Molecular mechanisms of drought stress tolerance that underlie the stay green trait in Sorghum bicolor

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Submitted for the Degree Master of Science by Research (2017)

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Abstract

Drought stress is a major issue for food security, affecting yield production, crop survival and agricultural land quality. *Sorghum bicolor* is a vital food crop for semi-arid and arid regions, and is also used as a biofuel and as animal fodder. It is extremely well adapted for drought-prone climates and is used extensively as a model for investigating drought tolerance mechanisms. Several varieties display a 'stay green' trait in which leaf chlorophyll content and photosynthetic activity is maintained for longer under water limiting conditions, improving survival rates and maintaining higher grain yields under stress.

Two-dimensional gel electrophoresis was used to compare the proteomes of a stay green sorghum variety (B35) and a senescent variety (R16). Alteration in the levels of several proteins were identified between the two lines. Identification and functional characterisation of differentially expressed genes and proteins could enhance our understanding of the processes that underlie the stay green trait in sorghum and identify targets for selective breeding.

SDIR1 homologs in several species have frequently been associated with drought tolerance and stay green mechanisms. Wheat transgenic lines overexpressing *SbSDIR1* were analysed for the acquisition of stay green associated characteristics. Transgenic lines were found to display differences in transpiration, ABA-induced gene expression, tiller development and senescence timing suggesting it plays a key role in these processes. Further validation of the phenotype of the *SbSDIR1* overexpressing lines and functional characterisation of other candidate genes will contribute to our understanding of the drought tolerance mechanisms in sorghum.

Contents

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Abbreviations

2-D GE – 2-dimensional gel electrophoresis

2-D DIGE – 2-dimensional difference gel electrophoresis

ABA – abscisic acid

AP2 – apetala 2

AQP – aquaporin

ARF – ADP-ribosylation factor

BiP – endosperm luminal binding protein

C – carbon

CGIAR - The Consultative Group on International Agricultural Research

CHAPS - 3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate

$C0_2$ - carbon dioxide

DAS – days after sowing

DRE – dehydration responsive element

DREB1 - dehydration-responsive element-binding 1A

DTT – dithiothreitol

ERF – ethylene response factor family

FC- fold change

GST - Glutathione s-transferase

HECT - Homology to E6-AP *C*-Terminus

HMG – high mobility proteins

Hr – hour

IAA - iodoacetamide

IEF – isoelectric focussing

IPG – Immobilized pH gradient

iTRAQ - isobaric tag for relative and absolute quantitation

IAA – Iodoacetamide

JUB1 – jungbrunnen 1

LEA – late embryogenesis protein

MALDI – TOF- MS - Matrix assisted laser desorption ionisation time-of-flight mass spectrometry

MAS – marker assisted selection

MS – Murashige and Skoog

N – Nitrogen

NAC - no apical meristem (NAM), ATAF1-2 and cup-shaped cotyledon (CUC)

NIL – near isogenic line

NL – Nonlinear

NptII – Neomycin phosphotransferase II

OE – overexpression

P5CS – Delta 1-pyrroline-5-carboxylate

PCR – polymerase chain reaction

PDI – protein disulphide isomerase

PEG – polyethylene glycol

PI – isoelectric point

PIP - plasma membrane intrinsic proteins

PTM – post-translational modification

QTL – Quantitative trait locus

RD26 – response to dehydration 26

RIL – Recombinant inbred line

RING - Really Interesting New Gene

RNAi – RNA interference

ROS – reactive oxygen specis

RQ – Relative Quantitation

SAM - S-Adenosyl-L-methionine

SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDD1 - Stomatal density and distribution 1

Stg QTL – Stay green associated quantitative trait loci

SDIR1 - salt and drought-induced RING finger 1

SDIRIP1 - SDIR1-INTEREACTING PROTEIN1

Till0 – main culm

Till1 – first tiller growth

T1 – First progeny of transformed plants

T0 – parent transformed plants

TCA cycle – tricarboxylic acid cycle

TE – transpiration efficiency

TIP – tonoplast intrinsic proteins

UPS – ubiquitin proteasome system

USP – universal stress protein

w/v – weight/volume

WUEMED - Water Use Efficiency in Mediterranean agriculture

Acknowledgments

I would most like to thank Professor Marc Knight for giving me the opportunity to conduct this research, and for his constant support and guidance throughout my studies.

I would also like to thank my co-supervisor Professor Angharad Gatehouse for her help and advice, and Dr Catherine Tetard-jones and Gillian Davison for teaching me the protocols for two-dimensional gel electrophoresis.

Thank you to all the members of the Knight research group for making Lab 19 such a great place to work. Particular thanks to Dr Heather Knight for her encouragement and support, and Rebecca Manning for her technical help and advice.

Lastly, I would like to thank my family and friends for their unwavering support and understanding throughout my studies, without whom this would not be possible.

Chapter 1

Introduction

Constant or intermittent drought conditions affect up to 45% of agricultural land (Ashraf and Foolad, 2007; Bot et al., 2000), and 80% of global cultivated land (providing 60% of world food production) depends on unreliable rain-fed irrigation methods (UNCTAD, 2011). Drought stress is considered a major threat to food security, particularly in the arid and semi-arid tropics (Boyer, 1982; Passioura, 2007), and in combination with salinity and extreme temperatures it has been estimated to cause greater than 50% yield loss for most major crops (Boyer, 1982; Qin et al., 2011). With a projected increase in global food demand of up to 70% by 2050, tolerance to drought conditions is a major focus of agricultural research and conventional crop breeding programmes (UNWWDR4, 2012).

Drought resistance mechanisms occur at the morphological, physiological, biochemical and cellular level, and have been previously described to fall into four categories; drought escape, drought recovery, drought tolerance and drought avoidance (Fang and Xiong, 2015; Luo, 2010). An example of a drought escape strategy could be a reduction in the duration of the life cycle to complete full development before water limiting conditions take hold, whilst drought recovery strategies allow a plant to recover after periods of complete desiccation and halted growth (Luo, 2010; Yue et al., 2006). Drought avoidance strategies improve plants water uptake and minimise water loss, whilst drought tolerance strategies allow a plant to maintain function under water limiting conditions (e.g. through osmotic adjustment) (Luo, 2010; Yue et al., 2006). Avoidance and tolerance strategies tend to be the main areas of focus in studies aiming to understand and improve drought resistance in crops (Luo, 2010; Yue et al., 2006). One example of a drought tolerance strategy is that of the ‘staygreen’ trait, in which leaf chlorophyll content and photosynthetic activity is maintained for longer despite drought stress conditions, contributing to higher grain yields under stress (Borrell and Hammer, 2000; Harris et al., 2007;).

A stay green phenotype is of great agronomic value in crop species. In addition to contributing to an increase in yield under drought conditions, desirable morphological traits associated with stay green include a greater number of grains per ear (Luche et al., 2015) and enhanced resistance to stem lodging (Adeyanju et al., 2016; Thomas and Howarth, 2000). Greater tolerance to abiotic stresses such as heat stress and submergence

have also been reported (Fukao et a., 2012; Luche et al., 2015), as well as greater tolerance to biotic stress such as spot blotch infection (Distelfeld et al., 2014; Joshi et al., 2007).

Sorghum (*Sorghum bicolor* L. Moench) is a C4 monocot crop which has been described as the 5th most important global cereal crop in terms of yield production (Kholova et al., 2013). It is primarily a vital food source in the semi-arid regions of Africa and Asia, but also has uses as an animal feed and as a biofuel (Anami et al., 2015; Kholova et al., 2013). It is well adapted to water limiting environments and is used extensively as a model species for drought tolerance studies (Dugas et al., 2011; Kholova et al., 2013), with functional genomics approaches facilitated by Sorghum's fully sequenced genome (Paterson et al., 2009) and resources such as the metabolic pathways database SorghumCyc (http://www.gramene.org/pathway/sorghumcyc.html; Dugas et. al, 2011). In more drought sensitive sorghum varieties, post-flowering drought conditions can cause lower yields, premature leaf senescence, stem lodging and rot (Harris et al., 2007; Thomas and Ougham, 2014). In general the crop is capable of growing well under water limited conditions, but across the 45000 sorghum accessions there is great genetic and phenotypic diversity (De Souza et al., 2015). Several sorghum lines exhibit the specific drought tolerance phenotype of 'stay green' (Rosenow et al., 1983; Sanchez et al., 2002; Thomas and Ougham 2014) as described in the 'B35' sorghum line which is derived from the Ethiopia line 'IS 12555' (Borrell and Hammer, 2000). These lines tend to exhibit greater levels of drought tolerance than elite growing varieties such as 'R16' which produce higher yields under normal growth conditions (Kassahun et al., 2010).

Furthering our understanding of the processes and mechanisms that underlie the stay green trait in sorghum will contribute to marker assisted breeding programmes to improve drought tolerance of cultivars, and could facilitate production of introgression lines more accurately targeted to stay green trait loci (Harris et al., 2007; Johnson et al., 2015b). This chapter explains the current literature on the stay green phenotype, divided into physiological studies, quantitative trait locus (QTL) analysis, transcriptomic studies, proteomic studies and targeted gene function analysis.

1.1 Morphological, physiological and biochemical features of stay green

The process of senescence constitutes degradation of chlorophyll and gradual reduction in photosynthetic productivity (Xu et al., 2000a). Crop varieties can be described to be exhibiting the 'functional stay green' trait when senescence processes as a whole are delayed or the rate of progression is decreased whilst photosynthesis continues (Thomas

and Howarth, 2000; Borrell et al., 2014a). This is distinct from 'cosmetic stay green' phenotypes in which only the process of chlorophyll catabolism is delayed or slowed (Thomas and Howarth, 2000; Thomas and Ougham, 2014). In the review paper by Thomas and Howarth, 2000, the functional stay green phenotype was classified into two types. Type A involves a late onset of senescence and normal rate of progression, and Type B involves a normal timing of senescence but slower progression through senescence. Different varieties of sorghum tend to display stay green behaviour that falls into the type A or type B functional categories (Thomas and Howarth, 2000).

Senescence can also be characterized by the transition in the predominant function of a leaf from the photosynthetically active 'carbon (C) capture phase' to the 'nitrogen (N) remobilization phase' in which nitrogen and other nutrients are remobilized from senescing leaves to the rest of the plant prior to leaf death (Thomas and Ougham, 2014). Functional stay greens such as sorghum therefore constitute a delay in this physiological shift in the leaf life cycle, either as a consequence of delayed initiation or slower progression of the nitrogen remobilization phase (Thomas and Howarth, 2000; Yoo et al., 2007). Interestingly, in species with high N content in grain a delay in C-N transition may be detrimental to the supply of nitrogen and essential minerals to the grain, compromising the quality of grain composition (Simmonds, 1995; Uauy et al., 2006). This negative effect of the stay green phenotype has also been recorded in soybean (Kumudini, 2002). Thomas and Ougham, 2014 highlight the importance of studying several varieties of sorghum in order to explore the potential genetic variation available in senescence initiation, independent of nutrient remobilization rate, and potential trade-offs between high yields and grain composition.

In addition to improving yield and size of grain, as a consequence of greater capability to continue normal grain development under drought stress, the stay green phenotype has also been associated with increased resistance to lodging and stem rot, and higher carbohydrate content in stems (McBee et al., 1983; Burgess et al., 2002; Thomas and Ougham 2014). Morphological features associated with the stay green trait are also characteristics which have been suggested to be contributing to greater water use efficiency – such as reduced tillering and reduced surface area of upper leaves, potentially contributing to water conservation pre-flowering (Borrell et al., 2014a; Borrell et al., 2014b; Kassahun et al., 2010). An increase in water availability and accessibility during grain filling under drought conditions contributes to a more successful yield (Vadez et al., 2011), and therefore is a possible explanation for the improved yields seen in stay green sorghum. Xu et al., 2000a observed substantially higher relative water content in the apical leaves under

severe drought in apical leaves of stay green compared to non-stay green lines (81% as opposed to 38%) which could be related to continued efficiency of stem transport under drought conditions, and altered transpiration efficiency (Vadez et al., 2011).

1.2 QTL analysis and genetic mapping

There are several studies that describe the physiological, developmental and morphological features of the stay green phenotype, but there is limited understanding of the molecular mechanisms and processes that are responsible for the trait. The following studies employed quantitative trait locus (QTL) analysis to investigate associations between the phenotypic trait and genomic loci, in order to identify potential candidate genes and processes contributing to the stay green characteristic.

Four QTLs for the stay green trait have been identified, following analysis on a recombinant inbred line (RIL) population produced from the cross between B35 (stay green line originating from Ethiopia) and Tx7000 (which is susceptible to post-flowering drought) (Xu et al., 2000b; Sanchez et al., 2002). A total of 53.5% of the phenotypic variation within the RIL population was explained by the genetic variation in all 4 stay green QTLs combined (Stg QTL; Stg1, Stg2, Stg3, Stg4) (Subudhi et al., 2000). These stay green QTL regions have been mapped to areas in the sorghum genome; both Stg1 and Stg2 have been mapped to chromosome 3, Stg3 is located on chromosome 2, and Stg4 on chromosome 5 (Sanchez et al., 2002; Subudhi et al., 2000; Xu et al., 2000b).

