The mechanisms of drought stress tolerance in the crop Sorghum bicolor

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Abstract

Drought stress can have a major impact upon plant survival and crop productivity. *Sorghum bicolor* is an important cereal crop grown in the arid and semi-arid regions of >98 different countries. It is well adapted to the harsh drought-prone environments in which it is grown however; relatively few studies have investigated the molecular basis of these adaptations. Breeding programs have lead to the identification of 'stay-green' varieties, so-called due to their ability to maintain green photosynthetic leaf area for longer under drought conditions. However, despite extensive breeding efforts to select for this trait we have very little understanding of the fundamental biological processes that underlie it.

Microarray analysis was used to identify gene expression changes in sorghum following heat stress, drought stress and combined heat and drought stress. These microarrays were additionally used to compare gene expression in stay-green (drought-tolerant) and senescent (drought-sensitive) sorghum lines. Ontological analysis of the genes expressed to higher levels in the stay-green lines identified key processes hypothesised to be associated with the trait. These include genes associated with proline and betaine biosynthesis, glutathione S-transferase (GST) activity and the regulation of stomatal aperture and density. Both proline levels and GST activity were found to be higher in the stay-green lines thus validating that the changes at the gene expression level result in changes at the protein level. Stay-green lines were also shown to have reduced transpiration and reduced numbers of stomata.

Two signalling genes, *DREB1A* and *SDIR1* were expressed to higher levels in the stay-green varieties. Transgenic lines overexpressing these genes were generated in order to test their function. Based on the gene expression data, putative mechanisms underlying two QTL for the stay-green trait (Stg1 and StgB) were generated. Further validation of these genes and processes could not only improve our understanding of drought tolerance mechanisms in sorghum, but also facilitate the improvement of future sorghum cultivars.

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Statement of Authorship

I certify that all of the work described in this thesis is my own original research unless otherwise acknowledged in the text or by references, and has not been previously submitted for a degree in this or any other university.

Statement of Copyright

List of abbreviations

The standard scientific conventions for protein and gene naming have been followed: wild type genes and proteins are in capitals and mutants are denoted by lower case, gene names are italicized whereas protein names are not.

Standard scientific abbreviations have been used for units of weight, length, amount, molarity, temperature and time.

Standard chemical element symbols, nucleic acid and amino acid codes are used.

ABA - Abscisic acid
ABF - Abscisic acid binding factor
ABI - Abscisic acid insensitive
ABRE - Abscisic acid-responsive elements
ACC - 1-aminocyclopropane-1-carboxylate
AHK - Arabidopsis histidine kinase
AP2 - Apetala 2
APX - Ascorbate peroxidase
ARF - Auxin response factor
ARR2 - Arabidopsis response regulator 2
BADH - Betaine aldehyde dehydrogenase
BES1 - Brassinosteroid insensitive1-ethyl methanesulfonate-suppressor1
bHLH - Basic heleix-loop-helix
bZIP - Basic-domain leucine zipper
CAT - Catalase
CBF - Cold regulated
CDNB -1-chloro-2,4-dinitrobenzene
CPK - Calcium-dependent protein kinase
CSGR - Comparative Saccharinae Genome Resource
CE - Coupling element
CIPK - CBL-interacting serine/threonine-protein kinase

CIM - Callus induction media

CMO - Choline monooxygenase

DAS - Days after sowing

DRE - Dehydration responsive element

DREB - Dehydration responsive element binding

ER - Endoplasmic reticulum

ERD - Early response to dehydration

ERF - Ethylene response factor

ETR - Ethylene resistant

FC - Fold change

FMOC - Fluorenylmethyloxycarbonyl

GLAM - Green leaf area at maturity

GO - Gene ontology

GTL1 - GT-like 1

GST - Glutathione S-transferase

HKT1 - High affinity K+ transporter 1

HPLC - High performance liquid chromatography

HSP - Heat shock protein

ICE - Inducer of CBF expression

IPT - Isopentenyl transferase

LEA - Late embryogenesis abundant

KAT1 - Potassium channel 1

LB - Luria-Bertani

MAP - Mitogen-activated protein

MAPK - Mitogen-activated protein kinase

MAS - Marker-assisted selection

MS - Murashige and Skoog

MV - Methyl viologen

OM - Osmotic medium

OPA - O-Phthaldialdehyde

OST1 - Open stomata 1

PAO - Pheide *a* oxygenase

PCA - Principle component analysis

PCR - Polymerase chain reaction

PDA - Phosphodiode array

PEA - Photosystem efficiency analyser

PEG - Polyethylene glycol

PIG - Particle inflow gun

PIP - Plasma membrane intrinsic protein

PRE - Proline responsive element

P5CS - Delta 1-pyrroline-5-carboxylate

PP2C - Protein phosphatase 2C

PYL - PYR1-like

PYR - Pyrobactin resistance1

QTL - Quantitative trait loci

RCAR - Regulatory components of aba receptors

RD - Response to dehydration

ROI - Reactive oxygen intermediates

ROS - Reactive oxygen species

RWC - Relative water content

SAG - Senescence-associated gene

SAMDC - S-adenosylmethionine decarboxylase

SDD1 - Stomatal density and distribution 1

SDIR1 - Salt-and-drought-induced RING finger

SE - Standard error

SEA - Singular enrichment analysis

SID - Senescence-induced degradation

SLAC1 - slow anion channel-associated 1

SnRK - SNF-related kinase

SNP - Single nucleotide polymorphism

SOD - Superoxide dismutase

SPDS - Spermidine synthase

Stg - Stay-green

T-DNA - Transfer DNA

TPP - Trehalose-6-phosphate phosphatase

TPS - Trehalose-6-phosphate synthase

UPLC - Ultra performance liquid chromatography

USP - Universal stress protein

UV - Ultra violet

WOX1 - Wuschel homeobox 1

WT - Wild type

ZFHD - Zinc finger homeodomain

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CHAPTER 1

Introduction

Environmental stress can have a major impact on plant survival and crop productivity. Due to their sessile nature plants are unable to avoid adverse conditions. Instead, changes to plant physiology and changes at the cellular, molecular and biochemical level are imperative to their survival. Some plants are better adapted than others to harsh conditions and, for agriculturally important crops; screening for certain beneficial physiological traits is common practice in breeding strategies. Such strategies have been successful for the generation of well-adapted varieties however; often the selection process is slow and the precise biological processes underlying the improved tolerance are not known. The identification of the causal genes and processes not only facilitates selection for the trait but also serves as a rich source of genes for introduction into other species.

This study aims to investigate the molecular mechanisms of drought stress tolerance in the crop *Sorghum bicolor* and in particular the mechanisms underlying the stay-green trait for drought tolerance. This chapter reviews topics relevant to the present study. Firstly, the problems associated with drought stress are described along with some general information about the crop sorghum. Then, general drought resistance mechanisms that are most pertinent to the work are defined. Finally, the stay-green trait is described, with a particular focus on the mechanisms shown to cause a stay-green phenotype. The chapter concludes with a summary of our current knowledge of the stay-green trait in sorghum.

1.1 Drought stress

Drought stress is the biggest single cause of yield reduction in crop species (Boyer, 1982). When in plentiful supply, water enters the roots and moves through the plant in the transpiration stream before evaporating from leaf stomata. This intake of water is vital for plant metabolism, nutrient transport and the maintenance of cell turgor. When water loss from the plant exceeds the ability of the roots to take up more water, however, these processes are inhibited, resulting in drought stress. Around 45% of the world's agricultural lands are subjected to continuous or frequent drought and this can reduce crop yields by up to 70% (Ashraf and Foolad, 2007). Drought stress is aggravated by temperature extremes and its affects are likely to worsen given the predicted increase in aridity in many areas as a result of global warming. At the same time, global demand for food is expected to increase by up to 70% by 2050 (UNWWDR4, 2012). It is therefore of vital importance that we investigate the processes by which plants can survive in arid environments in order to gain insights into how we can limit the problems of drought stress and improve crop yields in the future.

1.2 Sorghum as a study species

Sorghum (*Sorghum bicolor* [L.]) Moench) is an important C4 grain crop that is grown on arid and semi-arid soils of over 98 different countries. Worldwide annual production of sorghum grain is about 60 million tonnes, of which ~90% is from developing countries that are mostly in Africa and Asia (Sasaki and Antonio, 2009). Consequently, sorghum is the 5th most important cereal crop grown worldwide based on yields and is an important source of food, feed, fibre and fuel (Kumar et al., 2011; Kholova et al., 2013). Sorghum is well adapted to the harsh drought-prone environments in which it resides and is considered to be one of the most drought-tolerant crops grown in the US with more than 80% of hybrids grown under non-irrigated conditions (Sanchez et al., 2002). Nevertheless drought stress remains a major cause of sorghum yield reduction, particularly at the post-flowering stage, and considerable effort is underway to identify and select for new stress-tolerant varieties (Kassahun et al., 2010). Whilst numerous studies have investigated the physiological mechanisms underlying drought tolerance in sorghum, relatively little is known about the adaptations at the biochemical and

molecular level. A better understanding of how sorghum can survive under these conditions would considerably improve our understanding of plant drought tolerance mechanisms and could help to improve sorghum yields in the future. Such investigations will be facilitated by the fact that sorghum has a small recently sequenced genome (~730 Mbp) (Paterson et al., 2009) and a diverse germplasm collection

1.3 Drought resistance mechanisms

There are a number of known ways that plants can resist drought stress including drought escape, drought avoidance and drought tolerance strategies. As part of a drought escape mechanism plants may complete their lifecycle before physiological water deficits occur with successful reproduction before the onset of stress. Early maturing sorghum genotypes and the adjustment of planting dates to avoid periods of low rainfall are strategies commonly used by farmers however; this often results in reduced yields, which counteract the effectiveness of this approach (Rosenow et al., 1983).

A vast number of studies have investigated drought avoidance and drought tolerance mechanisms. The majority of these studies have been carried out using the model plant *Arabidopsis thaliana* due to its small and simple genome, which facilitates molecular and genetic investigations. Many investigations have focussed on key crops such as rice, maize and wheat although; comparatively few studies have been carried out on sorghum, despite its obvious agricultural importance. Some of the most well characterized drought avoidance and drought mechanisms that are most pertinent to the work in this thesis are discussed below.

1.3.1 Drought avoidance

Drought avoidance mechanisms involve maintaining cell turgor either through increasing water uptake via the roots or reducing water loss from transpiration and other non-stomatal pathways.

1.3.1.1 Regulation of stomatal numbers

Stomata are pores on the surface of the leaf that control the uptake of CO_2 for photosynthesis and the efflux of water through transpiration. Stomatal numbers are influenced by both endogenous and environmental factors and are regulated by precise signalling mechanisms (Casson and Hetherington, 2010). The aberrant expression of genes in these pathways results in a disruption to stomatal patterning. For example, transgenic Arabidopsis lines overexpressing *stomatal density and distribution 1* (*SDD1*) have a 25% reduction in stomatal density (Berger and Altmann, 2000). Similarly, Arabidopsis loss-of-function mutations in *GT-2 LIKE 1* (*GTL1*) result in reduced stomatal numbers, reduced transpiration and increased survival under water deficit conditions (Yoo et al., 2010).

1.3.1.2 Regulation of stomatal aperture

The alteration of stomatal aperture is a well-understood process that has been functionally linked to drought tolerance and water use efficiency. The opening and closing of the stomatal pore results from the shrinking and swelling of the two surrounding guard cells. This is tightly regulated and again requires the integration of endogenous and environmental cues (Kim et al., 2010). In response to drought, stomatal aperture is reduced in order to minimise water loss and this process is triggered by the plant hormone ABA (Kim et al., 2010). ABA stimulates a signalling pathway that leads to the activation of the kinase *open stomata 1* (*OST1*), which is a positive regulator of stomatal closure (Mustilli et al., 2002). This leads to the production of reactive oxygen species (ROS) and cytosolic calcium and the subsequent activation of ion channels such as SLAC1 (Geiger et al., 2009) and inhibition of cation channels such as KAT1 by phosphorylation (Sato et al., 2009). This results in the efflux of potassium and anions from the guard cells and the removal of organic osmolytes. Consequently, there is a reduction in guard cell turgor, which causes stomatal closure (Kim et al., 2010). The engineering of stomatal closure is an attractive approach to improve water loss under water-limited conditions. Numerous examples in the literature exist whereby the mutation or overexpression of a gene involved in the above signalling pathway results in guard cell closure and reduced water loss. As an example, transgenic plants overexpressing

MYB44 resulted in faster ABA-induced stomatal closure and increased drought tolerance. This was thought to be as a result of the down-regulation of negative regulators of ABA signalling (Jung et al., 2008).

1.3.1.3 Water transport

Water transport across membranes is vital for plant growth and development. This can occur via simple diffusion and via aquaporins. Aquaporins are membrane proteins that increase the permeability of cell membranes to water and other small molecules such as CO_2. Over 30 aquaporin isoforms have been identified in plants and these are involved in processes such as stomatal opening and closure, organ movement and the plant response to drought (Chaumont and Tyerman, 2014). The overexpression of PIP1 from *Vicia faba* in transgenic Arabidopsis resulted in enhanced survival following water withdrawal (Cui et al., 2008). In contrast, the overexpression of PIP1b in transgenic tobacco plants increased transpirational water loss and had no beneficial effects under stress (Aharon et al., 2003). The precise function of aquaporins therefore remains elusive and is likely to depend on the particular isoform and the timing and location of expression.

1.3.1.4 Root architecture

The possession and maintenance of a large, deep rooting system is of course essential for the extraction of soil water. The accumulation of ABA in the roots under drought stress restricts the production of the plant hormone ethylene thus preventing ethylene-induced growth inhibition (Chaves et al., 2003). This enables the maintenance of apical root growth under drought conditions. Rooting systems vary depending on the species and the genotype and increased root area is associated with increased survival under low water conditions (Mahajan and Tuteja, 2005).

1.3.2 Drought tolerance

Drought tolerance mechanisms function at the tissue or cellular level to stabilize and protect cellular and metabolic integrity. Following the perception of the stress, a signal is relayed to the nucleus via signalling networks involving second messengers such as reactive oxygen species (ROS), calcium, calcium-associated proteins and kinase cascades such as mitogen-activated protein (MAP) kinase cascades. This leads to the activation of transcriptional pathways, which in turn lead to changes in the flow of metabolites and the induction of genes associated with protection from cellular damage (see Figure 1.1) (Mahajan and Tuteja, 2005). The signalling pathways that lead to the induction of protective genes are complex and involve both ABA-dependent and ABA-independent pathways (see Figure 1.2). These are discussed briefly below.

1.3.2.1 Drought signal perception

The first step in the regulation of the drought stress response is the recognition of the stress. A change in the osmotic potential across a plasma membrane caused by a decrease of turgor pressure may trigger the water stress response. In yeast, the initial perception of water deficit is mediated by a two-component signal transducer (Sln1p, Ypd1p and Ssk1p) (Posas et al., 1996). The mutant phenotype of *sln1* in yeast was complemented by the overexpression of AtHK1 from Arabidopsis and activated a mitogen activated protein kinase (MAPK) (Urao et al., 1999). Loss of function mutations in the *AtHK1* gene resulted in sensitivity to drought stress and poor induction of drought-inducible genes thus together leading to the suggestion that AtHK1 functions as an osmosensor in Arabidopsis (Wohlbach et al., 2008). Following stress perception, a signalling cascade is triggered which leads to the activation of stress-inducible transcription factors.

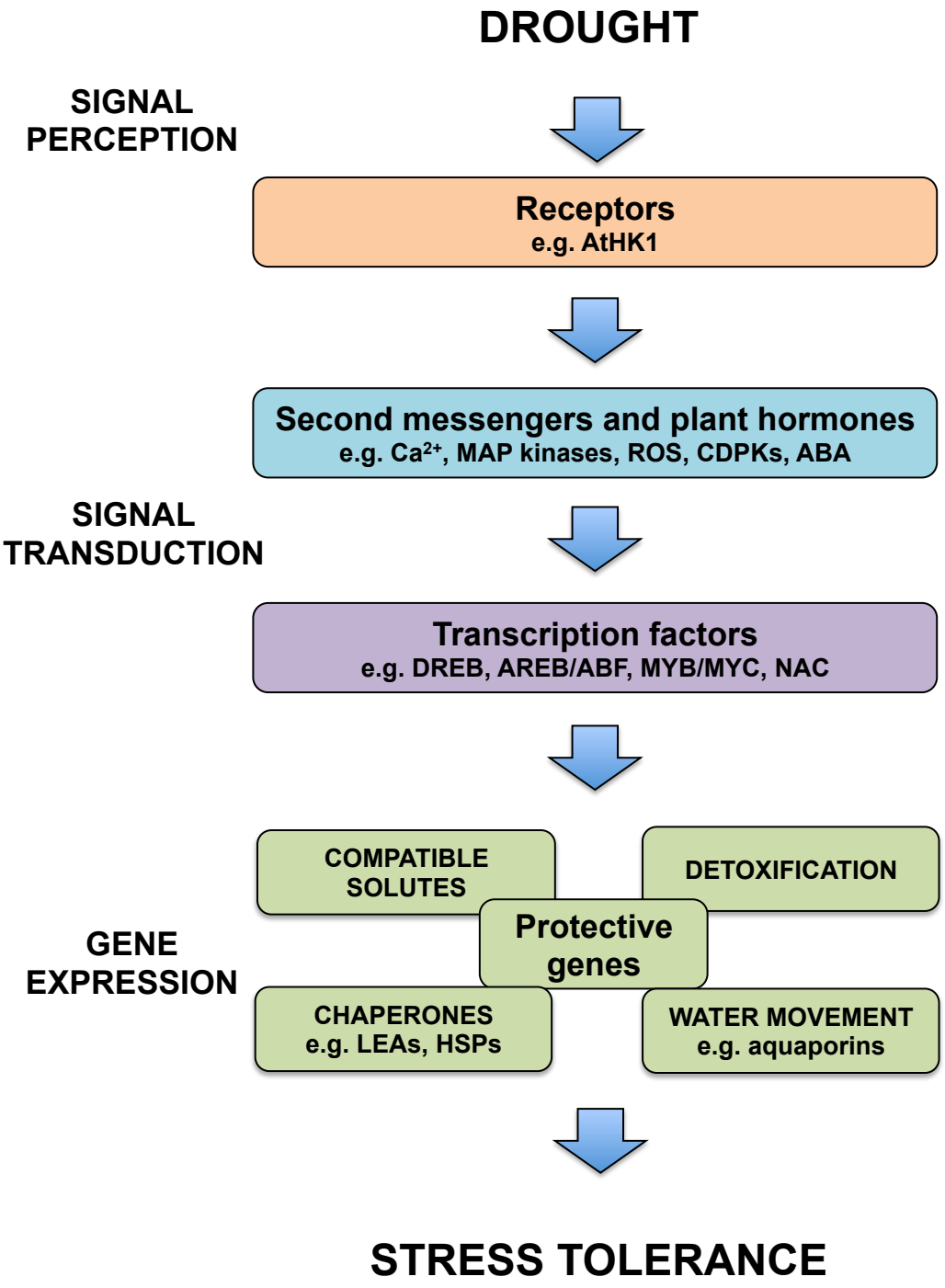


Figure 1.1 - Mechanisms of drought stress tolerance. Following perception of drought stress, the signal is transduced leading to the activation of transcription factors and the induction of genes that provide protection and ultimately stress tolerance.

1.3.2.2 Stress signalling

1.3.2.2.1 ABA signalling

As described earlier ABA is a plant hormone that is rapidly synthesised in response to low water conditions (Qin et al., 2011). ABA stimulates signalling pathways both to induce stomatal closure (see 1.3.1.2) and to induce the expression of stress-inducible protective genes (Yamaguchi-Shinozaki and Shinozaki, 2006). ABA-inducible genes contain multiple *cis*-elements termed ABREs (ACGTGG/TC) or the combination of an ABRE with a coupling element (CE) (Yamaguchi-Shinozaki and Shinozaki, 2006). This ACGT element was first identified in the wheat *Em* gene, which functions mainly in seeds during late embryogenesis (Guiltinan et al., 1990). A group of bZIP transcription factors termed AREBs/ABFs bind to this element. Amongst these are ABF2/AREB1, ABF4/AREB2 and ABF3. These are ABA responsive and require ABA for their activation (Uno et al., 2000). The overexpression of ABF3 or ABF4 in transgenic Arabidopsis resulted in improved drought tolerance and the expression of ABA-responsive genes (Kang et al., 2002). The AREBs/ABFs are activated by phosphorylation by the SnRK family of protein kinases (Umezawa et al., 2009). ABA binds to the PYROBACTIN RESISTANCE 1/PYR1-like protein/REGULATORY COMPONENTS OF THE ABA RECEPTOR (PYR1/PYL/RCAR) family of proteins. This results in the inactivation of group A PP2Cs including ABI1/ABI2 (Ma et al., 2009; Park et al., 2009). This inactivation of the PP2Cs allows the SnRK family of kinases to remain in their active form and phosphorylate and activate the AREB/ABF transcription factors and other factors such as ion channels (Umezawa et al., 2009). Once activated, the AREBs/ABFs induce the expression of genes containing the ABRE-element in their promoter. In rice and barley, homologues of AREB2/ABF4 have been identified, named TRAB1 and HvAB15 respectively. Expression of these genes was found to be upregulated following drought stress (Hobo et al., 1999). In sorghum 92 bZIP transcription factors have been identified by a comparative genomics approach. Of the 16 tested six were found to be upregulated following osmotic stress induced by PEG treatment suggesting these transcription factors may be involved in drought tolerance in sorghum (Wang et al., 2011).

1.3.2.2.2 DREB transcription factors

Not all stress-responsive genes are induced by ABA, suggesting the existence of an ABA-independent signalling cascade (Yamaguchi-Shinozaki and Shinozaki, 2006). DEHYDRATION RESPONSIVE ELEMENT BINDING (DREB) transcription factors bind to the dehydration-responsive element (DRE) (G/ACCGAC) in the promoters of many stress-inducible genes and induce their expression (Yamaguchishinozaki and Shinozaki, 1994; Stockinger et al., 1997; Liu et al., 1998). These DREB proteins belong to the ERF superfamily and contain an AP2 DNA binding domain (Sakuma et al., 2002). There are six DREB1 proteins (DREB1A-F) in Arabidopsis and eight DREB2 proteins (Akhtar et al., 2012). In Arabidopsis, DREB1A (CBF3), DREB1B (CBF1) and DREB1C (CBF2) are induced by cold whereas DREB1D (CBF4) is induced by dehydration (Liu et al., 1998; Haake et al., 2002). A gene named inducer of CBF expression 1 (*ICE1*) encodes a MYC-like bHLH protein that regulates the expression of DREB1A but not that of the other DREB1 genes (Chinnusamy et al., 2003). DREB2 genes are induced by high salinity and drought and are activated by post-translational modification (Liu et al., 1998). Constitutive overexpression of DREB1 transcription factors from a range of plant species including rice, maize, barley, wheat, apple (*Malus baccata),* and perennial ryegrass (*Lolium perenne* L.) results in the expression of stress-inducible genes and increased stress tolerance (Dubouzet et al., 2003; Ito et al., 2003; Shen et al., 2003; Qin et al., 2004; Xiong and Fei, 2006; Zhao and Bughrara, 2008; Yang et al., 2011). This indicates that similar regulatory systems are conserved in monocots and dicots.

1.3.2.2.3 Other transcription factors

Some drought-inducible genes do not contain either the DRE or the ABRE in their promoter. For example, promoter analysis of the early response to dehydration 1 (*ERD1*) gene identified two cis-elements, a MYC-like sequence CATGTG and 14-bp rps1 site sequence that are important for drought stress induction (Simpson et al., 2003). NAC transcription factors such as ANAC019, ANAC055 and ANAC072 are responsible for the induction of many stress-inducible genes and were found to be able to induce the expression of this ERD1 gene in combination with the ZFHD1 transcription factor (Tran

et al., 2004). Other NAC transcription factors that are involved in the drought response include RD26, which is induced by both dehydration and ABA and is responsible for the upregulation of stress-inducible genes (Fujita et al., 2004). The NAC transcription factor, JUB1 was found to increase tolerance to abiotic stress and delay senescence in transgenic Arabidopsis (Wu et al., 2012). Additionally, the overexpression of sorghum NAC1 in Arabidopsis resulted in increased survival following water withdrawal (Lu et al., 2013). MYC and MYB transcription factors are also required for the induction of other stress-inducible genes and these are synthesised after the accumulation of ABA *(Abe et al., 2003).*

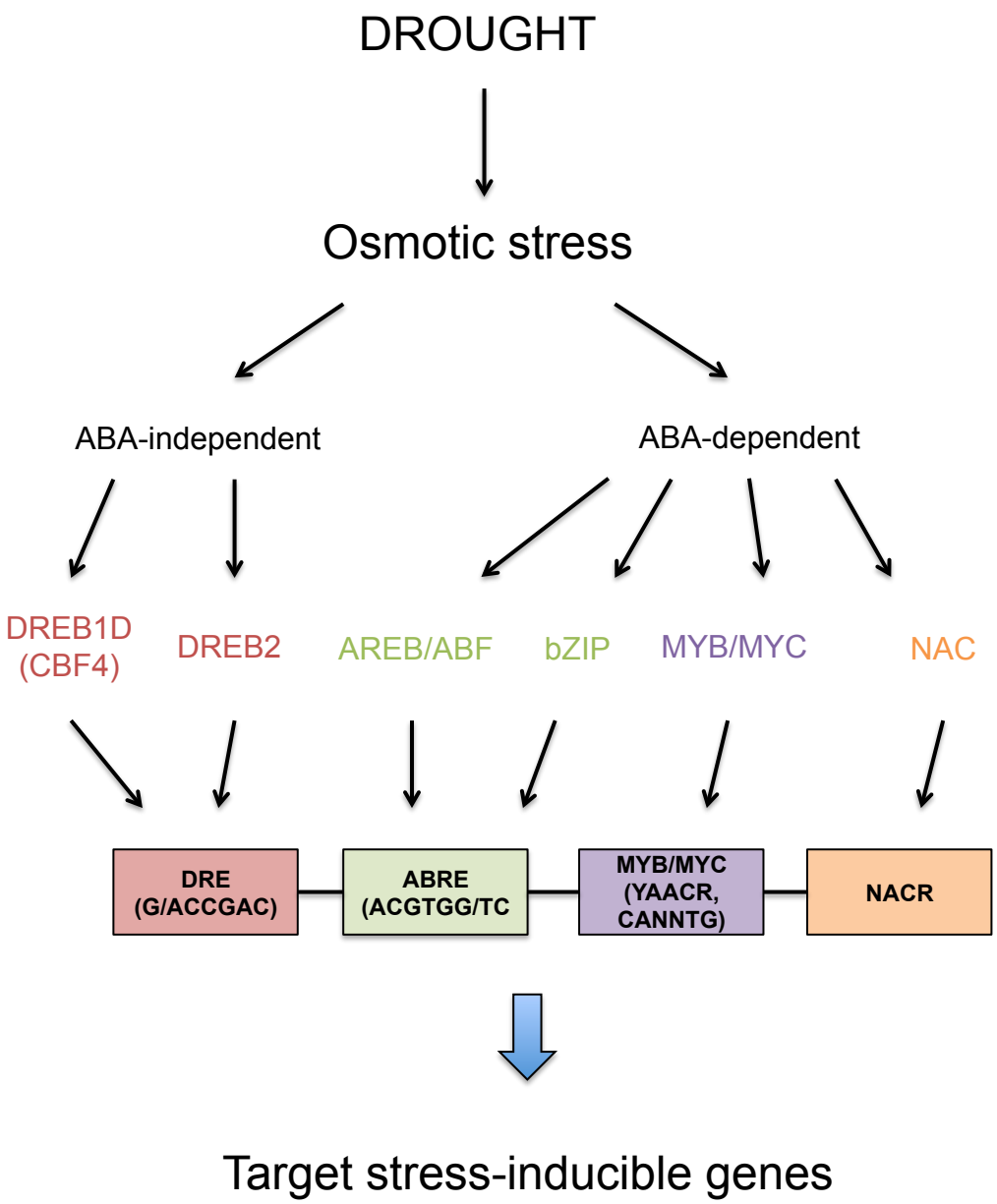


Figure 1.2 - Signalling pathways involved in the response to osmotic stress. Both ABA-independent and ABA-dependent pathways lead to the induction of different families of transcription factors. These bind to specific *cis*-elements in the promoters of stress-inducible genes.

1.3.2.3 Compatible solutes

The above network of signalling pathways leads to the expression of genes that have protective properties and help to confer drought tolerance. For example, several genes that are involved with the biosynthesis of compatible solutes are upregulated. Compatible solutes are low molecular weight, soluble compounds that are non-toxic at high cellular concentrations. These include amino acids such as proline, aspartic acid and glutamic acid, quaternary amino acids such as betaine and sugars such as trehalose. The accumulation of compatible solutes within cells in response to stress lowers the osmotic potential thus enabling a favourable water potential gradient for water uptake and maintenance of cell turgor. This helps to maintain high relative water content and improves root capacity for water uptake thus facilitating drought avoidance (Mahajan and Tuteja, 2005).

In addition to osmotic adjustment, compatible solutes are thought to facilitate the detoxification of reactive oxygen species and the stabilization of membranes and proteins (Mahajan and Tuteja, 2005). Some of the main compatible solutes are discussed in more detail below.

1.3.2.3.1 Amines

Glycine betaine accumulates in some crop species including spinach, barley, wheat and sorghum in response to water stress (Wood et al., 1996; Ashraf and Foolad, 2007). It is abundant mainly in the chloroplast where is plays a role in osmotic adjustment and in the protection of the thylakoid membrane. However, there is no evidence for the production of glycine betaine in Arabidopsis or rice. Betaine is synthesised via a two-step oxidation: choline is converted to betaine aldehyde by choline monooxygenase (CMO) which is then converted to glycine betaine via betaine aldehyde dehydrogenase (BADH) (Ashraf and Foolad, 2007). Transgenic lines overexpressing such components of the biosynthetic pathway showed better survival than wild type in response to stress (Hayashi et al., 1997; Sakamoto et al., 1998; Holmstrom et al., 2000).

Proline accumulates in a range of plants, including sorghum, in response to stress and drought-tolerant varieties have been shown to accumulate proline to higher levels than drought-sensitive varieties (Wood et al., 1996; Hsu et al., 2003; Su et al., 2011). Transgenic lines overexpressing pyyroline-5-carboxylate synthase (*P5CS*), which plays a key role in proline biosynthesis are more tolerant to drought stress compared to wild type (Ashraf and Foolad, 2007). The increase in solute concentration however is not always large enough to impact upon osmotic adjustment suggesting that proline has an additional protective role. Indeed, it is thought that proline is also able to stabilize membrane and protein structures and scavenge harmful free radicals under stress conditions (Ashraf and Foolad, 2007). Proline has additionally been suggested to act in a signalling pathway to induce the expression of stress-inducible genes that possess proline-responsive elements (PRE) in their promoters (Satoh et al., 2002).

Polyamines including putrescine, spermidine and spermine have also been associated with stress tolerance. Transgenic Arabidopsis plants overexpressing genes involved with spermidine synthase, which is involved in the biosynthesis of spermidine, were found to be more tolerant to a range of stresses including drought (Kasukabe et al., 2004).

1.3.2.3.2 Sugars

Trehalose is amongst the sugars synthesised in response to drought. Trehalose is a disaccharide of glucose and is able to stabilize membranes and macromolecules under drought conditions. Trehalose is formed from UDP-glucose and glucose-6-phosphate in reactions catalysed by trehalose-6-phosphate phosphatase (TPP) and trehalose-6-phosphate synthetase (TPS) (Penna, 2003). The overexpression of the *E. coli* trehalose biosynthesis genes *OTSA* and *OTSB* in transgenic rice resulted in reduced photo-oxidative damage and increased survival under drought conditions compared to wild type. The transgenic lines also exhibited increased photosynthetic capacity at saturating light levels under non-stress conditions. The concentration of trehalose within cells remained low suggesting that trehalose increases photosynthetic capacity under both stress and non-stress conditions but does not function in osmotic adjustment (Garg et al., 2002). Trehalose therefore also plays an important role in the drought response.

1.3.2.4 Detoxifying genes

Drought stress can lead to a disruption to cell membranes and the impairment of the electron transport chain in the chloroplast. This can result in the production of reactive oxygen species (ROS), which can cause lipid peroxidation, protein and lipid modification and ultimately cell death and senescence. ROS accumulation during stress depends on the balance between ROS production and ROS scavenging. The ability to reduce the damage caused by ROS therefore constitutes an important drought tolerance mechanism (Miller et al., 2010). Enzymes that are essential for ROS detoxification include: ascorbate peroxidases (APX), glutathione S-transferases (GSTs), superoxide dismutases (SOD) and catalases (CAT). These are found in all cellular compartments thus demonstrating the requirement for these enzymes for cell survival (Miller et al., 2010). The altered expression of these genes can positively influence drought tolerance. For example, stress-tolerant sorghum varieties have been found to have higher anti-oxidant enzyme activity (Jagtap et al., 1998). The overexpression of a rice cytosolic APX in Arabidopsis also exhibited increased tolerance to salt stress, as indicated by the maintenance of chlorophyll content (Lu et al., 2007).

1.3.2.5 Late embryogenesis abundant proteins

High temperature, salinity and drought stress can cause denaturation of enzymes and other proteins. LATE EMBRYOGENESIS ABUNDANT PROTEINS (LEAs) are hydrophilic globular proteins, which accumulate during seed desiccation and in response to stress. As a result of their hydrophilic nature, they act to stabilize enzyme complexes and membrane structures and prevent protein denaturation. Constitutive expression of HVA1, a group 3 LEA from barley conferred tolerance to soil water deficit in transgenic wheat (Sivamani et al., 2000). Similarly, the overexpression of a wheat LEA resulted in increased dehydration tolerance in transgenic rice (Cheng et al., 2002).

1.3.2.6 Heat shock proteins

HEAT SHOCK PROTEINS (HSPs) are molecular chaperones that are induced in response to stress. They provide cellular protection by controlling the proper folding and

refolding of both structural and functional proteins. The higher expression of many HSPs is positively correlated with stress tolerance ((Vierling, 1991). For example, the overexpression of HSP17.6A in transgenic Arabidopsis increased survival compared to wild type following water withdrawal (Sun et al., 2001).

1.4 Stress and senescence

Photosynthesis is one of the first processes to be affected by drought stress. This can either be as a direct effect i.e. as a result of reduced CO_2 influx due to stomatal closure or a secondary effect due to oxidative stress, which can negatively affect the photosynthetic machinery (Chaves, 1991). Leaf senescence is likely to ensue and this characterized by the progressive loss of chlorophyll, desiccation, a loss of photosynthetic efficiency and eventually leaf abscission. This drought-induced leaf senescence reduces further water loss and enables the remobilization of nutrients to youngest leaves (Lim and Nam, 2007). This reduction in photosynthesis under drought conditions has the knock-on effect of reducing carbon assimilation. For crops, this can have a significant impact on grain filling and ultimately can drastically reduce crop yields. The ability of crop plants to delay senescence under conditions of low water availability is of vital importance for the maintenance of substantial crop yields under adverse conditions (Thomas and Howarth, 2000).

In addition to water availability, senescence is influenced by a range of environmental signals and also by developmental cues. These signals are integrated by the plant and result in changes in hormone levels, levels of reactive oxygen species and changes in the expression of senescence-associated genes including transcription factors. Together these changes determine the senescence outcome (Figure 1.3). The whole process requires meticulous control and is regulated via a series of interlinking signalling pathways. There is considerable overlap between the genes involved in stress-induced senescence and the genes induced by developmental cues although the programs are not identical (Lim and Nam, 2007). Some of the processes known to regulate senescence are discussed briefly below.

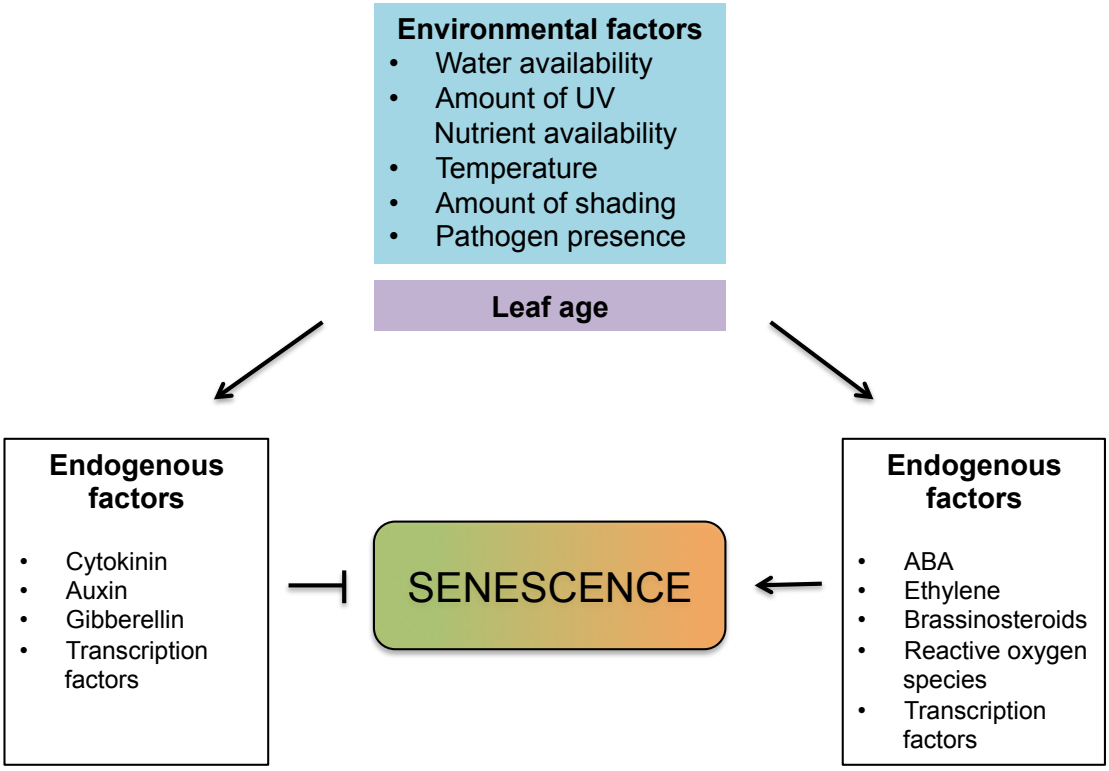


Figure 1.3 - A model for the regulatory pathways involved in leaf senescence. Senescence is an intricately controlled process that requires the fine balance between different endogenous signals. These signals are influenced by both environmental factors and also by developmental factors such as leaf age.

1.4.1 Hormonal regulation of senescence

1.4.1.1 ABA

The upregulation of genes associated with ABA biosynthesis is an intrinsic feature of both drought-induced and developmentally regulated senescence (Lim and Nam, 2007). The exogenous application of ABA is known to accelerate senescence of detached leaves incubated in the light (Nooden and Obermeyer, 1981). Additionally, a number of senescence associated genes (SAGs) are ABA-inducible (Weaver et al., 1998). Whilst the precise role of ABA in leaf senescence is not known, it has been suggested that ABA

induces the accumulation of H_2O_2 in senescing rice leaves and that this in turn accelerates senescence (Hung and Kao, 2004).

1.4.1.2 Cytokinin

Cytokinins are well known as senescence-delaying plant hormones. The exogenous application of cytokinins represses the expression of senescence-associated genes (Weaver et al., 1998). Additionally, a gain-of-function mutation in Arabidopsis in which the cytokinin receptor gene AHK3 is expressed constitutively, results in a delayed senescence phenotype (Kim et al., 2006). Overexpression of a proteolysis-insensitive version of ARR2, another gene involved in cytokinin signalling, also resulted in delayed senescence (Kim et al., 2012). Senescence is therefore closely associated with cytokinin signalling. Furthermore, senescence is delayed in a range of transgenic plants overexpressing isopentenyl transferase (IPT), an enzyme that catalyses the rate limiting step in cytokinin biosynthesis, under the control of a senescence-associated promoter (Gan and Amasino, 1995; Gregersen et al., 2013). Transgenic tobacco plants overexpressing IPT under the control of the stress-inducible promoter P_{SARK} exhibited delayed drought-induced senescence and were able to maintain leaf water potential during drought. This resulted in increased yields under drought stress and was associated with increased tolerance to oxidative stress and the enhanced expression of stress-induced transcripts such as HSPs and LEAs (Rivero et al., 2007).

1.4.1.3 Auxin

The expression of genes associated with the biosynthesis of the plant hormone auxin increases during age-dependent leaf senescence and this is associated with an increase in auxin levels (Lim and Nam, 2007). The exogenous application of auxin represses the transcription of some senescence-associated genes thus leading to the suggestion that auxin is a negative regulator of leaf senescence (Lim and Nam, 2007). This is supported by the fact that disruption of ARF2, a repressor of the auxin signalling pathway causes a delay in leaf senescence (Ellis et al., 2005).

1.4.1.4 Other

Exogenous application of ethylene is able to induce the expression of senescence-associated genes (Grbic and Bleecker, 1995; Weaver et al., 1998). Furthermore, reduced expression of ACC synthase, which encodes the rate-limiting step in ethylene biosynthesis, resulted in a delayed senescence phenotype in maize and increased drought tolerance (Young et al., 2004). Similarly, a mutant of the ethylene receptor ETR1 in Arabidopsis exhibited delayed senescence compared to wild type (Grbic and Bleecker, 1995). As a result, ethylene is thought to play a role in the promotion of senescence.

Brassinosteroids are also implicated in leaf senescence. A mutant of BZR2/BES1, a transcription factor that positively regulates brassinosteroid signaling, displayed a delayed-senescence phenotype (Yin et al., 2002).

Gibberellins also have a significant impact on leaf senescence. Delayed leaf senescence with exogenous application of gibberellins has been reported in many plant species including *Dioscorea rotundata* and *Catharanthus roseus* (Sarwat et al., 2013). The application of endogenous gibberellic acid to *Paris polyphylla* also slowed down the senescence of shoots and the degradation of proteins and chlorophyll (Li et al., 2010).

1.4.2 Role of transcription factors

Transcription factors are important for the regulation of the senescence process. In particular, NAC transcription factors are known to play an important role. For example, the overexpression of the NAC transcription factor JUB1 in Arabidopsis delays senescence and increases tolerance to various abiotic stresses (Wu et al., 2012). Conversely, senescence is delayed under drought conditions in Arabidopsis lines with a loss-of-function mutation in the NAC protein NTL4 (Lee et al., 2012). Other important NAC transcription factors include VND-INTERACTING2 (VNI2). Overexpression of VNI2 in Arabidopsis delays senescence and is associated with the upregulation of stress-responsive genes such as *COR15A*, *COR15B* and *RD29A*. The overexpression of RD29A or COR15A resulted in a delayed senescence phenotype suggesting that VNI2 regulates leaf longevity by regulating the expression of these genes (Yang et al., 2011).

Another member of the NAC protein family, NAMB-1, was shown to be present in wild ancestral wheat but lost in modern cultivated wheat. Reduction in RNA levels of this gene using RNAi resulted in senescence being delayed by over three weeks indicating a role for this gene in regulating senescence (Uauy et al., 2006). Transgenic wheat overexpressing a different NAC transcription factor also exhibited delayed senescence and increased grain yields (Zhao et al., 2015).

WRKY transcription factors have also been implicated in the senescence program. For example, WRKY53 is induced during leaf senescence and its overexpression in Arabidopsis produces an early senescence phenotype suggesting that WRKY53 is a positive regulator of leaf senescence (Miao et al., 2004).

1.4.3 Regulation of senescence by reactive oxygen species

Reactive oxygen species (ROS) play a pivotal role in the natural progression of senescence and both facilitate the breakdown of cellular components and act as a signal to promote the senescence process (Bhattacharjee, 2005). The production of ROS causes the induction of senescence-associated genes (Miao et al., 2004) and transgenic tobacco plants that have reduced levels of ROS are more drought-tolerant and have a delayed senescence phenotype (Rivero et al., 2007). The NAC transcription factor NTL4 is triggered following drought stress and induces the expression of respiratory burst homolog genes (Atrboh) thus resulting in the accumulation of ROS. Loss-of-function *ntl4* mutants exhibit both delayed drought-induced and natural senescence and have reduced levels of ROS. This suggests that ROS accumulation is essential for the progression of senescence induced by both stress and developmental signals (Lee et al., 2012).

1.5 Stay-green

1.5.1 Types of stay-green

There are numerous examples in the literature whereby certain plant genotypes or crop varieties exhibit delayed senescence and as a result have been termed 'stay-green'. These 'stay-green' plants have been classified into five categories. In Type A stay-green the initiation of senescence is delayed but then progresses at the normal rate. In Type B stay-green, senescence is initiated at the same time but then progresses at a reduced rate. In Type C chlorophyll breakdown is impaired but the rest of the senescence-related events proceed at a comparative rate to wild type. In Type D the plant dies in the middle of senescence and so appears green and in Type E plants accumulate higher levels of chlorophyll which takes longer to degrade during senescence (Thomas and Howarth, 2000). Types C and D are known as 'cosmetic' stay-green because despite their green appearance they are no longer photosynthetically active. Types A and B on the other hand are functional stay-green and both chlorophyll *and* photosynthetic efficiency are retained (Thomas and Howarth, 2000).

1.5.1.1 Cosmetic stay-green

Cosmetic stay-green arises as a result of alterations in the chlorophyll degradation pathway. For example, the *senescence induced degradation* (*sid*) mutant in pasture grass, *Festuca pratensis* has permanently green leaves, which is thought to be due to a perturbation in a key enzyme in the chlorophyll a catabolic pathway (KingstonSmith et al., 1997; Thomas et al., 1999). A similar cosmetic stay-green mutant is the *sgr* mutant in rice which is thought to arise due to altered activity of PHEOPHORBIDE a OXYGENASE (PAO) an enzyme involved in chlorophyll degradation (Jiang et al., 2007). Other cosmetic stay-green examples include the *CytG* mutant in in soybean (Guiamet et al., 1991) and the *chlorophyll retainer* (*cl*) mutant in pepper (Efrati et al., 2005).

1.5.1.2 Functional stay-green

In plants with a functional stay-green phenotype both chlorophyll *and* photosynthetic efficiency are retained for longer. Examples of functional stay-green plants include transgenic cotton plants overexpressing an Arabidopsis 14-3-3 protein, GF14λ. The transgenic lines maintained higher photosynthetic rates under conditions of low water availability. The mechanisms behind this are not known although it was hypothesised that GF14λ may influence ABA-induced gene expression (Yan et al., 2004). Similarly, constitutive overexpression of a gene encoding the ER-resident molecular chaperone BiP in soybean and tobacco results in delayed drought-induced senescence through an as yet unknown mechanisms (Valente et al., 2009). Stay-green maize lines with a functional stay-green phenotype have also been identified and have been suggested to be associated with increased cytokinin content and reduced ABA in the leaves (He et al., 2005). Two stay-green wheat cultivars, CN12 and CN18, were also found to have delayed onset of senescence, which was associated with reduced H_2O_2 levels (Chen et al., 2010).

1.6 The stay-green trait in sorghum

Sorghum is extremely stress-tolerant compared to other crops however; drought stress is still a limiting factor on sorghum yield production. In India sorghum is cultivated in two distinct seasons: June to October (rainy season) and October to February (post-rainy season) (Kholova et al., 2013). Around 5.5 million hectares of sorghum are grown in the post-rainy season on residual soil moisture. This is the best environment for food production however; the crops face increasing moisture stress as the season progresses (Kassahun et al., 2010). Post-flowering stress occurs during the grain filling stage and results in premature leaf senescence and the associated reduction in carbon assimilation and grain filling. Other symptoms of post-flowering stress include stalk lodging and increased infection by charcoal rot, a soil-borne pathogen. Ultimately, this culminates in reduced yields (Rosenow et al., 1983; Kassahun et al., 2010).

Drought-tolerant sorghum varieties with a functional stay-green phenotype have been identified and selected in the field. These can be classified as either Type A or Type B

stay-green. These varieties are able to avoid premature senescence and maintain green photosynthetic leaf area for longer under conditions of low soil moisture at the post-flowering stage (Rosenow et al., 1983) (Figure 1.4). This increases the potential period for grain development which has an obvious positive impact on grain yields under drought conditions (Borrell et al., 2000) (Figure 1.5). There is little, if any, negative impact on yields under well-watered conditions (Borrell et al., 2000). Stay-green in additionally associated with resistance to charcoal rot and reduced lodging (Tenkouano et al., 1993).

Figure 1.4 - Photograph showing the stay-green trait in sorghum the field in Petancheru, India. The photograph was taken at the post-flowing stage with no supplemental irrigation. Plants on the right have the stay-green trait (B35) whereas plants on the left are senescent varieties (R16) (Kumar et al., 2011).

Several sorghum genotypes exhibit the stay-green trait including B35, SC56 and E36-1 (Rosenow et al., 1983; Kebede et al., 2001; Haussmann et al., 2002; Sanchez et al.,

2002). B35 (also known as BTx643) originated from Ethiopia and is the best characterized stay-green variety with a number of physiological studies having been carried out on this variety or its derivatives (Crasta et al., 1999; Xu et al., 2000; Kassahun et al., 2010; Vadez et al., 2011). E36-1 also originated in Ethiopia but is unrelated to B35 (Haussmann et al., 2002).

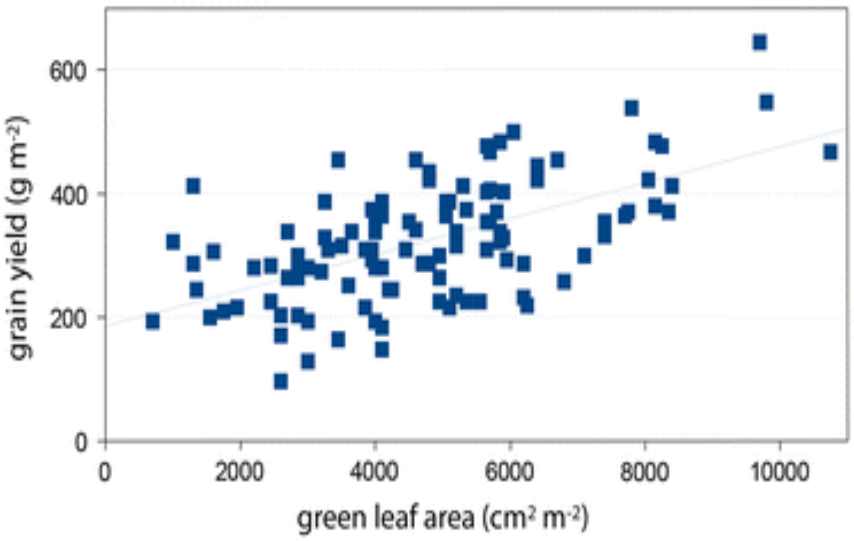


Figure 1.5 - Scatter plot showing the relationship between green leaf area at 25 days after flowering and grain yield in a set of 160 recombinant inbred lines grown during the post-rainy season at Petancheru, India. (Borrell et al., 2014a)

1.6.1 Physiological studies

The stay-green trait can be identified in the field by withholding irrigation prior to flowering so that moisture stress develops just after flowering and intensifies during grain filling. Under these drought conditions, there are clear differences in chlorophyll content between stay-green and senescent lines (Xu et al., 2000a). The B35 stay-green variety has been shown to reach maturity with 40% green leaf area compared to the senescent variety R16 that had 0% at the same time (Kassahun et al., 2010). In the absence of

stress there are also differences in chlorophyll content between the B35 stay-green line and senescent lines and these differences first become visible at around 50 days after sowing (Figure 1.6). Stay-green in the B35 sorghum line therefore involves both delayed drought-induced and developmental senescence. The stay-green trait in E36-1 however, is only visible under drought-stressed conditions (Thomas and Howarth, 2000).

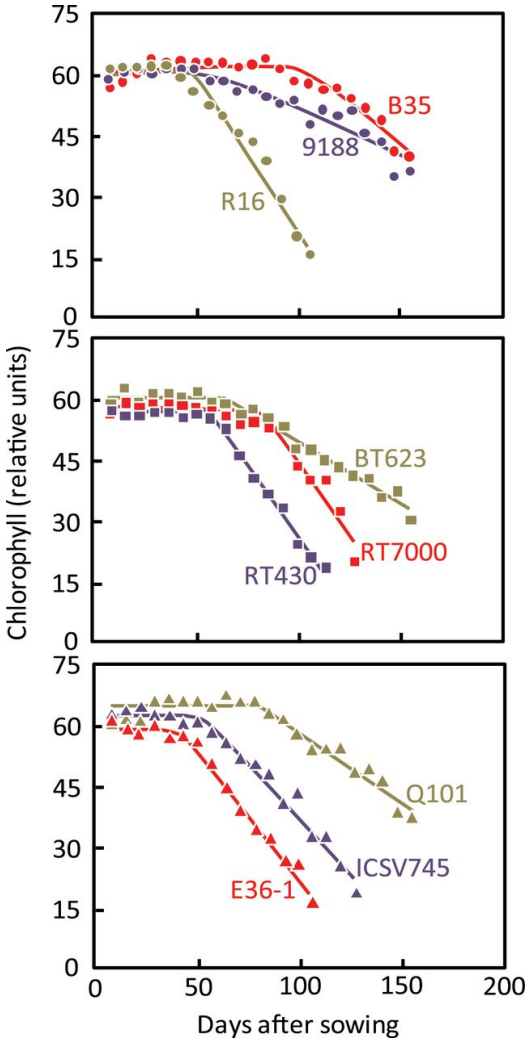


Figure 1.6 - Chlorophyll content in stay-green and senescent sorghum lines under well-watered conditions. B35, BT623, Q101 and E36-1 are stay-green lines and 9188, R16, RT7000, RT430 and ICSV745 are senescent. Developmental senescence is delayed in all stay-green lines apart from E36-1 (Thomas and Ougham, 2014).

The biological basis of the stay-green trait remains unclear although a number of studies have improved our understanding of stay-green physiology. For example, the relative water content in apical leaves of stay-green lines was shown to be about 80% compared to 38% in non-stay-green lines under conditions low water availability (Xu et al., 2000a) suggesting that stay-green sorghum lines are better able to retain water under drought conditions. There are also differences in transpiration efficiency between stay-green and senescent lines under both drought-stressed and fully irrigated conditions (Vadez et al., 2011; Borrell et al., 2014a). Stay-green in sorghum is additionally associated with morphological changes. For example, stay-green lines have been shown to have a reduced canopy size at flowering due to reduced tillering and reduced size of upper leaves. It has been suggested that these changes contribute to reduced water usage prior to flowering meaning that more water is available at the post-flowering stage when soil moisture stress usually develops. The increased grain yield has been suggested to arise at least in part due to reduced pre-flowering water usage (Borrell et al., 2014a). There is additionally some limited evidence that stay-green lines have modified root architecture which could facilitate water extraction (Borrell et al., 2014a). The stay-green trait is therefore hypothesised to arise as an emergent consequence of genes acting earlier in development (Borrell et al., 2014a; Borrell et al., 2014b).

Figure 1.7 - Photographs showing tillering in stay-green (Stg1) (left) and senescent (Tx7000) (right) varieties. Pictures from (Borrell et al., 2014a).

1.6.2 Stay-green QTL in sorghum

Given the obvious agricultural importance of the stay-green trait in sorghum there has been considerable effort to identify quantitative trait loci (QTLs) that underlie it. Such QTL mapping has lead to the identification of six main regions in the sorghum genome that are associated with the trait. These have been termed Stg1-4 and StgA and StgB (Tuinstra et al., 1997; Subudhi et al., 2000; Tao et al., 2000; Xu et al., 2000b; Kebede et al., 2001; Haussmann et al., 2002; Sanchez et al., 2002). These QTL have been introgressed individually into the high-yielding but senescent R16, S35 and Tx7000 lines (Kassahun et al., 2010; Vadez et al., 2011). Each of these introgression lines show increased yields under drought conditions compared to their senescent parent thus confirming that all six of these regions contribute to the trait (Harris et al., 2007; Borrell et

al., 2014b). The influence of the particular QTL however was shown to depend on the particular genetic background (Vadez et al., 2011). For example, Stg1 and Stg3 have reduced tillers in the S35 background but not in the R16 background (Kassahun et al., 2010). Stg QTLs 1-4 were additionally found to affect water extraction, transpiration efficiency and green leaf area (Vadez et al., 2011). The Stg QTL regions however remain large and between them consist of ~2000 genes. The exact causal genes within these regions have yet to be identified. The identification of these would be particularly useful given that the introgression of whole QTLs involved with stress tolerance often brings alongside undesirable agronomic characteristics.

1.7 Summary

Sorghum is an extremely important crop, particularly in developing countries. It is well adapted to the hot, dry conditions in which it lives however; despite our extensive knowledge of the drought stress response in the model plant Arabidopsis relatively little is known about the sorghum response to drought. In particular, the stay-green trait for drought tolerance in sorghum is poorly understood. Whilst physiological studies have provided some insight into the trait, the precise molecular mechanisms and biological processes that underlie it remain to be discovered. A greater insight into this trait would be greatly beneficial not only to improve our understanding of drought tolerance mechanisms in sorghum, but also to facilitate the improvement of future sorghum cultivars by either marker-assisted selection (MAS) or transgenic approaches.

1.8 Thesis aims

The aim of this study was to:

- Identify genes and processes associated with the sorghum response to abiotic stresses such as drought stress, heat stress and drought and heat stress combined (Chapter 3).
- Identify gene expression differences between stay-green and senescent sorghum lines in order to identify genes and biological processes that may underlie the trait (Chapter 4).
- To validate these identified biological processes using biochemical and physiological analyses (Chapter 5).
- To confirm the function of candidate regulatory genes through the production and analysis of transgenic plants (Chapter 6).
- To assess the linkage of the identified candidate genes and processes with the known Stg QTL regions (Chapter 7).
- To identify gene expression differences between a Stg QTL introgression line and the senescent parent (Chapter 7).

CHAPTER 2

Materials and Methods

2.1 Reagents

2.1.1 Chemicals

All chemicals and media were supplied by one of the following companies unless otherwise stated:

Melford Laboratories Ltd (Ipswich, UK),
Fisher Scientific UK Ltd (Loughborough, UK),
Bioline (London, UK),
Sigma-Aldrich Ltd (Poole, UK).

2.1.2 Enzymes

All DNA and RNA modifying enzymes were purchased from Bioline, Fisher Scientific UK Ltd, Applied Biosystems (Forster City, USA), Qiagen (Crawley, UK), Promega (Southampton, UK) or New England Biolabs Ltd. (NEB) (Hitchin, UK).

2.1.3 Antibiotics

All antibiotics were purchased from Melford Laboratories Ltd. Antibiotics were filter sterilized using a 0.22 μm filter (Millipore Corporation, Bedford, USA) attached to a syringe (VWR International Ltd, Lutterworth, UK), prior to addition to liquid media. The concentrations used are listed in (Table 2.1).

Table 2.1 - Concentrations of antibiotics used for both bacterial and plant culture plates

Antibiotic	Stock concentration (mg/ml)	Working concentration (µg/ml)	Stock Solvent
Spectinomycin	50	50	Water
Kanamycin	100	For bacteria: 100 For plants: 50	Water
Ampicillin	100	100	Water
Rifampicin	50	100	DMSO
Geneticin® (G418)	30	30	Water
Timentin®	200	200	Water

2.2 Bacterial strains and growth conditions

2.2.1 Bacterial strains

Escherichia coli (E. coli) strains (DH5α) were obtained from Bioline (London, UK).

Agrobacterium tumefaciens (*A. tumefaciens*) strain C58C1 (Holsters et al., 1978) was used for stable transformation of Arabidopsis and was propagated in house.

2.2.2 Bacterial growth media

E. coli and *A. tumefaciens* were grown either on solid agar plates consisting of 1.5% (w/v) micro agar and 2% (w/v) Luria-Bertani (LB) medium (Sigma-Aldrich) or liquid media made from 2% (w/v) LB. All growth media was sterilised prior to use by autoclaving at 121 °C for 20 min and allowed to cool to 50 °C prior to the addition of appropriate antibiotics.

2.2.3 Bacterial growth conditions

Bacteria were incubated either at 37 °C *(E. coli)* or 29 °C *(A. tumefaciens).* Solid agar plates were incubated statically whilst liquid media cultures were shaken at 200 rpm.

2.3 Plant materials and growth conditions

2.3.1 Seed material

Arabidopsis (*A. thaliana*) ecotype Columbia (*Col-0*) seeds were obtained from Lehle seeds (Round Rock, Texas, USA).

Sorghum (S. bicolor) seeds of variety R16, E36-1, S35 and B35 were used for the majority of experiments and were obtained from Dr Santosh Despande, (ICRISAT, Petancheru, India). The Sorghum inbred line Tx430 was used for Sorghum transformation and was provided by Professor Ian Godwin (University of Queensland, Australia). Stg QTL introgression lines in the R16 or S35 background were also obtained from Dr Santosh Despande (See Appendix E.1 for genotype names and numbers of each of these lines).

2.3.2 Seed sterilization

2.3.2.1 Ethanol sterilization

Arabidopsis seeds were sterilized by shaking (Labnet Vortex Mixer, Labnet International Inc., Woodbridge, New Jersey, USA) in a 1.5 ml microfuge tube with 70% ethanol (v/v) for 5 min. Seeds were then transferred to sterile filter paper (Whatman™ International Ltd, Kent, UK) and air dried in a sterile laminar flow hood before being sprinkled onto solid agar medium (see 2.3.3.1).

2.3.2.2 Bleach sterilization

Arabidopsis seeds collected from plants dipped in *A. tumefaciens* solution were first surface sterilized with ethanol as described above. They were then shaken in a solution containing 10% (v/v) sodium hyperchlorite (NaOCl) and 0.25% (v/v) sodium dodecyl sulphate (SDS) for 10 min. The seeds were then washed six times in sterile water (10 min per wash), spread directly onto agar plates (see 2.3.3.1) and left to dry in a sterile laminar flow cabinet.

2.3.3 Growth conditions

2.3.3.1 Arabidopsis growth conditions

Arabidopsis seeds were grown on solid 1 X MS medium agar plates (Murashige and Skoog, 1962). This comprised of 0.8% (w/v) plant tissue culture grade agar (Sigma-Aldrich) and 1 X Murashige and Skoog salts (Duchefa Biochemie BV, Haarlem, Netherlands). All growth media was sterilized by autoclaving at 121 °C for 20 min. The pH was adjusted to 5.8 using 0.1 M KOH before autoclaving. If required, appropriate antibiotics were added to the liquid medium after autoclaving and cooling to 50 °C.

Plates containing seeds were stratified at 4 °C in the dark for a minimum of 48 hr to ensure uniform germination and growth. They were then transferred to a Percival (CU-36L5D, CLF plant climatics, Emersacker, Germany) at a temperature of 20 °C and 16/8 h photoperiod with a light intensity of 150 µmol $m^{-2}s^{-1}$. After 7 days they were transferred to hydrated peat plugs (Jiffy Products International, Moerdijk, Norway). Individual seedlings were grown on small (41 mm diameter) peat plugs, whilst large peat plugs (44 mm diameter) were used to grow up to three plants per plug for *A. tumefaciens* dipping (see 2.9.1). Seedlings transferred to peat plugs on trays were covered in cling film, and grown at a temperature of 20 °C and 16/8 h photoperiod with a light intensity of 150 µmol $m^{-2}s^{-1}$. The film was removed after two days. Peat plugs were watered with 100 mg/L intercept (Everris International, Ipswich, UK) and Baby Bio (Bayer Garden, Cambridge, UK), following the manufacturer's instructions, until the siliques had developed. If seeds needed to be collected from individual plants, the Aracon system (Beta tech, Ghent, Belgium) was used to contain each plant.

2.3.3.2 Sorghum growth conditions

For the majority of experiments, sorghum seeds were imbibed in water overnight and germinated on the surface of soaked peat plugs in a closed plastic container in a growth chamber at 28 °C day and 23 °C night, 12 h photoperiod. The lids were removed following germination. This occurred 3-4 days after sowing (DAS). If mature plants were required, at ~20 DAS the peat plugs were transferred to 6" pots. At 30 DAS plants were transferred to 8" pots. New Horizon Organic and Peat Free Compost (William Sinclair Horticulture Ltd, Lincoln, UK) was used for potting up.

For sorghum transformation (see 2.9.2.2), the sorghum tissue culture room was controlled at 27 °C with a luminescence of approximately 100 µmol $m^{-2}s^{-1}$ with a 16/8 h photoperiod.

2.4 Molecular biology techniques

2.4.1 DNA extraction

2.4.1.1 gDNA extraction from Arabidopsis

The method was adapted from (Edwards et al., 1991). Briefly, a single leaf from 2-3 week old plants was transferred to a microfuge tube and flash frozen in liquid nitrogen. The sample was ground in 400 µl of Edwards extraction buffer (Appendix A.1). The tube was then spun for 1 min in a microcentrifuge at full speed (15000 *g*). An aliquot of the supernatant (300 µl) was transferred to a fresh tube. The supernatant was then mixed with 300 µl of isopropanol and incubated at room temperature. The tube was spun again for 5 min and the supernatant was removed and discarded. The pellet was dried using a vacuum desiccator (5031 eppendorf UK Ltd, Stevenage, UK) before resuspension in 50 µl of TE buffer (Appendix A.2).

2.4.1.2 gDNA extraction from sorghum

The method was adapted from (Dellaporta et al., 1983). Samples were ground using liquid nitrogen cooled mortar and pestles, and then added to a 1.5 ml microfuge

tube. To extract the gDNA, 750 µl of Dellaporta extraction buffer was added (Appendix A.3) and samples were heated at 65 °C for 10 min. To precipitate the DNA, 200 µl of 5 M potassium acetate was then added and vortexed before incubating on ice for 20 min. Samples were then spun down in a microcentrifuge for 10 min at 13000 *g* and the supernatant pipetted into a fresh microfuge tube. An equal amount of isopropanol was added to the supernatant, mixed and then centrifuged at 12000 *g* for 10 min. The supernatant was removed from the resulting pellet. To wash the DNA, 80% ice-cold ethanol was then added to the pellet. It was then centrifuged for 3 min at 12000 *g* and the supernatant was removed. The pellet was left to air dry for 20 min at room temperature then slowly rehydrated on ice for 30 min in 50 µl TE (see Appendix A.2).

