Kiwifruit (NZ$807 M) (NZIER, 2016).

1.2 Polyploidy

Polyploidy (or whole genome duplication) is defined as the presence of greater than two full sets of chromosomes in an organism's genome. Polyploidy is more common in plants than animals (Otto & Whitton, 2000) and has been shown to have an important role in plant evolution. Polyploidisation is commonly viewed as a major force of evolution due to its role in plant speciation and diversification (Adams & Wendel, 2005; Heslop-Harrison et al., 2022; Madlung, 2013; Otto & Whitton, 2000; Soltis et al., 2009).

Two different types of polyploidy are commonly recognised – allopolyploidy and autopolyploidy. Autopolyploidy occurs when there is a genome duplication within a species while allopolyploidy occurs when genome expansion is the result of combining two different species genomes (hybridisation) (Stebbins, 1950).

1.2.1 The formation of polyploids

A variety of mechanisms for the formation of polyploids in nature have been proposed since the discovery of polyploidy. Polyploidy most commonly occurs in plants via the production and fusion of unreduced reproductive cells. Production of unreduced gametes is a heritable feature in many plant species (d'Erfurth et al., 2008). In addition, the production of unreduced gametes is influenced by environmental factors such as temperature, water deficit and nutrient shortage (Ramsey & Schemske, 1998). For example, in the arctic there is a high prevalence of allopolyploids, particularly where plants are reclaiming previously frozen landscapes (Brochmann et al., 2004).

1.2.2 Genetic and genomic consequences of polyploidy

Studies have shown that polyploidy results in a number of short- and long-term consequences in both the genome and transcriptome (Wendel et al., 2018). Short-term consequences of polyploidy may include the spread of transposable elements through the sub-genomes, the loss or silencing of a copy of a duplicated gene also known as a homoeologue. (Parisod et al., 2010; Senerchia et al., 2015). Homoeologues are pairs of genes or chromosomes brought together in an allopolyploid that existed in a common ancestor of the parental species (Glover et al., 2016). Whereas in the longer term, polyploidy can cause vast rearrangements of the genome including a decrease in the number of chromosomes (Woodhouse et al., 2014), neofunctionalization (new gene function) or subfunctionalisation (sharing of gene function between replicates) (Renny-Byfield & Wendel, 2014; Yoo et al., 2014). These changes to the genome can occur at different rates and to different extents in various species due to the diverse nature of polyploid systems (Soltis et al., 2016).

1.2.3 Sub-genomes and gene expression

Polyploidy has a major effect on transcriptome expression. Due to the presence of multiple copies of the same genes, four different fates of these duplicated genes have been observed in allopolyploids (Doyle et al., 2008; Prince & Pickett, 2002; Roulin et al., 2013).

1- Both homoeologues function
2- One homoeologue is silenced, and the other functions as it did in the parental species
3- Both homoeologues diverge and each completes part of the original function (subfunctionalisation)
4- One copy gains a new role (neofunctionalisation)

In addition, expression levels of a polyploids duplicated genes may differ from the original parental levels (Buggs et al., 2014).

A common consequence of polyploidy is sub-genome dominance, where one sub-genome is more dominant in gene expression than the other. Examples of this have been observed in *Tragopogon* species which are young polyploids (80 years) and *Brassica rapa* which is an ancient polyploid (13 million years) (Cheng et al., 2012; Chester et al., 2012).

Many studies have presented data that demonstrating epigenetic changes are a common consequence of polyploidisation. The type of epigenetic reprogramming that occurs most frequently is differences in DNA methylation of cytosines (Diez et al., 2014; Springer et al., 2016; Vicient & Casacuberta, 2017). It was also proposed that DNA methylation of transposable elements explains unbalanced homoeologue expression bias in polyploids resulting in differences in gene expression between sub-genomes of a polyploid (Freeling et al., 2012). This model proposes that when the genomes of two species come together in an allopolyploid, the regions surrounding genes in one sub-genome are likely to have greater methylation content than the other. This causes the genes of one sub-genome to be expressed at lower levels and this sub-genome with suppressed gene expression may be under less

selective constraint and more prone to gene loss (Schnable et al., 2011). DNA methylation will be discussed in greater detail in a later section of this report.

1.2.4 White clover has a polyploid genome

White clover is an allotetraploid (Williams et al., 1998) (AABB-type genome, 2*n*=4x=32) which exhibits disomic inheritance. This means that WC formed following the hybridisation of two different species and that within WC's genome, there exist two different sub-genomes.

Extant relatives of white clover's progenitor species are *T. occidentale* Coombe (Western clover) and *T. pallescens* Shreb (pale clover) (Ellison et al., 2006; Williams et al., 2012). These parental species are diploid (2*n*=2x=16) and exist in very specific and disparate niches. *T. occidentale* (creeping: spreads along the ground via stolons) lives within ~100m of the shore in a niche on the coasts of western Europe (Coombe, 1961). Meanwhile, *T. pallescens* (non-creeping) lives in European alpine niches between 1800 and 2700 m altitude (Raffl et al., 2008).

White clover's genome is relatively compact with 1C genome size of 1093 Mega base pairs (Mbp) (Bennett & Leitch, 2011) arranged in 32 (16 homologous pairs; 8 homoeologous groups) small, similar-sized chromosomes. Draft genomes for WC and both progenitors have been generated (Griffiths et al., 2019). Insights gained from Griffiths et al. (2019) include confirmation of the progenitors and the estimation that WC originated between ~15,000 and 28,000 years ago in the last glaciation, when the alpine and coastal progenitors were likely co-located in glacial refugia (Griffiths et al., 2019). They also found that the progenitor sub-genomes had both been retained and maintained in a single organism, with no evidence of inter sub-genome recombination (Griffiths et al., 2019). This provides WC with a genomic toolbox comprised of the genetic material of both progenitor species, potentially conferring greater

adaptability to environmental changes and stresses. Polyploids in general are thought to have greater adaptability to different environments and stresses due to their increased genetic variation (Tossi et al., 2022; Van de Peer et al., 2017). It also appears both WC's sub-genomes are transcriptionally active, and the relative expression of a gene in one sub-genome to its homoeologue in the other sub-genome is consistent for most genes across a range of plant tissues (Griffiths et al., 2019). This situation is like that identified in wheat, as it was found that ~72% of homoeologous genes in wheat showed stable expression ratios across 15 tissues (Ramírez-González et al., 2018). Griffiths et al. (2019) found that there were stable sub-genome expression ratios across tissues, however there were a few genes that showed tissue-specific switching between homoeologous gene copy expression. Of these differentially expressed genes, flavonoid biosynthesis genes were over-represented, suggesting that some allopolyploidy-associated changes in transcription have an adaptive role (Griffiths et al., 2019).

1.3 Plant stress

Plants experience a variety of stresses; however, they are sessile meaning they are unable to simply move away from these stresses. This has resulted in plants developing highly complex responses to cope with a wide range of challenges. These stress responses involve changes at the transcriptomic, cellular, and physiological levels.

Plants encounter both biotic and abiotic stresses. Biotic stresses are the result of damage done to the plant by another living organism such as pathogens, insects, and grazing animals. By contrast, abiotic stresses are caused by non-living environmental factors, such as drought, waterlogging, salinity, and extreme temperatures (Audil et al., 2019).

Abiotic stress is a major concern and causes losses in crop plants worldwide, especially as climate change becomes more of an issue. Studies have suggested that climate change could become the largest worldwide threat to biodiversity over the next few decades (Leadley et al.,

2010). Drought has been described as the most damaging climate hazard facing our global population (Allen et al., 2018; Kogan, 1997). Additionally, drought has been described as the largest cause of loss in agricultural production as it has caused >34% of crop and livestock loss in low- and lower-middle-income countries (FAO, 2021). NZ droughts such as the ones in 1998, 2008 and 2013 have had substantial impacts on New Zealand's agricultural industry (Kamber et al., 2013). The 2013 drought had costs to the NZ economy estimated at NZ$1.5 billion (Frame et al., 2020). Global climate change is predicted to cause increases in the frequency and severity of drought events in the future due to decreased levels of rain and increased levels of evaporation (Allen et al., 2018; Dey et al., 2019; Naumann et al., 2018). Therefore, a key challenge in plant science lies in improving our understanding of the responses of plants to drought and how plant breeding can help to maintain/increase productivity in the future.

1.3.1 Drought trials

A common strategy to better understand how drought affects plant physiology, and functional traits is through the application of controlled water deficit experiments in glasshouses or controlled growth rooms. Research in applying soil water deficits date back at least 50 years, but there has not been any agreement on a gold-standard method over this period (Munns et al., 2010).

The simplest method for achieving drought is by withholding irrigation. This method has the disadvantage of the soil drying out very fast and may not replicate natural conditions (Poorter et al., 2012). The most prevalent method for achieving drought is to weigh individual pots as the soil dries out, and add specific water quantities to balance water loss, until the target soil water measurement is reached (Earl, 2003). This method is effective in simulating drought stress for plants of different sizes but can be quite time-consuming and complex, particularly if done with large experimental sizes and if done by hand.

One of the important aspects to establishing a drought trial is to ensure the conditions replicate those that the plants would encounter in nature. This ensures that the phenotypic and molecular changes in response to the stress will be as similar as possible to those that would naturally occur. For example, droughts are often associated with higher temperatures and longer day length.

1.3.2 Plant traits and drought response

Plants have evolved many strategies to overcome water deficit or drought stress conditions including mechanisms at the morphological, physiological, biochemical, cellular and molecular levels (Fang & Xiong, 2015). This means that a variety of drought-related traits can be used to assess the drought tolerance and resistance of plants.

1.3.2.1 Morphological characteristics

Plants respond to drought through changes in their morphological characteristics. They can adapt to volatile environments through phenotypic changes in the leaves, stems and roots. Morphological changes in response to drought are reasonably simple to observe. Common symptoms of water deficit in plants includes decreased plant height, leaf wilting, and changes in number and area of leaves (Yang et al., 2021).

Decreases in plant height during drought are caused by many factors including reduced cell expansion, increased leaf senescence and shedding and impaired cell replication (Yang et al., 2021).

Leaves are an important organ in observing water deficit in plants. Plant leaves usually adapt to drought through smaller leaf areas, increased leaf thickness, and higher leaf tissue density (Werner et al., 1999). Changing leaf area is one of the most easily observed morphological changes in response to drought and it directly affects yield, photosynthesis and other important plant processes. Multiple studies have shown evidence of decreases in plant leaf area under drought conditions. For example, ten different cowpea genotypes showed a

steep leaf area decrease under water deficit stress in a 2004 study (Anyia & Herzog, 2004). Under water deficit, decreases in leaf area are in part caused by lowering leaf turgor pressure and photosynthetic rates (Rucker et al., 1995).

Wilting and leaf rolling are other phenotypic characteristics associated with drought. Wilting occurs when there is insufficient plant hydration and subsequent loss of water pressure in the plant leaves. Leaves can return to a normal turgor pressure and therefore be no longer wilted when there is a decrease in transpiration at night (Hsiao et al., 1984). Wilting is an important response to water deficit because it allows leaves to receive lower levels of sunlight, resulting in less water loss and better overall water status. Wilting and leaf rolling reduce the amount of radiation from the sun hitting the leaves, therefore reducing leaf surface temperature and protecting plants from excess water loss (Fang & Xiong, 2015).

Plant roots also play an important role in drought stress. These organs help plants to survive drought conditions by fully making use of water stored in the soil (Gowda et al., 2011). Many studies have shown that root morphology can change when a plant has experienced drought. Both *Cunninghamia lanceolata* of the Cupressaceae (cypress) family and maize have shown that in response to drought, root branching was decreased and instead root growth was focussed downwards resulting in deeper rooting to allow for greater water absorption in the deep soil (Yang et al., 2018; Zhan et al., 2015).

1.3.2.2 Photosynthetic capacity

Photosynthesis is one of the main plant processes affected by drought stress. The products of photosynthesis form the basis of plant energy production and therefore growth. Photosynthetic rate is a reliable way to measure the growth and potential yield of plants (Yang et al., 2021). Rates of photosynthesis and respiration decrease with the decrease of soil hydration. Photosynthesis is reliant on stomatal organs, therefore distribution and aperture size of stomata impacts on photosynthetic rate.

Stomata are pores in the leaves of plants that are crucial organs for exchanging gas and water with the external environment. The stomata play an important role in controlling the level of CO_2 absorption and transpiration (Haworth et al., 2021). Guard cells that surround the stomata are environmentally responsive and open/close the stomata depending on the environmental stimuli (Franks & Farquhar, 2006). Stomatal closure reduces the level of leaf intake of CO_2 and prevents water loss via transpiration due to reduced leaf turgor pressure and/or reduced water potential. In wheat it was found that drought decreased stomatal conductance, increased stomatal resistance, and decreased both photosynthetic and transpiration rates (Ashraf et al., 2017).

There are three different photosynthetic pathways – the C_4 pathway, the C_3 pathway, and the Crassulacean acid metabolism (CAM) pathway. WC is a C_3 plant (Smith et al., 1984). The C_4 pathway is significantly better than the C_3 pathway under drought stress. This is because those plants have a greater water use efficiency and under water deficit as they have a metabolic pump which concentrates CO_2 in the bundle sheath cells allowing separate fixation of CO_2 (Chaves et al., 2003). The stomata of CAM plants open at night to absorb CO_2 and close during the day. This makes these plants better at dealing with drought stress because there is lower water loss via transpiration and evaporation during the day. When challenged by water stress, some plants can switch their photosynthetic pathway from the C_3 cycle to the CAM cycle (Winter & Holtum, 2014). These plants are considered facultative CAM species and this process has not been observed in WC.

1.3.2.3 Osmotic regulation

Osmotic regulation is an important way for plants to reduce osmotic potential and reduce stress under water deficit. Osmotic adjustment is done in three ways – the decrease of intracellular water, the decrease of cell volume, and the increase of cell contents. The initial effect of a decrease in osmotic potential allows plant cells to absorb more water due to the

differences in water potential, thus maintaining the turgor pressure for cell growth (Osakabe et al., 2014). Osmotic regulation helps the plant to maintain normal activities during drought stress and minimises damage to plant functions. Osmotic regulating substances such as proline and mannitol play an important role in the drought response as they help to osmotically regulate the cells (Yang et al., 2021).

Accumulation of flavonoids such as quercetin and kaempferol glycosides are associated with higher drought tolerance in WC (Ballizany et al., 2014). In addition, hydroxycinnamic acid's role in stress tolerance has been widely documented (Shahidi & Chandrasekara, 2010). Nichols et al. (2015) further found increased accumulation of quercetin glycoside, kaempferol glycoside and hydroxycinnamic acid under drought conditions in WC. Increased accumulation of these phenolic compounds was associated with reduced leaf senescence, lower decreases in yield and maintenance of photosynthetic rates in drought resistant clover varieties (Nichols et al., 2015).

1.3.2.4 Reactive oxygen species

Reactive oxygen species (ROS) are produced when oxygen is not fully reduced in the metabolic process. When plants are under drought stress, ROS production and clearance is out of balance resulting in greater numbers of free ROS in the plant cells. This causes the cells to suffer oxidative stress and oxidative damage, resulting in a variety of harmful effects including protein denaturation and DNA strand breakage (Cruz de Carvalho, 2008).

There are endogenous enzymatic and non-enzymatic antioxidant protection systems that plants use to protect themselves from ROS damage. These systems are important in the drought stress response. Non-enzymatic ROS scavenging systems mainly include substances (e.g. ascorbate, mannitol and flavonoids) which react with ROS or appear as substrates of enzymes in the ROS scavenging system (Dvořák et al., 2021).

1.3.2.5 Stress signal transduction

The signal transduction pathways leading from plants sensing environmental stimuli to them responding to these stimuli are key in the drought response.

The decrease of leaf water potential and turgor pressure affects the synthesis, transportation and distribution of plant hormones such as ABA (abscisic acid) (a critical chemical messenger of drought) and cytokinin. These plant hormones are a kind of chemical signal that can play a regulatory role as they can transmit cell signals between cells and different parts of the plant (Yang et al., 2021).

ABA is an important signal molecule in plant water stress. It plays a critical role in the information relay between the aboveground and underground parts of the plant. ABA production in the roots during drought stress transmits the signal to other parts of the plant, resulting in leaf senescence and stomatal closure to reduce water loss (Campalans et al., 1999).

1.3.3 Transcriptional changes

Transcriptome analysis is an active area within research into drought resistance in plants. There is a very complicated process in response to drought stress, that involves many genes and signalling pathways. It has been shown that under drought conditions, there is the alteration of gene expression of a large number of genes with a wide range of functions (Bartels & Sunkar, 2005). These differentially expressed genes are often related to the morphological and physiological drought responses described in this report. Sensors on plant membranes perceive external drought stimuli and these signals are passed through multiple signal transduction pathways leading to the differential expression of drought-responsive genes and therefore drought adaption within the plant (Zhu, 2002).

The main drought responsive genes can be split into three groups (Fang & Xiong, 2015). Firstly, there are protein coding genes that are involved in transcriptional regulation and signalling cascades e.g. protein kinases and transcription factors. Secondly, there are proteins

that provide protection to structures such as cellular membranes e.g. antioxidants. Thirdly, there are proteins that are involved in water and ion uptake e.g. sugar transporters and aquaporins (Hirayama & Shinozaki, 2010; Shinozaki & Yamaguchi-Shinozaki, 2007).

In WC, there has been prior investigation into transcriptional changes associated with drought stress. Exogenous treatment with the auxin phytohormone caused increased ABA and jasmonic acid content in WC, as well as transcriptional changes in a range of genes including those involved in the drought stress response resulting in increased drought tolerance in WC (Zhang et al., 2020). Endogenous polyamine transcription has been shown to regulate drought stress tolerance through antioxidant pathways (Li et al., 2015). Li et al. (2016) found that greater drought tolerance was conferred by increased proline metabolism and increased transcription of flavonoid and other phenols (Li et al., 2016). Additionally, it has been described that drought stress induced transcription of a range of antioxidant enzymes in WC (Zhang et al., 2015). However, investigation into sub-genome differential expression in WCs drought response has not been investigated. This is a relevant area of research as it has been previously found that there was biased expression of a gene involved in the raffinose family of oligosaccharides (RFO) in response to frost stress in one of WC's progenitor sub-genomes (Fechete et al., 2024) although WC shows overall even sub-genome expression levels (Griffiths et al., 2019). Further investigation may reveal roles of WC's sub-genomes in the drought response.

Transcriptomic expression is controlled by a variety of factors, and a major influencer of gene expression is epigenetic control via mechanisms such as DNA methylation.

1.4 Epigenetics and DNA methylation

Epigenetics is an area of research that has gained much attention in recent years. It is the study of heritable changes in gene activity or function, without any changes to the DNA

sequence itself (Waddington, 2012). Although most cells within an organism or between clones (apart from sex cells) contain identical genetic information, different cells can have vastly different phenotypes and gene expression profiles. Epigenetic regulation is a central mechanism underlying these differences (Allis & Jenuwein, 2016). There are a variety of different types of epigenetic regulation which work at different levels, such as DNA methylation and histone modifications (Figure 1.2). These all involve proteins that are associated with the DNA strand and can affect the transcription of a range of genes (Abi Khalil, 2014).

Figure 1.2. The three main epigenetic mechanisms. DNA methylation, histone modifications and micro-RNA based mechanisms (from Abi Khalil, 2014)

DNA methylation was first discovered in mammals in 1948 (Hotchkiss, 1948) and its role in gene regulation and cell differentiation was demonstrated by several studies during the 1970s and 1980s (Compere & Palmiter, 1981; Holliday & Pugh, 1975). Since then, DNA methylation has been the focus of a range of studies in both animal and plant genetics.

Although DNA methylation occurs on different residues, cytosine methylation is the most common and the most well-studied. This is when DNA methylation occurs at the C5 position of cytosine to form 5-methylcytosine (5-mC). It influences the chromatin structure and as a result, the accessibility of genetic information. In mammals, DNA methylation mainly occurs at CG dinucleotides with ~70-80% of CG dinucleotides experiencing cytosine DNA methylation throughout the genome (Ehrlich et al., 1982). However, in plants, DNA methylation occurs in all sequence contexts of cytosine bases: CG, CHG, and CHH (where H = A, T or C) (Henderson & Jacobsen, 2007). In *Arabidopsis thaliana,* DNA methylation was measured for these sequence contexts and it was found that there were approximately 24%, 6.7% and 1.7% methylation for each CG, CHG and CHH, respectively (Cokus et al., 2008).

Addition of methylation to cytosine bases is catalysed by a family of DNA methyltransferases that use S-adenosyl-L-methionine (SAM) as the methyl donor (Karr et al., 1967). Active removal of methyl marks from DNA (demethylation) involves a base excision repair pathway (Zhu, 2009). In plants, an RNA-directed DNA methylation (RdDM) pathway is another key process for *de novo* DNA methylation (Zhang et al., 2018).

1.4.1 Bisulphite conversion-based approaches to determine DNA methylation

There are multiple groups of methods for determining the distribution of 5-mC in DNA. Two of the main groups are 1) Differential sensitivity to chemical conversion (e.g., bisulphite conversion-based approaches); and 2) differential enzymatic cleavage of DNA.

Bisulphite conversion-based approaches for the analysis of DNA methylation falls under the differential sensitivity to chemical conversion category. This is because the method

relies on unmethylated cytosines being more susceptible to conversion to uracil than methylated cytosines, when treated with sodium bisulphite (Clark et al., 2006). This method is usually coupled with DNA sequencing to identify and locate the converted and unconverted cytosines in the genome.

The detection of 5-mC through the use of sodium bisulphite to chemically convert the DNA prior to sequencing was first reported in 1992 (Frommer et al., 1992) and was optimised in 1994 (Clark et al., 1994). Frommer et al. (1992) and Clark et al. (1994) were the first to demonstrate the use of bisulphite conversion in the detection of 5-mC. Since these publications, bisulphite sequencing (BS) has been widely used and validated thoroughly to the extent that this method is currently considered the gold standard for DNA methylation analysis. BS provides an efficient approach for identifying 5-mC at single base-pair resolution that is also qualitative and quantitative.

Frommer et al. (1992) found that the amination reactions of cytosine and 5-mC were very different after treatment with sodium bisulphite as unmethylated cytosines were converted to uracil residues while 5-mCs were immune to this conversion. Bisulphite sequencing uses these findings as it allows unmethylated cytosines to be recognised as thymine in PCR reactions following sodium bisulphite treatment, while 5-mCs remain recognised as cytosines (Figure 1.3). The methylation status can be measured by either direct sequencing of PCR products (detection of average methylation status) or by sub-cloning (allowing single molecule resolution) (Figure 1.3). Since treatment with sodium bisulphite causes DNA strands to lose their complementarity, this method can independently identify methylation on the separate strands (the amplification products can be measured individually).

Figure 1.3. Methylation analysis using bisulphite genomic sequencing. After treatment with sodium bisulphite, unmethylated cytosine residues are converted to uracil whereas 5-mC remains unaffected. After PCR amplification, uracil residues are converted to thymine. DNA methylation status can be determined by direct PCR sequencing or cloning sequencing (from Li and Tollefsbol (2011)).

Many different methods using the principles of BS have arisen since its first use. One example of this is reduced representation bisulphite sequencing (RRBS), which was first described by Meissner et al. (2005) as a method for large-scale high-resolution analysis of

DNA methylation. This method is based on treatment with restriction enzymes followed by size selection of restriction fragments to generate a 'reduced representation' of the genome (Meissner et al., 2005).

While BS sequencing is viewed as the gold standard, this method does come with some limitations. As BS requires single-stranded DNA, there is the possibility of incomplete denaturation or annealing. This impacts on subsequent ease of analysis and it means that you can't be completely certain that an unconverted cytosine is in fact 5-mC or if it is an experimental artefact (Meissner et al., 2005).

BS involves harsh reaction conditions which can cause damage to the DNA, resulting in decreased yield. Grunau et al. (2001) estimate that >90% of BS input DNA is degraded in the first hour of a bisulphite reaction (Grunau et al., 2001). That means that in the following stages of the process, considerable DNA PCR amplification is required for sequencing to make up for the lost DNA. This can also cause a bottleneck and result in minimal representation of certain genomic regions. Non-proofreading Taq polymerase is required in the PCR stage because most proof-reading enzymes stop at uracil residues in the template strand. This can lead to errors in the PCR amplification steps.