Near isogenic lines (NILs) containing each of the 4 stay green QTLs *individually* in a Tx7000 background have been produced to investigate the individual contribution of the QTLs to the stay green trait (Harris et al., 2007). Each line displayed features of the stay green phenotype at varying levels of intensity, including universally higher grain yield than the non-stay green line, suggesting each QTL region has an important influence on determining the overall stay green phenotype (Harris et al., 2007; Borrell et al., 2014b). In the paper by Vadez et al., 2011, each QTL was also introgressed into different non-stay green lines in addition to Tx7000 (R16 and S35) and each line showed varying effects of the same QTLs in different genetic backgrounds. The number of QTLs identified, and the variability of their contribution to the stay green phenotype in different genetic contexts highlights the complexity of the trait (Borrell et al., 2014b).

There is a close association between the stay green phenotype and plant response to stress, as is illustrated in the co-localization of QTLs for stay green with QTLs for

temperature and drought stress (Xu et al., 2000b; Vadez et al., 2011; Emebiri, 2013; Thomas and Ougham, 2014). Another example of QTL co-localization was found in studies on RIL populations produced from an original cross between lines with different nodal root angles (narrow vs. wide angle). These nodal root angle QTLs have been found to overlap with stay green QTLs, suggesting modified root architecture is likely to be a contributor to the stay green trait observed in this population, potentially influencing water extraction capabilities (Vadez et al., 2011; Borrell et al., 2014a).

The knowledge gained from QTL studies such as these can have agronomic application in marker assisted selection (MAS) techniques, which attempts to introduce QTL regions for stay green into the non-stay green high yielding varieties (Sanchez et al., 2002). Due to the genetic linkage between the stay green trait and stress response (highlighted by the overlapping QTL regions), selection for stay green can simultaneously lead to inheritance of stress tolerance features (Vadez et al., 2011; Thomas and Ougham, 2014).

1.3 Transcriptomics studies

QTL analysis can identify regions of genetic variation which are closely associated with variation in the phenotype of interest. This can be used to identify genomic regions that could potentially contain genes that contribute to the stay green trait. However, transcriptomic studies are able to provide further insight into the molecular and physiological basis of the trait by identifying differential expression of specific genes across different varieties or in response to a change in environment (Buchanan et al., 2005; Dugas et al., 2011; Johnson et al., 2015b). This technique can reveal more about the processes involved in the stay green phenotype, without needing to identify the genes underlying the QTLs. Several studies have used transcriptomic approaches to analyse the change in gene expression in Sorghum as a result of abiotic stress such as osmotic stress and abscisic acid (Buchanan et al., 2005; Dugas et al., 2011; Johnson et al., 2014). Others have compared the transcriptome of stay green and non- stay green Sorghum under normal conditions (Johnson et al., 2015b). RNA sequencing has been used in addition to microarray data to give a more complete impression of the altered expression of genes between lines or treatment, producing a 'global transcriptome profile' (Dugas et al., 2011).

In the paper by Johnson et al., 2015b, the transcriptome of B35 (stay green) and R16 (senescent) was compared in plants grown in non-stress conditions. In the B35 line, 1038 genes were upregulated and 998 genes were downregulated compared to R16. Gene ontological analysis was then used to elucidate the genetic categories and pathways in

which significant proportions of differentially expressed genes were involved (Dugas et al., 2011; Johnson et al., 2015b). Enriched categories included 'response to osmotic stress' and 'water transport', which corroborates evidence of the close association between the stay green trait and osmotic stress, observed in the co-localization of stay green QTLs with those of drought stress (Xu et al., 2000b; Vadez et al., 2011; Emebiri, 2013; Thomas and Ougham 2014).

Genes of particular interest that were upregulated in B35 and grouped in the 'response to osmotic stress' category included *DREB1A* (*dehydration-responsive element-binding 1A*) transcription factor, *SDIR1* (*salt and drought-induced RING finger 1*) ubiquitin ligase – both of which will be discussed in greater detail later in this review – and *P5CS2* (*delta-1-pyrolline-5-carboxylate synthase 2*) (Johnson et al., 2015b). *P5CS2* is known to be involved in the biosynthesis of the osmoprotectant proline (Kishor et al., 1995; Ashraf and Foolad, 2007), the accumulation of which is thought to contribute to osmotic adjustment under stress, as well as contributing to stress responses such as free-radical scavenging, and membrane and protein stabilization (Smirnoff and Cumbes, 1989; Mishra and Dubey, 2006; Ashraf and Foolad, 2007). Further confirmation of the role of *P5CS2* in contributing to the stay green trait was found when comparing the transcriptomic data to the QTL analysis of earlier studies. *P5CS2* has been found to fall within the *Stg1* QTL (Johnson, 2015a), indicating that there is both an expression level difference between stay green and non-stay green, and a correlation between genetic variation in/around this gene and variation in the phenotype (Subudhi et al., 2000; Johnson, 2015a) The correlation between increased *P5CS2* expression and actual proline levels was confirmed by measurements of ~1.8-fold greater total proline content in B35 compared to R16 (Johnson et al., 2015b). Therefore, *P5CS2* has an important role in both drought tolerance and the stay green phenotype.

1.4 Proteomics

Transcriptomic studies cannot be used as definitive indicators of gene translation and protein production due to the lack of correlation between mRNA and actual protein levels (Carpentier et al., 2008; Ngara and Ndimba 2014b, Velez-Bermudez and Schmidt, 2014). This non-linearity between mRNA and protein expression can partly be explained by variability in mRNA degradation rates (Salekdeh et. al., 2002; Jedmowski et al., 2014), and differential translational efficiency between mRNA transcripts (Bailey -Serres, 1999; Mustroph et al., 2009). Transcriptome studies also cannot account for the post-transcriptional modifications (PTMs) that occur extensively in plants, including

phosphorylation, ubiquitination and *N*-linked glycosylation, which can have profound effects on protein function, stability and localisation (van Wijk, 2001; Gong et al., 2015). To address this problem, several studies have used proteomic analysis to further investigate abiotic stress response mechanisms, some of which are described below.

1.4.1 Proteomic studies on abiotic stress response in crop species

In plant proteomics, 2-dimensional gel electrophoresis (2-D GE) continues to be the most widely used technique for separation and identification of proteins. Several studies have analysed the change in protein expression as a response to different levels of drought stress in comparison to normal growth conditions. A review by Wang et al. 2016 listed 440 unique proteins as being drought-responsive based on proteomics studies on the leaves of 25 different plant species. These drought-responsive proteins were found to be involved in in a range of different functions, including signalling, gene expression regulation, protein metabolism and turnover, photosynthesis, photorespiration and carbohydrate metabolism. Other proteins had roles in reactive oxygen species scavenging pathways (e.g. superoxide dismutase, peroxidases), osmotic regulation (e.g. late embryogenesis proteins or 'LEAs') and membrane trafficking (e.g. aquaporins) (Wang et al.,2016). For example, a study in Barley by Vitamavas et al., 2015 used 2-dimensional difference gel electrophoresis (2-D DIGE) to analyse the change in protein expression under drought stress. Glutathione s-transferase isoforms (GSTs, known to be involved in processes of cell division and stress response) were found to accumulate under osmotic stress, as well as LEAs (involved in membrane and enzyme stabilization) and chaperones (involved in the prevention of protein degradation, and facilitating correct protein folding) (Vitimavas et al., 2015). Changes in the expression of several tricarboxylic acid (TCA) cycle enzymes (some up- and some down-regulated), suggested a severe disruption of aerobic metabolism, with upregulation of glutathione peroxidase potentially indicating a subsequent response to ROS accumulation (Vitamavas et al., 2015). More recently, studies have used iTRAQ (isobaric tag for relative and absolute quantitation) based proteomic techniques, for example in tobacco (Xie et. al., 2016) and apple (Zhou et al., 2015) potentially benefiting from increased sensitivity and reproducibility.

Several proteomic studies have used comparisons between lines of differing drought tolerance phenotypes to gain insights into tolerance mechanisms. In the paper by Zang and Komatsu in 2007, the proteome of a drought-sensitive rice variety was compared to that of a drought-tolerant variety after mannitol treatment. Upregulated proteins in the tolerant

line included a 26S proteasome regulatory subunit (required for the correct functioning of the proteasome, facilitating removal of polyubiquitinated proteins), a lipid transfer protein, and BiP (endosperm luminal binding protein, involved in the correct folding of proteins) (Zang and Komatsu, 2007). Another study by Benesova et al., in 2012 comparing maize cultivars found evidence of an increase in polysaccharide hydrolysis inhibitors in the more drought tolerant line compared to the drought-sensitive line, possibly reflecting an increase in lignification as a drought tolerance mechanism. Enhanced cell wall synthesis was suggested to be a response to changes in tugor pressure, where maintained mechanical strength minimizes further dehydration and cell wall loosening allows continued gradual growth and development even under stressed conditions (Benesova et al., 2012; Wang et al., 2016). In both apple (Zhou et al., 2015) and maize (Benesova et al., 2012) more tolerant varieties have been shown to have increased levels of light-harvesting chlorophyll a/b-binding proteins compared to the sensitive lines in response to drought (Wang et al., 2016). It was suggested that enhanced levels of photosynthesis-related proteins reflect that photosynthetic efficiency is protected and maintained in these lines under stressed conditions, which is a typical feature of the stay green phenotype. In this way, reactive oxygen species (ROS) homeostasis is also protected by continued quenching of excitation energy through the light-harvesting complex system (Benesova et al., 2012; Wang et al., 2016; Zhou et al., 2015).

Proteomic comparisons between transgenic and wildtype lines have also provided interesting insights into drought tolerance mechanisms. This is evident in the paper by Paul et. al., 2015 in which the drought-stressed proteome of a rice line overexpressing *DREB1A* (*Dehydration responsive element-binding 1 A*), compared to a wildtype line was analysed by 2-dimensional gel electrophoresis (2-D GE). Stress-induced upregulation of carbohydrate metabolism-related proteins, including UDP-glucose pyrophosphorylase, was observed in the transgenic line in contrast to the wildtype. It was suggested that the increase in plant height and root exploration of the drought-stressed transgenic line could be explained by an effectively sustained carbohydrate and energy metabolism despite water limitations.

As previously mentioned, PTMs have a significant influence on protein function and several proteomic studies have focussed on protein modification changes in response to stress. The response of phosphoproteins to drought have been investigated in rice (Ke et al., 2009), wheat (Zhang et al., 2014) and maize (Bonhomme et al., 2012; Hu et al., 2015). These types of studies investigate the proteome to a greater level of complexity, and give

additional insight into alterations and regulation of protein function under stress, beyond that of quantitative proteomics methods.

1.4.2 Proteomic studies on abiotic stress response in sorghum

Several recent studies have used sorghum as a model crop species to investigate changes in the proteome in response to abiotic stress. A paper by Jedmowski et al., in 2014 described the processes of protein and amino acid metabolism to be significantly altered at the protein level in response to drought stress. In this study, a drought tolerant line (11434) and a drought sensitive line (11431) were compared using 2-D GE, and the tolerant line was found to have an upregulation of heat-shock and chaperone proteins during stress and after recovery. This could suggest a more reliable defence against stress-related disturbance to protein synthesis and folding processes in this line compared to the drought sensitive line. Other studies have looked at differences in the proteome response to salt stress between various sorghum cultivars (Swami et al., 2011; Ngara et al., 2012). These papers are described in greater detail in section 3.1.

1.5 Characterization and functional analysis of *SDIR1*, *DREB1*, *NAC* and *USP*

Transcriptomic and proteomic analyses identify proteins with association to drought tolerance phenotypes. These could be involved in signal perception (e.g. receptors), signal transduction (e.g. transcription factors) or directly in stress protection (e.g. chaperones, osmolytes) (Zang and Komatsu, 2007). Genes of interest require further characterization to investigate whether the correlations observed indicate a true function in stress tolerance mechanisms.

1.5.1 *SDIR1*

As previously mentioned, one of the genes found to be upregulated in B35 compared to R16 and belonging to the 'response to osmotic stress' category is *SDIR1*, encoding an E3 ubiquitin ligase (Zhang et al., 2007; Johnson et al., 2015b). Overexpression of *SDIR1* homologs in *Arabidopsis*, maize and rice increased drought tolerance in the transgenic lines, potentially involving changes in stomatal aperture and transpiration efficiency (Zhang et al., 2007, Zhang et al., 2008; Xia et al., 2012). It has therefore been hypothesized that *SDIR1* is involved in the aspect of the stay green phenotype in which water availability during grain filling is maintained under drought conditions (Vadez et al., 2011; Johnson, 2015a).

1.5.2 *DREB1*

The transcription factor *DREB1A* is also upregulated in B35 compared to R16 (Johnson et al., 2015b). The DREB transcription factors, from the ethylene response factor (ERF) family, bind to DNA at their AP2 (apetala 2) domain (Sakuma et al., 2002). DREB transcription factors are able to modify expression of stress responsive genes by binding to their promoters at the DRE ('dehydration responsive element') (Stockinger et al., 1997; Sakuma et al., 2002). Homologs in a number of different species have been shown to increase stress tolerance when overexpressed, for example in wheat and brassica, suggesting conservation of the stress response regulatory system (Shen et al., 2003; Savitch et al., 2005). Overexpression in Arabidopsis of *AtCBF3*, the Arabidopsis homolog of *DREB1A,* has been shown to cause accumulation of osmoprotectants such as proline and sugars (Gilmour et al., 2000).