2.4.1.3 Plasmid DNA extraction from *E. coli*

Small-scale bacterial plasmid DNA extraction was performed using the Wizard® Plus SV Minipreps DNA Purification System (Promega), according to the manufacturer's instructions. Briefly, 5 ml bacterial cultures were centrifuged and the bacterial pellet was resuspended and lysed in the presence of alkaline phosphatase. The supernatant was separated from the pellet by centrifugation and the plasmid DNA was bound to the column supplied. The column was then washed in an ethanol-based buffer and the DNA was eluted in nuclease-free water.

Large-scale bacterial plasmid DNA extraction was performed using the Qiagen Plasmid Maxi Kit according to the manufacturer's instructions in an analogous manner to above.

2.4.1.4 Extraction of DNA from an agarose gel

DNA fragments separated by gel electrophoresis (see 2.4.3) were excised from the gel using a scalpel blade whilst visualizing on a UV trans-illuminator (Ultra-Violet products Ltd., Cambridge, UK). The DNA was then purified using a QIAquick gel extraction kit (Qiagen), according to the manufacturer's instructions. In this method, the agarose gel slices were first dissolved in a buffer and the mixture was applied to a column with a silica membrane. Nucleic acids adsorbed to the membrane and impurities were washed away. The DNA was finally eluted in a low-salt buffer.

2.4.2 Polymerase chain reaction (PCR)

2.4.2.1 DNA polymerases and reaction mixes

For general PCR reactions either BioTaq (Bioline) or BioTaq Red Taq polymerases were used. For high fidelity applications, a proof reading Phusion DNA polymerase was used (Finnzymes, Keileranta, Finland).

Reaction mixes were made up according the manufacturer's instructions using the buffers and $MgCl_2$ provided.

For amplification of sorghum gDNA 5% DMSO was added to the reaction mixture to improve yield and specificity.

2.4.2.2 Oligo nucleotides

Primers were designed to be a minimum of 18 bp in length and to have a GC content of 40-60 % for optimal annealing. The Oligo Calc: Oligonucleotide Properties Calculator (www.basic.northwestern.edu/biotools/oligocalc.html) program was used to check for potential hairpin formation. A full list of oligo nucleotides used for PCR can be found in Appendix C.1.

2.4.2.3 Cycling conditions

PCR was performed using a 96 well Px2 thermocycler (Thermo Electron Corporation, Waltham, Massachusetts, USA). PCR conditions for different enzymes are listed in Table 2.2. The annealing temperature for new templates and primer pairs were optimized before use. Typically, annealing temperatures were chosen to be 5 °C lower than the melting temperature of the lowest from the primer pair. The resulting PCR products were analysed using gel electrophoresis (see 2.4.3).

Table 2.2 - PCR conditions for different Taq Polymerases

Cycle steps	Time and temperature		No. of cycles
	BioTaq	Phusion	
Initial denaturation	95 °C; 5 min	98 °C; 30 s	1
Denaturation	95 °C; 30 s	98 °C; 10 s	25-35
Annealing	50-60 °C; 30 s	50-60°C; 20 s	
Extension	72 °C; 2 min	72 °C; 30 s	
Final extension	72 °C; 2 min	72 °C; 10 min	1

2.4.3 Gel electrophoresis

DNA was separated by size using agarose gel electrophoresis. Gels were prepared by melting 1% (w/v) electrophoresis grade agarose (Sigma) in 0.5 X TBE buffer (see Appendix A.4) in a microwave oven. After cooling to ~50 °C, ethidium bromide (10 mg/ml) was added to a final concentration of 5 µg/ml. The molten gel was poured into a gel tank containing a comb and allowed to set.

TBE (0.5 X) was used as a running buffer and 5 X DNA sample-loading buffer (Bioline) was added to DNA samples before loading into wells. Gels were run at 35 mA for approximately 1 h. Nucleic acid bands were visualized using a UV trans-illuminator (Uvitech Limited, Cambridge, UK) at a wavelength of 254 nm. Fragment size was determined by comparing to a 1 kb molecular size standard (Bioline Hyperladder 1).

2.4.4 Cloning

2.4.4.1 Plasmids

All plasmid vectors are presented in Appendix B, including vector maps annotated with specific features.

2.4.4.2 Ligation

DNA fragments were ligated into a linearized vector using T4 DNA ligase (Promega) in the supplied buffer. A 1:3 molar ratio of linearized vector to insert was used. As a control, linearised vector alone was ligated and water was added instead of DNA. Ligation reactions were incubated overnight at 16 °C.

2.4.4.3 Phosphatase treatment

Phosphatase treatment was used to prevent re-annealing of a vector once it had been cut with a single enzyme (see Appendix B.5). Alkaline phosphatase was used according to the manufacturers instructions (Promega).

2.4.4.4 Gateway cloning

DNA to be cloned was PCR amplified using Phusion Taq Polymerase (see 2.4.2) to give a blunt-ended product. This was visualized using gel electrophoresis (see 2.4.3) and extracted from the gel (see 2.4.1.4). The fragment was then ligated into p-ENTR/D-TOPO using the p-ENTR/D-TOPO cloning kit according to the manufacturer's instructions (Life Technologies, Cat. No. K2400-20).

Gateway recombination using the LR Clonase II Enzyme Mix (Life Technologies, Cat. No. 11791-020) was used according to the manufacturer's instructions to create final binary vector constructs. One Shot TOP10 Chemically Competent *E. coli* cells (Life Technologies) were then transformed with the resulting plasmids (see 2.4.4.7.1).

Plasmids were checked for the incorporation of the correct insert by restriction digestion (see 2.4.4.6) and then sequencing (see 2.4.5).

2.4.4.5 Gibson Assembly

The DNA fragment to be cloned was ordered as a gBlocks® gene fragment (Integrated DNA Technologies, Coralville, Iowa, USA). The fragment was cloned into a linearized

vector using a Gibson Assembly® Cloning Kit (NEB, Cat. No. E5510S) according to the manufacturer's instructions. Briefly, the linearized vector was incubated in a reaction with a DNA fragment containing overlapping ends and the Gibson Assembly Master Mix. A 5' exonuclease in the master mix generated long overhangs. A polymerase then filled in the gaps of the annealed single strands and a DNA ligase sealed the nicks. A molar ratio of 3:1 of insert to vector was used. Cells were transformed with 2 µl of the reaction (see 2.4.4.7.1)

2.4.4.6 Restriction digests

Restriction digests were carried out to obtain fragments either for diagnosis or for cloning. Digests were carried out using NEB restriction enzymes and buffers and these were incubated for a minimum of 2 hr at the temperature recommended by the manufacturer (usually 37 °C). For single enzyme digestions, enzymes were added so that they comprised a maximum of 1/10th of the total reaction volume. Digests were then run on a gel to determine the size of the insert (see 2.4.3).

2.4.4.7 Transformation

2.4.4.7.1 Transformation of E. coli

Aliquots (25 µl) of either One Shot TOP10 Chemically Competent *E. coli* cells (Life Technologies) or α-select silver cells (Bioline) were transformed with plasmid DNA. DNA (2.5 µl) was added to thawed cells on ice and incubated for 20 min. The cells were then heat shocked at 42 °C for 30 s before being returned to ice for a further 2 min. SOC media (250 µl) (Life Technologies) was added to the cells and they were then incubated with shaking at 220 rpm for 1 hr at 37 °C. Cells were then plated onto LB containing the appropriate antibiotics (see Table 2.1) and incubated overnight at 37 °C.

2.4.4.7.2 Transformation of A. tumefaciens

2.4.4.7.2.1 Competent cell production

Cultures containing 5 ml of LB supplemented with appropriate antibiotics were inoculated with a single *Agrobacterium* colony (from a fresh LB plate) using a sterile wire loop. This was grown overnight at 28 °C in a shaking incubator. The next day, 4 ml of the overnight culture was added to 100 ml of LB in a sterile 500 ml flask and this was shaken vigorously (250 rpm) at 28 °C until the culture reached an OD_{600} of 0.5 to 1.0. The culture was chilled on ice and the cells were centrifuged at 3500 *g* for 5 min at 4 °C. The supernatant was discarded and the cells were resuspended in 2 ml of ice-cold 20 mM $CaCl_2$ solution. The cells were then dispensed into 0.1 ml aliquots in 1.5 ml pre-chilled microfuge tubes. The cells were flash frozen in liquid nitrogen and stored at -80 °C.

2.4.4.7.2.2 Transformation

Aliquots containing 100 µl of C58C1 *A. tumefaciens* cells were allowed to thaw on ice. To this, 1 µg of plasmid DNA was added and these were incubated on ice for 30 min. The cells were then heat-shocked for 5 min at 37 °C before being returned to ice for a further 2 min. Liquid LB media (1 ml) was then added to each aliquot and the cells were incubated with shaking for 4 hr at 28 °C. The cells were then briefly spun down in a microcentrifuge and the cell pellet was resuspended in 100 µl of LB before being spread on LB agar containing the appropriate antibiotics for selection (see Table 2.1). Plates were incubated at 29 °C for 48 hr to allow colonies to develop.

2.4.5 Sequencing

2.4.5.1 Sequencing reactions

For sequencing of plasmids containing cloned fragments, DNA was isolated using the Miniprep method (see 2.4.1.3). All sequencing reactions were carried out by the DNA sequencing laboratory (School of Biological and Biomedical Sciences, Durham University).

2.4.5.2 Sequence alignments

Analysis of chromatograms was carried out using SnapGene (www.snapgene.com). Sequences were aligned using Clustal W (www.ebi.ac.uk/Tools /msa/clustalw2/).

2.4.6 RNA extraction

The RNeasy Plant Total RNA kit (Qiagen) was used to extract total plant RNA, from sorghum leaves and Arabidopsis tissue, according to the manufacturer's instructions.

For the extraction of RNA to be used for microarray analysis, which requires a higher quantity and purity of RNA, the miRNeasy Extraction kit (Qiagen) was used, again according to the manufacturer's instructions. Tissue was ground in liquid nitrogen using a pre-cooled mortar and pestle and the tissue was homogenized using a QIAshredder column (Qiagen). In all cases on-column DNase digestion using RNase free DNase (Qiagen) was carried out. Concentrations of RNA were determined using the nanodrop technique (see 2.4.7.1). The RNA was eluted in RNase free water and stored at -80°C.

2.4.7 Nucleic acid quantification

2.4.7.1 UV-VIS spectrophotometer

DNA or RNA concentrations were determined by measuring the optical density of samples at 260 nm using a ND-1000 UV-Vis spectrophotometer (Nanodrop technologies, Delaware, USA). Water or elution buffer was used as a zero reference.

2.4.7.2 Bioanalyzer

The quality and integrity of the RNA to be used for microarray experiments (see 2.5) and Fluidigm qPCR (see 2.4.9.2) was determined using the Agilent 2100 bioanalyzer (Palo Alto, CA) and the Agilent RNA 6000 Nano Kit. These were used in accordance with the manufacturer's instructions.

2.4.8 cDNA synthesis

cDNA was produced from RNA using the Applied Biosystems High Capacity cDNA synthesis kit again according to the manufacturer's instructions. A total volume of 10 µl was made up with 1 µg total RNA and nuclease-free water. A master mix was made up containing (per reaction): 2 µl 10 x RT buffer, dNTP Mix (100 mM), 1 µl Multiscribe^{TM} Reverse Transcriptase and 4.2 µl of nuclease free water. Aliquots of this master mix (10 µl) were added to each diluted RNA sample to give a total volume of 20 µl. Controls with no RNA and no reverse transcriptase enzyme were all set up in parallel. The samples were then transferred to a Px2 thermocycler and run on the following program: 25 °C for 10 min, 37 °C for 120 min and then 85 °C for 5 s. The resulting cDNA was diluted 1:50 with nuclease free water before use in qPCR (see 2.4.9.1) and then stored at -20 °C until needed.

2.4.9 Gene expression measurements using qPCR

2.4.9.1 Applied Biosystems system using SYBR green.

The relative transcript level of genes of interest was determined by qPCR using the Applied Biosytems 7300 real time PCR machine and Go Taq qPCR master mix (Promega). Diluted cDNA (5 µl) (see 2.4.8) was added to 10 µl of SYBR green master mix containing (per reaction): 7.5 µl of 2 X GoTaq qPCR master mix, 0.9 µl of each forward and reverse primer and 0.7 µl of nuclease free water. The GoTaq qPCR master mix contains ROX reference dye to account for optical differences between the wells. The diluted cDNA and master mix were added to wells of a 96-well plate (STARLAB UK, Milton Keynes, UK). For each sample to be tested, three replicate wells were set up to give three technical replicates. At least three biological replicates were also carried out for each experiment. *Sb03g038910.1* was used as an endogenous control when testing Sorghum gene expression because its expression was found to be unchanging in microarray analysis following various stress treatments (Johnson et al., 2014). PEX4 (At5g25760.1) was used as an endogenous control when testing Arabidopsis gene expression (Moffat et al., 2012). A full list of qPCR primers can be found in Appendix C.2. All qPCR primers were designed using Primer3 (http://primer3.ut.ee) with an amplicon size of 75-150 bp. Gene expression levels were analysed using the ΔΔCt method

(Applied Biosystems). The algorithm described in the Applied Biosystems user bulletin in 2007 entitled, 'Relative Quantitation (RQ) algorithms, Applied Biosystems Real-Time PCR Systems Software' was used for the statistical analysis of the qPCR data (Moffat et al., 2012). Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to the Student's t-test.

2.4.9.2 Fluidigm system using TaqMan probes

qPCR validation of the microarray data was carried out using Fluidigm 96 Dynamic arrays (Fluidigm, San Francisco, California, USA). Assays were run in triplicate to give three technical replicates. Three biological replicates were also carried out. The setup was performed in accordance with the 'Fluidigm® 96.96 Real-Time PCR Workflow' (PN68000088) (http://fgl.salk.edu/ BioMark/pdf/ 96.96%20Real-Time%20PCR%20Workflow%20Quick%20 Reference%20rev%20C1.pdf). RNA (1 µg) was used as input in a 20 µl reverse transcription reaction. The SuperScript III First-Strand Synthesis SuperMix Kit (Applied Biosystems) was used for first-strand cDNA synthesis and the TaqMan PreAmp Master Mix (ABI PN4391128) was used for pre-amplification of the cDNA. Custom designed 20 X Custom TaqMan® Gene Expression Assays (Applied Biosystems) were used for amplification of the cDNA (see Appendix C.3 for full list of TaqMan probes). Data was collected using the Fluidigm Real-Time PCR analysis Software v3.0.2. TaqMan probes were designed using the Primer Express® Software (Life Technologies). The average (mean) raw expression value of the three technical replicates was calculated. Relative quantification was then calculated for each biological replicate using the Comparative Ct Method ($\Delta\Delta C_T$ Method) (Livak and Schmittgen, 2001). *Sb04g028990.1* was used as an endogenous control because its expression was found to be unchanging following various treatments (Johnson et al., 2014).

2.4.10 Luciferase assay

Relative promoter activity was determined by cloning different promoters upstream of the firefly luciferase gene (see 2.4.4 and Appendix B.5 & B.6 for vector map and cloning strategy). The resulting constructs were bombarded into sorghum leaf tissue on MS agar

plates (see 2.9.2.1). The plates were then sealed and incubated in the Percival for 96 hr to allow for expression. The transformed leaves were sprayed with luciferin (5 mM potassium luciferin (Melford Laboratories Ltd) in 0.01% (v/v) Triton-X-100). Each plate was then imaged for 60 min using a Photek photon-counting camera (Photek, Hastings, UK).

2.5 Microarray processing and analysis

2.5.1 Experimental design

2.5.1.1 Combined stress

Seeds of *Sorghum bicolor* R16 variety were imbibed overnight and surface sown on soaked peat plugs (see 2.3.3.2). Plants were subjected to control (no treatment), heat, drought or combined heat and drought conditions (six plants per treatment). Drought stress was applied to the drought and combined stress plants by withholding water after 12 DAS (see 2.7.1.1). The remaining plants (control and heat shock) were well watered. The F_v/F_m of all of the plants was measured daily using a FluorCam (see 2.7.2.2). At the point when the F_v/F_m of the drought-stressed plants first started to differ from the well-watered controls, either a heat shock by incubation in the dark at 50 °C for 3 hr (heat and combined treatment) or control treatment at 28 °C (control and drought treatment) was initiated. The youngest three leaves were sampled and tissue was pooled for each treatment set. All plants were sampled at the same time following the heat treatment. Experiments were carried out in triplicate to give three biological replicates. All treatments were carried out at the same time for each replicate to reduce circadian variation. Samples were stored at -80 °C.

2.5.1.2 Mature sorghum

At 30 days after sowing (DAS) sorghum seedlings were transferred to 8” pots (see 2.3.3.2). From this point the photosynthetic efficiency of leaf 2 and 4 was monitored using a portable photosystem efficiency analyser (PEA) machine (see 2.7.2.2). A sample from leaf 10 was taken when the average photosynthetic efficiency of leaves 2 and 4 first started to differ between the B35 and R16 varieties, as indicated by a reduced ratio of

variable fluorescence (F_v) to maximal fluorescence (F_m) in R16. This occurred at around 45 DAS. At this stage the plants were at the booting stage and had ten leaves. Leaf 10 was sampled and the tissue was pooled from six plants of each variety. Plants were maintained under well-watered conditions throughout. Experiments were carried out in triplicate, with samples taken on different occasions, to give three biological replicates. Samples were taken at the same time of day for each biological replicate to reduce variation due to circadian/diurnal factors. Tissues samples were harvested into liquid nitrogen and stored at -80°C.

2.5.1.3 Young sorghum

Sorghum varieties R16 (senescent), S35 (senescent), B35 (stay-green) and E36 (stay-green) were grown until 12 DAS (see 2.3.3.2). Drought was imposed by withdrawing water at 12 DAS, while control plants were well watered (six plants of each variety per treatment). Leaf tissue samples of the youngest three leaves were taken when the photosynthetic efficiency of one of the varieties first started to differ from the controls, as measured using a FluorCam (see 2.7.2.2).

2.5.2 Preparation of microarrays

All microarray processing was carried out at Unilever. Custom expression microarrays (4X44K format) for sorghum were designed and submitted for manufacturing using the Agilent Technologies eArray web-based application (https://earray.chem.agilent.com/earray/). Briefly, Sbicolor release 79 coding sequences were downloaded from the sorghum GDB database (http://www.plantgdb.org/SbGDB/). Based upon these 29289 coding sequences, 28585 microarray probes (60 mer oligonucleotides) were designed (with a 3' bias). In addition, for ten of the longest coding sequences, ten tiling probes were also designed. These probes were randomly laid out onto the 4X44K microarray design format by eArray, along with default Agilent control probes (Agilent Technologies UK Ltd., Wokingham, Berkshire, UK), and ten additional replicate probes of 100 randomly selected sorghum coding sequences.

2.5.3 cRNA synthesis and labelling

A schematic for the production of cRNA is shown in Figure 2.1. All products were obtained from Agilent Technologies UK Ltd and used according to the manufacturer's protocol unless stated otherwise. Total RNA was isolated using the miRNeasy Extraction Kit (Qiagen). The integrity of the RNA was confirmed with analysis by the Agilent 2100 bioanalyzer and the Agilent RNA 6000 Nano Kit (see 2.4.7.2). RNA (1µg) was added to 1.2 µL of T7 promoter primer and 5 µL of a "spike-in" control and made to a total volume of 11.5 µL with nuclease free water. The primer and template was denatured at 65 °C for 10 min. The One-Color Low RNA Input Linear Amplification Kit PLUS was used for the synthesis of cDNA as follows: 5 x First Strand Buffer, DTT (to 10mM), dNTP mix (to 0.5 mM), Moloney murine leukaemia virus (MMLV) reverse transcriptase (1 µL stock to 20 µL reaction) and RNaseOut (0.5 µL of stock to 20 µL reaction) were added to the denatured template. The cRNA was synthesized by incubation at 40 °C for 2 h and then denaturation at 65 °C for 15 minutes. Transcription Buffer (X4), DTT (to 7.5mM), NTP mix (8 µL stock to 80 µL reaction), PEG (to 4 %), RNaseOUT (0.5 µL to 80 µL), inorganic pyrophosphate (0.6 µL to 80 µL reaction), T7 RNA Polymerase (0.8 µL to 80 µL reaction) and Cyanine 3-CTP (10mM) (2.4 µL to 80 µL reaction) were added. The synthesis of the cRNA was performed by incubation at 40 °C for 2 h. The labelled cRNA was purified using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol and quantified using a UV-VIS Spectrophotometer.

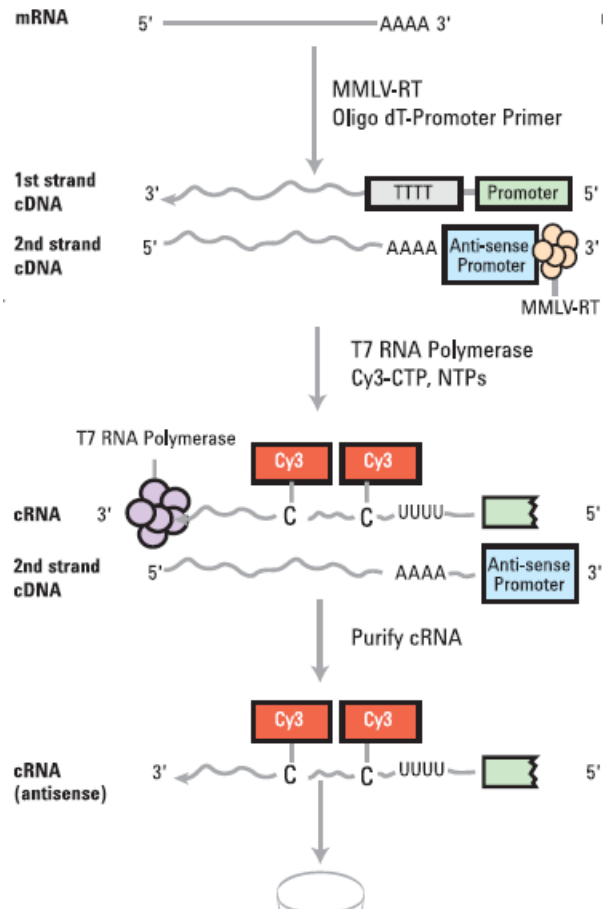


Figure 2.1 - Schematic depicting the production of cRNA for the microarray experiments taken from the Agilent protocol for single colour labelling (http://www.chem.agilent.com/library/usermanuals/public/g4140-90041_one-color_tecan.pdf).

2.5.4 Hybridization and washing

The Agilent Hybridization Kit (Cat. No. 5188-5242) was used with the sorghum custom oligo Arrays. Labelled total RNA (2 µg) was added to 10 X blocking Agent, 25 X

fragmentation buffer and nuclease free water to a total volume of 55 µl. The RNA was fragmented by incubation at 60 °C for 30 min. Fragmentation was stopped by the addition of 55 µl 2 X GE Hybridization Buffer HI-RPM. The hybridization was performed for 17 h at 65 °C with shaking at 10 rpm. Slides were then washed for 1 min in Wash Solution 1, 1 min in pre-warmed (37 °C) Wash Solution 2 and then 20 s in acetonitrile. Slides were then incubated for 30 s in Agilent Stabilization and Drying Solution. The slides were scanned with the Agilent G2565BA Microarray Scanner System.

2.5.5 Feature extraction and analysis

The Agilent G2567AA Feature Extraction Software (v.9.1) was used to extract the data and check the quality of the arrays. The extracted data was analysed using GeneSpring GX 7.3.1 (Agilent Technologies). Controls, spots of poor quality (not detected) and gene probes which were not present in all three replicates in either the control or treatment samples were excluded from the analysis. This yielded approximately 21000 probes for each control vs. treatment comparison. For the sorghum stay-green experiments, genes with an average fold-change of >2 ($p<0.05$) (Moderated T-test with Benjamini-Hochberg correction) were selected for further analysis. For the combined stress analysis, genes with a fold-change of >2 in all 3 reps were selected for further analysis.

2.6 Bioinformatics

Singular Enrichment Analysis (SEA) of Gene Ontology (GO) terms was carried out using agriGO (http://bioinfo.cau.edu.cn/agriGO/) and redundant GO terms were removed using REVIGO (http://revigo.irb.hr/) (medium similarity). Hierarchical clustering of normalized gene expression was carried out on conditions and entities using GeneSpring default settings. The Sorghum Cyc metabolic pathways database (http://pathway.gramene.org/gramene/sorghumcyc.shtml) was used to identify sorghum genes involved in particular biosynthetic pathways. Genes within known QTLs for stay-green were identified using the Comparative Saccharinae Genome Resource (CSGR)-QTL (http://helos.pgml.uga.edu/qtl/) (Zhang et al., 2013). Known promoter motifs were identified using the PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php) and

PLACE databases (http://www.dna.affrc.go.jp/PLACE/). Over-represented promoter motifs were identified using a web-based tool (http://element.mocklerlab.org).

2.7 Plant physiology

2.7.1 Stress and hormone treatments

2.7.1.1 Drought stress

i. For the combined stress microarray experiment (see 2.5.1.1) and GST activity measurements in sorghum, (see 2.8.4), drought stress was induced by withholding water from 14 DAS. Samples were taken when the F_v/F_m, which gives an indication of photosynthetic efficiency, first started to differ between the well-watered and drought-stressed plants (see 2.7.2.2). This was usually after around three days of stress induction.

ii. For proline quantification (see 2.8.2) and sorghum gene expression analyses, seedlings were grown on peat plugs with constant watering up to 14 DAS (see 2.3.3.2). Drought stress was then induced by subsequent watering with 10% polyethylene glycol (PEG [6000]) (w/v) solution. The same volumes of either water (control) or PEG were applied to each batch of plants. Samples of leaf tissue (the three youngest leaves) were taken immediately prior to stress induction and every two days thereafter until eight days following stress induction. This method was used to better synchronise the initiation of the osmotic stress.

iii. For Arabidopsis gene expression analyses, seeds were germinated on MS agar (see 2.3.3.1) and then, after eight days, transferred to a sterile well of a 6-well culture plate (~15 seedlings per well) containing 3 ml of water. The plate was kept in the percival overnight to allow the seedlings to recover after the transfer to water from agar. The next day, 3 ml of 0.8 M mannitol (to give a 0.4 M final concentration) was added to the wells, except to control wells to which 3 ml of water was added. Seedling samples were taken at 0 hr, 2 hr, and 5 hr time points.

iv. For sorghum gene expression analyses, six sections of sorghum leaf blades (approx. 5 cm in length) were cut from seedlings at 14 DAS and placed in 9 cm petri dishes containing 25 ml of water. The plates were incubated overnight in a growth chamber to equilibrate. The following day, the water was removed from the dishes using a syringe and replaced with either water (control) or 0.66 M mannitol. Samples were taken at the following time points: 0 hr, 2 hr, 6 hr and 24 hr.

2.7.1.2 Heat stress

For the combined stress microarray experiment (see 2.5.1.1) heat stress was administered by heating whole plants to 50 °C for 3 hr in an incubator in the dark. Control plants were incubated at 28 °C for 3 hr in the dark.

2.7.1.3 Salt stress

See the method described in 2.6.1.1 part iv. However in this case, the water was replaced with 25 ml 200 mM NaCl and samples were taken at 0 hr, 2 hr, 6 hr and 24 hr time points.

2.7.1.4 ABA

See the method described in 2.6.1.1 part iv. In this case, the water was replaced with 10 ml 100 µM ABA. This high (non-physiological) concentration of ABA was used in order to ensure penetration through the thick waxy cuticle of the sorghum leaves. Water containing 0.1% ethanol was used as a control.

2.7.1.5 Oxidative stress

Methyl viologen (MV) was used to induce oxidative stress. Leaf discs of 0.8 cm diameter were cut from sorghum leaves at around 14 DAS. The leaf discs were floated adaxial side up in 5 ml water and allowed to equilibrate overnight in the dark. The water was then removed and replaced with MV at the following concentrations: 0 µM, 2.5 µM and 5 µM. Six leaf discs of each variety were used for each concentration tested.

2.7.2 Measurements of photosynthetic activity

2.7.2.1 Chlorophyll measurements

A chlorophyll assay was carried out as a non-subjective, quantitative measurement of bleaching in response to MV treatment (see 2.7.1.5). The leaf discs were blotted dry and transferred to a 1.5 ml microfuge tube. Acetone (400 µl) was added to each microfuge tube and the tubes were then incubated overnight in the dark at room temperature. Samples were then homogenised using a micropestle (pellet pestle motor, New Jersey, USA) and vortexed to resuspend the material. Tubes were then centrifuged at 20000 *g* to separate the plant material from the solubilised chlorophyll in the supernatant. The supernatant was collected, the pellet re-suspended in another 400 µl of acetone for further chlorophyll extraction, vortexed and centrifuged again. The steps were repeated until a total of 1.2 ml of acetone had been used to extract the chlorophyll. This pooled supernatant was made up to a volume of 1.5 ml with distilled water (resulting in an acetone concentration of 80% (v/v). The chlorophyll content was measured at OD 663 and OD 645 nm using a spectrophotometer. 80% acetone was used as a blank. The following equation was used to calculate chlorophyll concentration (Hipkins and Baker, 1986):

$$\textit{Chlorophyll concentration } (g/cm^2) = \frac{((20 \times A645) + (6.02 \times A663)) \times V}{\textit{Leaf area}}$$

V = Volume of 80% acetone

2.7.2.2 Fv/Fm measurements

Damage due to stress can be observed by a reduced ratio of variable fluorescence (F_v) to maximal fluorescence (F_m) of plant photosystems (Maxwell and Johnson, 2000; Oxborough, 2004). This was measured in seedlings (up to 14 DAS) using a FluorCam 700mf (Photon Systems instruments, Brno, Czech Republic) on the F_0, F_m and Kautsky effect setting. Plants were dark adapted for 30 min prior to measurements. F_v/F_m was measured in mature plants using a portable Photosystem Efficiency Analyser (PEA) machine (Hansatech, Norfolk, UK).

2.7.3 Excised-leaf water-loss assay

Mature Arabidopsis plants, ~3 weeks old, were transferred from trays to perspex boxes and then covered with a lid to maintain high humidity conditions the night before the experiment was carried out. The following day, leaves were detached and weighed abaxial side up at different time intervals at room temperature. Leaves of a similar size and developmental age were selected from at least eight plants of each genotype. The experiment was repeated three times to give three biological replicates.

Similarly, sorghum seedlings were maintained in high humidity conditions before the experiment by placing the tray of plants into a cellophane bag. Leaf three was excised and weighed abaxial side up at room temperature.

2.7.4 Stomatal conductivity measurements

Stomatal conductivity was measured at the University of Lancaster using the Li-Cor® system (http://www.licor.com/env/). The LI-6400XT Portable Photosynthesis System was used with the 6400-15 Extended Reach 1 cm Chamber (LiCor, Lincoln, Nebraska USA). Arabidopsis plants at ~5 weeks old were used and leaves of the same developmental stage were measured. Measurements were taken throughout the day and measurements of the different genotypes were alternated. The following parameters were used: light intensity = 150 µmol $m^{-2}s^{-1}$, CO_2 = 550 µmol, temperature = 22 °C, flow rate = 100 µmol/s.

2.7.5 Stomatal density measurements

Impressions of the abaxial surface of leaves were made using dental resin (President Jet Light Body, Coltène/Whaledent, Burgess Hill, UK). Clear nail varnish was applied to the set impression after removal from the leaf, and the varnish impressions were viewed on a Zeiss Axioskop inverted microscope with a Retiga 2000R camera. Impressions were made of a ~3 x 3 cm area in the middle of leaf 9 at 50 DAS. Arabidopsis impressions were taken from leaves at a similar developmental stage of three-week-old plants. Stomatal counts were taken from four areas per leaf from eight separate plants, to give a total of 32 measurements for each genotype.

2.7.6 Root growth measurements

Arabidopsis seedlings were germinated in a line on vertical, square MS agar plates (see 2.3.3.1.). In order to account for plate-to-plate variation, the plates were divided into four so that each of the four genotypes tested were represented on each plate. Root lengths were measured after ten days using ImageJ software.

2.8 Biochemical techniques

2.8.1 Spermidine quantification

Spermidine levels were quantified using UPLC (Waters Acquity H-Class UPLC® system with fluorescence (FLR) and photodiode array (PDA) detectors) (Waters, Wilmslow, UK). Sorghum leaf tissue was freeze-dried using a lyophiliser. Dried tissue (0.1 g) was then homogenised in cold 5% (w/v) perchloric acid (2.5 ml) and centrifuged at 1000 *g* for 30 min at 4 °C. Cadaverine was used as an internal standard and was added to 1.5 ml of the supernatant to give a final concentration of 100 µM. To the extract, 1 ml of 2 M NaOH was added and then vortexed. Spermidine was derivatized by the addition 10 µl of benzoyl chloride. Finally, 2 ml of saturated NaCl solution was added to the extract along with 2 ml diethyl ether. The resulting solution was vortexed again and the phases separated by centrifugation at 200 *g* for 20 min at 4 °C. The upper ether phase was transferred to a vial and this was dried under a nitrogen stream, before being

resuspended in 300 µl methanol. The derivitised compounds were separated on the UPLC with a 100 mm x 2 mm C18 column (Waters), and detected at 254 nm. The spermidine concentration in the samples was determined using a standard curve with standards of 0, 10, 50 and 100 µM concentrations.

2.8.2 Proline quantification

Proline levels were also quantified using UPLC (Waters Acquity H-Class UPLC® system with fluorescence (FLR) and photodiode array (PDA) detectors) (Waters). Leaf tissue was ground in liquid nitrogen and lyophilized overnight. Lyophilized tissue (0.04 g) was extracted in 1.5 ml of 0.1 N HCl by grinding and then centrifugation at 17000 *g* for 20 min at 4 °C. The extracts were then sequentially derivatised with OPA (o-Phthaldialdehyde) reagent and FMOC (Fluorenylmethyloxycarbonyl) (Sigma). OPA reagent consists of 260 mM N-Isobutyryl-L-cysteine (IBLC) (Sigma) and 170 mM OPA (Sigma) in 1 M potassium borate buffer (pH 10.4). The following reactions were set up in a HPLC vial: 10 µl sample, 10 µl OPA reagent, 20 µl FMOC (5 mM in acetonitrile) and 60 µl of 100% methanol. Separations were performed on a Cortecs C18 column, 100 mm x 2.1 mm, 1.6 M µM column (Waters) and elution was achieved at 40 °C. Mobile phase A was made up of 20 mM sodium acetate, pH 6.0. Mobile phase B was made up of acetonitrile: methanol: water in a 45:45:10 (v/v/v) ratio. A flow rate of 400 µl/min was used. Automated HPLC injection added 3 µl of the sample for analysis and samples were run for 20 min. For OPA detection the excitation and emission wavelengths were 340 nm and 455 nm respectively. For FMOC detection the excitation and emission wavelengths were 266 nm and 305 nm.

2.8.3 Betaine quantification

Betaine extraction and quantification was carried out using UPLC as described in (Bessieres et al., 1999). Lyophilized tissue (0.04 g) was ground in 2 ml of sterile water. The samples were then centrifuged at 17000 *g* for 20 min at 4 °C and the supernatants were diluted with nine volumes of acetonitrile: methanol: water (45:45:10). A cation exchange resin (Amberlite IR-120 Plus) was applied to the column and washed with 10 ml of water before application of the extract. The column was again washed with 10 ml

of water and eluted with 5 ml 4 M NH_4OH. The extract was then concentrated by freeze-drying, before being transferred to a HPLC vial. Columns (2.1 mm x 150 mm) (Ascentis silica, Sigma) were packed with silica particles of 2.7 µm diameter. The columns were kept at 30 °C. Mobile phase A was made up of acetonitrile. Mobile phase B was made up of 10 mM ammonium formate, (pH 3.0): acetonitrile in a 90:10 (v/v) ratio. A gradient was applied for 16 min at a flow rate of 400 µl/min from 15% to 25% mobile phase B then up to 40% by 20 min before going down to 15% B after 25 min. UV detection was carried out at 192 nm.

2.8.4 Measurements of glutathione S-transferase (GST) activity

GST activity assays with the 1-chloro-2,4-dinitrobenzene (CDNB) substrate were carried out as described in Habig *et al* (1974) with minor modifications. Sorghum tissue was ground in three volumes of GST extraction buffer (See Appendix A.5) and then centrifuged at 17000 *g* for 15 min at 4 °C to remove cell debris. Protein samples were kept on ice. Assays were set up consisting of 900 µl 0.1 M potassium phosphate buffer (pH 6.5), 25 µl 40mM CDNB substrate, 50 µl of 100 mM glutathione (final assay concentration 5 mM) and 25 µl of supernatant (enzyme solution). Assays were carried out at 30 °C and activity was measured by following the change in absorbance of the reaction mixture at 340 nm. Control incubations in which the enzyme solution was omitted were also performed. A minimum of five technical replicates was carried out and the experiment was repeated three times to give three biological replicates. The amount of protein used in each assay was quantified using the Bio-Rad DC™ protein assay (Bio-Rad, Hercules, California, USA) and a nanodrop (See 2.4.7.1.).

2.9 Plant transformation

2.9.1 Transformation of *A. thaliana*

2.9.1.1 Transformation

The floral dip method of transformation was used for stable transformation of Arabidopsis (Clough and Bent, 1998). Wild-type Arabidopsis seeds were grown on MS media (see 2.3.3.1) for seven days. Large (44 mm) peat plugs were set up with three

seedlings per plug. When the plants began to flower, the bolts were clipped to allow multiple secondary stems to develop. The final clipping was carried out seven days before transformation, producing an abundance of stems with flowers. Overnight cultures containing 5 ml of LB media with *A. tumefaciens* containing the correct plasmid and appropriate antibiotics were set up (see 2.2.2). An aliquot (1 ml) of the 5ml overnight culture was then added to a 200 ml overnight culture, again with the appropriate antibiotics for selection. The culture was incubated overnight at 28 °C with agitation at 150 rpm. The *A. tumefaciens* cells were then pelleted by centrifugation at 3500 g for 20 min at room temperature. The bacterial pellet was then resuspended in 200 ml of 5% sucrose solution (w/v) and 0.05% Silwet L-77 (v/v). The flower stems of each Arabidopsis plant were dipped in the sucrose solution before being placed on their side in a tray lined with tissue paper. The tray was covered in cling film and returned to the growth chamber overnight. The following day the plants were transferred to a fresh tray and stood upright.

2.9.1.2 Selection of transformants

Seeds (T_1) were collected from the transformed Arabidopsis plants. These seeds were bleach sterilized (see 2.3.2.2) before germinating on large MS agar plates containing kanamycin and timentin antibiotics (see Table 2.1 for antibiotic concentrations). The plates were put in the light for 6-8 hr, wrapped in foil and left in the dark for 48 hr. The plates were then kept in constant light until transformed seedlings could be identified by the presence of dark green leaves and roots which were able to penetrate the agar (usually after 5-6 days) (Harrison et al., 2006). Primary transformants (T_1) surviving the selection were transferred to peat plugs and grown to maturity under normal conditions in the growth room. The seeds from this generation (T_2) were harvested separately. All investigations compared the transformed Arabidopsis lines to distinct Col-0.

2.9.2 Transformation of *S. bicolor*

2.9.2.1 Transient transformation

2.9.2.1.1 Preparation of gold particles

Gold particles were prepared by the addition of 60 µg of 1.6 µm gold particles to 1 ml 100 % ethanol. The particles were vortexed for 1 min and then pelleted by spinning in a micro centrifuge for 10 s. The supernatant was removed and the particles were washed in this way a further three times and after the last wash spun for 1 min. MilliQ water (1 ml) was then added and the particles were resuspended by vortexing for 1 min. The particles were spun and the supernatant removed. A final 1 ml of water was added and the particles resuspended.

An aliquot of gold particles (50 µl) was dispensed into a microfuge tube and 5 µg of plasmid DNA was added before vortexing. To this, 50 µl 2.5 M $CaCl_2$ was added and then vortexed hard for 30 s. Then, 20 µl 0.1 M spermidine free-base was added and again vortexed hard for 10 s before being placed in a continuous vortex for 3 min. The tube was pulse spun and the supernatant was removed. After this, 250 µl 100% ethanol was added to the particles and vortexed. The particles were spun and the supernatant removed. Finally, 125 µl of 100% ethanol (v/v) was added to the particles and they were vortexed to resuspend the particles. An aliquot of this suspension (20 µl) was dispensed onto each of 5 macrocarrier discs within a silver macrocarrier ring. The DNA/gold suspension was allowed to dry on the macrocarriers.

2.9.2.1.2 Firing at the plant tissue

Sorghum leaf blades from plants 7-20 days old were cut and placed onto 1 X MS plates to cover an area of 16 cm^2 in the centre of the plate. The PDS-1000/He™ system (Bio-Rad) was used for bombardment and the procedure was carried out according to the manufacturer's instructions. The 1100 psi rupture discs were used and the plates were placed on the 3rd shelf. The plates were then sealed with micropore tape (3G) and kept in the growth chamber for 96 hr to allow for expression.

2.9.2.2 Stable transformation

Stable Sorghum transformation was carried out as described by Liu *et al (Liu et al., 2014)* in the lab of Professor Ian Godwin (University of Queensland, Australia).

2.9.2.2.1 Harvesting of Sorghum immature seeds

Immature seeds were collected from healthy Sorghum panicles (Tx430 variety) 12-15 days after pollination. All work was carried out under a laminar flow hood. The immature seeds were sterilized by shaking at 200 rpm for 5 min in 70% ethanol. The seeds were rinsed in sterilized water and then soaked in a solution containing 4% (w/v) sodium hypochlorite with 3 to 5 drops of Tween20. They were then shaken at 200 rpm for 10 min before being rinsed at least five times with sterilized water until the bleach had completely washed away. The seeds were then placed into a sterile Petri dish in the laminar flow hood and allowed to dry for 20-30 min.

2.9.2.2.2 Preparation of target tissue for particle bombardment

Immature embryos were isolated from the seeds using sterile forceps and a surgical blade within the laminar flow hood. Immature embryos that were between 1.1-2.2 mm in length were selected. The immature embryos were placed with the scutellum side up on callus induction media (CIM) (see Appendix A.6) (maximum 25 immature embryos per 90 x 15 mm Petri dish). The plates were sealed with parafilm and kept in the dark at 26-28 °C to allow callus formation. After 9-11 days in the dark, immature embryos that had formed compact, globular, white embryogenic callus were selected. Embryos (6-8 per plate) were placed onto the centre of a Petri dish filled with osmotic medium (OM) (see Appendix A.7) for 2-3 hr before bombardment.

2.9.2.2.3 Preparation of gold particles and bombardment

Gold particles were prepared by the addition of 60 µg of 1.6 µm gold particles to 1 ml 100% ethanol. The particles were vortexed thoroughly for 5 min and then allowed to

stand for 15 min. The particles were then centrifuged at 16000 *g* for 10 s, the supernatant removed and the particles were washed a further three times in sterilized water. The particles were finally resuspended in 1 ml of sterilized 50% glycerol.

Plasmid DNA (5 µg) containing a selectable marker and 5 µg of plasmid DNA containing the target gene was added to a 50 µl aliquot of gold particles. This was then vortexed for 1-2 min before the addition of 50 µl of 2.5 M $CaCl_2$ and 20 µl of 0.1 M spermidine. The solution was vortexed for 1-2 min before precipitation on ice for 5 min. The particles were then pelleted for 10 s at 800 *g*. The supernatant was removed and the particles were resuspended in 35 µl of 100% ethanol by vortexing.

A particle inflow gun (PIG) was used to bombard the callus tissue (Finer et al., 1992). The gold particle suspension (50 µl) was applied to the centre of the syringe filter for each shot of the PIG and this was screwed into the top of the vacuum chamber. The particles were bombarded onto a Petri dish containing the target tissue under a baffle see Liu *et al.* for operating instructions (Liu et al., 2014). The distance from the filter holder to the target cells was set at 18.5 cm.

2.9.2.2.4 Post-bombardment recovery and selection

The bombarded immature embryos were kept on OM for 3-4 h in the dark, before subculturing onto CIM. They were then allowed to recover on CIM at 27 °C in the dark for 3 days. The embryos were then subcultured onto selective regeneration medium (Appendix A.8) and subcultured fortnightly until plantlets were 3 cm long. The individual plantlets were then subcultured onto selective rooting medium (Appendix A.9) for 3-4 weeks without additional subculture.

2.9.2.2.5 Potting out plantlets

The lids of Petri dishes were opened to allow plantlets to have exposure to air. Sterilized water was added to the Petri dishes daily to cover the selective rooting medium. After 2-4 days the plantlets were transferred to soil and maintained in a glasshouse with temperature control (18-28 °C).

CHAPTER 3

Microarray analysis investigating the effect of drought, heat and combined stress on sorghum gene expression

3.1 Introduction

As discussed in Chapter 1, abiotic stresses are major limiting factors for crop growth and yield production. In particular, drought stress and high temperatures can result in drastic reductions in crop yields (Boyer, 1982; Wang et al., 2003). These abiotic stresses are, however, rarely presented individually and crops are often subjected to multiple stress types simultaneously (Mittler, 2006). The combination of drought and heat has been shown in sorghum, wheat and other grasses to result in an even greater detrimental effect than when each stress is imposed individually (Craufurd et al., 1993; Savin and Nicolas, 1996; Machado and Paulsen, 2001). For this reason, it is important to gain an understanding of the mechanisms by which crops respond, not only to individual stress, but also to their combination. This will be particularly important in the face of climate change given the predicted increase in land area affected by these combined stresses in the future (Ahuja et al., 2010).

Analysis of changes to transcript levels can be a valuable way to gain insight into the genes and processes involved in providing stress tolerance. The transcriptional response of a range of species to individual stress types has been studied extensively and has identified a multitude of important genes and processes (Kreps et al., 2002; Zhang et al., 2005; Qin et al., 2008; Wang et al., 2011). Interestingly, it has been found that the combination of drought and heat stress in Arabidopsis and tobacco, results in a unique transcriptional response that cannot be simply extrapolated from the effect of

each stress imposed individually (Rizhsky et al., 2002; Rizhsky et al., 2004). This suggests that plants have a novel response to combined stress.

Sorghum is grown in arid and semi-arid countries and as a result is able to thrive when subjected to high temperatures and low water availability. A variety of studies have been undertaken to investigate the physiological basis of this tolerance (Xu et al., 2000; Sanchez et al., 2002; Srinivas et al., 2009; Borrell et al., 2014) however, until recently, molecular characterization has been relatively limited. The transcriptomic response of sorghum to osmotic stress, induced by PEG, has been reported (Dugas et al., 2011) although, there are no published reports using *bona-fide* drought-treated samples. No transcriptomic analysis of the sorghum response to heat stress or indeed combined heat and drought has been reported. Such an analysis would provide a greater understanding of the stress response in a crop that is well adapted to hot and arid environments and could provide important insights not gained from other species.

This chapter focuses on microarray experiments carried out to investigate the response of sorghum to drought stress, heat stress and their combination.

Aims of the research described in this chapter:

- To test the quality and efficiency of custom-designed microarrays (see 3.2.1.2)
- To investigate gene expression changes following drought, heat and combined heat and drought stress in sorghum (see 3.2.1.3)
- To identify biological processes that are associated with the response to each of these stresses and in particular the response to combined stress (see 3.2.1.3)
- To validate gene expression differences using qPCR (see 3.2.2)
- To validate identified biological processes using biochemical analyses (see 3.2.3).

3.2 Results

The results of this chapter are published in Johnson et al. (2014)

3.2.1 Microarray analysis of sorghum subjected to drought, heat and combined drought and heat stress

3.2.1.1 Sample selection

To investigate gene expression changes, sorghum seedlings were either untreated (control) or subjected to drought stress, heat stress or combined drought and heat stress (see 2.5.1.1 for detailed method). Briefly, drought was administered by withdrawing water from 14 days after sowing (DAS) (drought and combined) whilst the remaining (control and heat) plants were well watered throughout. Heat shock was administered (heat and combined) at the point at which the F_v/F_m, which gives an indication of photosynthetic efficiency, first started to significantly drop in the drought-stressed plants, with respect to the well-watered controls. This occurred following four days of water withdrawal (Figure 3.1). This ensured that the drought-treated plants were experiencing *bona fide* stress when the combined heat stress was executed. The experiment was carried out three times to give three biological replicates and a total of 12 samples.

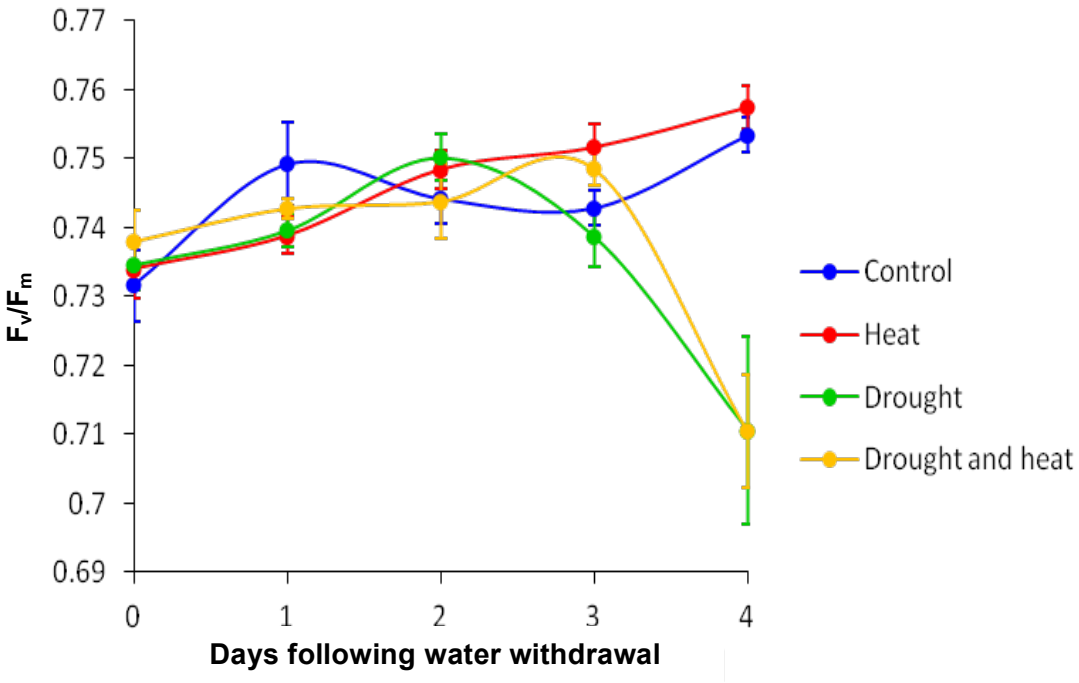


Figure 3.1 - The F_v/F_m (variable fluorescence/maximal fluorescence of Photosystem II) of sorghum seedlings that were either well watered (control and heat) or subjected to drought stress (drought and combined drought and heat). Drought stress was imposed by withdrawing water from 14 DAS. Photosynthetic efficiency was measured daily, using a FluorCam, following the initiation of water withdrawal (see Materials and Methods section 2.6.2.2).

3.2.1.2 Quality control of RNA and hybridised slides

Total RNA was extracted from the tissue and samples were run on a bioanalyzer to check the quality and integrity of the RNA. A representative bioanalyzer result is shown in Figure 3.2 and high quality RNA was confirmed by the presence of defined peaks of ribosomal RNA. The RNA samples were then labelled with Cy3 and hybridized to sorghum customized microarray chips containing 28585 gene probes. Initial quality control analyses were performed on the scanned arrays and the data was analysed using GeneSpring GX 11 software (Agilent Technologies, CA, USA). Principle

Component Analysis (PCA) was used to summarize the data set and to identify predominant gene expression profiles. The samples showed distinct clustering, with samples that had been treated in the same way clustering together (Figure 3.3). There was therefore strong consistency across biological replicates.

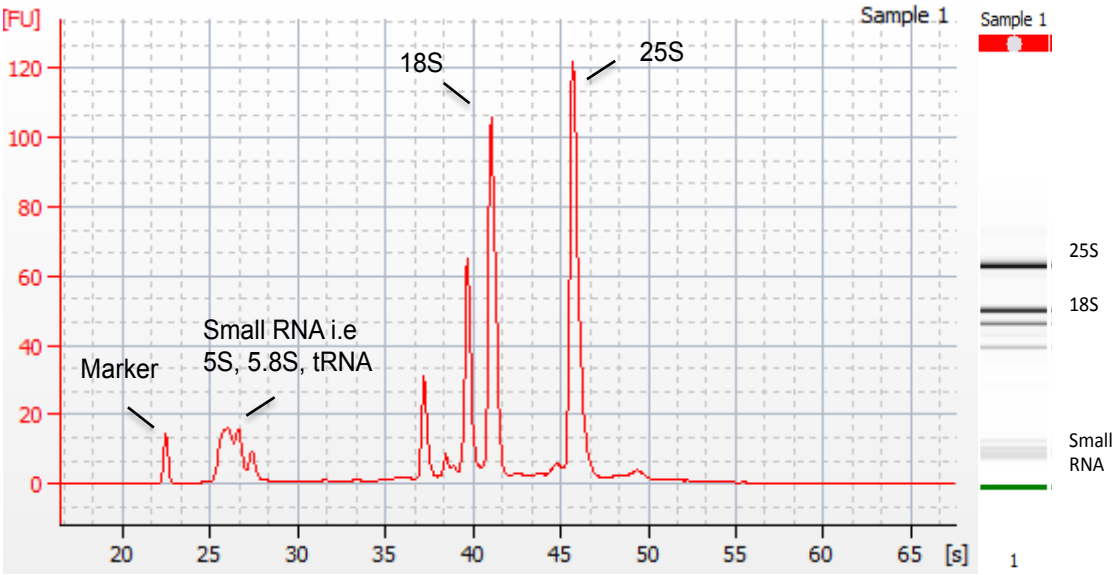


Figure 3.2 - Representative electrophoreogram of an RNA sample used for microarray analysis. Each sample was labelled with fluorescent dye and separated on a gel over time. The x-axis shows the length of time and the y-axis shows fluorescence. The gel image on the right-hand side shows the relative intensity of the different RNA constituents. Smaller molecules migrate faster than larger molecules and so appear at the left-hand site of the graph. The larger peaks represent the 18S and 25S ribosomal subunits (labelled). The smaller peaks (between 35 and 40 s) represent chloroplast RNA. Small RNA can be seen as small peaks between 25 s and 30 s. This includes 5S and 5.8S subunits and tRNAs. Defined peaks indicate the presence of high quality RNA.

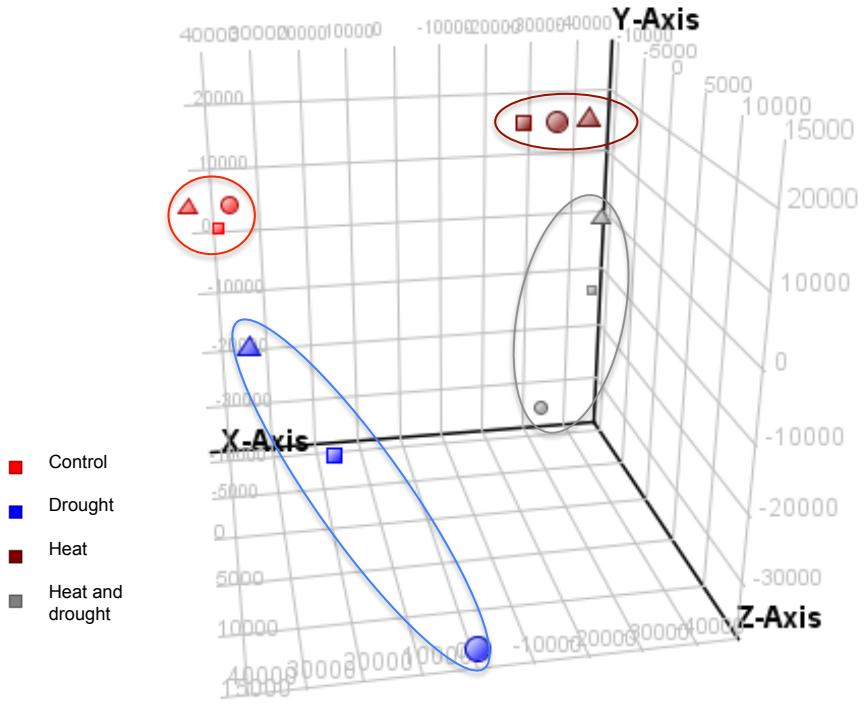


Figure 3.3 - Principle component analysis (PCA) plot showing the predominant gene expression profiles of each sample. The different colours represent the different treatments whilst the different shapes represent the three different biological replicates. The X-Axis represents 39.97% of the variance, the Y-axis represents 25.69% and the Z-axis represents 10.06%.

3.2.1.3 Identification of gene expression differences and functional classification of genes

The raw data was pre-processed and normalized as described in the materials and methods section (see 2.5.5). Spots of poor quality and gene probes that were not present in all replicates in at least one out of the four treatments were removed from the analysis. This resulted in around 21,000 filtered gene probes. Genes that were differentially expressed by >2 fold in all three replicates, when compared to the respective control samples were selected for further analysis (see Appendices D.1 – D.7 for full gene lists).

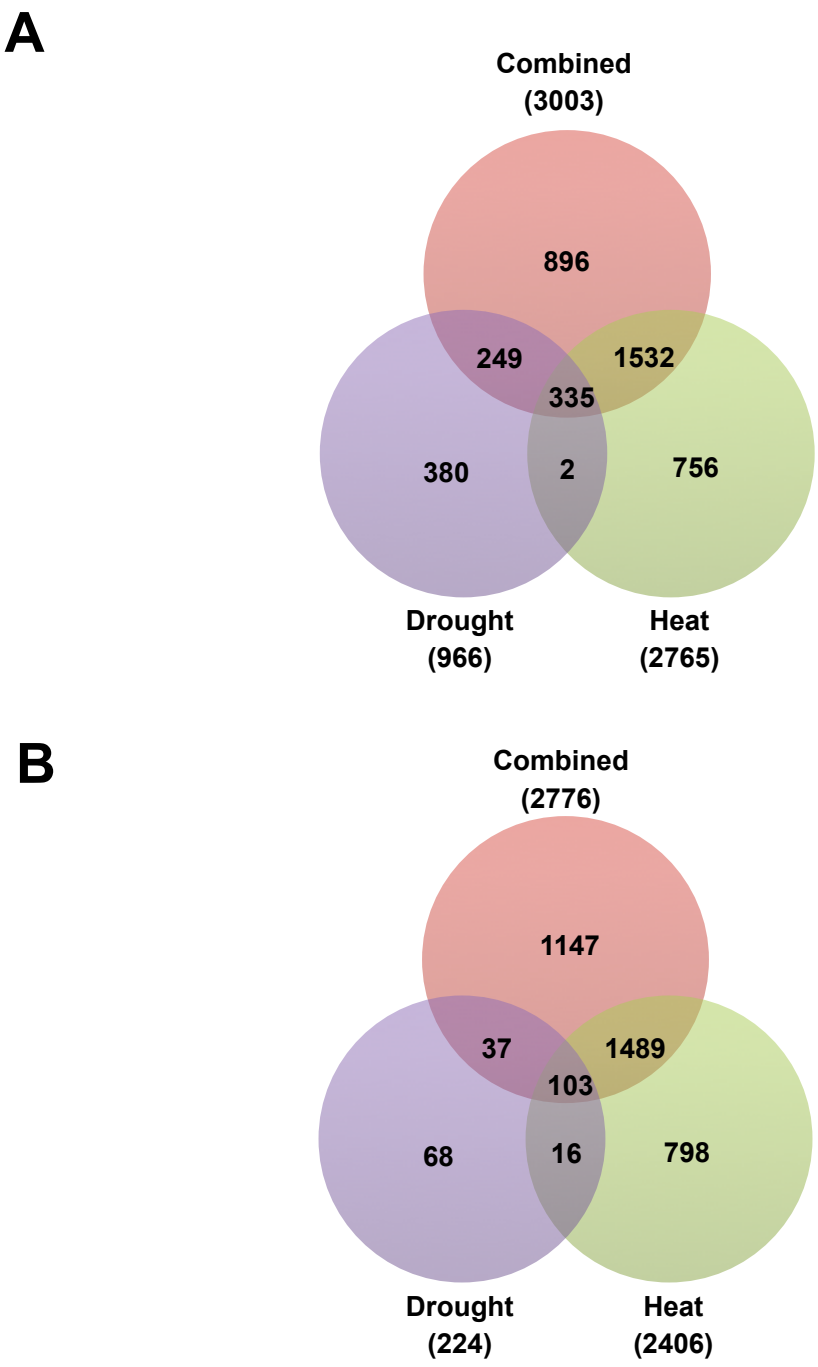


Figure 3.4 - Venn diagrams showing the number of transcripts [A] up-regulated or [B] down-regulated by either heat, drought or combined heat and drought treatments in sorghum leaf tissue (compared to control non-stressed plants). Only transcripts with a change of >2 fold in all three replicates were included.

3.2.1.3.1 Gene expression changes in response to drought

3.2.1.3.1.1 Water withdrawal

As shown in Figure 3.4, 996 transcripts were found to be upregulated and 224 downregulated following drought stress, when compared to the unstressed plants. This equates to approximately 4% of genes on the chip. Amongst the most highly elevated transcripts were those encoding late embryogenesis abundant (LEA) proteins. Other highly elevated genes included delta 1-pyrroline-5-carboxylate 2 (*P5CS2*), which is involved in the metabolism of the compatible solute proline and, high affinity K+ transporter 1 (*HKT1*), a sodium ion transmembrane transporter involved in maintaining cellular Na+ homeostasis (see Appendix D.1 for full list of genes). Interestingly, 380 transcripts were found to be upregulated only in response to drought stress i.e. were not also upregulated in response to heat, or heat and drought in combination (see Appendix D.2). Amongst these transcripts were two genes encoding LEA proteins. This could suggest that different LEA genes may have different, unique roles in the stress response i.e. some may function solely in the drought response whereas others may play a more general role.

In order to explore the biological processes and molecular functions that are enriched within these differentially expressed gene sets, gene ontology (GO) analysis was carried out. The AgriGO gene ontology tool (http://bioinfo.cau.edu.cn/agriGO/) was used to group genes into broad functional categories based on their GO annotations. Singular Enrichment Analysis (SEA) was then carried out to identify particular GO categories that were enriched within the data set ($P<0.05$). The REVIGO tool (http://revigo.irb.hr/) was then used to condense and visualise these GO terms and the resulting pie charts are shown in Figure 3.5. In total, 92 GO categories exhibited significant ($p<0.05$) enrichment amongst the genes upregulated by drought (see Appendix D.8 for the full list of GO terms). As would be expected, the analysis showed an enrichment of genes involved in 'response to stress' and in particular 'response to water deprivation'. Genes associated with 'response to ABA' were also enriched, which is understandable given the central role of ABA in the drought stress response. Other examples of enriched GO categories include regulation of photosynthesis, fluid transport and amino acid metabolism.

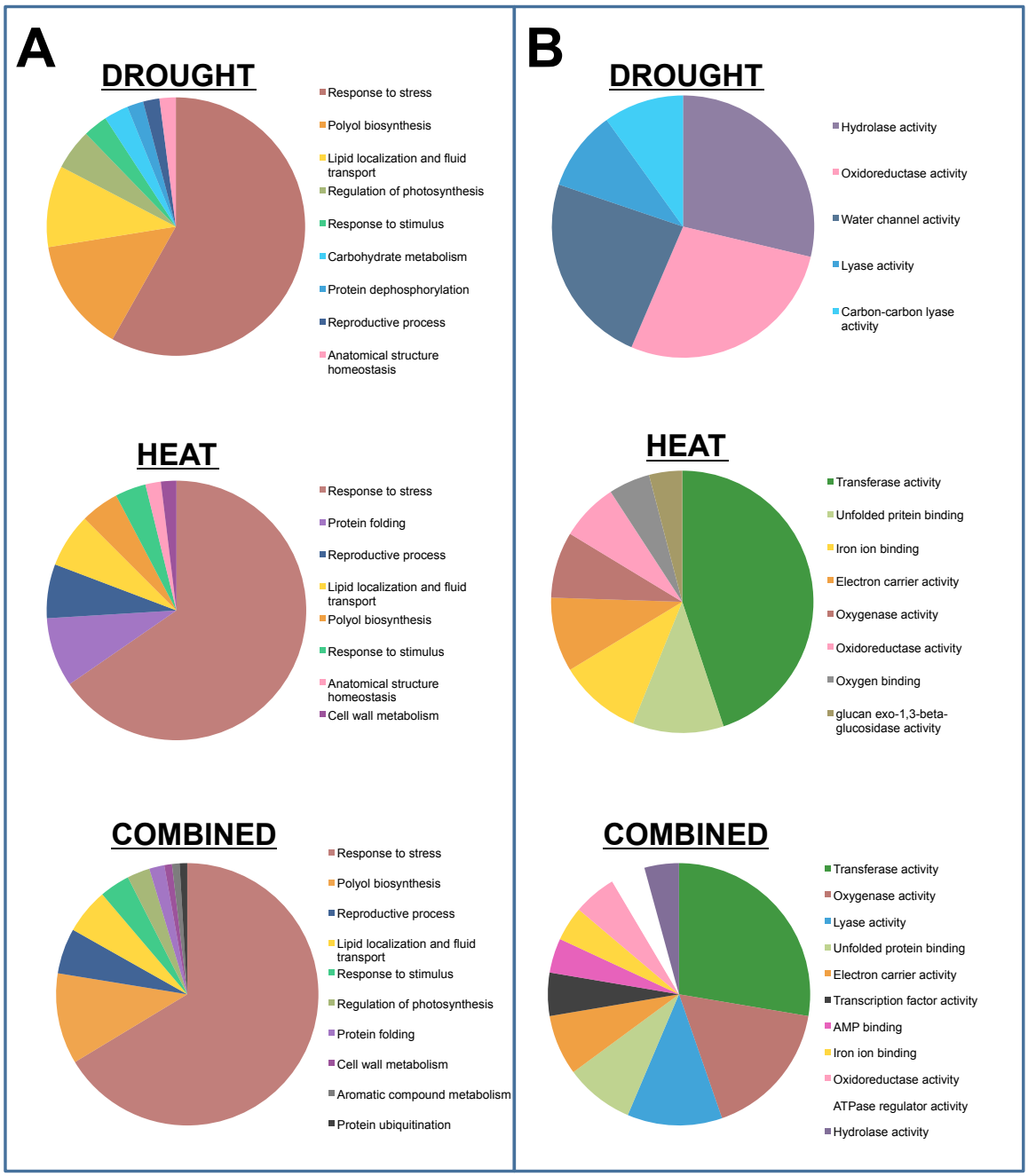


Figure 3.5 - Pie charts showing summarised Gene Ontology (GO) analysis of the genes up-regulated by either drought, heat or combined drought and heat stress. [A] shows biological process GO terms and [B] shows molecular process GO terms. Only GO terms enriched with a p-value of <0.05 were selected and summarized using REVIGO.