There are many advantages to this method as it is highly accurate and resolves DNA methylation to a single-nucleotide resolution. When whole genome BS (WGBS) sequencing is used, it completely covers the genome. However, WGBS is expensive and is computationally demanding due to the large amount of data and base conversion associated with this method. Bisulphite-converted DNA is also fragile which limits the storage potential of the DNA due to high degradation.

1.4.2 DNA methylation in plants and stress responses

As plants are sessile and unable to escape the environments they are growing in, they require fast-responding short-term strategies based on the manipulation of already existing

genome. Epigenetic changes such as DNA methylation are a key mechanism that allow these changes through regulation of gene expression (Bartels et al., 2018; Gibney & Nolan, 2010).

Investigation into DNA methylation has revealed that both hypo-methylation and hyper-methylation contribute to the plant stress adaptation (Thiebaut et al., 2019). The location of DNA methylation has an influence on the pattern of gene expression (Zhang et al., 2018). For example, research has shown that DNA methylation in or near the promoter region of genes is associated with low or no transcription (Phillips, 2008). This is because the methyl marks on the promoter DNA prevent the binding of transcription activators or promote the binding of transcription repressors (Phillips, 2008). By contrast, DNA methylation in the body of genes has been positively associated with gene expression in mammals and in some plant species (Liang et al., 2014).

To respond to both biotic and abiotic environmental stresses, many signalling pathways are induced as well as short-term changes in transcription. Epigenetic mechanisms are a key controlling factor that can change gene expression levels during stress and then return expression to a normal level once the stress is no longer present (Boyko & Kovalchuk, 2008). Abiotic stress can cause demethylation of stress response gene promoter regions and therefore transcriptional activation. For example, an aluminium-tolerance associated protein was upregulated in tobacco plants exposed to aluminium stress and this was associated with DNA demethylation (Choi & Sano, 2007; Wada et al., 2004). It was also found that cadmium stress in rice was associated with altered methylation and subsequent differential gene expression in a range of stress response pathways (Feng et al., 2016). Furthermore, Garg et al (2015) showed that DNA methylation regulates stress-responsive gene expression levels in rice, supporting the idea that DNA methylation is important in abiotic stress response (Garg et al., 2015).

1.4.3 DNA methylation in the plant drought response

Several studies have identified drought-induced differential gene expression in plants that DNA methylation plays a role in (Wang et al., 2011; Zheng et al., 2017). Research into drought-response mechanisms, including DNA methylation, is important for future development of drought-resistant plant varieties.

Wang et al. (2014) analysed changes in the drought tolerant *Citrillus colocynthis'* (of the Cucurbitaceae family) transcriptome after drought stress exposure and found more than 2500 significantly differentially expressed genes. These differentially expressed genes included transcription factors, stress signalling factors, detoxification genes and some involved in DNA methylation (Reddy et al., 2004; Wang et al., 2014). This provides evidence of the importance of DNA methylation in abiotic stress responses.

A study conducted by Zheng et al. (2017) found that when rice had been exposed to drought stress, some stress-responsive genes gained stress-associated DNA methylation profiles which were able to be inherited by offspring for multiple generations. It was also found that a rice variety with greater drought resistance had higher transmission levels of methylation marks to the subsequent generation (Zheng et al., 2013)

Wang et al. (2011) compared DNA methylation in response to drought stress in a drought-tolerant rice cultivar and a drought-sensitive rice cultivar. They reported that 12.1% of the methylation differences between the two were drought-induced. They also found that drought stress resulted in overall hypo-methylation in both cultivars, although locations of DNA methylation differed between the two (Wang et al., 2011).These differences in methylation patterns in response to drought stress may have caused the observed differences in gene expression and therefore differences in drought tolerance. This study also found that there were some loci that retained their drought-associated methylation pattern once the drought stress was no longer present (Wang et al., 2011).

In WC, a previous study has shown that DNA methylation plays a significant role in responding to drought stress (González et al., 2016). It was found that white clover plants that were subjected to global demethylation were limited in their ability to respond to drought stress, leading to less control of growth under drought stress.

1.4.4 Plant epigenetic memory

Changes in DNA methylation in response to an environmental stress usually last for a short time and revert to the non-stress state once the plant is no longer experiencing said stress. However, some studies have shown that a small proportion of changes in DNA methylation can be maintained for the rest of the plants life (somatic memory) and some can even be passed on to offspring (transgenerational inheritance) (Gallo-Franco et al., 2020). Epigenetic memory is seen in plants more often than animals which reinforces the idea that, due to their general immobility, plants require a variety of internal ways to adapt to their environment (Takeda & Paszkowski, 2006).

Many plant species have been shown to exhibit plant memory/priming after initial exposure to a stress, helping in response to subsequent stresses. White clover exhibits memory retention of drought stress. González et al. (2016) demonstrated that once a plant was subjected to drought stress, the same individuals were able to respond to the following drought event through changes in biomass of new growth. They also found evidence that this memory was related to DNA methylation, as global demethylation negated these effects. (González et al., 2016). Other species such as rice have demonstrated short-term memory of drought stress, with a series of drought stresses revealing slower loss of water over subsequent droughts therefore ensuring better adaptation to drought (Li et al., 2019). A later study revealed that this stress memory was linked to DNA methylation, with DNA methylation likely regulating genes helping the plants cope with subsequent drought stresses (Kou et al., 2022).

Transgenerational inheritance allows for modifications of gene expression across many subsequent generations (Whitelaw & Whitelaw, 2006). There is evidence that transgenerational inheritance of DNA methylation changes are more frequent in plants than in animals (Takeda & Paszkowski, 2006). A reason for this is because plants generate germline cells later in their life than animals, therefore allowing the inheritance of epigenetic marks that were accumulated throughout the plant's life.

A range of studies have demonstrated that stress-induced epigenetic marks can be stably transmitted from parents to offspring. For example, Feng et al. (2016) found DNA methylation changes related to cadmium stress in rice plants and that some of these were heritable and increased the stress-tolerance of progeny (Feng et al., 2016). Similarly, it was found that epigenetic signatures related to cadmium stress could be transgenerationally inherited to some extent in rice (Cong et al., 2019). In comparison, it has been found that alterations in DNA methylation induced by mild drought in *A. thaliana* were not passed on to progeny (Van Dooren et al., 2020). This suggests that the level of stress may have an impact on the inheritance of the associated epigenetic marks.

Evidence of transgenerational inheritance of DNA methylation marks has not been investigated so far in white clover. What has been described as epigenetic inheritance in this species was only investigation of methylation of cloned stolons from single individual rather than inheritance across generations (González et al., 2016).

Diversity through epigenetic variations is an addition to traditional genetic breeding and is a potential source for further genetic improvement of plants (Gallusci et al., 2017). However, the molecular machinery behind epigenetic memory and transgenerational inheritance is not well known and requires further investigation before it can be effectively used in the breeding process.

1.5 Project aims and hypotheses

In recent years there has been increased interest in understanding how plants respond to drought stress, particularly as climate change progresses. WC is a species of interest due to its importance in NZ agriculture and agriculture worldwide. There are many different cultivars and ecotypes of white clover which have varying levels of drought tolerance, therefore, understanding these drought responses and mechanisms underlying them is a key area of research. As WC is an allopolyploid, investigation into the drought response of the progenitor species can also provide insight into WC's drought response. DNA methylation has been shown to have a role in plant stress responses, but there are gaps in knowledge, particularly when it comes to WC. Therefore, investigation into the role of DNA methylation in WC's drought response and whether this plays a role in plant memory is a key area of research.

This research aimed to investigate phenotypic responses of WC and related species to drought stress (Chapter 2) and the role that DNA methylation plays in WC's drought response (Chapter 3). This research was addressed by investing the following hypotheses:

- Different clover species, cultivars and ecotypes show differing drought responses (Chapter 2)
- Plants that have been 'primed' by an initial drought exposure display greater phenotypic tolerance in their second drought exposure (Chapter 2)
- There is differential DNA methylation depending on whether plants have experienced no drought stresses, one drought stress or two drought stresses (Chapter 3)
- Areas of the genome that are differentially methylated between treatment conditions are related genes involved in the drought stress response (Chapter 3)

2 Short term drought trial

2.1 Introduction

Drought is a major abiotic stress of interest, as it is one of the most damaging climate hazards facing our global population (Allen et al., 2018). Droughts are becoming increasingly frequent and severe with the progression of climate change, therefore investigating plant responses to drought and mechanisms underlying tolerance is an important area of research. White clover's (WC) drought response is of particular importance in New Zealand as this species is one of the principal sources of livestock feed in this country and the third most important plant after perennial ryegrass and pine based on its contribution to New Zealand's economy (NZIER, 2016). Improving understanding of WC's drought response may assist in plant breeding and finding ways to increase/maintain productivity in the future.

Plants have many mechanisms to overcome drought stress conditions, including at a morphological, physiological, biochemical and molecular level. Therefore, a range of drought-related traits can be used to assess the drought tolerance and resistance of plants. Morphological characteristics associated with drought include decreased plant height, leaf wilting and decrease in leaf production and leaf area. Photosynthetic capacity is another main aspect impacted by drought stress. Photosynthetic and respiration rates decrease with the decrease of soil relative water content. This process is reliant on stomata, so these organs also have an impact on photosynthetic rate.

A controlled environment drought trial was used to investigate WC's phenotypic responses to short-term drought. The species and cultivars included in this thesis were selected with a specific purpose to provide interesting information for analysis. *T. pallescens* and *T. occidentale* are WC's two progenitor species and have reference genomes (Griffiths et al., 2019). *T. occidentale* is a coastal species in shallow, sandy soils with natural salt stresses. This

species has been shown to increase drought tolerance when hybridised with WC (Hussain et al., 2016). *T. repens* S10 is the inbred and previously sequenced individual so the DNA from this can be easily aligned to the reference genome. 880 is a new synthetic WC made from a cross between the two progenitor species (*T. pallescens* and *T. occidentale*). The inclusion of this species provided insight into the sub-genome behaviour in WC. The response of these sub-genomes may be more like those of the progenitor species than WC due to the differences in time since hybridisation. *T. repens* ecotype 'Tian Shan' is another WC cultivar of interest as it is an ecotype which is UV and drought tolerant. This species may better cope with drought than the WC S10. *T. repens* cv 'Grasslands KopuII' is a large-leaved dairy WC cultivar which was bred for irrigated pastures. This cultivar was expected to struggle under drought conditions.

The aim of the controlled drought trial was to provide phenotypic information on WC and relatives during drought stress, and insight into how these different plants respond to two back-to-back drought stress periods.

2.2 Materials and Methods

A short-term drought trial was a central experiment of this thesis. It provided a range of phenotypic and methylation data.

This drought trial aimed to emulate a drought which may occur naturally in a New Zealand summer in terms of temperature and day length. Therefore, any effects on the phenotype and genotype could be related back to naturally occurring drought conditions and imply how white clover would react in the field.

2.2.1 White clover genotypes and preparation for drought trial

The drought trial included six different species and cultivars of white clover.

1. *T. repens* S10 (an inbred line of white clover which had been through 10 generations of self-pollination)

2. *T. repens* cv ‘Grasslands KopuII’
3. *T. repens* ecotype ‘Tian Shan’
4. 880 (synthetic *T. occidentale* × *T. pallescens* hybrid)
5. *T. pallescens* (alpine progenitor species of *T. repens*)
6. *T. occidentale* (coastal progenitor species of *T. repens*)

Nine replicates of each genotype were included in the drought trial. These replicates were clones which were trimmed to as uniform a size as possible before the beginning of the drought trial. The clones were created by propagating stolons from a single original plant.

The clones were all raised in a glasshouse and were re-potted into their final experiment pots on 21/07/21.

The soil composition used in the trial was 75% Aokautere topsoil silt loam, 25% 5 mm washed river sand, 2 g/L Osmocote® Pro ¾ (DaltonsTM, New Zealand). These were mixed using a rotary hoe and transferred to 15 L pots. The plants were then allowed to adjust to the new soil in the glasshouse for ~3 weeks before being moved to the controlled growth room where the experiment was run. There was an additional four-week adjustment period inside the controlled growth room before the drought trial began.

2.2.2 Controlled growth room conditions

The humidity was maintained at ~60% using a Delonghi AriaDry Pure Dehumidifier. Light intensity was maintained between 100-500 mmol/m^2/s during the day. Temperature was maintained at 22°C during the day and 15°C overnight. Day length was 16 hours and night length was 8 hours.

2.2.3 Growth room layout

The layout in the growth room was randomised using Deltagen (Jahufer & Luo, 2018) to correct for differences between conditions and ensure that the main difference between plants was the treatment type. The experimental design was a randomised complete block and

was generated using Deltagen software (Jahufer & Luo, 2018). This design was used to account for any spatial effects within the growth room. Figure 2.1 shows the layout of the controlled growth room and the positions of the pots. Figure 2.2 shows what the growth room looked like when it was set up.

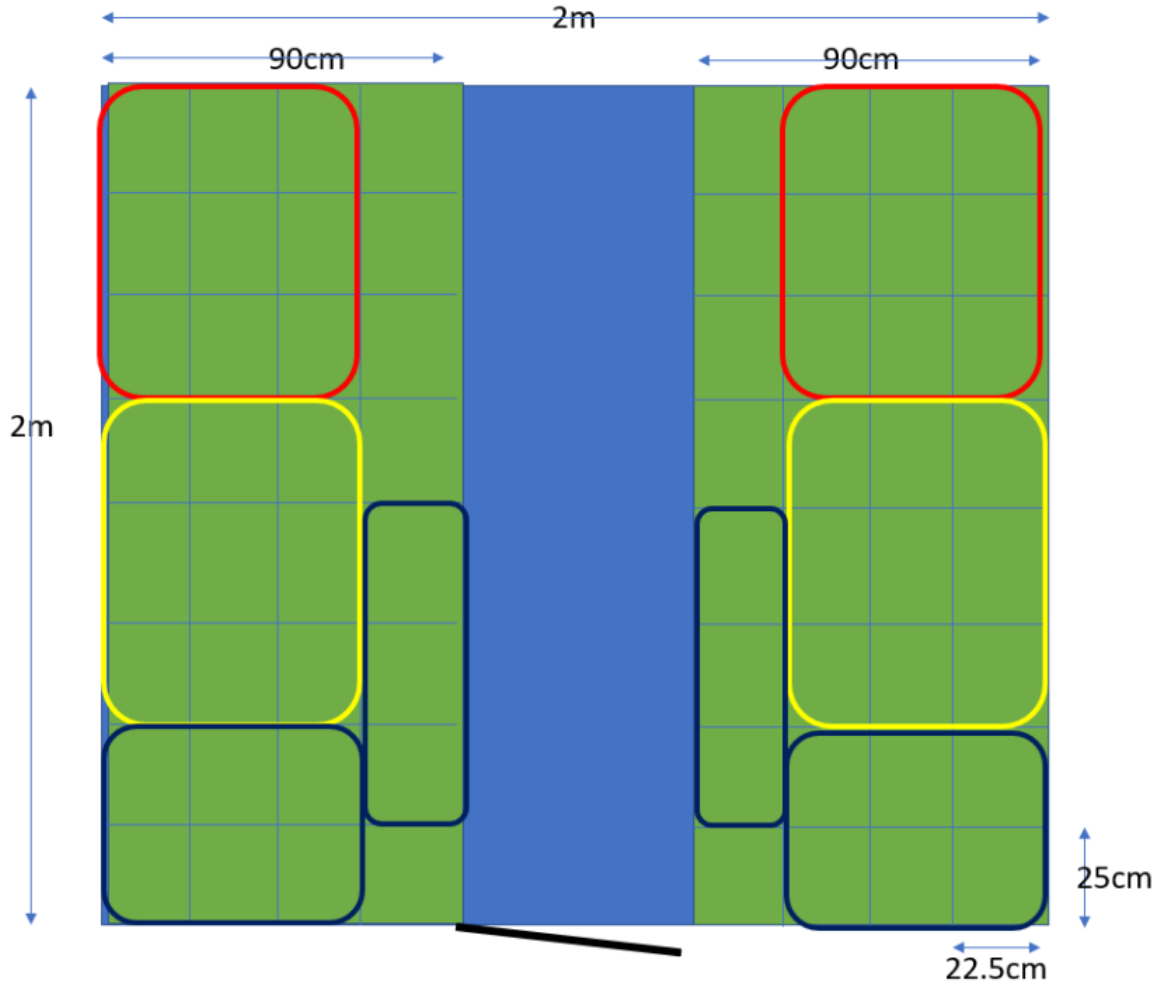

Figure 2.1. Layout of the controlled growth room. Benches are shown in green and the walkway between them is shown in blue with the door indicated by the slightly angled black line. The grid illustrates positions of replicates. Red = placement of replicate 1 pots, yellow = placement of replicate 2 pots and dark blue = placement of replicate 3 pots.

Figure 2.2. Image showing the growth room layout as it was for the duration of the drought trial.

2.2.4 Drought trial timeline

The drought trial comprised different drought regimes. A set of plants were watered the entire trial (watered controls), a set underwent a single four-week drought, and another set was exposed to a double drought involving two consecutive four-week droughts with a four-week recovery period after each drought. Each genotype had nine replicate pots. Therefore, for each genotype there were three replicates which were watered controls throughout. There were three replicates which were double-droughted and had water withheld for both drought periods. There were three replicates which were single-droughted and had water withheld for only the second drought period and were watered throughout the first drought period (Figure 2.3). The inclusion of double-droughted replicates aimed to determine whether an initial drought could help the plants to adapt and better cope with a second drought through plant memory or a 'priming' effect.

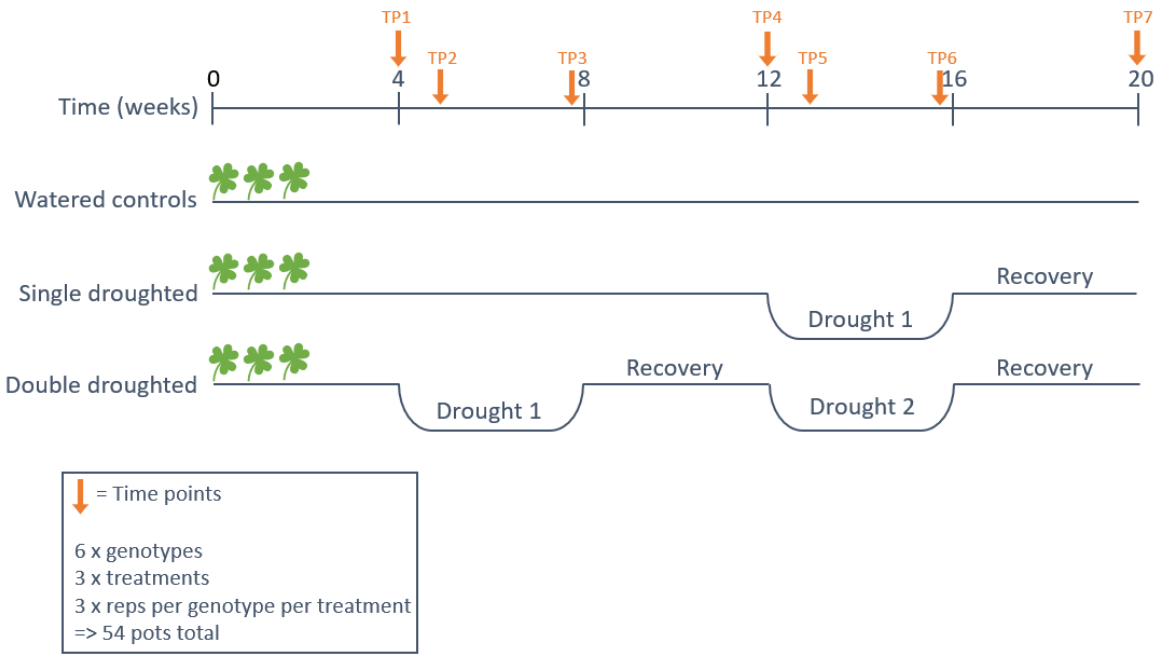

Figure 2.3. Structure of the drought trial. For the first four weeks all plants were watered normally as they adjusted to the growth room conditions. After these four weeks, time point 1 (TP1) measurements and sampling occurred. Subsequently, watering of the double-droughted was withheld until the plants reached permanent wilting point. After they reached permanent wilting point, they were watered 2% of their total weight each time they wilted. 1 week into permanent wilting point, the plants were again sampled, and measurements were taken (TP2). The plants were held at permanent wilting point for 4 weeks and at the end of these four weeks, another set of samples and measurements were taken (TP3). All plants were then watered normally again for four weeks of recovery. At the end of this recovery period TP4 samples and measurements were taken. The second drought then commenced and for this drought, water was withheld from both the double-drought and single-drought pots. Again, the plants reached permanent wilting point and after one week of this, TP5 measurements and sampling occurred. They were held at permanent wilting point for four weeks total with sampling and measurements again taken at the end of these four weeks (TP 6). All plants were then watered normally for four weeks of recovery. At the end of these four weeks, another set of samples and measurements were taken (TP7).

Drought was induced for each plant by withholding irrigation until the plant reached permanent wilting point. This was the point at which the plant was wilted in the evening and did not recover overnight (Kirkham, 2005). Plants within this experiment reached permanent wilting point at different rates depending on a range of factors such as genotype and plant size. This meant that the length of time between TP1 and TP2, and between TP4 and TP5 (Figure

2.3) was variable. Consequently, the treatment stage for individual plants became staggered through the trial. However, plants were held at the critical phases (permanent wilting point and recovery) for the same length of time. When a plant reached permanent wilting point, the pot was weighed, and the pot was watered with 2% of its total weight. This was repeated for the length of the drought phase. During the first drought period, only plants designated to undergo a double-drought were droughted and the watered controls and plants to undergo a single-droughted were watered as required to maintain hydration. Once the double-droughted plants had been held at permanent wilting point for four weeks (TP3; Figure 2.3), they were watered as required for four weeks to allow recovery. After this, the second drought phase commenced (TP4; Figure 2.3). This second drought phase (TP5 to TP6; Figure 2.3) involved both the double-droughted plants and single-droughted plants entering their second and first four-week drought, respectively (TP6). This was followed by another four-week recovery period where all plants were watered as required (TP7; Figure 2.3).

Non-droughted or drought-recovery plants were hand-watered until water ran through the pot approximately every 3-4 days to ensure sufficient hydration.

2.2.5 Samples and measurements

Phenotypic measurements were taken at the seven time points throughout the experiment (Figure 2.3). TP1 was pre-drought and TP2 was one week into permanent wilting point for the group of plants designated to experience two droughts. TP3 was after four weeks at permanent wilting point andTP4 was after the four-week recovery phase for the double-droughted plants. TP5 was one week into permanent wilting point of drought phase two where double-droughted and single-droughted plants underwent their second and first drought, respectively. TP6 was after four weeks at permanent wilting point and TP7 was after the second four-week recovery phase.

At each of these time points, a range of phenotypic measurements were taken from all plants including the watered controls. This included three faster-responding traits and four slower-responding traits.

The faster-responding traits that were measured were relative water content (RWC), stomatal conductance and photosynthetic rate. The slower-responding traits were leaf area, node emergence rate, plant height and internode length.

2.2.6 Faster-responding phenotypic measurements

2.2.6.1 Relative water content (RWC)

The RWC of three separate trifoliate leaves were measured from each pot included in the drought trial at each of the time points.

RWC was measured using the following equation:

$$RWC(\%) = 100\left(\frac{FW - DW}{TW - DW}\right)$$

Where FW was the fresh weight of a most recent fully emerged leaf chosen from a random stolon, TW was the turgid weight of the leaf after being saturated in water for 12 hours and gently blotted dry, and DW was the dry weight of the leaf after being dried in an 80°C oven for 48 hours (Kwak et al., 2011). All weights were measured on leaves that had been removed from the plant, and FW was measured immediately after removal to ensure the water content measurements were as accurate as possible.

2.2.6.2 Gas exchange measurements

Stomatal conductance (g_s) and photosynthetic rate per unit leaf area (A) were measured on one randomly chosen most recent fully emerged leaf per pot at each time point in the drought trial. This was done using a LI-COR 6400 infrared gas exchange system (LI-COR Biosciences Ltd, Nebraska, United States).

One at a time these leaves that were still attached to the plant were acclimated in the leaf chamber of a LI-COR 6400 under the following conditions:

- 415 ppm CO_2
- 60% relative humidity
- Temperature was set to match room 22°C
- PAR of 400 µmol photons $m^{-2}s^{-1}$ red/blue light

After 15 minutes of acclimatisation, net photosynthesis per unit leaf area (A) and stomatal conductance (g_s) were measured.