1.5.3 *NAC*

NAC (no apical meristem (NAM), ATAF1-2 and cup-shaped cotyledon (CUC)) proteins are transcription factors which share a highly conserved NAC domain at the N-terminus involved in DNA-binding and containing a nuclear localisation signal (Nakashima et al., 2012; Wang et al., 2013), the structure of which has been determined in Arabidopsis (Ernst et al., 2004) and rice (Chen et al., 2011). Several *NAC* transcription factors have been highlighted as potential contributors to the regulation of both drought stress responses and senescence processes, with evidence of abscisic acid (ABA) and methyl jasmonic acid induced expression (Fujita et al., 2004; Nakashima et al., 2012).

There are ~110 members of the *NAC* family in Arabidopsis (Riechmann et al., 2000), and several of these *NAC* genes have been studied extensively in connection with abiotic stress tolerance. For example, overexpression of *RD26* (*RESPONSE TO DEHYDRATION* 26) in Arabidopsis transgenic lines have resulted in increased expression levels of stress-inducible genes (Fujita et al., 2004). Arabidopsis *JUB1* (*JUNGBRUNNEN 1*) overexpression lines were shown to produce a delayed senescence phenotype, and greater tolerance of abiotic stress associated with ROS metabolism regulation (Wu et al., 2012).

Several NAC transcription factors have been associated with the regulation of senescence initiation and progression, and have been hypothesised to contribute to the delayed senescence phenotype associated with the stay green trait (Guo and Gan, 2006). There are several examples of *NAC* genes exhibiting positive senescence regulation, for example in

Arabidopsis (Guo and Gan, 2006; Yang et al., 2011; Lee et al., 2012) and rice (Sperotto et al., 2009), as well as negative regulators in Arabidopsis (Wu et al., 2012) and wheat (Zhao et al., 2015).

1.5.4 *USP*

Universal stress proteins (USPs) can be found in bacteria, archaea and eukaryotes, and have roles in plant stress response including defence against superoxide generating agents (Nachin et al., 2005). Transcriptomic studies show evidence of *USP* upregulation when sorghum is exposed to heat stress (Johnson et al., 2014). The role of USPs in drought stress response is also illustrated in transgenic studies in which tomato lines overexpressing *USP* under drought stress conditions are shown to have reduced stomatal aperture and increased expression of genes for the maintenance of photosynthetic components. This reflects a stay green-like response of increased water conservation and maintained photosynthetic activity under osmotic stress (Loukehaich et al., 2012).

In sorghum, evidence of upregulation of a *USP* gene has also been found in microarray analysis in which R16 senescent lines were compared to R16 lines containing the *StgB* stay green QTL. It has been suggested that this USP could have a function that contributes to the stay green phenotype, and specifically to the genetic variation underlying that particular QTL (Johnson, 2015a). This USP gene was *Sb01g037580.1* and was grouped in the gene ontology (GO) category 'response to osmotic stress' (Johnson, 2015a).

1.6 Summary

Drought stress is a major food security issue. Investigation into the mechanisms of response and tolerance is essential to inform future crop breeding programmes. Sorghum in particular is an interesting crop to study considering the wealth of genetic diversity among cultivars and the evidence of stay green phenotypic traits (Mace et. al., 2013).

Several studies have made observations and measurements of the physiological, developmental and morphological features associated with the stay green phenotype in sorghum. However, there is still limited understanding of the molecular mechanisms and processes responsible for these characteristics. Studies have employed QTL analytical techniques to follow the correlations between genetic variation and stay green phenotype variation in populations in order to narrow down the potential genomic location of genes and loci involved in the trait. Discovery of the co-localization of these QTLs with those of abiotic stress responses have confirmed the close association between stay green and

stress tolerance in sorghum (Xu et al., 2000b; Vadez et al., 2011; Emebiri, 2013; Thomas and Ougham 2014; Johnson et al., 2015b). Transcriptomic studies have also been used to identify the directional changes in gene expression levels as a response to applied abiotic stresses, or between different sorghum lines (Buchanan et al., 2005; Dugas et al., 2011; Johnson et al., 2014; Johnson et al., 2015b). Comparison of these results to the genetic mapping of QTLs has identified candidate genes/processes that could form the genetic basis of the stay green trait.

Whilst the transcriptome of B35 stay green and R16 senescent sorghum lines have been compared, a complementary proteomic comparison has yet to be conducted. Genes of interest identified in the transcriptomic comparison between these lines, such as *SbSDIR1* ubiquitin ligase (Johnson et al., 2015b), require further characterization to investigate their respective functions.

Project plan

General aim:

To investigate the underlying mechanisms and pathways involved in the stay green trait in Sorghum, in relation to drought tolerance.

Specific aims of my project:

1. To conduct a proteomic comparison between the B35 stay green line and the R16 senescent line to compare the difference in the levels of specific proteins between these lines. This data will be analysed alongside previous transcriptomic data for these lines. Candidate proteins for involvement in the processes and mechanisms that form the basis of the stay green trait can be selected for future study.

2. To investigate the function of sorghum *SbSDIR1* in relation to the stay green trait and drought stress response. Work by a previous student Stephanie Johnson (Johnson, 2015a) found *SbSDIR1* to be upregulated in stay green B35 lines compared to non-stay green R16 lines in sorghum, and overexpression in other species have shown increased drought tolerance characteristics (Xia et al., 2012; Zhang et al., 2007; Zhang et al., 2008). An *SbSDIR1* overexpression construct was produced by Stephanie Johnson and used to transform wheat at NIAB. These were tested for evidence of the stay green phenotype such as improved drought tolerance, water use efficiency, stomatal conductivity, production of compatible solutes and expression of stress genes.

Chapter 2

Materials and Methods

2.1.1 Reagents

All chemicals were supplied by the following list of companies:

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

2.1.2 Sorghum seed source

Sorghum bicolor seeds of the cultivars B35 and R16 were supplied by Dr Santosh Despande, (ICRISAT, Petancheru, India).

2.1.3 Sorghum growth conditions

Following overnight imbibition in water, sorghum seeds were germinated on 44mm hydrated peat plugs (Jiffy Products international, Moerdijk, Norway) within sealed plastic containers. Growth chambers were set to a 12 hour (hr) photoperiod, with 28°C/23°C day/night temperature cycles. Containers were opened following evidence of germination, normally 3-4 days after sowing (DAS). Sorghum plants were grown for 14 days after imbibing, and the whole aerial part of the part was harvested for protein extractions.

2.1.4 Source of Fielder Wheat seed transformed with the overexpression construct pEW304-*SbSDIR1*

A construct for overexpressing the sorghum gene *SbSDIR1* (Sb01g039740.1) was produced by Stephanie Johnson. Agrobacterium-mediated transformation of Fielder wheat (*Triticum aestivum L.*) with the overexpression construct (pEW304-*SbSDIR1*) was then conducted by Dr Emma Wallington at NIAB (Huntington Road, Cambridge, CB3 0LE), and the second generation of transformed seeds were collected.

2.1.5 Wheat growth conditions

Seed dormancy was broken by heat treating dry seeds at 32^{o}C for 6 days, followed by cold treatment at 4^{o}C overnight. Seeds were then imbibed overnight and germinated on wet

tissue in petri dishes, at 20^{o}C in the dark. Germinated seedlings were transferred to 44mm hydrated peat plugs and grown at 20^{o}C with a photoperiod of 16 hours.

2.2 Molecular biology techniques

2.2.1 RNA extraction

RNA was extracted from mature wheat plants grown for 12 days, and all 3 leaves were harvested for RNA extraction. Leaf tissue was disrupted under liquid nitrogen and total RNA was extracted using RNeasy Plant Total RNA kit (Qiagen, Crawley, Uk) following the manufacturers protocol.

2.2.2 cDNA synthesis

Full length cDNA was synthesised from RNA extractions using M-MLV reverse transcriptase and oligo dt primers (Promega, Southampton, UK) following the manufacturers protocol.

2.2.3 Real time polymerase chain reaction (PCR)

Applied Biosytems 7300 Real Time PCR Machine (Applied Biosystems, Forster City, USA) was used to analyse relative transcript levels of the *SbSDIR1* gene. A reaction mix containing 7.5µl of FastStart SYBR Green Master mix (Roche Diagnostics GmbH, Mannheim, Germany), 0.9µl of forward primer (5µM) and 0.9µl of reverse primer (5µm) was added to 5µl of cDNA (50 x dilution), with 3 technical replicates per reaction. The ADP-ribosylation factor (ARF) gene Ta. 2291 (Paolacci et al., 2009) was used as an invariant reference gene for wheat RNA. Relative transcript levels were calculated using the algorithm described in the Relative Quantitation (RQ) Algorithms in the Applied Biosystems Real-Time PCR Systems Software (Applied Biosystems Real-Time PCR Systems, 2007). Values represent the relative quantitation (RQ) estimates as calculated using the 2(-Delta Detla C(T)) method (Livak and Schmittgen, 2001). The RQ_{MIN} and RQ_{max} error bars are calculated from the Student's t test, and represent the limits of acceptable error at 95% confidence as described in Knight et al., 2009. A list of real time real time PCR primers is included in Appendix A.

2.3 Physiological experiments for analysing the phenotype of wheat transgenics

2.3.1 Excised leaf water-loss assay

Wheat plants were grown for 18 days under normal conditions (see section 2.1.5). For the final night prior to the experiment the plants were exposed to conditions of high humidity by covering them with a transparent plastic bag. The following day, leaves of the same

developmental stage (leaf 4) were detached and weighed at regular intervals over a period of 5 hours. The excised leaves were positioned flat with the abaxial side up throughout, and maintained at room temperature. One leaf from six individual plants was measured for each genotype, and the full experiment was repeated 3 times to produce 3 biological replicates.

2.3.2 Assay for tolerance to polyethylene glycol (PEG) induced stress.

Leaves of the same developmental stage (leaf 4) were excised from mature wheat plants (25 days old) and cut into sections of approximately 1cm in length. Leaf sections from 6 individual plants per genotype were pooled and mixed, then divided into 6 equal groups. Grouped leaf sections were subsequently floated adaxial side up in 7ml of 0, 15 and 25% solutions of PEG (w/v) in 6-well plates, with two wells allocated to each unique treatment and genotype combination. Assays were maintained at 20°C with a 16hr photoperiod. Visible changes in chlorophyll degradation and senescence progression were documented photographically over the course of 7 days.

2.3.3 Light and dark induced senescence assay

Leaves of the same developmental stage (leaf 6) were excised from mature 28 days-old wheat plants and cut across the leaf blade into two equal halves. Leaf sections from 6 individual plants per genotype line were pooled and mixed, then divided into two equal groups with equal numbers of leaf tip and base sections. These sections were then placed adaxial side up on wettened tissue in 6ml petri dishes (2 plates per genotype line) and sealed with micro-pore tape. One plate from each line was then covered in two layers of tin foil. All plates were incubated at 20°C under a 16-hour photoperiod. Visual changes in senescence progression between genotype lines and between light and dark exposed plates were then documented photographically over the course of 10 days.

2.3.4 ABA-induced gene expression analysis by real time PCR

Leaves of the same developmental stage (leaf 5) were excised from mature 25 days-old wheat plants and cut into sections of approximately 1 cm in length. Sections from 6 individual plants from each genotype line were pooled and mixed together. Leaf sections were floated adaxial side up in 7ml of water in 6-well plates and allowed to equilibriate overnight in the dark. The water was then replaced with 7ml of 100µM ABA solution, with 0.1% ethanol solution as a control treatment. Plates were incubated at 20°C in the dark, and samples were collected and frozen in liquid nitrogen at the intervals of 12 and 24 hours

following treatment. One harvested sample equated to approximately 1.5 sectioned leaves, and were subsequently used for RNA extraction and real time qPCR (see sections 2.2.1 and 2.2.3).

2.4 Proteomics

2.4.1 Total protein extraction

Total proteins were extracted from the whole aerial part of sorghum plants grown for 14 days using the 10% TCA (trichloroacetic acid)/acetone extraction method described in Carpentier et al., 2005. Each individual sample consisted of a single plant. Samples were then resuspended in resolubilization buffer containing 7M urea, 2M thiourea, 30mM Tris, 1% protease inhibitor mix and 2% (w/v) CHAPs (3-[(cholamidopropyl)dimethylammonio]-1-propanesulfonate) at pH 8.5. Proteins were further purified using 2-D Clean-Up kit (GE Healthcare, Little Chalfont, UK) following the manufacturer's instructions, and readjusted to pH 8.5 by the addition of resolubilization buffer at pH10. Protein content was quantified using 2-D Quant Kit (GE Healthcare) also following the manufacturer's instructions.

2.4.2 Preparation of samples for coomassie stained gels

For the non-labelled protein samples intended for use in coomassie stained 2-dimensional SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) gels, 250µg of protein was added to Destreak Rehydration buffer (GE Healthcare) containing 0.5% IPG (Immobilized pH Gradient) buffer pH 3-10 (GE Healthcare), up to a total volume of 340µl.