3.2.1.3.1.2 Water withdrawal vs. PEG treatment

Previous work carried out by (Dugas et al., 2011), using next generation sequencing, has identified transcript changes in sorghum (BTx623) subjected to osmotic stress, induced by PEG treatment. In order to determine whether there are differences in the sorghum response to different types of osmotic stress i.e. PEG treatment compared to the gradual water loss imposed here; the differentially expressed transcripts identified in both studies were compared. Approximately one third of the drought-induced transcripts identified here were in common with those identified by Dugas et al (2011) (Figure 3.6). However, 902 and 807 transcripts were unique to either the PEG treatment or the water withdrawal treatment respectively. Ontological analysis of the genes unique to the PEG treatment showed an enrichment of genes associated with 'response to reactive oxygen species' (Table 3.1). Interestingly, GO analysis of the genes unique to the gradual water withdrawal showed a strong enrichment of genes associated with wax biosynthesis (Table 3.2). Different biological processes therefore seem to be associated with the two different stress types.

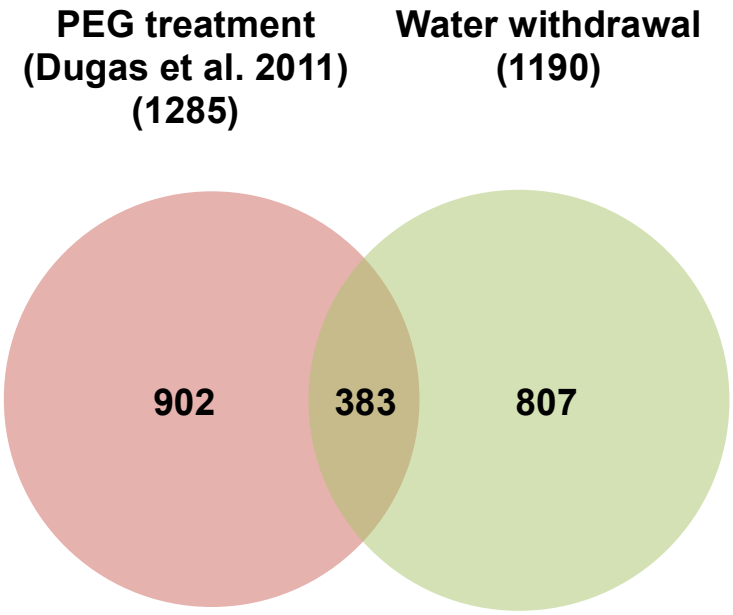


Figure 3.6 - Venn diagram showing the number of transcripts differentially expressed in response to the gradual drought stress imposed here and in response to the PEG treatment imposed by Dugas et. al (2011). Only transcripts with a change of > 2 fold are included.

Table 3.1 - Gene Ontology (GO) terms enriched (p<0.1) in the differentially expressed genes following the sorghum PEG treatment pertained by Dugas et al. (2011) but not in the drought treatment imposed here

GO ACCESSION	GO Term	P-value	% Count in Selection	% Count in Total Genome
GO:0050896	response to stimulus	0.000	39.6	28.1
GO:0006950	response to stress	0.000	23.7	14.7
GO:0009642	response to light intensity	0.000	3.0	0.7
GO:0010035	response to inorganic substance	0.000	5.5	2.2
GO:0042221	response to chemical stimulus	0.000	19.9	13.1
GO:0006805	xenobiotic metabolic process	0.001	1.2	0.1
GO:0009408	response to heat	0.001	3.9	1.3
GO:0009410	response to xenobiotic stimulus	0.002	1.2	0.1
GO:0009607	response to biotic stimulus	0.002	10.0	5.5
GO:0051707	response to other organism	0.004	9.3	5.1
GO:0009644	response to high light intensity	0.005	2.1	0.5
GO:0000302	response to reactive oxygen species	0.008	2.8	0.9
GO:0005576	extracellular region	0.008	6.4	3.1
GO:0006026	aminoglycan catabolic process	0.023	1.1	0.2
GO:0009055	electron carrier activity	0.023	7.6	4.2
GO:0051704	multi-organism process	0.027	10.7	6.6
GO:0005385	zinc ion transmembrane transporter activity	0.029	0.8	0.1
GO:0009628	response to abiotic stimulus	0.029	14.2	9.6
GO:0071577	zinc ion transmembrane transport	0.029	0.8	0.1
GO:0006030	chitin metabolic process	0.032	1.1	0.2
GO:0009719	response to endogenous stimulus	0.041	10.9	6.9
GO:0061134	peptidase regulator activity	0.083	1.7	0.4

Table 3.2 - Gene Ontology (GO terms) enriched (p<0.1) in the differentially expressed genes following the drought treatment imposed here but not in the PEG treatment imposed in Dugas et al. (2011)

GO ACCESSION	GO Term	P-value	% Count in Selection	% Count in Total Genome
GO:0010025	wax biosynthetic process	0.065	1.50	0.25
GO:0032787	monocarboxylic acid metabolic process	0.065	5.26	2.35
GO:0042221	response to chemical stimulus	0.068	18.80	13.05
GO:0016740	transferase activity	0.081	25.26	18.90
GO:0043765	T/G mismatch-specific endonuclease activity	0.081	0.60	0.03
GO:0003824	catalytic activity	0.084	58.95	51.25
GO:0050896	response to stimulus	0.084	35.19	28.12

3.2.1.3.2 Gene expression changes in response to heat

Following heat stress, 2765 sorghum transcripts were upregulated and 2406 were downregulated (~18% of the genes on the chip in total) (Figure 3.4) (see Appendix D.3 for full list of genes). As would be expected, amongst the transcripts most highly upregulated in response to heat were a large number of genes encoding heat shock proteins (HSPs) and universal stress proteins (USPs). Some of these were found to be unique to heat stress, with five elevated only in response to this stress type i.e. not expressed in response to heat and drought combined, or drought alone (see Appendix D.4 for full list of genes unique to the heat stress response). The up-regulation of these HSPs was accompanied by the unique up-regulation of two heat shock factors (HSFs), which are known to regulate the expression of HSPs (Wang et al., 2003). GO analysis of the heat up-regulated genes shows an enrichment of the following categories: response to heat, response to high light, response to reactive oxygen intermediates (ROIs) and protein folding (see Figure 3.5 and Appendix D.9 for full list of GO categories).

3.2.1.3.3 Gene expression changes in response to combined heat and drought

Following the combined heat and drought stress, 3003 transcripts were upregulated and 2776 were downregulated (~20% of gene spots in total) compared to the untreated control (Figure 3.4) (see Appendix D.5 for full lists of genes). Out of the total 5779 (both up and down) gene expression changes, a large proportion (60%) was shared with the heat stress response and 13% were shared with the response to drought (Figure 3.4). Many of the GO categories enriched following combined stress are in common with those enriched following drought or heat alone (Figure 3.5). For example, lipid localization, fluid transport, regulation of photosynthesis and protein folding are all enriched in the combined stress gene set (Appendix D.10).

Interestingly, a number of genes, 896 and 1147, were found to be significantly up- or down-regulated, respectively, *only* in response to combined heat and drought stress (Figure 3.4) (see Appendix D.6 for full gene list). Amongst the elevated transcripts were a number of ion transporters, transcription factors and signalling genes. In addition, transcripts encoding two HSP and three LEA proteins were upregulated in response to

the combined stress treatment only. Functional classification of these differentially expressed genes shows an enrichment of, amongst others, genes associated with 'response to stress', 'response to stimulus' and also 'amine metabolism' (Figure 3.7) (Appendix D.11). Interestingly, within the 'amine metabolism' category are genes associated with polyamine biosynthesis and in particular spermidine synthase 3 (*SPDS3*) (*Sb10g020570.1*) and spermidine synthase 1 *(SPDS1) (Sb02g009730.1*).

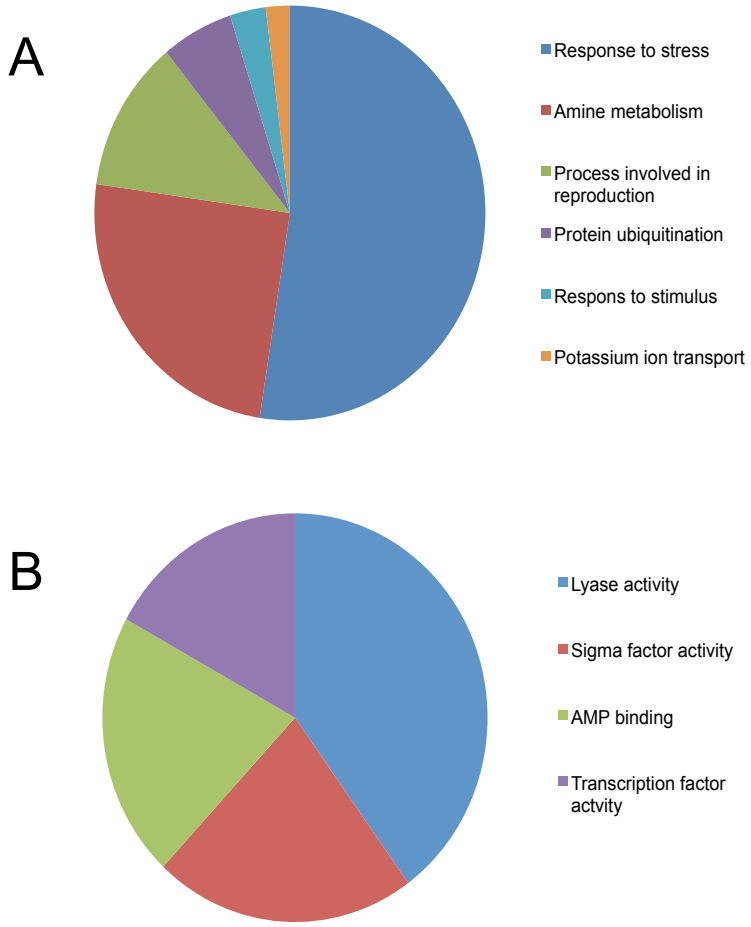


Figure 3.7 - Analysis of genes upregulated only in response to combined heat and drought treatment. Ontological analysis showing [A] enriched biological process and [B] molecular function GO terms ($p<0.05$). Ontological terms were summarized using the REVIGO tool.

3.2.2 qPCR validation of microarray data

In order to validate the robustness of the microarray analysis, qPCR was carried out on tissue from an additional biological replicate. qPCR primers were designed to genes that were shown in the microarrays to be upregulated by one treatment type only i.e. upregulated by either drought, heat *or* the combined stress. This was to validate the specificity of gene expression following these particular treatments. All of the genes tested showed similar expression patterns to those indicated by the arrays (Figure 3.8). The qPCR therefore validated the results obtained by the microarray analysis, confirming the high sensitivity of the high-density microarrays and the robustness of the experimental system.

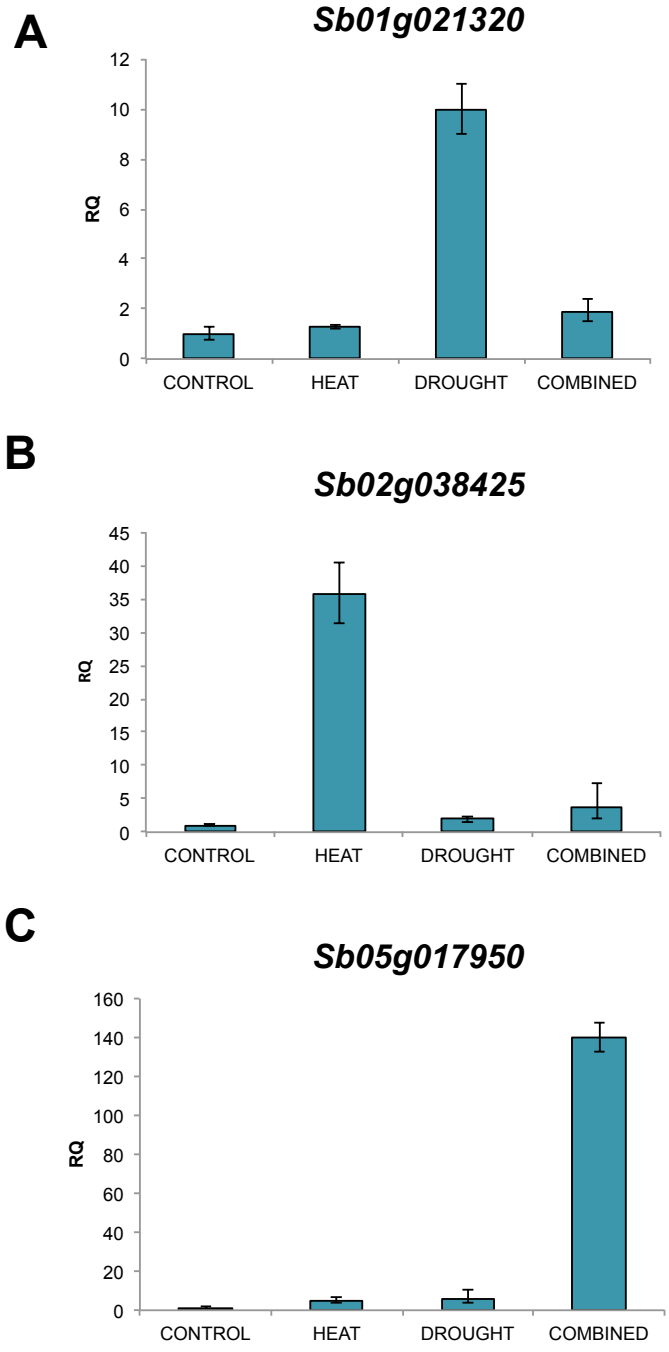


Figure 3.8 - Relative transcript abundance of genes representative of the gene sets identified as being upregulated preferentially by either [A] drought stress, [B] heat stress or [C] combined heat and drought stress. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to the Student's t-test.

3.2.3 Quantification of spermidine levels

Genes associated with spermidine biosynthesis were found to be upregulated specifically in response to the combined heat and drought treatment. Changes at the gene expression level do not always correlate with changes at the protein or biochemical level. In order to determine whether the identified gene expression changes result in changes in actual spermidine levels, amounts of spermidine were quantified using HPLC following each treatment type (Figure 3.9). Surprisingly, whilst genes associated with spermidine biosynthesis were found to be upregulated following the combined stress treatment only, levels of spermidine were actually found to be highest following the heat stress treatment.

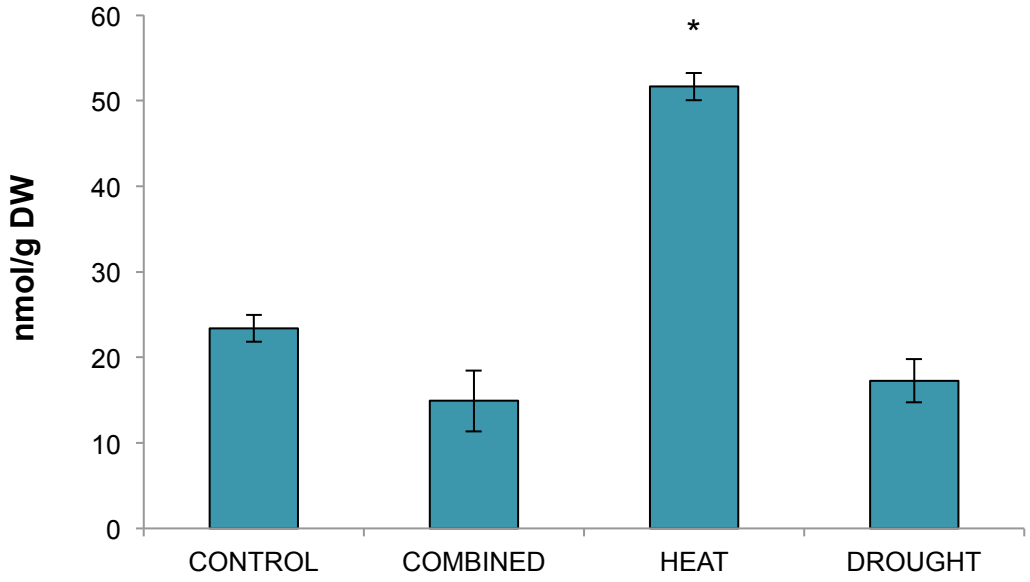


Figure 3.9 - Amount of spermidine in leaf tissue following no treatment (control), heat treatment, drought treatment and combined heat and drought treatment. See Materials and Methods section 2.7.1 for spermidine extraction and quantification procedure. Error bars show standard error (SE) of three technical replicates (*$p<0.05$).

3.3 Discussion

3.3.1 Microarray analysis has identified genes associated with the sorghum response to stress

The microarray analysis has identified genes that are differentially expressed in response to drought, heat and combined drought and heat conditions in sorghum. In response to drought the expression of ~3.5% of the sorghum genes was changed by >2-fold. Many of the genes identified were in known pathways for drought tolerance. For example, a number of genes associated with osmotic adjustment, the maintenance of protein and membrane integrity, alteration in photosynthetic machinery and response to ABA were all changed in response to drought. The utility of the microarray system for identifying stress-responsive transcripts was therefore authenticated by the detection of genes previously found to be stress responsive (Seki et al., 2001; Kreps et al., 2002; Qin et al., 2008).

Heat shock resulted in >2-fold changes in expression of 15% of the genome. All previous studies investigating the plant transcriptional response to multiple stresses have similarly found heat to induce the largest number of genes (Prasch and Sonnewald, 2013; Rasmussen et al., 2013). The large number of gene expression changes is perhaps not surprising given the acute, severe nature of the heat shock and is comparable to studies in other species (Zhang et al., 2005; Qin et al., 2008). It should be noted that heat stress simultaneously induces dehydration stress due to vapour pressure changes; therefore, the effect of this stress is not solely due to the change in temperature.

Some genes appear to be required for universal stress responses and their expression levels were changed in response to all stress types, both single and combined. For example, a number of HSPs and glutathione S-transferases were upregulated by all three stress types. Many HSPs bind to unfolded or denatured proteins and act as ATP-independent molecular chaperones to prevent protein aggregation (Sun and MacRae, 2005). Glutathione S-transferases are involved in the detoxification of substrates such as the products of lipid peroxidation (Gill and Tuteja, 2010). These genes may be involved

in providing general stress protection and could be good candidates for conferring tolerance to multiple stresses.

Previous studies have shown that different transcripts encoding ROI-detoxifying enzymes are induced following different stress types, suggesting that ROI-detoxification occurs through different routes in different situations (Rizhsky et al., 2002). Here, it was found that different genes encoding HSPs and late embryogenesis abundant proteins (LEAs) were upregulated in response to the different stress types. LEAs have protective functions during cellular dehydration through hydration buffering, ion trapping, antioxidant protection, stabilization of sensitive enzymes and membrane protection (Goyal et al., 2005). It is possible that while some LEAs and HSPs have a more general role in the stress response, others are more specific and function in the response to one stress-type only.

3.3.2 Different types of osmotic stress result in the induction of different sets of genes

The number of expression changes identified here in the R16 genotype, in response to drought, were similar to previous studies in which the expression of ~ 4% of the sorghum genome was changed when the BTx623 genotype was subjected to osmotic stress by PEG treatment (Buchanan et al., 2005; Dugas et al., 2011). Out of the total 1190 drought-induced gene changes 32% were shared with the PEG-induced gene changes identified by Dugas *et al.* (2011) using a next generation sequencing transcriptomic approach. The different patterns of gene expression between the two studies may be due to the different genotypes used or the different methods of stress induction. Gene ontology analysis of the genes *only* changed by the PEG treatment, showed an enrichment of the 'response to reactive oxygen species' category. In comparison, gene ontology analysis of the genes *only* changed by the water withdrawal treatment imposed here, showed an enrichment of the 'wax biosynthesis' category. Large quantities of ROS are generated as an early response to stress (Beffagna et al., 2005) therefore reducing ROS levels could be seen as a short term solution to drought. The induction of wax biosynthesis genes on the other hand could result in increased epiculticuar wax that would result in reduced water loss and therefore could be seen as a longer-term strategy

for survival. The slightly different treatments i.e. an osmotic shock using PEG compared to water withdrawal results in the induction of different response pathways and genes.

3.3.3 The sorghum response to combined heat and drought is not simply a merger of the responses to the single stresses

The combined stress response resulted in 5779 gene changes of which a large proportion were in common with the heat-regulated gene set (60%) and around 13% were shared with the response to drought. Such overlap is understandable: whilst there will be unique challenges presented to the plant when stresses are combined, there is still the need to attend to fundamental issues arising from each stress individually. Many of the GO categories enriched by combined stress share elements of those enriched following the other stress types. For example, protein folding is enriched in both the heat and combined stress responses and regulation of photosynthesis and water channel activity is also enriched in the drought stress response. This is understandable given the overlap in gene expression and the fact that similar stresses would require similar downstream processes. The fact that more genes were found to be in common with the heat stress response is likely to be due the acute nature of the heat shock treatment that results in a more synchronised induction of genes.

Interestingly, there is also evidence for specificity in that there are clear sets of genes that are only changed in response to the combined stress treatment. This suggests that the sorghum response to combined heat and drought is not simply an additive effect. This unique response is perhaps to be expected given the partly opposing physiological changes that would normally ensue following this stress type. For example, in response to drought stress, stomata usually close to reduce water loss by transpiration whereas in response to heat stomata tend to open to allow for evaporative cooling (Rizhsky et al., 2002). These opposing physiological changes may be reflected at the transcriptional level in the form of specific sets of differentially expressed genes that are only changed in response to the combined stress. Amongst these are a number of signalling components such as the mitogen activated protein kinase, *MAP20* and the calcium-dependent protein kinase*, CPK16,* which may be involved in transducing the combined stress signal. In addition, a number of transcription factors are only elevated in response

to the combined stress. A gene encoding the NAC transcription factor *ATAF1* for example, is upregulated, a homolog of which has been found to be induced by drought, salinity and wounding in Arabidopsis (Wu et al., 2009). It is possible that this gene has evolved an alternative role in the combined stress response in sorghum.

3.3.4 Genes associated with spermidine biosynthesis are upregulated following combined stress only

Previous studies investigating combined stress have shown that some metabolites, such as sugars, accumulate during stress combination whilst levels of other metabolites such as proline decrease (Rizhsky et al., 2002). Here, we found that S-adenosylmethionine decarboxylase (*SAMDC*), spermidine synthase 1 *(SPDS1) and* spermidine synthase 3 *(SPDS3)*, which are involved in the biosynthesis of the metabolite spermidine, were upregulated exclusively in response to combined heat and drought stress. This suggests that spermidine may play a protective role in the combined stress response. Spermidine has been implicated in tolerance to multiple stresses including high and low temperature, oxidative stress and salinity (Kasukabe et al., 2004; Kusano et al., 2008) and has been suggested to play a role in ROI scavenging and membrane protection (Groppa et al., 2001). Interestingly, one of the uniquely elevated transcription factors identified here, WOX1, has been shown to physically interact with SAMDC suggesting a possible way in which the activity of this enzyme is regulated by combined stress (Zhang et al., 2011). Given the existing literature relating to the role of spermidine in the plant stress response and given that genes involved with spermidine biosynthesis were upregulated, the levels of spermidine following each stress type were quantified using HPLC. Surprisingly, spermidine levels were found to be higher following heat stress and unchanged following the other stresses. This discrepancy could be due to the time delay between gene induction and spermidine biosynthesis. A time course following stress treatment would be required to determine whether this is the case or not. It Alternatively, given that there are a number of genes within the spermidine biosynthesis pathway, perhaps the ones that are upregulated following combined stress are simply not sufficient to increase spermidine levels. This could be due to substrate levels acting as a limiting factor. Putrescine, a different polyamine, is a precursor to spermidine (Kusano et al., 2008) therefore in the future it could be interesting to additionally measure levels of this

metabolite. Spermidine is metabolised into spermine (Kusano et al., 2008) therefore it could be interesting to measure the levels of this polyamine also. There is therefore currently not sufficient evidence to suggest a role for spermidine in the combined stress response. This does however highlight the need to validate gene expression data with either functional analysis of genes using a transgenic approach or with biochemical analysis.

3.3.5 Conclusions

Some genes in sorghum are required for universal stress responses whereas others play a more specific role. This allows plants to properly respond to the specific environmental conditions encountered. We must bear in mind however, that this microarray experiment presents only a 'snapshot'. A time course study would enable visualization of the dynamics of the transcriptional response.

The aim of this chapter was two-fold: firstly, to identify stress-induced transcripts in sorghum and secondly to test the quality and efficiency of custom-designed microarrays. Here it has been shown that these microarrays are useful for the detection of differentially expressed genes therefore; these same microarrays could now be used for the identification of genes underlying the stay-green trait (see Chapter 4).

CHAPTER 4

Microarray analysis comparing gene expression in stay-green and senescent sorghum lines.

4.1 Introduction

An important way in which we can improve our understanding of plant stress tolerance mechanisms is through the analysis of beneficial physiological traits. As reviewed in Chapter 1, the stay-green trait in sorghum confers drought tolerance at the post-flowering stage. Sorghum varieties that possess this trait are able to maintain greater photosynthetic leaf area for longer under drought conditions. This increases the potential period for grain development, which has an obvious positive impact on grain yields under stress conditions (Rosenow et al., 1983; Borrell et al., 2000a; Sanchez et al., 2002). Several sorghum genotypes have been identified that exhibit the stay-green trait including B35, SC56 and E36-1 (Rosenow et al., 1983; Kebede et al., 2001; Haussmann et al., 2002; Sanchez et al., 2002). Of these, B35 is the best characterized with a number of physiological studies being carried out on this variety or its derivatives (Crasta et al., 1999; Xu et al., 2000a; Kassahun et al., 2010; Borrell et al., 2014a; Borrell et al., 2014b).

Previous studies investigating stay-green in sorghum have identified differences in chlorophyll content, transpiration efficiency, relative water content (RWC) and nitrogen status when comparing stay-green and senescent lines (Crasta et al., 1999; Xu et al., 2000a; Borrell and Hammer, 2000b; Kassahun et al., 2010; Vadez et al., 2011) Other studies have identified differences in tillering and leaf size which could impact upon pre-flowering water usage (Borrell et al., 2014a; Borrell et al., 2014b). In addition, other studies suggest stay-green lines are more drought tolerant at the seedling stage and are

better able to tolerate heat stress (Jagtap and Bhargava, 1995; Jagtap et al., 1998; Burke et al., 2010). This trait is however, undoubtedly complex and despite these advancements the physiological and molecular basis of the stay-green trait remains unclear.

One promising avenue by which this problem can be addressed is through the use of functional genomics tools. As described in Chapter 3 transcriptomic analyses, including microarrays, are a valuable way in which mechanistic insights into biological phenomena can be obtained. For example, the comparison of gene expression in different samples can provide insight into the actual biological processes that are perturbed following a specific treatment or between different genotypes. In sorghum, a number of transcriptomic experiments have lead to the identification of stress-induced transcripts (Buchanan et al., 2005; Dugas et al., 2011; Johnson et al., 2014). The release of the sorghum genome sequence (Paterson et al., 2009) and the development of a metabolic pathways database, SorghumCyc (http://pathway.gramene.org/gramene/sorghumcyc.shtml) have greatly facilitated these studies. Microarray analysis could therefore be a powerful approach for elucidating some of the molecular and biochemical pathways involved in conferring the stay-green trait in sorghum.

This chapter focuses on microarray experiments carried out to compare gene expression between stay-green (drought-tolerant) and senescent (drought-sensitive) lines in an attempt to identify biological processes and molecular pathways underlying the stay-green trait.

Aims of the research described in this chapter:

- To compare gene expression between both stay-green lines (B35, E36) and the senescent variety R16 at 45 days after sowing (4.2.1)
- To validate the gene expression data using qPCR (4.2.1.3)
- To carry out ontological analysis of this gene expression data and to use this to identify important processes and genes underlying the stay-green trait (4.2.1.4 & 4.2.1.5)

- To compare gene expression between both stay-green lines (B35, E36-1) and the senescent lines (R16, S35) at 14 days after sowing under both control and drought conditions (4.2.2).

4.2 Results

Many of the results described in this chapter are published in (Johnson et al., 2015)

4.2.1 Microarray analysis comparing gene expression in stay-green and senescent lines at a mature age (45 days after sowing)

4.2.1.1 Sample selection

B35 and E36-1 varieties were selected as sources of stay-green for the microarray analysis based on the fact that the majority of genetic and physiological studies so far have been carried out on these lines (Rosenow et al., 1983; Jagtap et al., 1998; Kebede et al., 2001; Haussmann et al., 2002; Burke et al., 2010; Vadez et al., 2011). E36-1 is thought to be a unique source of stay-green; while some stay-green QTLs map to the same loci as in B35, others were only identified from E36-1 (Haussmann, 2002). R16 was used as a senescent variety. It is high yielding but very senescent and has been used as the recurrent parent in a number of mapping studies (Kassahun et al., 2010; Vadez et al., 2011).

Tissue samples were taken prior to flowering at around 45 days after sowing under well-watered conditions. At this developmental stage the flag leaf was just beginning to emerge and there were around ten leaves. At this time point there were higher levels of chlorophyll fluorescence (Figure 4.1), therefore at least one known element of the trait was manifesting at this time point (Thomas and Howarth, 2000). Samples were taken prior to flowering because it is thought that the increase in grain yield in the stay-green varieties following stress at the post-flowering stage can be attributed to the emergent consequence of genes acting at the pre-flowering stage (Borrell et al., 2014a; Borrell et al., 2014b). In addition, when testing for stay-green in the field, irrigation is normally withheld at this time point i.e. just prior to flowering (Xu et al., 2000; Kassahun et al., 2010) therefore conceivably any drought tolerance mechanism should be in place and can be captured by the microarrays. Tissue was taken under well-watered conditions. This is because many previously observed differences in physiology between stay-green and senescent lines were identified under non-stressed conditions i.e. differences in chlorophyll content and transpiration efficiency (Kassahun et al., 2010; Vadez et al.,

2011; Borrell et al., 2014a). Samples were harvested on four separate occasions to provide four biological replicates (Figure 4.2).

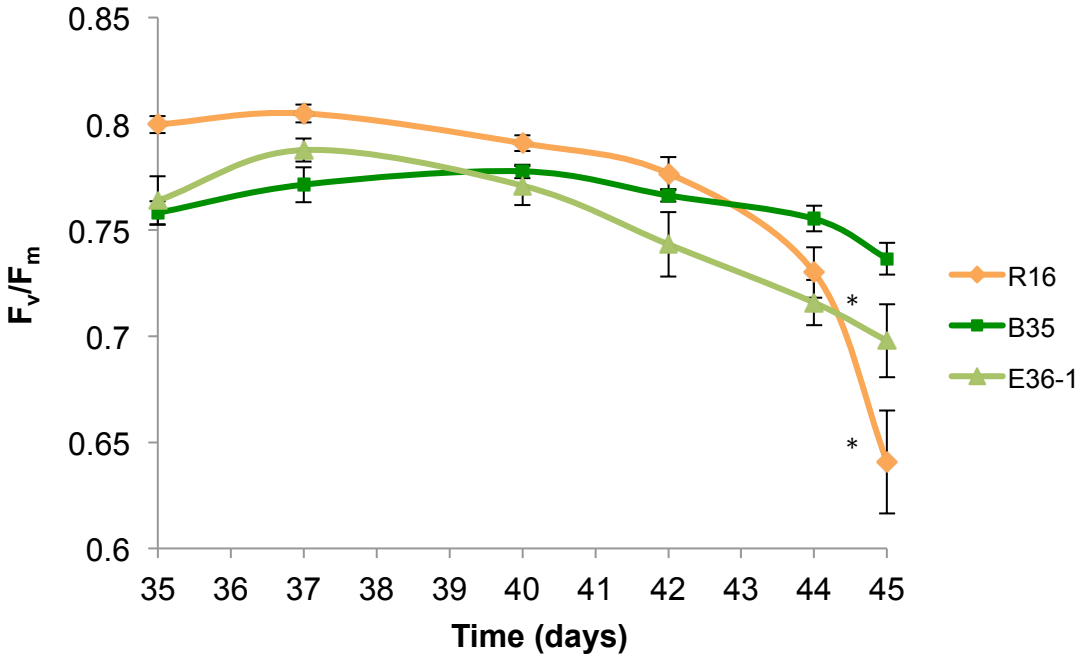


Figure 4.1 - Measurements of F_v/F_m in stay-green (B35 and E36-1) and senescent (R16) sorghum varieties from 35 days after sowing (DAS). Measurements were taken from the middle of leaf 2 and 4 of each plant. The average of multiple measurements is shown. Error bars represent SE (n=8). This was carried out for each of the four biological replicates and the graph above shows a representative result (*$p<0.05$).

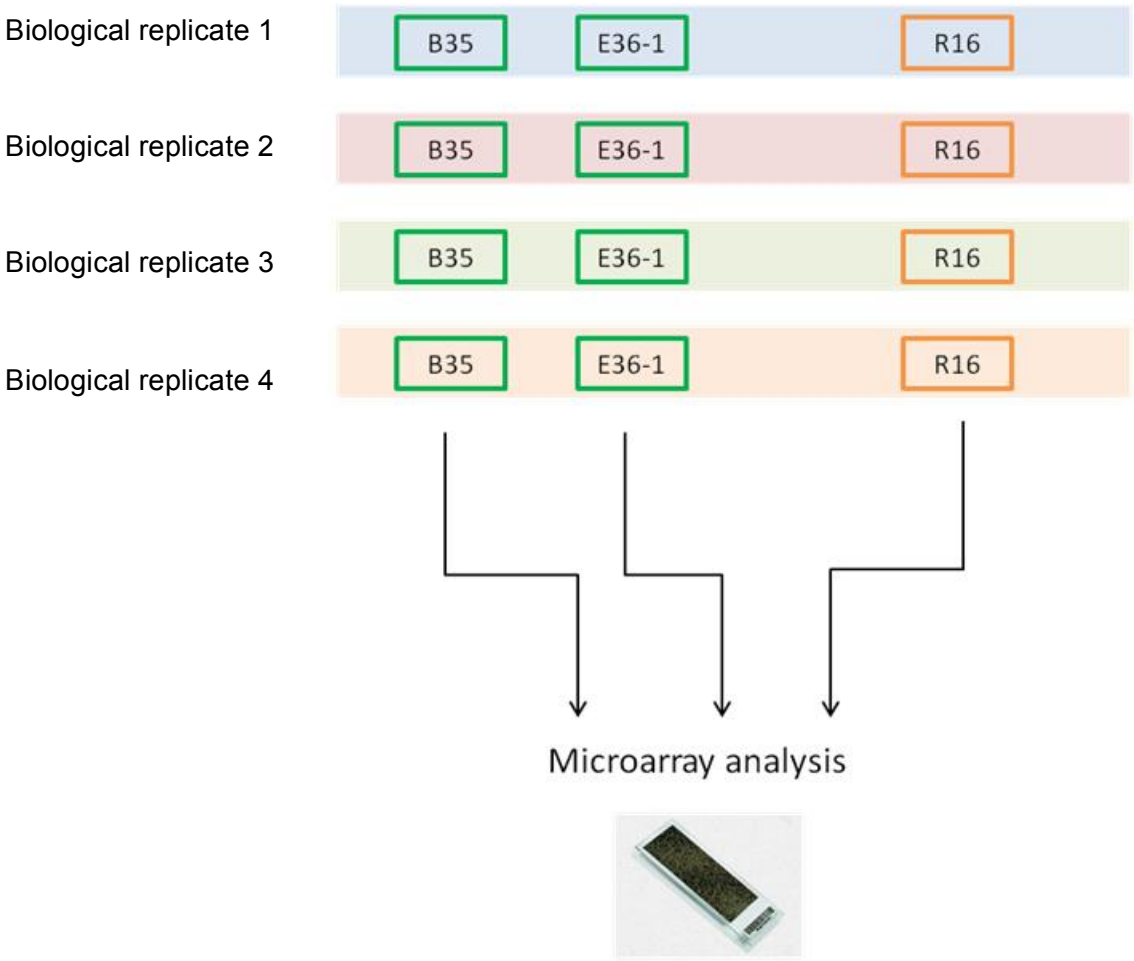


Figure 4.2 - Experimental design for microarray analysis carried out on sorghum leaf tissue at 45 DAS. Green boxes signify stay-green varieties and orange boxes signify senescent varieties.

4.2.1.2 Identification of differentially expressed genes

RNA was extracted from the 12 samples and the high quality and integrity of the RNA was confirmed using a bioanalyzer (see Figure 3.2 for an example electrophoreogram). The RNA samples were then labelled with Cy3 and hybridized to the same sorghum customized microarray chips described in Chapter 3. Initial quality control analyses were

performed on the resulting scanned arrays. While the majority of the arrays were of good quality, two samples (B35 and E36-1 from biological replicate 1) were excluded from any further analysis due to poor quality control values. Despite this, there were still at least three biological replicates remaining for each genotype. The extracted data was then analysed using GeneSpring GX 11 software (Agilent Technologies, CA, USA). Principle Component Analysis (PCA) was again used to summarize the data set and to identify predominant gene expression profiles. The samples showed distinct clustering, with samples of the same variety clustering together (Figure 4.3). There was therefore strong consistency across biological replicates.

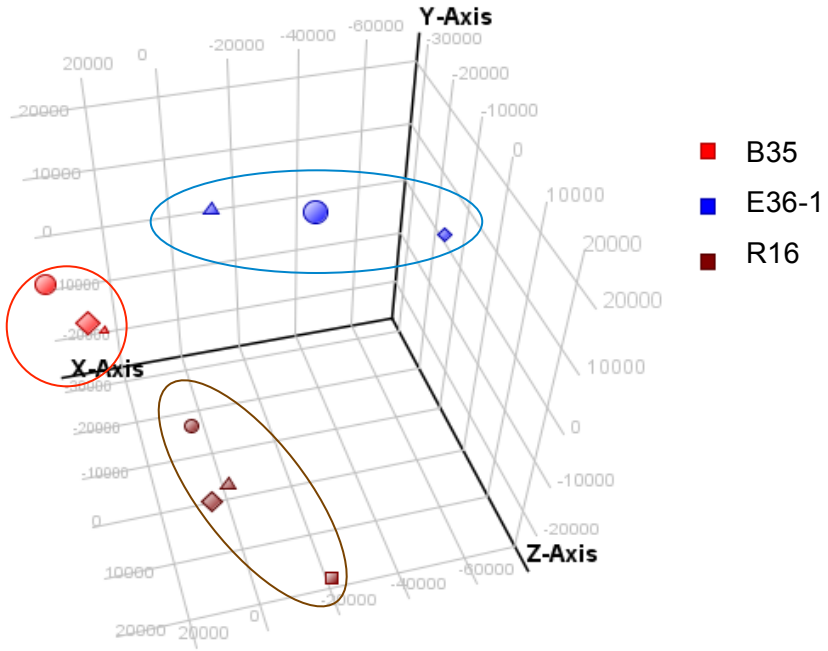


Figure 4.3 - Principle component analysis (PCA) plot showing the predominant gene expression profiles of each sample. The different colours represent the different varieties whilst the different shapes represent the different biological replicates. The X-Axis represents 35.5% of the variance, the Y-axis represents 17.78% and the Z-axis represents 17.7%.

The raw data was pre-processed and normalized as described in the Materials and Methods section (see 2.5.5). Gene expression levels in each stay-green line (B35 and E36-1) was compared to that of the senescent R16 line and differentially expressed genes were identified based on an absolute fold change of >2 and a p-value cut-off of <0.05 (Moderated T-test with Benjamini-Hochberg correction). As shown in Figure 4.4, 1038 genes were expressed to higher levels (FC>2, p<0.05) in B35 compared to R16 and 998 genes were expressed to lower levels. These gene expression changes constitute 3.6% and 3.4% of total genes on the chip for the higher expressed and lower expressed genes respectively. A similar number of gene expression changes were identified when comparing E36-1 with R16 with 1090 genes being expressed to higher levels and 1143 genes expressed to lower levels, accounting for ~8% of genes on the chip in total (Figure 4.4). Genes that were differentially expressed in *both* B35 and E36-1 vs. R16 comparisons totalled 827 while 993 were unique to B35 and 1406 were unique to E36-1 (Figure 4.5). See Appendices D.12 - D.17 for full gene lists.

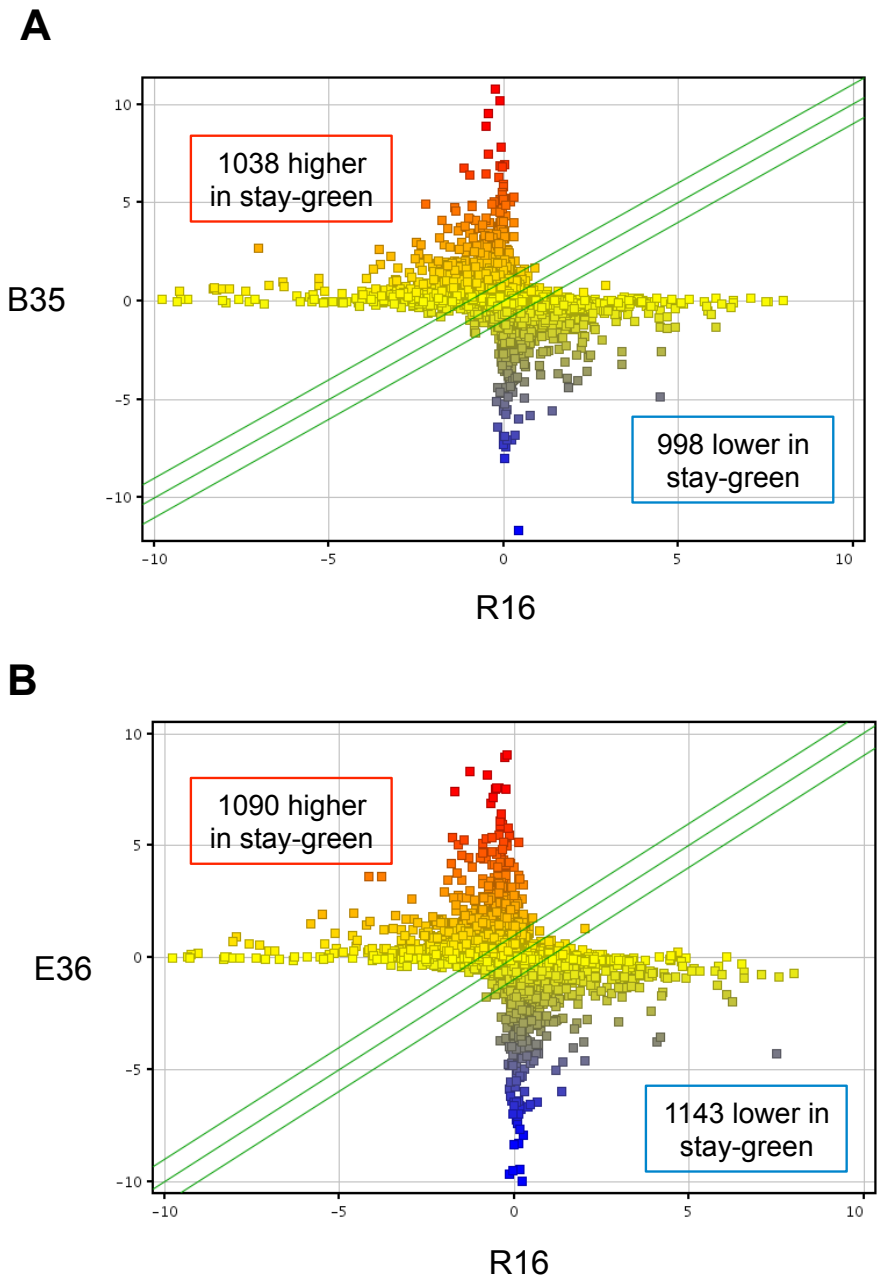


Figure 4.4 - Scatter plots showing the distribution of expression of filtered genes in [A] the stay-green (B35) line compared to the senescent (R16) line and in [B] the stay-green (E36) line compared to the senescent (R16) line. Axes denote normalized gene expression and squares represent individual genes. The green lines mark a 2-fold cut-off value. Differentially expressed genes are based on a 2-fold cut-off and a p-value<0.05. Colour corresponds to normalized gene expression with red representing high relative expression and blue representing low relative gene expression.

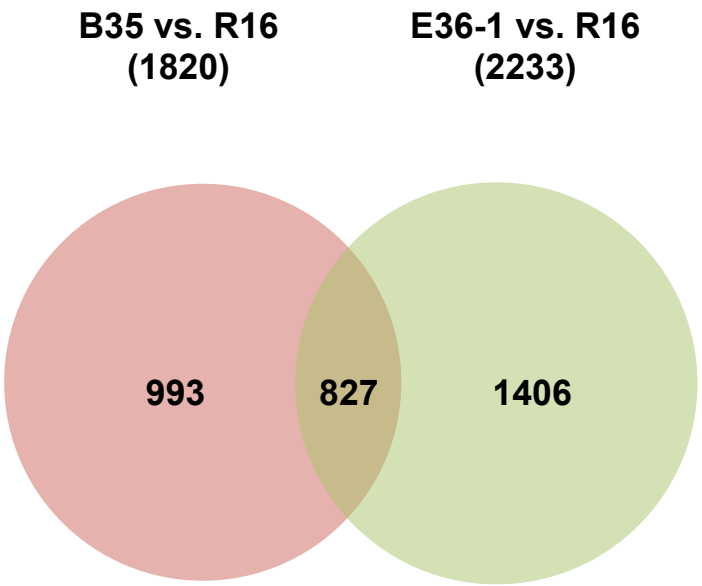


Figure 4.5 - Venn diagram showing total numbers of differentially expressed genes in both B35 vs. R16 and E36-1 vs. R16 comparisons. Fold change >2 and $p<0.05$.

4.2.1.3 Validation of microarray data using qPCR

In order to validate the robustness of the microarrays, qPCR analysis was carried out on an additional three biological replicates using the Fluidigm system for large-scale qPCR (see Materials and Methods section 2.4.9.2). Probes were designed to 87 randomly chosen genes and gene expression levels were compared between B35 and R16. As a result, 80% of the genes tested showed a similar expression pattern in both the qPCR and microarray analysis (Figure 4.6). Similar to what has been previously reported, for genes with large increases or decreases in magnitude, microarrays underestimated the magnitude of the changes (Etienne et al., 2004), implying that qPCR is more accurate when fold changes are large. Nevertheless, the qPCR confirmed the results obtained by the microarray analysis, confirming the robustness of the experimental system.

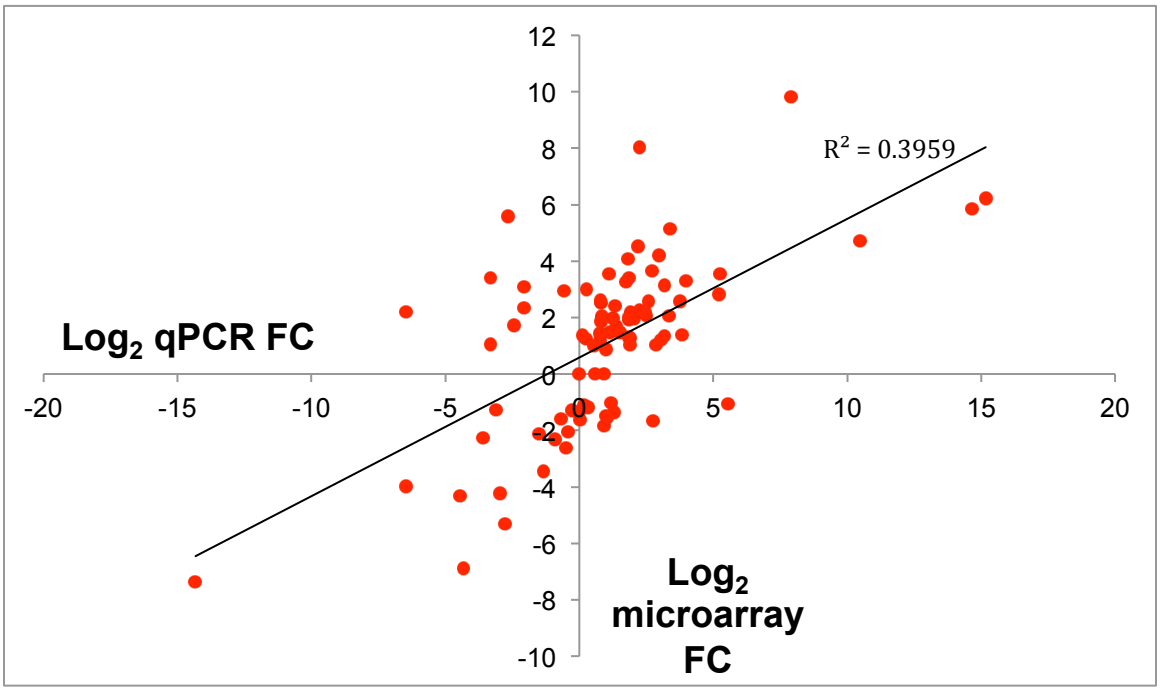


Figure 4.6 - Scatter plot comparing the fold changes of 87 genes obtained by microarray analysis and by qPCR in B35 vs. R16 at 45 DAS. qPCR was carried out using the Fluidigm system (see Materials and Methods 2.4.9.2).

4.2.1.4 Functional classification of genes

4.2.1.4.1 B35 vs. R16

In order to explore the biological processes and molecular functions that are enriched within these differentially expressed gene sets, gene ontology (GO) analysis was carried out. In the same way as described in Chapter 3, the AgriGO gene ontology tool was used to group genes into broad functional categories and then Singular Enrichment Analysis (SEA) was carried out to identify enriched ($p<0.05$) GO categories. The REVIGO tool (http://revigo.irb.hr/) was then used to condense and visualize these GO terms. Pie charts showing the GO terms enriched in the genes expressed at higher levels in B35 compared to R16 are shown in Figure 4.7. Tables showing full lists of enriched GO terms can be found in Appendix D.18.

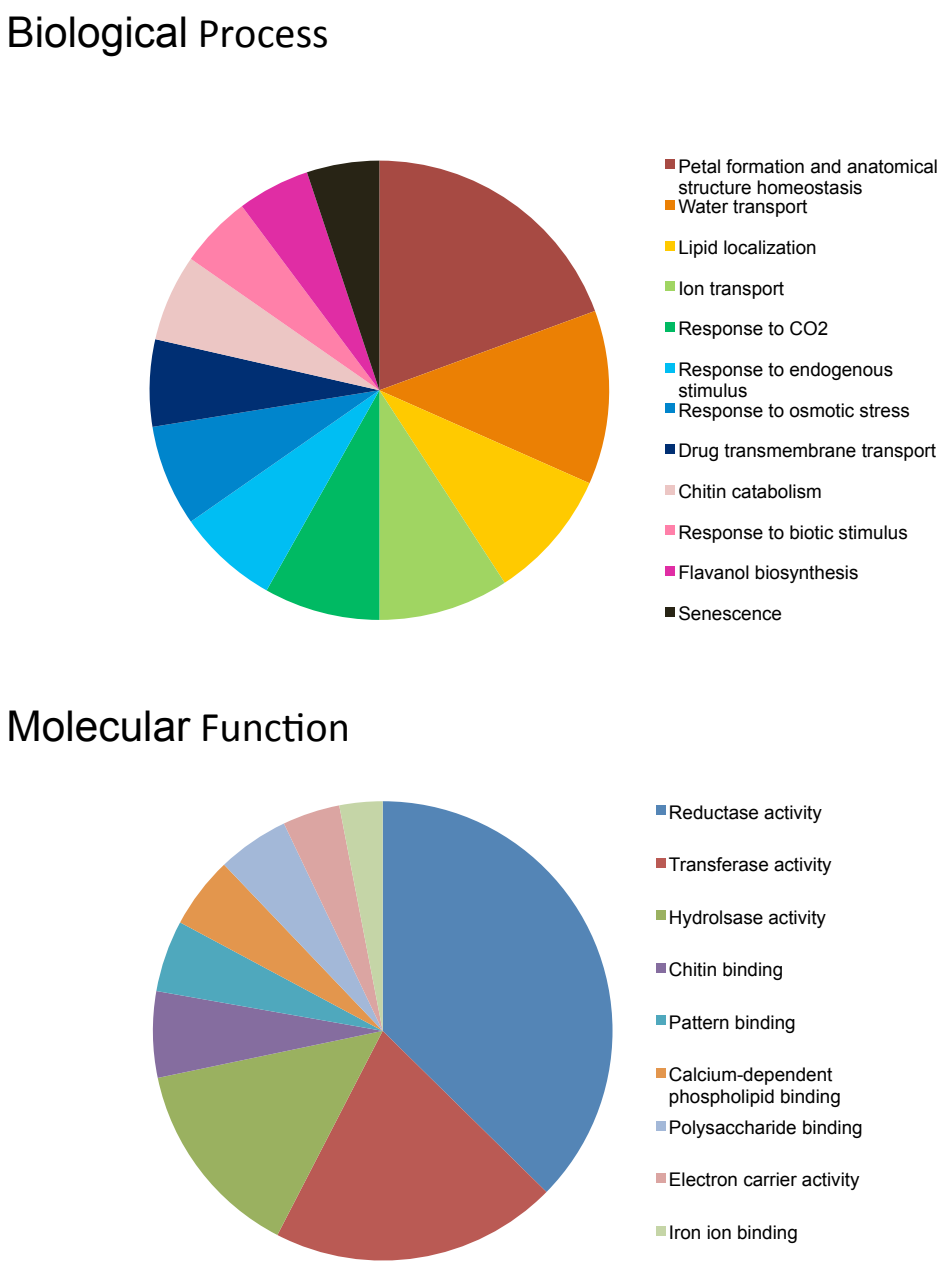


Figure 4.7 - Pie charts showing significantly enriched GO terms ($p<0.05$) for the 1038 genes expressed to higher levels in the stay-green (B35) variety compared to senescent (R16) variety (fold change (FC)>2, $p<0.05$). Significant GO terms were identified using AgriGO and then condensed and visualized using the REVIGO tool (see Materials and Methods section 2.5.6).

GO categories enriched within the genes expressed to higher levels in B35 include developmental processes such as 'post-embryonic morphogenesis' and 'anatomical structure homeostasis' (Figure 4.7 and Appendix D.18). Notably, processes that are known to be associated with the plant response to low water availability were also enriched, including 'response to osmotic stress' and 'water transport'. In order to determine whether genes associated with other known stress stimuli were differentially expressed, the percentage of genes in the input list that are associated with these stimuli was compared to the percentage of genes in the genome that are associated with that same stimulus. Interestingly, the 'response to osmotic stress' category was found to be the only strongly enriched stimulus, with nearly 5% of the genes in the input list belonging to this category. The response to wounding category showed slight enrichment however there was no enrichment of genes associated with any of the other stress stimuli (Figure 4.8).

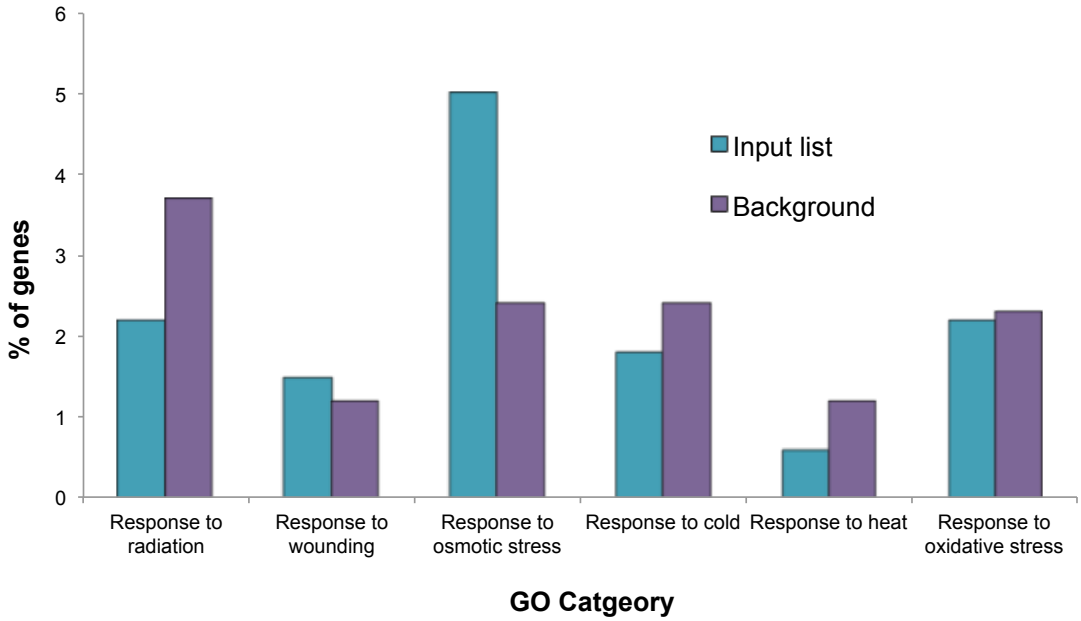


Figure 4.8 - Singular enrichment analysis of genes that are expressed to higher levels (FC>2, p<0.05) in the stay-green (B35) variety compared to the senescent (R16) variety. The bar chart shows the percentage of the genes expressed to higher levels in B35 that are associated with different stress stimuli (Input list) compared to the percentage of genes in the genome involved with that same stimulus (Background).

The 42 genes that were involved specifically in the plant 'response to osmotic stress' category were identified and their expression levels analysed (Figure 4.9). This list contains a dehydration-responsive element-binding (*DREB1A)* transcription factor, a ubiquitin ligase called salt and drought-induced RING finger 1 (*SDIR1*) and a CBL-interacting serine/threonine-protein kinase 1 (*CIPK1*). Other up-regulated genes include trehalose-6-phosphate synthase (*TPS*) and delta-1-pyrroline-5-carboxylate-synthase (*P5CS2*), which are known to be important for the biosynthesis of trehalose and proline, respectively (Goddijn and van Dun, 1999; Ashraf and Foolad, 2007). The genes that are associated with the 'water transport' GO category encode different plasma membrane intrinsic protein 2B (PIP2B) isoforms. These proteins are known to be aquaporins which function as water channels within membranes in a range of plant species (Maurel et al., 2008). Whilst not all of the enriched processes shown in Figure 4.7 and listed in Appendix D.18 will necessarily be causal to the stay-green phenotype, it is possible that the higher expression of genes involved with the plant response to osmotic stress in B35 may be contributing to its ability to maintain green leaf area for longer under drought conditions.

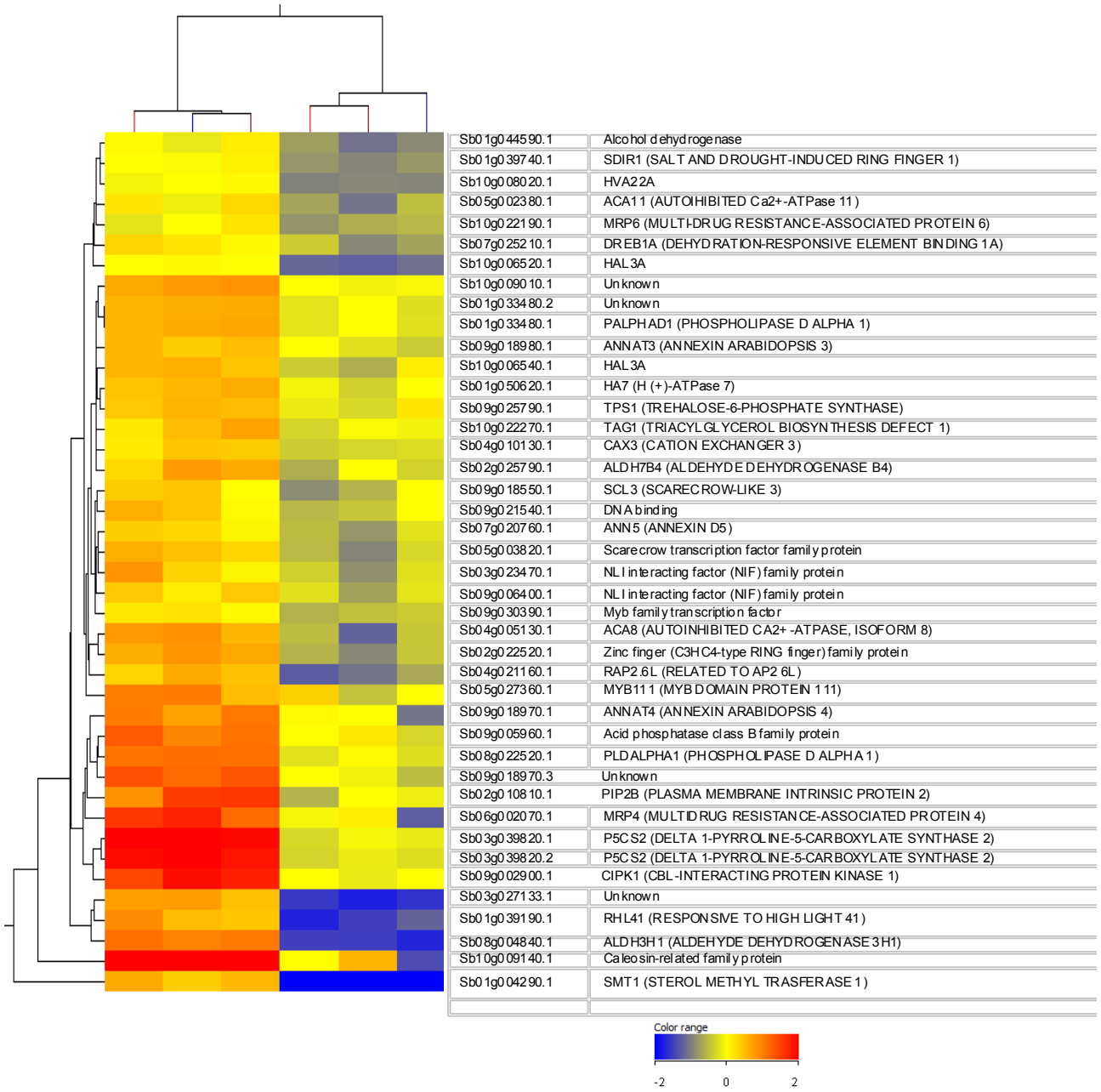


Figure 4.9 - Heat map showing the 42 genes expressed at higher levels in the stay-green (B35) variety (FC>2, p<0.05) when compared to the senescent (R16) variety that are also associated with the 'response to osmotic stress' GO category. Colour denotes normalized gene expression. Hierarchical clustering was carried on both samples and genes.

Additional enriched GO categories included 'senescence', 'lipid localization' and 'response to endogenous stimulus'. In order to identify which stimuli in particular the 'response to endogenous stimulus' category refers to, a more detailed GO analysis was carried out. The percentage of genes in the input list that were associated with different hormone stimuli was compared to the percentage of genes in the genome that are associated with that same stimulus. As shown in Figure 4.10 there was an enrichment of genes associated with the response to auxin, ABA, jasmonic acid, brassinosteroids and ethylene when compared to the background genome.

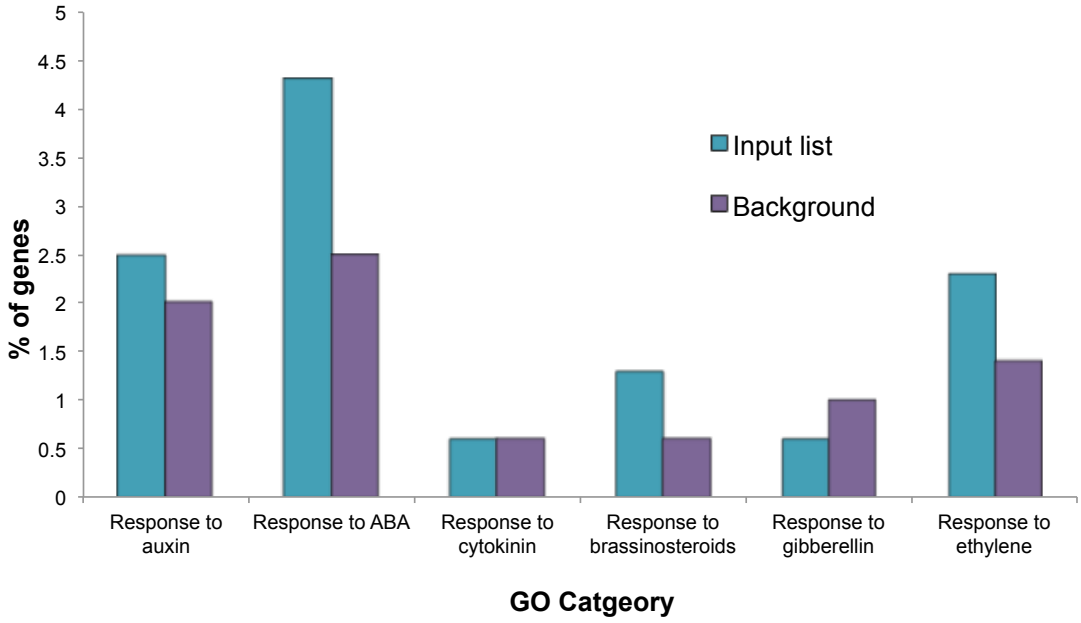


Figure 4.10 - Singular enrichment analysis of genes that are expressed at higher levels (FC>2, $p<0.05$) in the stay-green (B35) variety compared to the senescent (R16) variety. The bar chart shows the percentage of the genes expressed to higher levels in B35 that are associated with different hormone stimuli (Input list) compared to the percentage of genes in the genome involved with that same stimulus (Background).