After all gas exchange analyses were conducted, leaves were removed from the plant and photographed. Leaf area was calculated using GIMP 2.8.22 (GNU Image Manipulation Program). Leaves were then dried (in an 80°C oven for 24 hours) and weighed (as backup in case leaf areas weren't informative). Photosynthesis per unit leaf area was calculated.

The LI-COR 6400 machine was checked for leaks regularly by blowing on the leaf chamber.

2.2.7 Slower-responding phenotypic measurements

2.2.7.1 Leaf area

Leaf area was measured for three randomly chosen most recent fully emerged leaves per pot at each time point.

This was measured by taking a photo of these leaves immediately post harvesting with them placed on grid paper with a 6 cm^2 box outlined. GIMP 2.8.22 (GNU Image Manipulation Program) was used to determine leaf area based on the number of pixels in the image using the following equation:

$$Leaf\ area(cm^2) = 6\ cm^2 \left(\frac{pixels_{leaf}}{pixels_{6cm^2}}\right)$$

Where $pixels_{leaf}$ was the number of pixels the leaf takes up and $pixels_{6cm2}$ was the number of pixels the 6 cm^2 box takes up.

2.2.7.2 Node emergence rate

Node emergence rate was measured for three randomly chosen stolons per pot. The chosen stolon was marked at the most recent fully emerged leaf at TP1 and then again at each following time point, counting the number of new nodes emerged at each time point. The node emergence rate was calculated using the following equation:

$$Node\ emergence\ rate\ (nodes/day) = \frac{no.new\ nodes}{days\ since\ last\ measurement}$$

2.2.7.3 Plant height

Plant height was measured for each pot at each time point in the drought trial. This was done by measuring from the top of the soil to the highest point of the plant using a ruler.

2.2.7.4 Internode length

Internode length was measured for three randomly chosen stolons per pot at each time point. This was done for each plant except *T. pallescens* (non-stoloniferous). Internode length was measured by using a ruler to find the distance between the node of the most recent fully emerged leaf and the second most recently fully emerged leaf.

2.2.8 Statistical analysis of whole dataset

Statistical methods were used to generate data for visualisation and interpreting of the phenotypic results. This was first done on the dataset which included all genotypes, and each time point was run separately.

The computer program Deltagen (Jahufer & Luo, 2018) was used to generate univariate models for each time point. A linear mixed model analysis was used with the fixed terms being Treatment, Species and Treatment:Species. The random terms were specified as treatment:replicates, treatment:replicates:row, treatment:replicates:column and species:sample. Below is the model formula:

```
Formula: I(Phenotypic trait) ~ Treatment + Species + Treatment:Species + (1 |
        Treatment:Replicates) +  (1 | Treatment:Replicates:Row) + (1 |
             Treatment:Replicates:Column) +  (1 | Species:Sample)
```

Using Deltagen, modelling results and Best Linear Unbiased Estimator (BLUE) results for each phenotypic trait were generated at each time point. The BLUE results showed the standard error of differences, LSD (least significant difference) values and generated a table showing the BLUE mean, standard error, degrees of freedom and 95% confidence intervals. BLUE results for phenotypic traits were generated when looking at treatment:species, treatment and species individually at each separate time point (1-7). All results were collated for further data analysis and visualisation.

2.2.9 Statistical analysis of individual genotype data

For further investigation into the phenotypic responses of the different treatment conditions to drought within each genotype, the data was split by genotype (species/cultivar/ecotype) prior to statistical analysis. This meant that the experimental design of the drought trial was not incorporated as this method split up the design.

Deltagen (Jahufer & Luo, 2018) was again used to generate univariate models for each species at each time point. A linear mixed model analysis was used with the fixed terms being treatment and the random terms being replicates and sample. Below is the model formula:

```
Formula: I(Phenotypic trait) ~ Treatment + (1 | Replicates) + (1 | Sample)
```

Using Deltagen, modelling results and BLUE results were generated for each phenotypic trait for each species at each time point (Jahufer & Luo, 2018). The BLUE results showed the standard error of differences, LSD values and generated a table showing the BLUE

mean, standard error, degrees of freedom and 95% confidence intervals. All results were collated for further data analysis and visualisation.

2.2.10 Data visualisation

Visualisation of data from the whole experimental design and from when the design was split by genotype was done on R studio using ggplot. For both data sets, plotting of the BLUEs revealed phenotypic patterns of the genotypes and treatment conditions across the seven time points.

2.3 Results

2.3.1 Overview

All phenotypic data gathered in the drought trial, which included every genotype (species, cultivar, ecotype), were presented as an overview to observe patterns in the data as a whole prior to evaluating individual genotypes. Broad patterns and characteristics of the different treatment conditions (double-droughted, single-droughted and watered controls) were evident among the species and cultivars.

Of the four slower-responding phenotypes, three (leaf area, node emergence rate and plant height) showed reduced values when the plants experienced drought (Figure 2.4), while the fourth phenotype (node length) showed a gradual decrease throughout the drought trial regardless of drought condition.

Figure 2.4A shows leaf area trends across the drought trial with decreased values when plants experienced water deficit. At TP3 and TP6 (four weeks into each drought period) there were significant differences ($p<0.05$) in leaf area between the droughted plants and the watered plants. At TP3, only plants selected for double-droughting experienced the water deficit whereas at TP6 both double- and single-droughted plants were in water deficit. A dip in leaf area of the droughted plants also occurred at TP5 when droughted plants had reached

permanent wilting point for one week, but this was not statistically significant (p>0.05). The leaf area of the watered plants remained consistent throughout the drought trial at ~5-6 cm^2. Leaf size recovered well after drought periods as there were no significant differences (p>0.05) between treatment conditions at TP5 and TP7 (four weeks post drought).

Plants experiencing drought had significantly (p<0.05) lower node emergence rates than the watered plants (Figure 2.4B) implying that drought exposure influenced plant growth rate. The double-droughted plants had significantly lower (p<0.05) node emergence rates than the watered and single-droughted plants at TP 2 and TP3 (during the first drought phase). Similarly, at TP5 and TP6 (during the second drought phase), double-droughted and single-droughted plants had a significantly lower (p<0.05) node emergence rate than the watered controls. The droughted plants recovered to equal or greater node emergence rates than the watered controls at TP4 and TP7 (after four weeks of watering). At TP7, the double-droughted plants recovered to a significantly greater value than the single-droughted plants.

Figure 2.4C shows that plant height trends through the drought trial reflected those of leaf area (Figure 2.4A) and node emergence rate (Figure 2.4B). There were significant differences (p<0.05) in plant height during both drought periods (TPs 2, 3, 5 and 6) between watered and droughted plants. Additionally, there was good recovery of droughted plants after four-week recovery periods as there were no significant differences (p>0.05) between treatment conditions at TP4 and TP7.

Internode length (Figure 2.4D) displayed different trends to the other slower-responding phenotypic traits as there was a gradual decrease in internode length over the course of the drought trial. There were no clear patterns of differences between treatment conditions and TP3 is the only time point which displayed any significant differences (p<0.05).

These slower-responding phenotypes showed patterns of decreasing leaf area, node emergence rate and plant height for droughted plants when the data were viewed as a whole.

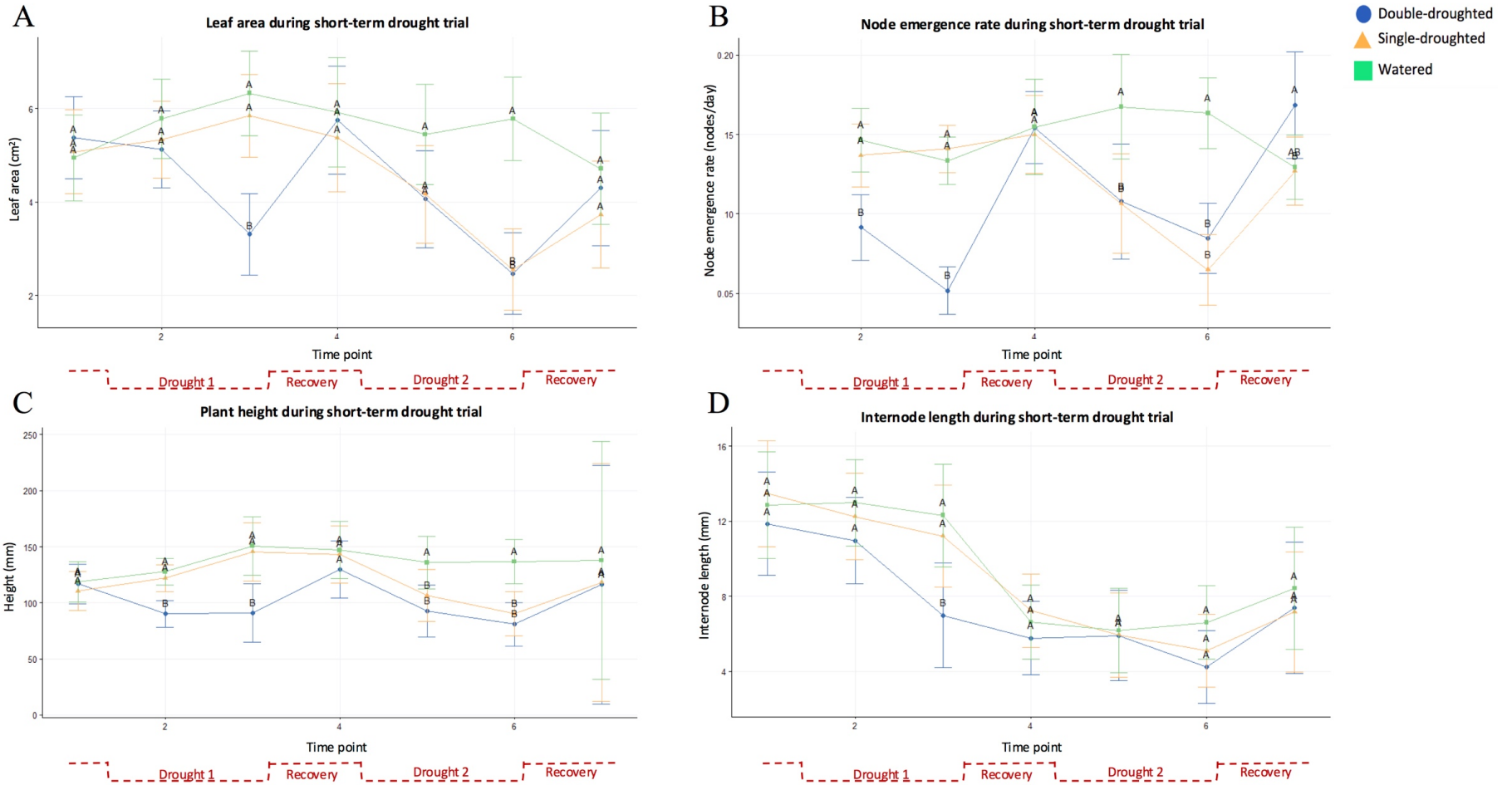

Figure 2.4. Plots showing slower responding drought related phenotypes across the time points. The data are combined for all species and individuals which were involved in the drought trial, split by treatment condition. A) plot showing leaf area over time; B) plot showing node emergence rate over time; C) plot showing plant height over time; D) plot showing internode length over time. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significance determined by LSD values at each individual time point.

When plants experienced water deficit, there were decreases in three faster-responding phenotypes – stomatal conductance, photosynthetic rate and relative water content (RWC) (Figure 2.5). There was a clear pattern of significant decreases (p<0.05) in RWC when plants were experiencing drought (Figure 2.5A). During the first drought period (TP2 and TP3), the double-droughted plants had significantly lower RWC values than the watered plants. Additionally, during the second drought period (TP5 and TP6), both the single- and double-droughted plants had significantly lower (p<0.05) RWC values than the watered equivalents. The droughted plants RWC values returned to the same level as watered controls after four weeks of recovery (TP4 and TP7). There seemed to be a slight upwards trend over time. The RWC of the droughted plants dropped quickly after the onset of water deficit as the RWC values one week into permanent wilting point (TP2 and TP5) were a similar value to the RWC four weeks into permanent wilting point (TP3 and TP6).

Gas exchange measurements (stomatal conductance and photosynthetic rate) both showed a general downwards trend over the course of the drought trial, although it was also possible to observe some treatment condition-related trends too (Figures 2.5B and 2.5C). At TP2 and TP3 (during the first drought period), double droughted plants had significantly lower (p<0.05) stomatal conductance and photosynthetic rate than the watered plants. Similarly, at TP5 (one week into the second drought period) the single and double-droughted plants showed significantly lower (p<0.05) stomatal conductance and photosynthetic rate than the watered controls. At TP4 and TP7 (after four weeks of recovery) there were no significant differences (p>0.05) for both phenotypes indicating that the droughted plants recovered back to the watered levels of gas exchange when they had normal watering resumed.

These faster responding phenotypes showed decreases in RWC and gas exchange measurements when plants experienced a water deficit when viewing the data as a whole, regardless of genotype.

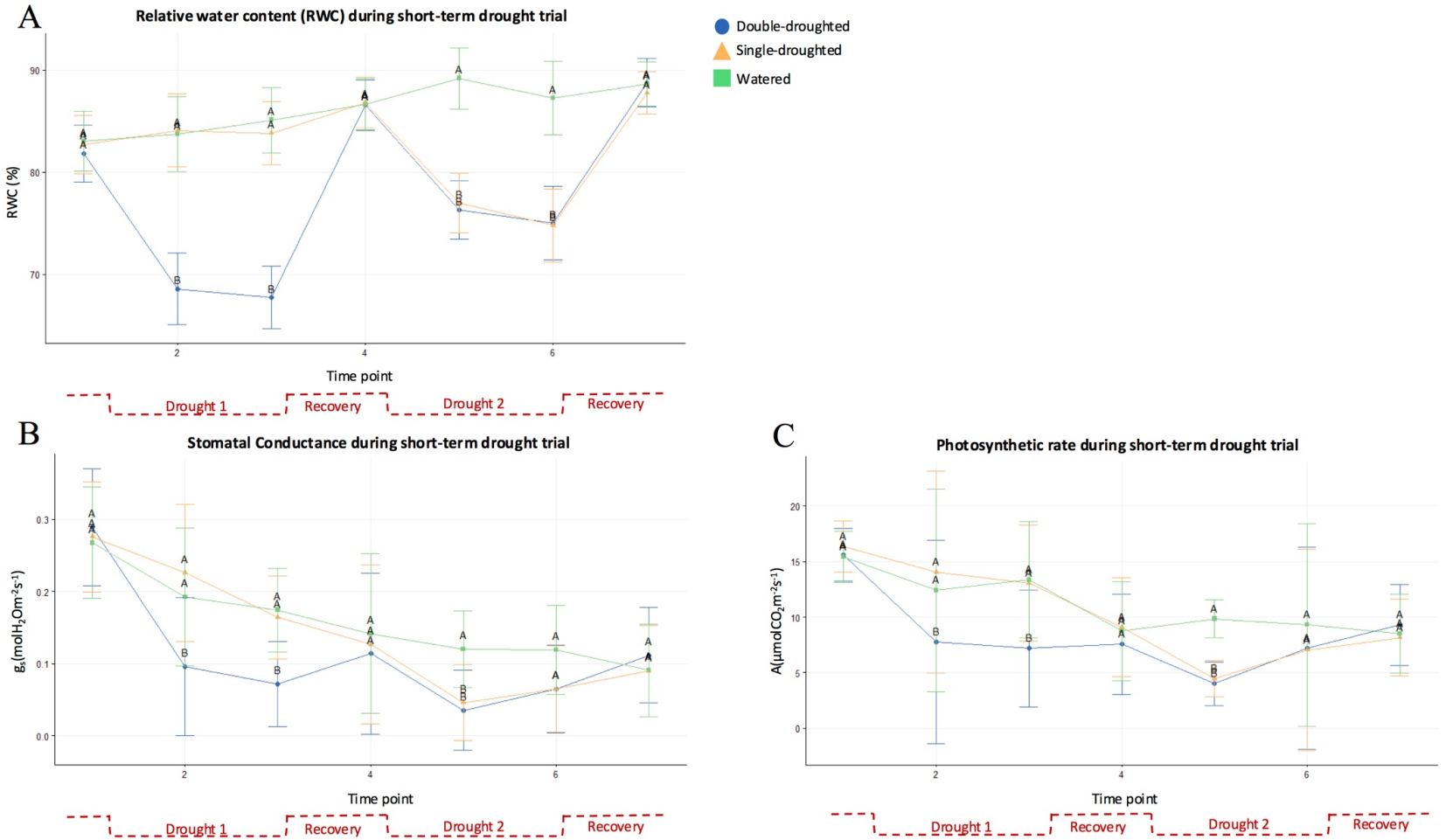

Figure 2.5. Plots showing faster responding drought related phenotypes across the time points. The data includes all genotypes which were involved in the drought trial, split by treatment condition. A) plot showing relative water content over time; B) plot showing stomatal conductance (g_s) over time; C) plot showing photosynthetic rate (A) over time. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significance determined by LSD values at each individual time point.

When investigating at leaf area split by genotype (species/cultivar/ecotype) regardless of treatment condition, Figure 2.6 shows that there were three groupings within this drought trial.

The largest-leaved group in Figure 2.6 only includes the cultivar *T. repens* cv 'Grasslands KopuII'. This cultivar had significantly larger leaf areas ($p<0.05$) than the other genotypes throughout the whole drought trial. The leaf area values for this genotype ranged from 10.50 cm^2 to 16.19 cm^2. The mean leaf area for this cultivar was impacted by the phases of the drought trial. There was a drop in leaf area in TP6 which was after four weeks of drought in the second drought phase. This is likely due to 2/3 of the plants experiencing drought (both double- and single-droughted treatment conditions) whereas at TP3 only 1/3 of the plants were experiencing drought (only double-droughted plants).

T. repens S10 and *T. repens* ecotype 'Tian Shan' are the middle leaf area grouping (Figure 2.6). These genotypes had statistically the same leaf areas ($p>0.05$) for the first three time points and from TP4 onwards *T. repens* S10 had significantly higher leaf areas than *T. repens* ecotype 'Tian Shan'. The leaf areas for these genotypes ranged from 3.52cm^2 to 7.83cm^2.

The group with the smallest leaf areas across the drought trial included 880, *T. occidentale* and *T.pallescens*. These three genotypes were not significantly different ($p>0.05$) from each other at any time point of the drought trial. They also had the smallest range of leaf areas as a group compared to the other larger leaved genotypes. The leaf area values ranged from 0.67 cm^2 to 2.40 cm^2 (Figure 2.6).

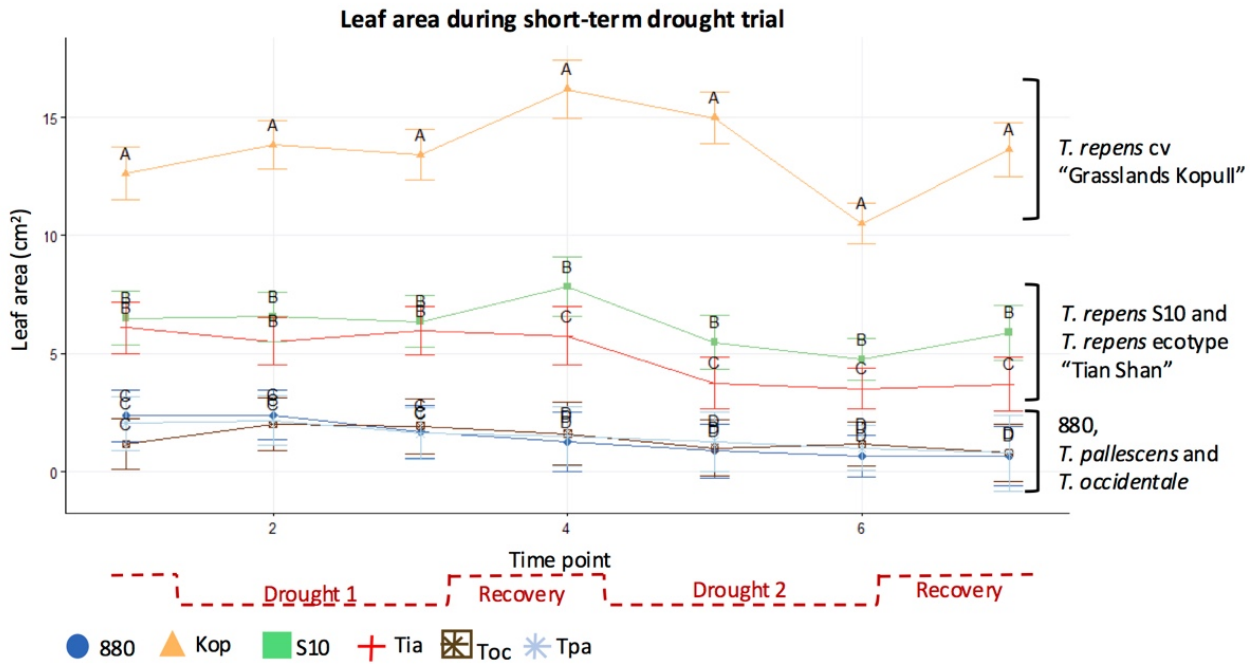

Figure 2.6. Plot showing leaf area in cm squared across the drought trial time points. The data are split by genotype. Blue circles represent 880 (synthetic hybrid); green squares represent *T. repens* S10; Brown boxes represent *T. occidentale*; yellow triangles represent *T. repens* cv 'Grasslands KopuII'; Red crosses represent *T. repens* ecotype 'Tian Shan'; Light blue stars represent *T. pallescens*. Data points represent Best Linear Unbiased Estimate means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significance determined by LSD values at each individual time point.

2.3.2 Leaf area analysis of individual genotype data

Two phenotypes were chosen to be investigated in more detail – leaf area and relative water content. These were chosen because from Figures 2.4A and 2.5A, these two phenotypes showed clear patterns related to the different phases of the drought trial. Both showed significant decreases ($p<0.05$) while plants were experiencing water deficit compared to watered equivalents and there weren't any obvious downwards trends over time as some other phenotypes such as the gas exchange measurements demonstrated (Figures 2.5B and 2.5C). Figures of the other phenotypes measured in this drought trial are included in the supplementary material.

To view phenotypic results when looking at each individual species, the model analysis was done separately on each genotype (species/cultivar/ecotype). This enabled a more in-depth investigation into the differences between treatment condition within each genotype.

The first phenotype investigated in more depth was leaf area (Figure 2.7). The three smaller-leaved genotypes show decreasing leaf area over the course of the drought trial while the three medium – larger leaved genotypes showed patterns of decreases in leaf area while plants were experiencing a water deficit.

Figures 2.7A, 2.7B and 2.7C show results for the smaller leaved genotypes which were 880, *T. occidentale* and *T. pallescens* respectively (Figure 2.6). These three plots all showed a general trend of decreasing leaf area over the course of the drought trial. During the first drought period (TP2 and 3), double-droughted plants of both *T. occidentale* (Figure 2.7B) and *T. pallescens* (Figure 2.7C) show significantly lower (p<0.05) leaf area compared to the watered controls. Throughout the drought trial for 880 (Figure 2.7A) and time points 4 onwards for *T. occidentale* and *T. pallescens* there are no easily discernible individual treatment condition-based trends.

Figures 2.7D, 2.7E and 2.7F show results for the three medium-large leaved cultivars/ecotypes (Figure 2.6) which were *T. repens* S10, *T. repens* cv 'Grasslands KopuII' and *T. repens* ecotype 'Tian Shan' respectively. These graphs detailed treatment condition-based trends with decreases in leaf area during water deficit.

T. repens S10 (Figure 2.7D) showed significantly lower (p<0.05) leaf area measurements for plants which were experiencing drought at TPs 3, 5 and 6 (during both drought periods) compared to the plants which were being watered. There were also increases in leaf area of the droughted plants back towards the watered levels at the recovery time points – TP4 and TP7. Although there were increases of the double-droughted and single-droughted plants after the second drought period, at TP7 the droughted plants were still exhibiting

significantly lower (p<0.05) leaf areas than the watered controls. There were also significant differences (p<0.05) between watered controls and single droughted leaf areas in the first half of the drought trial even though none of these plants were experiencing a water deficit.

T. repens cv 'Grasslands KopuII' showed a clear pattern of decreased leaf area when plants were experiencing water deficit (Figure 2.7E). The droughted plants exhibited significantly decreased (p<0.05) leaf areas at TP3 and TP6 (after four weeks of drought during both drought periods) compared to the watered equivalents. There were also significant differences (p<0.05) at TP4 and TP7 – after four weeks of drought recovery. After the double-droughted plants experienced their first drought, they recovered to a significantly larger (p<0.05) leaf area than the watered equivalents (TP4), but there was not a similar trend when the single droughted plants had just been through their first drought as they had a significantly smaller leaf area than the watered and double-droughted treatment conditions at TP7.