2.4.3 Labelling and preparation of protein samples for Difference Gel Electrophoresis (DIGE)

Protein samples intended for use in DIGE were labelled using Cy-Dye DIGE Fluor Minimal Labelling Kit (GE Healthcare) using 50ug of protein per sample, according to the manufacturer's instructions. B35 and R16 sorghum samples were labelled with Cy-3 and Cy-5 alternately, and control samples were labelled with Cy-2. One labelled sample of each genotype and one control were combined for separation on each separate gel. For Gels 1 and 2, B35 and R16 samples were labelled with Cy-3 and Cy-5 respectively, with the opposite labelling for gels 3 and 4.

2.4.4 2-Dimensional gel electrophoresis

Protein extracts (both fluorescent labelled and non-labelled) were primarily separated in the first dimension by IEF (Isoelectric focussing) on 18 cm pH 3-10 Nonlinear (NL)

Immobiline DryStrips strips (GE Healthcare), using the IEF unit Ettan IPGphor II (Amersham Biosciences, GE Healthcare, Little Chalfont, UK). The overnight programme used is detailed in Table 2.1. Once completed, the strips were equilibrated by 20-minute incubation in 10ml of equilibration buffer (6M urea, 30% glycerol, 2% SDS and 50mM Tris pH 8.8) with 1% (w/v) dithiothreitol (DTT) added. This was followed by a second 20-minute incubation in 10ml equilibration buffer with 2.5% (w/v) iodoacetamide (IAA) added. SDS-PAGE was run in the second dimension using 12.5% 1mm polyacrylamide gels. Gels used for separating non-labelled proteins were stained using Coomassie Brilliant Blue G-250 and scanned using an Image Scanner (Amersham Biosciences, GE Healthcare). DIGE gels were scanned using the Ettan DIGE Imager (Amersham Biosciences, GE Healthcare) using the final exposure times of cy2:1, cy3:0.1, cy5:0.2.

Table 2.1- 1EF programme for IPG strips ph3-10 NL 18cm

Step	**Volts**	**Volt Hours**
1. Rehydration step	30	10
2. Focussing step & hold	500	500
3. Focussing gradient	1000	800
4. Focussing gradient	8000	13500
5. Focussing step & hold	8000	20000

2.4.5 Statistical analysis of 2D-gels

Aligned gel scans were analysed using Progenesis Samespots software 4.0 (Nonlinear Dynamics, Newcastle, UK). Gels were aligned to the reference gel image, and spot volumes were normalized to the total spot intensity. In DIGE analysis the spot volumes were normalized to the internal standard in each gel to minimize inaccuracy due to gel-to-gel variation. Each detected spot was confirmed manually to avoid artefacts. Differentially expressed proteins between the two lines were identified using the criteria of a 2-fold or greater change in gene expression ($ratio_{B35/R16}$, ANOVA $p<0.05$).

Chapter 3

Comparison between the proteomes of stay green and senescent sorghum varieties using 2-dimensional gel electrophoresis

3.1 Introduction

Transcriptomic data reveals changes at the gene expression level in response to abiotic stress, but gene transcript levels often do not correlate directly to actual protein level due to factors such as protein degradation and post-translational modifications (Carpentier et al., 2008; Gong et al., 2015; Jedmowski et al., 2014; Ngara and Ndimba 2014b; van Wijk 2001). Proteomic studies are required to investigate changes at the level of the end-product; the protein. In plant proteomics, 2-dimensional gel electrophoresis (2-D GE) continues to be the most widely used technique for separation and identification of proteins. Rice has been used extensively as a model crop species for proteomic and abiotic stress studies, as previously described (see section 1:2:2) (Chitteti et al., 2007; Zang and Komatsu, 2007; Paul et al., 2015). However, this study instead utilizes the drought tolerant crop sorghum to analyse drought stress response mechanisms. Ngara and Ndimba 2014a champion sorghum as a superior model for drought tolerance mechanisms as an alternative to rice, considering it's natural drought tolerance and use as a staple food crop in arid and semi-arid regions (Rosenow et al., 1983; Ngara and Ndimba 2014a). It is also a C4 crop species, in contrast to C3 rice, and this method of carbon fixation helps to maintain photosynthetic activity under stressed conditions (Buchanan et al., 2005; Ngara and Ndimba 2014a).

A few papers have described analysing the proteome-level responses of sorghum to abiotic stress. The proteome of Egyptian landraces of Sorghum lines were compared using 2-D GE in a paper by Jedmowski et al., in 2014, focussing on a drought tolerant line 11434 and drought sensitive line 11431. The drought treatment was sufficient to cause disruption to levels of proteins involved in photosynthesis, glycolysis and the TCA cycle for both lines. Of particular note was the response of proteins involved in protein and amino acid metabolism. For example, methionine synthase and S-Adenosyl-L-methionine synthase (SAM synthase) are upregulated in both lines, but only remain upregulated in the tolerant

line following recovery (Jedmowski et al., 2014). Interestingly, upregulation of SAM synthase was also found in the drought tolerant rice transgenic line overexpressing *DREB1A* compared to wildtype (Paul et al., 2015), and overexpression of SAMs in transgenic *A. thaliana* has been shown to increase drought tolerance (Kim et al., 2015). SAM synthase and methionine synthase are both involved in the biosynthesis of S-adenosyl-L methionine (SAM) via the 'SAM cycle', components of which have roles in methyl-group donation, ethylene biosynthesis (a hormone involved in senescence), gene expression regulation, cell wall metabolism and lignin biosynthesis (Boerjan et al., 1994; Cruz et al., 1992; Jedmowski et al., 2014; Kim et al., 2015; Krannich et al., 2015). SAM also has a major role in polyamine production, and sorghum microarray data has previously indicated upregulation of the polyamine spermidine synthase under combined heat and drought stress (Gill and Tuteja, 2010; Jedmowski et al., 2014; Johnson et al., 2014). Additionally, Hsp60, a chaperone protein disulphide isomerase (PDI) and 40S ribosomal protein S3 were all upregulated either during stress or after recovery in the tolerant line, but not the sensitive sorghum line, indicating a greater efficiency of protein synthesis, assembly and regulation of aggregation associated with drought tolerance (Jedmowski et al., 2014).

Two independent papers using different cultivars of sorghum have studied proteomic changes in response to salt stress (Swami et al., 2011; Ngara et al., 2012). In both cases, there was evidence of upregulation of GSTs, ATP synthases and peroxidases in response to increased salinity – similar to the proteomic response to drought stress found in several other crop species (Zang and Komatsu 2007; Vitamavas 2015; Xie et al., 2016).

In this study, the full proteome of the stay green sorghum line B35 and the senescent line R16 are compared by 2-D GE, firstly using Coomassie Blue staining and secondly using CyDye DIGE fluor dyes in the more sensitive 2-dimensional difference gel electrophoresis (DIGE) method. This data will complement previous transcriptomic data which compared the differences in gene expression between the same lines at the same developmental stage (Johnson et al., 2015b), and any differentially expressed proteins discovered can be investigated for correlation to changes previously found at the transcription level.

Aims;

- To compare the total proteins expressed in the stay green and senescent lines grown under normal conditions using 2D gel electrophoresis (2-D GE).

- To identify differentially expressed proteins between the two lines using Matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF-MS).
- To use previous microarray data to identify any correlation between protein level and gene expression level differences between the two lines, and identify candidate genes and processes that potentially underlie the stay green trait.

3.2 Comparative analysis of the full proteomes of the stay green line B35 and the senescent line R16 grown under normal conditions.

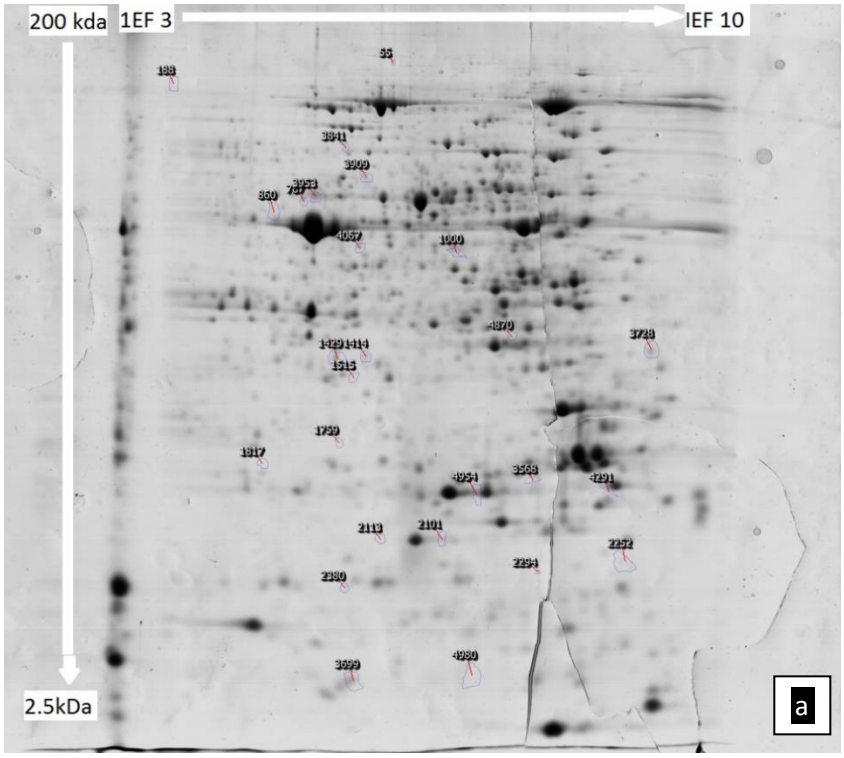
3.2.1 Comparison of total protein extractions of B35 (stay green) and R16 (senescent) sorghum plants at 14 days old using 2D gel electrophoresis visualized using Coomassie Blue staining.

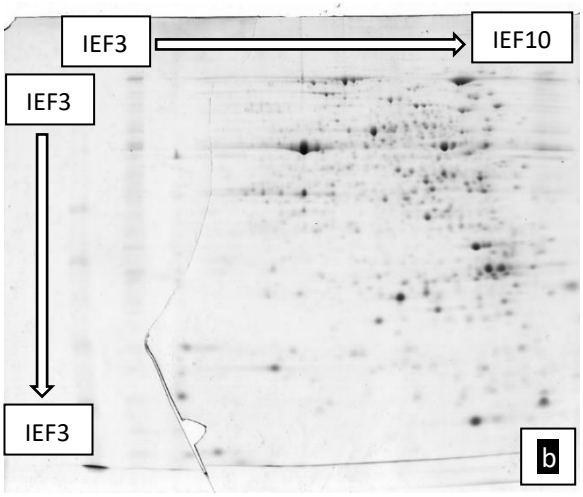
In this study, the full proteomes of two sorghum cultivars with different drought tolerance phenotypes were compared using 2-D GE with Coomassie Blue staining (section 2.3). Sorghum plants were grown for 14 days (5 leaf stage) under normal conditions, and the whole aerial part of the plants were harvested (section 2.1.2). Total proteins were extracted from 4 replicates using a TCA/acetone extraction method (section 2.3.1). Each replicate sample consisted of a single plant. Gel scans were analysed using Progenesis Samespot software, and differentially expressed proteins between the different lines were selected using the criteria of a 2-fold threshold change in relative signal intensity, ANOVA $p \leq 0.05$ (Swami et al., 2011) (section 2.3.3).

The drought tolerant 'stay green' sorghum line used in this study is 'B35' (or BTx632) and is of Ethiopian origin, derived from accession IS 12555 (Rosenow et al., 1983; Kassahun et al., 2010). It exhibits a phenotype of delayed senescence under drought treatment, and maintains greater chlorophyll content and percentage of photosynthetically active leaf area compared to 'senescent' drought sensitive varieties such as R16 (Rosenow et al., 1983; Kassahun et al., 2010). The stay green phenotype of B35 has been studied extensively, and B35 and R16 were used in the paper by Johnson et al., 2015b which conducted a transcriptomic comparison between the two lines under normal conditions. By using these same varieties at the same developmental stage, the proteomics data generated in this study can be used to complement this transcriptomic data and give further insight into both the gene transcript level and protein level disparities between stay green and non-stay green sorghum.

The 2-D GE gels presented in this report were stained using Coomassie Brilliant Blue G-250, and serve as a pilot study to test for the suitability of the total protein extraction method prior to use in the more sensitive DIGE analysis (see section 3.2.2). Progenesis Samespot software 4.0 (Nonlinear Dynamics) was used to align the gels to the reference gel image, detected spots were validated manually to avoid artefacts and spot volumes were normalized to total spot intensity.

A total of 919 protein spots were detected on the 2-D GE gels, and the comparative analysis using Progenesis SameSpots identified 27 of these spots as having a 2-fold or greater change in expression level between the two lines, statistically significant to a level of 95% confidence ($ratio_{B35/R16}$ ANOVA $p<0.05$). Twelve protein spots were upregulated in B35, and 15 protein spots were downregulated in B35 compared to R16 (see Table 3.1). Figure 1a displays the reference gel image following separation by SDS-PAGE, with the differentially expressed protein spots numbered. Evidence of clearly defined spots over a range of isoelectric points (PIs) and molecular weights suggests that the protein extraction and analysis methods used are sufficient for analysis of total proteins from sorghum seedlings, with minimal degradation.