4.2.1.4.2 E36-1 vs. R16

The lists of genes that were differentially expressed in E36-1 compared to R16 can be found in Appendices D.14 & D.15. The ontological analysis for the E36-1 vs. R16 comparison can be found in Appendix D.19. Given that B35 is the most well characterized stay-green line and has revealed interesting ontological results it was decided that the E36-1 line would not be investigated further in order to maximise characterization of B35.

4.2.1.5 Analysis of known drought associated processes

As reviewed in Chapter 1 a number of pathways and processes can contribute to plant stress tolerance. However, if only a small number of genes associated with a particular process are differentially expressed, they might not appear in the ontological analysis despite their potentially large impact. For this reason, a more subjective approach was additionally employed and the expression of genes involved in processes known to be involved in drought tolerance was analysed in both the stay-green and senescent lines.

4.2.1.5.1 Compatible solutes

Compatible solutes are known to accumulate in plants under low water conditions and confer protective properties (Wood et al., 1996; Hsu et al., 2003; Ashraf and Foolad, 2007; Su et al., 2011). Well-studied compatible solutes include the amino acid proline and the quaternary amino acid glycine betaine (Ashraf and Foolad, 2007). Others include the amino acids aspartic acid and glutamic acid and the sugar trehalose (Goddijn and van Dun, 1999; Chen and Jiang, 2010). The SorghumCyc metabolic pathways database (http://pathway.gramene.org/gramene/ sorghumcyc.shtml) was used to identify all sorghum genes involved in the biosynthesis of these known compatible solutes. The expression of these genes was then compared between B35 and R16 (Figure 4.11). None of the genes in the aspartic acid or glutamic acid pathways were found to be differentially expressed (either up or down) by >2 fold. However, three genes associated with the biosynthesis of proline were expressed to higher levels in B35. In addition, nine genes that are associated with the biosynthesis of betaine were differentially expressed

by >2 fold; three were expressed to higher levels in B35 and five were expressed to lower levels. Only one gene associated with trehalose biosynthesis, *TPS* (*Sb02g033420.1*) was expressed to higher levels in B35.

4.2.1.5.2 Polyamines

Polyamines are thought to provide cellular protection under stress conditions (see Chapter 1). Genes associated with the biosynthesis of the polyamine spermidine were also found to be upregulated following heat and drought stress suggesting a role for spermidine in the drought stress response (see Chapter 3). The expression levels of genes involved in polyamine biosynthesis were analysed in both varieties, again using the SorghumCyc database. No genes in this pathway were differentially expressed between the varieties.

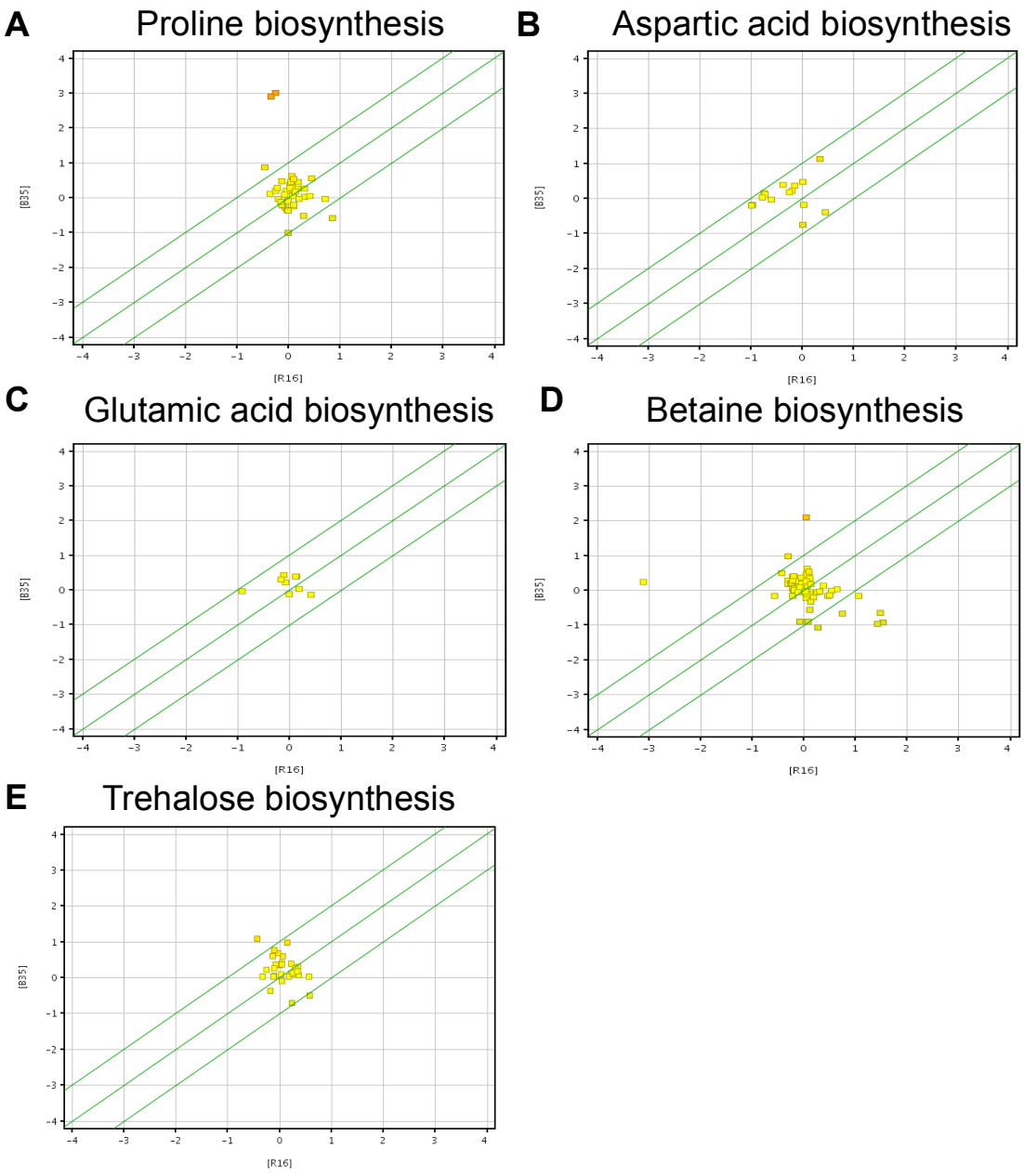


Figure 4.11 - Scatter plots showing the distribution of expression of filtered genes involved with [A] proline biosynthesis [B] aspartic acid biosynthesis [C] glutamic acid biosynthesis [D] betaine biosynthesis and [E] trehalose biosynthesis. Genes were identified using SorghumCyc metabolic pathways database. Axes denote normalized gene expression (X-axis shows R16 values and Y-axis shows B35 values) and squares represent individual genes. The green lines mark a 2-fold cut-off value.

4.2.1.5.3 Oxidative stress tolerance

Minimising the harmful effects of reactive oxygen species (ROS) can limit cellular injury following drought stress and thus can delay senescence (Jaleel et al., 2009). In order to determine whether any ROS detoxification pathways were enhanced in the stay-green lines, genes involved in known ROS detoxification pathways were identified and their expression levels analysed. Whilst there were no differentially expressed superoxide dismutases or catalases, two ascorbate peroxidase genes were expressed to higher levels (172 and 118 -fold) in B35. A number of glutathione S-transferases (GSTs) were also differentially expressed with 14 genes expressed to higher levels and six expressed to lower levels (Figure 4.12).

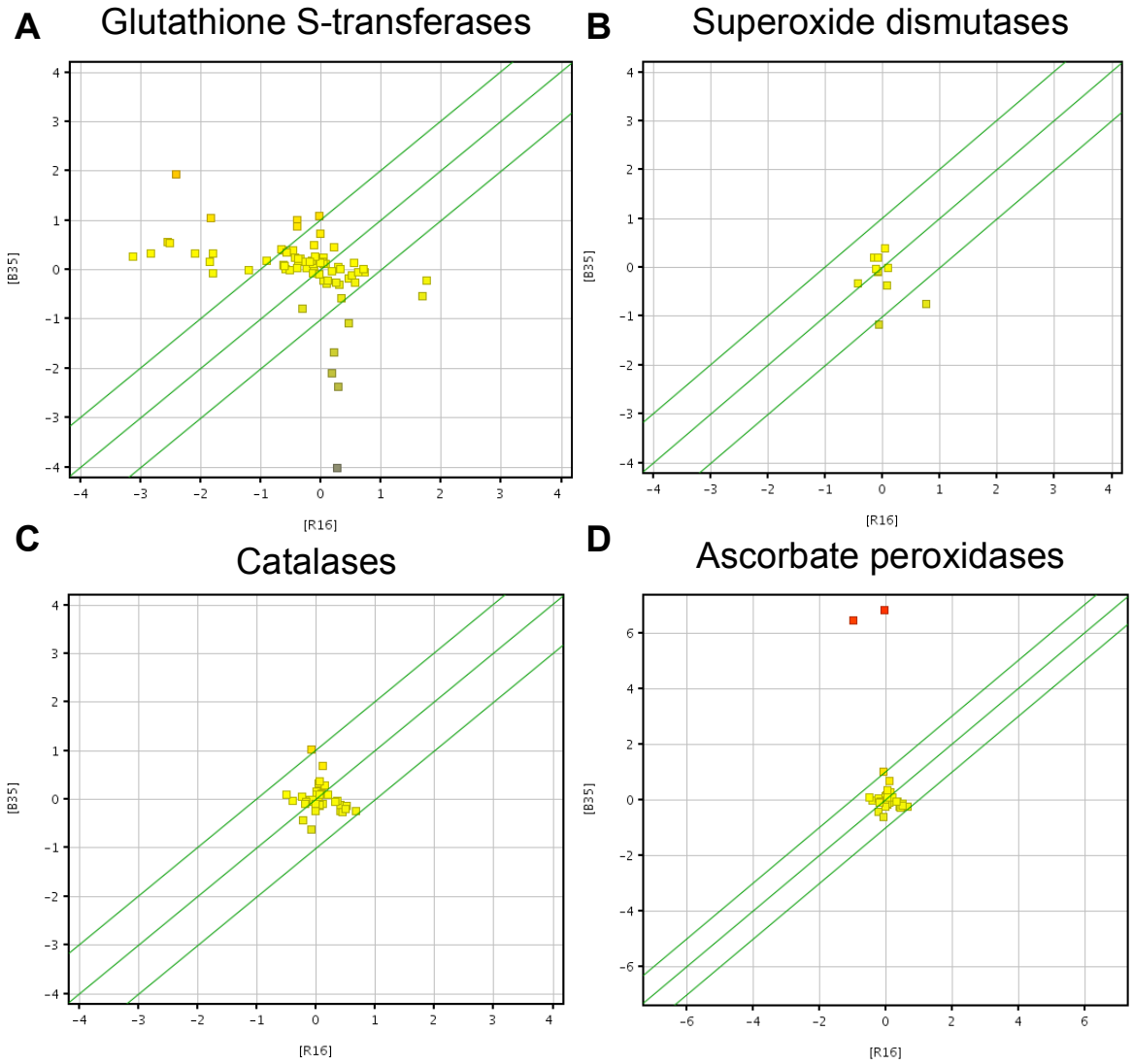


Figure 4.12 - Scatter plots showing the distribution of expression of filtered genes associated with the oxidative stress response including [A] glutathione-S-transferases [B] superoxide dismutase [C] catalases and [D] ascorbate peroxidases. Genes were identified using SorghumCyc metabolic pathways database. Axes denote normalized gene expression (X-axis shows R16 values and y-axis shows B35 values) and squares represent individual genes. The green lines mark a 2-fold cut-off value.

4.2.1.5.4 Regulation of transpiration

The regulation of water loss via transpiration is a key way in which plants can avoid low water status. The amount of water transpired depends on both stomatal numbers and aperture (Ingram and Bartels, 1996; Sanchez et al., 2002). Given that there are known differences in transpiration efficiency between stay-green and senescent lines (Vadez et al., 2011), all genes associated with the stomatal movement and development GO categories were analysed. Out of 163 genes analysed 17 were differentially expressed (Table 4.1). Amongst these was a gene called stomatal density and distribution 1 (*SDD1)* and two slow anion channel 1 (*SLAC1*) homologs.

Table 4.1 - Genes differentially expressed in B35 compared to R16 (>2 fold; p-value <0.05) at 45 DAS that are associated with the stomatal movement and development GO categories

SbID	FC (abs)	P-value	Regulation	Annotation
Sb09g029920.1	3.15	0.0147	up	SLAH2 (SLAC1 HOMOLOGUE 2)
Sb09g029910.1	2.51	0.0028	up	SLAH2 (SLAC1 HOMOLOGUE 2)
Sb08g022520.1	3.71	0.0010	up	PLDALPHA1 (PHOSPHOLIPASE D ALPHA 1)
Sb06g002070.1	7.16	0.0241	up	ATMRP4 (ARABIDOPSIS THALIANA MULTIDRUG RESISTANCE-ASSOCIATED PROTEIN 4)
Sb03g003280.1	3.22	0.0147	up	CKX5 (CYTOKININ OXIDASE 5)
Sb03g002810.1	9.29	0.0065	up	CKX5 (CYTOKININ OXIDASE 5)
Sb02g020470.1	3.60	0.0017	up	SDD1 (STOMATAL DENSITY AND DISTRIBUTION)
Sb01g033480.2	2.28	0.0049	up	
Sb01g033480.1	2.29	0.0051	up	PLDALPHA1 (PHOSPHOLIPASE D ALPHA 1)
Sb01g019000.1	8.19	0.0156	up	CKX6 (CYTOKININ OXIDASE/DEHYDROGENASE 6)
Sb10g001960.4	9.00	0.0002	down	
Sb10g001060.1	2.05	0.0101	down	XLG3 (extra-large GTP-binding protein 3)
Sb07g022530.1	2.60	0.0077	down	CKX7 (CYTOKININ OXIDASE 7)
Sb03g028240.1	3.95	0.0025	down	JAR1 (JASMONATE RESISTANT 1)
Sb03g012785.1	2.80	0.0026	down	PHS1 (PROPYZAMIDE-HYPERSENSITIVE 1)
Sb03g012770.1	3.70	0.0016	down	PHS1 (PROPYZAMIDE-HYPERSENSITIVE 1)
Sb03g011640.1	5.69	0.0097	down	MYB61 (MYB DOMAIN PROTEIN 61)

4.2.1.5.5 NAC transcription factors

As reviewed in Chapter 1, NAC transcription factors play a role in both drought tolerance and senescence processes. For this reason the expression of NAC transcription factors was analysed in B35 and R16. All sorghum genes with similarity to the Arabidopsis NAC NTL4 (At3g10500.1) were identified using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A total of 96 genes were identified in sorghum as also containing the NAM super family domain and of these, six were found to be significantly ($p<0.05$) differentially expressed between the B35 and R16 varieties (Table 4.2).

Table 4.2 - Genes containing the NAM super family domain that were differentially expressed in B35 compared to R16 (>2 fold; $p<0.05$) at 45 DAS.

SbID	FC (abs)	P-value	Regulation	Annotation
Sb10g009670.1	2.18	0.0012	down	ANAC008 (Arabidopsis NAC domain containing protein 8)
Sb07g005610.1	3.03	0.0001	down	DNA binding
Sb02g026510.1	4.27	0.0023	down	NAP (NAC-like, activated by AP3/PI); transcription factor
Sb10g027100.1	39.65	0.0000	down	DNA binding
Sb05g001590.1	2.38	0.0030	up	ANAC087
Sb03g037940.1	2.26	0.0008	up	ATAF2

4.2.2 Microarray analysis comparing gene expression in stay-green and senescent lines at the seedling stage (14 days after sowing)

Some studies have suggested that elements of the stay-green phenotype can be assessed at a younger growth stage (Jagtap and Bhargava, 1995; Jagtap et al., 1998; Burke et al., 2010) and others have suggested that the drought tolerance of the stay-green lines at the post-flowering stage arises as a result of genes acting much earlier (Borrell et al., 2014a). Given the need to identify genes causal to the phenotype, microarray analysis was also carried out on sorghum seedlings (around 14 days after sowing).

4.2.2.1 Sample selection

For this experiment four genotypes were compared; the stay-green lines B35 and E36-1 and the senescent lines R16 and S35. S35 is a highly senescent sweet sorghum variety which has also been used as a recurrent parent in a number of mapping studies (Vadez et al., 2011). In order to assess whether any elements of the stay-green phenotype are drought inducible, each variety was sampled under both well-watered conditions and following a period of water withdrawal.

Twelve seedlings of each of the four varieties were grown until 14 DAS. The plants were then split into two groups and randomized, with one group being maintained in a well-watered state and the other group not receiving any more water (see materials and methods 2.5.1.1). The F_v/F_m of all of the seedlings was measured daily following water withdrawal. Leaf samples were taken at the point when the F_v/F_m of the drought-treated seedlings first started to significantly differ from that of the well-watered controls (Figure 4.13). This occurred at around three days following water withdrawal. This was to ensure that drought stress was actually occurring at the time of sampling. Three biological replicates of this experiment were carried out (see Figure 4.14 for experimental design).

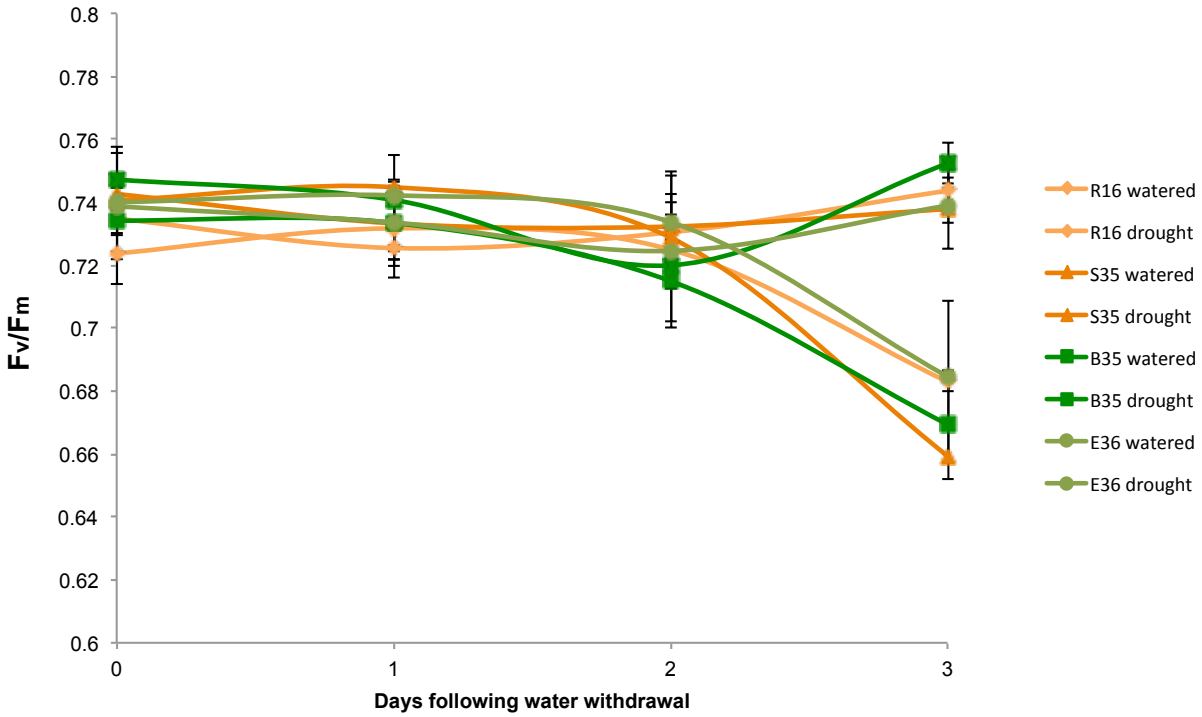


Figure 4.13 - The F_v/F_m (variable fluorescence/maximal fluorescence of Photosystem II) of sorghum seedlings that were either well watered or subjected to drought stress. Drought stress was imposed by withdrawing water from 14 DAS. Photosynthetic efficiency was measured daily, using a FluorCam, following the initiation of water withdrawal (see Materials and Methods section 2.6.2.2). Green lines signify stay-green varieties and orange lines signify senescent varieties.

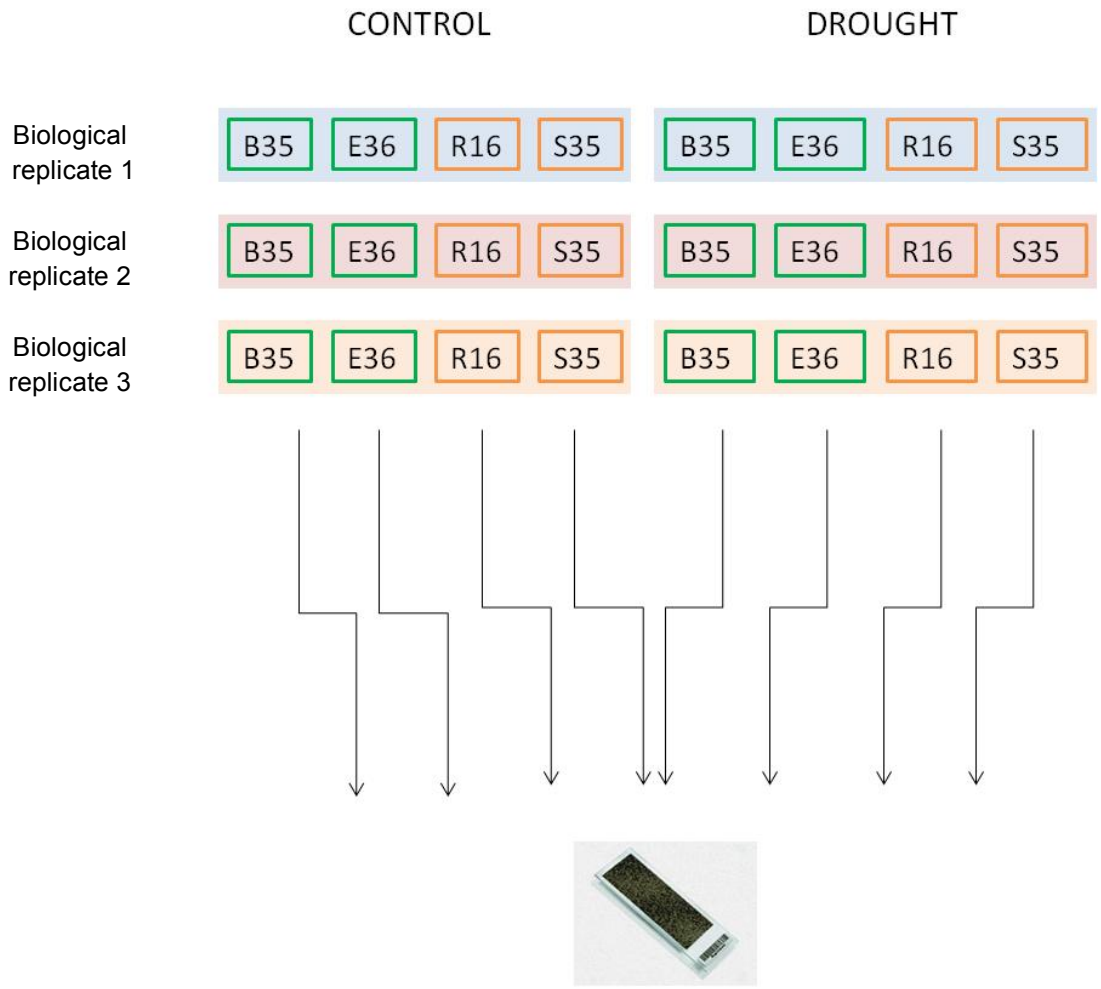


Figure 4.14 - Experimental design for microarray analysis carried out at 14 days after sowing. Green boxes signify stay-green varieties and orange boxes signify senescent varieties.

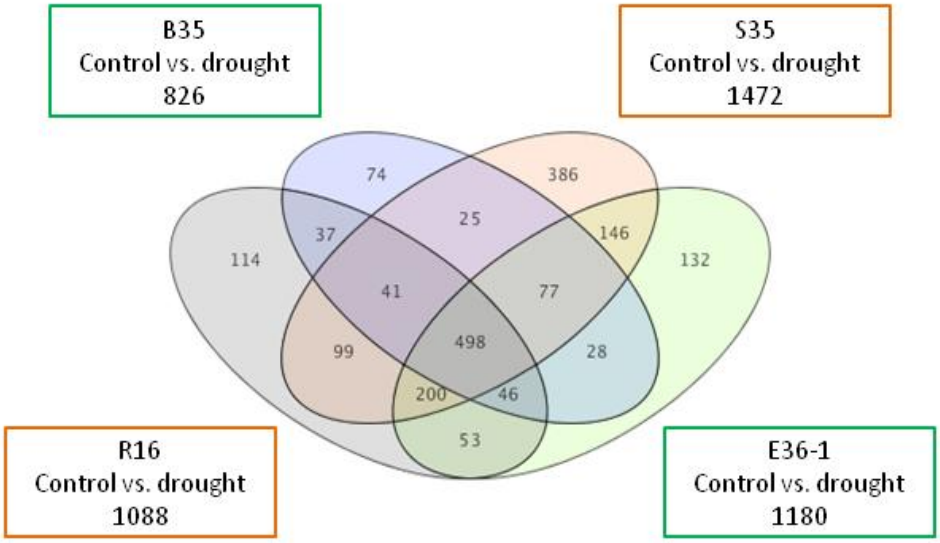
4.2.2.2 Drought-induced gene expression

The extracted RNA was labelled and hybridized to the Agilent custom-designed arrays. Genes were filtered based on presence in all three replicates in either the control or

drought-treated samples resulting in around 20,000 gene probes for each control versus drought treatment comparison.

As shown in Figure 4.15, only 546 transcripts were upregulated following drought stress in all four varieties. A number of differentially expressed transcripts were found to be unique to one particular variety. For example, 947 genes were upregulated and 386 downregulated by drought stress in S35 only. Genes unique to the drought response of the stay-green B35 and E36-1 lines totalled 153 and 137 respectively. All of the differentially expressed genes from this experiment can be found in Appendices D.20 – D.31.

UP-REGULATED



DOWN-REGULATED

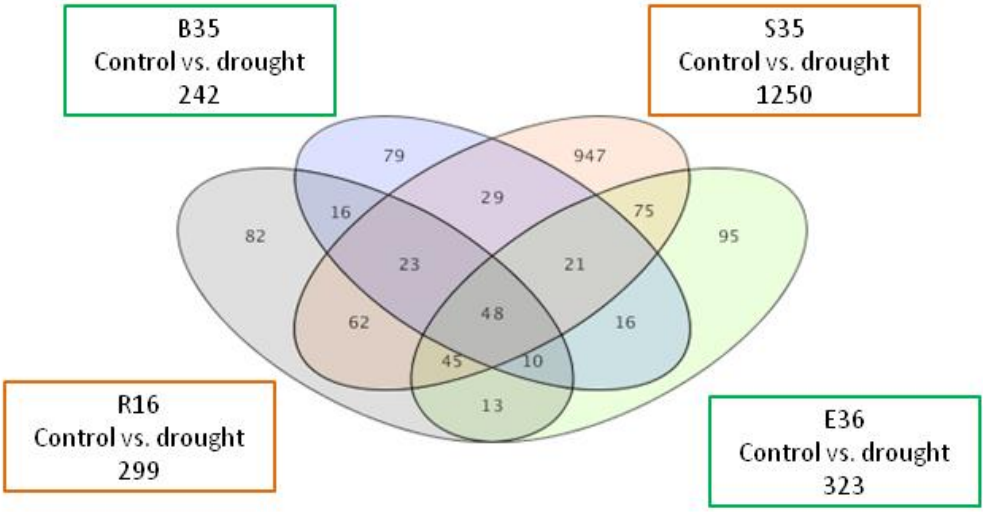


Figure 4.15 - Venn diagrams showing gene expression changes following drought stress at 14 DAS in 4 different sorghum varieties (B35, S35, R16 and E36-1). B35 and E36-1 are stay-green varieties as indicated by the green box and R16 and S35 are senescent varieties as indicated by the orange box. Only transcripts with a change of >2 fold in each treatment vs. control comparison in all three replicates were included.

4.2.2.3 Analysis of known drought-associated processes

A number of processes were identified in mature sorghum tissue as being expressed to higher levels in B35 compared to R16 (see 4.2.1.5). To determine whether any of these processes were also expressed to higher levels at the seedling stage, the expression of these genes was analysed in the younger tissue. As can be seen in Figure 4.16, genes associated with proline and betaine biosynthesis were additionally differentially expressed at the seedling stage along with genes encoding glutathione S-transferases and an ascorbate peroxidase. These processes therefore appear to be constitutively more active in B35 throughout its life.

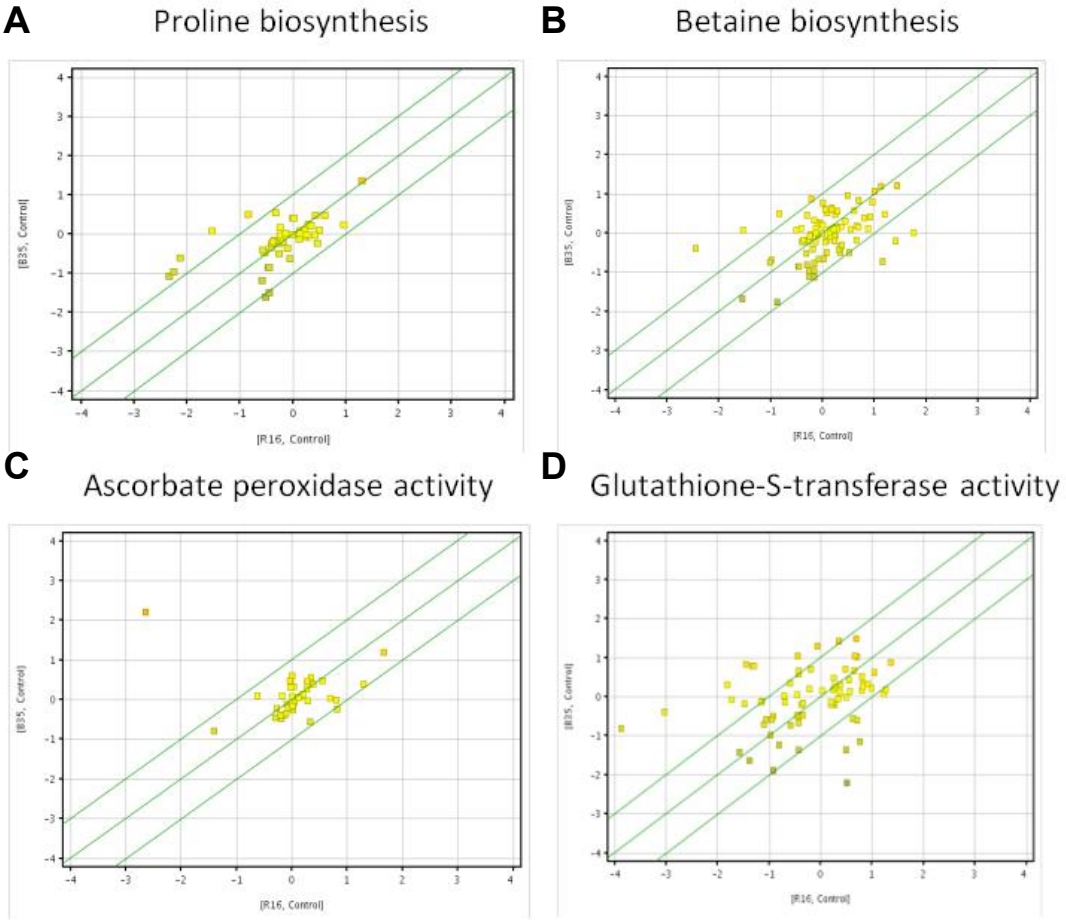


Figure 4.16 - Scatter plots showing the distribution of expression of filtered genes associated with [A] proline biosynthesis [B] betaine biosynthesis [C] ascorbate peroxidase activity and [D] glutathione-S-transferase activity in B35 and R16 varieties at 14 DAS. Genes were identified using SorghumCyc metabolic pathways database. Axes denote normalized gene expression (X-axis shows R16 values and y-axis shows B35 values) and squares represent individual genes. The green lines mark a 2-fold cut-off value.

4.3 Discussion

Microarray analysis was used to identify pathways, processes and genes that underlie the stay-green trait in sorghum. Some of the genes and processes expressed to higher levels in the B35 stay-green line compared to the senescent R16 line are discussed below, along with their biological contexts.

4.3.1 Osmotic adjustment

Three transcripts involved with the biosynthesis of proline (two different *P5CS2* transcripts and a glutamate S-semialdehyde dehydrogenase) were expressed to higher levels (FC>2) in B35 compared to R16. In addition, nine genes associated with betaine biosynthesis were differentially expressed between the varieties along with one gene associated with trehalose biosynthesis (trehalose 6-phosphate synthase). Proline, betaine and trehalose are organic solutes that are well known to accumulate within plant cells under water-limiting conditions. This accumulation lowers cellular water potential which ultimately allows plants to maintain cell turgor (Chaves et al., 2003). Proline and betaine have a number of additional protective properties including the ability to detoxify reactive oxygen species (ROS), protect membrane integrity and stabilize proteins (Wood et al., 1996; Hsu et al., 2003; Ashraf and Foolad, 2007; Su et al., 2011). As a result, drought tolerant varieties of a range of species, including sorghum, have been found to have higher levels of these solutes and transgenic lines that produce more proline and betaine have been found to be more drought-tolerant (Kishor et al., 1995; Hong et al., 2000) (Sivaramakrishnan, 1988; Hsu et al., 2003; Nayyar and Walia, 2003; Ashraf and Foolad, 2007). Stay-green varieties are known to have a higher relative water content (RWC) compared to other varieties (Xu et al., 2000b), which could be maintained due to higher levels of solutes. Taken together, these results suggest that the accumulation of compatible solutes may be contributing towards the drought tolerance of the stay-green lines.

4.3.2 Oxidative stress response

Genes that are involved in protection against ROS-induced damage were also expressed to higher levels in the stay-green line compared to the senescent line. For example, glutathione S-transferases (GSTs) represent an abundant class of differentially expressed genes (15 in total) along with two ascorbate peroxidases. GSTs are involved in the detoxification of substrates, such as peroxidised lipids, following drought stress (Jaleel et al., 2009). Drought-tolerant sorghum varieties have been found to have lower levels of lipid peroxidation under low water conditions compared to drought-sensitive varieties, and to have significantly higher levels of GST activity following salt stress (Jagtap and Bhargava, 1995; Jogeswar et al., 2006). Taken together, it is possible that the GSTs that are expressed to higher levels in B35 are providing protection against ROS and so are contributing to its drought-tolerant phenotype.

4.3.3 Regulation of transpiration

Genes that could be involved in regulating stomatal numbers or aperture were found to be differentially expressed between the stay-green and senescent lines. For example, a stomatal density and distribution (*SDD1*) gene that could potentially impact upon stomatal numbers was expressed to higher levels in B35, along with two *SLAC1* homologues that may play a role in regulating stomatal aperture (Berger and Altmann, 2000; Vahisalu et al., 2008). A gene called salt and drought-induced RING finger 1 (*SDIR1)* was also expressed to higher levels in B35. When homologs of this gene were over-expressed in a range of species, the resulting transgenic plants displayed reduced stomatal aperture and exhibited improved drought tolerance compared to wild type (Zhang et al., 2007; Zhang et al., 2008; Xia et al., 2012; Tak and Mhatre, 2013). Given that the sorghum stay-green lines have been reported to have improved transpiration efficiency compared to other lines (Kassahun et al., 2010; Vadez et al., 2011), it is possible that the differential expression of these genes, either on their own or in combination, could be contributing to differences in water loss and hence drought tolerance.

4.3.4 Transcription factors

Transcription factors including *DREB1A*, *RAP2.6* and a number of NAC transcription factors were expressed to higher levels in B35 compared to R16. The activation of transcription factors can cause a cascade of events resulting in the activation of a number of downstream stress-responsive genes. They are therefore thought to be excellent targets for the genetic engineering of stress tolerance. DREB transcription factors in particular are well known to play a role in the plant response to drought stress and a plethora of studies show increased stress tolerance in transgenic lines over-expressing homologs of this gene (Dubouzet et al., 2003; Qin et al., 2004; Xiong and Fei, 2006; Zhao and Bughrara, 2008; Yang et al., 2011). Similarly, the differential expression of the NAC transcription factors, NTL4 and JUB1, in Arabidopsis transgenic lines results in increased stress tolerance and delayed senescence (Lee et al., 2012; Wu et al., 2012). It is therefore possible that the differential expression of the transcription factors identified here could be contributing to the drought tolerance of the stay-green varieties.

4.3.5 Other

A number of genes associated with the 'response to endogenous stimulus' GO category were expressed to higher levels in the B35 stay-green line. These genes were found to be involved with the response to hormone stimuli including the response to auxin, ABA, brassinosteroids, ethylene and jasmonic acid (Figure 4.10). Previous studies have suggested that developmental differences between stay-green and senescent varieties may contribute towards the stay-green trait. For example, stay-green near-isogenic lines (NILs) have been shown to display reduced tillering and reduced size of upper leaves when compared to their senescent recurrent parent Tx7000 (Borrell et al., 2014a). It is possible that the gene expression differences identified here, that are associated with the response to plant hormones, may be contributing to these developmental differences. For example, auxin is known to play a role in shoot branching and leaf development (Dengler and Kang, 2001; McSteen and Leyser, 2005) therefore the auxin-related gene expression changes could have an impact on these processes. As reviewed in Chapter 1 ABA acts as an endogenous messenger in the plant stress response and is involved with regulating stomatal aperture and inducing stress-responsive genes (Fujita et al.,

2011) The differences in ABA-related gene expression identified here could therefore provide protective properties to the stay-green lines. Plant hormones also play a significant role in the senescence process; auxin, jasmonic acid, ABA and ethylene have all been shown to influence senescence (Grbic and Bleecker, 1995; Weaver et al., 1998; Woo et al., 2001). It is equally possible that the differential expression of genes associated with these plant hormones could be contributing to the phenotype. Whilst increased cytokinin levels have previously been shown to result in delayed senescence and drought tolerance in a range of species (Gan and Amasino, 1995) (see Chapter 1), there was no enrichment of genes associated with the 'response to cytokinin' GO category in the up-regulated gene set. This suggests that differences in cytokinin signalling are not responsible for the stay-green phenotype investigated here.

4.3.6 Gene expression changes at the seedling stage

Many of the potentially important biological processes identified at the older growth stage (45 DAS) were also identified at the seedling stage suggesting that they are constitutively more active throughout the life of the plant. For example, *P5CS2* and *SDIR1* was upregulated at both growth stages along with many of the genes encoding glutathione S-transferases and genes involved in betaine biosynthesis. There are, of course, gene expression differences that are unique to either growth stage. For example, some of the GSTs, the *DREB1A* and *RAP2.6* transcription factors and the potential regulator of stomatal density, *SDD1*, were only upregulated at the older growth stage. Other genes encoding GSTs and transcription factors were only upregulated at the seedling stage. Whilst it is likely that the key regulators of the trait are acting at the later growth stage it is possible that some are acting much earlier and place the plant in a better position to face the post-flowering drought stress. This has been previously hypothesised by Borrell *et al*. (2014a) and other studies have shown that the E36-1 stay-green line is additionally more tolerant to stress at the seedling stage (Jagtap and Bhargava, 1995).

In response to drought stress a number of genes were only upregulated in the stay-green B35 and E36-1 varieties and not in the senescent varieties (Figure 4.15). Amongst these genes were two putative peroxidases that may be involved in protection against

oxidative stress and three genes encoding heat shock proteins (see Appendices D.24 & D.28). It is interesting to note here that in this experiment 1387 genes were changed by drought stress in R16 whereas in the earlier combined stress experiment (see Chapter 3) 1190 genes were changed. Of these gene changes, only 568 genes were differentially expressed in *both* experiments. This may be as a result of the slightly different drought treatments received by the two sets of plants. For example, in this experiment the F_v/F_m on the plants averaged 0.65 at the time of sampling whereas in the earlier experiment the F_v/F_m was 0.71. Other than this the experimental set up was identical for both experiments. This highlights the fact that slightly different drought treatments can result in large differences at the gene expression level. In fact, this could be responsible for the differences in the drought-induced gene expression between the varieties. For example, the less well-adapted S35 variety may actually face more extreme drought stress due to weaker drought avoidance mechanisms thus resulting in the induction of a larger number of drought-tolerance genes (Figure 4.14).

4.3.7 Conclusions

Microarray analysis has identified a number of genes and biological processes that may be causal to the stay-green phenotype of the B35 line. However, as discussed in Chapter 3, changes at the transcriptomic level do not always correlate with changes at the protein/metabolic level. In fact, we cannot assume that translation has even occurred and, if it does, the protein may still require post-translational modifications in order to function. A more detailed analysis of these identified biological processes is therefore required before any firm conclusions can be made as to their role in the trait (see Chapter 5). In addition, a functional analysis of the identified signalling genes is required to assess whether the sorghum genes can function in the same way as the homologs from other species (see Chapter 6).

CHAPTER 5

An in-depth analysis into the biological processes underlying the stay-green trait

5.1 Introduction

The microarray results described in Chapter 4 highlighted a number of potential pathways and processes that may underlie the stay-green trait in sorghum. These include the biosynthesis of compatible solutes, the detoxification of reactive oxygen species (ROS) and the regulation of stomatal aperture/numbers. These processes were selected for further study based on what was already known about the stay-green phenotype and the known role of these processes in conferring drought tolerance in general. However, before we can make any conclusions about their role in conferring the stay-green trait, we must first determine whether the identified differences in gene expression result in actual differences in metabolism or plant physiology.

The research in this chapter therefore focuses on biochemical and physiological analyses of the stay-green lines in order to examine whether the gene expression changes identified at the transcriptomic level translate into changes at a protein and metabolic level.

Aims of the research described in this chapter:

- To quantify levels of the compatible solutes proline and glycine betaine in the stay-green and senescent lines (5.2.1).
- To quantify glutathione S-transferase and ascorbate peroxidase activity in the stay-green and senescent lines (5.2.2).
- To measure stomatal density and distribution in the stay-green and senescent lines (5.2.3).

5.2 Results

5.2.1 Osmotic adjustment

5.2.1.1 Proline

5.2.1.1.1 Validation of gene expression differences

As described in Chapter 4, three transcripts associated with the biosynthesis of proline were expressed to higher levels in B35 compared to R16. These include two different delta1-pyrroline-5-carboxylate synthase 2 (*P5CS2)* transcripts (*Sb03g039820.1* and *Sb03g039820.2*) and glutamate S-semialdehyde dehydrogenase (*Sb02g025790.1*). These genes were expressed to higher levels at both the mature stage (45 DAS) and the seedling stage (14 DAS) (Table 5.1).

Table 5.1 - Genes that are associated with proline biosynthesis that were expressed to higher levels in the stay-green (B35) variety compared to the senescent (R16) variety under well-water conditions

SbID	Gene name	Fold Change (Abs) in B35 vs. R16 at 45 DAS	Fold Change (Abs) in B35 vs. R16 at 14 DAS	Regulation
Sb03g039820.1	DELTA1-PYRROLINE-5-CARBOXYLASE-SYNTHESTASE 2 (P5CS2)	8.74	2.85	Up
Sb03g039820.2	DELTA1-PYRROLINE-5-CARBOXYLASE-SYNTHESTASE 2 (P5CS2)	8.55	2.52	Up
Sb02g025790.1	GLUTAMATE S-SEMIALDEHYDE DEHYDROGENASE	2.32	3.35	Up

Proline can be synthesised by either the glutamate or ornithine pathways in higher plants (Figure 5.1). The glutamate pathway is the major route and in this pathway proline is synthesised via the intermediates glutamate λ-semialdehyde and pyrroline 5-carboxylate. P5CS catalyses the first two reactions and as such represents the rate-limiting step (Kishor et al., 1995). The differential expression of *P5CS2* could therefore feasibly have an impact upon the amount of free proline.

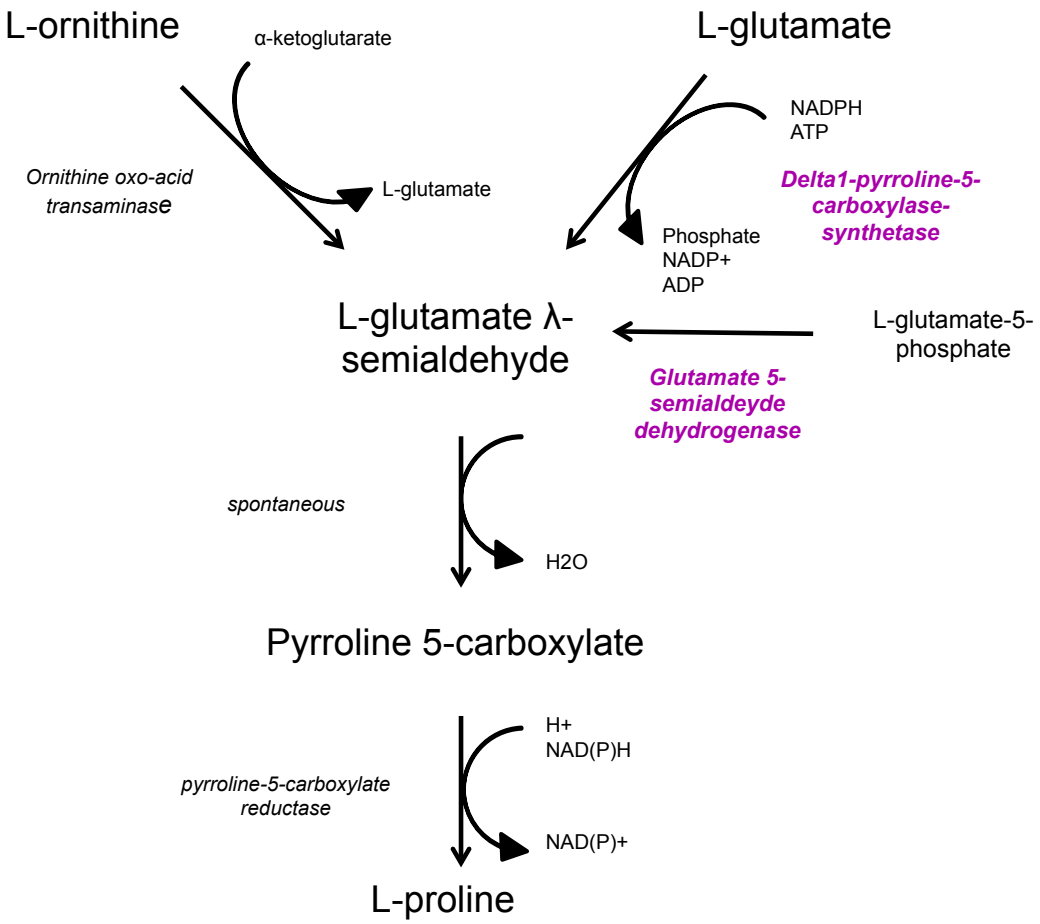


Figure 5.1 – Schematic showing the proline biosynthesis pathway in higher plants. The genes that encode the enzymes in purple are expressed to higher levels in the stay-green B35 line compared to the senescent R16 line. The pathway was adapted from the SorghumCyc Metabolic Pathways database (http://pathway.gramene.org/gramene/sorghumcyc.shtml).

Three *P5CS* genes have been identified in the sorghum genome. In order to confirm that only one of these genes is highly expressed in B35, qPCR with probes designed specifically for each gene, was carried out and gene expression was compared between B35 and R16. Of the three annotated *P5CS* genes, *P5CS2* (*Sb03g039820.1*) is the only one expressed to higher levels in the B35 line (Figure 5.2).

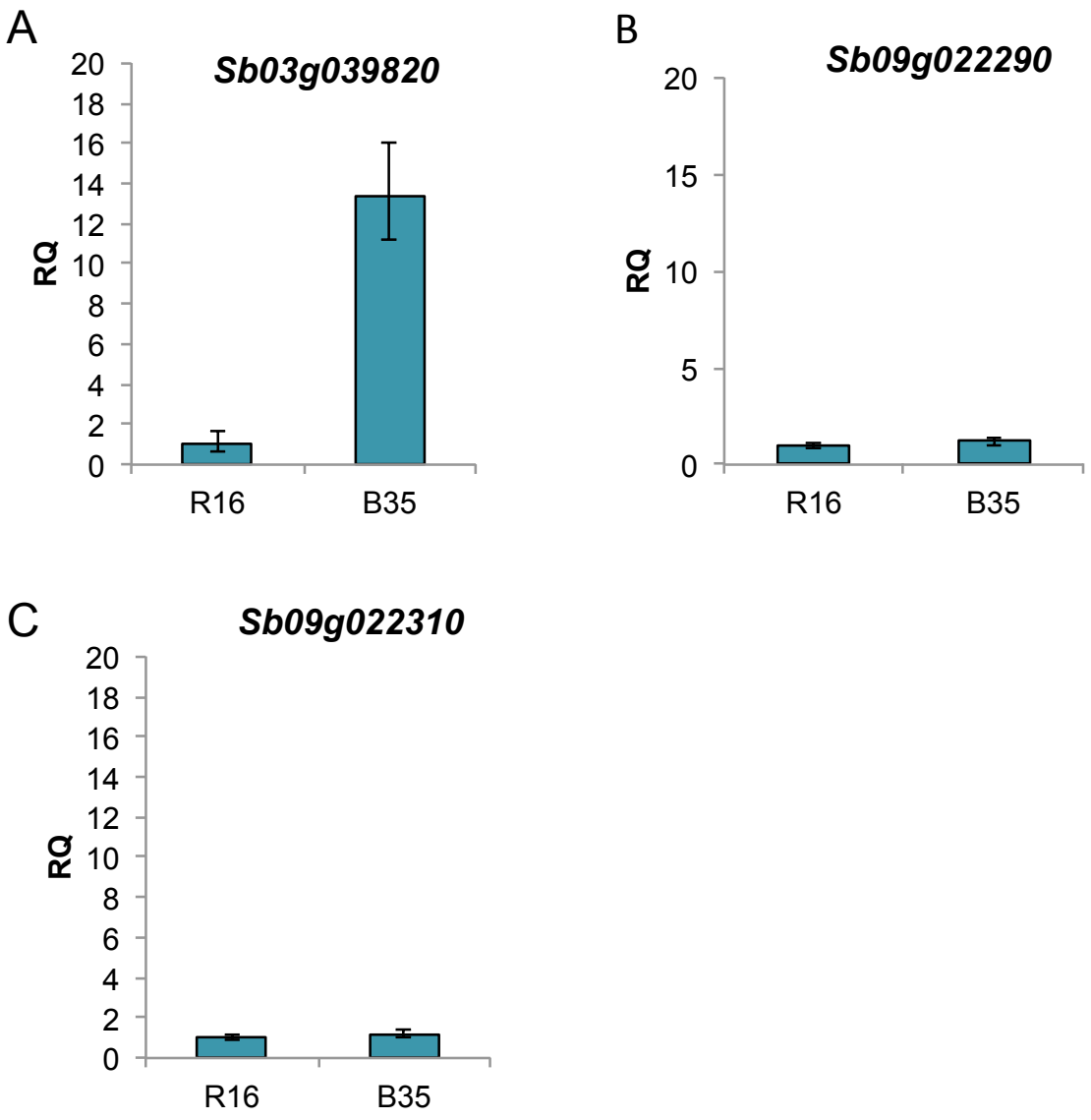


Figure 5.2 - Relative transcript abundance of [A] *Sb03g039820.1*, [B] *Sb09g022290.1* and [C] *Sb09g022310.1* in the senescent (R16) and stay-green (B35) varieties at 45 DAS. Each of these genes has been annotated as P5CS in the sorghum genome. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

5.2.1.1.2 Biochemical quantification of proline levels

In order to determine whether the observed differences in *P5CS2* gene expression result in an increase in actual proline levels, total proline content was quantified in B35 and R16 under both well-watered and osmotically-stressed conditions using HPLC. Proline levels were found to be ~1.8 fold higher in the B35 stay-green line compared to the R16 senescent line under well-watered conditions and ~1.5-fold higher under osmotically-stressed conditions. The differences in *P5CS* gene expression between the varieties therefore correlate well with changes in actual proline levels (Figure 5.3).

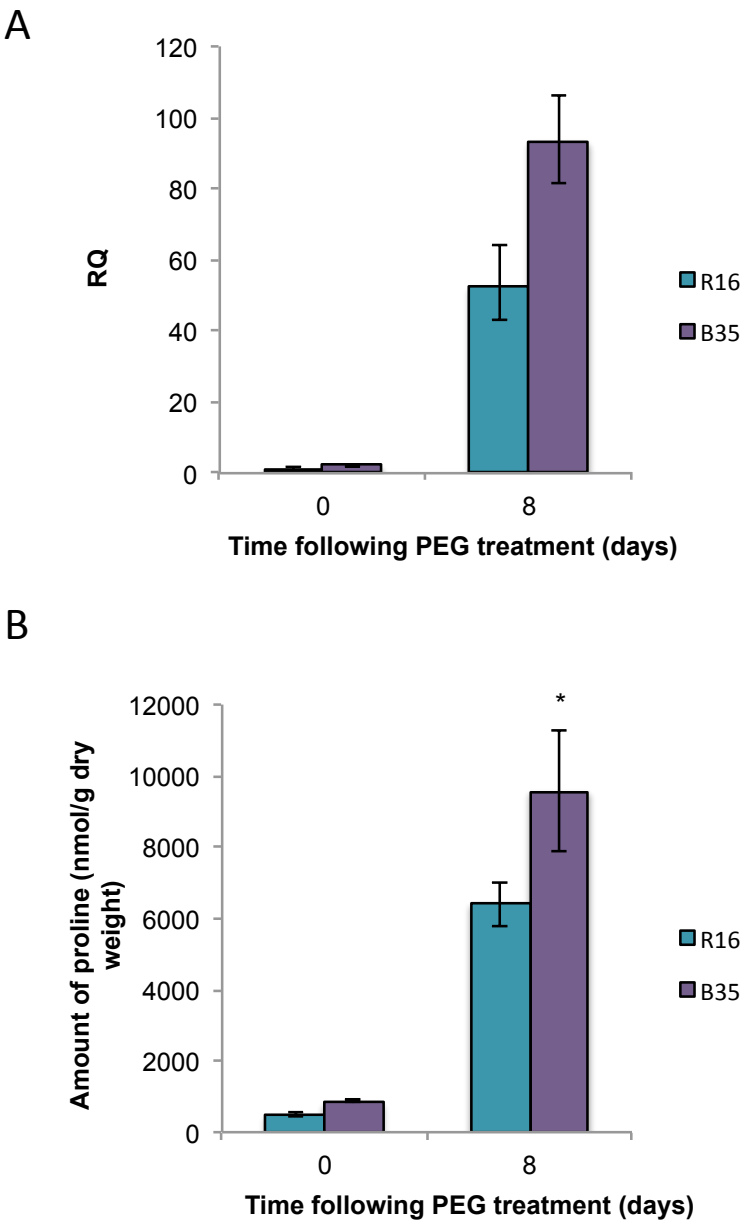


Figure 5.3 - [A] Relative transcript abundance of *P5CS2* (*Sb03g039820.1*) in the stay-green (B35) and senescent (R16) lines following PEG treatment. Four biological repeats of this experiment were carried out and the graph shows a representative result. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test. [B] Amount of proline in the stay-green (B35) and senescent (R16) sorghum varieties following PEG treatment at 14 DAS. Four biological repeats were carried out and the graph shows the average (*$p<0.05$ at 8 days). Error bars show SE (n=4).

5.2.1.2 Glycine betaine

5.2.1.2.1 Validation of gene expression differences

A total of nine genes associated with betaine biosynthesis were differentially expressed between the stay-green B35 and senescent R16 varieties. Out of these, eight were significantly ($p<0.05$) different and are shown in Table 5.2. One of these genes, choline monooxygenase (*CMO*) (*Sb10g028700.1*), is associated with the biosynthesis of glycine betaine, whereas the others are associated with the biosynthesis of β-alanine betaine. Choline monooxygenases function in the first step of the glycine betaine biosynthetic pathway (Figure 5.4) therefore the high expression of this gene could result in higher levels of free glycine betaine (Ashraf and Foolad, 2007).

Table 5.2 - Genes that were differentially expressed between the stay-green (B35) and senescent (R16) varieties that are involved with the biosynthesis of betaine.

SbID	Gene name	Fold Change (abs) in B35 vs. R16 at 50 DAS	Regulation
Sb07g026610.1	BASIC HELIX-LOOP-HELIX FAMILY PROTEIN	2.60	Down
Sb02g009120.1	AAO3 (ABSCISSIC ALDEHYDE OXIDASE 3)	6.54	Down
Sb06g025450.1	UNKNOWN	2.16	Down
Sb10g028070.1	EXLB2 (EXPANSIN-LIKE B2 PRECURSOR)	14.21	Up
Sb02g009130.1	AAO1 (ARABIDOPSIS ALDEHYDE OXIDASE 1)	5.26	Down
Sb10g028700.1	CMO (CHOLINE MONOXYGENASE)	2.77	Up
Sb02g009120.2	UNKNOWN	6.25	Down
Sb02g027360.1	EXLB2 (EXPANSIN-LIKE B2 PRECURSOR)	4.89	Up

The difference in the expression of choline monooxygenase between B35 and R16 was validated using qPCR (Figure 5.5). The qPCR showed similar fold changes to the microarray data thus confirming the higher expression of this gene in B35.

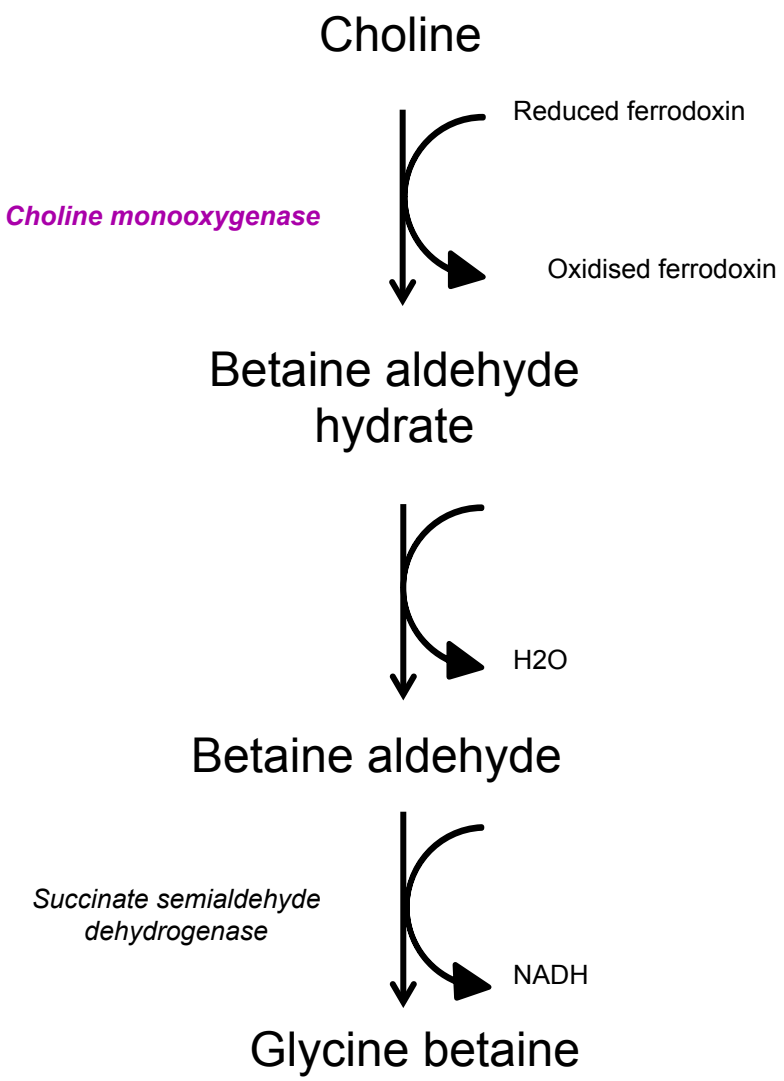


Figure 5.4 - Schematic showing the glycine betaine metabolic pathway in higher plants. The pathway was adapted from the SorghumCyc Metabolic Pathways database (http://pathway.gramene.org/gramene/ sorghumcyc.shtml).

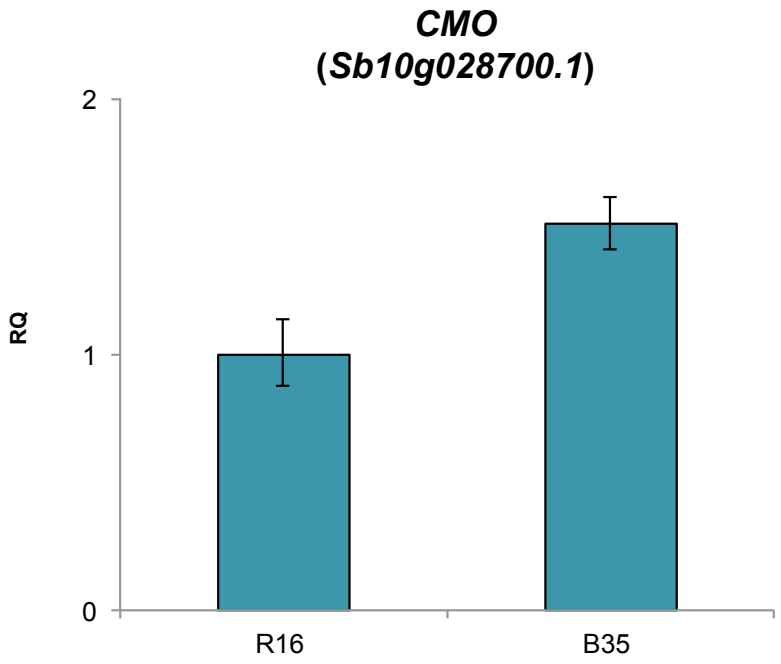
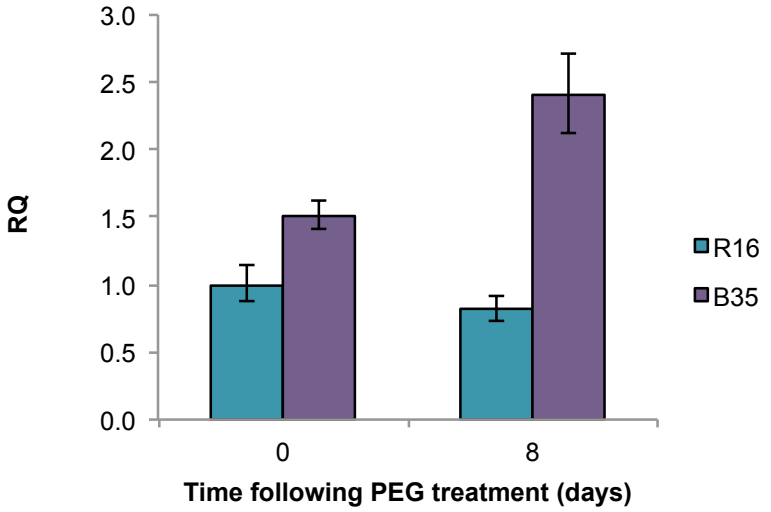


Figure 5.5 - Relative transcript abundance of *CMO* (Sb10g028700.1) Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

5.2.1.2.2 Biochemical quantification of glycine betaine levels

To determine whether the differences in the expression of *CMO* result in differences in the levels of glycine betaine, total glycine betaine levels were quantified under both well-watered and osmotically-stressed conditions using HPLC. Whilst slightly more glycine betaine was found in B35 under both conditions, these differences were small and not statistically significant. This suggests that the differences in the expression of this *CMO* gene are not sufficient to impact upon the amount of glycine betaine produced (Figure 5.6)

A



B

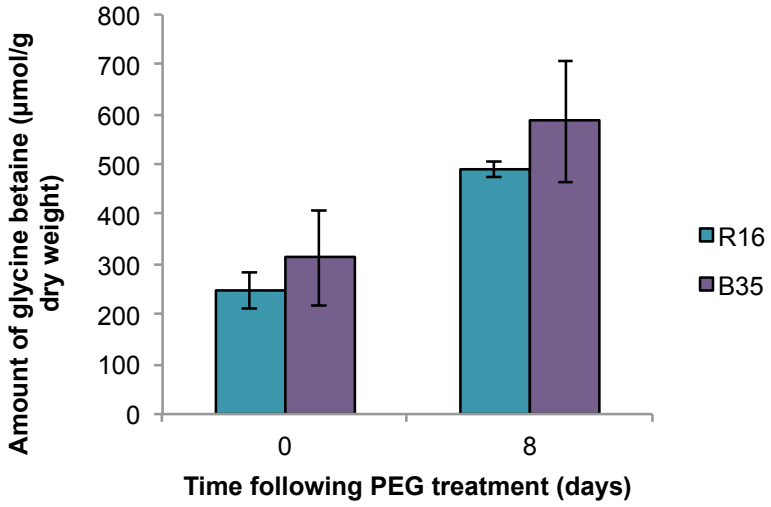


Figure 5.6 - [A] Relative transcript abundance of *CMO* (*Sb10g028700.1*) in the stay-green (B35) and senescent (R16) lines following PEG treatment. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test. [B] Amount of glycine betaine in the stay-green (B35) and senescent (R16) sorghum varieties following PEG treatment at 14 DAS. Three biological repeats were carried out and the graph shows the average (p=0.50 at 8 days). Error bars show SE (n=3)

5.2.2 Oxidative stress response

5.2.2.1 Ascorbate peroxidases

Two genes associated with ascorbate peroxidase (APX) activity (*Sb03g016083.1* and *Sb09g028890.1*) were expressed to higher levels in B35 at 45 DAS. Amino acid sequences of these sorghum genes were compared to sequences in Arabidopsis in order to gain an insight into their function. No similarity was found between the sorghum genes and known peroxidases in Arabidopsis or indeed other species. Instead the genes showed similarity to a protein kinase and an iron-sulphur cluster assembly protein respectively, suggesting the genes were incorrectly annotated in the SorghumCyc database. To confirm this, the sequences were checked for the presence of the peroxidase active site motif (APITLRLAWHSA) and the peroxidase heme-ligand motif (DIVVLSGGHTL) (Kunta and Louzada, 2004). Neither of the sequences contained these key motifs therefore these genes were not investigated further.