Figure 2.7F shows that for *T. repens* ecotype 'Tian Shan' there was a similar pattern to the other medium-larger leaved genotypes. The droughted plants had significantly lower (p<0.05) leaf areas than the watered equivalents at TPs 3, 5 and 6 (drought time points). But this genotype did not seem to recover to the same level as the watered controls after experiencing 4 weeks of recovery as seen by the significantly lower (p<0.05) droughted leaf areas at TP4 and TP7 when compared to the watered control plants.

Overall, it was possible to observe leaf area patterns associated with drought in larger-leaved clover types.

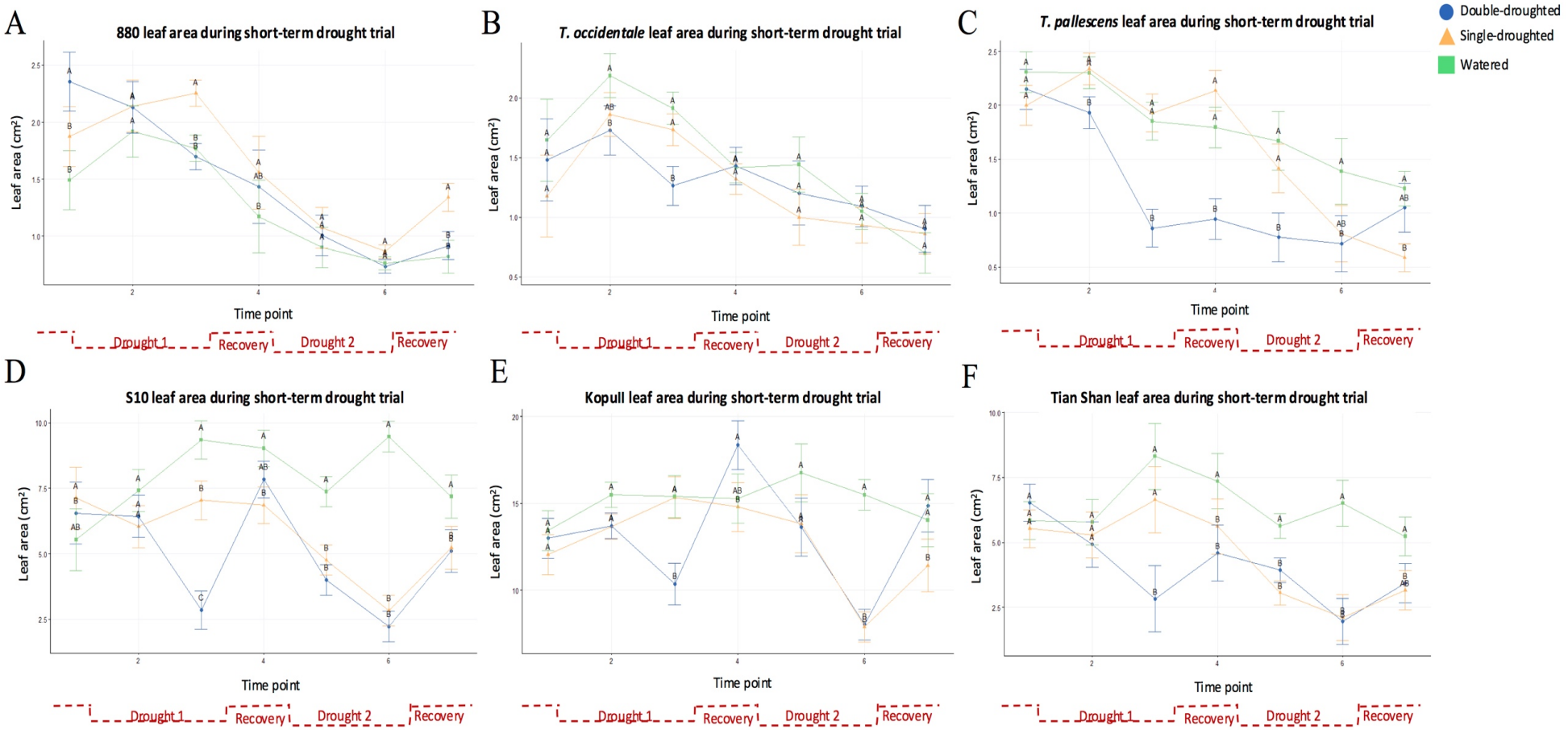

Figure 2.7. Plots showing leaf area across the drought trial time points. Each graph has the data split by treatment condition. A) Leaf area in 880 synthetic hybrid; B) Leaf area in *T. occidentale*; C) Leaf area in *T. pallescens*; D) Leaf area in *T. repens* S10; E) Leaf area in *T. repens* cv 'Grasslands KopuII'; F) Leaf area in *T. repens* ecotype 'Tian Shan'. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.

2.3.3 Relative water content analysis of individual genotype data

Relative water content (RWC) was the next phenotype investigated in more depth. Figure 2.8 shows the RWC results for each genotype included in the drought trial. The results were split by treatment condition.

The six different genotypes all demonstrated patterns of decreased RWC values when the plants were being droughted and for *T. repens* S10, *T. repens* ecotype 'Tian Shan' and *T. repens* cv 'Grasslands KopuII' it was possible to see RWC differences between the single- and double-droughted plants. The RWC results for 880 throughout the drought trial showed a clear pattern with decreased RWC values when plants were experiencing drought (Figure 2.8A). The droughted plants had significantly lower (p<0.05) RWC values than the watered equivalents at TPs 2, 3 and 6 (during drought periods). The droughted plants' RWC values dropped lower during the first drought period (to 61.0%) than they did in the second drought period (to 73.3%). Additionally, the RWC values recovered well after both drought periods as there were no significant differences (p>0.05) between treatment conditions at TP4 and TP7. At TP5 (one week into the second drought period) there was a significant difference (p<0.05) between the single- and double-droughted plants with the single-droughted RWC value being statistically the same as watered controls and double-droughted being significantly lower.

Figure 2.8B and 2.8C show results for *T. occidentale* and *T. pallescens* respectively (the progenitor species of *T. repens*). These species both demonstrated that RWC values dropped when plants were experiencing drought. For *T. occidentale*, the droughted plants had a significantly lower (p<0.05) RWC than the watered equivalents at TPs 3, 5 and 6 (during drought periods). Also, at TP5 (one week into the second drought period) the double-droughted RWC was significantly lower (p<0.05) than that of the single-droughted plants. For *T. pallescens* there were mainly significant differences between treatment conditions in the first half of the drought trial. The double-droughted plants had significantly lower (p<0.05) RWC

values than the watered equivalents at TP2 and TP3. A difference between the two progenitor species in the first drought period was that the *T. occidentale* species displayed its lowest RWC value at TP3 whereas the *T. pallescens* species had its lowest RWC value at TP2.

T. repens S10 showed a pattern of droughted plants experiencing decreases in RWC during drought periods and recovery to watered levels during the drought trial (Figure 2.8D). At TPs 2, 3, 5 and 6 (during the drought periods), the droughted plants had significantly lower (p<0.05) RWC values than the watered equivalents. There was a significant difference (p<0.05) at TP5 (one week into the second drought period) between the single-droughted and double-droughted treatment conditions, with the plants experiencing their first drought exhibiting a lower RWC than the plants going through their second drought. This genotype showed a similar pattern to *T. pallescens* in that the RWC value was lower one week into drought (TP2 and TP5) than it was four weeks into drought (TP3 and TP6).

T. repens cv 'Grasslands KopuII' showed a similar pattern to S10 in Figure 2.8E. The droughted plants had significantly lower (p<0.05) RWC values than the watered plants during the drought periods (TPs 2, 3, 5 and 6). There were also no significant differences (p>0.05) after four weeks of drought recovery (TP4 and TP7) as the RWC values of the droughted plants increased back up to watered levels. At TP6 (four weeks into second drought period) there was a significant difference (p<0.05) between the drought treatment conditions with the single-droughted plants exhibiting a significantly lower RWC than the double-droughted ones. This genotype showed a similar pattern to *T. occidentale* in that the RWC value was lower four weeks into drought (TP3 and TP6) than it was one week into drought (TP2 and TP5).

T. repens ecotype 'Tian Shan' again showed a similar pattern to *T. repens* S10 and *T. repens* cv 'Grasslands KopuII' in Figure 2.8F. The droughted plants had significantly lower (p<0.05) RWC values than the watered equivalents at TPs 2, 3, 5 and 6 (during both drought periods). And at TP5 (one week into the second drought period) there was a significant

difference (p<0.05) between the drought treatment conditions with the single-droughted plants exhibiting a significantly lower RWC than the double-droughted plants. This reflected the significant differences seen at TP5 for *T. repens* S10 and TP6 for *T. repens* cv 'Grasslands KopuII'. This genotype showed a similar pattern to *T. pallescens* in that the RWC value was lower one week into drought (TP2 and TP5) than it was four weeks into drought (TP3 and TP6).

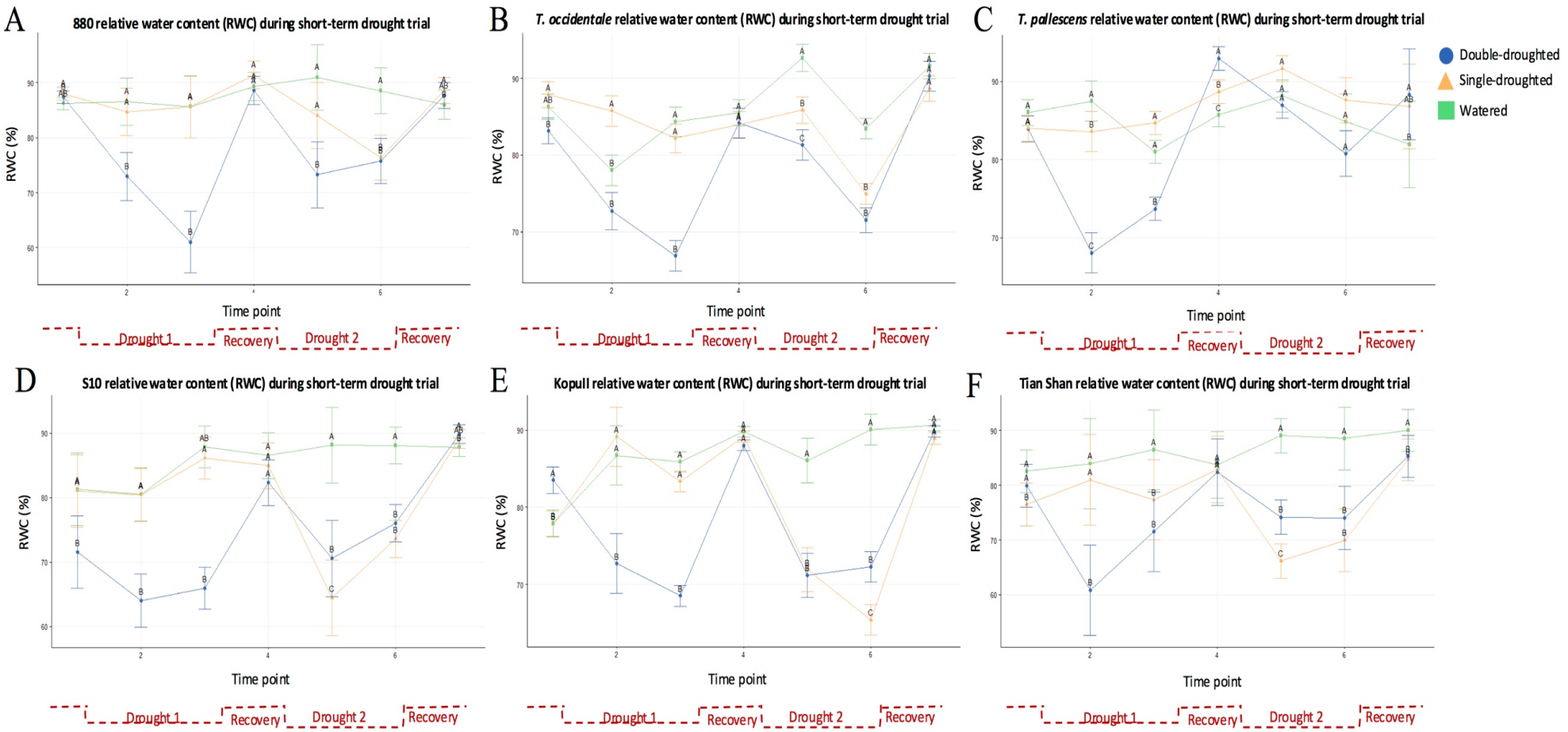

Figure 2.8. Plots showing relative water content (RWC) across the drought trial time points. Each graph has the data split by treatment condition. A) RWC in 880 synthetic hybrid; B) RWC in *T. occidentale*; C) RWC in *T. pallescens*; D) RWC in *T. repens* S10; E) RWC in *T. repens* cv 'Grasslands KopuII'; F) RWC in *T. repens* ecotype 'Tian Shan' ecotype. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.

2.4 Discussion

2.4.1 Overview of phenotypic results reveals overall trends

Investigating the overview of the phenotypic data generated through the drought trial revealed overall phenotypic trends of white clover and relatives in response to drought.

Two phenotypic aspects that showed interesting patterns in response to drought were RWC and leaf area. These phenotypes both showed clear decreases of drought exposed plants during drought periods (Figures 2.4A and 2.5A) while the phenotypes of the watered plants remained steady. This made these ideal phenotypes to investigate in more detail as they did not seem to not be impacted by any other factors, other than the watering regime.

In addition, node emergence rate showed clear patterns in response to drought when the data were not separated by genotype (Figure 2.4B) but this phenotype was not chosen for further discussion as when the data were separated by genotype there were fewer statistically significant differences.

Some of the other phenotypes investigated in this drought trial showed general declining trends over time such as internode length (Figure 2.4D), stomatal conductance (Figure 2.5B) and photosynthetic rate (Figure 2.5C). In addition, node emergence rate showed clear patterns in response to drought when the data was not separated by genotype (Figure 2.4B) but this phenotype was not chosen for further examination as when the data was separated by genotype, there were fewer significant differences to discuss This is discussed in more detail in the limitations section of this thesis. These trends suggest that there were some other factors impacting on the plant phenotypes during the drought trial, rather than just the drought parameters. Although there were these declining trends, it was still possible to interpret some drought-related trends.

2.4.2 Stomatal conductance and photosynthetic rate are important in the drought response

The stomata are key organs in plant drought response as they impact on water loss to the environment. It has previously been described that plants partially close their stomata during the early stages of drought in order to reduce water loss from transpiration (Hu & Xiong, 2014; Reddy et al., 2004). The results from this drought trial when looking at the overview of stomatal conductance measurements (Figure 2.5B) support this theory.

When the clover plants were exposed to drought, there was a trend of the droughted plants showing significantly lower ($p<0.05$) stomatal conductance measurements than the watered equivalents one week into drought. This indicated that the clover plants involved in this drought trial were able to respond to water deficit quickly. This quick reaction likely decreased the transpiration rates and was beneficial in protecting the plants from desiccation as described in (Chaves et al., 2011). Guard cells around stomatal pores have been characterised as very responsive to environmental conditions and the findings from this thesis support this. The fast reactions of stomatal pores allowed the clover plants to better maintain their water status and continue their normal physiological processes. Stomata size and distribution on leaf surfaces are related to drought resistance (Doheny-Adams et al., 2012) therefore, it would be interesting to investigate how the stomatal size and distribution impact on drought resistance and tolerance in different clover types.

Although there were visible trends when assessing this data from an overview (including all genotypes), the data from the individual species/cultivars/genotypes (Supplementary Figure D) did not clearly show these patterns. This suggests that there was too much noise at the individual genotype level, but looking at the data as a whole allowed the trends were to be identified.

Stomata regulate the CO_2 and H_2O in plant cells and therefore impact on photosynthesis. This means that photosynthetic rate is conditional on stomatal conductance. Therefore, it makes sense that the clover plants used in this drought trial showed significantly decreased photosynthetic rates ($p<0.05$) during drought phases compared to the watered equivalents when observing from the overview standpoint (Figure 2.5C). This means that during the drought phases, the plants photosynthetic rates were decreased, resulting in less water usage and less energy production. This likely allowed the plants to retain more moisture inside of their organs (Zargar et al., 2017). Photosynthesis is a critical plant process as it is the way in which energy is produced, therefore it has major effects on other plant processes such as growth (Zhang et al., 2022).

It was possible to see from growth-related phenotypes such as leaf area and node emergence rate that the growth processes were impacted by drought. At the overview level, both leaf area and node emergence rate showed clear patterns of significant decreases ($p<0.05$) in growth when plants had been droughted for four weeks compared to their watered equivalents (Figure 2.4). This was likely a knock-on effect from the decreases in stomatal conductance and photosynthetic rate (Zhang et al., 2022).

2.4.3 Leaf area during drought impacts on potential utilisation

When looking at Figure 2.6 there were clear differences in leaf area between the different genotypes used in the drought trial. Leaf area during drought showed interesting patterns at the individual genotype level (Figure 2.7). White clover has plastic leaf size depending on the environment (Seker et al., 2003). This means that when WC plants are exposed to stresses such as drought, new leaves grown during the stress exposure are smaller than ones which are grown in ideal environments. This can be clearly seen in this drought trial as WC S10, KopuII and Tian Shan all had significantly smaller ($p<0.05$) leaf areas than the watered equivalents when they were exposed to four weeks of drought. The smaller leaf size

likely occurred due to a decrease in transpiration, as more of the plants available resources were focussed on survival rather than growth.

Leaf area in plants can be used as a surrogate measurement for photosynthetic capacity. This is because leaves are the first organs which show visible signs of drought stress under water deficit (Anyia & Herzog, 2004). Leaf size is also associated with other morphological features such as root size and stolon density (Charlton & Stewart, 1999). Stolon density is a key feature of WC for persistence (Woodfield & Caradus, 1996). In this study we were able to see that after four weeks of drought, the leaf area of droughted plants was significantly lower than the watered equivalents, so it can also be interpreted from this information alone that root size and stolon density would have also decreased. This single phenotypic aspect which was relatively simple to measure provided insight on a range other plant phenotypes, meaning that this is a very valuable measurement. In the future, using leaf area as a cheap and easy trait for selection under water deficit is a viable option.

From this study there was an exception to the patterns of decreased leaf area during drought, as the smaller-leaved clover species/genotypes did not show clear patterns in response to drought. This supports the hypothesis that different clover species/genotypes show different responses to drought. There was an overt decreasing trend over time for *T. pallescens*, *T. occidentale* and 880, and it was hard to determine any drought-related trends (Figure 2.7). This may be due in part to leaf size changes being more subtle in smaller-leaved plants and therefore harder to get accurate measurements for. Therefore, it appears that leaf size/area measurements are most informative on medium to large leaved white clover cultivars/genotypes.

2.4.4 RWC during drought reveals trends and potential sub genome utilisation

The two progenitor species of WC showed different RWC trends during their first drought exposure (Figures 2.8B and 2.8C). *T. occidentale* reached the lowest RWC value at TP3 (after four weeks of drought) whereas *T. pallescens* reached the lowest RWC value at TP2

(after one week of drought). The other four genotypes all exhibited RWC phenotypes with the same pattern as one of WCs progenitor species when the plants were experiencing their first drought exposure. The synthetic hybrid 880 and KopuII exhibited the same pattern as *T. occidentale* (Figures 2.8B and 2.8E). S10 and alpine ecotype Tian Shan exhibited the same pattern as the alpine-derived *T. pallescens* (Figures 2.8C, 2.8D and 2.8F). These patterns were observable trends without statistical data backing them up, therefore the following interpretations should be viewed with caution. Although this data should be viewed with caution, it further supports the hypothesis that there are differences in drought response between clover genotypes/species.

These patterns of RWC in response to drought may relate to sub-genome utilisation. *T. pallescens* and *T. occidentale* are the parental species of 880 and the WC genotypes included in this study (Ellison et al., 2006; Williams et al., 2012). This means that all these clover types have two sub-genomes within their genome - one from each of these parental species. Following hybridisation, there are many genomic consequences from having two sub-genomes within one species. This can result in different sub-genomic regions being silenced and often one sub-genome is found to be more dominant in gene expression than the other (Cheng et al., 2012; Chester et al., 2012).

The findings in this study suggest that some drought responses in the genotypes of interest take after one of the parental species. Perhaps the genomic regions responsible for RWC/involved in the drought response were more highly expressed in one sub-genome than the other. Griffiths et al. (2019) found that there were a few white clover genes that showed tissue-specific switching between homoeologous gene copy expression. It is possible that during drought stress, similar switching between expression of homoeologues occurred, resulting in phenotypes matching one of the progenitors.

T. occidentale is a coastal species thought to confer drought tolerance (Hussain et al., 2016). This suggests that 880 and KopuII may be more drought tolerant than S10 and Tian Shan due to their response matching *T. occidentale*. However, KopuII is a large-leaved dairy cultivar which was bred for irrigated pastures rather than drought conditions therefore was not expected to be very drought tolerant. Investigation into sub-genome gene expression of these plants would help to further elucidate the role that the progenitor species genomes are playing in the drought response.

2.4.5 RWC results show that drought priming is beneficial for subsequent drought tolerance

Relative water content (RWC) is an important indicator of plant water status. During drought, RWC values decreased across almost all genotypes included in this thesis (Figure 2.8). In this thesis, RWC results revealed some interesting differences in the drought response between plants which were experiencing their first drought, and plants which had been pre-exposed to drought (primed). *T. repens* S10, *T. repens* cv 'Grasslands KopuII' and *T. repens* ecotype 'Tian Shan' all exhibited these differences between the single- and double-droughted plants (Figures 2.8D, 2.8E and 2.8F). This suggests that the plants which were pre-exposed to drought responded by exhibiting a significantly lesser reduction in RWC, indicating that the plant leaves lost less water during the same levels of drought stress. This mechanism indicates that plants that have been pre-exposed to drought have greater drought resistance than those facing drought for the first time. This supports the hypothesis that 'drought primed' plants display greater phenotypic tolerance in their second drought exposure. Given that a 'priming' effect was observed through RWC measurements, it is likely that there were other phenotypes exhibiting this pattern that were not discerned through this drought trial. Deeper investigations into other drought stress-related phenotypes may further reveal 'priming' effects.

Other examples of plant memory/priming in response to drought stress have been previously reported. *Arabidopsis thaliana* plants that were exposed to cycles of dehydration stress conditions and recovery were shown to retain memory of an earlier dehydration stress. It was found that *A. thaliana* plants that had been previously exposed to dehydration were able to retain leaf water content (RWC) to a greater extent than plants which experienced dehydration stress for the first time (Ding et al., 2012). This mimics the results in this thesis. Additionally, Ding et al. (2012) identified that there were also differences between trained (pre-droughted) and untrained (plants facing their first drought) plants at the transcription level, and that several ABA-inducible genes showed greater transcription rates during subsequent exposures compared to the initial drought exposure. A similar study showed that *Zea mays* (maize) exhibited reduced rates of water loss (RWC) when plants had been pre-exposed to dehydration. Further investigation found that this species also exhibited transcription memory in a subsequent dehydration stress. They found that there was modification of transcriptional responses by similar transcriptional memory patterns (Ding et al., 2014).

Previous studies have shown that the priming/training effect which leads to plants coping better to subsequent droughts has a root in transcriptional memory – differential expression of genes is underlying the phenotypic differences. It seems likely that the plant memory in white clover observed in this thesis may be caused by similar transcriptional differences.

DNA methylation is a key mechanism which underlies differences in gene expression, therefore it is possible that DNA methylation plays an important role in this plant memory we observed in white clover. It has been previously shown that DNA methylation played a role in white clovers drought response with a role in biomass of new growth (González et al., 2016). It would be interesting to investigate DNA methylation in the plants involved in this drought

trial to investigate whether there are differences in methylome between the different treatment conditions, and if related genes play a role in the drought response.

2.4.6 Limitations and next steps

There were a few limitations of the drought trial used to investigate the aims of this thesis. One of the main limitations could be seen through the phenotype results e.g. node length (Figure 2.4D), stomatal conductance (Figure 2.5B) and photosynthetic rate (Figure 2.5C) as all these phenotypes showed gradual decreases in values over the course of the drought trial, even in the watered controls. This suggests that the structure of the drought trial and the length of time in the growth room were not ideal for the plants. These gradual decreases in plant health/function made it harder to identify trends that were associated with drought.