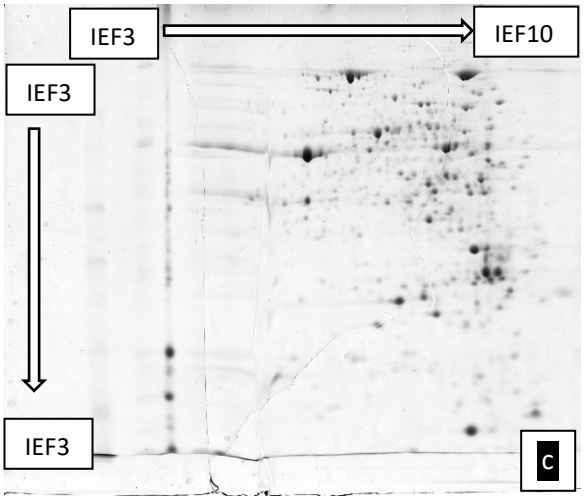


Figure 1*- Gel images of second dimension protein separation by SDS-PAGE. Proteins were separated in the first dimension on Immobiline DryStrip strips pH 3-10 (nonlinear) NL, then in the second dimension on 12.5% 1mm thick polyacrylamide gels and visualized using Coomassie Brilliant Blue G-250. Gels were scanned on ImageScanner (Amersham Biosciences) and analysed using Progenesis Samespot software 4.0 (Nonlinear Dynamics).* ***a.*** *Reference gel image following spot analysis. Numbered spots relate to the 27 differentially expressed proteins as identified using a selection criteria of FC ≤ 2, ANOVA P value ≤ 0.05* ***b.*** *Original gel scan for B35.* ***c.*** *Original gel scan for R16.*

Spot Number	Anova (p)	Fold change (FC)	Regulation in B35 compared to R16	Average Normalised Volumes	
				B35	R16
3728	7.38E-05	3.2	Down	1.32E+06	4.23E+06
3909	2.39E-04	4	Up	8.83E+06	2.23E+06
1414	0.002	2.5	Down	7.85E+05	1.95E+06
4954	0.003	2.6	Down	3.65E+06	9.37E+06
787	0.003	2.3	Down	5.57E+05	1.30E+06
3699	0.005	2.7	Down	3.62E+06	9.66E+06
1817	0.005	3.3	Up	1.55E+06	4.76E+05
1515	0.005	2.4	Up	1.65E+06	6.75E+05
4291	0.007	5.2	down	6.30E+05	3.26E+06
2380	0.009	2.3	down	4.06E+05	9.47E+05
2252	0.012	3.7	up	1.00E+07	2.74E+06
1759	0.013	2.2	up	8.74E+05	4.02E+05
860	0.016	2.3	up	8.93E+06	3.93E+06
3953	0.019	2.6	down	1.80E+06	4.63E+06
2113	0.024	3.1	down	4.94E+05	1.54E+06
3841	0.026	2.4	up	1.06E+06	4.43E+05
4870	0.03	3.3	up	3.02E+06	9.12E+05
1429	0.033	2.2	down	3.82E+06	8.26E+06
4980	0.035	3.8	up	1.14E+07	3.04E+06
1000	0.035	2.1	up	6.28E+06	2.95E+06
3568	0.035	2	up	2.13E+06	1.07E+06
2294	0.037	2.9	down	6.89E+04	1.98E+05
4067	0.039	2.1	down	1.06E+06	2.19E+06
2101	0.041	2.2	down	1.05E+06	2.34E+06
188	0.042	2.1	down	1.26E+06	2.61E+06
55	0.044	2.1	up	2.39E+05	1.15E+05

Table 3.1 – Average normalised spot volumes and fold changes for each differentially expressed protein spot identified. Criteria for differentially expressed proteins was a fold change $\geq$ 2, statistically significant to a level of 95% confidence (ANOVA P value $\leq$0.05).

3.2.2 Comparison of the full proteome in B35 (stay green) and R16 (non-stay green) 14 day old plants using 2-dimensional difference gel electrophoresis (DIGE).

Having confirmed the suitability of the extraction method and the quality of the proteins obtained, the protein samples were then separated using DIGE. In DIGE analysis, one replicate sample from each variety can be separated on the same gel in the presence of an internal standard (mixture of all replications). In this way, each gel exposes both genotypes to identical running conditions, and subsequent normalization to the internal standard can account for between gel variation, minimizing the risk of false positives (Jedmowski et al., 2014). Accuracy is therefore improved upon compared to conventional staining methods, achieving greater sensitivity to true variation in protein levels. The results are highly reproducible, and technical replication is not required (Ettan DIGE System, User Manual, GE Healthcare, https://www.mcgill.ca/cian/files/cian/ge_dige_manual.pdf).

Protein samples were labelled with fluorescent dye prior to separation, and gel scans were again analysed using Progenesis Samespot software 4.0 (Nonlinear Dynamics). Gels were aligned to the refence image, and spot volumes normalized to the internal standard on each gel. This technique successfully identified only 329 true protein spots in total, suggesting the sensitivity of the labelling reaction was compromised. None of these identified protein spots met the criteria of a 2-fold threshold change in relative signal intensity between the two lines. Instead, potentially differentially expressed proteins were identified by the criteria of a 1.5 or greater fold change in spot intensity, statistically significant to a level of 95% confidence ($ratio_{B35/R16}$ ANOVA $p<0.05$). Two candidate proteins satisfy this criterion, as highlighted in Table 3.2, neither of which appear to correlate with the differentially expressed proteins identified in the Coomassie Blue stained gels (see figure 1). Figure 2 and Table 3.2 list all proteins with a statistically significant variation in signal intensity at ANOVA $p\leq0.05$. There is a potential correlation between spot #790 (figure2) at 1.4 fold change (FC) (ANOVA P value = 0.038) and #2252 (figure 1) which might illustrate corroboration between both techniques and could identify an upregulated protein in B35 that requires further functional analysis.

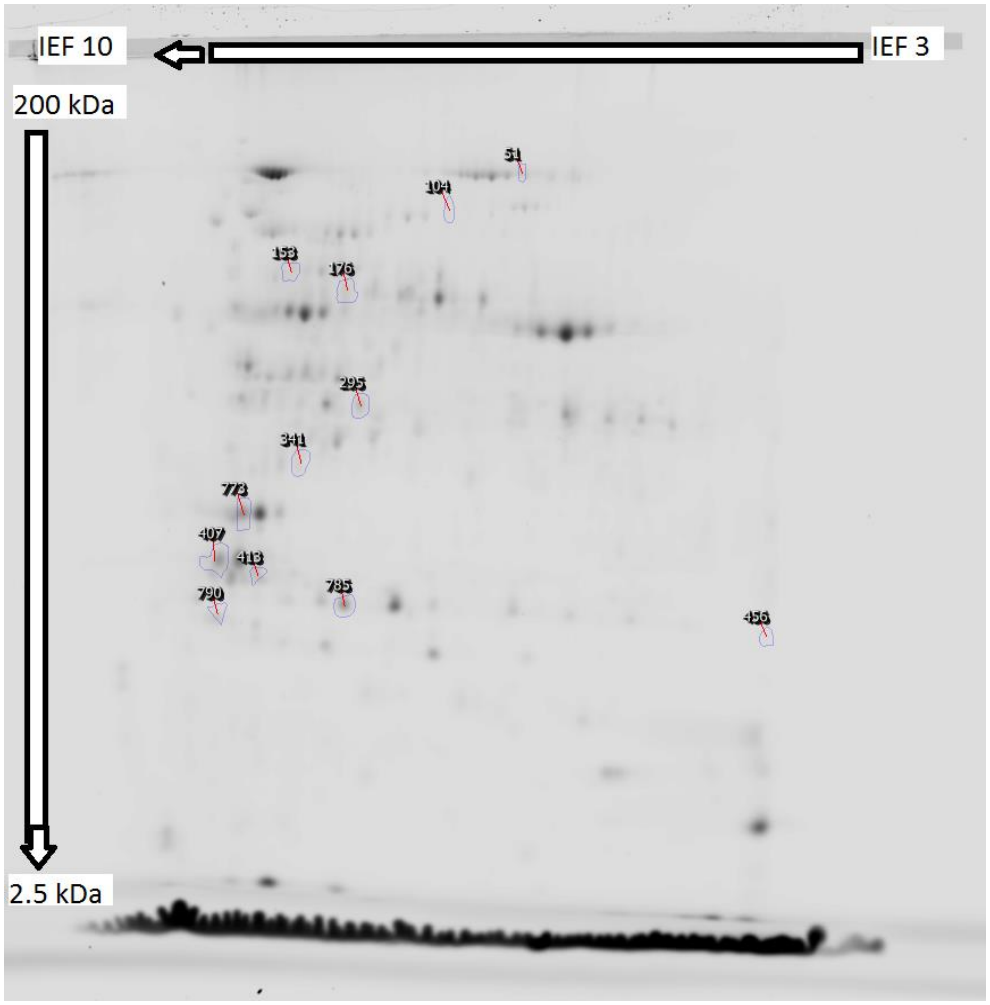


Figure 2 - Reference gel image of second dimension protein separation by SDS-PAGE. Proteins were fluorescently labelled then separated in the first dimension on IPG strips pH 3-10 NL, then in the second dimension on 12.5% 1mm thick polyacrylamide gel. Gels were scanned on (Ettan DIGE Imager (GE Healthcare, Amersham Biosciences) and analysed using Progenesis Samespot software 4.0 (Nonlinear Dynamics). Numbered spots relate to the differentially expressed proteins as identified using a selection criteria of ANOVA P value ≤ 0.05.

Spot Number	Anova (p)	Fold change (FC)	Regulation in B35 compared to R16	Average Normalised Volumes	
				B35	R16
341	0.004	1.2	Up	0.925	0.79
295	0.013	1.2	Up	0.871	0.698
104	0.016	1.3	Up	1.048	0.804
153	0.018	1.3	down	0.783	1.024
407	0.023	1.2	Up	1.014	0.83
773	0.031	1.2	down	0.866	1.073
785	0.033	1.1	Up	0.942	0.826
790	0.038	1.4	Up	1.282	0.888
413	0.04	1.7	Up	1.196	0.724
456	0.041	1.9	Up	1.115	0.575
176	0.042	1.3	down	0.931	1.214
51	0.042	1.3	Up	0.863	0.689

Table 3.2 – Average normalised spot volumes and fold changes for each differentially expressed protein spot identified. Criteria for differentially expressed proteins was a fold change ≥ 1.1, statistically significant to a level of 95% confidence (ANOVA P value ≤0.05).

3.3 Discussion

The proteomic comparison in this study has found evidence of protein level differences between the B35 stay green and R16 senescent sorghum lines when grown under normal conditions. The previous transcriptomic comparison also found significant differences between transcript levels of several genes in the two lines under normal conditions (Johnson et al., 2015b). By combining these two studies, correlation between protein level and transcript level variation could be identified, and candidate proteins investigated for their roles in stay green and drought tolerance mechanisms.

3.3.1 Conclusions from the 2-D GE analysis visualised using Coomassie Blue stain, comparing the proteomes of B35 and R16

Of the 27 proteins found to be significantly differential expressed between the two lines, 12 proteins were more highly expressed in B35 compared to R16. In the paper by Johnson et al., 2015b, expression levels of 1038 genes were higher in the B35 tolerant line compared to the senescent line, including a *DREB1A* (*dehydration-responsive element-*

binding 1A) transcription factor and a *SbSDIR1* (*salt and drought-induced RING finger 1*) E3 ubiquitin ligase. Identification of the proteins of interest in this proteomic study will reveal the degree of correlation between these two omics studies, revealing differences between the two lines at both the protein and transcript level. It could also indicate the level at which genes and processes contributing to the stay green phenotype may be regulated, i.e. either pre- or post-transcription.

Fifteen proteins were found to be lower in B35 compared to R16. Previous proteomic studies found proteins involved in damage and repair to be expressed to lower levels in the tolerant crop varieties than the more sensitive lines, indicating a greater efficiency of stress protective mechanisms and minimization of subsequent damage. This is evident in the proteomic comparison between two Egyptian sorghum lines where there was significant upregulation of an aspartate protease in the sensitive line, suggesting increased degradation activity, possibly as a manifestation of damage due to oxidative stress (Jedmowski et al., 2014). A study comparing the response of two maize cultivars to mild water deficit reported lower levels of ribosomal proteins in the drought tolerant cultivar compared to the sensitive line, suggesting potential differences in drought-induced photosynthesis regulation (Benesova et al., 2012). It would be interesting to discover whether the proteins found at lower levels in B35 also have similar functions to those proteins found at lower levels in other tolerant cultivars, or if different proteins and processes are downregulated in this particular sorghum stay green line compared to R16. Isolation and functional characterization of the target proteins in this experiment is required to fully explore the significance of the proteomic differences between B35 and R16.