5.2.2.2 Glutathione S-transferases

5.2.2.2.1 Validation of gene expression differences

A total of 26 genes encoding glutathione S-transferases (GSTs) were differentially expressed between the stay-green B35 and senescent R16 varieties (Table 5.3). Some of these genes were differentially expressed at only one of the growth stages tested whereas others were differentially expressed at both. While some of the identified GSTs were expressed to lower levels in B35, the majority are expressed to higher levels. The difference in the expression of some of the GSTs at 45 DAS was validated using qPCR. The qPCR data showed similar fold changes to the microarrays thus confirming the increased expression of these genes in B35 (Figure 5.7).

Table 5.3 – Glutathione S-transferase genes that were differentially expressed in the stay-green (B35) variety compared to the senescent (R16) variety (FC>2, p<0.05) under well-watered conditions

SbID	Gene name	Fold Change (abs) in B35 vs. R16 at 50 DAS	Fold Change (abs) in B35 vs. R16 at 14 DAS	Regulation
Sb01g000230.1	GSTT1 (GLUTATHIONE S-TRANSFERASE THETA 1)	4.8	3.93	Down
Sb08g006680.1	GSTZ2	3.68	6.82	Down
Sb01g022070.1	ERD9 (EARLY-RESPONSIVE TO DEHYDRATION 9)	28.8	-	Down
Sb01g022080.1	ERD9 (EARLY-RESPONSIVE TO DEHYDRATION 9)	18.62	-	Down
Sb02g027080.1	GSTU7 (GLUTATHIONE S-TRANSFERASE TAU 7)	19.37	-	Down
Sb04g022250.1	GSTF13	3.92	-	Down
Sb03g045830.1	GSTT7 (GLUTATHIONE S-TRANSFERASE TAU 7)	-	2.52	Up
Sb08g007310.1	GSTF13	4.67	3.26	Up
Sb05g001525.1	GSTU7 (ARABIDOPSIS THALIANA GLUTATHIONE S-TRANSFERASE TAU 7)	-	4.42	Up
Sb03g015070.1	GSTF13	7.38	5.29	Up
Sb10g022780.1	GSTU25 (GLUTATHIONE S-TRANSFERASE TAU 25)	-	5.68	Up
Sb03g031780.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	20.7	6.62	Up
Sb01g030790.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	2.33	8.58	Up
Sb02g003090.1	GSTU19 (GLUTATHIONE S-TRANSFERASE TAU 19)	-	8.69	Up
Sb01g031020.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	-	11.16	Up
Sb01g030990.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	-	32.43	Up
Sb01g001230.2	UNKNOWN	8.36	-	Up
Sb01g001230.1	GSTF13	8.68	-	Up
Sb01g030980.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	4.03	-	Up
Sb01g031000.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	8.98	-	Up
Sb01g031040.1	ERD9 (EARLY-RESPONSIVE TO DEHYDRATION 9)	3.34	-	Up
Sb03g025210.1	ERD9 (EARLY-RESPONSIVE TO DEHYDRATION 9)	2.2	-	Up
Sb03g031790.1	GSTU18 (GLUTATHIONE S-TRANSFERASE TAU 18)	4.45	-	Up
Sb03g045790.1	GSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)	10.5	-	Up
Sb03g045840.1	GSTU8 (GLUTATHIONE S-TRANSFERASE TAU 8)	2.04	-	Up
Sb05g026490.1	GSTU18	2.12	-	Up

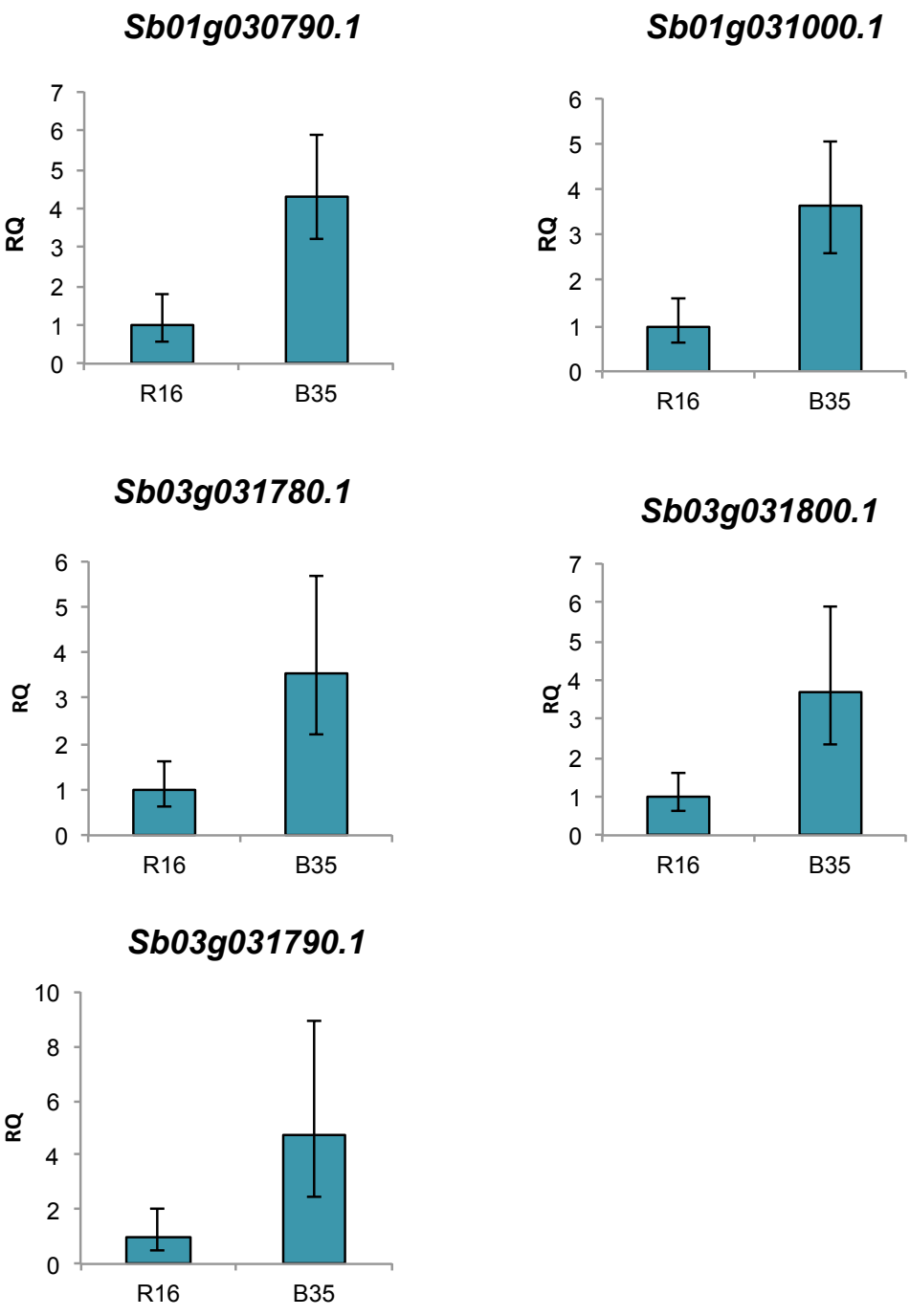


Figure 5.7 - Relative transcript abundance of GSTs in the B35 stay-green sorghum line and the R16 senescent sorghum lines at 50 DAS measured using qPCR. [A] *Sb01g030790.1*, [B] *Sb01g031000.1*, [C] *Sb03g031780.1*, [D] *Sb03g031800.1* and [E] *Sb03g031790.1* The Fluidigm system was used for qPCR measurements (See Materials and Methods) and error bars show standard deviation of three biological replicates.

5.2.2.2.2 Quantification of GST activity

In order to determine whether the differences in GST gene expression result in an actual increase in enzyme activity, an assay was carried out to test GST activity in the stay-green and senescent lines. A model substrate, 1-chloro-2,4-dinitrobenzene (CDNB), was used according to the method of (Habig et al., 1974). To ensure that the differentially expressed GSTs identified here have activity towards CDNB, the amino acid sequences of the sorghum GSTs were first compared to those of known GSTs in Arabidopsis. Homologs of the sorghum genes in Arabidopsis all show high activity to CDNB thus validating the use of this particular assay (Dixon et al., 2009). GST activity was shown to be higher in the B35 stay-green line under both well-watered and drought stressed conditions (Figure 5.8).

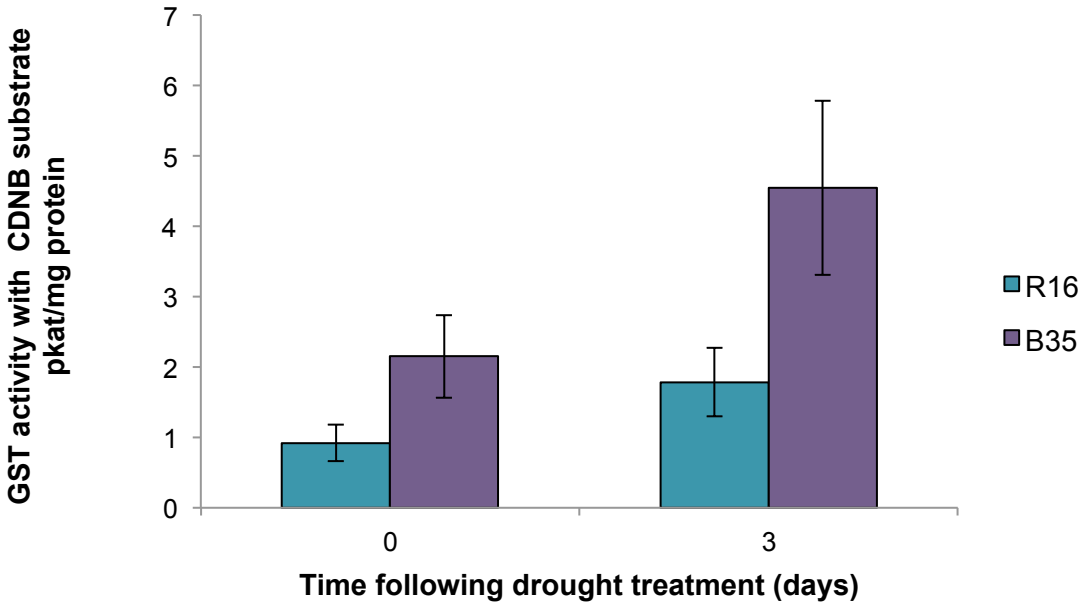


Figure 5.8 - GST activity towards the CDNB substrate in the stay-green (B35) and senescent (R16) varieties at 14 DAS under both well-watered and drought-stressed conditions. Graph shows the average of three biological replicates and error bars show SE, n= 3.

5.2.2.2.3 MV treatment

GSTs are known to be able to counteract the effects of oxidative stress (Gill and Tuteja, 2010). If GST activity were constitutively higher in the B35 line, as appears to be the case, we would perhaps expect this line to be more tolerant to oxidative stress. To test this, leaf discs of both varieties were incubated with methyl viologen (MV) (paraquat), a herbicide known to induce O_2 radical production. Leaf discs of both varieties displayed extensive chlorophyll degradation however; discs of the B35 variety retained slightly more chlorophyll. This was quantified by measuring total chlorophyll content. Whilst differences between the varieties at 1 µM MV were small, differences at 2.5 µM were more significant with B35 retaining more chlorophyll (Figure 5.9). This suggests that B35 is more tolerant to oxidative stress induced by MV.

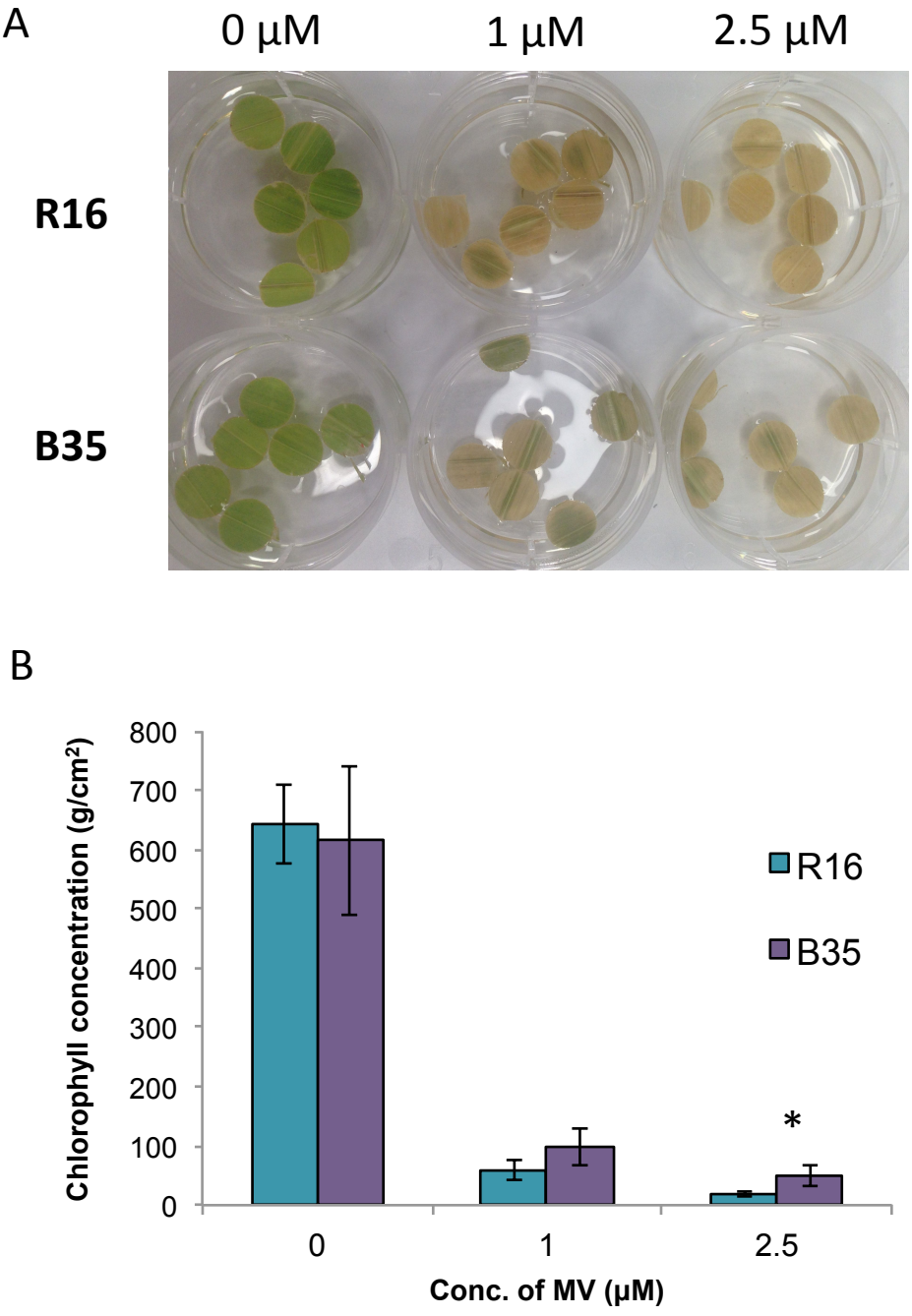


Figure 5.9 - [A] Leaf discs of the stay-green (B35) and senescent (R16) varieties treated with different concentrations of methyl viologen (MV). Discs were taken from plants at 14 DAS. Image shows a representative photograph taken following 48 hr of treatment. [B] Average chlorophyll concentration of leaf discs following 48 hr of MV treatment. Graph shows the average of four biological replicates and error bars show SE. (*$p<0.05$).

5.2.3 Regulation of transpiration

5.2.3.1 Excised-leaf water-loss assay

As described in Chapter 4, a number of genes that are associated with the regulation of stomatal development and stomatal aperture were differentially expressed between the stay-green and senescent lines. Stomata regulate water loss from the leaf, therefore if these gene expression changes are influencing stomatal behaviour, we would expect to see differences in transpiration between the varieties. Differences in transpiration efficiency have been previously reported when comparing stay-green introgression lines with their senescent parents R16 and S35 at a mature growth stage (Kassahun et al., 2010; Vadez et al., 2011). To assess whether there are differences in transpiration between B35 and R16 at a younger age, an excised leaf water loss assay was carried out. After 200 min of leaf detachment the fresh weight loss was 62% and 73% for R16 and B35 respectively (Figure 5.10). This suggests that the stay-green line B35 is better able to retain water.

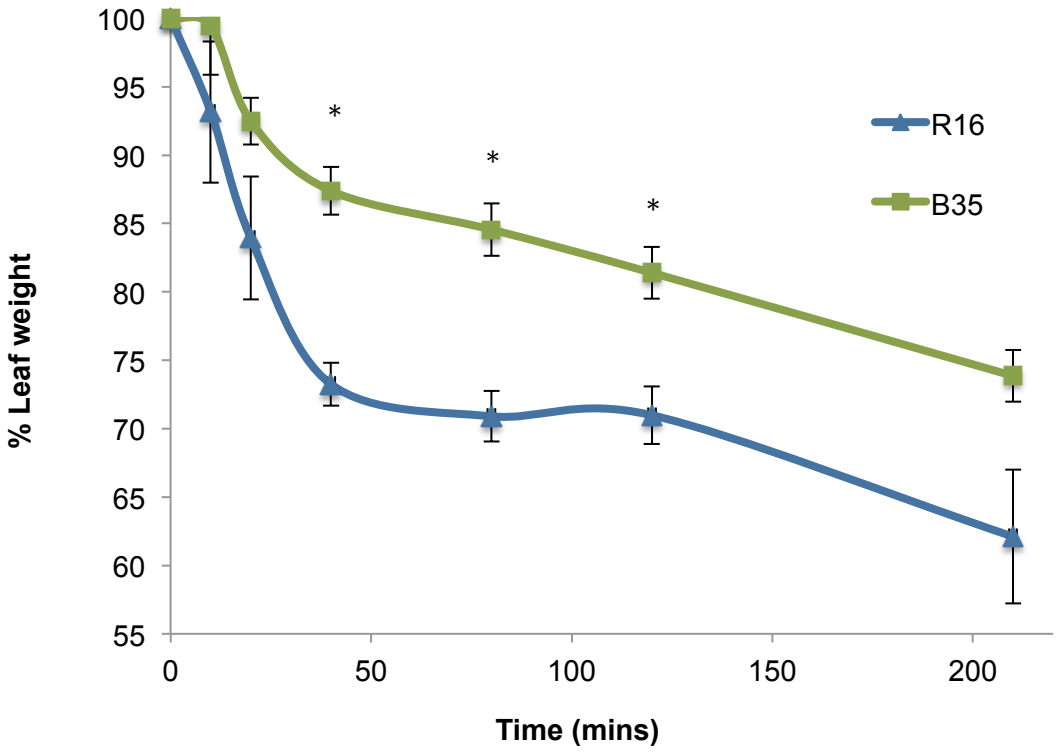


Figure 5.10 - Excised-leaf water-loss assay at 14 DAS. Leaves of the same developmental stag es were excised and weighed at various time points after detachment. Each data point represents the mean of duplicate measurements. Error bars represent SE (*n* = 8 each). This experiment was carried out four times and the graph shows a representative result (*p<0.05). See Materials and Methods section 2.6.3.

5.2.3.2 Stomatal density measurements

Differences in transpiration between genotypes can be due to differences in stomatal numbers and/or differences in stomatal aperture. The total numbers of stomata were counted in a defined area in leaves of the same developmental age in R16 and B35. All external factors, such as light levels and CO_2 concentration were kept constant for all plants to ensure any differences are due to differences in the varieties, not in the growth conditions. B35 was found to have fewer stomata than R16 (Figure 5.11). This suggests one way in which B35 is able to reduce water loss is via reduced stomatal numbers.

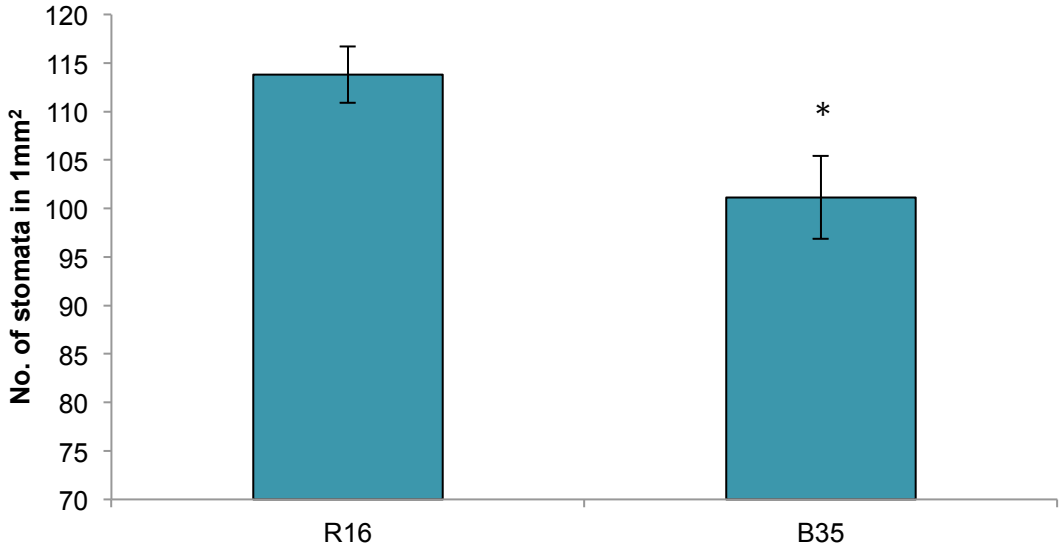


Figure 5.11 – Stomatal numbers of leaves at the same developmental stage taken from the stay-green (B35) and senescent (R16) varieties at 50 DAS. Mean values are shown with error bars representing the SE (n=32) ($p<0.05$).

5.3 Discussion

5.3.1 Proline

Genes associated with proline biosynthesis were expressed to higher levels in B35. One of these genes, *P5CS*, encodes an enzyme that acts as the rate-limiting step in the pathway (Kishor et al., 1995). Not only was the expression of this gene shown to be ~8-fold higher in the stay-green line but this higher expression resulted in constitutively higher levels of free proline. Proline is known to accumulate in plants, including sorghum, under low water conditions and is known to have a number of protective properties including a role in osmotic adjustment, detoxification of reactive oxygen species (ROS), protection of membrane integrity, and stabilization of proteins (Wood et al., 1996; Hsu et al., 2003; Ashraf and Foolad, 2007; Su et al., 2011). In addition, evidence has suggested that proline is able to induce the expression of stress-responsive genes which possess proline responsive elements (PRE) in their promoters (Satoh et al., 2002). As a consequence, the overexpression of *P5CS* genes and the accumulation of proline have been shown to result in drought tolerance in a wide range of transgenic species (Kishor et al., 1995; Hong et al., 2000). In addition, proline concentrations have also been shown to be generally higher in stress-tolerant genotypes of plants, including sorghum (Sivaramakrishnan, 1988; Hsu et al., 2003; Nayyar and Walia, 2003; Ashraf and Foolad, 2007). The proline accumulation identified here could therefore be an important way in which stay-green lines are able to withstand drought stress for longer and therefore maintain their green leaf area.

The maintenance of cell turgor via osmotic adjustment is particularly important during cell growth and leaf expansion. Sorghum plants with a better capacity for osmotic adjustment have been shown to have a larger leaf area and have better leaf retention during grain filling (Tangpremsri et al., 1995). Stay-green sorghum lines have been shown to have higher relative water content (RWC) than senescent lines (Xu et al., 2000a). It is possible therefore that the high proline accumulation identified here in the stay-green variety, is contributing to the maintenance of high relative water content. This means that when fresh water availability is reduced at later growth stages, the stay-green varieties are better adapted to cope. Some evidence has suggested stay-green lines have modified root architecture (Borrell et al., 2014). Whilst not investigated

specifically in this study, it is also plausible that if *P5CS2* is additionally expressed to higher levels in the roots, osmotic adjustment could enable better root growth, which could facilitate water uptake. Stay-green sorghum plants are able to save water in the period prior to flowering (Crasta et al., 1999; Xu et al., 2000). The accumulation of compatible solutes such as proline could again be associated with conferring these phenotypes.

5.3.2 Betaine

Out of the nine differentially expressed genes that are associated with betaine biosynthesis one gene is associated with the biosynthesis of glycine betaine and the rest are associated with the biosynthesis of β-alanine betaine. Whilst there is some evidence that β-alanine betaine functions in the drought stress response, the majority of previous studies have focussed on glycine betaine (Ashraf and Foolad, 2007). Glycine betaine is a quaternary amino acid that accumulates in response to stress in a number of plants including sugar beet, spinach, barley and wheat (Rhodes and Hanson, 1993). It is abundant mainly in the chloroplast where it plays a role in osmotic adjustment and in the maintenance of photosynthetic efficiency under both heat and drought stress conditions (Jolivet et al., 1982; Holmstrom et al., 2000). Drought tolerant genotypes accumulate more glycine betaine than drought sensitive genotypes (Ashraf and Foolad, 2007) and transgenic lines overexpressing components of the biosynthetic pathway display delayed senescence in response to stress (Hayashi et al., 1997; Sakamoto et al., 1998; Holmstrom et al., 2000) .

A gene encoding choline monooxygenase (CMO), the first step in the biosynthesis of glycine betaine, was expressed to higher levels in the B35 stay-green line compared to the senescent R16 line. However, this does not translate into significant differences in actual glycine betaine levels (Figure 5.6). This could be due to a number of reasons. Firstly, the amount of the choline substrate may be limited or its transport into the chloroplast where it is metabolised may be restricted (McNeil et al., 2000). Alternatively, the up-regulation of this gene may simply not be great enough to cause a significant increase in the amount of the metabolite. A number of genes have been annotated as

encoding a CMO in the sorghum genome; therefore, the one identified here might not have a major function.

Previous studies have generated transgenic lines overexpressing CMO genes. These transgenic plants accumulated only low levels of the metabolite (~18 µmol/g) despite a large induction of the gene. Even though the increase in the level of this metabolite was small, the transgenic plants still exhibited increased drought tolerance (Huang et al., 2000; McNeil et al., 2000). Perhaps similarly here the B35 line could accumulate only slightly more glycine betaine than R16 yet this could still be sufficient to contribute towards its enhanced drought tolerance. A greater number of biological repeats are required to determine whether the small increase in glycine betaine observed in B35 compared to R16 (Figure 5.6) is significant.

Despite no evidence for significant differences between the stay-green and senescent lines, it is clear that the amounts of glycine betaine are increased in both varieties following osmotic stress. Not all plants accumulate this metabolite so this result confirms previous findings that glycine betaine is accumulated in sorghum in response to stress (Wood et al., 1996).

5.3.3 Glutathione S-transferases (GSTs)

A total of 26 genes putatively encoding GSTs were differentially expressed between the stay-green and senescent lines. These gene expression differences resulted in higher GST activity towards the CDNB substrate in the B35 stay-green line under both well-watered and drought-stressed conditions. GSTs are a well-characterized enzyme family that are involved in stress tolerance (Marrs, 1996). Abiotic stresses lead to the production of ROS, which are highly reactive, and cause damage to proteins, lipids and DNA and ultimately results in oxidative stress. GSTs help to counteract this by catalysing the transfer of glutathione (GSH) to xenobiotic substrates such as products of lipid peroxidation thus enabling their detoxification (Gill and Tuteja, 2010). Interestingly, the B35 line was better able to tolerate oxidative stress induced by MV at 2.5 µM; the higher GST activity in this line may be contributing to that tolerance. Stress-tolerant varieties of plants, including sorghum have increased GST activity (Jogeswar et al., 2006; Galle et

al., 2009) and the over-expression of GSTs can result in tolerance to a variety of stresses including low temperature, salt stress and dehydration (Yu et al., 2003; Zhao and Zhang, 2006; Ji et al., 2010). It is therefore plausible that the higher GST activity identified here is contributing towards drought tolerance in the B35 stay-green line. The fact that levels are constitutively higher could mean that the plants are already primed and ready to face the stress.

Whilst there are differences in GST activity between the lines, this difference is small in view of the marked increase in mRNA levels. There are 99 GSTs in total in the sorghum genome, many of which could have similar substrate specificity. Only a few of these are expressed to higher levels in B35 meaning that total GST activity may not dramatically increase. Furthermore, not all GSTs have high activity towards CDNB (Dixon et al., 2009) so the effect of some of the genes might not be taken into account in the assay. Other substrates such as alachlor or ethacrynic acid could be used to circumvent this problem in the future (Takesawa et al., 2002)

ROS accumulation aids the breakdown of the cellular machinery during senescence (Gregersen et al., 2013). The activation of GSTs could conceivably reduce the effects of ROS and slow down this process. In this case, the higher levels of GSTs could be responsible for the delayed senescence in sorghum at the post-flowering stage. Indeed delayed senescence mutants have increased tolerance to oxidative stress (Woo et al., 2004). We must bear in mind however that GST expression has only been investigated at two growth stages and GST activity has only been measured at one growth stage. Measurements at other growth stages are required to better link GST activity with physiological function. For example, higher GST activity in B35 at the post-flowering stage could be further indicative of a role for these enzymes in delaying physiological senescence. Different genes encoding GSTs were found to be differentially expressed at the two developmental stages investigated. It will therefore be important to confirm that the increase in the expression of these genes results in an increase in GST activity at both stages. We must also bear in mind that GSTs are not just involved in the stress response. Many are induced by hormone treatments such as salicyclic acid and auxin and have been implicated in hormone transport and maintaining homeostasis (Marrs, 1996). Interestingly, the overexpression of a GST in rice resulted in improved root

growth under stress (Takesawa et al., 2002). A role for these GSTs in regulating sorghum development can therefore not be ruled out.

While many of the GSTs identified were expressed to higher levels in B35, some were expressed to lower levels. Previous studies have shown that *down* regulating a GST can actually improve adaptation to drought (Chen et al., 2012). The importance of the genes expressed to lower levels in B35 must not be overlooked. The only way to truly gauge the role of these GSTs would be to characterize each gene individually using a transgenic approach.

5.3.4 Regulation of transpiration

As discussed in Chapter 1, stomata are pores in the leaf surface through which plants exchange CO_2, water vapour and other constituents (Casson and Hetherington, 2010). Water loss can be restricted by reducing the aperture of the stomatal pore or by decreasing the number of stomata on the epidermis. In Chapter 4, genes that were differentially expressed between the stay-green and senescent varieties were found to be associated with one of these processes (see Table 4.1). Differences in transpiration between stay-green and senescent lines have been previously identified (Vadez et al., 2011) and this was again confirmed here using a simple assay (Figure 5.10). It would be interesting to confirm these differences through more accurate measures of stomatal conductance i.e. using an infra-red gas analyser (IRGA) to accurately measure gas exchange or by using thermal imaging to visualise whole plant conductance.

SDD1 was expressed to higher levels in B35. The overexpression of SDD1 in maize resulted in reduced stomatal numbers with a concomitant increase in drought tolerance (Liu et al., 2015). Here, stomatal counts revealed that the B35 stay-green line similarly has reduced stomatal numbers compared to the senescent R16. This suggests the B35 variety is able to reduce water loss via reduced stomatal numbers, possibly through the higher expression of SDD1. Differences in stomatal aperture may also be contributing to the differences in water loss observed between the varieties (Figure 5.10). It would be interesting to measure and compare stomatal apertures between the varieties through the use of epidermal peels in the future.

A reduced number of stomata/reduced stomatal aperture has a trade-off in that stomata also control the uptake of CO_2 required for photosynthesis and dry matter accumulation. This means that reduced water loss via the stomata can actually negatively impact on yields (Chaves et al., 2009). In sorghum, there have been some reports of B35 having slightly reduced yields compared to other varieties under well-watered conditions despite yields being increased under stressed conditions at the post-flowering stage (Borrell et al., 2000). This could be because prior to flowering the reduced stomatal numbers in B35 constrain assimilation, yet help with water conservation, meaning that more water is available in the dry period at the post-flowering stage. This water conservation could help survival and hence boost yields under stress compared to other varieties. We must bear in mind however that stomatal counts were carried out at only one developmental stage. Stomata form in early leaf development and mature by the time the leaf reaches 10-60% of the final size (Ticha, 1982). At the time of measuring the leaves were reaching maturity so formation of new stomata will have slowed. However, it will still be important to measure stomatal density at other growth stages, particularly post-flowering. This would enable us to be better correlate stomatal numbers with physiology. For example, perhaps there are only fewer stomata in B35 at early growth stages thus allowing water conservation. There may not be any differences at the post-flowering stage thus enabling the uptake of CO2 for photosynthesis and grain production.

5.3.5 Conclusions

The analysis in this chapter has shown that many of the gene expression differences between the stay-green and senescent lines, including those associated with proline biosynthesis, GST activity and stomatal activity, do result in changes at the metabolic and physiological level. However, an understanding of the precise role of individual genes is also required (see Chapter 6).

CHAPTER 6

An in-depth analysis of a transcription factor and a signaling gene that may underlie the stay-green trait

6.1 Introduction

The microarray results presented in Chapter 4 identified genes encoding transcription factors and signalling proteins that are expressed to higher levels in the B35 stay-green line compared to the senescent R16 line. However, a functional analysis of these genes is required before any conclusions can be drawn as to their role in conferring the stay-green trait. Two interesting candidate genes, which were selected for this analysis, are described below.

6.1.1 SALT-AND-DROUGHT-INDUCED RING FINGER1 (*SDIR1*)

SDIR1 was originally identified in an Arabidopsis microarray data set as a stress inducible really interesting new gene (RING) finger protein. It was later shown to be a functional E3 ligase that is associated with intracellular membranes (Zhang et al., 2007). Overexpression of SDIR1 in Arabidopsis was shown to enhance drought tolerance whereas *sdir1* mutant lines were more sensitive to drought stress. The drought tolerance of the overexpression lines was attributed, at least in part, to a reduction in transpiration caused by reduced stomatal aperture. The overexpression lines also exhibited other ABA-associated phenotypes, such as salt hypersensitivity in germination. SDIR1 is therefore thought to be a positive regulator of the ABA-dependent stress-signalling pathway (Zhang et al., 2007). Later studies showed that this gene is conserved in rice, maize, tobacco and grapevine and the overexpression of these genes similarly

enhanced drought tolerance and caused a reduction in stomatal aperture (Zhang et al., 2008; Gao et al., 2011; Xia et al., 2012; Liu et al., 2013; Tak and Mhatre, 2013).

SDIR1 (Sb01g039740) expression was found to be ~2-fold higher in both the B35 and the E36-1 stay-green sorghum lines when compared to the senescent R16 line at both growth stages tested. Given current knowledge of SDIR1 in other species, it could be hypothesised that the higher expression of *SDIR1* in the stay-green lines could be contributing to their drought tolerance by reducing the rate of transpiration via reduced stomatal aperture. This is supported by the fact that there are known differences in transpiration between stay-green and senescent lines (Vadez et al., 2011) (see Chapter 5). To test this hypothesis, a functional characterization of the sorghum *SDIR1* gene is required.

6.1.2 DREB1A

Transcription factors can regulate a series of downstream stress-responsive genes and are therefore excellent candidate genes for engineering for stress tolerance. As reviewed in Chapter 1, DREB transcription factors were identified due to their ability to bind the dehydration responsive element (DRE), A/GCCGAC, in the promoters of many stress-inducible genes in Arabidopsis and to activate their transcription (Yamaguchishinozaki and Shinozaki, 1994; Stockinger et al., 1997; Liu et al., 1998). These DREB proteins belong to the ERF superfamily and contain an AP2 DNA binding domain (Sakuma et al., 2002). There are six DREB1 proteins (DREB1A-F) in Arabidopsis and eight DREB2 proteins (Akhtar et al., 2012). The DREB1 proteins can be distinguished by the presence of a consensus nuclear localization signal (NLS) sequence and a DSA motif after the AP2 domain (Jaglo et al., 2001). In Arabidopsis, DREB1A (CBF3), DREB1B (CBF1) and DREB1C (CBF2) are induced by cold whereas DREB1D (CBF4) is induced by dehydration (Liu et al., 1998; Haake et al., 2002). The overexpression of these genes in Arabidopsis resulted in increased tolerance to stresses including freezing, drought and high salinity, with concomitant increased expression of downstream target genes (Liu et al., 1998; Kasuga et al., 1999; Haake et al., 2002). DREB1A overexpressing lines also accumulated osmoprotectants such as proline and

various sugars (Gilmour et al., 2000). More than 40 targets of these DREB1 genes have been identified using microarrays (Maruyama et al., 2004).

DREB transcription factors have been identified in a range of plant species other than Arabidopsis suggesting conservation of regulatory systems. The overexpression of DREB homologues from rice, maize, barley, wheat, apple (*Malus baccata)* and perennial ryegrass (*Lolium perenne L*.), resulted in the expression of stress-inducible genes and increased stress tolerance in Arabidopsis (Dubouzet et al., 2003; Qin et al., 2004; Xiong and Fei, 2006; Zhao and Bughrara, 2008; Yang et al., 2011). DREB homologues have also been expressed in rice, brassica and wheat and additionally the transgenic lines displayed increased stress tolerance when compared to wild type (Ito et al., 2003; Shen et al., 2003; Savitch et al., 2005) These observations suggest the DRE/DREB regulon can be used to improve tolerance in a range of species.

The expression of a putative DREB transcription factor (*Sb07g025210*) (annotated as *DREB1A*) was found to be ~2.5 fold higher in the B35 stay-green sorghum line compared to the senescent R16 line (see Chapter 4). Given the well-known role of DREB transcription factors in the plant stress response, it is possible that this *DREB* gene may be contributing to the drought tolerance of the B35 sorghum line. Presently, no DREB transcription factors in sorghum have been characterized.

This chapter focuses on a characterization of *SbSDIR1* and *SbDREB1A*.

Aims of the research presented in this chapter:

- To determine whether *SbSDIR1* and *SbDREB1A* are able to function in the same way as homologous genes from other species. This was carried out by:

    1. Examining amino acid homology and gene expression profiles in response to stress (6.2.1.2, 6.2.1.3, 6.2.2.2 & 6.2.2.3)

    2. Creating Arabidopsis transgenic lines overexpressing these genes and analysing their phenotype (6.2.1.4, 6.2.1.5 & 6.2.2.4)

    3. Creating sorghum transgenic lines overexpressing these genes (6.2.3)

6.2 Results

6.2.1 SDIR1

6.2.1.1 Validation of microarray data

The gene expression data obtained by microarray analysis (see Chapter 4) was validated using real-time qPCR. As shown in Figure 6.1, the expression of *SDIR1* was found to be ~2-fold higher in the B35 stay-green line compared to the R16 senescent line at both 14 DAS and 50 DAS.

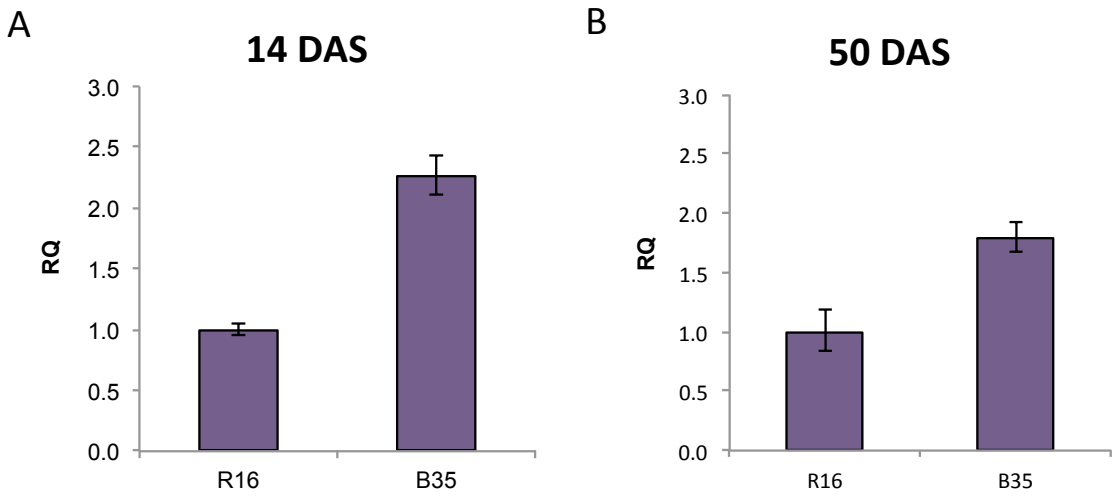


Figure 6.1 - Relative transcript abundance of *SbSDIR1* (*Sb01g039740*) in the senescent (R16) and stay-green (B35) varieties at [A] 14 DAS and [B] 50 DAS. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.1.2 Sequence alignment

The SbGDB database was used to identify the coding sequence of the sorghum *SDIR1* gene (http://www.plantgdb.org/SbGDB/). The amino acid sequence of SbSDIR1 was compared to the amino acid sequences of known SDIR1 proteins using Clustal W (www.ebi.ac.uk/Tools /msa/clustalw2/) (Figure 6.2). Putative transmembrane domains

were detected using the SMART program (www.smart.embl-heidelberg.de) and were conserved in the sorghum amino acid sequence. A RING finger domain required for the E3 ligase activity of SDIR1 in Arabidopsis (Zhang et al., 2007) was also conserved in the sorghum sequence. SbSDIR1 therefore shows strong homology at the amino acid level to SDIR1 from other species and contains all of the domains required for protein function.

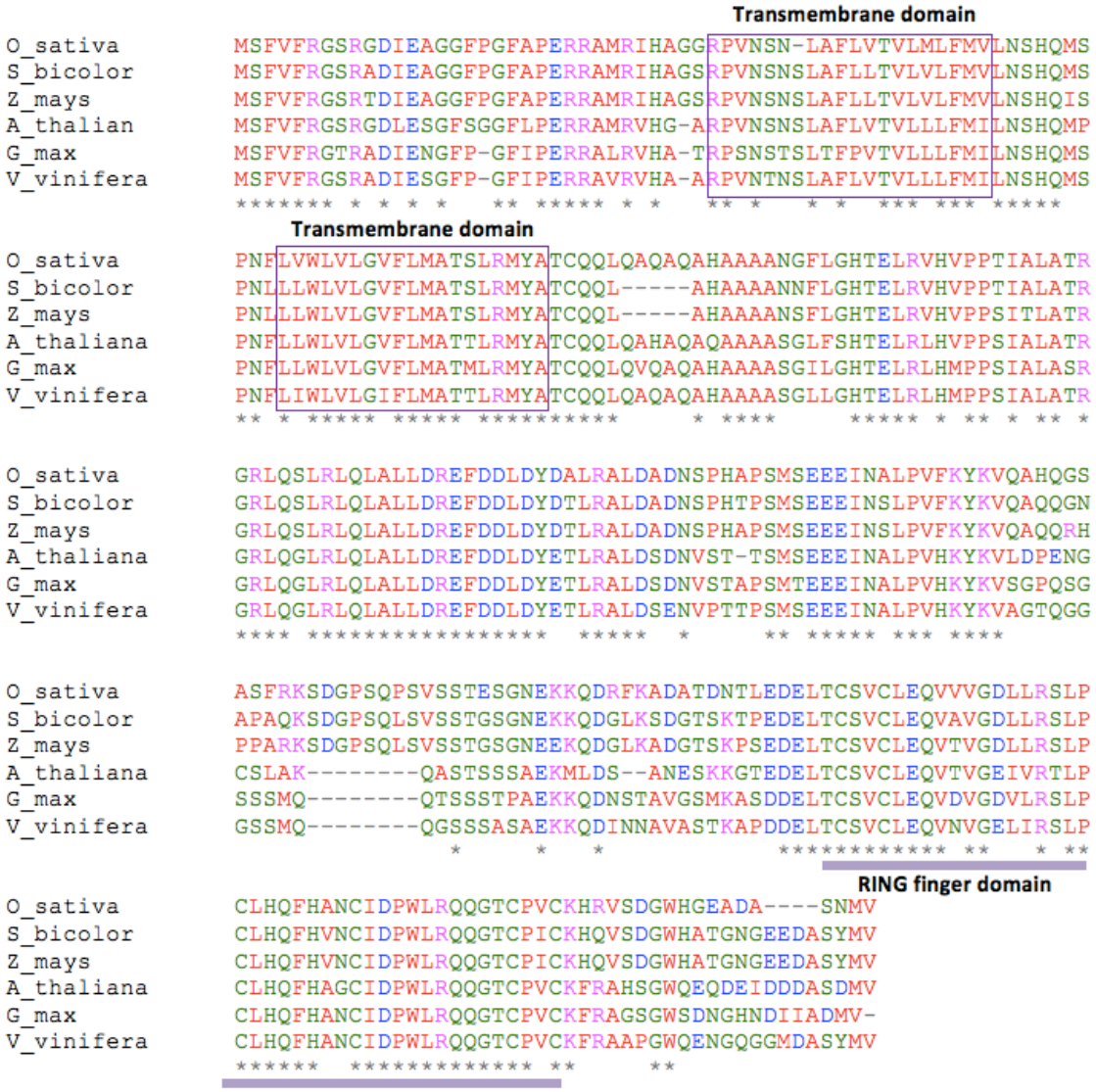


Figure 6.2 - Alignment of amino acid sequences of SDIR1 homologs from *O. sativa* (Os03g0272300), *Z. mays* (JN247666), *A. thaliana* (At3g55530), *G. max* (Bt096672) and *V. vinifera* (NC_012019.3). The locations of two putative transmembrane domains are indicated along with the position of the C3H2C3 RING finger domain.

6.2.1.3 Stress-induced expression in sorghum

SbSDIR1 expression was analysed in response to various stress treatments using qPCR (Figure 6.3). No changes in gene expression were observed following osmotic stress imposed by mannitol. However, the expression of *SbSDIR1* was increased by ~5-fold in response to osmotic stress imposed by PEG, over a period of 96 hr. This suggests that *SbSDIR1* is involved with the response to gradual soil drying, as simulated by PEG treatment, as opposed to the sharp osmotic shock imposed by mannitol. *SbSDIR1* showed a small increase in expression in response to NaCl (~1.5-fold) after 2 hr. The expression of *SbSDIR1* was also increased (~1.7-fold) in response to ABA treatment. This is similar to findings in Arabidopsis, grapevine and maize (Xia et al., 2012; Tak and Mhatre, 2013). *SbSDIR1* therefore displays similar gene expression patterns to those in other species.

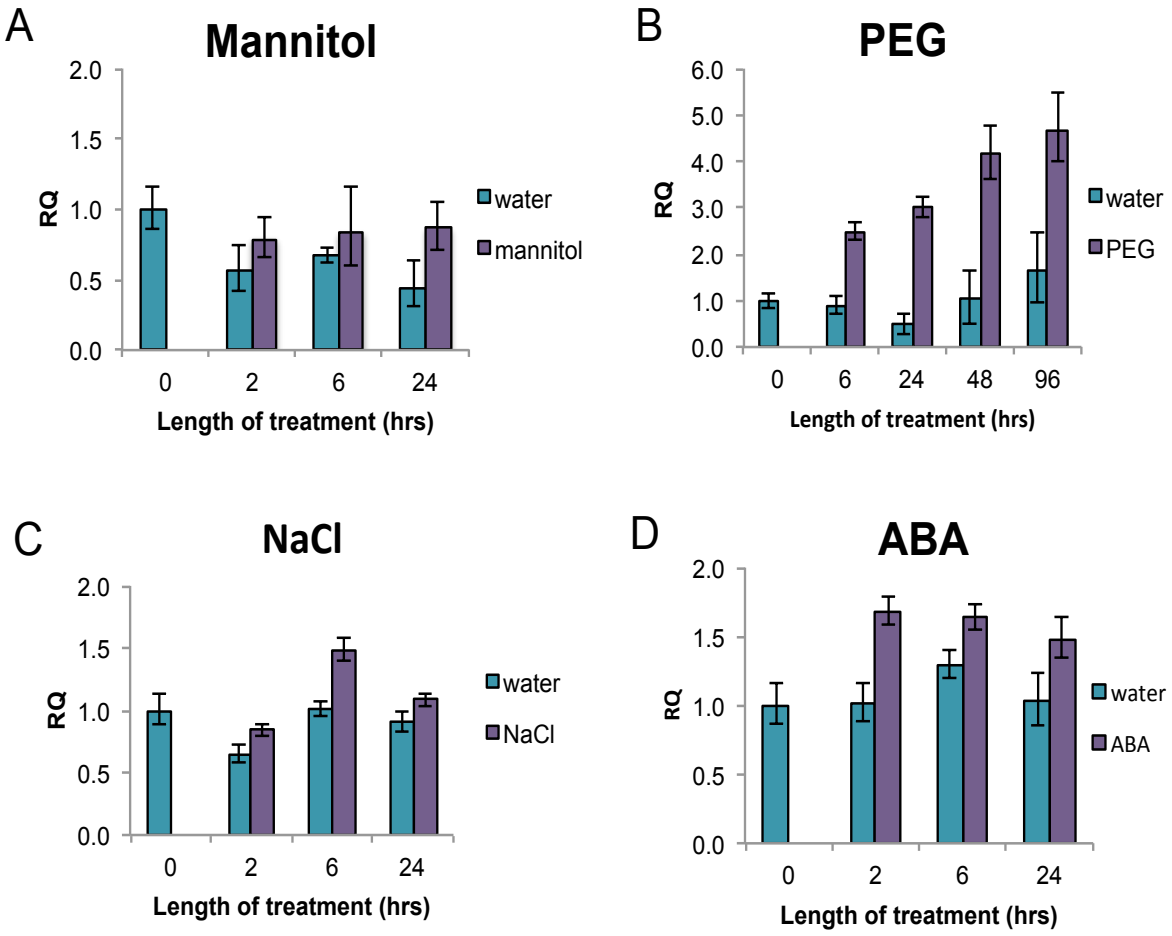


Figure 6.3 - Relative transcript abundance of *SbSDIR1* (*Sb01g039740*) in B35 following different stress treatments. Sorghum leaves were subjected to [A] mannitol (0.66M), [B] PEG (10%), [C] NaCl (200mM) and [D] ABA treatment (100 µM). For treatment details see Materials and Methods (2.6.1). Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

A homolog of SDIR1 in grapevine was shown to be upregulated in response to heat (Tak and Mhatre, 2013). To determine whether the expression of SbSDIR1 is also changed by heat shock, expression levels were analysed in the microarray data described in Chapter 1. *SbSDIR1* was found to be upregulated by ~2.5 fold following a 3 hr heat shock at 50 °C (Figure 6.4).

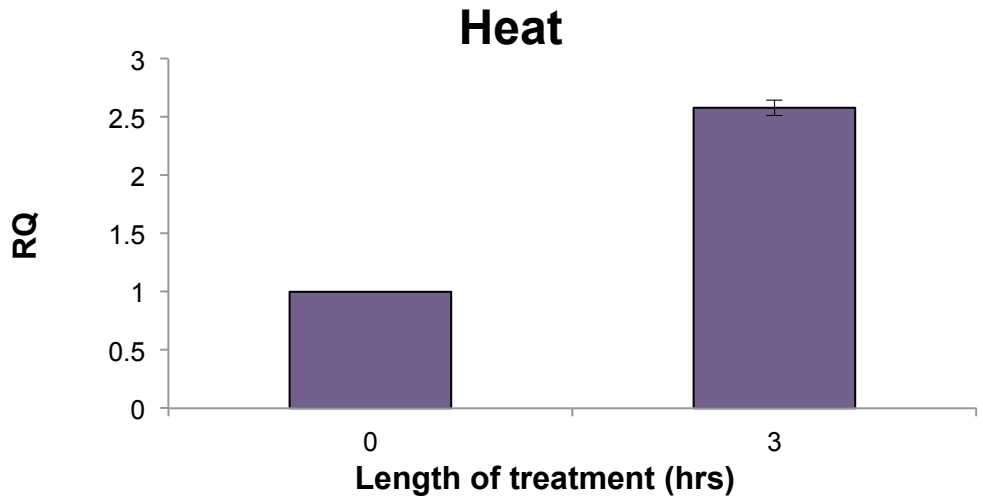


Figure 6.4 - Relative transcript abundance of *SbSDIR1* (*Sb01g039740*) in the R16 variety at 14 DAS following a 3 hr heat shock. Bar chart shows normalised gene expression data obtained from the microarray analysis (See Materials and Methods 2.5.5). Error bars show SE of normalized expression data from three biological replicates.

6.2.1.4 Overexpression in Arabidopsis

6.2.1.4.1 Generation of overexpression lines

Transgenic Arabidopsis plants overexpressing *SbSDIR1* under the control of the cauliflower mosaic virus 35S promoter were produced. The full-length *SbSDIR1* coding sequence was PCR amplified from sorghum cDNA and cloned into the pENTR/D-TOPO Gateway entry vector (See Appendix B.1 for vector map). *SbSDIR1* was then sub-cloned downstream of the CaMV 35S promoter of the binary gateway destination vector pK2GW7 (see Appendix B.2 for the vector map of the resulting construct). *Agrobacterium tumefaciens* was transformed with the construct and was used to transform *Col-0* Arabidopsis plants via the floral dip method (see Materials and methods section 2.8.1). Transgenic plants from the T_2 generation were used for further analysis. Overexpression of *SbSDIR1* was confirmed in six independent transgenic lines by real-

time qPCR. As shown in Figure 6.5 all of the lines tested exhibited increased *SbSDIR1* expression compared to wild-type *Col-0*.

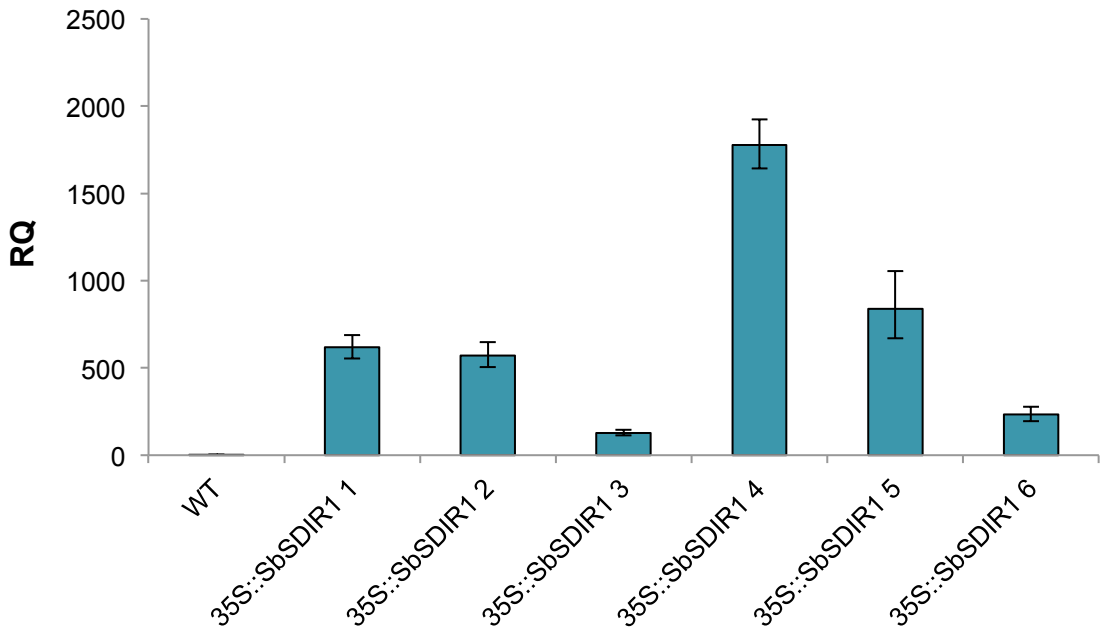


Figure 6.5 - Analysis of *SbSDIR1* expression levels in Arabidopsis overexpressing 35S::SbSDIR1 in the *Col-0* background using real-time qPCR. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.1.4.2 Measures of transpiration and stomatal conductivity

Physiological assays were carried to determine whether the overexpression of *SbSDIR1* resulted in a similar phenotype to that described by previous studies. An excised-leaf water-loss assay was carried out on three independent transgenic lines and wild-type Col-*0*, using the same method employed by Zhang et al. (2007) (see Materials and Methods section 2.6.3). As shown in Figure 6.6, the 35S::SbSDIR1 overexpression lines lost water more slowly than wild type. The extent of the phenotype correlates with *SbSDIR1* expression, with the highest expresser (35S::SbSDIR1 4) having the greatest

difference to wild type. The lowest expresser (35S::SbSDIR1 3) (not shown) loses water only slightly more slowly than wild type.

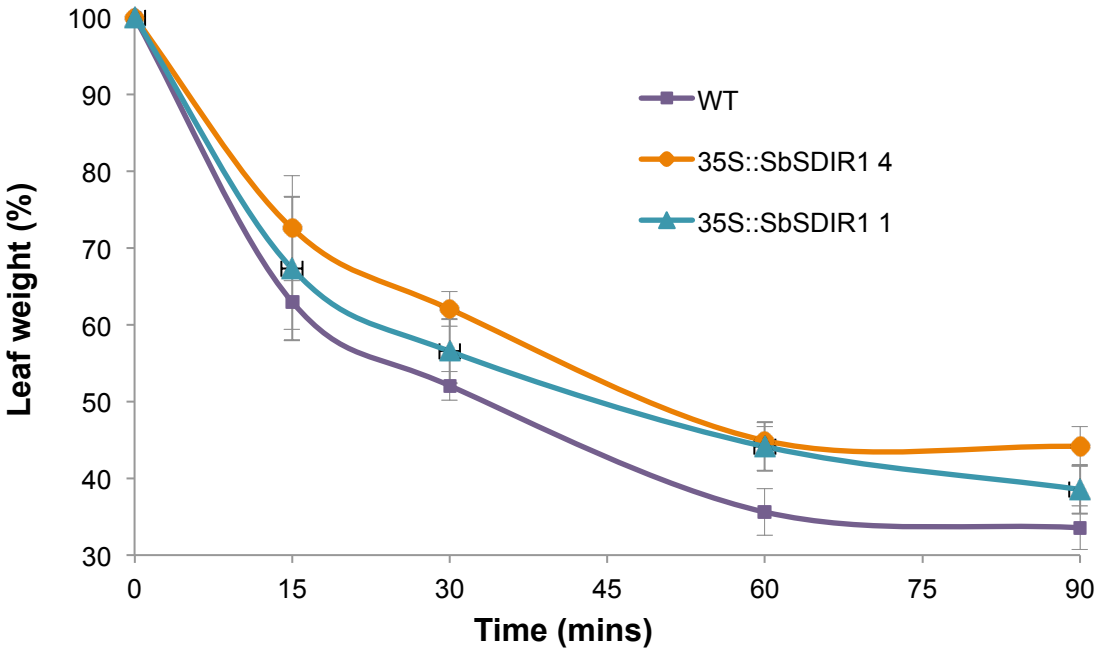


Figure 6.6 - Excised-leaf water-loss assay comparing water loss in wild type (*Col-0*) and 35S::SbSDIR1 overexpression lines. Leaves of the same developmental stages were excised and weighed at various time points after detachment (See Materials and Methods section 2.6.3). Each data point represents the mean of duplicate measurements. Error bars represent SE (n = 8). This experiment was carried out four times and the graph shows a representative result.

The differences in water loss between SDIR1 overexpression lines and wild type in other species are thought to be due differences in stomatal aperture (Zhang et al., 2007; Zhang et al., 2008; Xia et al., 2012; Liu et al., 2013). If this is the case for the 35S::SbSDIR1 transgenic lines, we would expect a reduction in stomatal conductivity in these lines compared to wild type. This was tested at the University of Lancaster using the Li-Cor® system (http://www.licor.com/env/). As shown in Figure 6.7, stomatal conductance was reduced compared to wild type in the highest expressing line,

35S:SbSDIR1 4. Conductance was reduced to a lesser extent in 35S::SbSDIR1 1 and there was no difference in conductance between the lowest expressing line (35S::SDIR1 3) and wild type.

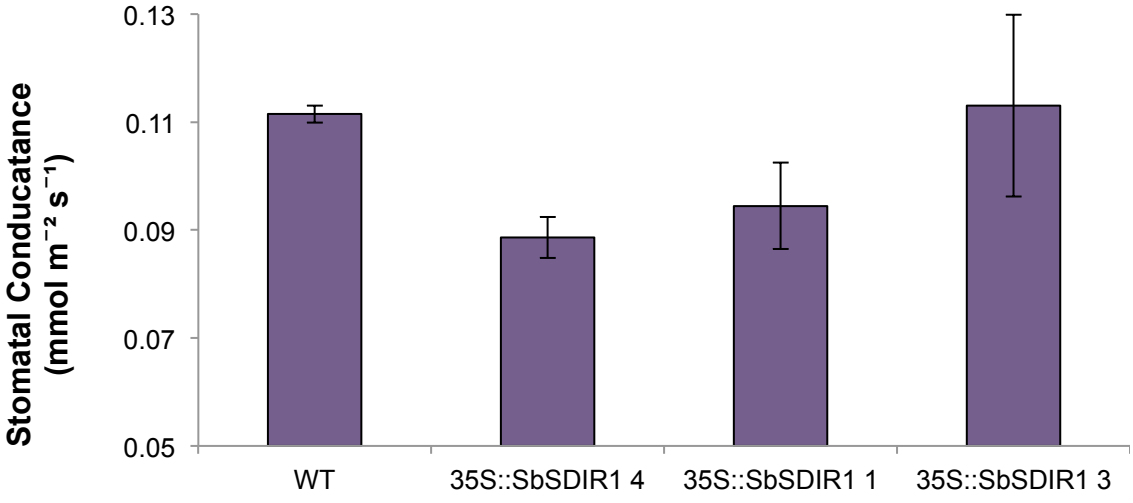


Figure 6.7 - Stomatal conductance of 35S::SbSDIR1 transgenic lines compared to wild type (*Col-0)*. Conductance was measured using the Li-Cor® system (see Materials and Methods section 2.6.4). Measurements were taken from ~5 week old plants under well-watered conditions. Leaves of approximately the same developmental stage were used. Error bars show SE (n = 3) ($p>0.05$ for all comparisons).

6.2.1.4.3 Stomatal density measurements

Differences in transpirational water loss can arise due to either differences in stomatal aperture or due to differences in stomatal numbers. To verify that the differences identified here are due to differences in stomatal aperture, stomatal numbers in each of the varieties were determined. As shown in Figure 6.8, all of the lines have similar numbers of stomata. This suggests that the differences in water loss and stomatal conductivity between the 35S:SbSDIR1 overexpression lines and wild type are due to differences in stomatal aperture.

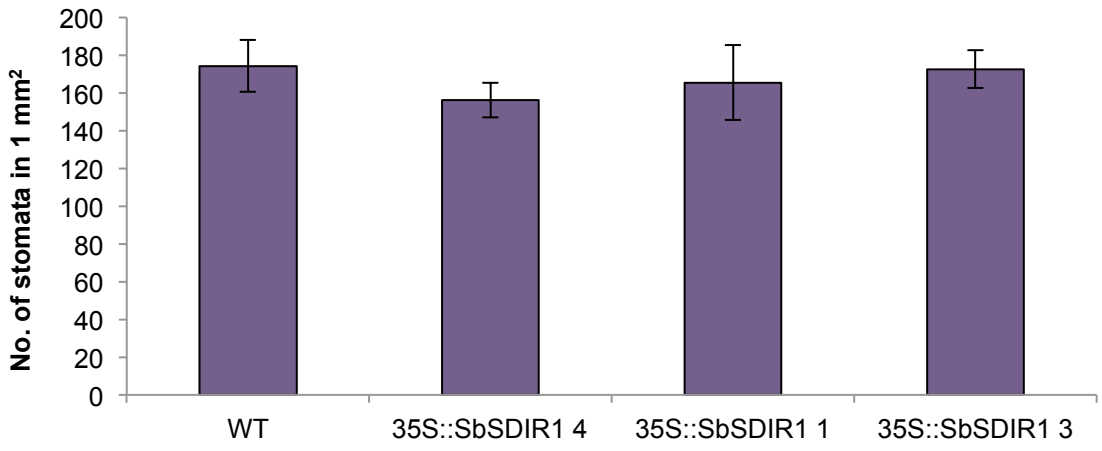


Figure 6.8 - Stomatal numbers in 35S::SbSDIR1 transgenic lines compared to wild-type *Col-0*. Leaves were taken from ~5 week old Arabidopsis. See Materials and Methods section 2.6.5. Leaves of approximately the same developmental stage were used. Error bars show SE (n = 20).