Another limitation of the drought trial that may have contributed to the decline of the watered plants phenotypes over time was the watering regime. When the plants were being watered normally (watered controls, or droughted plants during recovery/non-droughted periods), there was no specific system to ensure plants were getting the right amount of water. In the future, it would be beneficial to have a percentage field capacity to aim for to provide more structure to the watering regime. As it was, some of the plants may have been getting overwatered, particularly those with less robust root systems.

One of the most important next steps to this research is to investigate mechanisms underlying the differences in phenotypic response we observed. One of the most interesting findings from the phenotypic responses of white clover and relatives to drought stress in this thesis was the priming/plant memory that was observed in the RWC results (Figure 2.8). DNA methylation may be a mechanism by which the plants remembered prior stresses, therefore DNA methylation analysis of the plants used in this drought trial is an important next step. This would provide relevant insights into plant memory mechanisms.

3 DNA methylation

3.1 Introduction

As illustrated in the previous chapter, there were a range of phenotypic responses to drought in white clover. There was also evidence of plant memory or 'priming' in the relative water content data (Figure 2.8). The next step in this research was to investigate how the plants were able to retain memory of the previous drought stress. Perhaps DNA methylation was a mechanism behind this observed memory?

DNA methylation is a type of epigenetic regulation that most commonly occurs on cytosine bases to form 5-methylcytosine (5-mC). It influences chromatin structure and impacts on the accessibility of genetic information (Ehrlich et al., 1982). In plants, DNA methylation has been observed in all cytosine base sequence contexts: CG, CHG and CHH (where H = A, T or C) (Henderson & Jacobsen, 2007). This means that DNA methylation can be widely spread throughout the genome, and impact on transcription of a wide range of genes. The location of DNA methylation impacts gene expression patterns (Zhang et al., 2018). For example, DNA methylation in promoter regions has been shown to silence gene expression (Phillips, 2008), whereas DNA methylation in the gene body has been positively correlated with gene expression in some plant species (Liang et al., 2014).

Recent studies have found that DNA methylation contributes to the adaptation of plants to stress, including to drought stress (Thiebaut et al., 2019). In *Citrillus colocynthis* of the Cucurbitaceae family, where there were over 2,500 differentially expressed genes in response to drought including stress signalling factor genes and detoxification genes. There was also differential expression of genes involved in DNA methylation, suggesting that DNA methylation played a role in this species drought response (Wang et al., 2014). In rice, exposure to drought conditions led to several stable DNA methylation changes in stress-responsive genes

(such as plant development and stress response pathway genes) which were passed on to multiple generations of offspring. Furthermore, varieties with greater drought resistance were found to pass on a higher proportion of methylation marks transgenerationally (Zheng et al., 2017). These studies support the idea that DNA methylation is important in plant drought responses and show that the inheritance of these drought-tolerant methylation marks in subsequent generations is possible.

Therefore, investigation into whether DNA methylation marks are associated with the drought response in white clover is a key area of interest. Furthermore, it is important to investigate the role that DNA methylation plays in the plant's memory to drought. If there was evidence of plant memory via DNA methylation in white clover, then that would have implications in the future breeding of more drought-tolerant and drought-resistant varieties.

3.2 Materials and Methods

3.2.1 Sample collection and storage

During the previously described drought trial (Chapter 2), leaf samples of white clover (WC) S10 were collected at TP (time point) 5 and TP7 to investigate DNA methylation patterns (Figure 3.1). WC S10 was chosen for investigation as this genotype showed significant differences in RWC and leaf area phenotypes during drought exposure (Figure 2.7D and Figure 2.8D), suggesting that this provided a good starting place to investigate whether DNA methylation was associated with the phenotypic differences. In addition, this was the same genotype that was the DNA source for an updated PacBio HiFi/Hi-C proximity analysis-derived white clover reference genome (Griffiths et al., unpublished), allowing for accurate placement of loci. TP5 was chosen due to the RWC results showing significant differences between treatment conditions at this time (Figure 2.8D), and TP7 was chosen to investigate how the genome responded after recovery from drought.

A total of 18 leaf samples were obtained, which included three replicates for each of the three treatment conditions (watered, single-droughted and double-droughted) at the two different time points. From each plant, at least three youngest fully mature clover leaves were collected into 5 mL Eppendorf tubes and snap frozen immediately in liquid nitrogen. These samples were stored at -80°C until used.

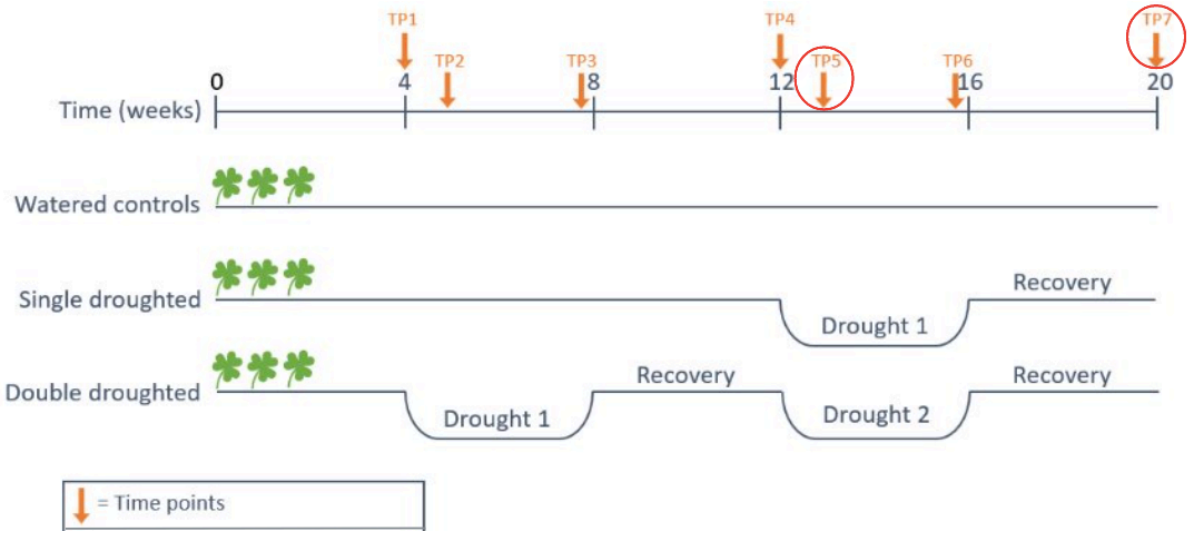

Figure 3.1. Structure of the drought trial. TP5 samples (circled in red) were taken in the second drought period after the plants had been held at permanent wilting point for one week. TP7 samples (circled in red) were taken in the second recovery period after the plants had been watered normally for four weeks.

3.2.2 DNA extractions

DNA extractions were conducted using a modified Whitelock DNA extraction method (Anderson et al., 2018). Steps 1-4 of the Anderson et al. (2018) method were skipped. These steps involved grinding tissue using a 96-well plate method. Instead, leaf tissue was ground in mortar and pestles with liquid nitrogen. For each sample, 50 to 100 g of ground tissue were transferred to 50 mL falcon tubes along with two 5/32 inch (3.97 mm) stainless steel beads per tube to assist in mixing of the ground tissue with the subsequent reagents.

Steps 5-8 of the Anderson et al. (2018) method were conducted in individual Eppendorf tubes rather than 96-well plates. These steps involved using buffers and a proteinase to break down the plant tissue allowing for DNA isolation.

The remaining steps 9 onwards were following according to the Anderson et al. (2018) protocol. These steps involved further use of buffers to isolate and purify the sample DNA.

Following DNA extraction, all samples were assessed for DNA integrity (quality and quantity) by resolving the samples via electrophoresis with a 1Kb plus DNA ladder (Invitrogen) on a 0.8% (w/v) Lithium Borate agarose gel for 30 mins at 100 V. The gel and DNA were stained with SYBR safe™ (Invitrogen, USA) and visualised with UV light (Gel DocTM, Bio-Rad, CA, USA). Samples were also quantified with a Qubit dsDNA Broad Range Assay Kit (Life Technologies, Thermo Fisher Scientific Inc.) and a Qubit 2.0 Fluorometer (Life Technologies, Thermo Fisher Scientific Inc.) following the manufacturer's instructions.

3.2.3 Methylation sequencing

The NEBNext Enzymatic Methyl-seq kit (EM-seqTM) (NEB, UK) was used on each sample. This kit combined NEBNext® UltraTMII reagents (NEB, UK) with two enzymatic steps that converted unmethylated cytosine to thymine to construct Illumina libraries that accurately represented 5-mC within the genome.

Illumina libraries were then sequenced on an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA) at AgResearch Invermay (AgResearch, Mosgiel, New Zealand). Paired-end reads were generated with read lengths of 150 bp or higher.

3.3.4 DNA methylation data processing

Data quality was checked using FastQC, followed by trimming of low-quality bases and adapter sequences with Trimmomatic (v0.39). Reads were aligned to the reference genome (Griffiths et al., unpublished) using Bismark-Bowtie2 (v0.24.2). Duplicate reads were removed, and methylation calling was carried out with CGmaptools (v1.0). Finally, differential

methylation analysis was conducted using the R DSS package to identify significant methylation changes ($p<0.05$) between the different treatment conditions (double-droughted, single-droughted and watered) and the different time points (TP5 and TP7).

3.2.5 DNA methylation data visualisation

Data were combined for all cytosine sequence contexts (CG, CHG and CHH, where H = C, T or A) and filtered based on maximum distance from closest gene (5 kb). The data were further filtered by methylation difference (0.1) to ensure that only cytosine sites with a proportional difference in methylation level of at least 0.1 between the treatment conditions and/or time points were included in further analysis. Rstudio was used to generate upset plots based on the comparisons of interest.

Rstudio was also used to generate scatter plots displaying differentially methylated cytosines (DMCs) along *T. repens* chromosomes. Scatter plots shown in this thesis include those containing areas with ≥10 DMCs associated with a single gene. A selection of areas with ≥10 DMCs associated with a single gene were chosen for further examination to provide information on whether areas of high DMC content were associated with genes involved in drought stress response. This aimed to provide a proof of concept that DMCs were involved in white clovers drought response and likely had a role in gene expression.

3.3 Results

3.3.1 DNA methylation after one week of drought

Investigation into the methylation profiles of WC S10 plants one week into drought was conducted using data from TP5 samples (Figure 3.1). This allowed for investigation into the methylation profiles of plants that were experiencing their first drought and plants that were experiencing their second drought.

The upset plot (Figure 3.2) shows total numbers of unique differentially methylated cytosines (DMCs) for all the treatment condition comparisons at TP5. Compared to the watered controls, single-droughted plants had 393,795 hypo-methylated and 352,639 hyper-methylated cytosine sites. Double-droughted plants had 415,461 hypo-methylated and 373,169 hyper-methylated cytosines compared to the water controls. Double-droughted plants had 417,878 hypo-methylated and 423,277 hyper-methylated sites as compared to the single-droughted plants. The upset plot (Figure 3.2) shows that all these one-on-one comparisons had between 352,639 and 423,277 unique DMCs. The two comparisons with the greatest number of DMCs were between the double-droughted and single-droughted S10 plants.

When investigating common DMCs across multiple comparisons, there were 125,397 and 122,267 DMC loci that were hypo- and hyper-methylated in double-droughted plants compared to both the single-droughted and watered controls. This means that there were ~250,000 cytosines that were differentially methylated in double-droughted plants compared to both other treatment conditions one week into this drought period. In addition, there were 72,944 and 60,042 DMCs that were hypo- and hyper-methylated in single- and double-droughted plants when compared to the watered controls. These DMCs were differentially methylated in both drought treatment conditions when compared to the watered controls.

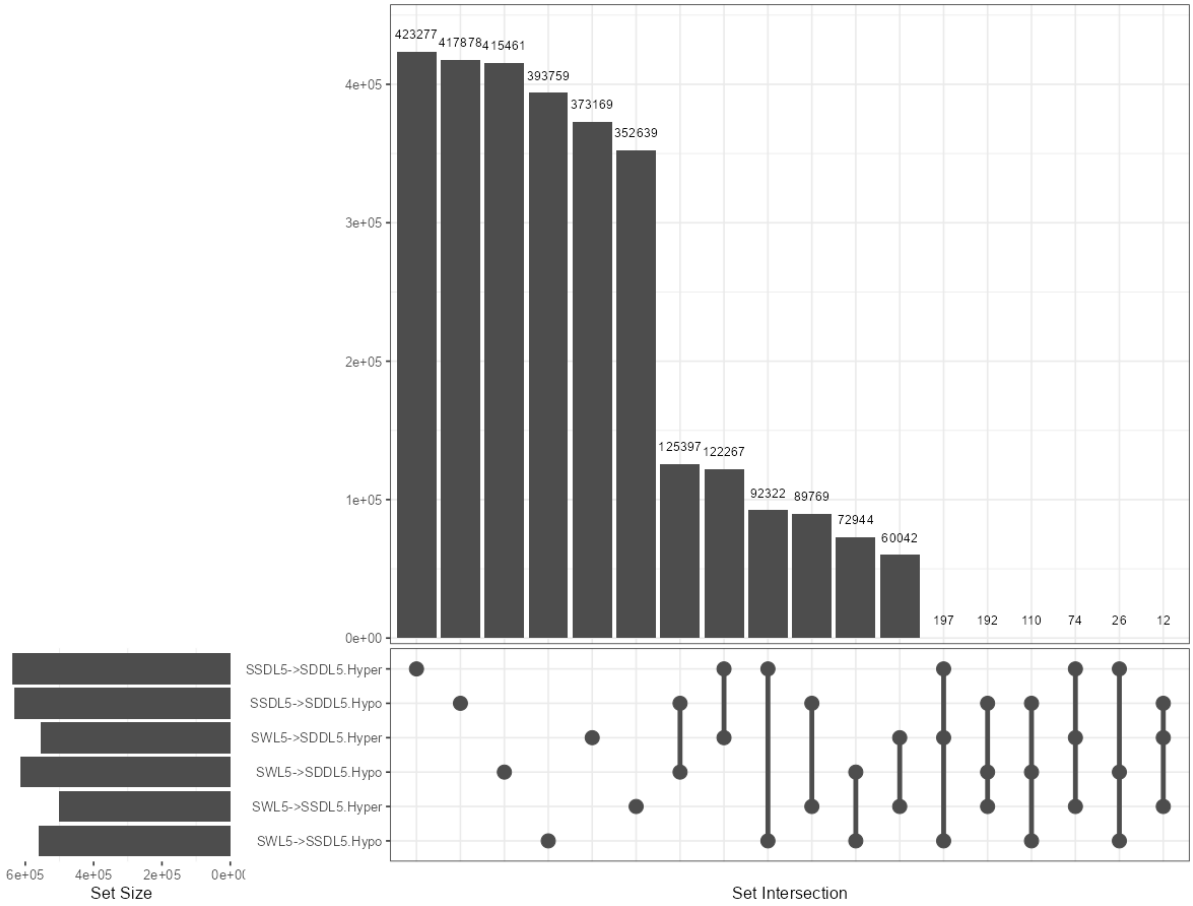

Figure 3.2. Upset plot showing numbers of differentially methylated cytosines (DMCs) for all comparisons at TP5, where droughted plants had been at permanent wilting point for one week. SWL5 = watered, SSDL5 = single-droughted and SDDL5 = double-droughted. Differential methylation was determined based on the second set in the comparison relative to the first, hence SSDL5->SDDL5 hyper = identification of loci hypermethylated in double-droughted plants relative to single-droughted plants at TP5.

3.3.2 DNA methylation after four weeks of drought recovery

DNA methylation analysis was conducted on samples collected at TP7 after four weeks of drought recovery post drought period 2. This allowed for comparison of DNA methylation profiles of plants that had recovered from their first drought and plants that had recovered from their second drought.

The upset plot (Figure 3.3) shows numbers of unique DMCs for all the treatment condition comparisons at TP7. Compared to the watered controls, the single-droughted plants had 352,313 hypo-methylated and 352,779 hyper-methylated cytosines. The double-droughted plants had 363,067 hypo-methylated and 334,820 hyper-methylated cytosine loci compared to

the watered controls. The double-droughted plants had 392,087 hypo- and 363,708 hyper-methylated loci as compared to the single-droughted plants. The upset plot (Figure 3.3) shows that all one-on-one comparisons had between 334,820 and 392,087 differentially methylated cytosines. The two comparisons that had the greatest number of unique DMCs were between the double-droughted and single-droughted S10 plants, similar to the pattern seen at TP5 (Figure 3.2).

When analysing common DMCs across multiple comparisons, there were 87,110 and 82,476 DMCs that were hypo- and hypermethylated, respectively, in double-droughted plants compared to both the single-droughted ones and the watered controls. This means that there were ~170,000 cytosines that were differentially methylated in double-droughted plants compared to the other two treatment conditions four weeks after post-drought recovery. In addition, there were 63,975 and 57,506 DMC loci that were hypo- and hypermethylated, respectively, in single- and double-droughted plants when compared to the watered controls. This means that there were ~110,000 cytosines that were differentially methylated in both drought treatment conditions compared to the watered controls.

There were 95,464 cytosines that were hyper-methylated in single-droughted plants compared to both double-droughted and watered ones. There were also 84,827 cytosines that were hypo-methylated in single-droughted plants compared to both double-droughted and watered ones. These DMCs represent loci that were significantly differentially methylated in single-droughted plants compared to both watered and double-droughted ones after four weeks of recovery.

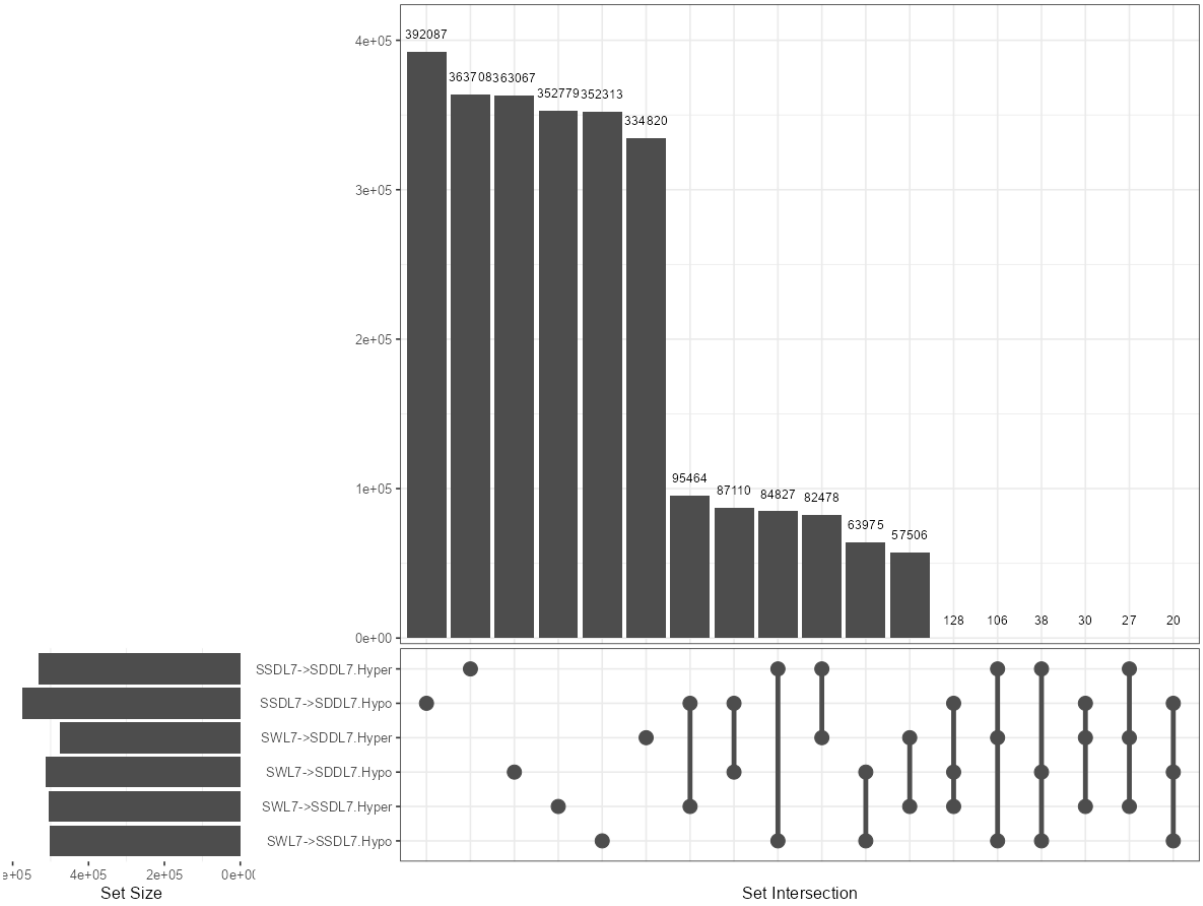

Figure 3.3. Upset plot showing numbers of differentially methylated cytosines (DMCs) for all comparisons at TP7, where droughted plants had been in recovery for four weeks. SWL7 = watered, SSDL7 = single-droughted and SDDL7 = double-droughted. Differential methylation was determined based on the second set in the comparison relative to the first, hence SSDL7->SDDL7 hyper = identification of loci hyper-methylated in double-droughted plants relative to single-droughted plants at TP7.

3.3.3 Sub-genome differential DNA methylation

The number of DMC loci were calculated in the different progenitor-derived chromosomes to investigate differences in DNA methylation between the *T. pallescens* sub-genome and the *T. occidentale* sub-genome at TP5 and TP7.

The number of DMC loci commonly differentially methylated in both drought treatment conditions compared to the watered controls at TP5 (Figure 3.2) were calculated. In the *T. occidentale* sub-genome chromosomes there were 45,038 DMCs and in the *T. pallescens* sub-genome there were 45,486 DMCs. This was a 1.0% difference in the number of DMC loci between the two sub-genomes.

At TP7 the number of commonly differentially methylated cytosines in both drought treatment conditions compared to the watered controls (Figure 3.3) were also calculated. In the *T. occidentale* sub-genome there were 41,531 DMCs and in the *T. pallescens* sub-genome there were 41,890 DMCs. Like at TP5, there was a 1.0% difference in number of DMC loci between the two sub-genomes.

3.3.4 DNA Methylation across time points

Analysis was conducted on single-droughted samples collected at TP5 and TP7 compared back to watered equivalents. This allowed investigation into similarities and differences in DNA methylation between plants that were experiencing their first drought and that were recovering from their first drought, and how these differed to the watered controls.

The upset plot (Figure 3.4) shows numbers of unique DMCs for all the comparisons at both TP5 and TP7, and between the two time points. All the one-on-one comparisons had between 385,901 and 453,016 unique DMCs. The comparison with the largest number of DMCs were loci which were hypo-methylated in single-droughted plants compared to watered at TP5.

A small proportion of DMC loci were common across the different comparisons. Compared to watered controls, there were 7,958 DMCs that were hypo-methylated in single-droughted plants at both TP5 and TP7 and 7,308 that were hypermethylated in single-droughted plants at both TP5 and TP7. These loci contained cytosines that were differentially methylated compared to watered controls after the onset of drought stress and remained differentially methylated even after the drought stress was no longer present. These DMCs were investigated further to determine their locations on the chromosomes and identify areas of high DMC representation.

Figure 3.5 shows a range of scatter plots that display chromosomal regions of DMCs that were hyper- or hypo-methylated in single-droughted plants at both TP5 and TP7 (when

compared to watered controls). The plots showed many DMCs, and several areas with ≥10 DMCs associated with a single gene are indicated with arrows (Figure 3.5). These areas of interest were chosen to be used as examples to investigate whether regions that had higher DMC content were associated with genes involved in the drought response process. This aimed to provide a proof of concept that DMCs were involved in white clovers drought response and likely had a role in gene expression.