3.3.2 Conclusions for DIGE comparison of B35 and R16 proteome.

A total of 329 proteins were detected using DIGE analysis, and only 2 proteins showed a significant difference between B35 and R16 at FC $\geq$ 1.5 (ANOVA P value = 0.05), both of which were upregulated in the stay green line compared to the senescent line. The previous Coomassie Blue stained 2D gels (described above in section 3.1.1) were used to separate 250µg of protein per sample, five times as much protein that was loaded onto the DIGE gels (at 50µg of protein per sample). CyDye fluorescent dyes are known to be significantly more sensitive than Coomassie Blue dye, and have been shown to detect approximately four times as many spots from the same initial protein load (Tonge et al., 2001). Considering 919 protein spots were detected on the Coomassie Blue stained 2D gels

following separation of 5 times as much protein, we would expect a similar number of spots to be detected in the DIGE analysis. The weakness of the fluorescence signal and the low spot number detection, suggests that the CyDye is inadequately labelling the proteins. Consequently, it is unlikely that this DIGE analysis properly represents the differential expression of proteins between the stay green and senescent line, and the method should be improved prior to protein identification by MALDI-TOF-MS. One possible explanation is that the protein concentration in the lysate for the labelling reaction may have been measured incorrectly. 2D Quant Kit (GE Healthcare) was used to quantify the proteins in each sample and is a method compatible with thiourea containing buffers (see section 2.4.1), but duplicates of the assay were not conducted due to limited sample volume. By confirming the protein concentration by duplication prior to the labelling reaction, a sufficient ratio of protein:dye can be ensured. Another option is to add more dye to ensure sufficient labelling despite any competition from contaminants that may still be present (Ettan DIGE System User Manual GE Healthcare https://www.mcgill.ca/cian/files/cian/ge_dige_manual.pdf). Alternatively, technical replicates of the Coomassie Blue stained 2-DE could be conducted, and these could be used instead to identify differentially expressed proteins by MALDI-TOF-MS.

3.4 - Summary

In conclusion, progress has been made towards conducting a full proteome comparison between the stay green sorghum line B35 and the senescent line R16. This is an essential study to complement a previous transcriptomic comparison between the same lines, in order to observe changes at both the protein and transcript level. The suitability of the protein extraction method has been confirmed by a pilot study using 2-D GE with Coomassie Blue staining which picked up proteins in the stay green line which were higher and lower compared to the senescent line. 2-D DIGE was subsequently used to confirm differential protein expression. Weak signal intensity of the protein spots in the DIGE analysis suggests an inadequate level of labelling that was insufficient for identifying differentially expressed proteins using the required criteria. Modifications to the labelling method, or additional technical replicates of the Coomassie Blue stained gels are required before differentially expressed proteins can be confirmed reliably, prior to identification by MALDI-TOF-MS. Genes and proteins expressed at different levels in the stay green line compared to the non-stay green line can later be investigated for their potential function in stay green mechanisms.

The next step in analysing the differences between B35 and R16 at the protein expression level would be to expose these plants to drought-stress conditions and then extract the proteins for DIGE analysis. In this way, differences in response to water-limiting conditions at the protein level can also be analysed in addition to differences under normal conditions, giving further insight into the potential biological processes involved in the stay green phenotype of B35. Changing the intensity of the drought treatment used may reveal more about response strategies in the two lines. For example, the thesis by Stephanie Johnson (Johnson, 2015a) described the sorghum transcriptome response following osmotic stress imposed by water withdrawal, and compared this to the response following (polyethylene glycol) PEG-induced osmotic stress as described in the paper Dugas et al., 2011. It was found that genes associated with 'response to reactive oxygen species' were enriched following PEG-treatment, whereas treatment by water-withdrawal indicated an enrichment of genes associated with wax biosynthesis. It was suggested that treatment by PEG could be considered a more immediate and severe osmotic-stress treatment compared to gradual water withdrawal, explaining the differences in response observed here (Johnson, 2015a). Stress-induced changes in the transcript abundance of seven aquaporin genes in grapevine have also been found to differ significantly depending on the severity of the stress imposed, where all 7 genes decreased in expression within the leaf following moderate stress but were all upregulated following severe stress (Galmés et al., 2007). It would be interesting to explore the differences in response to moderate and extreme drought conditions between B35 and R16. Additionally, there is substantial evidence for the differences between initial and later stage drought responses, so analysis of the sorghum plants at multiple time-points following drought treatment could reveal temporal variation in transcriptome or proteome response between stay green and senescent lines (Gong et al., 2015). For example, the initial drought response phase in Arabidopsis 30 minutes post-treatment is characterized by a unique transcriptome profile, which differs from that of the acclimation phase several hours later (Gong et al., 2015; Kilian et al., 2007). Analysing the proteome following a period of recovery could also reveal differences between the mechanisms and processes involved in restoration of normal growth and development after stress, as analysed at the transcriptome level in sorghum in a paper by Jedmowski et al., in 2014.

As previously mentioned (see section 1.2.2), post-translational modifications (PTM) have significant influence over protein activity, binding, transportation, signalling and localisation (van Wijk 2001; Gong et al., 2015). Phosphorylation is an important PTM, and

analysis of the differences in drought-responsive phosphoproteins between stay green and senescent sorghum lines could provide further information on the behavioural and functional differences of proteins in addition to expression level variation. For example, a paper by Zhang et al., in 2014 found differences in the phosphorylation level of high mobility proteins (HMG) between drought tolerant and sensitive wheat lines, and these proteins are associated with transcriptional regulation via chromatin modification (Zhang et al., 2014; Wang et al., 2016). Phosphoproteomics studies in maize and wheat have found an increase in phosphorylation of E3 ubiquitin ligases as a response to drought stress conditions (Hu et al., 2015; Wang et al., 2016; Zhang et al., 2014) which corroborates evidence of upregulation of the E3 ubiquitin ligase *SbSDIR1* in B35 sorghum compared to R16 (Johnson et al., 2015b) (see chapter 4).

Chapter 4

Analysis of transgenic wheat lines overexpressing *SbSDIR1*

4.1 Introduction

Differentially expressed genes in drought tolerant crop lines compared to drought sensitive lines could potentially be involved in the stress tolerance mechanisms, and could reveal processes and pathways that underlie those phenotypes. Whilst some of these genes could have direct roles in protection from cellular damage and maintenance of osmotic homeostasis, others might be involved in signalling and regulatory pathways (Zang and Komatsu, 2007). For example, genes involved in post-translational modifications such as phosphorylation and ubiquitination are major players in signalling responses to drought (Gong et al., 2015; Lyzenga and Stone, 2012; Wang et al., 2016). Sorghum microarray data revealed the E3 ubiquitin ligase gene *SbSDIR1* (salt and drought dependent 1) to be upregulated in the stay green variety B35 compared to the senescent R16 variety under normal unstressed conditions (Johnson et al., 2015b). E3 ubiquitin ligases are integral to ubiquitin-dependent protein degradation, a process which influences a wide variety of plant growth and developmental processes such as senescence, hormone regulation, photo-morphogenesis and pathogen response (Devoto et al., 2003; Gao et al., 2011; Xie et al., 2002). Considering the significant upregulation of this gene in B35 compared to R16 (Johnson, 2015a; Johnson et al., 2015b), and its known role in drought stress tolerance as implicated by several previous studies (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007), the function of *SbSDIR1* in relation to drought tolerance and the stay green phenotype is investigated in greater detail in this chapter.

The ubiquitin-proteasome system (UPS) is the principle mechanism for protein turnover, and principally involves three consecutively acting enzymes for ubiquitin attachment, and the 26S proteasome for proteolysis (Xia et al. 2012). The universally expressed ubiquitin protein is attached to target proteins as part of a polyubiquitin chain or as a single or multiple mono-ubiquitination. The topology of the chain or singular attachment determines the fate of the target protein (Chen et al., 2009; Cheng et al. 2012; Lyzenga and Stone 2011. It could be labelled for destruction by the 26S proteasome, or involved non-proteolytic functions such as gene silencing via chromatin modification, DNA repair or membrane

trafficking (Chen et al., 2009; Cheng et al. 2012; Lyzenga and Stone 2011). The coupling of ubiquitin involves a conjugation enzyme cascade starting with E1 catalysing the ATP-dependent ubiquitin activating step, followed by E2 (ubiquitin-conjugating enzyme) and E3 (ubiquitin-ligase) (Cheng et al., 2012; Dametto et al., 2015). Specificity to a target protein is regulated at the substrate recruitment stage by E3 ligases in conjunction with accessory proteins such as F proteins (Dametto et al., 2015). The three major types of E3 ubiquitin ligases are 'Homology to E6-AP *C*-Terminus' (HECT), 'Really Interesting New Gene' (RING) and U-box type, which differ in their mechanisms of ubiquitin transfer (Dametto et al., 2015; Lyzenga and Stone 2011). RING- type E3 ligases are of particular interest for this chapter, and are defined by a motif consisting of an octet of conserved zinc-binding cysteine and histidine residues in a 'cross-brace' system, as described in Freemont et al., 1993 (Dametto et al., 2015; Lyzenga and Stone 2011; Xia et al., 2012).

Substrates that are targeted for degradation via the UPS include transcription factors, hormone receptors, hormone biosynthesis enzymes and effector proteins involved in stress tolerance, as well as damaged or misfolded proteins (Cheng et al., 2012; Lyzenga and Stone 2011). An increasing number of studies suggest the UPS system plays an important role in abiotic stress responses. Early observations found that expression of polyubiquitin genes in maize and tobacco was stress regulated (Christensen et al., 1992; Genschik et al., 1992). Arabidopsis proteomic studies have used immunoaffinity chromatography to specifically isolate and analyse Ub-related proteins, and have the found majority of these proteins to be associated with abiotic-stress response (Manzano et al., 2008; Igawa et al., 2009,). More recent studies focus on stress-regulated E3 ubiquitin ligases, and their potential roles in tolerance mechanisms.

E3 ubiquitin ligases are the most abundant of the three protein ubiquitination enzymes within eukaryotes, and are highly diverse. This diversity enables specific interaction with a wide range of substrates (Cheng et al., 2012; Xia et al., 2012). An increasing number of studies suggest a role for E3 ubiquitin ligases in ABA-mediated stress responses in several model plants. In Arabidopsis, overexpression of the RING-type E3s *AIRP1, XERICO* and *RHA2b* all enhance drought tolerance and reduce transpiration water loss via ABA-mediated stomatal closure (Ko et al., 2006; Li et al., 2011; Ryu et al., 2010). Overexpression of the rice genes *CTR1* and *BIRF1*, the soybean gene *DSR7*, and the pepper genes *DTR1*, *AIP1* and *RING1* have all been shown to increase tolerance to dehydration stress and

enhance ABA-dependent stress response (Joo et al., 2016; Lim et al., 2014; Li et al., 2016a; Lim et al., 2015; Liu et al., 2008; Park et al., 2016). Functional analysis of the pepper gene *Rma1H1* in the paper by Lee et al., in 2009 suggested that this stress-induced E3 may regulate the ubiquitin-dependent degradation of the aquaporin PIP2;1. Reduction in aquaporins may help the plant to maintain tugor pressure by minimizing water transport across membranes via the symplastic pathway (Alexandersson et al., 2005).

There is also evidence of RING-type E3 ubiquitin ligases playing *negative* roles in drought response. *DIS1* overexpression lines in rice displayed lower survival rates under water limiting conditions (Ning et al., 2011), and therefore appear to promote drought stress sensitivity. Ning et al., 2011 speculate that *DIS1* may interfere with drought tolerance mechanisms by disrupting H_2O_2-dependent stomatal control, via suppression of the gene *OsMT-14b* which has a potential role in ROS elimination, and concurrent suppression of the RNA-binding protein *OsGRP2A* (Kim et al., 2008; Ning et al., 2011; Yang et al., 2009). A drought sensitive phenotype was also observed in hot pepper lines overexpressing *AIR1*, accompanied by differential expression of stress genes and impaired stomatal closure mechanisms (Park et al., 2015).

Transcriptomic comparison between the B35 stay green sorghum and the R16 senescent line revealed a higher level of expression of the E3 ubiquitin ligase *SbSDIR1* (salt- and drought-induced RING finger1) under normal conditions in both 14 and 50 days old plants (Johnson et al., 2015b). Homologs of this gene have previously been studied in several other species following initial identification in Arabidopsis as a stress responsive RING finger protein, specifically associated with salt and drought stress response (Zhang et al., 2007). Analysis of the amino acid sequence of the sorghum *SbSDIR1* gene confirmed the presence of putative N-terminal transmembrane domains and a C-terminal C3H2C3 RING domain, features also identified in Arabidopsis, grapevine, tobacco, rice and maize homologs (Johnson, 2015a; Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). This distinctive RING motif has been shown to be required for E3 ubiquitin ligase activity in both Arabidopsis and rice (Gao et al., 2011; Zhang et al., 2007). There appears to be strong conservation of DNA and amino acid sequence across several different dicot and monocot species, and the putative ubiquitin ligase function may also be conserved in the sorghum gene although further functional analysis is required (Johnson, 2015a). *SDIR1* and the homologs mentioned above all appear to be induced by

drought, but the sorghum and grapevine genes are additionally induced in response to salt, ABA and heat, and the rice gene is suppressed by cold treatment ((Johnson, 2015a; Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). Spatial expression analysis of the maize homolog indicated highest expression in the aerial tissues, in agreement with that observed in Arabidopsis, but in rice the predominant expression appears to be in the roots (Gao et al., 2011; Xia et al., 2012; Zhang et al., 2007). It has been hypothesised that differential expression patterns may be a result of a slight divergence in regulation and function of *SDIR1* in a monocot compared to dicot species (Xia et al., 2012).