6.2.1.4.4 Gene expression analyses

In addition to improving drought tolerance by reducing stomatal aperture, SDIR1 is thought to function in the ABA signalling pathway and to influence downstream gene expression in Arabidopsis (Zhang et al., 2007). Overexpression of an SDIR1 homolog in tobacco resulted in an increase in the expression of delta 1-pyrroline-5-carboxylate synthase1 *(P5CS1*), which is involved with the biosynthesis of proline (Tak and Mhatre, 2013). Proline levels were also found to be higher in the maize *SDIR1* overexpression lines under drought conditions (Xia et al., 2012). Given that the B35 stay-green line has higher *P5CS* expression and higher levels of proline (see chapter 5), it was of interest to see whether the overexpression of *SbSDIR1* could result in an increase in *P5CS* expression. The expression of *P5CS1* in wild-type Arabidopsis and in each of the 35S::SbSDIR1 lines was quantified using real-time qPCR. As shown in Figure 6.9, there was no correlation between *SbSDIR1* and *P5CS1* expression in Arabidopsis under well-watered conditions. To assess whether there were differences in gene expression under

osmotically-stressed conditions, both wild type and the highest expressing 35S::SbSDIR4 line were subjected to osmotic stress imposed by mannitol. The expression of *P5CS1* was again tested. Surprisingly, *P5CS1* was expressed to lower levels in 35S::SbSDIR4 following drought stress (Figure 6.10).

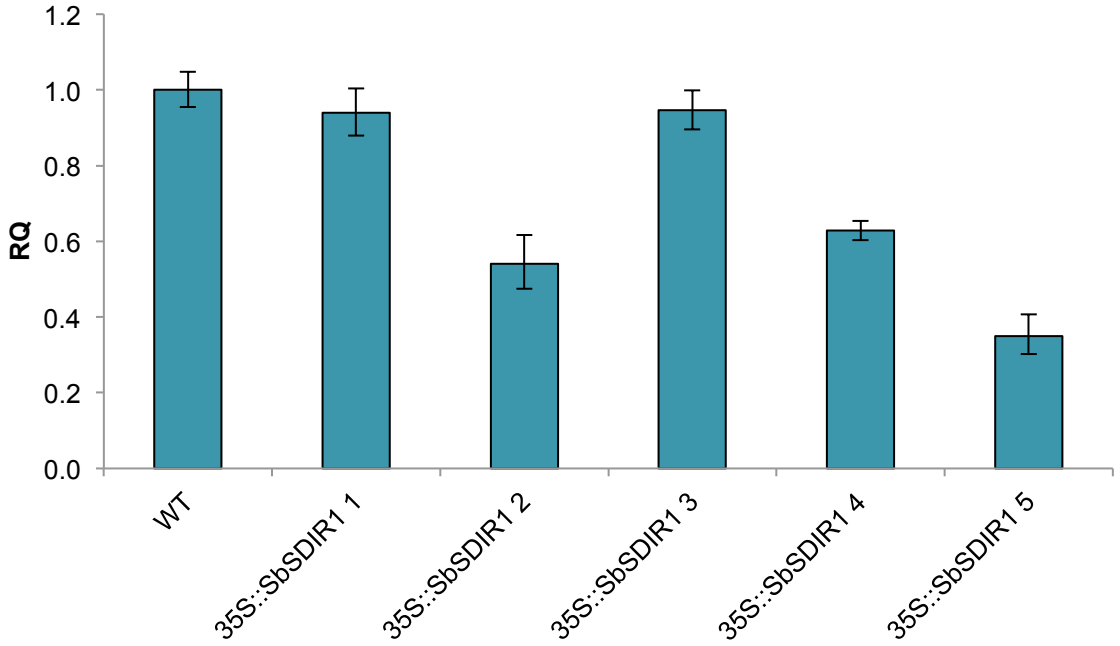


Figure 6.9 - Analysis of *P5CS1* expression levels in 35S::SbSDIR1 transgenic lines and in wild-type (*Col-0*) using real-time qPCR. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

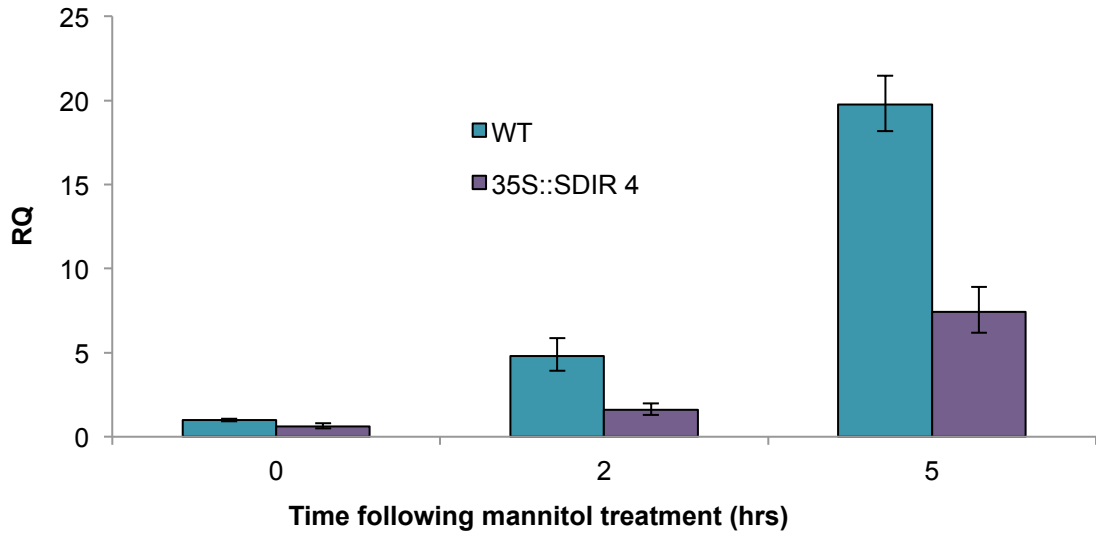


Figure 6.10 - Analysis of *P5CS1* expression levels in the 35S::SbSDIR1 4 transgenic line and in wild type (*Col-0*), following mannitol (0.4 M) treatment, using real-time qPCR. See Materials and Methods section 2.6.1.1 for treatment details. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.1.5 Mutant complementation in Arabidopsis

To determine whether SbSDIR1 could complement the Arabidopsis *sdir1* mutant, *sdir1-2* homozygous mutant plants were transformed with the 35S::SbSDIR1 construct. The *sdir1-2* mutant seeds were obtained from the Nottingham Arabidopsis Stock Centre (NASC) (http://arabidopsis.info) (SALK_114361). In order to confirm that these plants were homozygous for the T-DNA insertion, gDNA was extracted from the leaves and a PCR screening method was performed, using two reactions per plant. Firstly, a pair of gene-specific primers that flanked the putative T-DNA insertion, and secondly a gene-specific primer and a left border primer. Using this method all of the plants were verified as homozygous for the *sdir1-2* mutation before they were transformed with the 35S::SbSDIR1 construct. Transgenic plants from the T_2 generation were used for further

analysis. Expression of SbSDIR1 was verified by real-time qPCR in five independent transgenic lines (Figure 6.11).

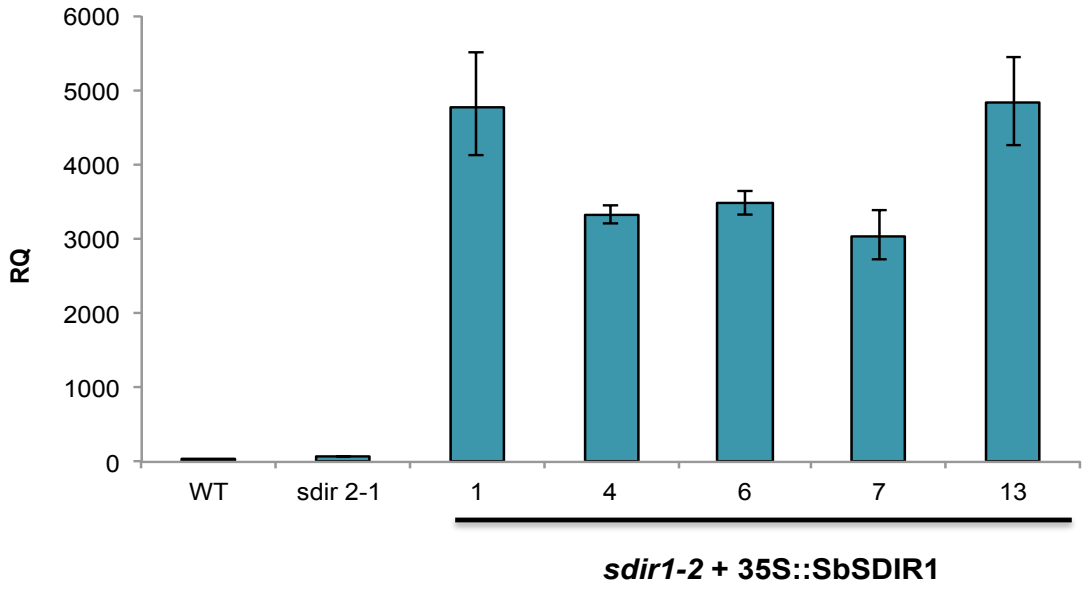


Figure 6.11 - Analysis of *SbSDIR1* expression levels due to 35S::SbSDIR1 in the *sdir1-2* mutant background using real-time qPCR. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.1.5.1 Measures of transpiration

Previous studies showed that the *sdir1* mutant line lost water more quickly than wild type in an excised-leaf water-loss assay (Zhang et al., 2007). To determine whether SbSDIR1 is able to complement that phenotype, a similar water loss assay was carried out on the lines expressing 35S::SbSDIR1 in the *sdir1-2* mutant background. The three transgenic lines tested lost water more slowly than wild type (Figure 6.12). However, no phenotype was observed for the *sdir1-2* mutant. This assay was repeated on three separate occasions and similar results were obtained.

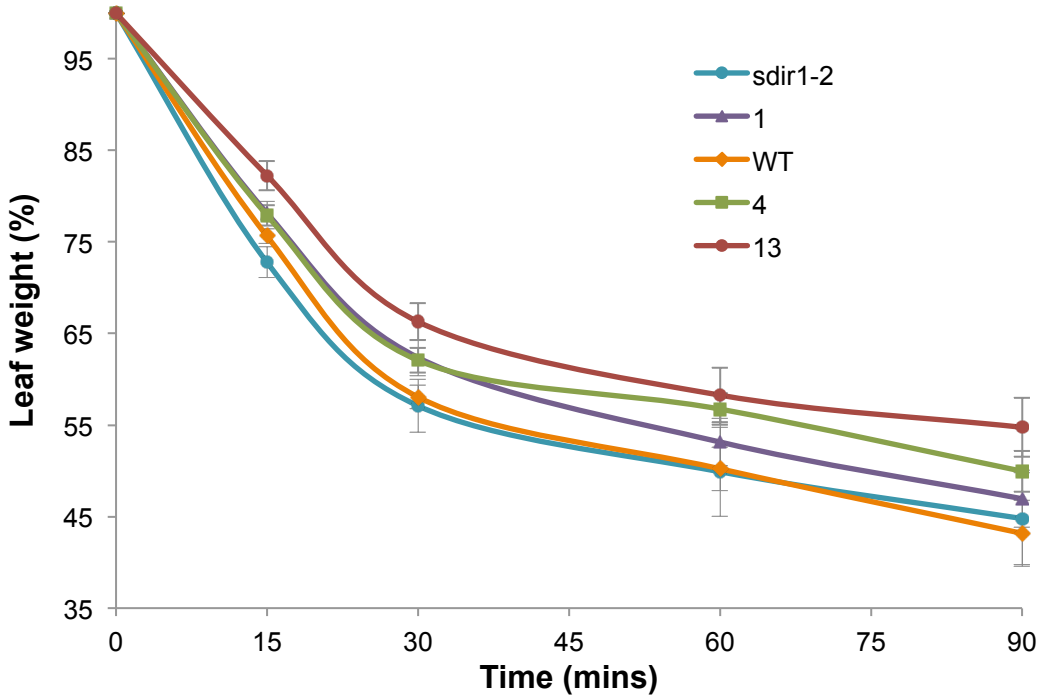


Figure 6.12 - Excised-leaf water loss assay comparing water loss in wild type (*Col-0*), *sdir1-2* and three lines containing 35S::SbSDIR1 in the *sdir1-2* mutant background (labelled 1, 4 and 13). Leaves of the same developmental stages were excised and weighed at various time points after detachment. Each data point represents the mean of duplicate measurements. Error bars represent SE (*n* = 8). This experiment was carried out three times and the graph shows a representative result.

6.2.1.5.2 Root phenotype analysis

The *sdir1-2* mutant line has been shown to have a longer primary root than wild-type and the 35S::SDIR1 overexpression lines have been shown to have a shorter root (Zhang et al., 2007). To determine whether SbSDIR1 is able to complement this element of the phenotype, root lengths were compared in wild type, *sdir1-2* and both the 35S::SbSDIR1 4 overexpression line and a complemented mutant line (named comp 4 here). There was no difference in the length of the roots of the 35S::SbSDIR1 4 line compared to wild type. The *sdir1-2* mutant had longer roots on average than wild-type, as previously

described (Zhang et al., 2007). This root phenotype was complemented by the expression of the 35S::SbSDIR1 construct (Figure 6.13).

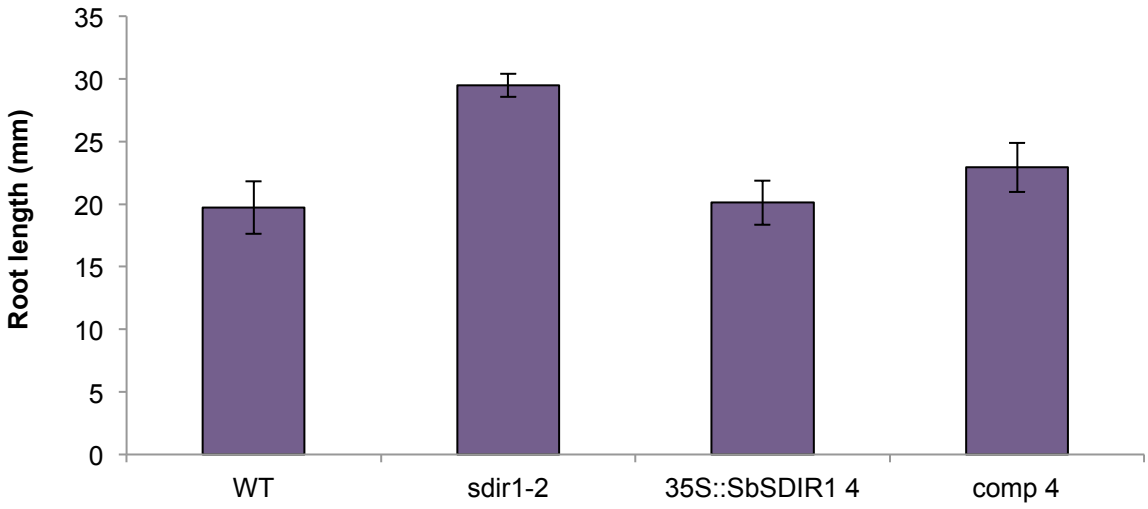


Figure 6.13 - Root length measurements in wild type (*Col-0*), *sdir1-2*, 35S::SbSDIR1 4 and 35S::SbSDIR1 in the *sdir1-2* mutant background (comp 4). Bar chart showing average root lengths. Seedlings were grown on vertical plates containing MS and measurements were taken after ten days (see materials and Methods 2.6.5). Each data point represents the mean of duplicate measurements. Error bars represent SE (n=35).

6.2.2 DREB1A

6.2.2.1 Validation of microarray data

The gene expression differences obtained by the microarray analysis were validated using real-time qPCR. As shown in Figure 6.14, the expression of *SbDREB1A* was ~4 fold higher in B35 compared to R16 at 50 DAS, thus validating the microarray data. *SbDREB1A* was not expressed to higher levels at 14 DAS.

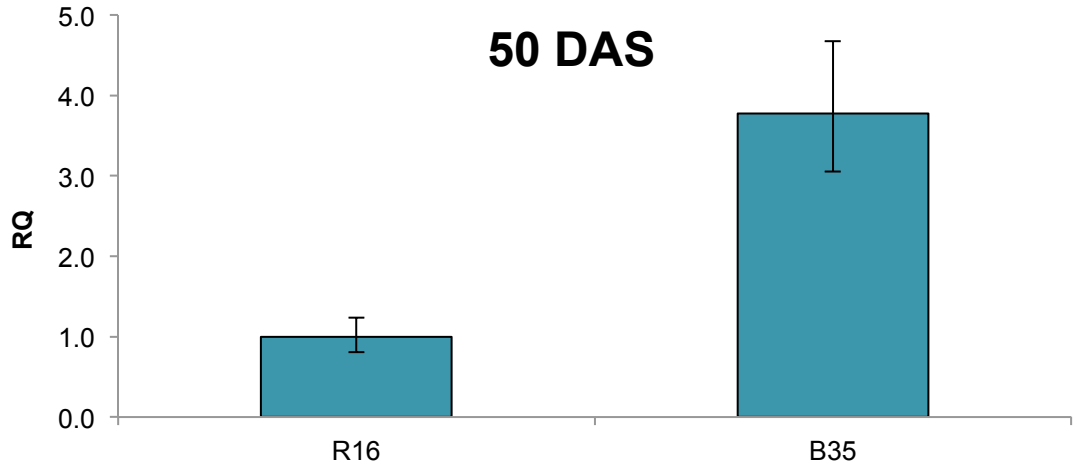


Figure 6.14 - Relative transcript abundance of *SbDREB1A* (Sb07g025210) in the senescent (R16) and stay-green (B35) varieties at 50 DAS. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.2.2 Sequence alignment

The sequence of SbDREB1A was obtained from the SbGDB database (http://www.plantgdb.org/SbGDB/). The SbDREBA cDNA was 723 bp in length with an open reading frame of 241 amino acids. When compared to Arabidopsis proteins using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), the SbDREB1B amino acid sequence shared strongest overall amino acid identity with DREB1D (38%, Total identity score:

128). When compared to proteins from other species, SbDREB1A shared strongest amino acid identity (77%, Total identity score: 244) with DREB1B from maize and 53% identity (Total identity score 192) to CBF3 from barley. The sequence was aligned to previously studied DREB homologues from other species using Clustal W (www.ebi.ac.uk/Tools/msa/clustalw2/) (Figure 6.15). The amino acid sequence contains the conserved putative AP2 DNA-binding domain and a putative activation domain rich in acidic amino acid residues at the C-terminal region. The sequence also contains the typical DREB1-type nuclear localization signal (NLS) consensus, KR/KPAGRT/KKFRETRHP, before the ERF/AP2 domain and a DSA sequence after the domain, which were identified in DREB1-type proteins from various species (Jaglo et al., 2001). The 14th and 19th conserved amino acids in the ERF/AP2 domain have been reported to be important in determining the target DNA sequences in Arabidopsis (Sakuma et al., 2002), although the 19th is less important (Qin et al., 2004). SbDREB1A was shown to have the conserved valine although E19 was replaced by another valine. SbDREB1A therefore contains many of the DREB1 conserved regions therefore is likely to be a homolog of DREB1.

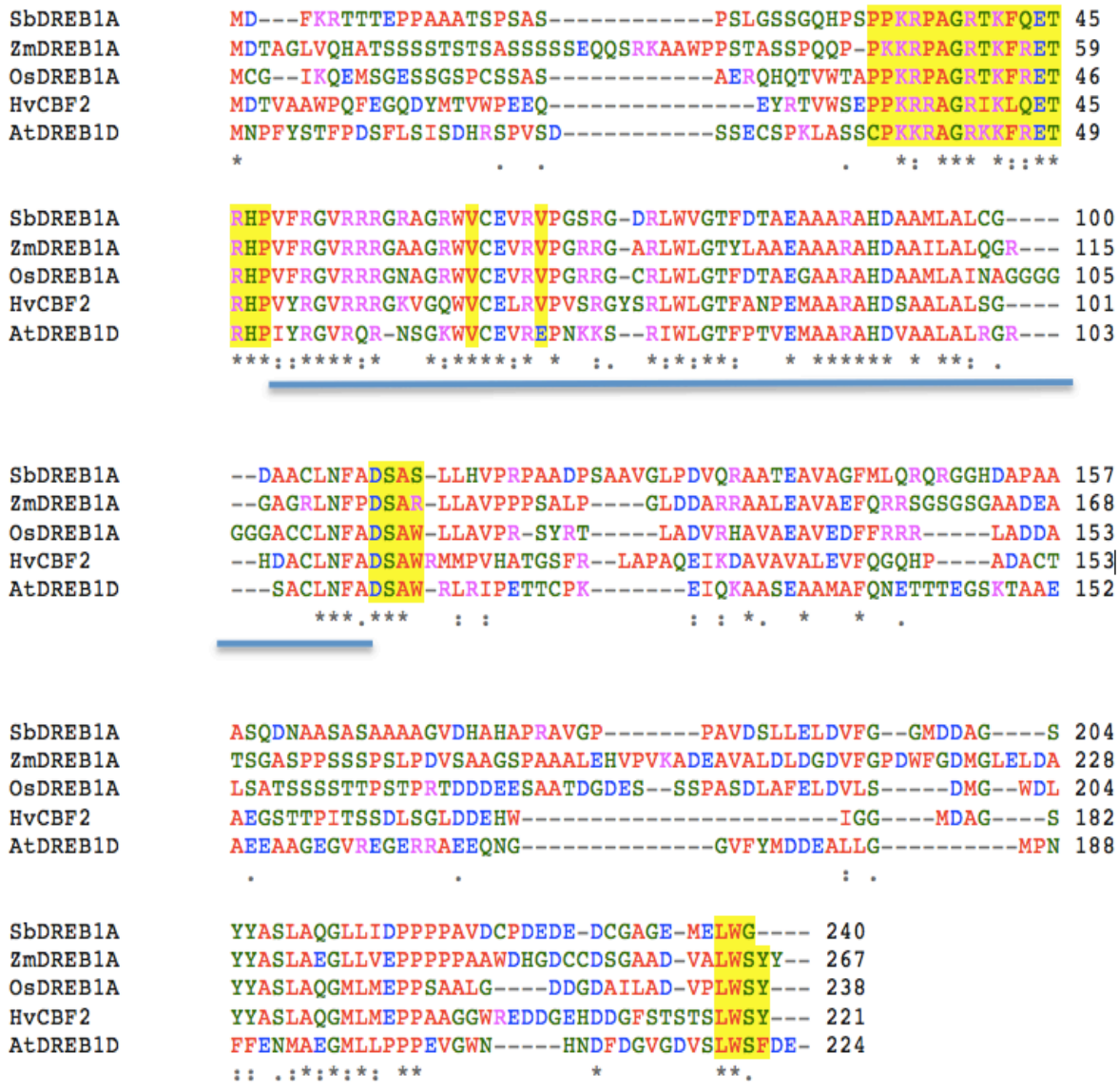


Figure 6.15 - Alignment of amino acid sequences of DREB1 homologs from *S. bicolor* (Sb07g025210), *Z. mays* (NM_001112181.1), *O. sativa* (AF300970), *H. vulgare* (AF442489) and *A. thaliana* (AT5G51990). The conserved AP2 DNA binding domain is underlined. Other conserved residues suggested to be important for DREB1 activity are highlighted. These include the KR/KPAGRT/KKFRETRHP nuclear localization signal and the DSAW and LSWY motifs.

6.2.2.3 Stress-induced expression in sorghum

The expression of *SbDREB1B* was examined in sorghum leaves in response to treatment with osmotic stress induced by mannitol and PEG, salt stress and ABA. *SbDREB1A* expression was strongly induced by osmotic stress induced by mannitol over 24 hr. It was also induced by osmotic stress induced by PEG and by NaCl treatment over 6 hr. SbDREB1A was additionally induced by ~7 fold in response to 24 hr ABA treatment. SbDREB1A therefore shows similar expression patterns to Arabidopsis DREB1D (CBF4) (Haake et al., 2002).

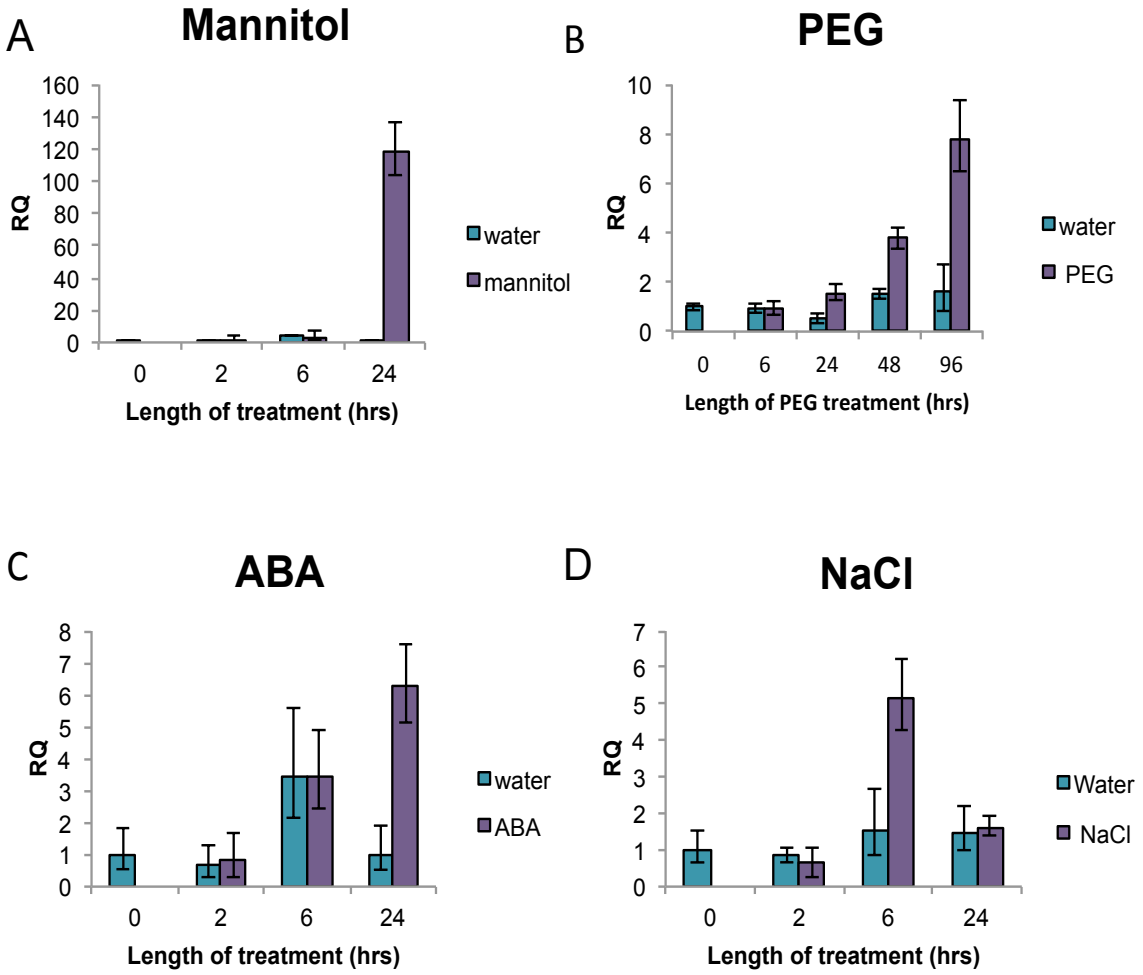


Figure 6.16 - Relative transcript abundance of *SbDREB1A* (*Sb01g039740*) in R16 following different stress treatments. Sorghum leaves were subjected to [A] mannitol (0.66M), [B] PEG (10%), [C] ABA (100 µM) and [D] NaCl treatment (200mM). For treatment details see Materials and Methods section 2.6.1. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

The expression of *SbDREB1A* was examined in response to heat using the microarray data described in Chapter 3. *SDREB1A* was shown to be upregulated by ~100 fold following a 3 hr heat shock at 50 °C (Figure 6.17). This upregulation in response to heat is similar to findings for Arabidopsis DREB2 genes (Agarwal et al., 2006).

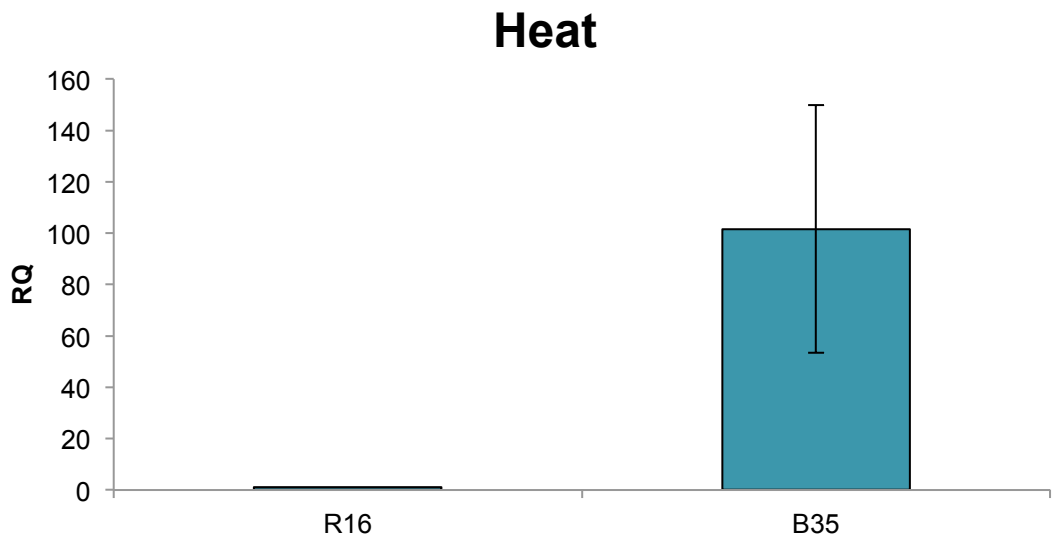


Figure 6.17 - Relative transcript abundance of *SbDREB1A* (*Sb01g039740*) in the R16 variety at 14 DAS following a 3 hr heat shock. Bar chart shows normalised gene expression data obtained from the microarray analysis (See Materials and Methods section 2.5.5). Error bars show SE of normalized expression data from three biological replicates.

6.2.2.4 Overexpression in Arabidopsis

6.2.2.4.1 Generation of overexpression lines

Transgenic Arabidopsis overexpressing *SbDREB1A* under the control of the cauliflower mosaic virus 35S promoter were produced, in the same way as described earlier (see 6.2.1.4.1). *SbDREB1A* was cloned downstream of the CaMV 35S promoter of the binary

gateway destination vector pK2GW7. *Agrobacterium tumefaciens* was transformed with the construct and was used to transform wild-type (*Col-0*) Arabidopsis plants via the floral dip method (see Materials and methods section 2.8.1). Transgenic plants from the T_2 generation were used for further analysis. Overexpression of *SbDREB1A* was confirmed in ten independent transgenic lines by real-time qPCR.

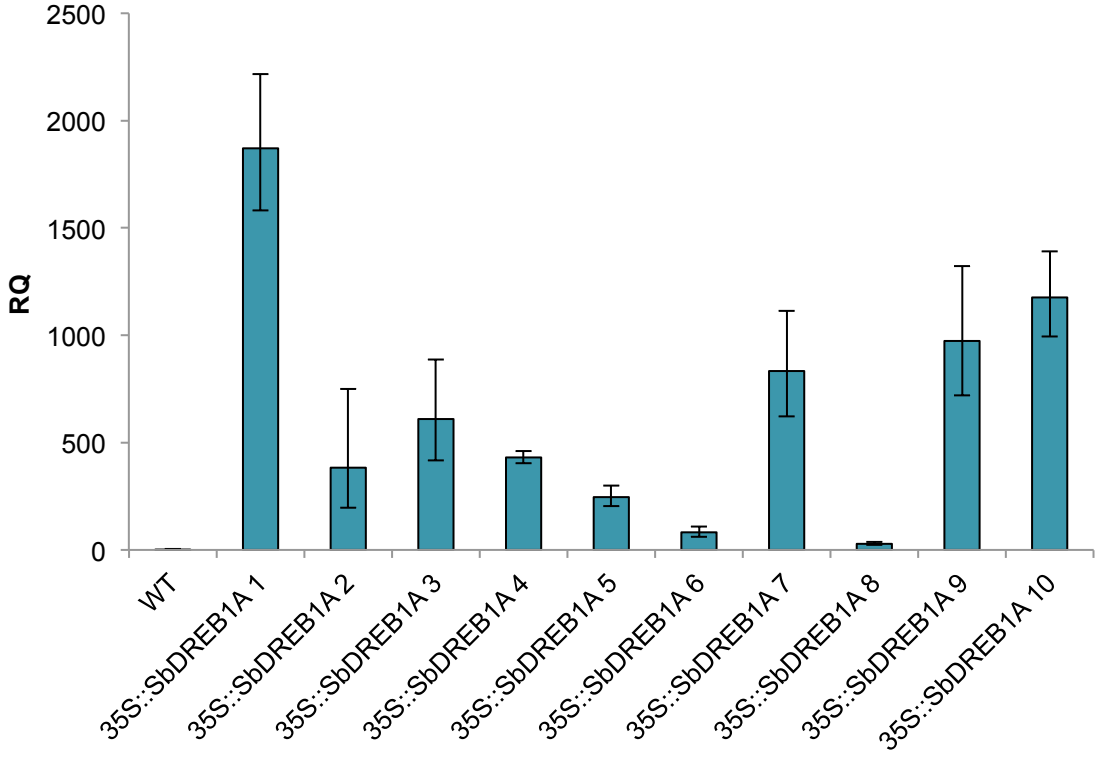


Figure 6.18 - Analysis of *SbDREB1A* expression levels in lines overexpressing the 35S::SbDREB1A construct in Arabidopsis *Col-0* background using real-time qPCR. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.2.4.2 Phenotype analysis

The overexpression of *DREB1* genes has previously been shown to result in growth retardation under normal conditions (Liu et al., 1998; Kasuga et al., 1999; Haake et al., 2002; Dubouzet et al., 2003; Qin et al., 2004; Savitch et al., 2005; Xiong and Fei, 2006). The 35S::SbDREB1A lines however, showed no growth retardation. Instead, they showed an unusual developmental phenotype in that excessive numbers of new leaves were produced (Figure 6.19).

Figure 6.19 - Growth phenotype of a transgenic plant overexpressing the 35S::SbDREB1A construct in the Arabidopsis *Col-0* background compared to wild type. Pictures were taken following ~6 weeks of growth.

6.2.2.4.3 Gene expression analyses

The overexpression of *DREB1* genes has previously been shown to result in an increase in the expression of downstream genes containing a DRE promoter element (Kasuga et al., 1999; Gilmour et al., 2000; Jaglo et al., 2001; Haake et al., 2002). Real-time qPCR was carried out test whether the overexpression of *SbDREB1A* could also induce the expression of genes known to contain a DRE element in their promoter in Arabidopsis. As shown in Figure 6.20, the expression of *KIN2* and *LTI78* was either unchanged or slightly reduced in the transgenic lines when compared to wild type. DREB2 proteins require post-translational modification in response to stress for their activation in plants (Liu et al., 1998). To see if this was the case here, the expression of DRE-containing genes was analysed in response to osmotic stress induced by mannitol treatment (Figure 6.21). The expression of *KIN2*, *LTI78*, *At1g16850* and *COR47* was lower in the 35S::SbDREB1A transgenic lines compared to wild type. The expression of *COR15a* however, was found to be increased compared to wild type, particularly in the 35S::SbDREB1A 7 transgenic line. Surprisingly, the expression of *P5CS1* was also increased in the transgenic lines despite the absence of the DRE in the promoter of this gene (Figure 6.21).

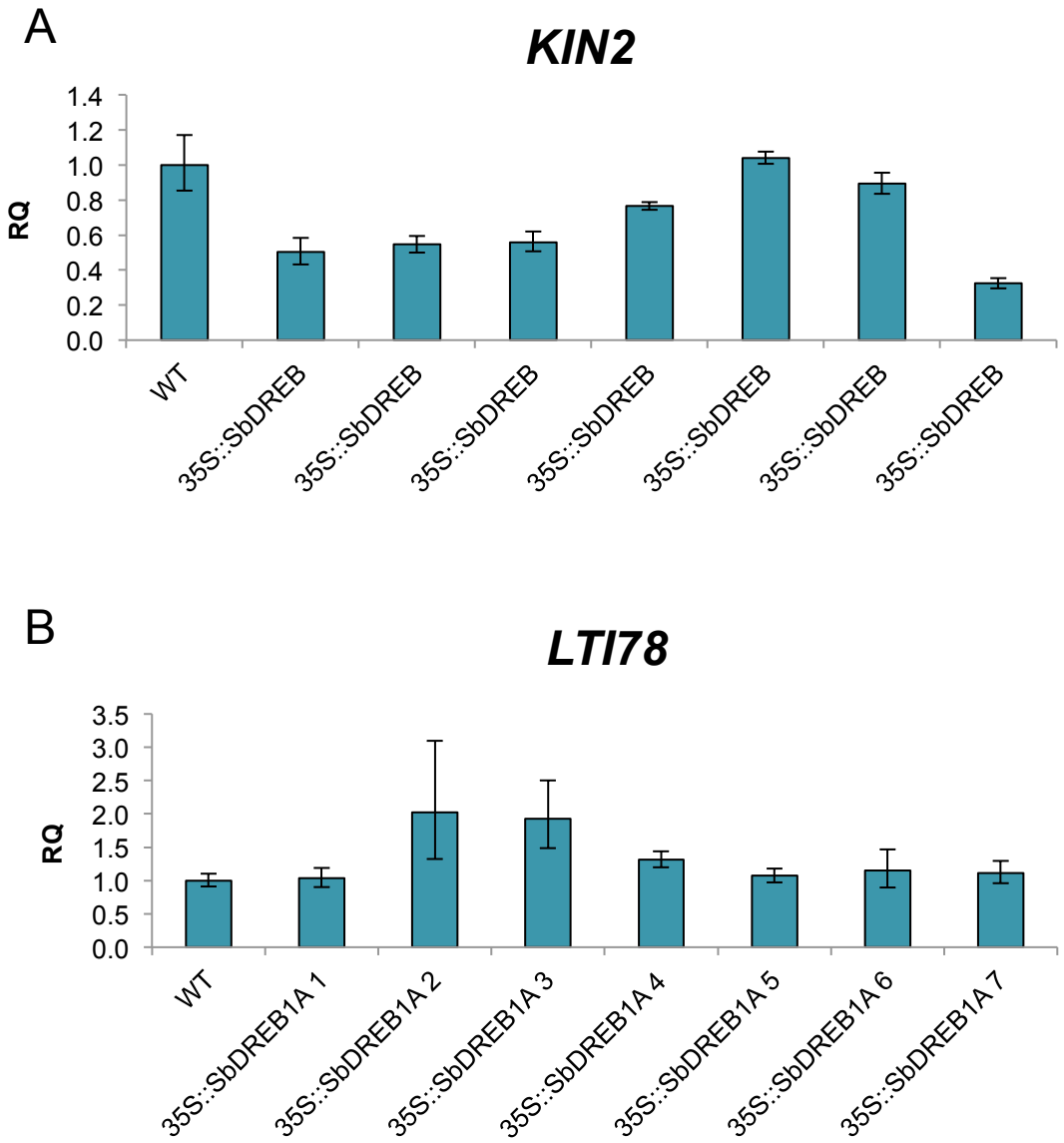


Figure 6.20 - Analysis of the expression levels of genes containing the DRE element in their promoter in lines overexpressing the 35S::SbDREB1A construct in Arabidopsis *Col-0* background using real-time qPCR. [A] Bar chart showing *KIN2* expression and [B] *LTI78* expression. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

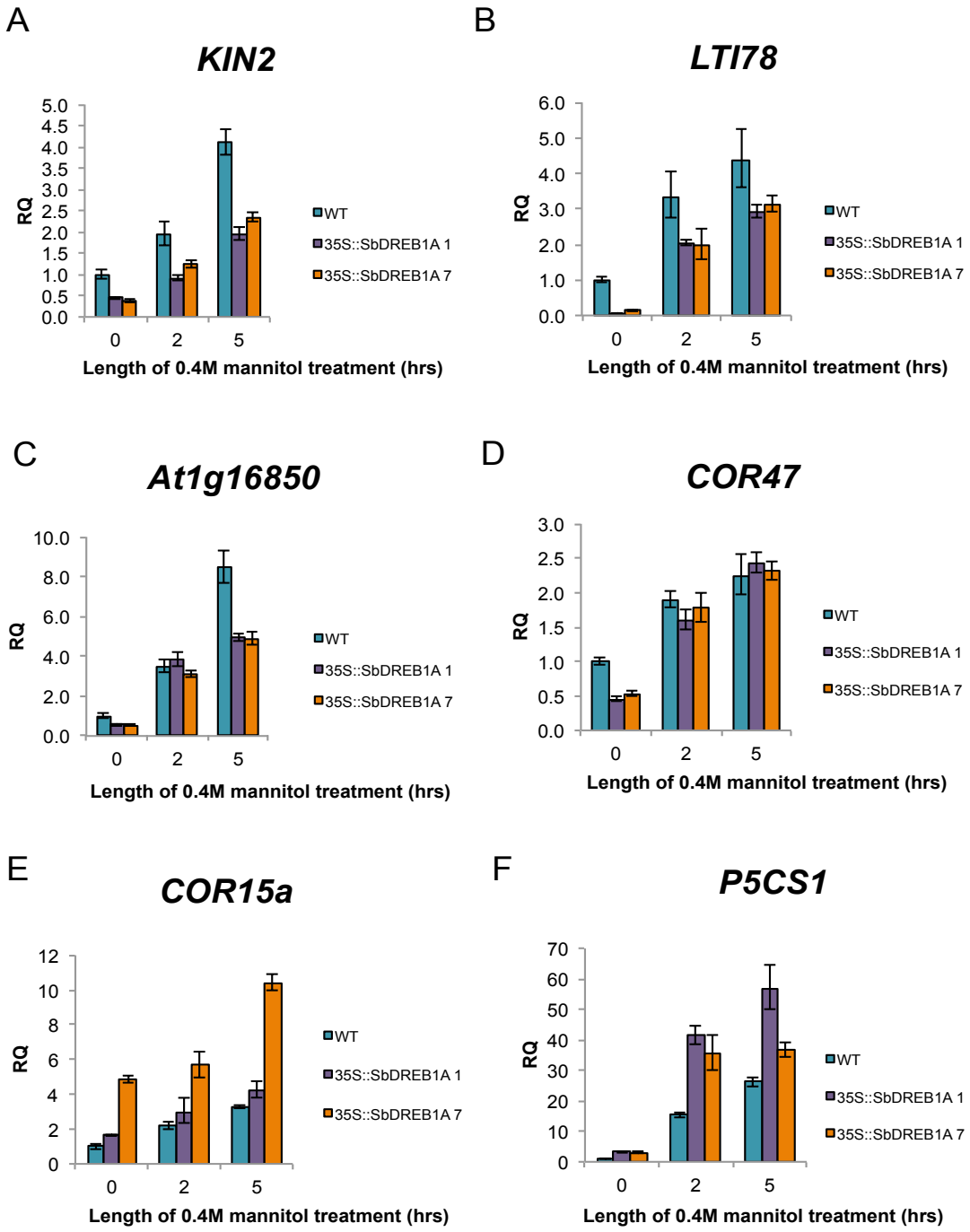


Figure 6.21 - Analysis of the expression levels of stress-inducible genes, following mannitol treatment, in lines overexpressing the 35S::SbDREB1A construct in Arabidopsis *Col-0* background using real-time qPCR. [A] *KIN2,* [B] *LTI78*, [C] *At1g16850*, [D] *RD47*, [E] *COR15a*, [F] *P5CS1* expression. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.2.3 Overexpression in sorghum

In order to test the function of both *SbSDIR1* and *SbDREB1A* in sorghum, stable sorghum transformation was attempted. This was carried out over a period of three months in Professor Ian Godwin's lab at the University of Queensland in Australia. Both genes were cloned downstream of the maize ubiquitin promoter of the binary Gateway destination vector pIPKb002 (see Appendix B.3 for vector map). Each construct was then co-bombarded with a Ubi::NPTII construct into immature sorghum calli (see Material and Methods section 2.8.2.2 for full method) (Liu et al., 2014). These calli were then cultured on selective regeneration media containing Geneticin® (G418). A total of 17 lines transformed with the Ubi::SbDREB1A construct and 15 lines transformed with the Ubi::SbSDIR1 construct were obtained following selection. To test for the incorporation of the constructs, real-time qPCR was carried out to test for either *SbSDIR1* or *SbDREB1A* overexpression. Neither of the transgenes were found to be overexpressed. The expression of the NPTII selectable marker was then measured in all of the lines. None of the lines transformed with the Ubi::SbSDIR1 construct had any NPTII expression whilst seven lines transformed with the Ubi::SbDREB1A construct showed NPTII expression (Figure 6.22).

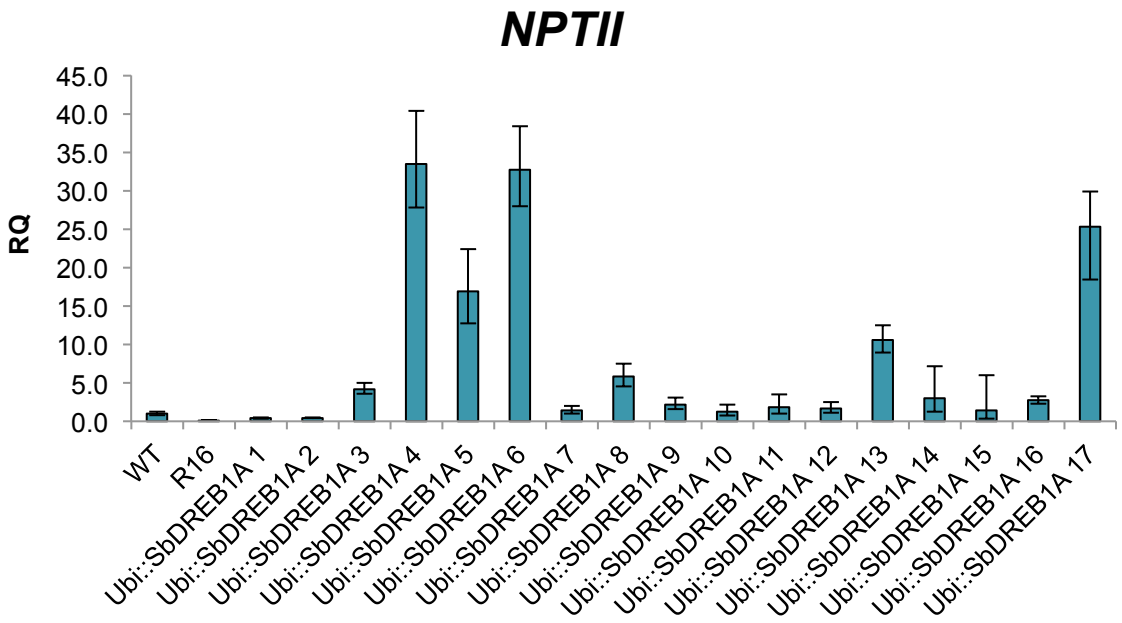


Figure 6.22 - Analysis of the expression levels of the NPTII selectable marker in the sorghum lines transformed with the Ubi::SbDREB1A construct. Error bars represent RQ_{MIN} and RQ_{MAX} and constitute the acceptable error level for a 95% confidence level according to Student's t-test.

6.3 Discussion

6.3.1 SbSDIR1 is a functional homolog of AtSDIR1

SDIR1 encodes a functional E3 ligase that confers drought tolerance in Arabidopsis, at least in part, by regulating transpirational water loss (Zhang et al., 2007). Homologues of this gene in rice, maize and grapevine have also been shown to encode functional E3 ligases and to have a role in conferring drought tolerance (Zhang et al., 2008; Xia et al., 2012; Tak and Mhatre, 2013). Here, the sorghum gene, *Sb01g039740,* was shown to encode a protein with strong homology to SDIR1 from other species and has all of the canonical domains required for E3 ubiquitin ligase activity (Figure 6.2). Based on this homology, this gene could play a similar role in conferring drought tolerance in sorghum. The ubiquitin ligase activity of the protein however was not tested here and it would be important to carry out *in vitro* assays to confirm this.

SbSDIR1 was shown to have similar gene expression profiles to those described for other species (Zhang et al., 2007; Gao et al., 2011; Xia et al., 2012; Tak and Mhatre, 2013). For example, it was found to be upregulated by osmotic stress induced by PEG and by salt stress (Figure 6.3). It was also slightly induced by ABA, which is similar to findings in grapevine and maize (Xia et al., 2012; Tak and Mhatre, 2013). This indicates that the function of *SDIR1* has been conserved in sorghum. Interestingly, *SbSDIR1* was also induced by heat, which is similar to findings in grapevine (Tak and Mhatre, 2013) (Figure 6.4). This suggests that *SbSDIR1* could have an additional role in conferring heat tolerance. *SbSDIR1* gene expression profiles were measured in the R16 senescent line. It would additionally be interesting to measure gene expression in response to these stress treatments in the B35 stay-green line. This would help to determine whether SDIR1 expression is upregulated to the same extent in the different varieties.

Arabidopsis transgenic lines overexpressing *SbSDIR1* exhibited a reduced water loss phenotype and the highest overexpressing lines showed reduced stomatal conductivity compared to wild type (Figures 6.6 & 6.7). This reduced water loss phenotype is similar to that described for all studied SDIR1 homologs, again suggesting that SDIR1 in sorghum is able to function in the same way as SDIR1 from other species. Despite this, stomatal conductance is lower only in the highest expressing lines (Figure 6.7). This

could be due to the small number of biological replicates used; due to time constraints at Lancaster University measurements were taken from only three plants. A greater number of replicates are required and could help to tease out any small differences and increase the significance of the results. Additionally, measurements were taken from only one leaf. It could be that whilst there are differences in conductivity at the whole plant level, measurements on only one leaf are not sufficient to see these differences. Whole plant imaging could be used in the future to see if this is the case. Furthermore, these measurements were taken under well-watered conditions only. Perhaps any differences are accentuated under drought-stress conditions. For example, the stomata in the 35S::SbSDIR1 lines may respond more quickly to the stress. This could be determined using epidermal peels to measure stomatal responsiveness to for example, mannitol or ABA (e.g. see Marchadier and Hetherington, 2014). No differences in stomatal numbers were found between the overexpression lines and wild type (Figure 6.8) thus suggesting that the observed differences in water loss are as a result of differences in stomatal aperture. Measuring stomatal apertures in epidermal peels would directly test this. As a control, it would also be important in the future to compare the transgenic lines to wild-type *Col-0* grown under the same selection system.

Loss-of-function *sdir1-2* mutant lines complemented with the 35S::SbSDIR1 construct showed a similar water loss phenotype to the overexpression lines (Figure 6.12). Previous work with the *sdir1-2* mutant line showed that it lost water more quickly than wild type (Zhang et al., 2007). However, this water-loss phenotype could not be replicated here (Figure 6.12). The previously described increase in root length in the *sdir1-2* mutant line on the other hand could be observed (Figure 6.13). The *sdir1-2* mutant line contains a T-DNA insertion in an intron (Zhang et al., 2007), meaning that the wild-type transcript might still be produced due to the T-DNA being spliced out alongside the intron. This would explain the unexpected SDIR1 transcript levels in the *sdir1-2* mutant line (Figure 6.11). Tissue-specific splicing of the intron could have resulted in the expected phenotype in the roots but not in the leaves. As a result, it would be interesting to repeat this experiment with a different known mutant line, *sdir1-1,* to confirm the water loss phenotype of this mutant.

6.3.2 SbSDIR1 could contribute to the stay-green trait in sorghum

Given that *SbSDIR1* is up-regulated by ~2 fold in the stay-green B35 line compared to the senescent R16 line, it is possible that it could be contributing to the known differences in transpiration between the varieties (Vadez et al., 2011) and hence to drought tolerance. It is thought that at the pre-flowering stage stay-green plants use less water, meaning that more is available when faced with drought stress at the post-flowering stage (Borrell et al., 2014a). It is possible that SbSDIR1 is contributing towards this water conservation. However, whilst SbSDIR1 appears to be able to reduce water loss in Arabidopsis, the only way to truly test its function would be to analyse the phenotype following its overexpression in sorghum. The sorghum transformation was unsuccessful here therefore it would be important to repeat this in the future.

The precise mechanism of SDIR1 action remains unclear. In Arabidopsis, it is thought that SDIR1 positively influences ABA signalling, due to the fact that its overexpression leads to ABA hypersensitivity and ABA-associated phenotypes. Consequently, it is thought that SDIR1 facilitates the degradation of a negative regulator of ABA signalling (Zhang et al., 2007). SDIR1 has recently been found to interact with SDIR1-INTERACTING PROTEIN1 (SDIRIP1), which acts upstream of the bZIP transcription factor ABI5 (Zhang et al., 2015). Although, given that 38 other clones were identified as interacting with SDIR1 using a yeast-two-hybrid approach (Zhang et al., 2015), it is likely that SDIR1 has many other, as yet uncharacterized, interacting partners. To better understand the mode of action of SbSDIR1 in sorghum, it would be important to identify these downstream targets. One approach could be to identify homologues of these 38 Arabidopsis proteins in sorghum and then confirm their interaction using *in vitro* pull-down assays.

Stress-responsive genes have previously been found to be upregulated in transgenic lines overexpressing SDIR1. For example, *P5CS1* was upregulated in tobacco and proline levels were found to be higher in maize (Xia et al., 2012; Tak and Mhatre, 2013). *P5CS1* was not found to be upregulated in the Arabidopsis 35S::SbSDIR1 expression lines here, although it is still possible that this gene is affected when *SbSDIR1* is overexpressed in sorghum. Unexpectedly, *P5CS1* was actually downregulated in the Arabidopsis overexpression lines compared to wild type, which could be an artefact of a

monocot gene being expressed in a dicot. The sorghum gene may be acting as a dominant negative and hence preventing the function of the wild type gene product. Transgenic tobacco lines overexpressing SDIR1 homologs from both maize and grapevine have increased levels of superoxide dismutase (*SOD*) and catalase (*CAT*) expression (Liu et al., 2013; Tak and Mhatre, 2013). Lines overexpressing the grapevine homolog additionally have higher levels of expression of a dehydrin (Tak and Mhatre, 2013). It would therefore be interesting to measure the expression of these stress-responsive genes in transgenic lines overexpressing the sorghum *SDIR1* gene. This would provide further insight into the downstream targets of SbSDIR1 and the processes it is involved with.

Maize SDIR1 overexpression lines are more tolerant to oxidative stress and have reduced ROS levels when exposed to drought. They also have increased expression of antioxidants and increased antioxidant activity (Xia et al., 2012). Similarly, transgenic tobacco overexpressing *SDIR1* from grapevine are better able to tolerate methyl viologen (MV) than wild type. It was shown in Chapter 5 that the B35 stay-green line is better able to tolerate MV than the R16 senescent line. Taken together, it is possible that the higher expression of *SbSDIR1* could be contributing to this enhanced oxidative stress tolerance via downstream target modification. Given that SbSDIR1 expression is also induced in response to heat stress (Figure 6.4), it is perhaps unlikely that it is only involved in regulating water loss in response to drought. This gene could therefore play an additional role in the oxidative stress response and in this way could also be involved in protection from heat stress. To test this, it would be interesting to investigate tolerance of *SbSDIR1* overexpression lines to oxidative stress.

6.3.3 SbDREB1A shows sequence homology to DREB1 and shows similar expression patterns

DREB transcription factors are well known to play a role in the plant stress response (Agarwal et al., 2006). Here, the sorghum gene *Sb07g025210 (SbDREB1A)* was shown to encode a protein that shares homology with previously characterized DREB transcription factors from other species (Figure 6.15). The amino acid sequence contains the conserved AP2 DNA binding domain and two signature motifs,

PKKP/RAGRxKFxETRHP and DSA, that are conserved amongst DREB1 genes across different species (Jaglo et al., 2001). In addition, the 14th amino acid in the AP2 domain, which is thought to be critical for DNA binding is also conserved (Sakuma et al., 2002). A glutamic acid at position 19 in the AP2 domain is thought to be important for DNA binding (Sakuma et al., 2002). This has been substituted with a valine in the SbDREB1A amino acid sequence however, this amino acid is thought to be less important and has also been substituted with a valine in other monocot DREB1 proteins (Qin et al., 2004). SbDREB1A shared strongest homology with DREB1D (CBF4) from Arabidopsis, which is known to play a role in the plant response to drought (Haake et al., 2002). Based on this homology, SbDREB1A could function in the same way as known DREB1 transcription factors from other species.

SbDREB1A was upregulated by osmotic stress induced by mannitol and PEG, NaCl and ABA (Figure 6.16). Arabidopsis DREB1A, DREB1B and DREB1C are induced by cold and not by drought stress (Liu et al., 1998). DREB1D on the other hand is upregulated by drought stress and not by cold (Haake et al., 2002). SbDREB1A therefore not only shows the strongest sequence homology to DREB1D but also shows similar patterns of expression. Interestingly, SbDREB1A was also induced by heat. This is similar to DREB2 genes (Lim et al., 2007) suggesting that SbDREB1A could also play a role in thermotolerance.

6.3.4 SbDREB1A induces the expression of some DRE-containing genes in Arabidopsis

More than 40 genes containing the DRE element have been identified as targets of DREB1 in Arabidopsis (Maruyama et al., 2004). In sorghum, 287 genes containing at least one DRE element (A/GCCGAC) 500 bp upstream of the transcription start site were expressed to higher levels in the stay-green B35 line when compared to the senescent R16 line. Amongst these are *SDIR1*, a number of *GST*s, late embryogenesis abundant proteins (*LEAs*) and stomatal density and distribution 1 (*SDD1*). It is possible that some or all of these genes are downstream targets of the highly expressed *SbDREB1A*.

The overexpression of *DREB1* genes in Arabidopsis has been shown to increase the expression of genes containing a DRE element (G/ACCGAC) in their promoter (Haake et al., 2002; Dubouzet et al., 2003; Qin et al., 2004; Xiong and Fei, 2006; Chen et al., 2007). However, the overexpression of *SbDREB1A* in Arabidopsis did not result in an increase in the expression of the majority of the genes tested. In fact, only *COR15a* showed increased expression (Figure 6.21). There could be a number of explanations for this. Firstly, perhaps *SbDREB1A* is simply not expressed to high enough levels to induce all of the genes tested. It was similarly found that when *ZmDREB1A* was over-expressed in Arabidopsis, only the highest overexpression lines showed increased expression of some of the DRE-containing genes (Qin et al., 2004). Alternatively, perhaps SbDREB1A binds preferentially to a slightly different DRE motif. For example, HvCBF2, a barley DREB protein, was shown to preferentially bind the GTCGAC sequence (Xue, 2003). Similarly, OsDREB1A, a rice DREB1 protein showed preferential binding to the GCCGAC sequence compared with ACCGAC (Dubouzet et al., 2003). Here, all of the DRE-containing genes tested, except *COR15a*, contained more ACCGAC motifs than GCCGAC motifs (Table 6.1). SbDREB1A could also preferentially bind the GCCGAC motif thus explaining why only *COR15a* was expressed to higher levels in the transgenic lines (Figure 6.21). To test this, transactivation assays could be carried out. For example, the Ubi::SbDREB1B construct could be co-bombarded into sorghum leaves with different promoter motifs fused to a luciferase reporter. The luciferase activity as a result of each promoter could then be compared to determine preferential binding activity. Microarray analysis comparing the 35S::SbDREB1A lines to wild type would also be useful to identify other downstream targets of this gene. Promoter analysis of the differentially expressed genes could then be used to identify specific binding motifs.

The overexpression of *SbDREB1A* resulted in the increased expression of *P5CS1,* which does not contain a DRE element (Table 6.1). The overexpression of *DREB1A* in Arabidopsis has also been shown to result in higher *P5CS1* expression compared to wild type with a concomitant increase in proline levels (Gilmour et al., 2000). As *P5CS1* does not contain a DRE element in its promoter, this effect is likely to be indirect. This indirect increase in gene expression has been previously observed in transgenic Arabidopsis overexpressing other DREB1 homologues (Wang et al., 2008).

Table 6.1 - Genes analysed in the 35S::SbDREB1A overexpression lines

Gene name	Gene ID	DRE (GCCGAC)	DRE (ACCGAC)
KIN 2	At5g15970	-	−131 to −126
LTI78	At5g52310	−131 to −126	−275 to −270 −225 to −220 −168 to −163
FL05-21-F13	At1g16850	−70 to −65	- 225 to −230 −133 to −138
COR 47	At1g20440	−966 to −961	−995 to −990 −161 to −156
Cor15a	At2g42540	−360 to −355 −183 to −178	−417 to −422
P5CS1	AT2G39800	-	-

The difference in downstream gene induction identified here compared to that previously described for other *DREB1* homologues could be due to differences in the SbDREB1A amino acid sequence. The PKKP/RAGRxKFxETRHP sequence is required for DREB1B to induce the expression of DRE-containing genes in Arabidopsis (Canella et al., 2010). SbDREB1A has a K3 to R3 substitution within this sequence, which could affect protein activity. Although, this is perhaps unlikely given that other monocot *DREB1* genes have the same substitution (Figure 6.15). The DSAW sequence is also thought to be important for DREB1 activity. In the SbDREB1A sequence W4 is substituted by S4, which could be responsible for differences in protein activity. However, a W4 to R4 substitution did not prevent ZmDREB1A from maize binding to the DRE element (Qin et al., 2004). It is possible that other residues, that are yet to be studied, are critical for DREB1 function. For example, particularly in the C-terminus, there are multiple residue differences between the sorghum sequence and that from other species (Figure 6.15). The C-terminus is thought to be important for the activation of transcription meaning that SbDREB1A might be able to bind the DRE element but then be unable to activate transcription. In this way SbDREB1A could have a dominant negative effect in Arabidopsis whereby it's binding to the DRE element could prevent the endogenous genes from activating transcription. Such an effect could account for the apparent decrease in the expression of many of the DRE-containing genes tested when compared to wild type (Figure 6.21).

Some DREB proteins, such as DREB2A, are degraded under non-stressed conditions meaning that their overexpression in Arabidopsis does not result in any phenotypic changes in the transgenic plants (Liu et al., 1998; Qin et al., 2008). Whilst perhaps unlikely given that the expression *COR15a* was increased in the transgenic lines, it is possible that SbDREB1A is rapidly degraded resulting in poor downstream gene induction. This could be tested using Western blots to detect SbDREB1A protein levels.

The overexpression of *SbDREB1A* in Arabidopsis gave an unexpected phenotype. However, this phenotype could be an artefact due to a monocot gene being overexpressed in a dicot i.e. perhaps SbDREB1A needs to be post-translationally modified or perhaps a binding partner is required. The only way to truly characterize the function of this gene is to overexpress it in sorghum. Whilst stable sorghum transformation was unsuccessful here, it will be necessary in the future for a full characterization of this gene.

6.3.5 Conclusions

SbSDIR1 and *SbDREB1A* both show sequence homology and similar expression patterns to the respective homologues in Arabidopsis. SbSDIR1 also appears to have a similar function to the Arabidopsis *SDIR1* gene. However, these genes can still not be linked conclusively to the stay-green phenotype. An investigation into their genetic linkage to known QTLs for the stay-green trait is required (see Chapter 7).

CHAPTER 7

Analysis of the stay-green QTL lines

7.1 Introduction

Quantitative trait loci (QTL) mapping has lead to the identification of regions in the sorghum genome that are associated with the stay-green trait (Sanchez et al., 2002). In this process, parents that differ in the trait are crossed to form an F_1 population, which have an intermediate phenotype. The F_1 population is then selfed to form a population of F_2 individuals. Each F_2 individual is then selfed for six additional generations, ultimately forming recombinant inbred lines (RILs) that are homozygous for a section of parental chromosome (Figure 7.1). The RILs are then scored for several genetic markers and for the phenotype (for mapping of the stay-green trait, individuals were given a score based on a visual rating of the green leaf area under post-flowering drought conditions (Sanchez et al., 2002)). In this way the probability that a particular genetic region is associated with the trait can be determined (Mauricio, 2001).

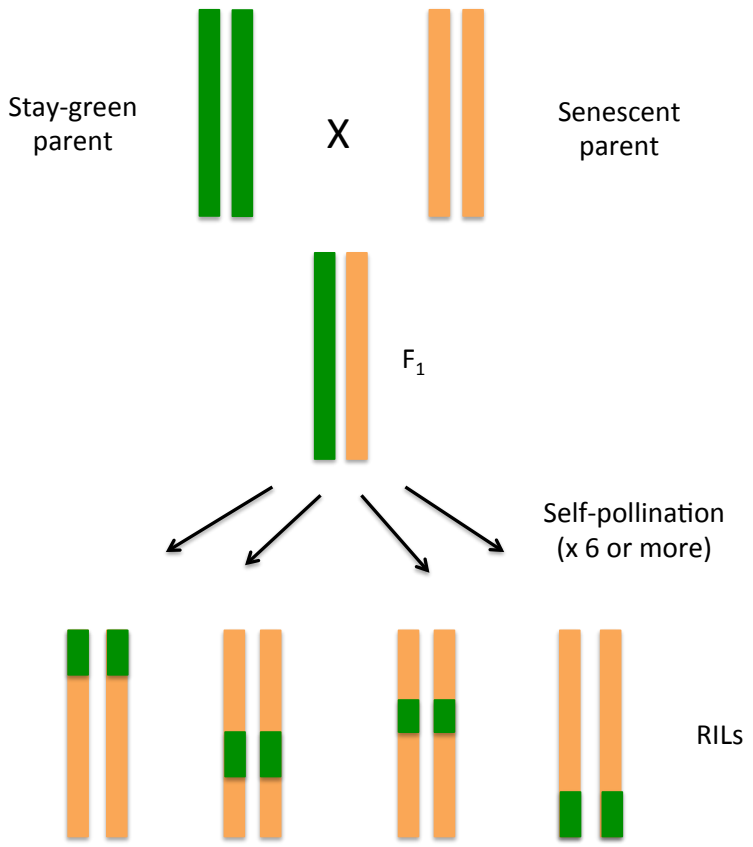


Figure 7.1 - Schematic depicting the basic strategy behind QTL mapping

Different studies, mostly using B35 as a source of stay-green, have lead to the identification of different numbers of QTLs for the stay-green phenotype (Table 7.1). Of these, initially four QTLs (Stg 1-4) were found to be consistent and are most often cited in the literature (Sanchez et al., 2002). Each of these has a different level of contribution towards the expression of the trait and Stg2 is thought to be the most important followed by Stg1 then Stg3 and lastly Stg4 (Xu et al., 2000; Sanchez et al., 2002). Another two QTLs, named StgA and StgB were also found to be consistent across multiple studies and show strong expression of the trait in the field (Kebede et al., 2001; Kassahun et al., 2010). To overcome difficulties in comparing QTL locations across studies, all of the QTLs were integrated onto a complete genome map (Mace and Jordan, 2011). The relative positions of the six main Stg QTL in the genome are shown in Figure 7.2. Each

of these regions have been introgressed from B35, both individually and in combination, into the high-yielding but senescent R16 and S35 lines (Vadez et al., 2011). These introgression lines show an intermediate phenotype between the stay-green and senescent parents and each single QTL introgression line shows increased post-flowering drought tolerance (Harris et al., 2007; Borrell et al., 2014b). This confirms that all six genetic regions are contributing towards the trait. Despite this, these regions remain large and between them contain ~2000 genes. The exact genes that are causal to the stay-green phenotype within these six regions have yet to be identified.