A region of chromosome 3P (chromosome 3, *T. pallescens*-derived subgenome) with a high number of hypomethylated cytosines at both time points, when compared to watered controls, was located ~500 bp upstream (Tr_Tp_82260) from a gene encoding a wall-associated receptor kinase. The arrow in Figure 3.5B showed a region in chromosome 4O (Chromosome 4, *T. occidentale*-derived subgenome) that had a high number of hypo-methylated cytosines. The closest gene to this region was ~4400 bp upstream and encoded an iron ascorbate-dependant oxidoreductase family protein (Tr_To_28183). In Figure 3.5C, there was a region in chromosome 7P (Chromosome 7, *T. pallescens*-derived subgenome) that had a high number of hypo-methylated DMCs. This region was inside a pectinesterase gene (Tr_Tp_33377). There were also three indicated examples of regions with a high number of hyper-methylated cytosines at both time points (Figure 3.5D and E). These were within chromosome 7P and 8P and were all located within genes. The region in chromosome 7P (Chromosome 7, *T. pallescens*-derived subgenome) encoded a soybean trypsin inhibitor (Kunitz) family of protease inhibitors (Tr_Tp_36731). The region near the beginning of chromosome 8P (Chromosome 8, *T. pallescens*-derived subgenome) was inside a gene which encoded a SUMO-activating enzyme subunit (Tr_Tp_55781) and the region towards the end of the chromosome was inside a gene which encoded a prolyl oligopeptidase N-terminal beta propeller domain protein.

There were also a few loci with opposite methylation patterns at the different time points. There were 5,601 DMCs that were hypo-methylated at TP5 and hyper-methylated at TP7 compared to watered controls and differentially methylated when compared against each other (double-droughted TP5 vs double-droughted TP7). Additionally, 4,877 DMCs were hyper-methylated at TP5 and hypo-methylated at TP7 when compared to watered controls and differentially methylated when compared against each other (Figure 3.4).

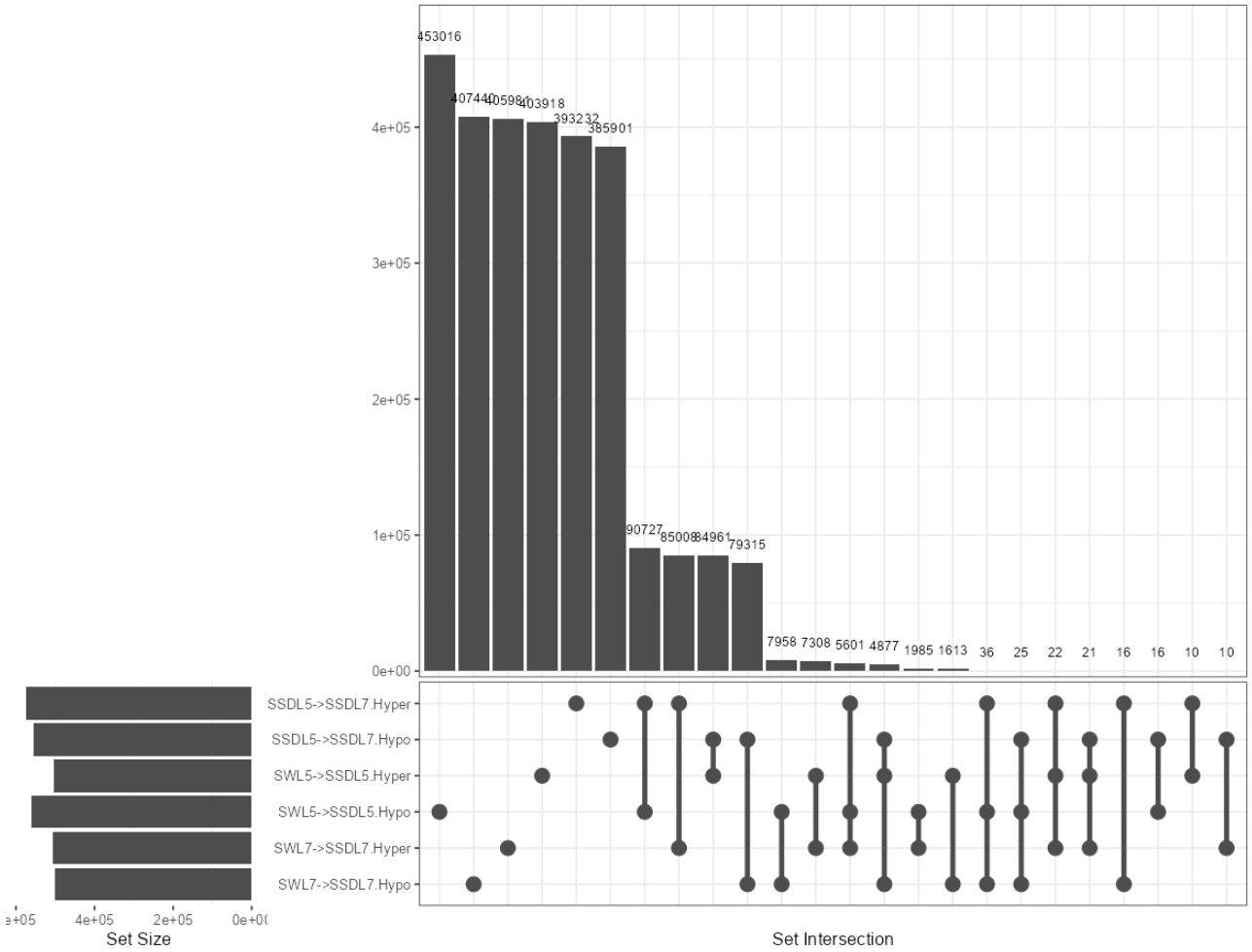

Figure 3.4. Upset plot showing numbers of differentially methylated cytosines (DMCs) for single-droughted S10 plants at TP5 (one week into their first drought period) and TP7 (after four weeks of drought recovery). SSDL5 = single-droughted TP5, SSDL7 = single-droughted TP7, SWL5 = watered TP5 and SWL7 = watered TP7. Differential methylation was determined based on the second set in the comparison relative to the first, hence SSDL5->SSDL7 hyper = identification of loci hyper-methylated in single-droughted plants at TP7 relative to single-droughted plants at TP5.

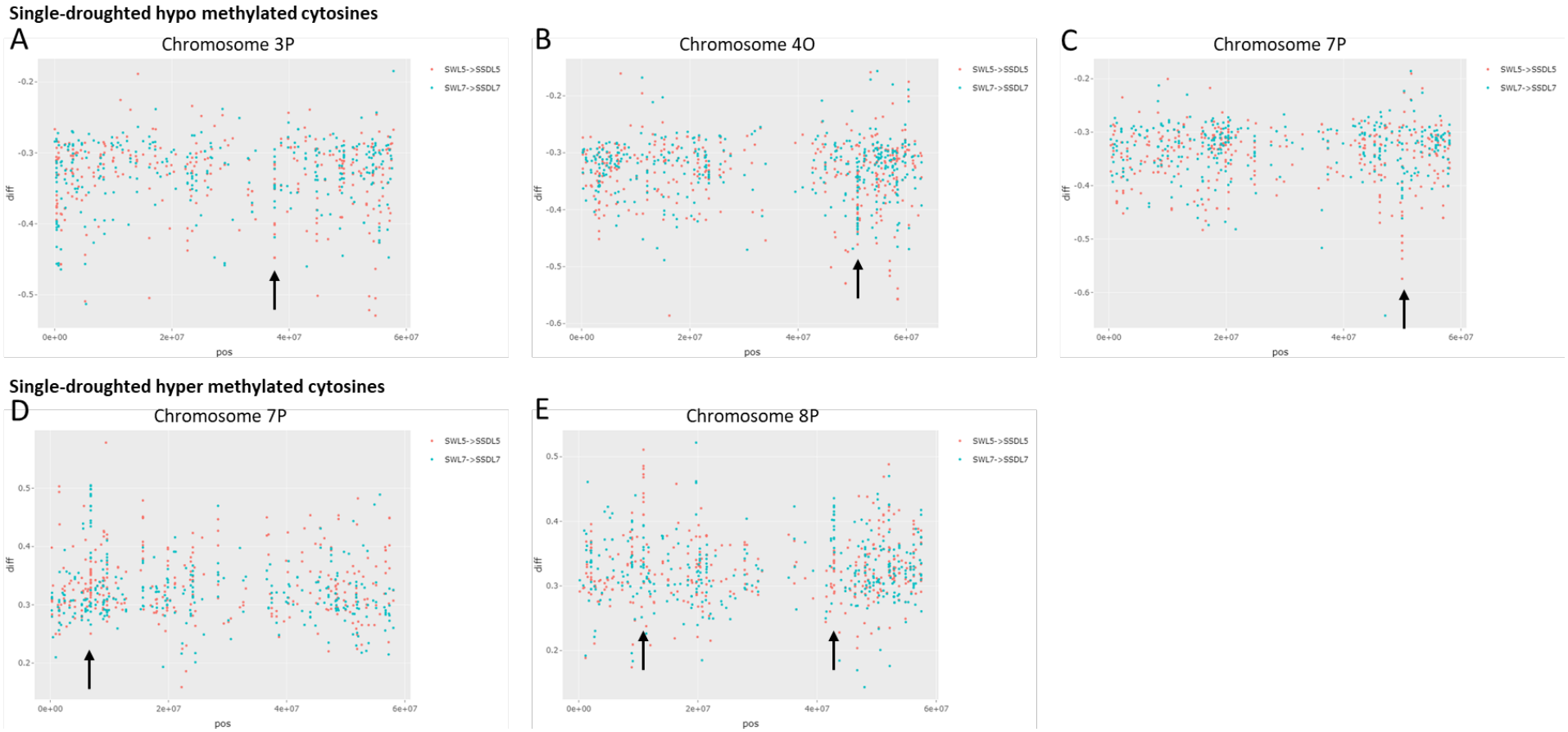

Figure 3.5. Scatter plots showing hypo- and hyper-methylated cytosines and their chromosomal locations (as mapped against the *Trifolium repens* genome) when single-droughted plants were compared back to watered controls at TP5 (red dots) and TP7 (blue dots). Each dot represents one differentially methylated cytosine, and the plots show levels of differential methylation along the positions of the chromosome. The arrows indicate genomic regions of interest. SWL5 = watered plants at TP5; SWL7 = watered plants at TP7; SSDL5 = single-droughted plants at TP5; SSDL7 = single-droughted plants at TP7. A) Hypo-methylated cytosines along chromosome 3P; B) Hypo-methylated cytosines along chromosome 4O; C) Hypo-methylated cytosines along chromosome 7P; D) Hyper-methylated cytosines along chromosome 7P; E) Hyper-methylated cytosines along chromosome 8P.

Analysis was also conducted on single-droughted samples collected at TP7 and double-droughted samples collected at TP5 compared back to watered equivalents. This facilitated investigation into differences in DNA methylation between plants that had recovered from their first drought and those that were experiencing their second drought.

The upset plot (Figure 3.6) shows numbers of unique differentially methylated cytosines (DMCs) for all the comparisons at both TP5 and TP7. All the one-on-one comparisons had between 485,761 and 597,625 unique DMCs.

A small proportion of DMCs were common across the different comparisons. Between 7,470 and 8,457 DMCs were found commonly differentially methylated between the different time points when compared against the equivalent watered controls. This included methylation in the same direction (hyper-methylation at both time points or hypo-methylation at both time points) and in the opposite direction (hyper-methylation at one time point and hypo-methylation at the other).

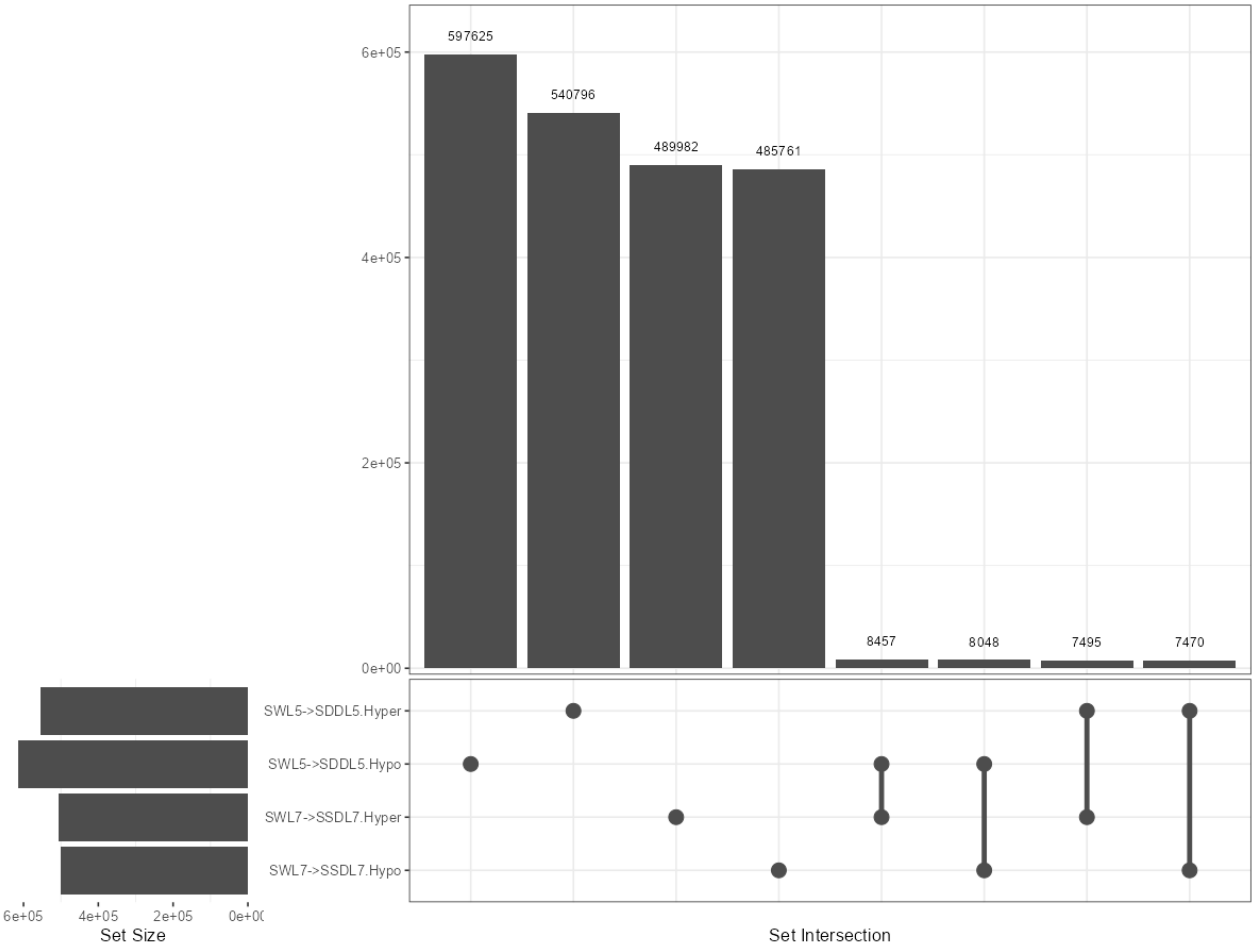

Figure 3.6. Upset plot showing numbers of differentially methylated cytosines (DMCs) for single-droughted S10 plants compared to watered controls at TP7 (four weeks of recovery after first drought stress) and double-droughted plants compared to watered controls at TP5 (one week into second drought stress). SDDL5 = double-droughted TP5, SSDL7 = single-droughted TP7, SWL5 = watered TP5 and SWL7 = watered TP7. Differential methylation was determined based on the second set in the comparison relative to the first, hence SWL5->SDDL5 hyper = identification of loci hyper-methylated in double-droughted plants at TP5 relative to watered plants at TP5.

Finally, analysis was conducted on double-droughted samples collected at TP5 and double-droughted samples collected at TP7 compared back to watered equivalents. This allowed investigation into differences in DNA methylation between plants that were experiencing their second drought and those that had recovered from their second drought.

The upset plot (Figure 3.7) shows numbers of unique differentially methylated cytosines (DMCs) for all the comparisons at times point 5 and 7 and between the two. All the one-on-one comparisons had between 385,345 and 479,698 unique DMCs.

A small proportion of DMCs were common across the different comparisons. When compared to the watered controls, there were 8,452 DMCs that were hypo-methylated in double-droughted plants at both TP5 and TP7 and 7,106 that were hyper-methylated in double-droughted plants at both TP5 and TP7. These DMCs were investigated in more depth to look at their locations on the chromosomes and identify areas of increased DMC representation.

Figure 3.8 shows a range of scatter plots that display chromosomal regions of DMCs that were hyper- or hypo-methylated in double-droughted plants at both TP5 and TP7 (when compared to watered controls). The plots show many DMCs, with some areas of greater DMC representation (≥10 DMCs associated with the same gene). Some of these examples were indicated with arrows. The arrow in Figure 3.8A indicated a region at the beginning of chromosome 1O (Chromosome 1, *T. occidentale*-derived subgenome) that had a high number of hypo-methylated cytosines at both time points when compared to watered controls. This location had a gene ~2,000 bp downstream (Tr_To_7753) encoding a protein belonging to the class I-like SAM-binding methyltransferase superfamily. The arrow in Figure 3.8B indicated a region at the beginning of chromosome 5P (Chromosome 5, *T. pallescens*-derived subgenome) with a high number of hypo-methylated cytosines. The closest gene to this region was ~1,700 bp downstream and encoded a mitogen-activated protein kinase (Tr_Tp_49701). In Figure 3.8C, there was a region in chromosome 7P (Chromosome 7, *T. pallescens*-derived subgenome) that had a high number of hypo-methylated DMCs. This region had a gene ~450 bp upstream belonging to the yippee family (Tr_Tp_35875). There were also three indicated examples of regions with a high number of hyper-methylated cytosines at both time points (Figures 3.8D, 3.8E and 8F). These were within chromosome 3P (Chromosome 3, *T. pallescens*-derived subgenome), 4P (Chromosome 4, *T. pallescens*-derived subgenome) and 7O (Chromosome 7, *T. occidentale*-derived subgenome). The region near the beginning of chromosome 3P had a gene ~1500 bp upstream that belonged to the protein kinase superfamily

(Tr_Tp_79773). The region in chromosome 4P was inside a gene involved in cellular manganese ion homeostasis (Tr_Tp_39782). The hyper-methylated region at the beginning of chromosome 7O had a gene ~2,000 bp upstream that belonged to the glycosyltransferase family (Tr_Tp_24759).

There were also smaller numbers of loci that were methylated in opposite directions at the different time points. There were 5,837 DMCs that were hypo-methylated at TP5 and hyper-methylated at TP7 (compared to watered controls) and were also significantly differentially methylated between the two time points. Additionally, there were 5,747 DMCs that were hyper-methylated at TP5 and hypo-methylated at TP7 (compared to the watered controls), that also showed differential methylation between the two time points (Figure 3.7)

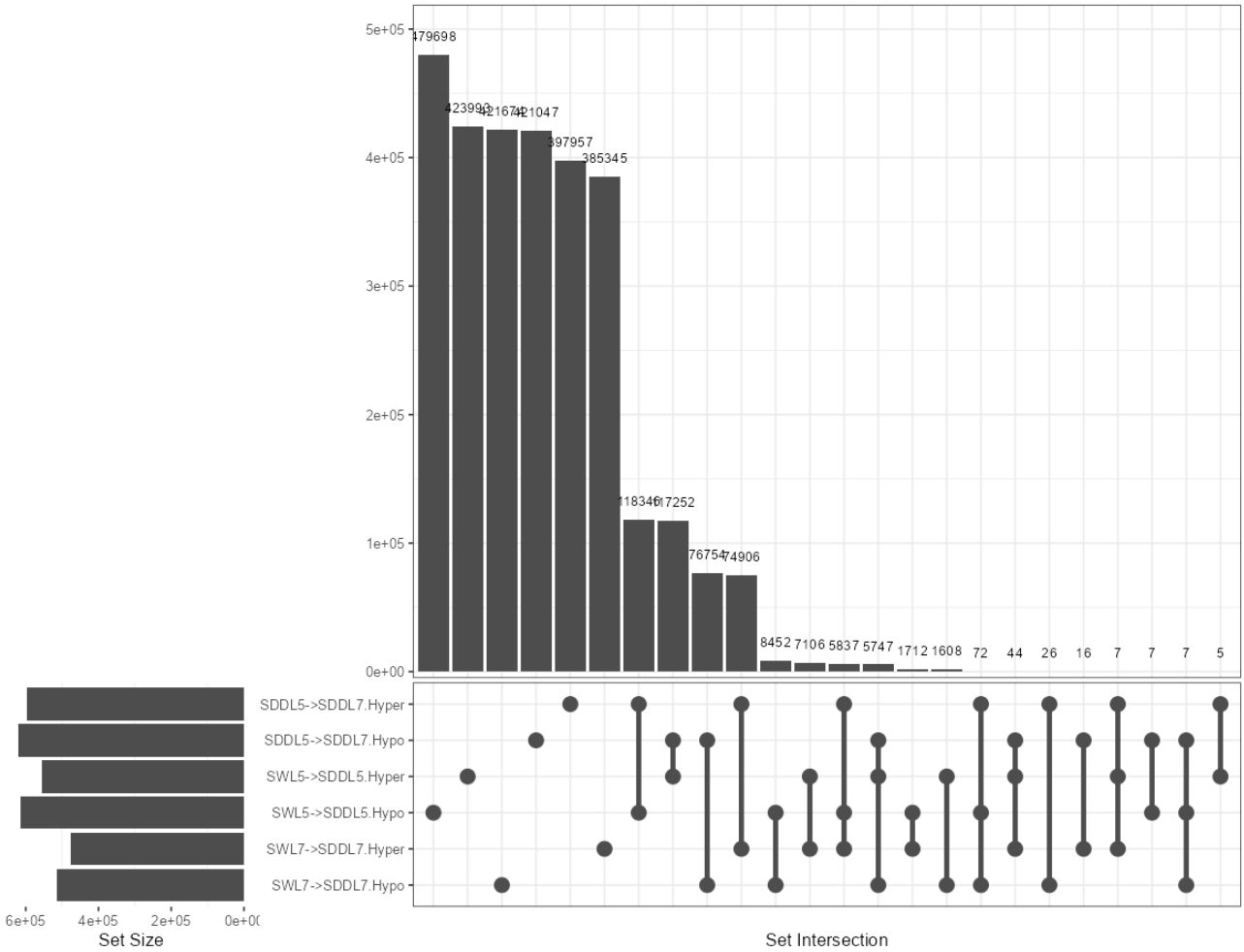

Figure 3.7. Upset plot showing numbers of differentially methylated cytosines (DMCs) for double-droughted S10 plants at TP5 (one week into second drought) and TP7 (four weeks of recovery after second drought). SDDL5 = double-droughted TP5, SDDL7 = double-droughted TP7, SWL5 = watered TP5 and SWL7 = watered TP7. Differential methylation was determined based on the second set in the comparison relative to the first, hence SDDL5->SDDL7 hyper = identification of loci hyper-methylated in double-droughted plants at TP7 relative to double-droughted plants at TP5.