Overexpression of *SDIR1* and its homologs has enhanced drought tolerance across several species including Arabidopsis, maize, tobacco, rice and grapevine (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). It has frequently been hypothesised that the altered transpiration rates within these transgenic lines explains the improved tolerance, supported by measurements of reduced rate of water loss and reduced stomatal aperture under normal and drought-stressed conditions (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). *SDIR1* could therefore be involved in ABA-controlled stomatal closure, consistent with observations of other ABA-related phenotypes in overexpression lines such as ABA-hypersensitivity at germination and post-germinative stages, confirmed by observations of expanded and greener cotyledons and inhibited root growth in comparison to control lines (Zhang et al., 2007; Zhang et al., 2015). It has previously been suggested that the sorghum *SbSDIR1* gene could function in a similar way, and overexpression of this gene in Arabidopsis lines also resulted in reduced rates of water loss from excised leaves, and reduced stomatal aperture compared to wild type lines (Johnson, 2015a). Considering stay green sorghum varieties are known to have reduced rates of transpiration, and that B35 stay green has higher levels of expression of *SbSDIR1* (along with *SDD1*, *GTL1* and *SLAC1* which are also involved in stomatal regulation) compared to R16 senescent line (Johnson, 2015a), it is possible that *SbSDIR1* may be contributing to the stay green phenotype through ABA-mediated stomatal control thus improving water conservation (Berger and Altmann 2000; Borrell et al., 2014; Geiger et al., 2009; Johnson, 2015a; Vadez et al., 2011; Yoo et al., 2010).

Understanding of the mechanism by which *SDIR1* contributes to drought tolerance and the stay green phenotype is still limited. Sequencing the promoter region of the sorghum

gene has revealed recognition sites for MYB, MYC, AREB and DRE elements, indicating potential for regulation by several different transcription factors (Johnson, 2015a). Consistent evidence using transgenic technology has suggested that *SDIR1* is a positive regulator of drought tolerance. Considering the various ABA-associated phenotypes observed in overexpression lines, it is likely that it acts partly through positive regulation of ABA signalling pathways, potentially targeting negative regulators for proteasomal degradation, or promoting mono-ubiquitination and stabilization of ABA signalling components (Zhang et al., 2007; Zhang et al, 2015). In addition to changes in stomatal regulation, expression of Delta 1-pyrroline-5-carboxylate 1 (*P5CS1*) has also been found to increase following overexpression of the Arabidopsis and grapevine homologs, and higher proline levels have been observed in maize overexpression lines, potentially suggesting a role in osmoregulation at the cellular level (Tak and Mahtre 2013; Zhang et al., 2015). *SDIR1* has been shown to function upstream of bZIP transcription factors ABF3 and ABF4, and to interact with SDIR1-INTEREACTING PROTEIN 1 (SDIRIP1) which in turn acts upstream of the bZIP ABI5 (Zhang et al., 2015). A further 38 other potential interacting partners were identified in the paper by Zhang et al., in 2015, which could represent other pathways of response that require further investigation. Considering *SDIR1* expression is also induced by heat stress in grapevine and sorghum, and overexpression of its homolog in maize enhances expression of antioxidant enzymes, it is also possible that *SDIR1* contributes to heat stress response pathways (Johnson, 2015a; Tak and Mahtre 2013; Liu et al., 2013).

To investigate the function of *SbSDIR1* within sorghum it would be best to overexpress the gene within sorghum itself rather than a different plant species. However previous attempts at stable sorghum transformation have been unsuccessful (Johnson, 2015a). This chapter instead analyses wheat lines transformed with an *SbSDIR1* overexpression construct, produced by Stephanie Johnson in Durham University and Dr Emma Wallington at NIAB (Huntington Road, Cambridge, CB3 0LE). The sorghum gene was expressed using a rice actin promoter (see construct diagram Appendix B). By overexpressing this monocot gene in another monocot system such as wheat, the observed function and phenotype is likely to be more representative of its true function within sorghum, rather than overexpressing it in a dicot system such as Arabidopsis, which has been done previously (Johnson, 2015a).

Wheat production represents 25.6% of total cereal crop production worldwide (based on figures for 2013), the leading producers being China, India, USA and Russia (Daryanto et al., 2016). Several wheat cultivars display the stay green characteristic of delayed senescence and extended photosynthetic capacity during grain filling (Chen et al., 2010). In this crop, stay green phenotypes are studied more extensively in the context of the trade-off between grain yield and quality, but stay green characteristics have also been linked to enhanced drought tolerance in the field (Tian et al., 2013; Checovich et al., 2016). The stay green cultivars CN12 and CN18 were found to have reduced H_2O_2 in flag leaves at the grain filling stage and larger grains under normal growth conditions (Chen et al., 2010). The CN17 stay green variety has been shown to be associated with prolonged chloroplast ultrastructure regeneration, possibly related to enhanced antioxidant capacity and high unsaturated fatty acid content (Luo et al., 2013). This chapter investigates whether overexpression of *SbSDIR1* in Fielder wheat (*Triticum aestivum*) can reproduce stay green characteristics within the transgenic lines.

Summary

This chapter focusses on the functional characterisation of sorghum *SbSDIR1* in relation to the stay green trait and drought stress response. This gene has been shown to be upregulated in stay green B35 lines compared to non-stay green R16 lines in sorghum when grown under normal conditions (Johnson et al., 2015b), and has been implicated in drought stress response strategies in several other papers (Gao et al., 2011; Xia et al., 2012; Zhang et al., 2007; Zhang et al., 2008; Zhang et al., 2015). Previous student Dr Stephanie Johnson produced an *SbSDIR1* overexpression construct, and fielder wheat plants have been transformed to produce transgenic lines at NIAB. The general aim for this experiment was to test these wheat lines for acquisition of stay-green and drought tolerance phenotypes.

The specific aims of this chapter are the following;

- To determine the *SbSDIR1* expression levels of the transgenic lines and identify a subset of lines that have higher transcript levels than the untransformed control line, over a range of different magnitudes.

- To measure any differences in the rates of transpirational water loss between these selected lines and the non-transformed control lines, which could potentially indicate change in stomatal conductivity.
- To examine the rate of senescence progression under normal conditions and osmotic stress between wheat lines.
- To investigate differences in the expression levels of ABA-regulated genes between the wheat lines, after treatment with exogenous ABA.

4.2 Results

4.2.1 Analysis of SbSDIR1 expression levels in wheat overexpression lines

The role of *SbSDIR1* in conferring stay green characteristics, and the extent to which its role and response pathway is conserved in other plant systems, was investigated by overexpressing the gene in Fielder wheat (*Triticum aestivum L*). T0 lines were assessed for qPCR copy number of the construct based on the selectable marker nptII (neomycin phosphotransferase II) by Dr Emma Wallington (see Appendix B). From the 36 transformed T0 lines, 14 lines with the lowest (but still multiple) qPCR copy number of the construct were selected for further analysis (see Appendix B for *SbSDIR1* copy number in all transformed lines). Along with the non-transformed control lines 81con1 and 81con2, T1 plants of the selected 14 lines were grown for 12 days under normal growth conditions (see section 2.1.5). RNA was extracted and *SbSDIR1* expression levels were analysed using real-time qPCR (see figure 3). Four transgenic lines with detectable *SbSDIR1* transcript levels across a range of different magnitudes were chosen for further study. These lines were 81.20 (the highest expresser), 81.5 (a low-level expresser), and 81.21 and 81.22 (intermediate level expressers).

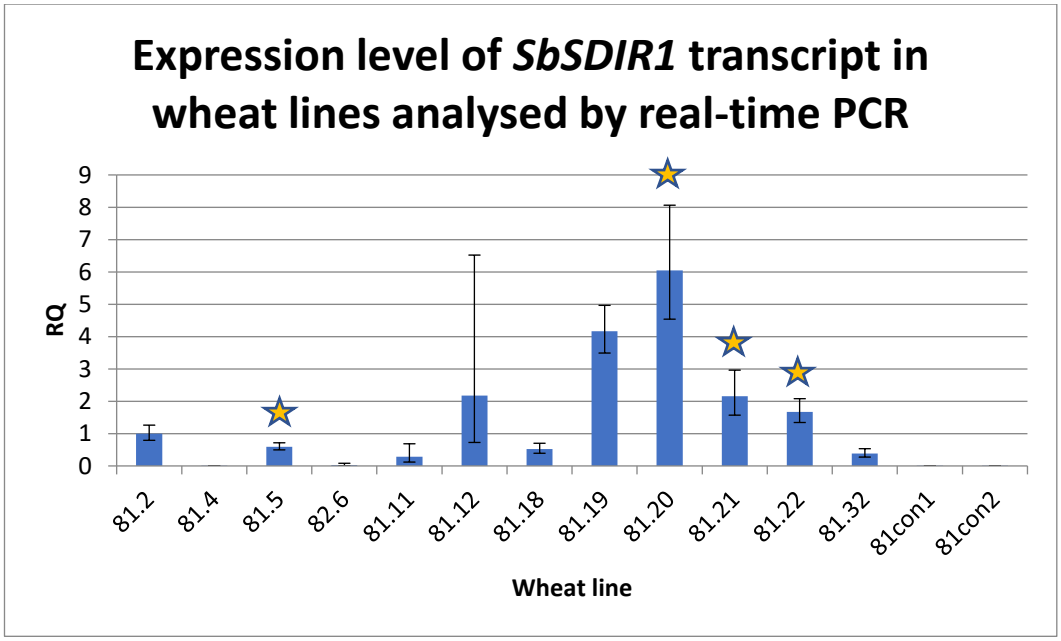


Figure 3 – Analysis of SbSDIDR1 expression levels in wheat transgenic lines overexpressing SbSDIR1 compared to the untransformed control lines 81con1A and 81con2A. Plants were grown for 12 days before RNA extraction. Transgenic lines chosen for further analysis are indicated by a star symbol. Error bars represent the RQ min/max range, based on a 95% confidence interval (student's t test).

4.2.2 Morphological and developmental phenotype

Wheat transgenic lines 81.5, 81.2, 81.21 and 81.22 where grown for 6 weeks alongside an untransformed control line 81con1A under normal conditions, as outlined in section 2.1.4. Six individual plants were grown per line, and the experiment was repeated twice to give two biological replicates. No obvious differences were observed between the transgenic lines and the control at the germination stage, and 6 week old plants appeared similar in height and leaf number (data not shown). At 25 DAS (days after sown), the percentage of plants with a primary tiller was recorded for each line (see figure 4). For this study, a primary tiller is defined as a first tiller growth (Till1) from the main stem (Till0). The control line showed no evidence of primary tiller development in any of the plants grown in either experiment. However, in the first experiment transgenic lines showed evidence of primary tiller emergence in 83.3%, 83.3%, 100% and 66.7% of plants for lines 81.5, 81.20, 81.21 and 81.22 respectively (see figure 4). In the second experiment only 16.7% of plants in transgenic lines 81.5 and 81.20 had developed a primary tiller, and none from the line

81.22. However, plants from the transgenic line 81.21 still showed primary tiller development in the majority of individuals (66.7%), having shown 100% tiller development in the first experiment. Figure 5 shows photographic evidence of the tillering phenotype of one wheat plant from the control line (2a) and one example of a tillered plant from the wheat transgenic line 81.21 (2b).

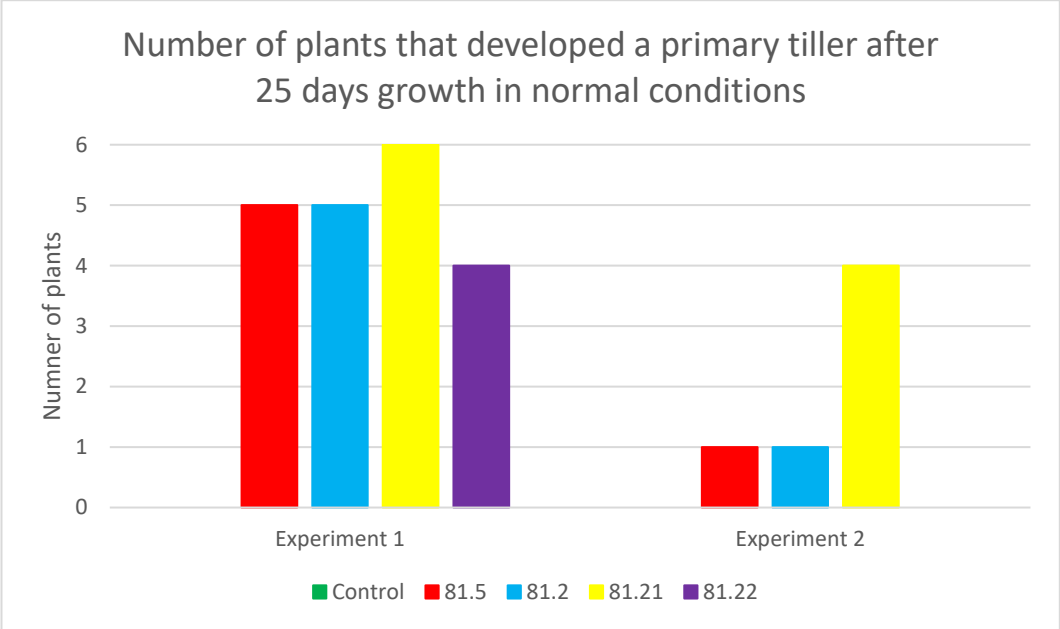


Figure 4 – Bar chart showing the number of plants showing emergence of primary a tiller at the early growth stage of 25 days old, in the untransformed control line compared to the transformed lines. Wheat plants were at the 4th leaf stage and grown under normal conditions (see section 2.1.4) and data was taken from two biological replicates of 6 individual plants.