Table 7.1 - Stg QTL mapping studies

Reference	Stay-green source	Senescent line	No. of QTLs identified
(Tuinstra et al., 1997)	B35	Tx7078	7
(Crasta et al., 1999)	B35	Tx430	7
(Xu et al., 2000)	B35	Tx7000	4
(Tao et al., 2000)	QL41	QL39	5
(Subudhi et al., 2000)	B35	Tx7000	4
(Kebede et al., 2001)	SC56	Tx7000	9
(Haussmann et al., 2002)	E36-1	IS9830/N13	5-8

One way in which the number of candidate genes within the Stg QTL intervals could be reduced is through the analysis of gene expression data. For example, genes that are differentially expressed between the B35 and R16 varieties may lie within the QTL interval itself and the differences in gene expression may arise as a result of sequence differences in key regulatory regions. These genes could therefore directly underlie the trait. Alternatively, differentially expressed genes may not lie within the QTL interval itself but instead act downstream of a regulatory QTL gene. Candidate genes within the QTLs could then be pinpointed through the prediction of upstream regulatory genes.

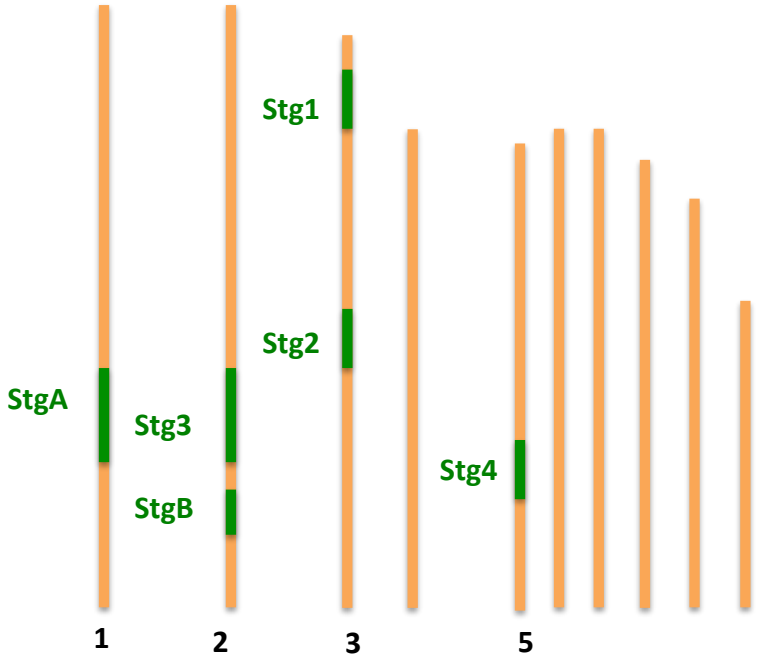


Figure 7.2 - Schematic showing the relative positions of the six main stay-green QTLs. Each line represents a different sorghum chromosome with the Stg QTL regions highlighted in green. The numbers represent the chromosome number.

This chapter focuses on experiments carried out to assess linkage of genes and processes with the known Stg QTL regions. This could help to reduce the number of candidate genes in the QTL intervals, which could greatly facilitate trait selection.

The aim of this chapter was to:

- Identify differentially expressed genes that lie within the stay-green QTL intervals (7.2.1)
- Determine whether any of the genes/processes taken forward for further analysis are genetically-linked to the Stg QTLs (7.2.2)
- Identify gene expression differences that are associated specifically with the StgB QTL region (7.2.3)

7.2 Results

7.2.1 Identification of differentially expressed genes that lie within known QTL regions

Genes lying within the Stg QTL intervals were identified by two methods. Firstly, Dr. Santosh Despande at ICRISAT, Petancheru, India, provided the position of the six main Stg QTLs and these co-ordinates were used to extract genes within these regions. Secondly, the Comparative Saccharinae Genome Resource (CSGR) (http://helos.pgml.uga.edu/qtl/) was used. This database contains genes found within all previously identified Stg QTLs both major and minor (see Table 7.1), not just the six shown in Figure 7.2 (Zhang et al., 2013). These lists of genes were compared to the list of genes that were differentially expressed between the stay-green B35 line and the senescent R16 line identified by the microarrays (Appendix D.12 & D.13). Out of the 2036 differentially expressed genes, 289 overlapped with the genes extracted from the CSGR database (see Appendix D.32 & D.33) and 165 overlapped with the genes within the six main QTLs (See Appendix D.34). Interestingly, these lists include *P5CS2,* which lies within the Stg1 QTL interval, *DREB1A*, which lies within a minor QTL on chromosome 7 and *RAP2.6*, which lies within a minor QTL on chromosome 4. Other potentially interesting genes that are differentially expressed between the varieties and that lie within the Stg QTL intervals include: a late embryogenesis abundant protein (*Sb02g036780.1*) within StgB, a senescence-related gene (*Sb03g026700.1*) and a heat shock protein (*Sb03g027330.1*) within Stg3. These gene expression changes could directly underlie the QTLs and could be caused by sequence polymorphisms in key regulatory regions.

7.2.2 Linkage of genes to particular QTL regions

Experiments were carried out to determine whether the higher expression of three of the genes that were focussed on in previous chapters (*P5CS2*, *SDIR1* and *DREB1A*) is associated with any of the Stg QTLs.

7.2.2.1 P5CS2

As discussed in Chapter 5, *P5CS2* was differentially expressed between the stay-green and senescent varieties and this differential expression is associated with differences in the amounts of free proline. *P5CS2* was found to lie within the Stg1 QTL interval. Differences in the expression of *P5CS2* between the varieties could be associated with polymorphisms in the upstream promoter region. To test this, 500 bp upstream of the start codon was amplified from the stay-green B35 variety and two senescent varieties, R16 and Tx7000 using PCR. The sequences were then compared (Figure 7.3). Sequence alignment identified three single nucleotide polymorphisms (SNPs) and a 22 bp deletion within the B35 line when compared with both senescent varieties. Two of the identified SNPs lie within known *cis*-element motifs. For example, a G to C SNP can be found in a potential C-box motif (Simpson et al., 2003) and an A to C SNP in a YACT motif (Gowik et al., 2004). A potential Myb element within the B35 upstream sequence is not present in the senescent R16 and Tx7000 varieties (Grotewold et al., 1994). It is thus possible that differences in the promoter sequence of *P5CS2* in B35 may be responsible for the differences in the expression of this gene. To test whether this is the case, constructs containing the luciferase reporter gene driven by either the B35 or R16 promoter were generated (see Appendix B.4-B.6). Each construct was then bombarded into sorghum leaf tissue on a MS plate (see Materials and Methods 2.9.2.1). Luciferase expression for each construct was quantified by measuring luminescence using a photon counting camera after spraying the leaves with luciferin (see Materials and Methods 2.4.10). Higher luminescence is indicative of higher luciferase expression and hence greater promoter activity. As can be seen in Figure 7.4, the leaves that were bombarded with the B35 promoter construct had slightly higher luminescence levels suggesting higher luciferase expression. However, this difference is small and not significant.

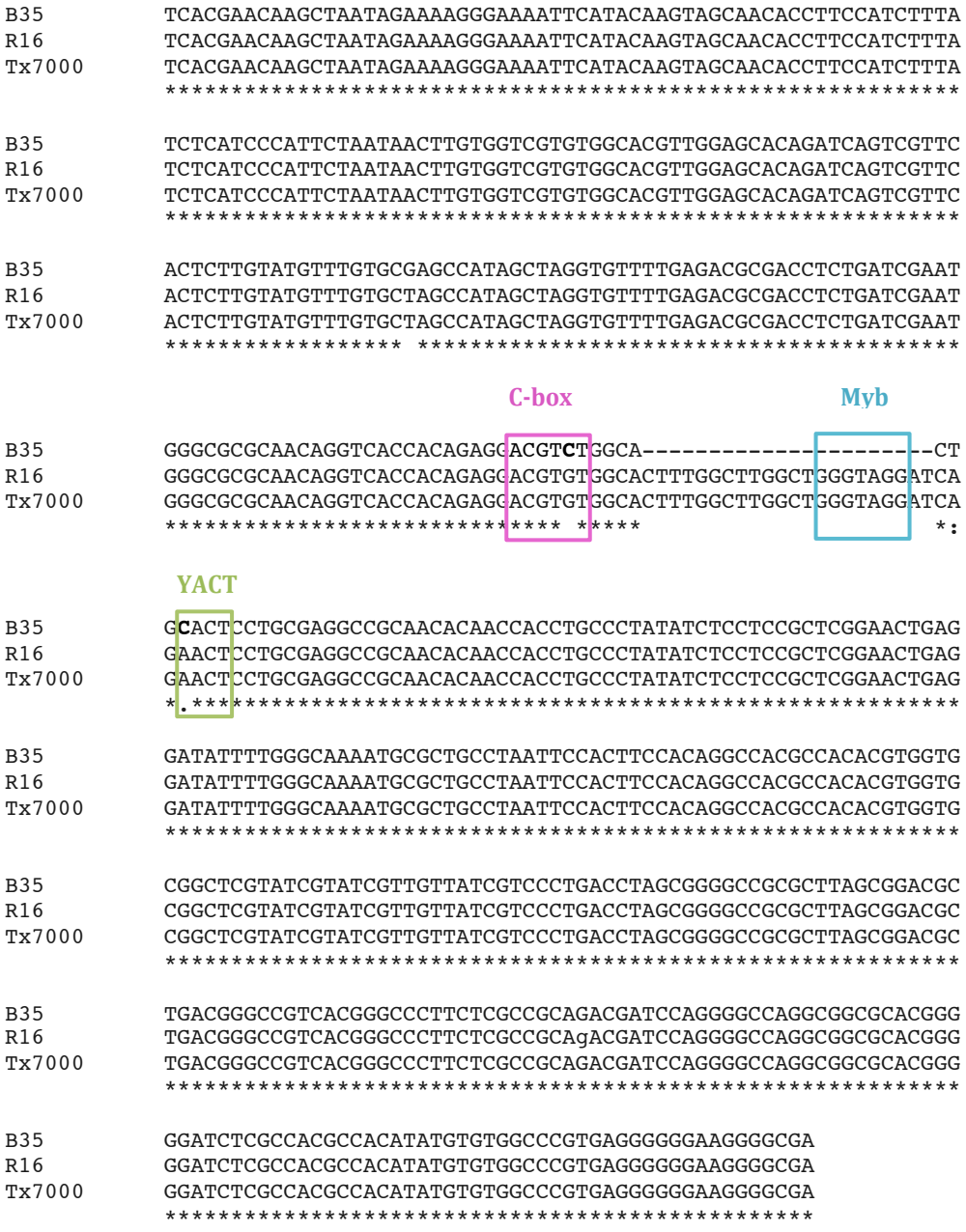


Figure 7.3 - Sequence alignment of -550 to -13 base pairs upstream of *P5CS2* in the stay-green (B35) and senescent varieties (R16 and Tx7000). The boxes highlight polymorphisms between the stay-green and senescent varieties that are within known cis-element motifs.

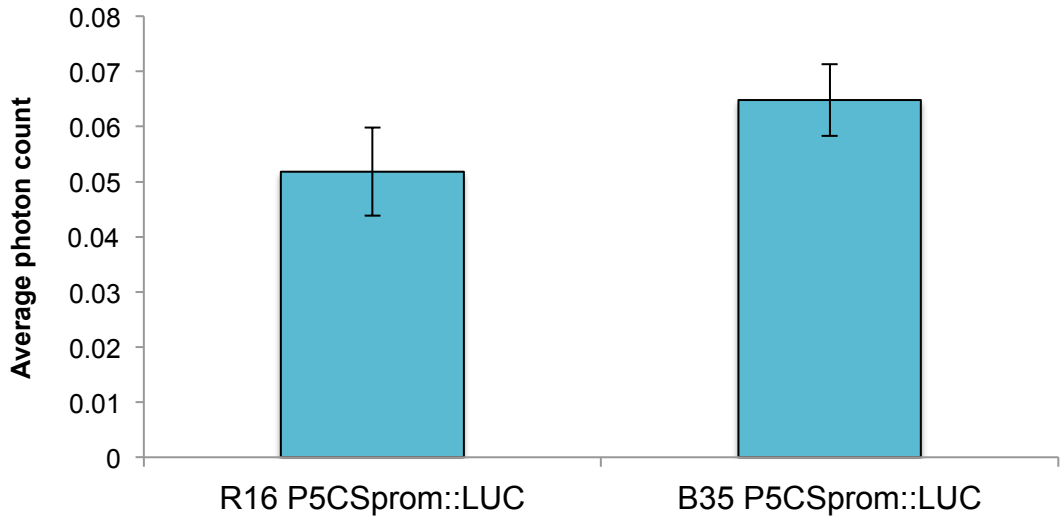


Figure 7.4 - Comparison of *P5CS* promoter activity from R16 and from B35 using a luciferase reporter construct. DNA 500 bp upstream of *P5CS2* in the stay-green (B35) and the senescent (R16) line was amplified and cloned upstream of the firefly luciferase reporter gene. Each construct was bombarded onto a square area (16cm^2) of sorghum leaf tissue (~8 DAS) on 1 X MS plates. Plates were sealed and incubated in the Percival for ~96 hrs. Each plate was then sprayed with luciferin and imaged for ~60 min using a photon counting camera. The average photon count for each plate was measured and the background luminescence subtracted. The graph shows the average of five plates for each construct and error bars show SE.

P5CS2 can be found within the Stg1 QTL region. It would be therefore be expected that if the identified polymorphisms are important for the expression of the trait, senescent lines introgressed with the Stg1 QTL from B35 would also contain them. Santosh Despande (ICRISAT, Petancheru, India) provided QTL introgression lines containing one or more Stg QTLs in the senescent R16 or S35 background (See Appendix E.1 for genotype numbers of the introgression lines). However, the putative promoter sequence of the Stg1 introgression lines was found to be identical to that of the senescent lines (data not shown). This suggests that these polymorphisms do not actually underlie the Stg1 QTL. Rather than underlying the QTL itself *P5CS2* may act downstream of a QTL

gene. To test this, *P5CS2* expression was analysed in lines containing different Stg QTLs in the senescent R16 or S35 backgrounds. Large-scale real-time qPCR was carried out on RNA extracted from leaf tissue taken at 50 DAS. The Fluidigm system was used for qPCR (see Materials and Methods section 2.4.9.2). As expected, *P5CS2* was expressed to higher levels in B35. However, whilst its expression was higher in nearly all of the QTL lines, it does not show linkage to any one line in particular (Figure 7.4). Multiple factors may be influencing the expression of this gene and this depends on the particular genetic background.

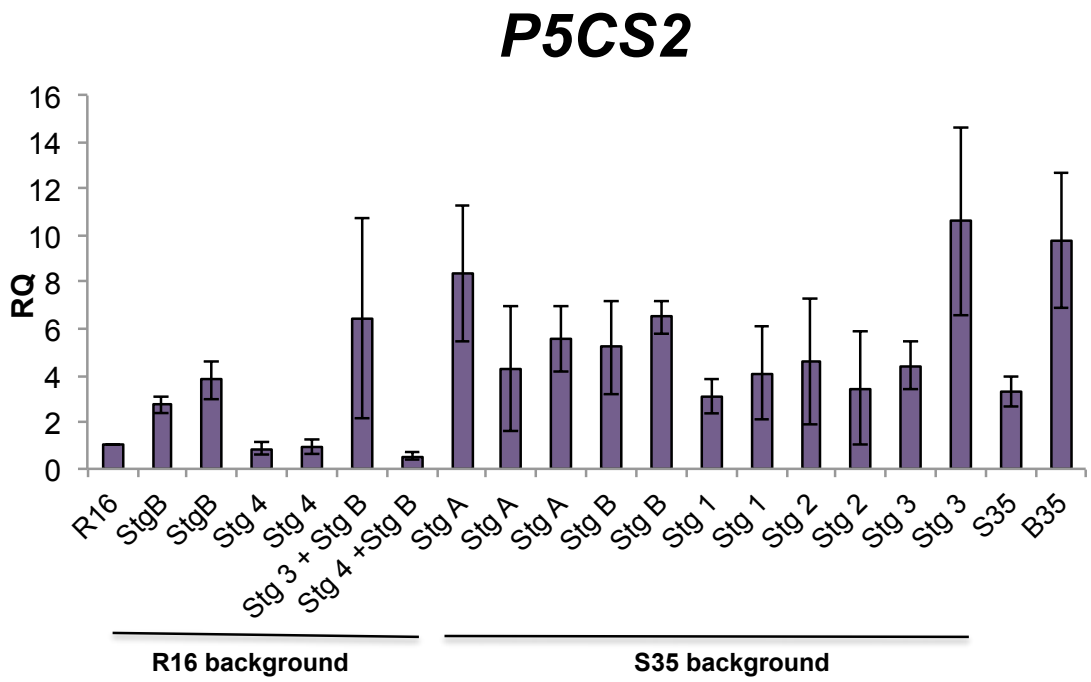


Figure 7.5 - Relative transcript abundance of *P5CS2* (*Sb03g039820.1*) in the senescent R16 and S35 lines, the stay-green B35 line and in introgression lines containing one or more Stg QTLs from B35 in the R16 or S35 background. Samples were taken from leaves at 50 DAS and qPCR was carried out using the Fluidigm system (see Materials and Methods section 2.4.9.2). Graph shows the average of three biological replicates and error bars show the standard error of the normalized expression values.

7.2.2.2 DREB1A

As discussed in Chapter 6, *DREB1A* was expressed to higher levels in the B35 stay-green line when compared to the senescent R16 line. *DREB1A* was also found to lie within a minor QTL on chromosome 7 (Haussmann et al., 2002). To determine whether any DNA sequence polymorphisms are responsible for the differences in the expression of this gene, the putative *DREB1A* promoter and coding sequence were PCR amplified from both the stay-green and senescent varieties and sequenced. The sequences were found to be identical. This suggests that *DREB1A* doesn't underlie the QTLs itself but instead could act downstream of another regulatory gene that does. To determine whether *DREB1A* acts downstream of one QTL in particular, gene expression was analysed in lines containing one or more QTLs in the senescent R16 or S35 backgrounds. As expected, *DREB1A* was expressed to higher levels in B35 compared to R16. Interestingly, it was also expressed to higher levels in both of the lines containing the Stg1 QTL and not in any of the other introgression lines (Figure 7.5). This suggests the Stg1 QTL is responsible for the higher expression of *DREB1A* in B35.

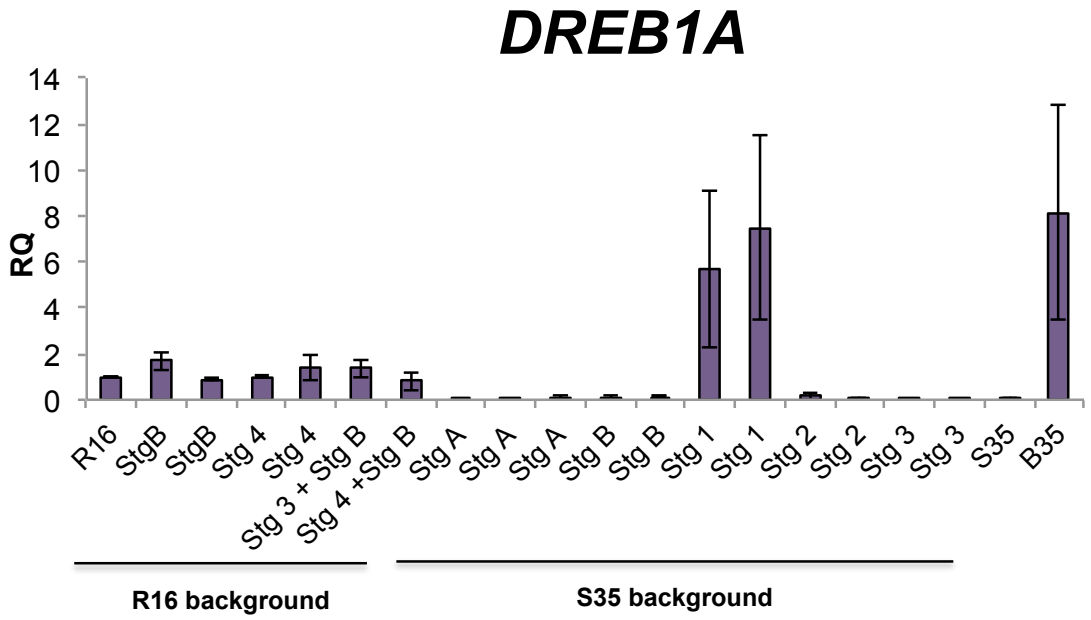


Figure 7.6 - Relative transcript abundance of *DREB1A* (*Sb07g025210*) in the senescent R16 and S35 lines, the stay-green B35 line and in introgression lines containing one or more Stg QTLs from B35 in the R16 or S35 background. Samples were taken from leaves at 50 DAS and qPCR was carried out using the Fluidigm system (see Materials and Methods section 2.4.9.2). Graph shows the average of three biological replicates and error bars show the standard error of the normalized expression values.

To explore possible regulators of *DREB1A* expression that lie within the Stg1 QTL, the putative *DREB1A* promoter was analysed for the presence of known *cis*-element motifs using the PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php) and PLACE databases (http://www.dna.affrc.go.jp/PLACE/). A number of known motifs were identified (Figure 7.6 & Table 7.2). Within the Stg1 QTL interval there is a gene encoding a bZIP transcription factor, which could potentially bind the ABRE element in the *DREB1A* promoter (Nakashima and Yamaguchi-Shinozaki, 2013). In Arabidopsis, MYB15 and ICE1 are known to regulate *DREB1* expression levels (Lata and Prasad, 2011). To identify potential homologs of these genes in sorghum, the amino acid

sequences were compared to all sorghum sequences using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Interestingly, a Myb transcription factor, MYB6 (Sb03g041360), showed 57% identity to MYB15 at the amino acid level and was found to lie within the Stg1 QTL interval. Whilst this gene itself is not differentially expressed by over 2-fold, it is possible that sequence polymorphisms could have impacted upon protein structure and hence altered the activity of this protein. This could have a knock-on effect on *DREB1A* expression.

Table 7.2 - Known *cis*-element motifs identified in the *DREB1A* promoter

Motif name	Description	Reference
Myb-related	Binding site of a GA-regulated *MYB* gene from barley	(Hosoda et al., 2002)
DRE-related	Binding site of barley CBF1 and CBF2	(Xue, 2003)
ABRE-related	Required for etiolation-induced expression of *ERD1* (early responsive to dehydration) in Arabidopsis	(Simpson et al., 2003)
GCC-box	Ethylene-responsive element	(Brown et al., 2003)
Myc-related	MYC recognition site found in the promoters of the dehydration-responsive gene *RD22* and many other genes in Arabidopsis	(Abe et al., 1997)
Myb-core	Binding site for ATMYB1 and ATMYB2, both isolated from Arabidopsis. ATMYB2 is involved in regulation of genes that are responsive to water stress in Arabidopsis	(Luscher and Eisenman, 1990)
W-box	Found in the promoter of the Arabidopsis *NPR1* gene. A cluster of WRKY binding sites act as negative regulatory elements for the inducible expression of *AtWRKY18*	(Yu et al., 2001)

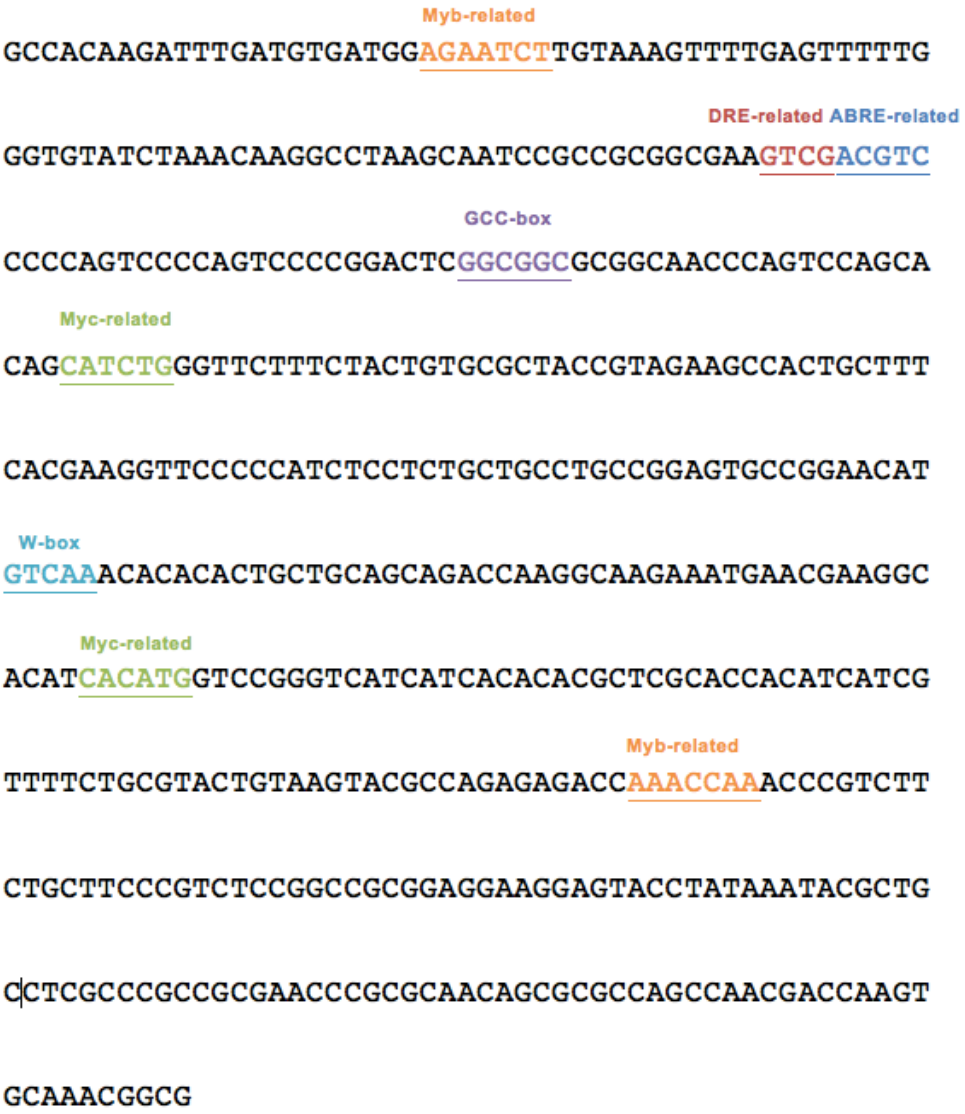


Figure 7.7 - DNA sequence 500 bp upstream of the start codon of *DREB1A* (Sb07g025210). Known *cis*-element motifs were identified using PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php) and PLACE (http://www.dna.affrc.go.jp/PLACE/) and are highlighted.

7.2.2.3 SDIR1

As discussed in Chapter 6, *SDIR1* is expressed to higher levels in the B35 stay-green line compared to the R16 senescent line. However, *SDIR1* cannot be found within a QTL interval suggesting that it acts downstream of another gene within a QTL. To assess whether this increased expression of *SDIR1* links to any of the QTLs in particular, the expression of this gene was also analysed in a range of QTL introgression lines. As expected the expression of *SDIR1* is higher in B35 compared to R16 however, expression is also significantly higher in both of the Stg1 introgression lines and in two of the StgB introgression lines (Figure 7.7), suggesting that the increased expression of *SDIR1* might be associated with these QTLs.

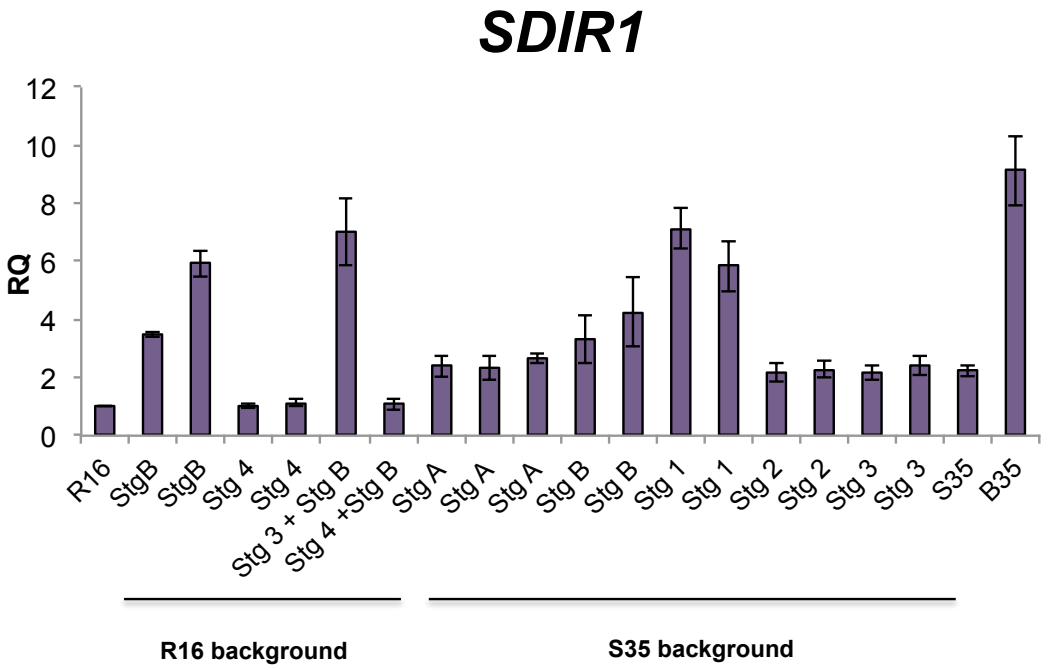


Figure 7.8 - Relative transcript abundance of *SDIR1* (*Sb01g039740*) in the senescent R16 and S35 lines, the stay-green B35 line and in introgression lines containing one or more Stg QTLs from B35 in the R16 or S35 background. Samples were taken from leaves at 50 DAS and qPCR was carried out using the Fluidigm system (see Materials

and Methods section 2.4.9.2). Graph shows the average of three biological replicates and error bars show the standard error of the normalized expression values.

As discussed in Chapter 6, the overexpression of *SDIR1* is associated with reduced water loss in a range of species (Zhang et al., 2007; Zhang et al., 2008; Xia et al., 2012; Tak and Mhatre, 2013) and the overexpression of *SbSDIR1* resulted in reduced stomatal conductivity in transgenic Arabidopsis (see 6.2.1.4). If *SbSDIR1* were involved with the regulation of transpiration in sorghum, we would perhaps expect to see reduced water loss in the QTL lines that express this gene to higher levels. To test this, excised-leaf water-loss assays were carried out on different QTL lines (see Materials and Methods 2.6.3). The percentage leaf weight for each line at 40 min following leaf detachment was calculated and then normalized to the R16 value. This was then correlated with *SbSDIR1* gene expression (Figure 7.8). There was a strong positive correlation between *SbSDIR1* gene expression and relative leaf weight following leaf detachment. This indicates that *SbSDIR1* reduces leaf water loss in sorghum as hypothesised in Chapter 6. Interestingly, both Stg1 lines showed the highest levels of gene expression and the lowest levels of water loss out of all of the lines tested (Figure 7.8). This suggests that Stg1 is largely responsible for the increased *SDIR1* expression in B35 and that this QTL could give rise to the stay-green phenotype by influencing transpirational water loss.

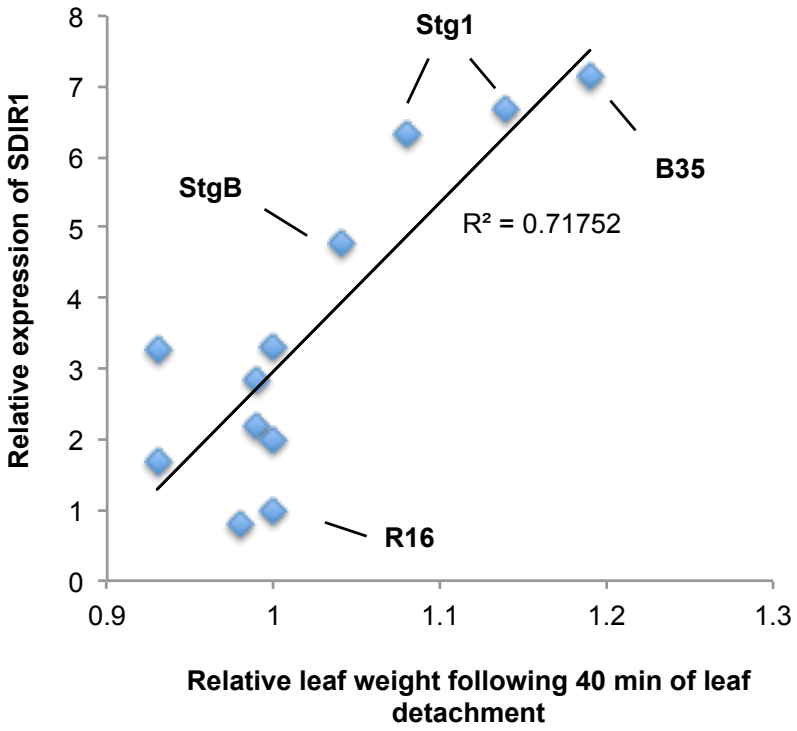


Figure 7.9 - Scatter plot showing the expression of *SDIR1* and the relative leaf weight following 40 min of leaf detachment measured in R16, B35 and different QTL introgression lines. All values are relative to the values obtained for R16. Relative gene expression values correspond to those shown in Figure 7.8 and were obtained using the Fluidigm qPCR system. An excised-leaf water-loss assay was carried out as described in the Materials and Methods section 2.6.3. These were carried out on R16, B35 and the Stg1 lines on three separate occasions. Ten QTL lines were measured and values obtained for Stg1 are indicated.

In the same way as described for *DREB1A*, the putative *SDIR1* promoter was analysed for the presence of known *cis*-elements to identify possible regulators of *SDIR1* within the Stg1 QTL. The promoter contains a number of known elements including two DRE elements, an ABRE and both MYB and MYC recognition sites (Figure 7.9 & Table 7.3). Within the Stg1 QTL there are two *MYB* transcription factors that could bind the Myb-binding sites and a bZIP transcription factor that could bind the ABRE element thus suggesting a possible way by which this gene is regulated.

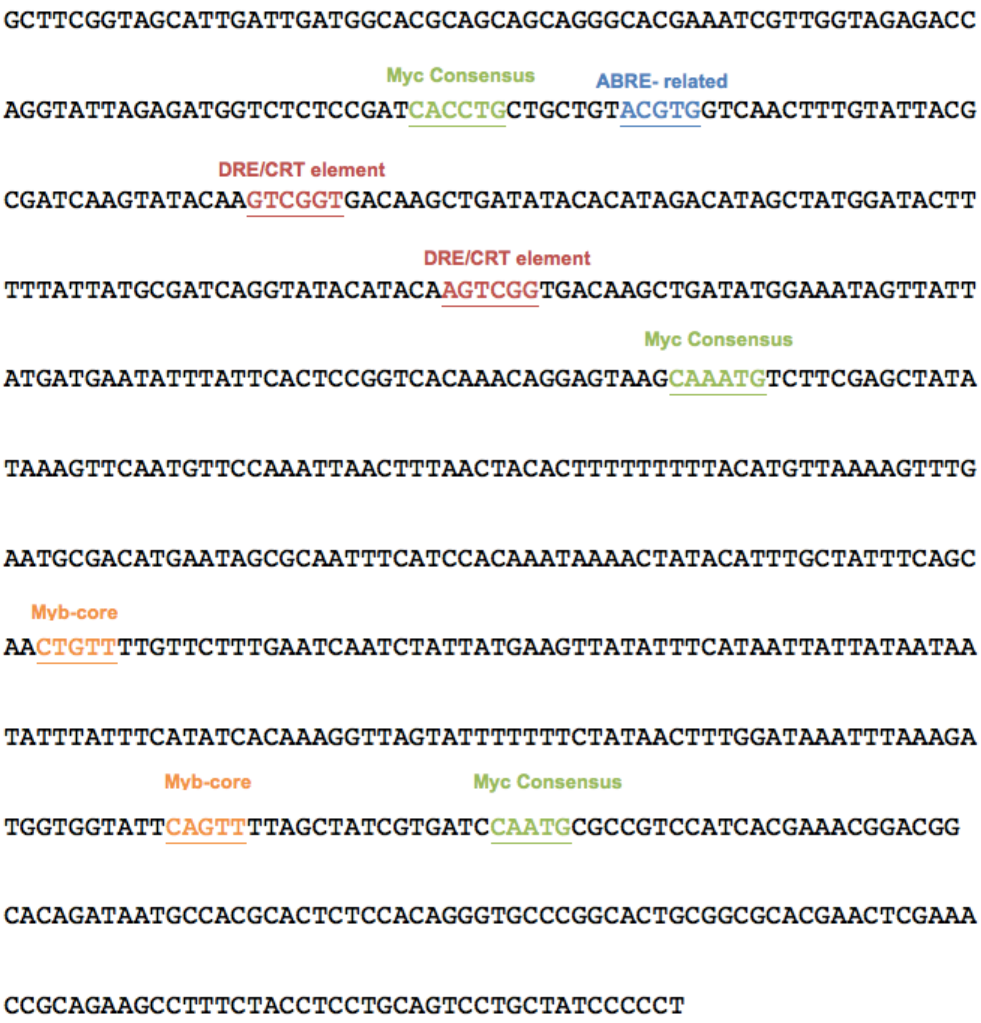


Figure 7.10 - DNA sequence 500 bp upstream of the start codon of SDIR1 (*Sb01g039740*). Known cis-element motifs were identified using PlantPAN (http://plantpan.mbc.nctu.edu.tw/seq_analysis.php) and PLACE (http://www.dna.affrc.go.jp/PLACE/) and are highlighted.

Table 7.3 - Known *cis*-element motifs identified in the DREB1A promoter

Motif name	Description	Reference
ABRE-like	Required for etiolation-induced expression of *ERD1* (early responsive to dehydration) in Arabidopsis	(Simpson et al., 2003)
DRE/CRT	Binding site of CBF transcription factors in Arabidopsis	(Yamaguchishinozaki and Shinozaki, 1994)
Myb core	Binding site for ATMYB1 and ATMYB2, both isolated from Arabidopsis. ATMYB2 is involved in regulation of genes that are responsive to water stress in Arabidopsis	(Luscher and Eisenman, 1990)
Myc consensus	MYC recognition site found in the promoters of the dehydration-responsive gene *RD22* and many other genes in Arabidopsis	(Abe et al., 1997)

7.2.3 Microarray analysis comparing gene expression in a senescent line (R16) and StgB QTL lines

The research described thus far has focussed on an identification of *all* processes that underlie the stay-green trait i.e. processes that underlie all six QTLs. In order to identify genes and processes that underlie a single QTL, microarray analysis comparing gene expression in R16 and in lines containing StgB in the R16 background was carried out. These lines are identical with the exception of the StgB interval therefore any gene expression changes should be directly related to this QTL. The StgB QTL was chosen for further study due to the fact that it has received comparatively little attention so far in the literature yet StgB introgression lines in the field display a strong stay-green phenotype (personal communication with Tom Hash, ICRISAT, Petancheru, India). Two StgB introgression lines were provided by Santosh Despande (K359-3 White-1 and K369-2-1).

7.2.3.1 Gene expression differences

Samples of leaf tissue were taken from the two StgB lines and from R16 at 50 DAS. Microarray analysis was carried out in the same way as described previously (see

Chapter 3). Gene expression in each StgB line was compared to gene expression in R16. Differentially expressed genes that were in common between the two StgB vs. R16 comparisons were taken forward for further study. As shown in Table 7.4, 113 genes were expressed to higher levels in *both* StgB lines when compared to R16 whereas 69 genes were expressed to lower levels. Of these, 58 were also expressed to higher levels and 29 to lower levels in B35. Two of the upregulated genes and six of the down-regulated genes were found to be located within the QTL interval itself.

Table 7.4 - Genes that were expressed to higher levels (FC>2 in all three biological replicates) in both StgB vs. R16 comparisons. Genes that were also expressed to higher levels in the B35 stay-green line are highlighted in purple and genes that lie within the StgB QTL interval are highlighted in red.

SbID	Average StgB1 vs. R16 FC	Average StgB2 vs. R16 FC	Annotation
Sb01g002760.1	7.34	6.14	ASA1 (ANTHRANILATE SYNTHASE ALPHA SUBUNIT 1)
Sb01g004140.1	7.40	9.10	N2, N2dimethylguanosine tRNA methyltransferase family protein
Sb01g004280.1	3.21	4.16	SMT1 (STEROL METHYLTRANSFERASE 1)
Sb01g004295.1	3.02	4.01	SMT1 (STEROL METHYLTRANSFERASE 1)
Sb01g004300.1	3.00	3.90	SMT1 (STEROL METHYLTRANSFERASE 1)
Sb01g015450.1	10.39	9.61	
Sb01g018470.1	8.68	7.50	Secretory protein, putative
Sb01g027330.1	44.68	47.82	Peroxidase, putative
Sb01g031740.1	10.47	7.06	Peroxidase, putative
Sb01g035990.1	7.82	15.82	Peroxidase, putative
Sb01g036360.1	9.11	4.79	CYP714A1
Sb01g045260.1	10.05	8.69	Protein kinase, putative
Sb01g046160.1	5.16	7.77	BGAL3 (betagalactosidase 3)
Sb02g019380.1	20.16	18.14	FLA2 (FASCICLINLIKE ARABINOGALACTAN 2)
Sb02g025240.1	5.86	7.97	Transcription factor
Sb02g028900.1	8.57	7.91	Sarcosine oxidase family protein
Sb02g033720.1	8.20	12.42	Protein kinase family protein
Sb02g033755.1	11.94	13.18	Meprin and TRAF homology domain-containing protein
Sb02g034940.1	13.97	12.61	Disease resistance protein (NBSLRR class), putative
Sb02g035050.1	10.75	8.80	Nodulin MtN21 family protein
Sb02g040480.1	9.76	5.04	ATMYB86 (MYB DOMAIN PROTEIN 86)
Sb02g042780.1	16.52	20.86	ATPME2
Sb03g000663.1	5.42	5.17	
Sb03g007960.1	5.20	4.32	Transferase, transferring glycosyl groups
Sb03g025570.1	9.48	10.57	Basic helix-loop-helix (bHLH) family protein
Sb03g033160.1	8.82	11.26	MYB37 (MYB DOMAIN PROTEIN 37)
Sb03g034120.1	19.63	15.01	Unknown protein
Sb03g034970.1	5.63	4.63	Oxidoreductase
Sb03g045980.1	167.94	40.01	GLP5 (GERMINLIKE PROTEIN 5)
Sb04g014800.1	3.07	2.68	Unknown protein
Sb05g002770.1	3.75	4.07	LACS6 (long-chain acylCoA synthetase 6)
Sb05g010060.1	4.00	5.07	Nitrate transporter, putative
Sb05g021320.1	218.63	206.50	Purple acid phosphatase family protein

Sb05g026930.1	3.61	4.45	Disease resistance protein (CCNBSLRR class), putative
Sb05g026940.1	3.28	4.03	RPP13 (RECOGNITION OF PERONOSPORA PARASITICA 13)
Sb05g026960.1	5.00	4.63	
Sb05g026960.4	4.65	4.35	
Sb05g026965.1	4.51	4.62	Disease resistance protein (CCNBSLRR class), putative
Sb05g026970.1	3.44	4.12	RPP13 (RECOGNITION OF PERONOSPORA PARASITICA 13)
Sb05g027450.1	5.98	5.43	Zinc finger (C2H2 type) family protein
Sb06g009610.1	11.87	9.74	ABA2 (ABA DEFICIENT 2)
Sb06g014840.1	4.12	4.02	
Sb06g014840.3	4.07	3.91	
Sb06g014865.1	47.54	34.32	
Sb06g025060.1	12.61	9.54	EGL3 (ENHANCER OF GLABRA 3)
Sb07g000550.1	9.00	16.26	CYP71A25
Sb07g002370.1	3.64	3.13	UGT72B3 (UDPGLUCOSYL TRANSFERASE 72B3)
Sb08g016320.1	7.74	21.18	FRS5 (FAR1related sequence 5)
Sb08g018440.1	7.39	8.22	Anion exchange family protein
Sb09g000760.2	10.22	10.85	
Sb09g006430.1	6.72	4.65	Unknown protein
Sb09g025890.1	9.92	13.12	Catalytic/ cation binding / hydrolase
Sb10g028440.1	12.83	16.24	Lectin protein kinase, putative
Sb10g029200.1	5.44	4.40	Myb family transcription factor
Sb10g030710.1	4.33	3.71	ATEXO70F1 (exocyst subunit EXO70 family protein F1)
Sb01g003890.1	48.68	60.95	
Sb01g004290.1	44.05	54.24	SMT1 (STEROL METHYLTRANSFERASE 1)
Sb01g016580.1	10.20	9.43	Receptor protein kinase-related
Sb01g023244.1	5.36	5.60	Lipase, putative
Sb01g031610.1	13.05	9.47	CYP71B37
Sb01g037070.1	22.21	21.15	Pentatricopeptide (PPR) repeat-containing protein
Sb01g037560.1	16.91	15.62	Mitochondrial import inner membrane translocase subunit
Sb01g037580.1	22.74	24.33	Universal stress protein (USP) family protein
Sb01g037590.1	10.42	9.61	Unknown protein
Sb01g039150.1	45.46	44.66	
Sb01g039190.1	25.08	25.03	RHL41 (RESPONSIVE TO HIGH LIGHT 41)
Sb01g040930.1	12.29	11.72	Hydrolase
Sb01g044590.1	3.28	4.19	Alcohol dehydrogenase, putative
Sb02g000920.1	150.21	136.81	Flavin-containing mono-oxygenase family protein
Sb02g001180.1	37.73	39.76	Unknown protein
Sb02g006860.1	10.63	10.26	Kelch repeat-containing protein
Sb02g025200.1	6.20	4.86	Nodulin MtN21 family protein
Sb02g027540.1	2.84	3.81	FAR1 (FATTY ACID REDUCTASE 1)
Sb02g027720.1	17.26	22.54	ATP binding / nucleotide binding / phenylalanine tRNA ligase
Sb02g028580.1	1463.20	1173.75	3betahydroxydelta5steroid dehydrogenase
Sb02g028860.1	4.24	4.17	Myosin heavy chain-related
Sb02g030030.1	26.78	20.57	UGT74F2 (UDPGLUCOSYLTRANSFERASE 74F2)
Sb02g030890.1	23.41	18.87	DNA photolyase
Sb02g030890.3	21.51	18.62	
Sb02g031570.1	39.61	58.22	Diaminopimelate decarboxylase
Sb02g031770.1	7.70	8.49	Auxin-responsive protein, putative
Sb02g031780.1	9.49	10.69	Auxin-responsive protein, putative
Sb02g032510.1	4.91	3.50	ATPUP5; purine transmembrane transporter
Sb02g034250.1	11.88	14.06	Targeting protein-related
Sb02g035460.2	103.12	90.02	
Sb02g035480.1	145.11	144.93	Glycosyl hydrolase family 17 protein
Sb03g002600.1	4.17	4.83	Glycosyl transferase family 14 protein
Sb03g006390.1	53.51	64.97	UGT84A2; UDP glycosyl transferase
Sb03g037720.1	14.62	19.56	ATOSM34 (osmotin 34)
Sb03g038280.1	12.25	18.24	Lipid transfer protein, putative
Sb03g042740.1	3.66	2.92	Unknown protein
Sb03g044040.1	628.50	616.62	Unknown protein
Sb05g001840.1	3.19	2.86	Harpin-induced protein-related / HIN1related
Sb05g024100.1	6.32	13.15	Jacalin lectin family protein

Sb05g026920.1	5.10	5.07	Disease resistance protein (NBSLRR class), putative
Sb06g021230.1	12.47	17.73	ATEP3; chitinase
Sb06g021240.1	25.09	33.77	ATEP3; chitinase
Sb06g021250.1	4.89	6.88	ATEP3; chitinase
Sb06g021260.1	5.58	9.32	ATEP3; chitinase
Sb06g022450.1	15.69	12.63	BGLU46 (BETA GLUCOSIDASE 46)
Sb07g023200.1	7.66	7.59	PLA2A (PHOSPHOLIPASE A 2A)
Sb08g023160.1	2.93	4.52	Catalytic
Sb09g000770.1	26.67	30.87	Nodulin MtN21 family protein
Sb09g000780.1	5.28	6.04	Nodulin MtN21 family protein
Sb09g018750.1	5.66	6.50	Catalytic/ cation binding / hydrolase
Sb10g024290.1	4.47	3.85	LPP2 (LIPID PHOSPHATE PHOSPHATASE 2)
Sb10g024350.1	22.76	28.11	Oxidoreductase, 2OGFe(II) oxygenase family protein
Sb10g024680.1	3.73	3.11	Tryptophan synthase-related
Sb10g025700.1	4.08	4.02	Endomembrane protein 70, putative
Sb10g028810.1	6.71	5.89	UDP glucoronosyl/UDP glucosyl transferase family protein
Sb10g030280.1	118.07	109.89	
Sb10g030650.1	84.28	68.44	ATEXO70F1 (exocyst subunit EXO70 family protein F1)
Sb10g030660.1	6.24	5.82	ATEXO70F1 (exocyst subunit EXO70 family protein F1)

Table 7.5 - Genes that were expressed to lower levels (FC>2 in all three biological replicates) in both StgB vs. R16 comparisons. Genes that were also expressed to lower levels in the B35 stay-green line are highlighted in purple and genes that lie within the StgB QTL interval are highlighted in red.

SbID	Average StgB1 vs. R16 FC	Average StgB2 vs. R16 FC	Annotation
Sb01g001893.1	4.44	4.08	Unknown protein
Sb01g012990.1	4.71	5.25	Ankyrin repeat family protein
Sb01g016030.1	5.43	4.11	Auxin-responsive protein, putative
Sb01g029470.1	3.66	4.02	NRT1.1; nitrate transmembrane transporter
Sb01g037640.1	3.12	3.51	Transcription co-activator
Sb01g038940.1	16.36	11.33	ESP3 (ENHANCED SILENCING PHENOTYPE 3)
Sb01g039100.1	18.14	10.50	Unknown protein
Sb01g044290.1	2.78	3.87	MIPS2 (MYO-INOSITOL-1-PHOSTPATE SYNTHASE 2)
Sb01g044290.2	2.73	3.80	
Sb01g044290.3	2.95	3.80	
Sb01g047500.1	4.31	7.35	BAM1 (BETA-AMYLASE 1)
Sb02g001730.1	4.60	9.39	
Sb02g023630.1	13.46	7.63	Armadillo/beta-catenin repeat family protein
Sb02g028200.1	8.36	5.78	TDT (TONOPLAST DICARBOXYLATE TRANSPORTER)
Sb02g030060.1	10.44	5.80	UGT74F2 (UDP-GLUCOSYLTRANSFERASE 74F2)
Sb02g030340.1	4.10	28.98	CBF4 (C- REPEAT-BINDING FACTOR 4)
Sb02g031720.1	8.14	5.47	Auxin-responsive family protein
Sb02g040640.1	2.38	2.59	TolB protein-related
Sb02g042790.1	4.30	5.06	Unknown protein
Sb03g028640.1	3.86	4.01	CYP72A14
Sb03g028930.1	9.04	8.43	Pectinesterase family protein
Sb04g004810.1	3.08	5.71	
Sb04g025270.1	5.95	7.79	J8; heat shock protein binding / unfolded protein binding
Sb04g031980.1	3.72	6.41	MATE efflux protein-related
Sb05g008580.1	3.76	2.33	

Sb05g021310.1	4.40	4.55	
Sb06g017230.1	3.06	2.61	PUB25 (PLANT U-BOX 25)
Sb06g034010.1	8.33	4.75	VND7 (VASCULAR RELATED NAC-DOMAIN PROTEIN 7)
Sb08g022660.1	21.14	11.76	
Sb09g000430.1	8.31	8.30	PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1)
Sb09g025510.1	22.07	16.01	O-methyltransferase family 2 protein
Sb10g008670.1	3.50	3.85	Proton-dependent oligopeptide transport (POT) family protein
Sb10g024260.1	14.36	18.58	Unknown protein
Sb10g028410.1	2.47	2.30	Transcription elongation factor-related
Sb10g029100.1	5.67	5.93	MBD02 (METHYL-CPG-BINDING DOMAIN PROTEIN 02)
Sb01g000230.1	4.40	3.80	ATGSTT1 (GLUTATHIONE S-TRANSFERASE THETA 1)
Sb01g000270.1	4.33	4.99	Unknown protein
Sb01g002730.1	3.71	2.96	Mitochondrial transcription termination factor family protein
Sb01g039140.1	20.81	13.84	
Sb02g001910.1	7.29	8.79	Unknown protein
Sb02g006320.1	6.63	4.45	AtSIP2 (Arabidopsis thaliana seed imbibition 2)
Sb02g026510.1	11.75	4.64	NAP (NAC-like, activated by AP3/PI)
Sb02g026820.1	4.01	5.74	ATRBP47C (ARABIDOPSIS THALIANA RNA-BINDING PROTEIN 47C)
Sb02g026820.2	4.89	6.43	
Sb02g027080.1	6.00	5.44	ATGSTU7 (ARABIDOPSIS THALIANA GLUTATHIONE S-TRANSFERASE TAU 7)
Sb02g028600.1	7.68	6.38	3-beta-hydroxy-delta5-steroid dehydrogenase
Sb02g029080.1	3.89	6.35	Glycosyl hydrolase family 17 protein
Sb02g029950.1	412.99	454.83	EIF3G2
Sb02g030880.1	19.28	17.85	Pentatricopeptide (PPR) repeat-containing protein
Sb02g035120.1	61.56	60.08	SYP132 (SYNTAXIN OF PLANTS 132);
Sb02g035160.1	6.08	4.90	Xyloglucan:xyloglucosyl transferase
Sb02g035230.1	8.70	6.51	Ankyrin repeat family protein
Sb02g035330.1	2.90	3.02	Receptor-like protein kinase, putative
Sb02g035700.1	3.64	3.29	IPP2 (ISOPENTENYL PYROPHOSPHATE:DIMETHYLALLYL PYROPHOSPHATE ISOMERASE 2)
Sb02g035960.1	5.07	4.03	Unknown protein
Sb05g008250.1	15.14	25.35	RPM1 (RESISTANCE TO P. SYRINGAE PV MACULICOLA 1)
Sb05g022920.1	21.02	19.91	Nucleic acid binding
Sb05g024830.1	3.63	4.04	BPM2 (BTB-POZ AND MATH DOMAIN 2)
Sb07g028620.1	2.18	2.44	AtSIP1 (Arabidopsis thaliana seed imbibition 1)
Sb09g000440.1	52.58	52.62	PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1)
Sb09g000450.1	105.69	143.67	PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1)
Sb09g001173.1	18.65	6.85	Unknown protein
Sb09g002620.1	3.21	3.22	Unknown protein
Sb10g024390.1	25.06	20.48	ATP-binding region, ATPase-like domain-containing protein-related
Sb10g025550.1	67.35	57.69	Serine carboxypeptidase S28 family protein
Sb10g026770.1	7.41	7.81	ATBPM1 (BTB-POZ and MATH domain 1)
Sb10g027100.1	62.51	61.73	DNA binding
Sb10g027485.1	17.11	11.21	
Sb10g027830.1	11.16	13.52	Protein kinase family protein

7.2.3.2 Ontological analysis

In order to determine whether any particular processes were enriched within these gene lists, gene ontology (GO) analysis was carried out as described previously (see Chapter

3). No biological process GO terms were significantly enriched ($p<0.05$) amongst either the up- or down-regulated gene set when compared to the background genome. However, 23 genes expressed to higher levels in the StgB lines were found to be associated with the 'response to stress' GO category. These include three genes encoding peroxidases (*Sb01g027330.1*, *Sb01g031740.1*, *Sb01g035990.1*), a gene encoding a universal stress protein (*USP*) and ABA deficient 2 (*ABA2*).

7.2.3.3 Promoter motif analysis

Promoter motif analysis was carried out to assess whether any of these genes lie downstream of a master regulator within the QTL. The lists of both up- and down-regulated genes were input into a web-based tool that identifies over-represented motifs across groups of promoters (http://element.mocklerlab.org). Sequences 500 bp upstream of the transcription start site was analysed, as the influence of the motif has been shown to decrease the further away from the transcription start site (Geisler et al., 2006). Only motifs with a p value of less than $1e^{-04}$ were considered significant. All over-represented motifs were then compared to those listed in the PLACE database (http://www.dna.affrc.go.jp/PLACE/) to check if they had previously been characterized in the literature. The results of the analyses are shown in Tables 7.6 and 7.7 on the following pages. Interestingly, there was a significant enrichment of stress-related motifs amongst the genes differentially expressed between StgB and R16. For example, the ABRE element is over-represented in the upregulated genes and a DRE-related element is over-represented amongst the downregulated genes.

Table 7.6 - ELEMENT motif analysis of all genes either up- or down-regulated in both StgB lines compared to R16. Published promoter elements (see Table 7.7) are underlined in blue.

	Motif	Length	Total Count	Promoter Count	Mean p-value
	UP-REGULATED				
4-mer	ATGC	4	526	108	9.64E-10
	GTAC	4	205	85	1.79E-08
	CGTA	4	268	90	3.25E-08

	ACGT	4	183	75	1.37E-07
	ATTA	4	671	103	3.98E-07
	TATA	4	477	98	2.23E-06
	TACA	4	475	105	1.14E-05
5-mer	CGTAC	5	116	48	3.18E-11
	ACGTA	5	104	46	2.06E-08
	CATGC	5	197	77	7.93E-08
	AGCTA	5	187	62	4.31E-07
	ATGCA	5	226	79	1.01E-06
	ATATA	5	445	83	1.36E-06
	TAATA	5	226	87	2.51E-06
	CCCCC	5	174	41	6.73E-06
	CTAGC	5	159	56	7.58E-06
	ATTAA	5	226	83	7.91E-06
	GTACA	5	121	67	1.47E-05
6-mer	CCCCCC	6	95	17	1.22E-12
	AGCTAG	6	105	39	4.29E-11
	ACGTAC	6	58	29	4.91E-10
	CGTACG	6	30	21	2.48E-07
	CTGGCA	6	44	36	1.10E-05
	AATTAA	6	104	54	2.48E-05
	AGTTAA	6	48	36	6.40E-05
7-mer	CCCCCCC	7	57	7	6.99E-12
	ACGTACG	7	35	18	8.92E-12
	AGCTAGC	7	58	20	5.65E-09
	CTAGCTA	7	55	19	3.51E-08
	ATGCATG	7	64	28	3.23E-06
	CACGTAC	7	22	16	2.46E-05
	CTACTGA	7	15	14	4.31E-05
	AGTTAAT	7	21	17	4.88E-05
	DOWN-REGULATED				
4-mer	CGGC	4	428	56	5.94E-08
5-mer	GGCAA	5	100	44	4.54E-06
	CCGGC	5	134	36	2.02E-05
	AGTGC	5	80	44	3.36E-05
6-mer	CGGCAA	6	38	18	7.82E-08
	TCGGCA	6	29	11	4.24E-06
	AGTGCC	6	26	21	4.13E-05
7-mer	TCGGCAA	7	22	5	7.06E-12
	CGGCAAA	7	24	5	4.42E-11
	CTTTGCC	7	20	6	1.19E-06

	GCCGAGA	7	17	6	1.35E-06
	CCGAGAG	7	15	4	3.45E-06
	CTCGGCA	7	15	3	4.88E-06
	AGCTAGC	7	34	17	1.11E-05
	CCGGCGC	7	22	12	3.02E-05
	CGAGAGC	7	16	8	3.72E-05
	CAACAAG	7	18	13	5.14E-05
8-mer	TCGGCAAA	8	20	3	9.00E-17
	CTCGGCAA	8	14	2	7.46E-11
	CGGCAAAG	8	15	2	1.16E-10
	CTCTCGGC	8	14	3	4.46E-10
	TCTCGGCA	8	11	3	9.60E-10
	CCGAGAGC	8	11	4	2.68E-08
	AGGAAGGA	8	16	11	4.98E-07

Table 7.7 - Published promoter elements identified from the analysis of the genes differentially expressed by >2-fold

Sequence	Description	Reference
UP-REGULATED		
ACGT	**ABRE-like sequence** Required for etiolation-induced expression of *ERD1* (early responsive to dehydration) in Arabidopsis	(Narusaka et al., 2003; Simpson et al., 2003)
YACT	Found in the cis-regulatory element of phosphoenolpyruvate carboxylase (*PPCA1*) of the C4 dicot *F. trinervia*	(Gowik et al., 2004)
GTAC	**CuRE (copper-response element)** Found in *CYC6* and *CPX1* genes in Chlamydomonas	(Quinn et al., 2000)
GTTAA	**GT-element** Critical for GT-1 binding to box II of *rbcS*	(Zhou, 1999)
CATG	**RY repeat (CATGCAY) or legumin box** Found in seed-storage protein genes in legume such as soybean	(Fujiwara and Beachy, 1994)
TATA	**TATA box** Found in the 5' upstream region of sweet potato sporamin A gene	(Grace et al., 2004)
DOWN-REGULATED		
AAAG	Core site required for binding of DOF proteins in maize	(Yanagisawa and Schmidt, 1999)
CCGAG	**DRE-related** DRE1 element found in maize *RAB17* gene promoter	(Kizis and Pages, 2002)
GGCAAA	Required for cell cycle regulation of the tobacco ribonucleotide reductase small subunit gene	(Chaboute et al., 2000)
CAACA	Binding consensus sequence of Arabidopsis transcription factor RAV1	(Kagaya et al., 1999)

7.3 Discussion

7.3.1 Genes within the QTL interval are differentially expressed between the B35 and R16 lines

A number of genes that were differentially expressed between the B35 and R16 lines (see Chapter 4) were found to be located within a Stg QTL interval. Some of these genes, including *P5CS2*, a *LEA* and a *HSP* are well known to be involved with conferring drought tolerance. These gene expression differences are likely to arise due to SNPs in regulatory regions such as in the promoter. These genes could therefore directly underlie the phenotype and provide good candidates for trait selection. This list is however still large and contains 289 genes. In the future, in order to reduce the numbers of candidates, it would be interesting to compare these genes to those found to be changed in response to drought stress in Chapter 3. Whilst the genes causal to the stay-green trait are not necessarily drought-inducible, this could provide a way to reduce candidate gene numbers. We must bear in mind however, that whilst many of the differentially expressed genes that have been previously discussed do not lie within the QTL intervals many may act downstream of these genes and therefore could act as diagnostic markers for trait selection. Additionally, genes that lie within the QTL interval yet are not differentially expressed could still be important for trait expression as SNPs in the coding sequence could impact on protein function or post-translational modification.

7.3.2 *P5CS2* is likely to act downstream of other QTL genes

P5CS2 was found within the Stg1 QTL interval and contained SNPs in the B35 upstream promoter region that could be responsible for the differences in the expression of this gene. These polymorphisms however, did not have a significant impact on promoter activity measured using a luciferase reporter (Figure 7.4). Importantly, this experiment was only carried out once with five replicates per construct. This experiment therefore requires repetition to increase the significance of the results. Furthermore, reproducibility depends on the efficiency of each bombardment event. An additional internal plasmid control is therefore additionally required in future experiments to normalize experimental variations. For example, a different reporter such as GUS or aequorin driven by a constitutive promoter could be co-bombarded along with the luciferase reporter construct.

The SNPs in the B35 promoter were not found in the Stg1 introgression line, which displays the stay-green phenotype (Vadez et al., 2011), suggesting that they are not important for the expression of the trait. It is possible that SNPs in the 5' or 3' UTR or in the introns, not investigated here, could be responsible for the differences in the gene expression and these may be present in the Stg1 introgression lines. Despite this, the increase in *P5CS2* expression in B35 compared to R16 was not mirrored in the Stg1 QTL line. This could be for a number of reasons. Firstly, *P5CS2* is on the edge of the Stg1 QTL so perhaps wasn't included in the introgression line provided. This would explain the absence of the SNPs in the promoter region and would suggest that *P5CS2* does not underlie the QTL. Secondly, multiple factors control gene expression therefore perhaps the high expression levels in Stg1 are 'dampened' by genes in the R16 genetic background. Alternatively, rather than acting within the Stg1 QTL, *P5CS2* may instead be acting downstream of one or more other QTL genes. All of the QTL lines, apart from Stg4 and, Stg3 and StgB combined, have increased expression of *P5CS2* compared to R16. This suggests multiple genetic regions are influencing the expression of this gene. The effect of these regions is likely to depend on the genetic background and on the combination of QTLs present (Vadez et al., 2011).