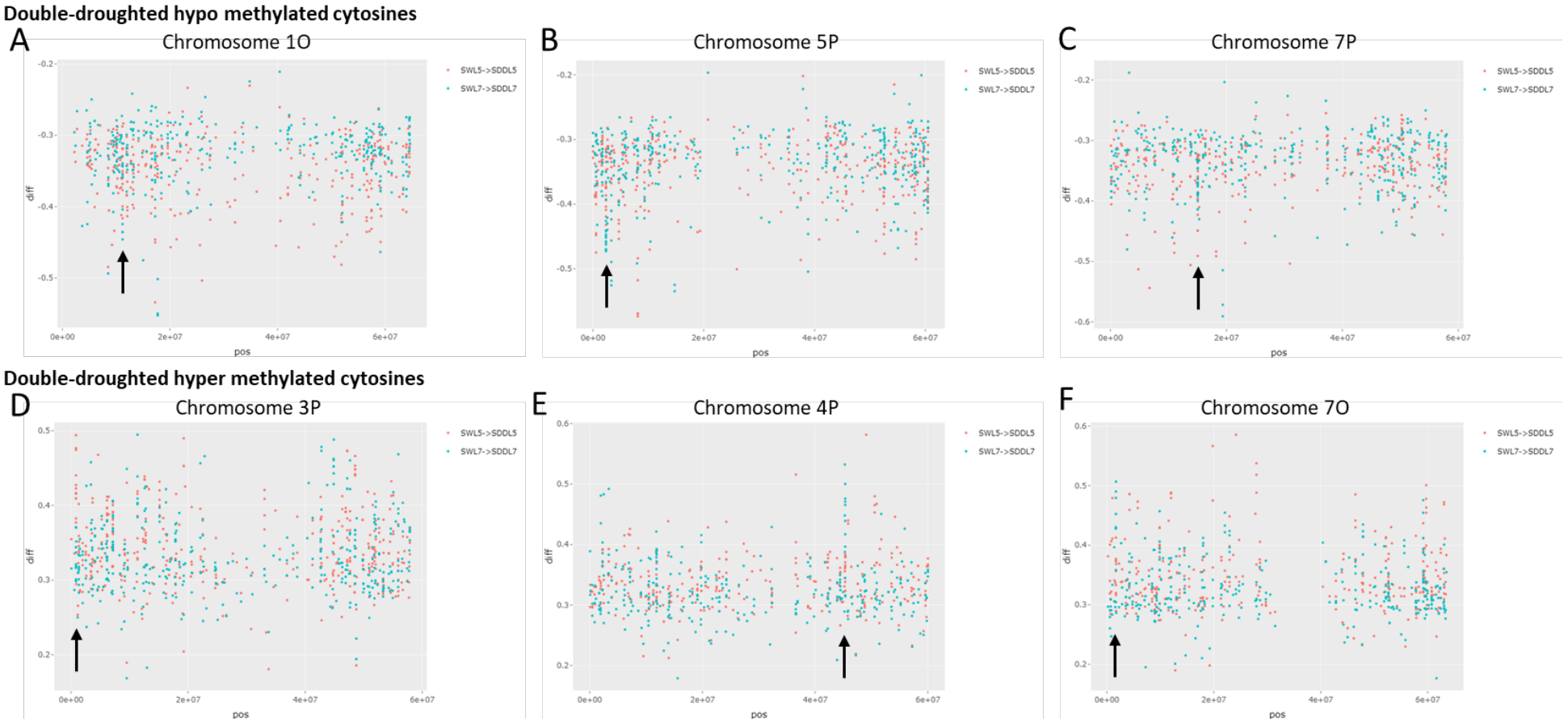

Figure 3.8. Scatter plots showing hypo- and hyper-methylated cytosines and their chromosomal locations (as mapped against the *Trifolium repens* genome) when double-droughted plants were compared back to watered controls at TP5 (red dots) and TP7 (blue dots). Each dot represents one differentially methylated cytosine, and the plots show levels of differential methylation along the positions of the chromosome. The arrows indicate genomic regions of interest. SWL5 = watered plants at TP5; SWL7 = watered plants at TP7; SDDL5 = double-droughted plants at TP5; SDDL7 = double-droughted plants at TP7. A) Hypo-methylated cytosines along chromosome 1O; B) Hypo-methylated cytosines along chromosome 5P; C) Hypo-methylated cytosines along chromosome 7P; D) Hyper-methylated cytosines along chromosome 3P; E) Hyper-methylated cytosines along chromosome 4P; F) Hyper-methylated cytosines along chromosome 7O.

3.4 Discussion

3.4.1 DNA methylation patterns differed between plants experiencing their first drought and those experiencing their second drought

When investigating differential DNA methylation patterns one week into the second drought period (TP5), there were found to be over 300,000 DMCs uniquely methylated for every individual comparison between treatment conditions (Figure 3.2). The comparisons that had the highest number of differentially methylated cytosines were when looking at single-droughted plants that were experiencing their first drought stress event vs double droughted plants that were experiencing their second drought event. This supports the hypothesis that there are differences in DNA methylation depending on the number of drought stresses a plant has experienced.

The differentially methylated loci between the plants experiencing their first and second drought event may be related to gene expression of genes that were underlying differences in phenotype that were observed between the two drought treatment conditions. At TP5, there were significant differences in RWC between the single-droughted and double-droughted plants (Figure 2.8), therefore it is possible that these DMCs may represent loci that were causing changes in gene expression resulting in these phenotypic differences.

3.4.2 DNA methylation patterns differed depending on whether plants were recovering from their first or second drought

At TP7 (after four weeks of drought recovery), there were again large amounts of differential methylation between single- and double-droughted plants, and when compared to the watered controls (Figure 3.3) further supporting the hypothesis that the number of drought stresses a plant experiences relates to differences in DNA methylation. This indicates that a drought event causes changes in DNA methylation even after the drought event is over and the plants have had time to recover. This implies that some drought-associated methylation marks

remain in place after the stress is no longer present, and that new methylation marks specific to previously droughted plants may also be put in place after the stress is no longer present. Investigation across time points is necessary to see if both are occurring.

3.4.3 WC's sub-genomes exhibited similar levels of differential methylation in response to drought stress

As described in 3.3.3, there were minimal levels (1.0%) of difference in overall number of DMCs between white clovers two sub-genomes (*T. occidentale* and *T. pallescens*) when comparing droughted plants against watered plants one week into the second drought period (TP5) and after four weeks of recovery (TP7). This indicated that there was an even spread of differentially methylated cytosines between the two sub-genomes in response to drought.

These findings correlate with recent research into abiotic stress responses in white clover (Fechete et al., 2024). Fechete et al. (2024) found that when investigating frost response in white clover S10, patterns of gene expression (numbers of upregulated genes) were very similar for the two WC sub-genomes. This suggested even sub-genome utilisation during frost stress events, supporting previous evidence of balanced activity between WCs two sub-genomes (Griffiths et al., 2019). Data from this thesis seems to support those previous findings, as there was found to be even differential methylation between WCs two sub-genomes during and after drought stress.

Although there were even patterns of gene expression in the two sub-genomes, Fechete et al. (2024) found that there were a small number of genes that showed homoeologue-specific expression in response to frost stress. One of these unevenly expressed genes was a gene involved in the biosynthesis of raffinose in the raffinose family of oligosaccharides (RFO) pathway that was biased towards the *T. pallescens* sub-genome. This pathway is linked to cold tolerance and has a role in protection against cold (Sengupta et al., 2015). This showed that while overall gene expression was even across the two sub-genomes, there were some stress

response specific genes that exhibited bias towards one of the sub-genomes. There may have been a similar pattern occurring in the S10 plants used in this thesis' drought trial. Although there was evidence of even differential DNA methylation between the sub-genomes, a more in-depth investigation may find that there were some drought-specific genes that exhibited differential methylation between the two sub-genomes.

Conducting transcriptome analysis on the S10 samples used in this thesis would allow more in-depth investigation into sub-genome expression in response to drought. Comparing transcriptome data and DNA methylation data would elucidate whether differential DNA methylation contributed towards biased sub-genome gene expression.

3.4.4 There were differences in DNA methylation across different drought trial time points as well as small proportions of DMCs that were retained post drought

When investigating DMC loci during the first drought exposure and comparing them to those after four weeks of recovery, there were ~800,000 loci that were differentially methylated between the two time points (Figure 3.4). The comparison with the highest number of DMCs was between single-droughted plants at TP5 and the watered controls. This indicated that there were lower overall levels of DNA methylation at TP5, and that a response to this first drought exposure may have been overall hypo-methylation. A study investigating DNA methylation and drought stress in the wild diploid forage legume *Medicago ruthenica* found that drought stress resulted in decreased methylation levels across the whole genome (Zi et al 2024). Decreased methylation levels associated with genes was also found to be related to increased gene expression levels in genes involved in metabolic pathways, flavonoid biosynthesis, proline metabolism and signal transduction which are all important in the stress response (Zi et al 2024). This may mean that the hypo-methylation observed in this thesis was associated with increased levels of expression of stress-related genes.

There were ~15,000 DMCs that were significantly differentially methylated when compared to watered controls during their first drought exposure, that remained differentially methylated to watered controls after four weeks of recovery (Figure 3.4). Similarly, when comparing plants that were experiencing their second drought against those that had recovered from their second drought stress, there were ~16,000 loci that were significantly differentially methylated than the watered controls in the same direction at both time points (Figure 3.7). These DMCs represent loci that may have had a role in the drought response of these plants and remained in place post drought. These may be related to important genes or regulatory regions that had a role in drought tolerance and in drought stress memory. It would be interesting to know the roles of genes that these loci were corresponding to, to investigate the roles of genes that were being differentially methylated during drought stress and maintained post stress.

When comparing DMCs during and after the first drought stress (Figure 3.4), and during and after the second drought stress (Figure 3.7), there were also ~10,000 And ~16,000 loci that were differentially methylated from the watered controls in opposite directions (hypo- vs hyper-) at each time point compared to watered controls (and were also significantly differentially methylated between each time point). These loci are more likely to represent methylation marks that were highly responsive to stress conditions and therefore the methylation associated with drought tolerance may not remain long enough to be inherited.

3.4.5 Areas of sustained hypo- and hyper-methylation during and after the first and second drought may have roles in the drought response

The ~15,000 loci and ~16,000 loci that were found to be significantly differentially methylated from the watered controls in the same direction for single-droughted plants at TP5 and TP7 (Figure 3.4) and double-droughted plants at TP5 and TP7 (Figure 3.7) are loci of interest. This is because these loci showed memory of drought stress/maintained methylation

signatures that were initiated during drought stress. These marks may have a role in regulation of gene expression that allows plants to be tolerant to current and future drought stresses.

Figures 3.5 and 3.8 showed areas of the WC genome/chromosomes that had differential methylation compared to watered controls in droughted plants during their first and second drought exposures, that were maintained after the plants were no longer being exposed to drought. The chromosome graphs revealed areas containing high levels of hypo- and hyper-methylation compared to watered controls across both time points. It was possible to determine whether these areas were inside of gene bodies, or alternatively what the closest gene was. These genes associated with DMCs of interest may have roles in the drought response and in plant memory/priming as these areas maintained their differential methylation signatures (compared to watered controls) even after the drought had finished.

For the genomic areas of interest during and after the first drought exposure (TP5 and TP7 of single-droughted plants) (Figure 3.5), it was possible to investigate some genes associated with the regions of hyper- and hypo-methylation as indicated by the arrows on the figure.

There was an area of hypo-methylation ~500 bp upstream of a wall associated receptor kinase gene (Figure 3.5A). This gene family plays critical roles in plant signalling networks and has been shown to be involved in ABA responses, calcium signalling and antioxidant defence. It has been shown that overexpression of receptor kinase genes increased drought tolerance and sensitivity to ABA and decreased ROS accumulation in rice (Feng et al., 2014). Another study found that a receptor-like protein kinase enhanced drought tolerance in rice via detoxification of reactive oxygen species (ROS) (Chen et al., 2013). It therefore makes sense that this gene would be differentially regulated during drought exposure. Hypo-methylation upstream of this gene may have allowed increased gene expression as there would be fewer methyl groups preventing the binding of transcription activators (Phillips, 2008).

There was an area of hypo-methylation in a pectinesterase gene (Figure 3.5C). It has been previously shown that over expression of pectinesterase in *Arabidopsis thaliana* resulted in inhibition of stomatal opening and decrease in water loss rate (Yang et al., 2020). Therefore, promotion of this gene during drought stress would assist in tolerance by reducing water loss. It is possible that hypo-methylation in this gene body impacted on gene expression.

Similarly, there were areas of hyper-methylation in the single-droughted plants maintained during and after drought. One of these areas of hyper-methylation was in the gene body of a soybean trypsin inhibitor (Kunitz) family of protease inhibitors gene (Figure 3.5D). Protease inhibitors have been shown to be induced in response to abiotic stresses such as drought and that this expression confers tolerance. Overexpression of a protease inhibitor resulted in higher RWC and enhanced antioxidant activity conferring reduced oxidative stress in one *Arabidopsis* study (Malefo et al., 2020). Another area where hyper-methylation was observed was in the gene body of a SUMO-activating enzyme subunit gene (Figure 3.5E). SUMO is a major molecular process that mediates plant tolerance to a wide range of stresses through protein modification. It has been previously shown that drought caused increases in abundance of SUMO conjugates (Catala et al., 2007). DNA methylation in gene body sequences has been positively correlated with gene expression in some plant species (Liang et al., 2014) so it is possible that the observed hyper-methylation caused increased protease inhibitor expression and SUMO-activating enzyme expression in response to drought.

For the areas of interest during and after the second drought exposure (TP5 and TP7 of double-droughted plants) (Figure 3.8), it was also possible to investigate some genes associated with regions of hypo-and hyper-methylation.

There was an area of hypo-methylation ~2,000 bp downstream of a gene encoding a class I-like SAM-binding methyltransferase superfamily protein (Figure 3.8A). This gene product transfers methyl groups to proteins and is involved in transcriptional regulation. It is

possible that the hypo-methylation downstream of this gene was impacting on expression, and therefore on addition of methyl groups to genomic regions, causing further differences in gene expression. Hypo-methylation was also observed ~1,700 bp downstream of a gene coding a mitogen-activated protein kinase (F3igure 8B). Protein kinases are positive regulators of the drought stress response and play an important role in stress response cascades involving ABA. Pepper plants with silenced mitogen activated kinase genes were found to be drought susceptible with increased transpiration rates, low leaf temperatures and decreased stomatal closure (Kim et al., 2021). Transgenic plants with overexpression of this gene were tolerant to drought stress with lower stomatal aperture (Kim et al., 2021). Expression of this gene is associated with increased drought tolerance; therefore, it is possible that hypo-methylation downstream of this gene was causing increased expression, and that maintenance of this hypo-methylation after the drought stress was no longer present helped to prime plants for future drought stress.

There were also areas of hyper-methylation that were observed during the second drought exposure, and maintained after the drought stress was no longer present. For example, hyper-methylation was observed ~1,500 bp upstream of a gene that belongs to the protein kinase superfamily (Figure 3.8D). This protein family plays an important role in signal transduction pathways. It is involved in stress sensing and signal transduction, particularly ABA signal transduction (Zhu et al., 2007). Hyper-methylation was also observed ~2,000 bp upstream of a gene that belongs to the glycosyltransferase family (Figure 3.8F). Glycosylation is important for secondary metabolite production, defence, and abiotic stress resistance. Multiple glycosyltransferases have been shown to have distinct roles in abiotic stress responses, including drought (Chen et al., 2024). These proteins function in stabilising and enhancing water solubility, inactivating or detoxifying natural products, promoting regulation of metabolic homeostasis and detoxifying exogenous substances (Yang et al., 2023). Hyper-

methylation was also observed in the gene body of a gene involved in cellular manganese ion homeostasis (Figure 3.8B). Manganese is important in photosynthetic activity as it is essential for the first step of photosynthesis (a water splitting reaction) (Alejandro et al., 2020). A study showed that foliar application of manganese to winter wheat plants (*Triticum aestivum* L.) resulted in decreased negative drought effects by increasing yield, water use efficiency, leaf area, photosynthesis rate and stomatal conductance (Karim et al., 2012). The DNA methylation upstream and in the gene body of both of these genes that have roles in abiotic stress response may have impacted on the expression levels of these genes. Increased expression during drought and maintenance of this after drought stress is no longer present may assist in drought tolerance in future stresses.

In all, these areas of interest reveal potential roles of DNA methylation in drought stress tolerance and suggest some gene types that may be involved in plant stress memory. This supports the hypothesis that areas of the genome with differential cytosine methylation between drought treatment conditions are related to genes involved in the drought stress response.

3.4.6 Future directions and limitations

There are many interesting possible future directions for this research. A few of which are outlined below.

DNA methylation analysis provides interesting results, but to get even more informative results it is important to also investigate gene expression through transcriptome and metabolome analysis. This would provide insights into changes in gene expression during and after the drought stress events and would provide information on whether the changes in DNA methylation that were observed also impacted on gene expression. This would provide greater insight into the role of DNA methylation in drought responses. A challenge of DNA methylation analysis is that it is hard to know which genes a hypo- or hyper-methylated cytosine is impacting expression of. This is because the genome is a 3D structure that folds

into itself, so it is possible that regulatory areas of a gene may be spatially nearby, but further away on the actual chromosome. This highlights the importance of generating transcription data for this research. Additionally, aligning transcriptome data with metabolite date provides a link to known physiological drought responses, such a proline and RFO production.

Another future direction of this research would be to investigate transgenerational inheritance of DNA methylation marks associated with drought. Evidence has been provided of plant stress memory via DNA methylation, therefore it would be interesting to take this a step further and investigate whether changes in DNA methylation associated with drought can be inherited by offspring. If this is the case, and it can be shown that transgenerational inheritance of drought-associated methylation marks impacts on subsequent drought tolerance, then it Could be possible to incorporate DNA methylation into the plant breeding process to generate more drought tolerant and drought resistant varieties.

Evidence was provided of even DNA methylation in WCs sub-genomes, however previous research indicated that there may be some genes that show bias towards a sub-genome (Fechete et al., 2024). Conducting transcriptome analysis on the S10 samples used in this thesis would allow more in-depth investigation into sub-genome expression in response to drought. Comparing transcriptome data and DNA methylation data would elucidate whether differential DNA methylation contributed towards biased sub-genome gene expression.

4 Conclusions

In this thesis, the aims were to investigate phenotypic responses of white clover and related species to drought stress. A drought trial was used to help gain understanding of how different clover species (such as white clover, *T. pallescens* and *T. occidentale*), a cultivar (WC KopuII), an ecotype (WC Tian Shan) and a synthetic hybrid (880) respond to a single drought event and two subsequent drought events compared to watered controls. Inclusion of the double-droughted treatment condition allowed insights into how plants that had been 'primed' by an initial drought exposure tolerated a second drought to be gained (Chapter 2). In addition, DNA methylation analysis of samples from the drought trial allowed investigation into how WCs genome responds to a single drought and two successive droughts compared to watered controls. It provided insights into areas of the genome that were differentially methylated between treatment conditions and whether those areas of the genome were related to stress-response genes (Chapter 3).

4.1 Findings

In Chapter 2, an overview of the phenotypic results revealed RWC, and leaf area measurements decreased during drought exposure, therefore providing good phenotypic data to analyse more closely. The overview also revealed general declining trends in some phenotypic data such as internode length and gas exchange measurements (Figure 2.4D, Figure 2.5C and Figure 2.5D). Phenotypic data revealed that stomatal conductance and photosynthetic rate also showed patterns related to drought stress (Figures 2.5C and D), likely reducing water loss to the environment. Gas exchange and photosynthetic rate are both critical processes for plant function and growth. Therefore, decreases in stomatal conductance and photosynthetic rate had implications on plant growth and this was shown through decreases in leaf area and node emergence rate during drought stress. Leaf area decreased during the drought periods (Figure 2.7), therefore impacting on yield and potential utilisation.

RWC (relative water content) was a key phenotype of interest in this thesis as it showed evidence of priming for increased drought tolerance. WC S10, WC KopuII and WC Tian Shan all showed significant differences in RWC during the second drought period where plants that were experiencing their first drought had significantly lower RWC values than those that had already experienced a previous drought stress event (Figure 2.8). This suggested the possibility of some sort of memory mechanism within WC to drought stress.

In Chapter 3, a possible mechanism of stress memory was investigated – DNA methylation. DNA methylation assays were conducted on WC S10 samples from TP 5 (one week into the second drought period) and TP7 (after four weeks of recovery post second drought period) of the drought trial. The DNA methylation results revealed that there were differences in the methylome between plants that were experiencing their first and second drought (Figure 3.2) and that there were also differences in the methylome between plants that were recovered from their first drought and their second drought (Figure 3.3). This may help to explain the phenotypic differences in RWC seen at TP5, as differences in DNA methylation likely resulted in differences in gene expression, therefore meaning that plants with different methylomes had different responses to drought stress.

It was also found that the sub-genomes of WC S10 showed similar levels of overall DNA methylation, fitting with previous data that WC evenly utilises both of its sub-genomes.

There was shown to be retention of some DNA methylation marks from drought to recovery. This retention of differential DNA methylation (compared to watered controls) may be involved (Figure 3.4 and Figure 3.7) in the plant stress memory/priming effect. This is because these methylation marks which were associated with drought stress conditions remained in place, even after the stress was no longer present. Further investigation into the spread of these DMCs across WCs genome found some areas that had high representation of hypo- or hyper-methylation were related to genes involved in drought stress responses (Figure

3.5 and Figure 3.8). This supported the idea that these areas of DMC retention play a role in drought stress response and potentially in memory of drought stress.

4.3 Limitations

Although this thesis had many interesting findings, there were also some limitations. One such limitation was the structure and length of the drought trial. A few phenotypes (such as internode length and gas exchange measurements) showed gradual decreases over the course of the drought trial even in the watered controls (Figure 2.5 and Figure 2.5). This indicated that something about the drought trial was not conducive to optimal plant health. This could have been due to factors such as the length of time they remained in the controlled growth room, the watering regime that was used or the pot size they were in, or the light conditions to which they were exposed. If this drought trial was to be redone, the structure would need to be reevaluated to ensure that the only stress the plants face is the one of interest (drought).

A limitation of DNA methylation analysis is that although it provides information on areas of the genome and specific cytosines that have differential methylation, it cannot be confirmed from DNA methylation results alone which genes are being impacted and how expression is being affected. This is partly because the genome is a 3D structure and pieces of the genome which are not close together on the DNA strand may be close together spatially and therefore impacting on expression of each other. This means that it is important for transcription data/gene expression data to be generated to fully understand the role and implications of differential DNA methylation.

4.4 Future directions

As previously mentioned, generating transcription/gene expression data is an important next step in this research. Generating transcription data for the same samples DNA methylation data was generated for would provide more information on how the differential DNA methylation that was observed impacted on gene expression. These data would also suggest

metabolic pathways to investigate through metabolomic analysis as this would provide a link between methylome to transcriptome to phenotype. Transcription data would also be able to provide more information on sub-genome utilisation within WC. It was found in this thesis that there was even sub-genome differential methylation in response to drought so it would be interesting to determine if this is the same for gene expression. It would also potentially provide more information on any genes/genomic areas that do have expression biased towards one progenitor sub-genome.

Another key next step in this research is to investigate transgenerational inheritance of DNA methylation marks associated with drought. It was shown that some DNA methylation marks were retained in the WC genome post drought stress, so it would be interesting to know if these are also retained in subsequent generations and whether this impacts on drought tolerance/resistance in the resulting generations. If transgenerational inheritance was found to benefit future generations, then this could provide a valuable new tool for plant breeding.