7.3.3 The increased expression of *DREB1A* and *SDIR1* in the B35 stay-green line is associated with the Stg1 QTL

DREB1A does not underlie a Stg QTL itself however, its high expression in B35 compared to R16 was found to be exclusively linked to the Stg1 QTL, as evidenced by high expression in both of the Stg1 QTL introgression lines tested (Figure 7.5). This suggests that *DREB1A* acts downstream of a gene in the Stg1 QTL. Interestingly, the *DREB1A* promoter was found to contain putative Myb-binding sites and a MYB transcription factor, *MYB6*, was found within the Stg1 QTL interval. This sorghum transcription factor shows strong sequence identity (57%) at the amino acid level to MYB15 in Arabidopsis. MYB15 is known to be involved in the regulation of DREB1 expression; transgenic lines overexpressing *MYB15* show reduced levels of *DREB1* expression whereas *myb15* loss-of-function lines show increased *DREB1* expression (Agarwal et al., 2006). It is therefore possible that MYB6 in sorghum is regulating

DREB1A activity. The expression of sorghum *MYB6* is 1.5-fold lower in B35 compared to R16. Whilst this fold change is small, it is possible that SNPs in the coding sequence of this transcription factor could affect its function i.e. through the introduction of a premature stop codon. This would reduce MYB6 activity and so could enable higher levels of *DREB1A* expression in B35. To test this it would be interesting to sequence the *MYB6* coding region in both B35 and R16 in order to detect the presence or absence of SNPs. If SNPs were present, it would be important to determine whether this MYB is able to influence *DREB* expression and, if it can, to compare levels of activation with the MYB6 amino acid sequence from R16 and from B35. This could be analysed using transient expression assays with luciferase as a reporter for DREB1A expression.

SDIR1 was not found within a stay-green QTL interval despite the known role of this gene in conferring drought tolerance (see Chapter 6). However, it *is* known to be within a QTL for the maintenance of green leaf area at maturity (GLAM) (Srinivas et al., 2009). Given that in many environments in which sorghum is grown drought stress is inevitable at the mature stage (Kassahun et al., 2010; Kholova et al., 2013), it is possible that the higher expression of this gene enables these varieties to survive for longer under these conditions and hence maintain green leaf area for longer. It would therefore be interesting to compare the sequences of the key regulatory regions of this gene in the stay-green and senescent lines in order to ascertain whether SNPs in the DNA sequence could be responsible for the differences in gene expression between the varieties.

SDIR1 could also act downstream of a QTL gene. For example, amongst others the high expression of *SDIR1* in B35 was also found to be linked to the Stg1 QTL. The putative *SDIR1* promoter contains two DRE elements (Figure 7.10), which DREB transcription factors are known to bind to (Narusaka et al., 2003). Given that *DREB1A* is also exclusively linked to Stg1, perhaps SDIR1 lies downstream so that the increased expression of *DREB1A* leads to the increase in the expression of *SDIR1*. Other transcription factors within the Stg1 interval could also contribute to the increased expression of *SDIR1*. The interaction between DREB1A and SDIR1 could again be tested through a transient expression system. For example, a construct containing *DREB1A* driven by a constitutive promoter could be co-bombarded into sorghum leaves with a luciferase reporter construct driven by the *SDIR1* promoter. Luminescence counts

would give an indication of luciferase activity and therefore whether DREB1A is able to promote *SDIR1* expression. As discussed in Chapter 6 287 genes are expressed to higher levels in B35 and contain the DRE element in their promoter. It would be interesting to see whether any of these genes are also expressed to higher levels in the Stg1 QTL lines because this could indicate that they are also downstream of DREB1A and are involved in the same pathway.

7.3.4 StgB QTL microarray analysis

The comparison of gene expression between QTL introgression lines and the recurrent parent is a valuable way to identify genes associated with the QTL (Holloway and Li, 2010). By comparing varieties of the same genetic background, that differ solely in the QTL region, noise is reduced as in theory only genes associated with that region are affected. Here, genes associated with the response to stress were found to be expressed to higher levels in the StgB introgression lines. Amongst these was *ABA2*, which is involved in the conversion of xanthoxin to abscisic aldehyde in the ABA biosynthetic pathway (Gonzalez-Guzman et al., 2002). As reviewed in Chapter 1, ABA is accumulated under drought conditions and is critical for both stomatal closure leading to reduced transpirational water loss and for the induction of stress-responsive genes (Nakashima and Yamaguchi-Shinozaki, 2013). Transgenic plants overexpressing ABA2 have elevated ABA levels and exhibited increased tolerance to salinity (Lin et al., 2007). Additionally, constitutive expression of *NCED1*, which is involved with ABA biosynthesis in tomato, resulted in increased transpiration efficiency, leaf expansion and root hydraulic conductivity under drought conditions (Thompson et al., 2007). The constitutively higher expression of *ABA2* in the StgB lines could result in constitutively higher levels of ABA. This could then be associated with a reduction in transpiration and constitutively higher levels of downstream protective genes. The over-representation of the ABRE-binding element, which is known to be involved with ABA signaling (Narusaka et al., 2003), amongst the genes expressed to higher levels in StgB, is consistent with this and suggests these genes may be induced as a consequence of the increased ABA concentration. Interestingly, in one study out of all of the introgression lines tested StgB had the greatest transpiration efficiency compared to R16 (Kassahun et al., 2010). Even here, amongst the introgression lines tested one StgB line showed one of the highest

average relative leaf weights (Figure 7.8). This high transpiration efficiency could arise as a result of ABA-induced stomatal closure caused by greater ABA2 expression. Further supporting this, when sorghum genetic maps are aligned with those from maize, StgB lies in a similar position to a maize QTL that influences leaf ABA content (Lebreton et al., 1995). To test this theory, it would be interesting to measure and compare ABA contents in the StgB lines and in R16. Whilst ABA has been also associated with the promotion of senescence (see Chapter 1), this could be counteracted by its protective properties under drought conditions.

Another potentially interesting gene expressed to higher levels in the StgB lines (~25 fold higher) is *Sb01g037580.1*, which encodes a universal stress protein (USP). Universal stress proteins were originally discovered in bacteria and are required for defence against superoxide-generating agents (Nachin et al., 2005). USPs are thought to be ubiquitous in plants and transgenic tomato lines overexpressing a USP have reduced stomatal aperture and increased chlorophyll content when subjected to drought stress conditions. The effect of this overexpression is small under control conditions yet under drought stress conditions a large number of genes are expressed to higher levels in the transgenic lines compared to wild type, many of which are associated with the maintenance of the photosynthetic machinery (Loukehaich et al., 2012). It would therefore be very interesting to characterize the sorghum USP further i.e. through the production of transgenic lines, especially since the higher expression of this gene was consistent across both StgB and B35 genotypes (Table 7.4).

A gene encoding CBF4 (DREB1D) was found to be expressed to lower levels in both StgB lines and there was an enrichment of a DRE-related element amongst the down-regulated genes (Tables 7.5 & 7.7). This is surprising given that the overexpression of DREB1D transcription factors usually results in drought tolerance (see Chapter 6) and it would be important to validate these expression changes using qPCR. One possible explanation for this is that other CBF/DREB independent pathways are active in promoting drought tolerance in the StgB line so the plant shuts down this CBF/DREB pathway.

As can be seen in Tables 7.4 & 7.5, not all of the genes that were differentially expressed between StgB and R16 were also changed in B35. This could be due to slight

differences in experimental conditions; plants used for the StgB microarray experiment were grown over the autumn/winter whereas those for the initial B35 vs. R16 experiment were grown over the summer. Whilst the plants were grown in a temperature-controlled greenhouse, it is possible that changes in day length/outside light levels could have caused differences in gene expression between experiments. As already mentioned in Chapter 4 small changes in experimental conditions can dramatically alter gene expression. Alternatively, these differences could be due to the StgB QTL interacting differently with the different genetic backgrounds i.e. R16 compared to B35. Perhaps there is an additive effect when these genes are in the R16 background thus resulting in the differential expression of a greater number of genes.

Only eight of the 182 differentially expressed genes were actually within the StgB QTL interval itself. It is possible that polymorphisms in the regulatory regions of these genes are responsible for their differential expression. These genes could affect the expression of further downstream genes and thus directly underlie the QTL. However, it is also possible that polymorphisms in the coding region of other genes within the QTL could impact on protein function and hence alter the expression of downstream targets.

7.3.5 Conclusions

Previously, the identification of genes underlying the stay-green trait has been hampered by the large regions encompassed by the QTLs. This resulted in difficulties identifying candidate genes among the hundreds of genes in the QTL regions. Here, by combining QTL analysis with gene expression analysis, promising results relating to the Stg1 and StgB QTL have been obtained.

CHAPTER 8

Discussion

8.1 Implications of the work

Sorghum is an essential crop grown on arid and semi-arid soils of some of the world's poorest countries. It is well adapted to the harsh environments in which it lives however; relatively few studies have investigated the molecular basis of these adaptations. The research described in this thesis sought to improve our understanding of the mechanisms of stress tolerance in this important crop and, in particular, improve our understanding of the mechanisms underlying the stay-green trait for drought tolerance. This chapter will briefly recap the results of this work and provide suggestions for future investigations.

8.2 Identification of stress-induced transcripts

Transcripts that were either up- or down-regulated in response to drought stress, heat stress and combined heat and drought stress in sorghum were identified (see Chapter 3). Many of these transcripts were involved in similar processes to those already identified as important in Arabidopsis, suggesting conservation of mechanisms across species. Some genes were changed in response to one stress type only i.e. changed in response to the combined stress but not the individual stress types. This suggests that, similar to what has previously been found in Arabidopsis and tobacco (Rizhsky et al., 2002; Rizhsky et al., 2004), sorghum has a unique response to combined stress. There were however, some elements of the combined stress response, which were unique to sorghum, such as the upregulation of specific transcription factors and signalling genes. A functional characterization of the genes and pathways identified could lead to new

targets for the enhancement of plant stress tolerance, which will be particularly important in the face of climate change and the increasing prevalence of these abiotic stress types.

8.3 The stay-green trait is likely to be associated with improved drought avoidance and drought tolerance mechanisms

Genes that are associated with proline and betaine biosynthesis, glutathione S-transferase activity and the regulation of stomatal numbers and aperture were expressed to higher levels in the B35 stay-green line compared to the senescent R16 line (see Chapter 4). Both proline levels and GST activity was found to be higher in B35 compared to R16 under well-watered and drought-stressed conditions. In addition, B35 was found to lose water more slowly and have reduced numbers of stomata (see Chapter 5). Furthermore, two signalling genes, *DREB1A* and *SDIR1*, were expressed to higher levels in B35. *SbSDIR1* was shown to function in a similar way to *SDIR1* in Arabidopsis and is likely to play a role in the regulation of stomatal aperture (see Chapter 6).

Senescence is delayed in the stay-green lines under drought conditions at the post-flowering stage (Rosenow et al., 1983). One explanation for this is that the stay-green lines have better drought *avoidance* mechanisms (see Chapter 1) meaning that they don't experience drought stress until a later stage thus are able to stay-green for longer (Figure 8.1). The findings presented here suggest that stay-green lines have reduced transpiration, at least in part due to reduced stomatal numbers. The increased expression of genes such as *SDIR1* may also contribute to this by reducing stomatal aperture. This is supported by previous findings suggesting stay-green lines have increased transpiration efficiency (Vadez et al., 2011; Borrell et al., 2014a). This reduced transpiration could enable the conservation of water in the soil prior to flowering meaning that more water is available at the post-flowering stage for grain filling. Similar findings were reported for stay-green Miscanthus genotypes, whereby the stay-green genotype Sin-H6 appeared to have a lower leaf conductance (Clifton-Brown et al., 2002). Stay-green in pearl millet is also associated with reduced leaf conductance under both drought-stressed and well-watered conditions (Kholova et al., 2010). Previous studies have suggested that the reduced size of upper leaves and reduced tillering in the stay-

green lines prior to flowering could also contribute to reduced water usage meaning that more water is available in the soil profile during grain filling (Borrell et al., 2014a; Borrell et al., 2014b). Improved water extraction due to an improved rooting system could additionally contribute to improved drought avoidance (Borrell et al., 2014a). Stay-green lines are able to maintain a higher relative water content (RWC) when subject to drought stress (Xu et al., 2000a). Based on the results presented here, it is possible that the accumulation of proline could contribute to improved osmotic adjustment and hence enable the maintenance of cellular water content.

An alternative explanation for the delayed senescence is the stay-green lines face the same level of stress, yet are better able to cope with the stress-induced damage i.e. have improved drought *tolerance* (Figure 8.1). The higher GST activity identified here could help to provide that protection. Furthermore, perhaps the higher expression of *DREB1A* could lead to the higher expression of other genes with protective properties. The B35 line was shown here to be more tolerant to oxidative stress induced by methyl viologen (Chapter 5). In addition, previous studies have suggested that the stay-green lines are more tolerant to heat stress at the seedling stage (Burke et al., 2010). This suggests that the drought tolerance of the stay-green lines is not just a product of improved water conservation but could also be due to better cellular protective mechanisms. As a result, it is likely that the stay-green trait arises as a result of both improved drought tolerance *and* drought avoidance mechanisms (Figure 8.1).

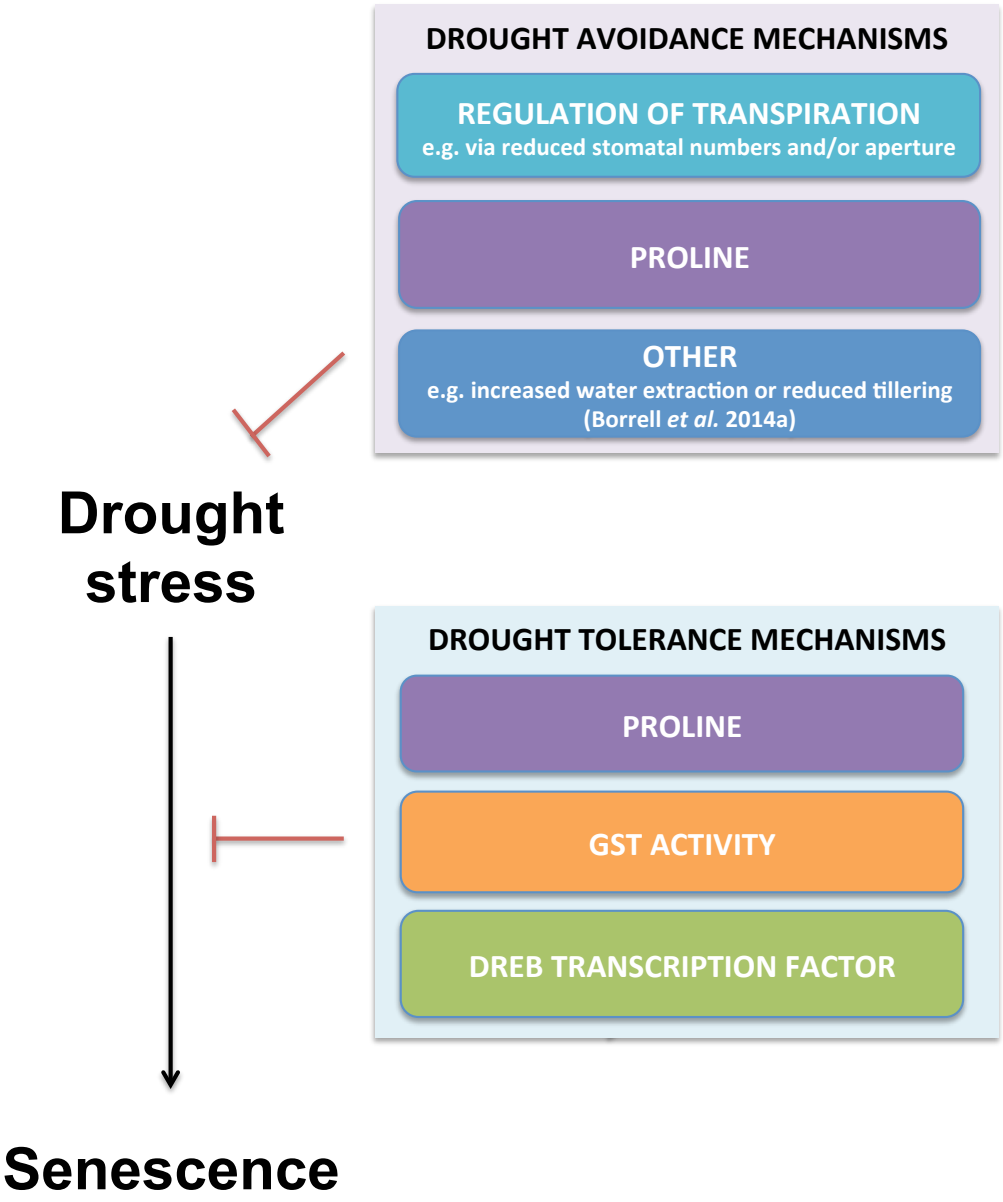


Figure 8.1 - Model showing the mechanisms that could underlie the stay-green trait in sorghum. Drought avoidance mechanisms including reduced transpiration and increased proline could mean that the stay-green lines don't experience drought stress until a later stage. As suggested by others, increased water extraction and reduced tillering could additionally contribute to the conservation of water (Borrell et al., 2014a). Drought tolerance mechanisms including increased proline and GST activity and the activation of *DREB1A* could additionally provide cellular protection. This means that even when the plants experience drought stress senescence is delayed.

In addition to delayed drought-induced senescence, stay-green lines also exhibit a delayed onset and a reduced rate of developmental senescence. This is evidenced in this work by the observed differences in chlorophyll content between the varieties (Chapter 4) and in the work of others (Xu et al., 2000a). It is possible that the same genes/processes that delay the drought-induced senescence also delay the developmental senescence. There is considerable cross talk between drought stress responses and leaf senescence. Among the 43 transcription factors reportedly induced during senescence, 28 are also induced by stress treatment (Lim and Nam, 2007). It would be interesting to identify homologs of these genes in sorghum and analyse their expression patterns in this microarray data. For example, a gene that is usually induced by senescence could be expressed to lower levels in the stay-green line indicating a potential role for that gene in conferring the trait.

Reactive oxygen species (ROS) accumulate following drought stress and during senescence. Reduced ROS accumulation or improved ROS scavenging could therefore delay both developmental and drought-induced senescence. Indeed, suppression of ROS production markedly delays leaf senescence and enhances drought tolerance (Rivero et al., 2007). The observed increase in GST activity could help to reduce the effects of ROS thus may contribute to the delay in both drought-induced and developmental senescence. It would be interesting to measure ROS levels in the stay-green and senescent varieties during senescence to determine whether there are additionally differences in ROS production. Alternatively, given that stay-green is such a complex trait, different genes/processes may be involved in regulating the delayed developmental and stress-induced senescence pathways separately. For example, the altered expression of one group of genes could result in the delay in drought-induced senescence whereas the altered expression of a different group of genes could cause the delay in developmental senescence.

8.4 Analysis of the Stg QTLs

The increased expression of both *DREB1A* and *SDIR1* in B35 compared to R16 was found to be associated with the Stg1 QTL (see Chapter 7). A gene showing homology to a negative regulator of *DREB* expression in Arabidopsis (MYB15) was found to lie within

the Stg1 QTL interval. As discussed in Chapter 7, a polymorphism in the coding region of this gene could impact upon protein function thus enabling higher levels of *DREB1A* expression. In addition *SDIR1* was found to have two DRE-elements in its promoter. This gives rise to the model shown in Figure 8.2. This model is, of course, highly speculative and as detailed in Chapter 7 a number of experiments are required before any firm conclusions can be made. Genes encoding late embryogenesis abundant proteins, GSTs and SDD1, which are expressed to higher levels in B35, also contain a DRE-element 500 bp upstream of the start of transcription. It is possible that these genes also lie downstream of DREB1A and are at least partially under the control of the Stg1 QTL. If this is the case we would perhaps expect higher GST activity and reduced stomatal numbers in the Stg1 lines and in the future it would be interesting to investigate this.

Genes that are up- and down-regulated in the StgB QTL lines compared to the senescent R16 parent were identified using microarrays. *ABA2* was expressed to higher levels in B35 (see Chapter 7), homologs of which are involved in ABA biosynthesis (Gonzalez-Guzman et al., 2002). One potential mechanism that could underlie the StgB QTL is shown below (Figure 8.3). However, as discussed in Chapter 7 this model requires validation and there may be other contributory genes/processes.

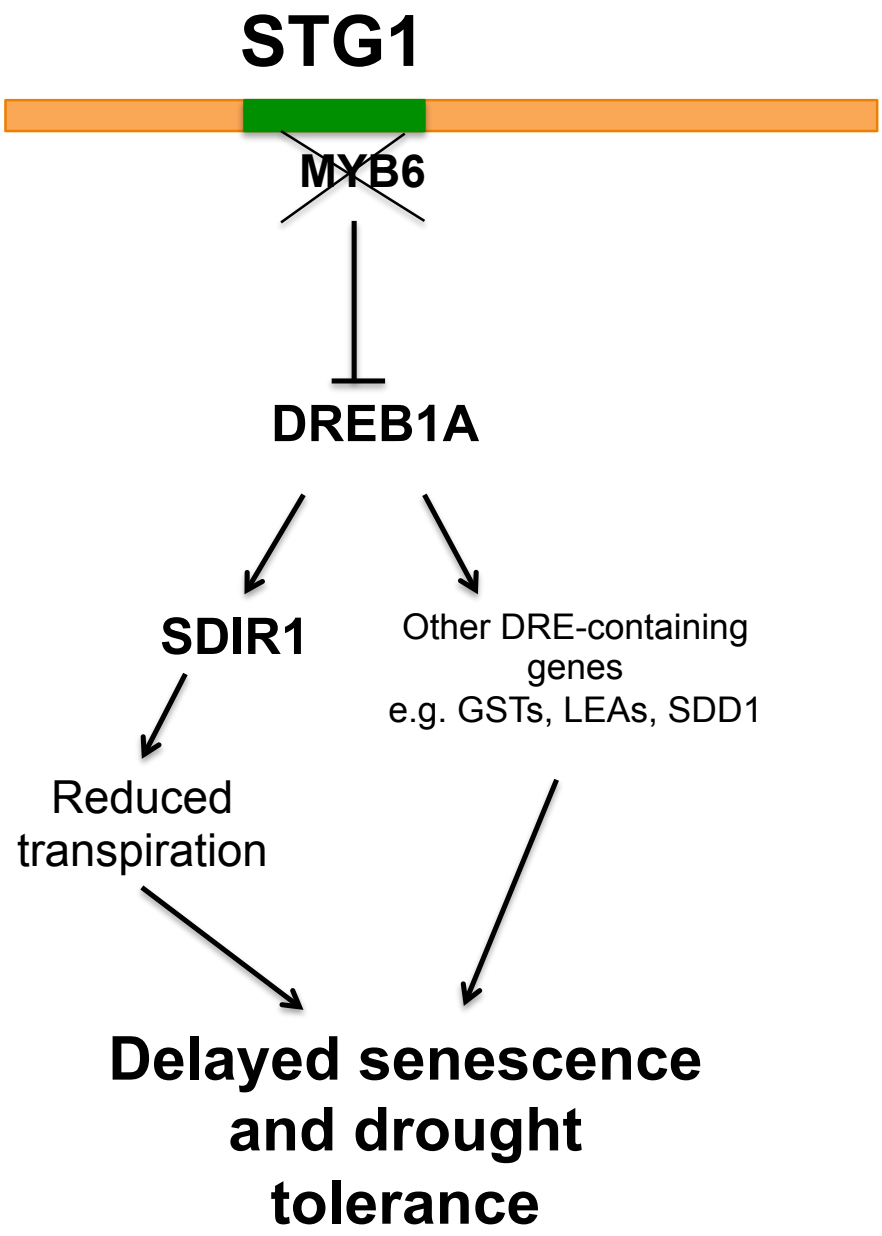


Figure 8.2 - Speculative model for the mechanism underlying the Stg1 QTL. Sequence polymorphisms in the MYB6 coding region (which shows homology to MYB15 in Arabidopsis) could alter the activity of this protein thus enabling higher expression of *DREB1A*. This could lead to the activation of protective genes including *SDIR1* thus resulting in reduced transpiration and delayed senescence and drought tolerance (see Chapter 7).

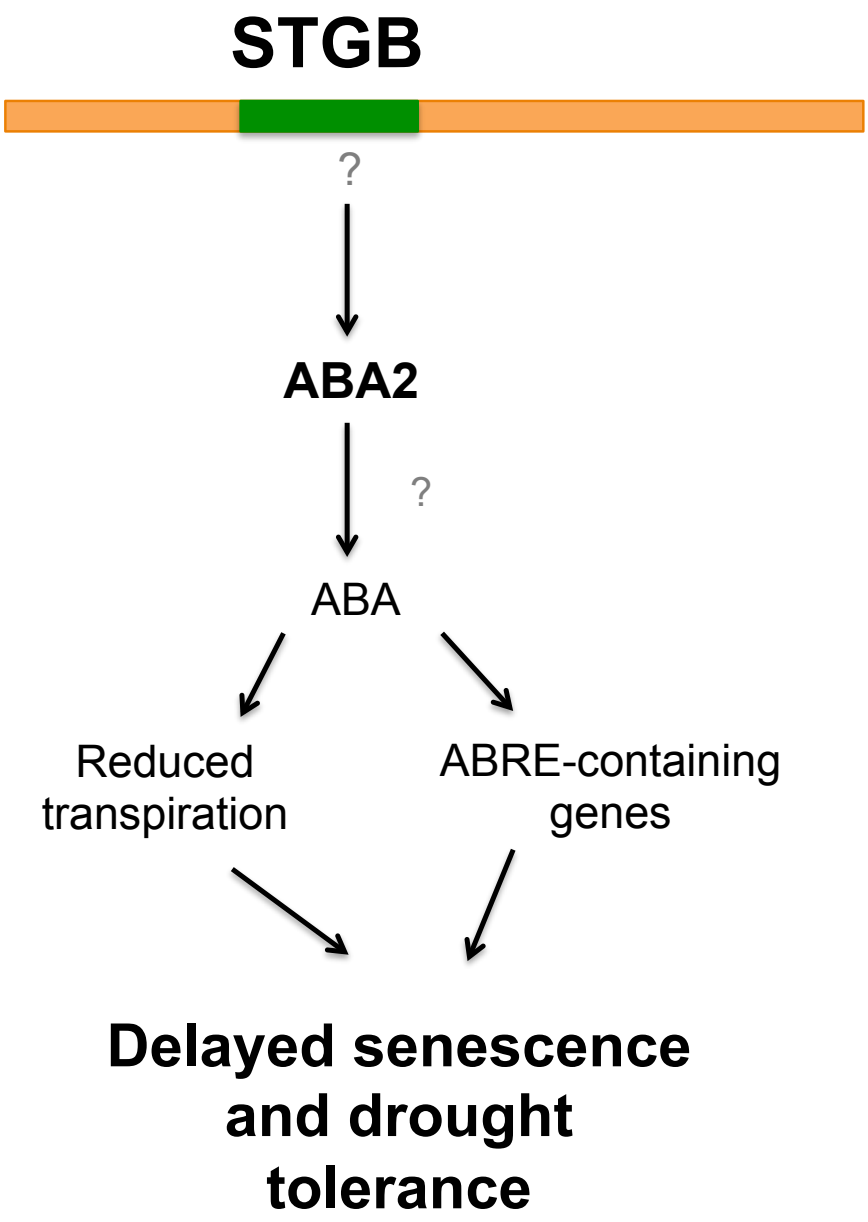


Figure 8.3 - Speculative model for the mechanism underlying the StgB QTL. Sequence polymorphisms in a gene within the QTL interval could affect the protein function of a regulator of *ABA2* expression. This could lead to increased ABA2 activity, which could enable the production of higher levels of ABA. The higher ABA could contribute to reduced transpiration and also the activation of protective genes containing an ABRE-element in their promoter. This could ultimately lead to the delayed senescence and drought tolerance phenotype in the StgB introgression lines.

8.5 Limitations of the approach taken

The approach taken has been successful insofar as a number of biological processes that could be important for trait expression have been identified. However, it is not without its limitations. Firstly, the sorghum genome was published relatively recently, in 2009 (Paterson et al., 2009). This means that the genome has not been extensively annotated; many of the gene annotations given have not been verified and genes have been incorrectly assigned to GO categories. For example, two genes were found here to be incorrectly categorized as peroxidases (see Chapter 5). This meant that the sequences of all genes discussed and taken forward for further analysis first had to be compared to the Arabidopsis genome to check for sequence homology. Additionally, a level of subjectivity was required for the selection of genes and processes for further study. Those taken forward were selected based on their known role in conferring drought tolerance in other species and their ease of measurement. However, this does mean that other genes with a less obvious role may have been over-looked, potentially due to poor gene annotation. Similarly, if only a single gene involved with a particular process was expressed to higher or lower levels then it wouldn't have been identified by the GO analysis. This means there are likely to be other genes that are contributing to the stay-green trait that have not been discussed here.

8.6 Future work

A number of specific future experiments have already been suggested in the relevant discussions section within each results chapter. This section will propose further general experiments that could further enhance our understanding of the stay-green trait.

8.6.1 Investigations into other candidate genes/processes

Many candidate genes and processes have been identified. However, due to the time constraints of the project not all of these could be taken forward for further investigation. Other genes that would be interesting to investigate in more detail in the future include *Sb10g027100.1*, which shows homology to the Arabidopsis NAC transcription factor

NTL4 and is significantly ($p<0.05$) down-regulated by ~40 fold in B35 compared to R16 (see Chapter 4). Given that the Arabidopsis NTL4 mutant exhibits delayed senescence and drought tolerance (Lee et al., 2012), it would be very interesting to characterize the sorghum gene through the use of transgenic lines and to assess linkage of this gene to the Stg QTLs. It would also be interesting to further characterize *Sb01g037580.1*, which encodes a universal stress protein (*USP*). Homologs of this gene have similarly been shown to confer drought tolerance and increased chlorophyll content when overexpressed (Loukehaich et al., 2012) and this gene shows linkage to StgB (see Chapter 7). Furthermore, a gene associated with trehalose biosynthesis, *TPS* (*Sb02g033420.1*), was expressed to higher levels in B35 (see Chapter 4). Trehalose provides protection to plants under stress conditions (Goddijn and van Dun, 1999). It would be valuable to measure and compare trehalose levels in the different varieties to determine whether the increase in the expression of this gene is sufficient to increase trehalose levels. Singular enrichment analysis (SEA) of the genes expressed to higher levels in B35 identified an enrichment of the 'water transport' GO category (see Chapter 4). The genes within this category encode aquaporins, which are involved in water transport. Aquaporin transcripts were found to be upregulated in Arabidopsis and rice under drought stress and have been suggested to facilitate water flow towards critical cell types. Studies in barley suggest the increased abundance of aquaporin transcripts in response to drought promotes residual growth of the leaf in response to stress (Maurel et al., 2008). For this reason, it would additionally be interesting to study these genes in more detail.

8.6.2 Investigations into the heat stress response

It has been suggested that stay-green sorghum lines also have improved heat tolerance compared to other lines (Burke et al., 2010). It would be interesting to investigate the basis of this heat tolerance and to assess whether it is associated with one Stg QTL in particular. Genes and processes that underlie heat tolerance may overlap with those underlying drought tolerance. For example, higher GST activity or increased *DREB1A* expression (Agarwal et al., 2006) could provide protection against both stress types. A number of heat shock proteins were expressed to higher levels in B35 and could confer protective properties (see Chapter 4). Genes that are heat-inducible may be involved

with providing heat tolerance. One way to gain an insight into the heat tolerance of the stay-green lines would be to compare the genes that are expressed to higher levels in B35 with the list of heat-inducible genes (see Chapter 3) and then to monitor the expression of these in the different varieties over time following heat stress. This could help to pinpoint genes that are causal to the phenotype.

8.6.3 Investigations into other stay-green lines/QTL introgressions

The majority of the work described has focussed on a comparison between the stay-green B35 variety and the senescent R16 variety. B35 was selected as a source of stay-green for this study due to the fact that most of the QTL mapping experiments have used this line (Tuinstra et al., 1997; Subudhi et al., 2000; Xu et al., 2000). However, it will important to investigate gene expression changes in other stay-green sources. Many of the genes taken forward for further investigation, including *SDIR1* and *DREB1A* were also expressed to higher levels in the stay-green E36-1 line. E36-1 is thought to be a unique source of stay-green; while some stay-green QTLs map to the same loci as in B35, others were only identified from E36-1 (Haussmann, 2002). This is reflected in the gene expression differences identified here in that 1442 genes were differentially expressed only in the E36-1 vs. R16 comparison and not in the B35 vs. R16 comparison (see Chapter 4). This suggests E36-1 may have some unique mechanisms of drought tolerance and it will be important to investigate these in the future.

There are multiple genetic differences between the B35 and R16 sorghum lines, not all of which will necessarily be associated with the stay-green trait. For this reason, the use of near isogenic lines (NILs), which differ genetically in one or more Stg QTL only will be of key importance for identifying the genes causal to the phenotype. Any gene expression differences between a QTL introgression line and the recurrent parent should be linked directly to those particular QTL. Through the analysis of upstream regulatory regions of the changed genes, causal genes within the QTL itself could be pinpointed. For example, the identification of significantly enriched promoter elements amongst the differentially expressed genes could lead to the identification of transcription factors within the QTL interval that could bind to these motifs. This approach has been informative for the StgB line (see Chapter 7) and so it would be interesting to carry out

similar analysis on the other QTL lines. To facilitate the identification of causal genes within the QTL interval, up- and down-regulated genes could be clustered based on their expression levels. Genes that cluster together will have similar expression levels therefore are likely to be regulated in the same way. Promoter motif analysis of these clusters could then aid the identification of regulatory genes that may lie within the QTL.

8.6.4 Investigations at different developmental stages

In this work gene expression changes at two developmental stages have been investigated: 14 DAS and 50 DAS. Many of the genes investigated further such as *P5CS2* and *SDIR1* were changed at both stages. However, there were of course genes that were only changed at one developmental time point. Considering the complexity of the trait, it is likely that some genes/processes are present throughout the life of the stay-green plants whereas others are important at one developmental stage only. It would be interesting to follow gene expression throughout development up until the post-flowering stage to better understand how different patterns of expression might lead to the delayed senescence. For example, some additional genes may be differentially expressed only at the post-flowering stage and these could also play an important role.

8.6.5 Investigations of stay-green roots

In this study gene expression in the leaves was investigated because the stay-green phenotype is most visible in this tissue. However, given that stay-green is an adaptation to a lack of soil water and that this signal is detected by the roots, it would also be important to investigate changes in root gene expression. There has been limited work on roots in sorghum. Some evidence suggests that some Stg QTLs in sorghum overlap with QTLs for nodal root angle and it has been suggested that this could enhance water uptake in the stay-green lines (Mace et al., 2012; Borrell et al., 2014). It will therefore be important to investigate the role of the roots and root signalling in conferring the phenotype.

8.6.6 Sorghum transformation

The development of a stable sorghum transformation system will be essential for the characterization of any candidate genes. The method developed by Liu *et al.* (2014) and carried out at the University of Queensland was not successful in producing transgenic lines here, perhaps due to the lethality of the genes tested (see Chapter 6). It will be important to develop a sorghum transformation system in Durham to test candidate gene function in the future. Alternatively, a monocot such as wheat, which has a well-developed transformation system, could be transformed with the sorghum genes. Compared to Arabidopsis transformation the transferal of a monocot gene into another monocot is likely to give a better representation of gene function.

8.6.7 Investigations into protein changes and post-translational modifications

The majority of the work described herein has focussed on gene expression. As discussed in Chapter 7, the causal gene within the Stg QTL will not necessarily have altered gene expression. Instead the molecular polymorphism causing the physiological QTL could be in the coding region, leading to variations in protein stability, enzymatic activity or post-translational modification, or possibly even introduce a premature stop codon. For this reason, it would additionally be interesting to carry out a proteomics study to complement the transcriptomic data. Additionally, a study of changes in metabolites would give an indication of changes in enzymatic activity.

8.6.8 Full genome sequencing

Full genome sequencing of the Stg QTL introgression lines and the R16 recurrent parent would enable the identification of polymorphisms that could be responsible for the trait. This would complement the gene expression data as the identified SNPs could then be linked directly to the changes in downstream gene expression. Research into the stay-green trait in sorghum is ever-evolving and so it will be important to continue to compare my gene expression data to any improved mapping data/sequencing data obtained by other groups.

8.7 Conclusions

In conclusion, this work has identified genes, pathways and processes that are associated with stress tolerance in sorghum. In particular, insights into the mechanisms underlying the stay-green trait have been obtained. Further investigation into these identified processes and into their linkage with the known Stg QTLs will be essential. This will both enhance our knowledge of this important crop and facilitate selection for the trait in the future.

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APPENDIX A

Solution and media recipes

A.1 Edward's extraction buffer

200 mM Tris-HCL, pH 7.5,
250 mM NaCl,
25 mM EDTA, pH 8.0,
0.5% SDS.

A.2 TE buffer

10 mM Tris, pH 8.0,
1 mM EDTA.

A.3 Dellaporta extraction buffer

50 mM Tris pH 8,
10 mM EDTA, pH8
100 mM NaCl,
1% SDS,
10 mM β-Mercaptoethanol,

A.4 TBE running buffer

1.1 M Tris
900 mM borate
25 mM EDTA (pH 8.0)

A.5 GST extraction buffer

100 µM Tris-HCl, pH 7.5,
2 mM EDTA,
14 mM β-Mercaptoethanol,
5% PVPP (w/v).

A.6 Callus induction medium (CIM)

MS powder with Gamborg vitamins (Murashige and Skoog, 1962)
30 g/L sucrose
8 g/L agar
1 g/L L-proline,
1 g/L L-asparagine,
1 g/L potassium dihydrogen- phosphate (KH_2PO_4),

1 µM $CuSO_4$,
1 mg/L 2,4-D.

A.7 Osmotic medium (OM)

MS powder with Gamborg vitamins (Murashige and Skoog, 1962)
30 g/L sucrose
8 g/L agar
0.2 M D-sorbitol
0.2 M D-mannitol.

A.8 Regeneration medium

MS medium with
1 mg/L BAP,
1 mg/L IAA,
1 µM $CuSO_4$.

A.9 Rooting medium

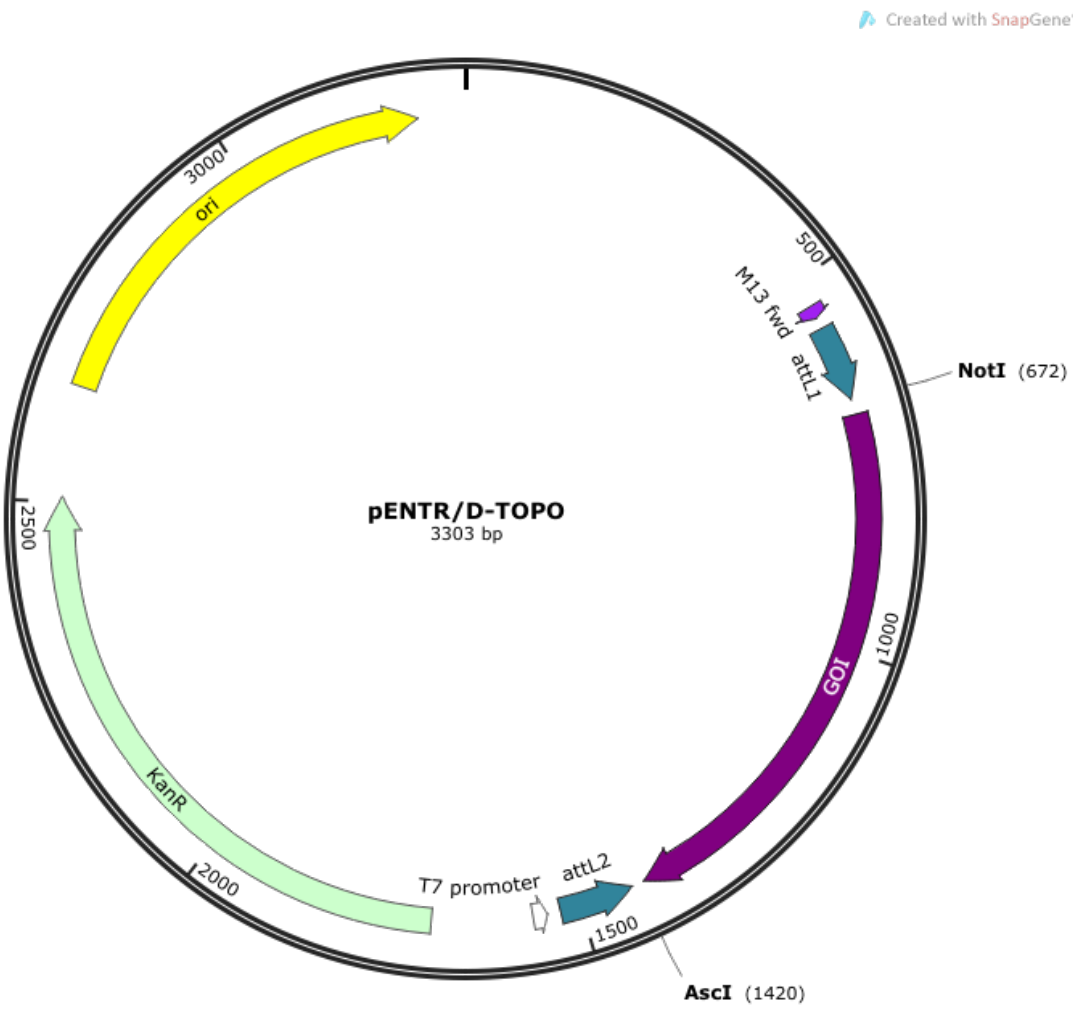
MS medium with
1 mg/L NAA,
1 mg/L IAA,
1 mg/L IBA,
1 µM $CuSO_4$.

APPENDIX B

Vector maps and cloning strategies

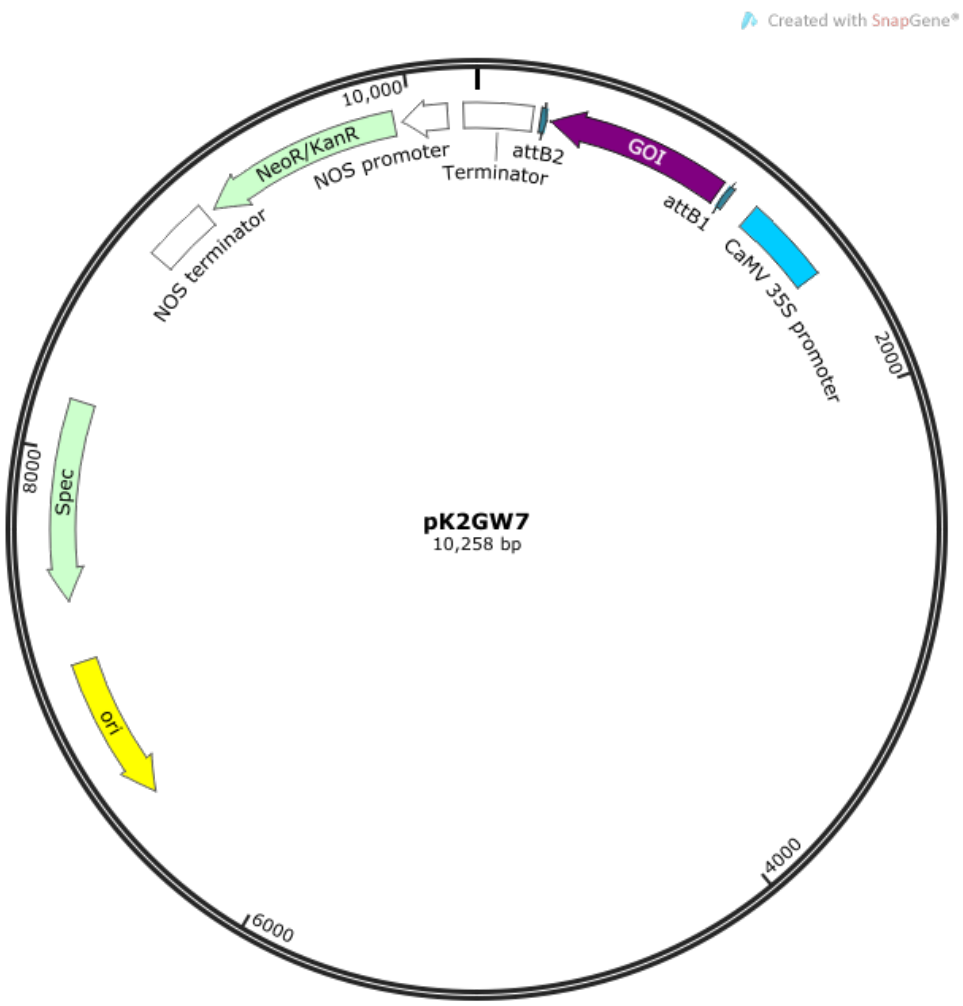
B.1 pENTR/D-TOPO

Vector map of pENTR/D-TOPO with gene of interest (GOI) incorporated. Not1 and Asc1 restriction sites were used to check for the incorporation of the correct insert.



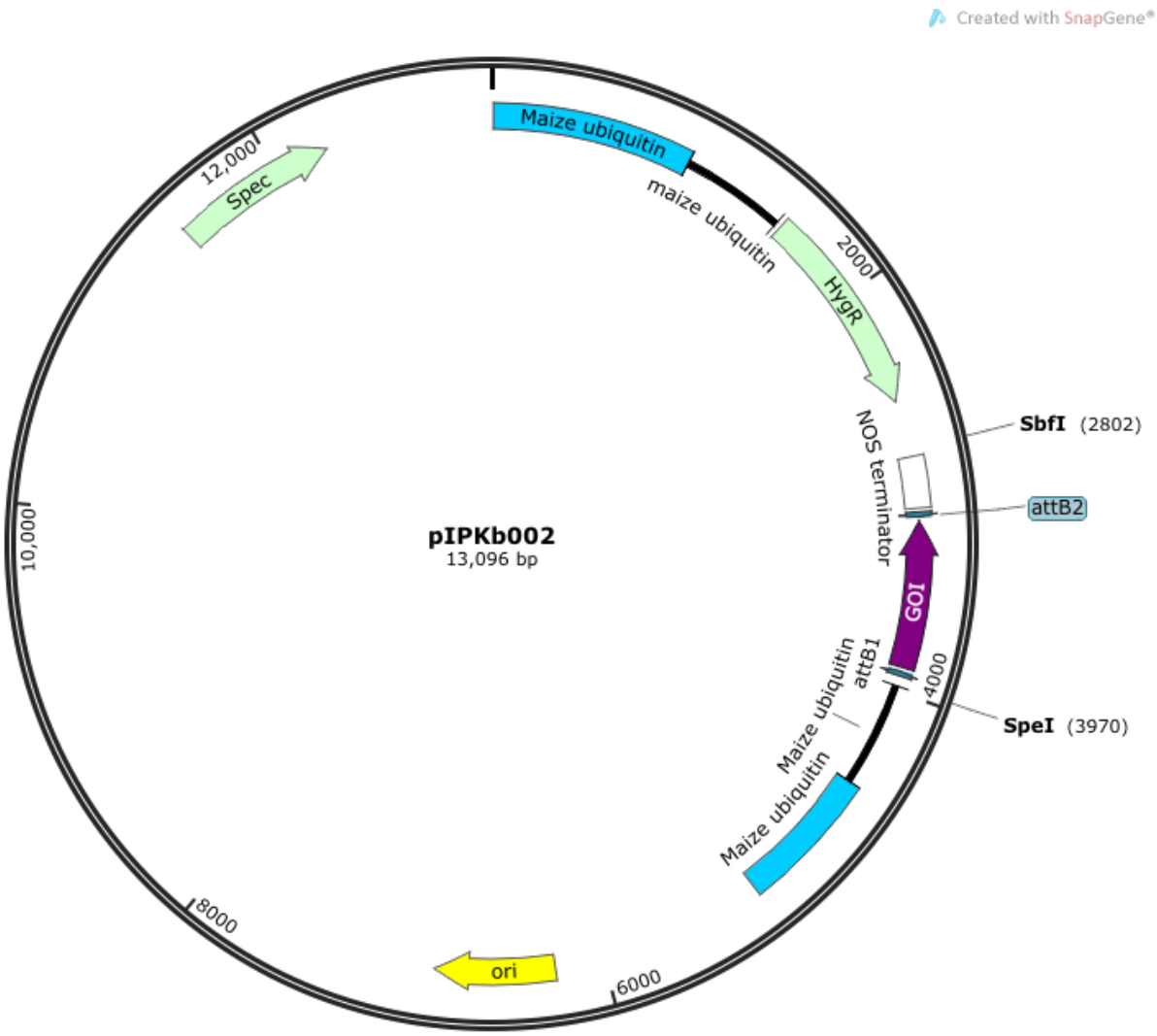
B.2 pK2WG7

Vector map of pk2GW7 with GOI incorporated. This plasmid was used for over-expression in stable transgenic Arabidopsis lines. The plasmid was produced using Gateway recombination (see Materials and Methods 2.4.4.4).



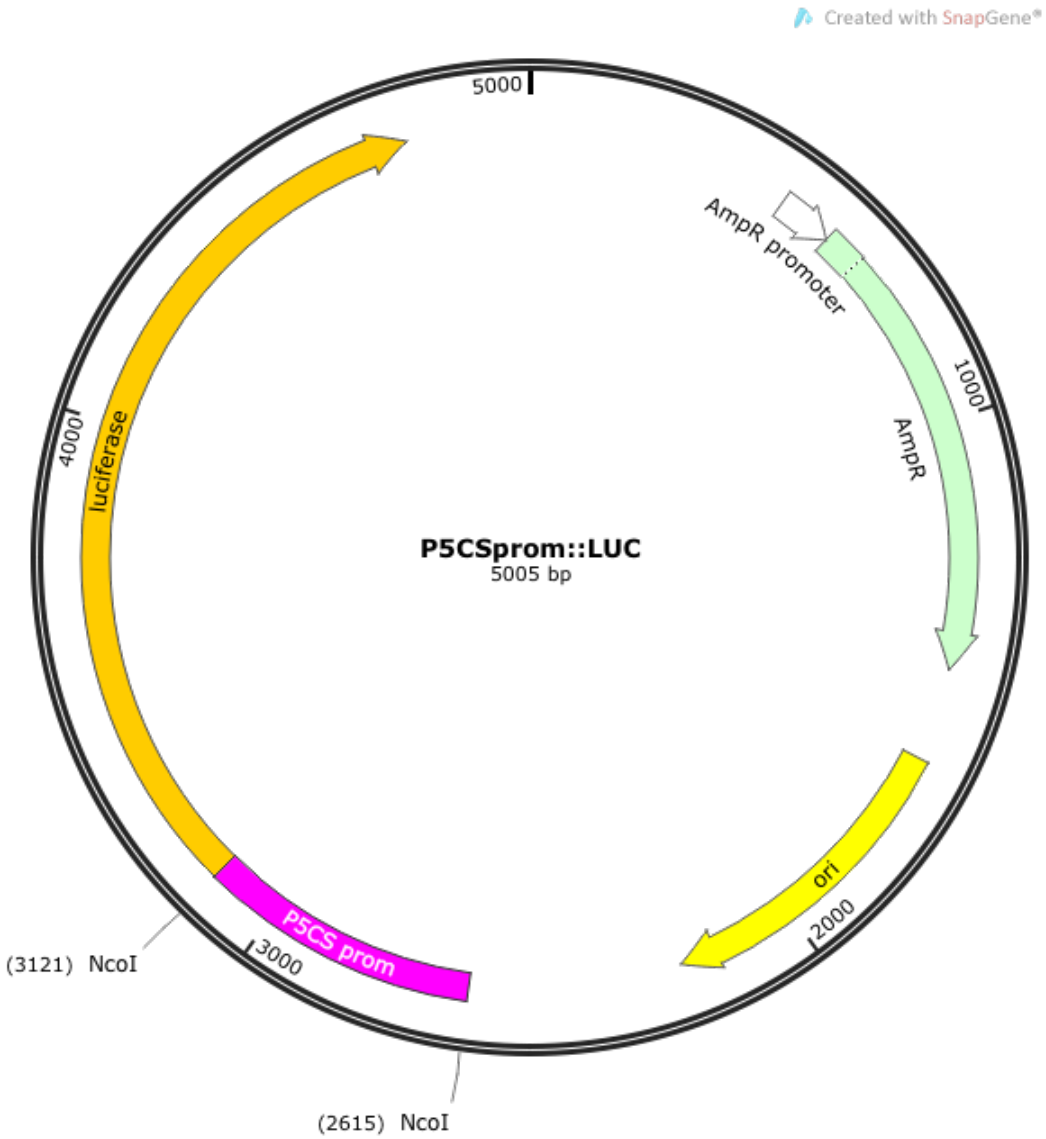
B.3 pIPKb002

Vector map of the destination vector pIPKb002 with GOI incorporated. This plasmid was used for over-expression in stable Sorghum transgenic lines. SbfI and SpeI were used to check for the incorporation of the correct insert. The plasmid was produced using Gateway recombination (see Materials and Methods 2.4.4.4).

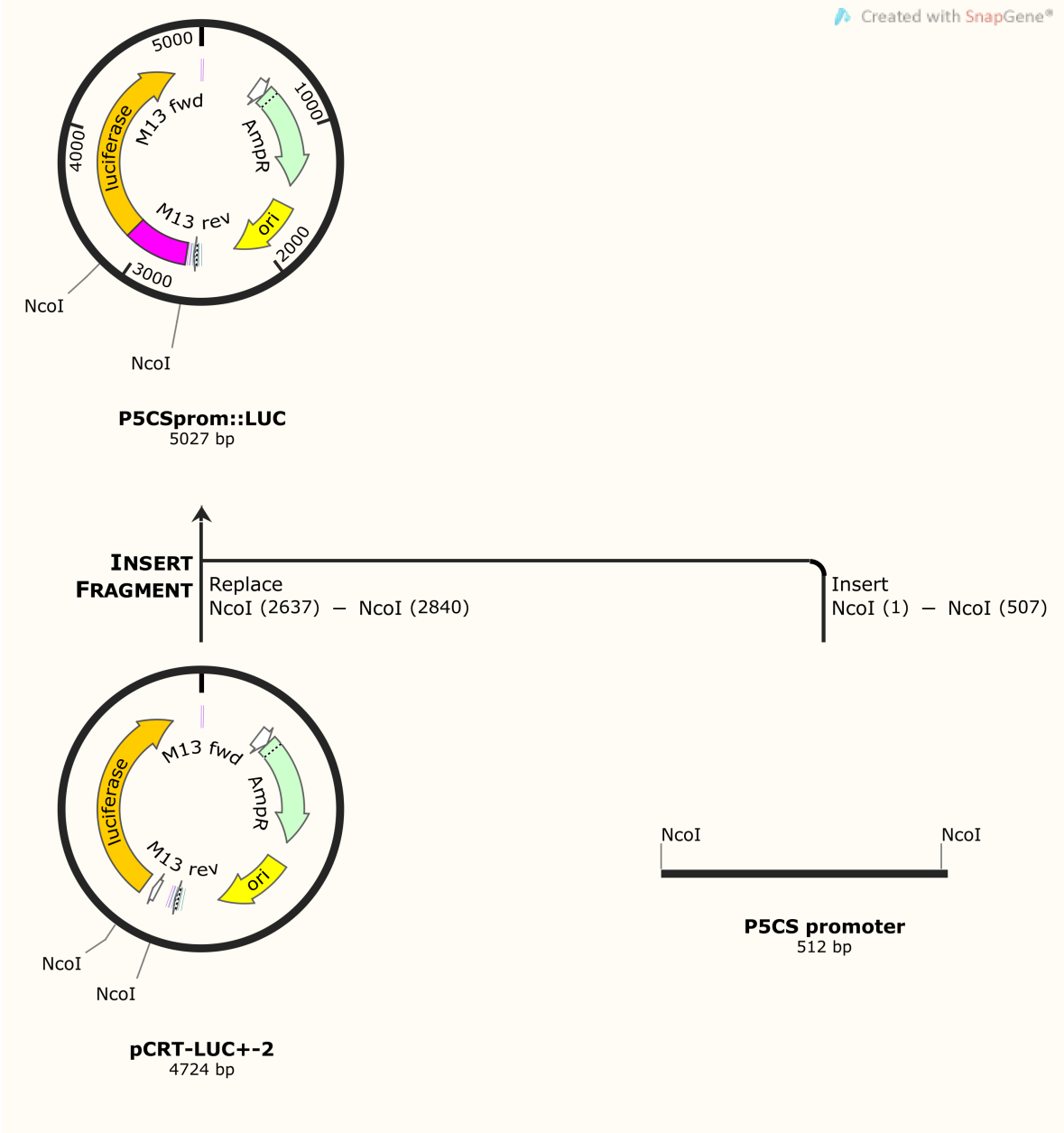


B.4 P5CS promoter::luciferase reporter construct

Vector map showing the P5CS promoter::luciferase reporter construct. This was used to compare the activity of the P5CS promoter from the R16 and B35 varieties (see Chapter 7).

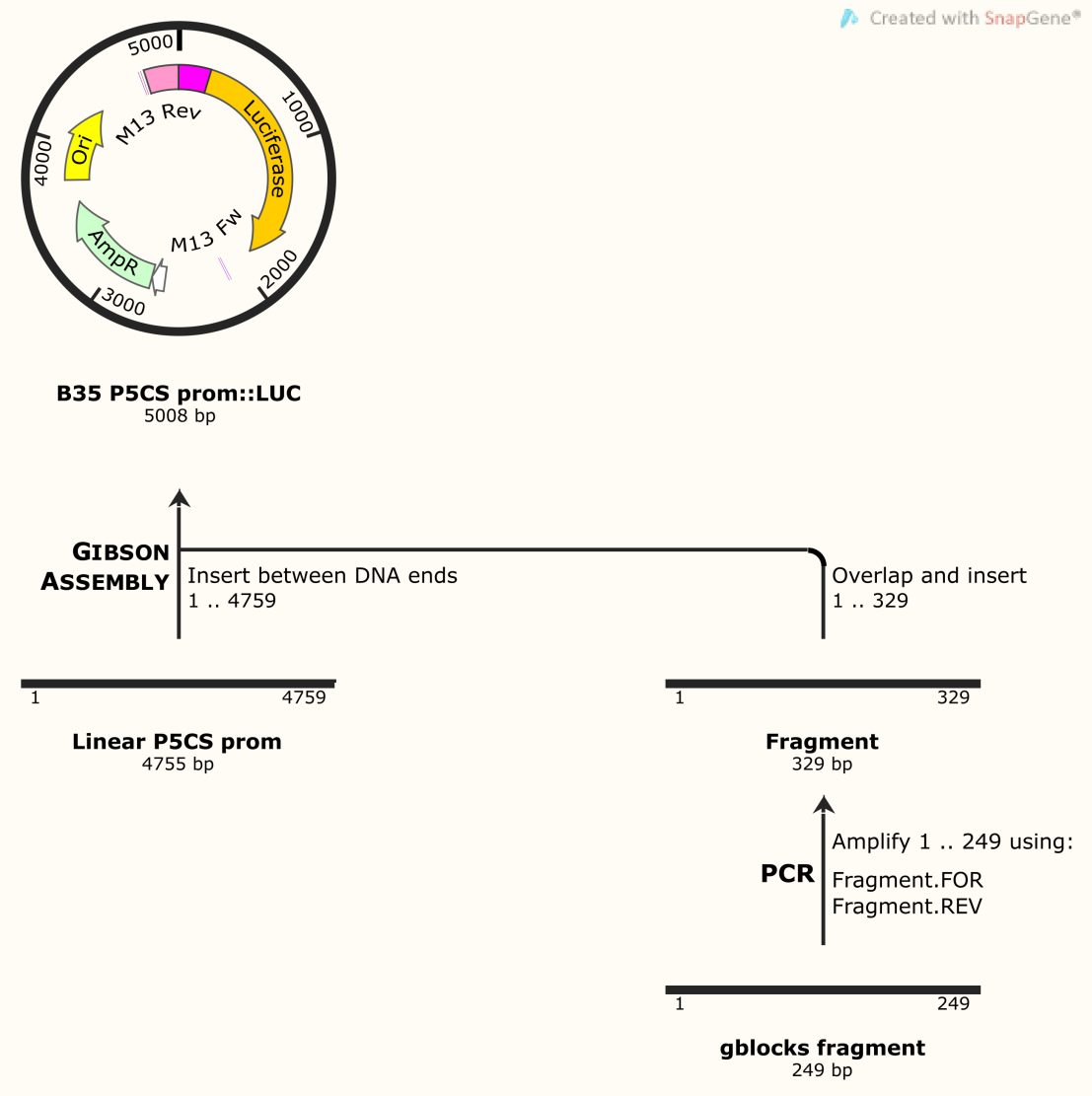


B.5 Cloning strategy for the incorporation of the R16 P5CS promoter upstream of the luciferase reporter.



Appendix B.6 Cloning strategy for the incorporation of the B35 promoter construct upstream of the luciferase promoter.

The R16 P5CSprom::LUC construct was linearized using BstXI and BsaA1 restriction sites. A pre-designed gblocks fragment (ordered from Integrated DNA Technologies) was then cloned into the vector using Gibson's assembly (see Materials and Methods 2.4.4.5) to give the B35 P5CSprom::LUC construct.



APPENDIX C

Oligonucleotides

The following files can be found on the enclosed CD-ROM.

C.1 Primers used for PCR and sequencing

C.2 Primers used for qPCR using the Applied BioSystems System

C.3 Taqman primers and probes used for qPCR using the Fluidigm system

APPENDIX D

Gene lists and GO terms

The following files can be found on the enclosed CD-ROM.

D.1 Genes differentially expressed in response to drought (>2 fold in all three replicates)

D.2 Genes differentially expressed in response to drought only (>2 fold in all three replicates)

D.3 -Genes differentially expressed in response to heat (>2 fold in all three replicates)

D.4 Genes differentially expressed in response to heat only (>2 fold in all three replicates)

D.5 Genes differentially expressed in response to combined heat and drought (>2 fold in all three replicates)

D.6 Genes differentially expressed in response to combined heat and drought only (>2 fold in all 3 reps)

D.7 Genes differentially expressed in response to all three stress types (>2 fold in all three replicates)

D.8 Gene Ontology (GO) terms enriched ($p<0.05$) in the genes differentially expressed in response to drought

D.9 Gene Ontology (GO) terms enriched ($p<0.05$) in the genes differentially expressed in response to heat

D.10 Gene Ontology (GO) terms enriched ($p<0.05$) in the genes differentially expressed in response to combined heat and drought

D.11 Gene Ontology (GO) terms enriched (p<0.05) in the genes differentially expressed in response to combined heat and drought only

D.12 Genes upregulated (>2 fold; P-value <0.05) in B35 vs. R16 at 50 DAS

D.13 Genes downregulated (>2 fold; P-value <0.05) in B35 vs. R16 at 50 days DAS

D.14 Genes upregulated (>2 fold; P-value <0.05) in E36 vs. R16 at 50 DAS

D.15 Genes downregulated (>2 fold; P-value <0.05) in E36 vs. R16 at 50 DAS

D.16 Genes upregulated (>2 fold; P-value <0.05) in B35 vs. R16 AND E36 vs. R16 at 50 DAS

D.17 Genes downregulated (>2 fold; P-value <0.05) in B35 vs. R16 AND E36 vs. R16 at 50 DAS

D.18 Gene Ontology (GO) terms enriched (p<0.05) in the genes upregulated in B35 vs. R16 at 50 DAS

D.19 Gene Ontology (GO) terms enriched (p<0.05) in the genes upregulated in E36-1 vs. R16 at 50 DAS

D.20 Genes upregulated (>2 fold in all three replicates) in B35 following drought stress at 14 DAS

D.21 Genes downregulated (>2 fold in all three replicates) in B35 following drought stress at 14 DAS

D.22 Genes upregulated (>2 fold in all three replicates) in R16 following drought stress at 14 DAS

D.23 Genes downregulated (>2 fold in all three replicates) in R16 following drought stress at 14 DAS

D.24 Genes upregulated (>2 fold in all three replicates) in E36 following drought stress at 14 DAS

D.25 Genes downregulated (>2 fold in all three replicates) in E36 following drought stress at 14 DAS

D.26 Genes upregulated (>2 fold in all three replicates) in S35 following drought stress at 14 DAS

D.27 Genes downregulated (>2 fold in all three replicates) in S35 following drought stress at 14 DAS

D.28 Genes upregulated in all three replicates (>2 fold) following drought in B35 only

D.29 Genes downregulated in all three reps (>2 fold) following drought in B35 only

D.30 Genes upregulated in all three replicates (>2 fold) in B35 vs. R16 at 14 DAS

D.31 Genes downregulated in all three replicates (>2 fold) in B35 vs. R16 at 14 DAS

D.32 Genes upregulated in B35 vs. R16 (FC>2; P-value<0.05) and in a known stay-green QTL (based on CSGR annotation)

D.33 Genes downregulated in B35 vs. R16 (FC>2; P-value<0.05) and in a known stay-green QTL (based on CSGR annotation)

D.34 Genes differentially expressed in B35 vs. R16 (FC>2; P-value<0.05) and in a known stay-green QTL (based on Stg QTL locations provided by Santosh Despande

APPENDIX E

Stg QTL introgression lines

Genotype	Introgression name	Number assigned
StgB	K359-3 White-1	1
StgB	K369-2-1	2
Stg4	K260-2-1	3
Stg4	k279-1-1	4
Stg4	K271-1-1	5
Stg3+StgB	K375-1-1	6
Stg3+StgB	K382-1-1	7
Stg4+StgB	K456-1-1	8
QTL from E36-1	RSG4008	9
QTL from E36-2	RSG04012	10
R16	IS18482	11
B35	IS40606	12
E36-1	IS30469	13
StgA	S35SG06003	14
StgA	S35SG06005	15
StgA	S35SG06010	16
StgB	S35SG06011b	17
StgB	S35SG07003	18
Stg1	S35SG06032	19
Stg1	S35SG06040	20
Stg2	S35SG06028	21
Stg2	S35SG06029	22
Stg3	S35SG06014	23
Stg3	S35SG06020	24
S35	IS36556	25