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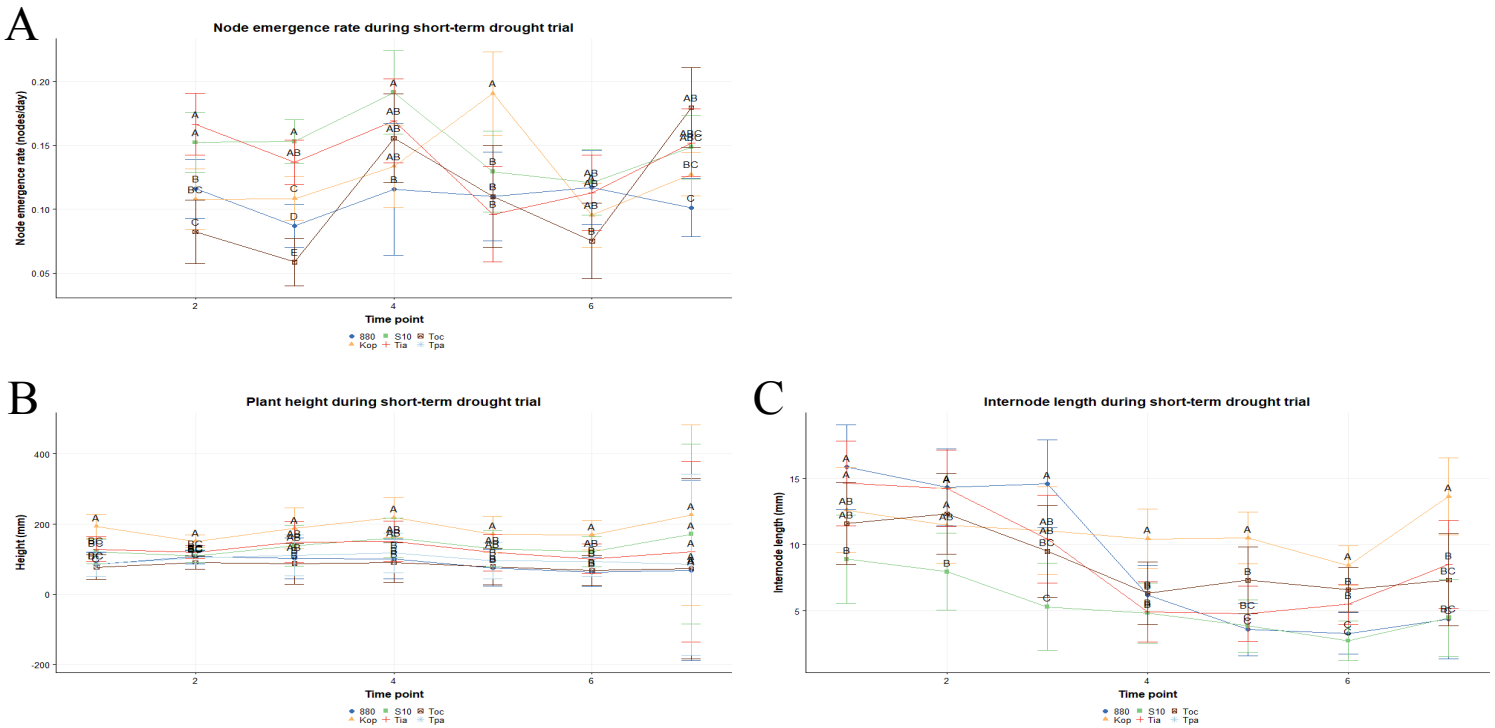
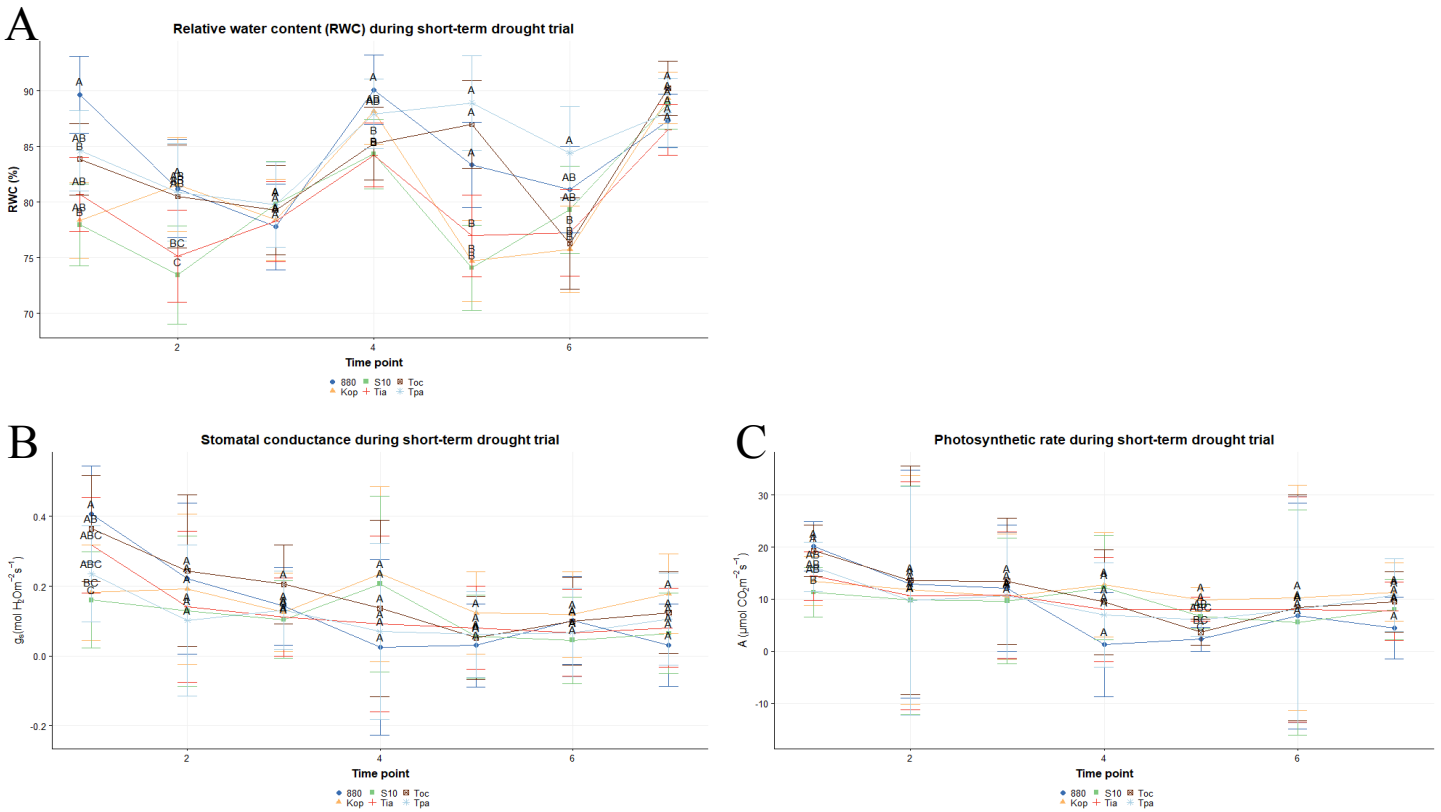
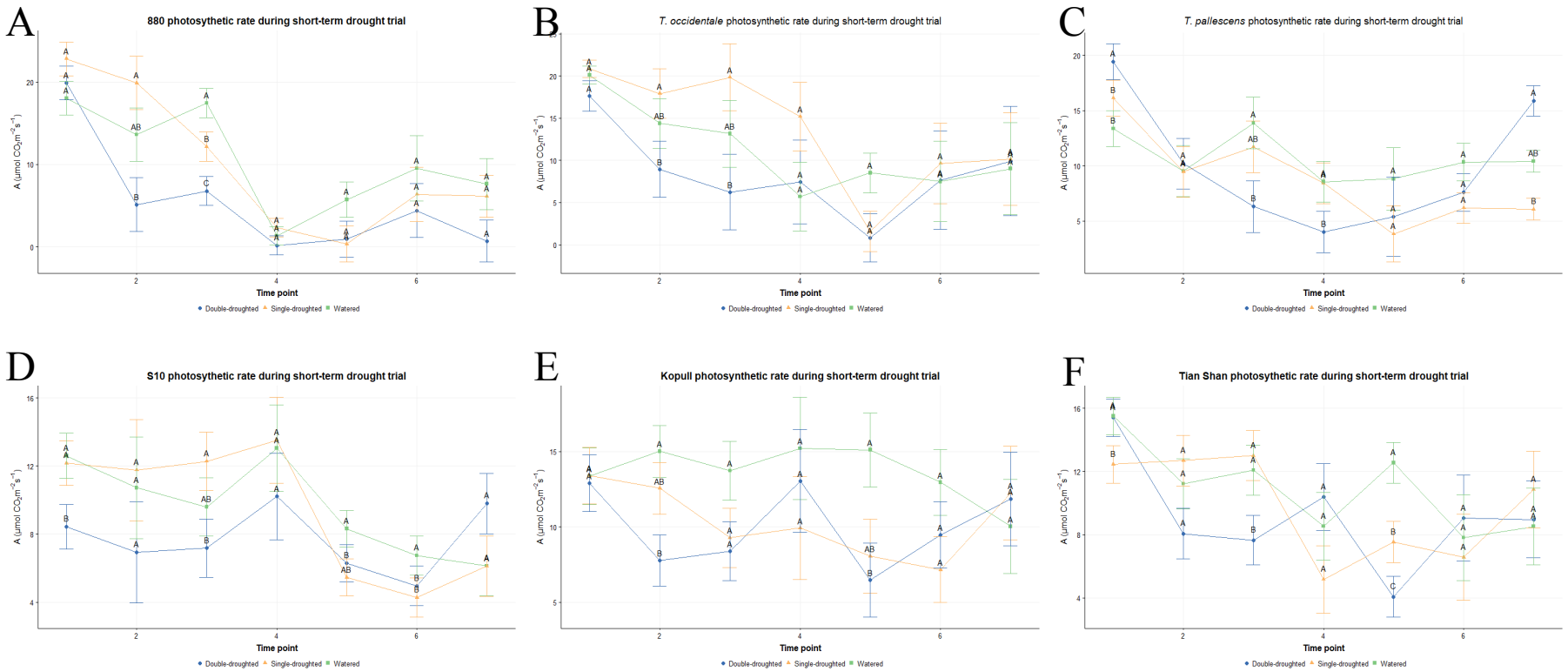
6 Supplementary materials



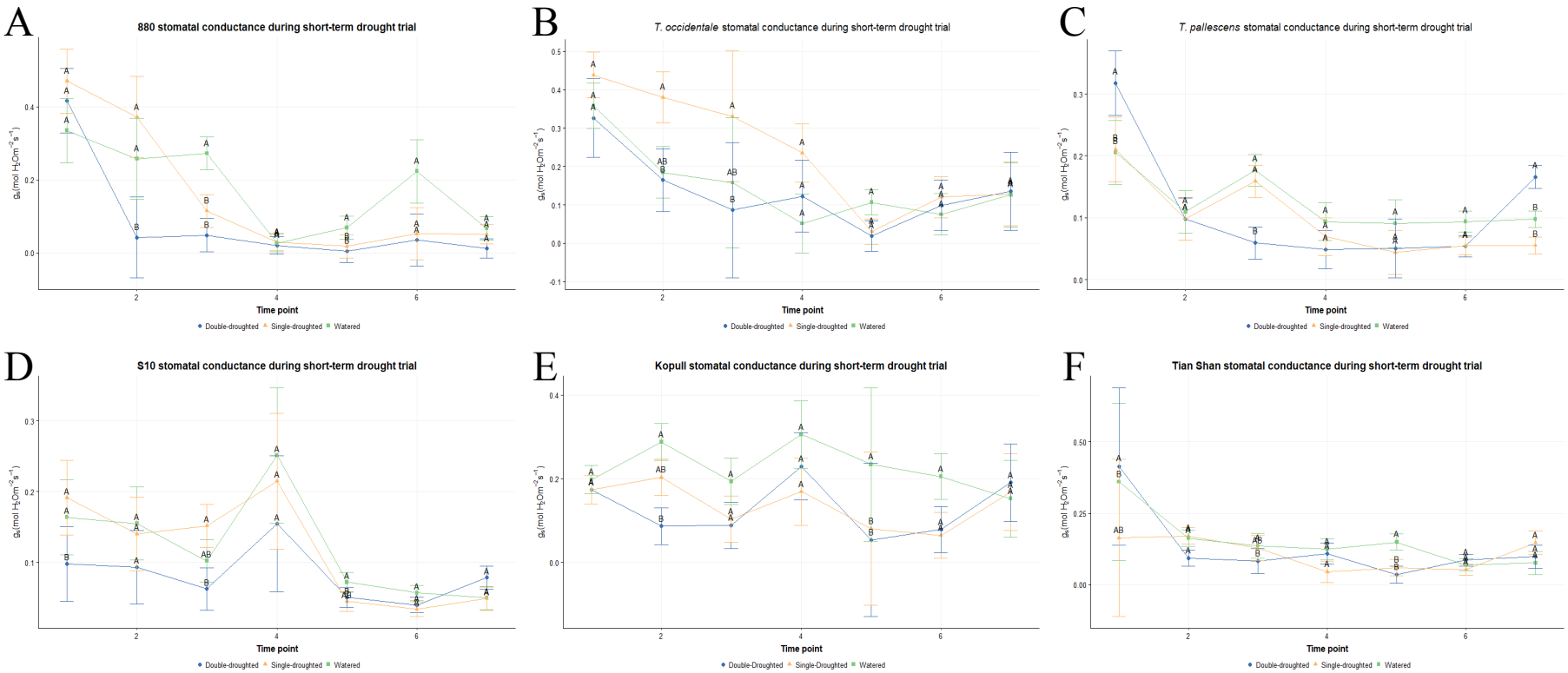
Supplementary Figure A. Plots showing slower responding drought related phenotypes across the time points. The data is split by genotype. A) plot showing node emergence rate over time; B) plot showing plant height over time; C) plot showing node length over time. Blue circles represent 880 (synthetic hybrid); green squares represent *T. repens* S10; Brown boxes represent *T. occidentale*; yellow triangles represent *T. repens* cv 'Grasslands KopuII'; Red crosses represent *T. repens* ecotype 'Tian Shan'; Light blue stars represent *T. pallescens*. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significance determined by LSD values at each individual time point.



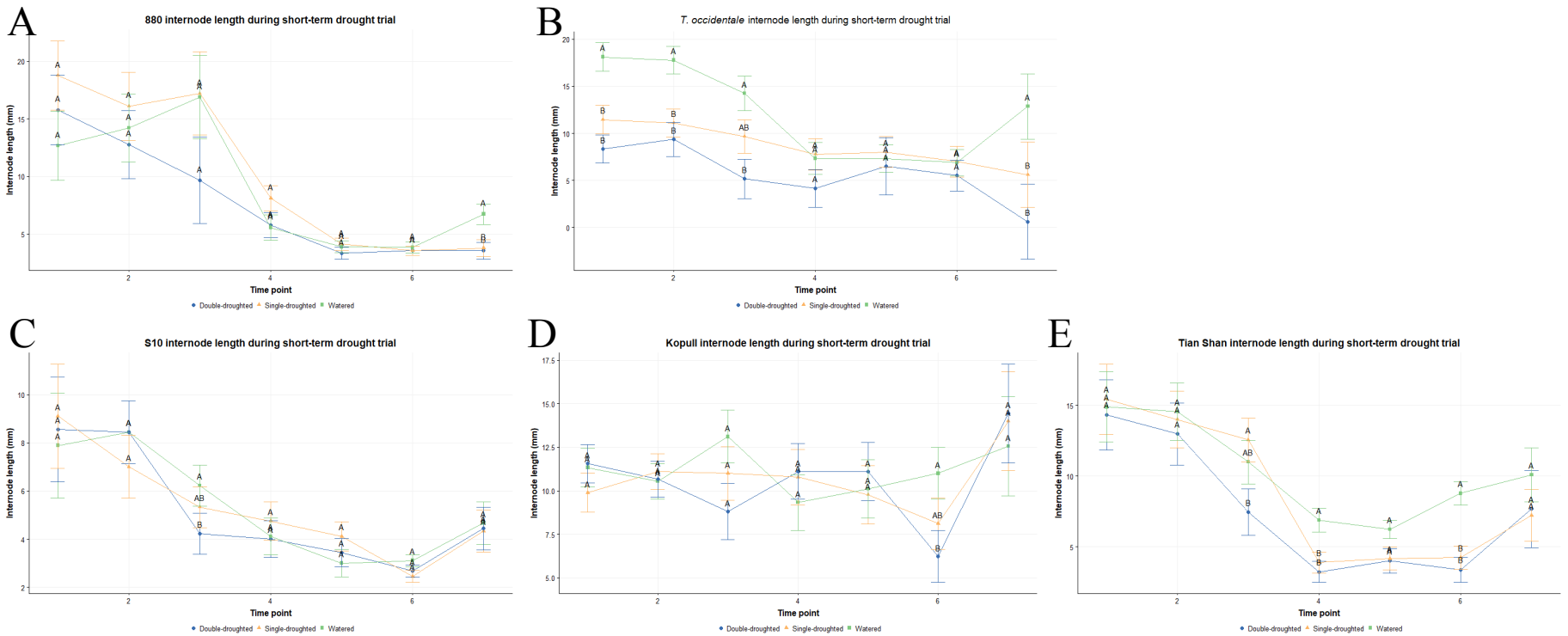
Supplementary Figure B. Plots showing faster responding drought related phenotypes across the time points. The data is split by genotype. A) plot showing relative water content over time; B) plot showing stomatal conductance (g_s) over time; C) plot showing photosynthetic rate (A) over time. Blue circles represent 880 (synthetic hybrid); green squares represent *T. repens* S10; Brown boxes represent *T. occidentale*; yellow triangles represent *T. repens* cv 'Grasslands KopuII'; Red crosses represent *T. repens* ecotype 'Tian Shan'; Light blue stars represent *T. pallescens*. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significance determined by LSD values at each individual time point.



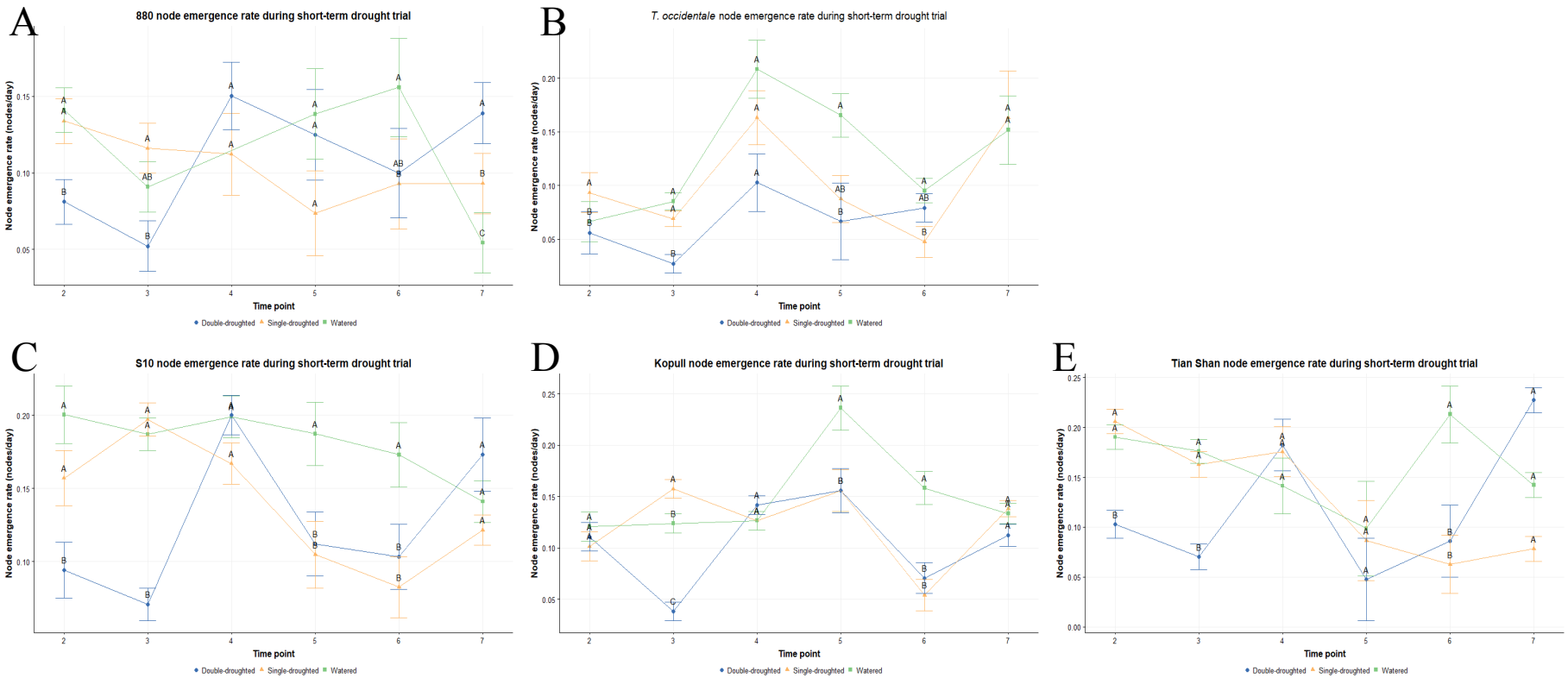
Supplementary Figure C. Plots showing photosynthetic rate (A) across the drought trial time points. Each graph has the data split by treatment condition. A) photosynthetic rate in 880 synthetic hybrid; B) photosynthetic rate in *T. occidentale*; C) photosynthetic rate in *T. pallescens*; D) photosynthetic rate in *T. repens* S10; E) photosynthetic rate in *T. repens* cv 'Grasslands KopuII'; F) photosynthetic rate in *T. repens* ecotype 'Tian Shan'. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.



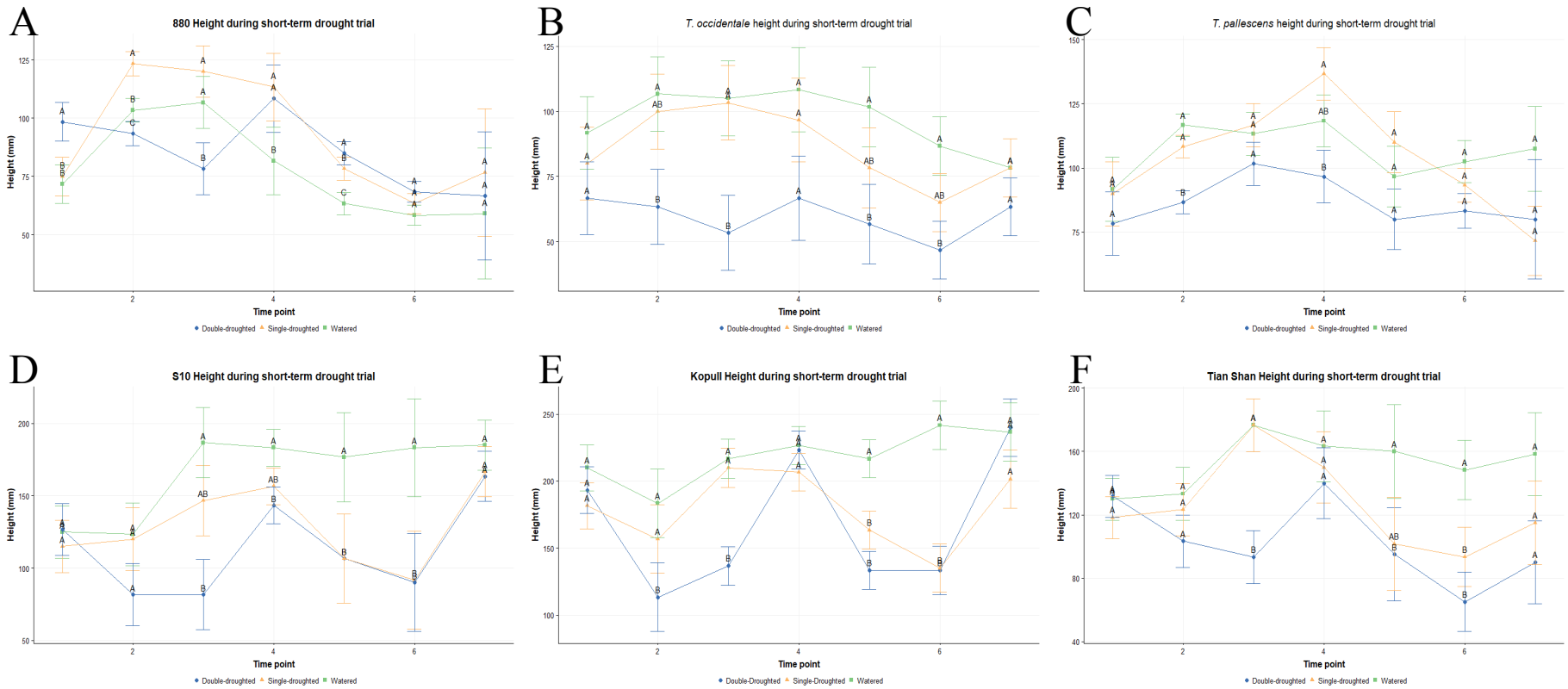
Supplementary Figure D. Plots showing stomatal conductance (g_s) across the drought trial time points. Each graph has the data split by treatment condition. A) photosynthetic rate in 880 synthetic hybrid; B) photosynthetic rate in *T. occidentale*; C) photosynthetic rate in *T. pallescens*; D) photosynthetic rate in *T. repens* S10; E) photosynthetic rate in *T. repens* cv 'Grasslands KopuII'; F) photosynthetic rate in *T. repens* ecotype 'Tian Shan'. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.



Supplementary Figure E. Plots showing internode length across the drought trial time points. Each graph has the data split by treatment condition A) internode length in 880 synthetic hybrid; B) internode length in *T. occidentale*; C) internode length in *T. repens* S10; D) internode length in *T. repens* cv 'Grasslands KopuII'; E) internode length in *T. repens* ecotype 'Tian Shan'. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.



Supplementary Figure F. Plots showing node emergence rate across the drought trial time points. Each graph has the data split by treatment condition. A) node emergence rate in 880 synthetic hybrid; B) node emergence rate in *T. occidentale*; C) node emergence rate in *T. repens* S10; D) node emergence rate in *T. repens* cv 'Grasslands KopuII'; E) node emergence rate in *T. repens* ecotype 'Tian Shan'. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.



Supplementary Figure G. Plots showing plant height across the drought trial time points. Each graph has the data split by treatment condition. A) plant height in 880 synthetic hybrid; B) plant height in *T. occidentale*; C) plant height in *T. pallescens*; D) plant height in *T. repens* S10; E) plant height in *T. repens* cv 'Grasslands KopuII'; F) plant height in *T. repens* ecotype 'Tian Shan'. Blue circles represent double-droughted plants, yellow triangles represent single-droughted plants and green squares represent watered controls. Data points represent Best Linear Unbiased Estimated means and error bars are 95% confidence intervals (p=0.05) from Linear Mixed Model. Letters above data points represent statistical significances determined by LSD values at each individual time point.