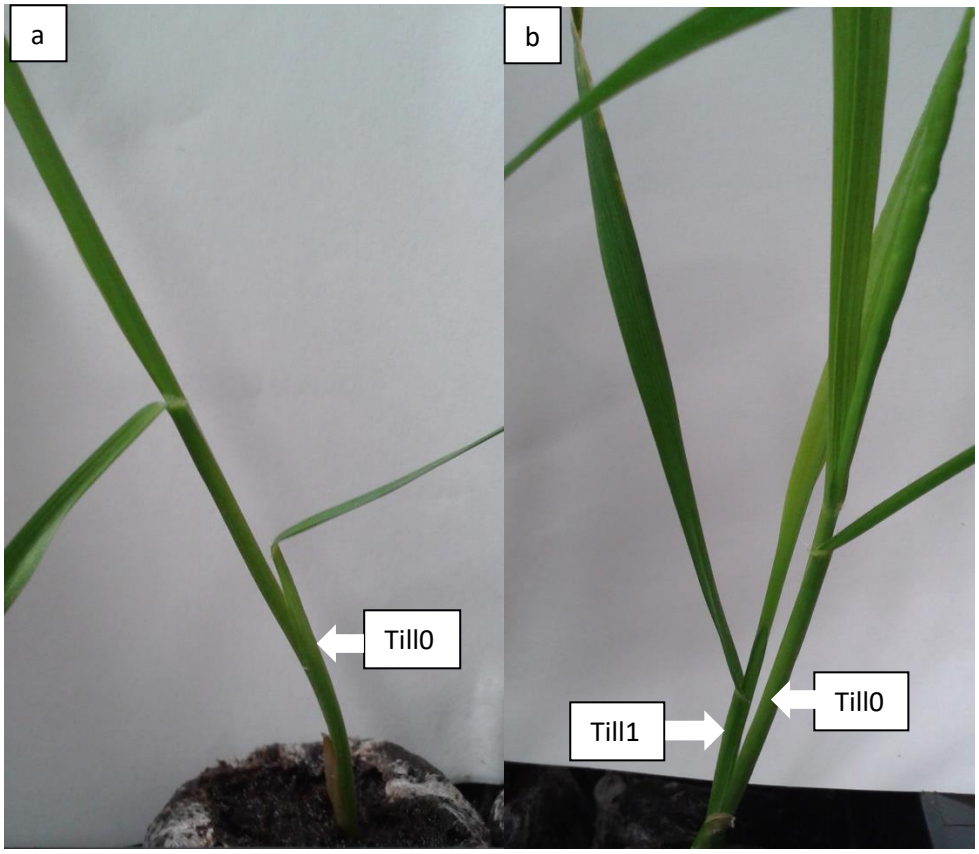


Figure 5 – Photographs showing the tillering phenotype in an individual plant from the transformed wheat line 81.21 (a) compared to the untransformed control line (b) after 25 days growth under normal conditions (see section 2.1.4). This transformed line showed the highest occurrence of a second tiller at this growth stage compared to the other transformed lines (see figure 4) whereas the control line still displayed only one tiller at this growth stage in 100% of the plants. Main stem is labelled Till0 and the first primary tiller is labelled Till1.

4.2.3 Excised leaf water loss assay

Stay green sorghum lines have been reported to have improved transpiration efficiency compared to senescent lines when grown in both well-watered and water-limiting conditions (Borrell et al., 2014a; Vadez et al., 2011). This characteristic potentially contributes to stay green drought tolerance by improving water conservation and maintaining supply during grain filling, even under drought conditions. Differences in transpiration can be partly attributed to differences in ABA-mediated stomatal control (Leung and Giraudat, 1998; Zhang et al., 2007). Sorghum *SbSDIR1* has previously been

suggested to be involved in stomatal regulation, particularly considering the differences in stomatal aperture reported in Arabidopsis *SbSDIR1* overexpression lines (Johnson, 2015a). *SbSDIR1* has previously been overexpressed in Arabidopsis, producing transgenic lines with decreased stomatal conductivity under well-watered conditions, and reduced rate of water loss from excised leaves, in comparison to control lines (Johnson, 2015a). To investigate whether overexpression of *SbSDIR1* in wheat lines can produce stay green characteristics of improved transpiration efficiency, the loss of water from excised leaves of plants grown under normal conditions was measured over the course of 5 hours. Leaves of the same developmental stage were cut from 18 day old plants and placed abaxial side up. Leaf weight was measured at regular intervals over 5 hours, and percentage weight loss was recorded (see figure 6).

This experiment was conducted on 6 individual plants per line, and the experiment was repeated 3 times to give 3 biological replicates. In all experiment lines 81.5 (the lowest transgenic expresser of *SbSDIR1*) and 81.20 (the highest transgenic expresser of *SbSDIR1*) showed relatively faster rates of water loss and greater percentage relative weight loss after 5 hours compared to the control line. Figure 6 displays the average leaf weight loss over 5hours from all 3 experiments. At 300 minutes there was no significant difference between the mean leaf weight loss of any of the genotypes ($F=0.908$, $P=0.495$, 4 df). At the final time point of 300 minutes the control line, 81.5 and 81.20 showed 69%, 60% and 55% leaf weight respectively. While not statistically significant, these results suggest that transgenic lines 81.5 and 81.20 are less able to retain water, with line 81.20 showing the fastest rates of water loss compared to the control line. Line 81.22 appeared to have a similar capacity for water retention as the control line reaching 68% leaf weight at 300 minutes compared to the control at 69%. Interestingly, line 81.21 shows a non-statistically significant reduced rate of water loss compared to the control at 73% leaf weight at 300 minutes.

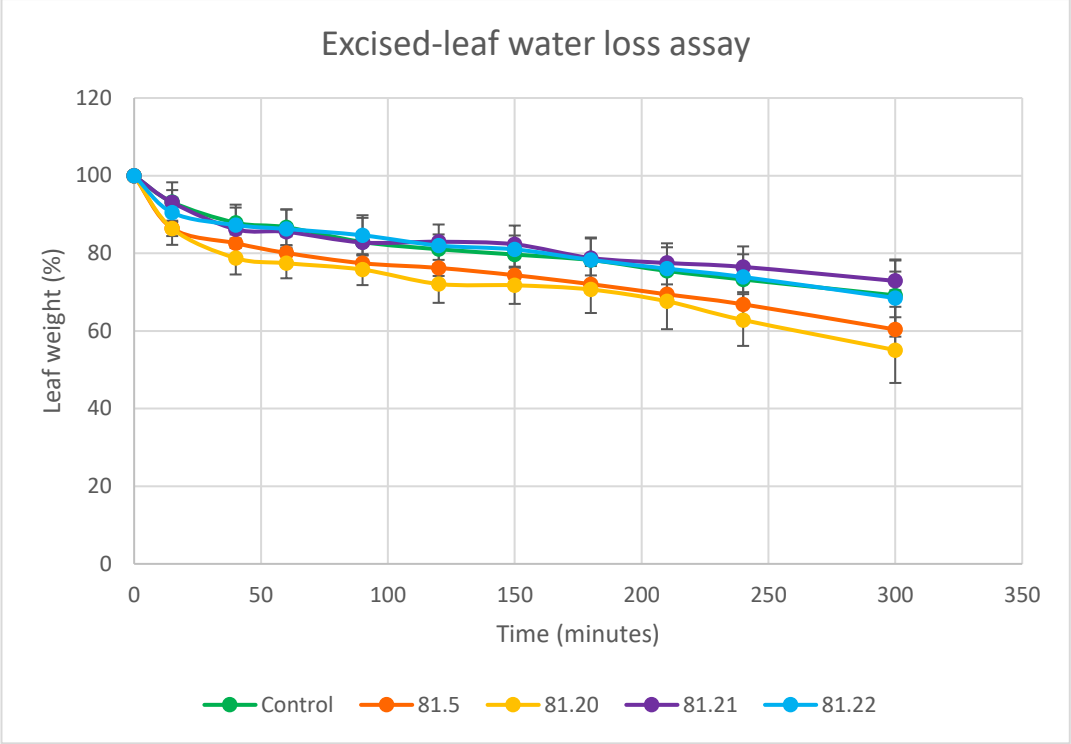


Figure 6 – Excised-leaf water loss assay on wheat lines overexpressing SbSDIR1. Leaf 4 from 18 day old plants (grown in normal conditions) were cut, placed abaxial side up at room temperature, and weight was measured at regular intervals over a 5 hour period and percentage weight loss compared to the original weight at 0 minutes calculated. Six individual plants per line were used in each biological replication of the experiment, and 3 experiments were conducted. The graph plots an average of all 3 biological replicates.

4.2.4 ABA-induced gene expression

Expression of *SbSDIR1* has been found to be induced in response to ABA treatment in sorghum (Johnson, 2015a), and overexpression of *OsSDIR1* in rice has been found to confer ABA hypersensitivity at germination and in later vegetative growth stages (Zhang et al., 2007). Several other ABA-associated phenotypes have been observed in *SDIR1* overexpression lines including stunted early growth, salt sensitivity at germination, and changes in stomatal conductance (Gao et al., 2011; Zhang et al., 2007). To assess the wheat overexpression lines for changes in ABA-sensitivity, and investigate the potential role of *SbSDIR1* in regulating ABA-related gene expression, the transcript abundance of known wheat ABA-induced genes were analysed following ABA treatment.

The genes *TaGBF1* and *TaNAC29* were selected for this experiment. The bZIP transcription factor *TaGBF1* is known to be a blue-light responsive component and is involved in abiotic stress responses, particularly salt stress (Sun et al., 2015). It has been shown to be induced in wheat by ABA exposure and overexpression lines have displayed ABA hypersensitivity phenotypes (Sun et al., 2015). The wheat NAC transcription factor *TaNAC29* is induced by ABA treatment, in addition to exposure to salt and drought stress (Xu et al., 2015). NAC transcription factors have previously been found to play essential roles in leaf senescence in connection with stay green phenotypes (Guo and Gan, 2006).

Transgenic lines 81.5, 81.20, 81.21 and 81.22 were grown for 25 days alongside the control line. Leaves of the same developmental stage were excised and cut into segments, then floated adaxial side up in solutions of 100μM ABA, with ethanol controls for comparison. These explants were incubated at 20°C under a 16 hour photoperiod, and samples for RNA extraction were taken at 12 hours and 24 hours following ABA/ethanol control treatment. Transcript abundance of *TaGBF1* and *TaNAC29* was analysed using real-time PCR.

Across all wheat lines the expression of *TaNAC29* was increased to its peak value following 12 hours of ABA treatment compared to the control line in the ethanol treatment, corroborating earlier evidence that this gene is ABA-induced (figure 7(a)) (Xu et al., 2015). Following 12 hours of ABA treatment, the trend in expression level suggests that the transgenic lines have similar transcript abundance of *TaNAC29* compared to the control line, with no statistically significant differences (figure 7(c)). After 24 hours of ABA treatment, the expression level of *TaNAC9* remains upregulated in all ABA treated lines compared to the control treated untransformed line (figure 8 (a)). Although not statistically significant, line 81.21 shows higher expression levels than the ABA treated untransformed line suggesting an increased duration of ABA-induced expression in this line (figure 8 (c)). In the ethanol control treated group, the line 81.21 shows a statistically significant upregulation of *TaNAC29* compared to the untransformed line, at both 12 hours (figure 7 (b)) and 24 hours (figure 8 (b)) following treatment. This suggests that in the absence of exogenous ABA this line already displays induction of *TaNAC29* expression.

Across all wheat lines the expression of *TaGBF1* is increased to it's peak value following 12 hours of ABA treatment compared to the control line in ethanol treatment, in

agreement with earlier evidence that this gene is ABA-induced (figure 9 (a)) (Sun et al., 2015). Following 12 hours of ABA treatment, data for *TaGBF1* transcript abundance is impaired by large and overlapping large error bars, leaving any trend uninterpretable with no statistically significant differences between lines (Figure 9 (a), (b), (c)). After 24 hours of ABA treatment initial upregulation of *TaGBF1* in all wheat lines has begun to reduce with no statistically significant differences between transformed lines and the control (Figure 10 (a)). Although statistically not significant, the line 81.21 does appear to have sustained a higher level of *TaGBF1* expression compared to the control line in ABA treatment at the 24-hour time point (Figure 10 (c)), suggesting that ABA-induction of this gene may have a longer duration of response in this line. There is also a non-statistically significant higher expression level of *TaGBF1* in the non-ABA treated 81.21 line compared to the control at the 24 hour time point (figure 10 (b)), suggesting a possible upregulation of this gene even in the absence of ABA. Interestingly, 81.21 also shows the lowest rate of water loss in the excised leaf assay compared to all plant lines including the control (see figure 6).

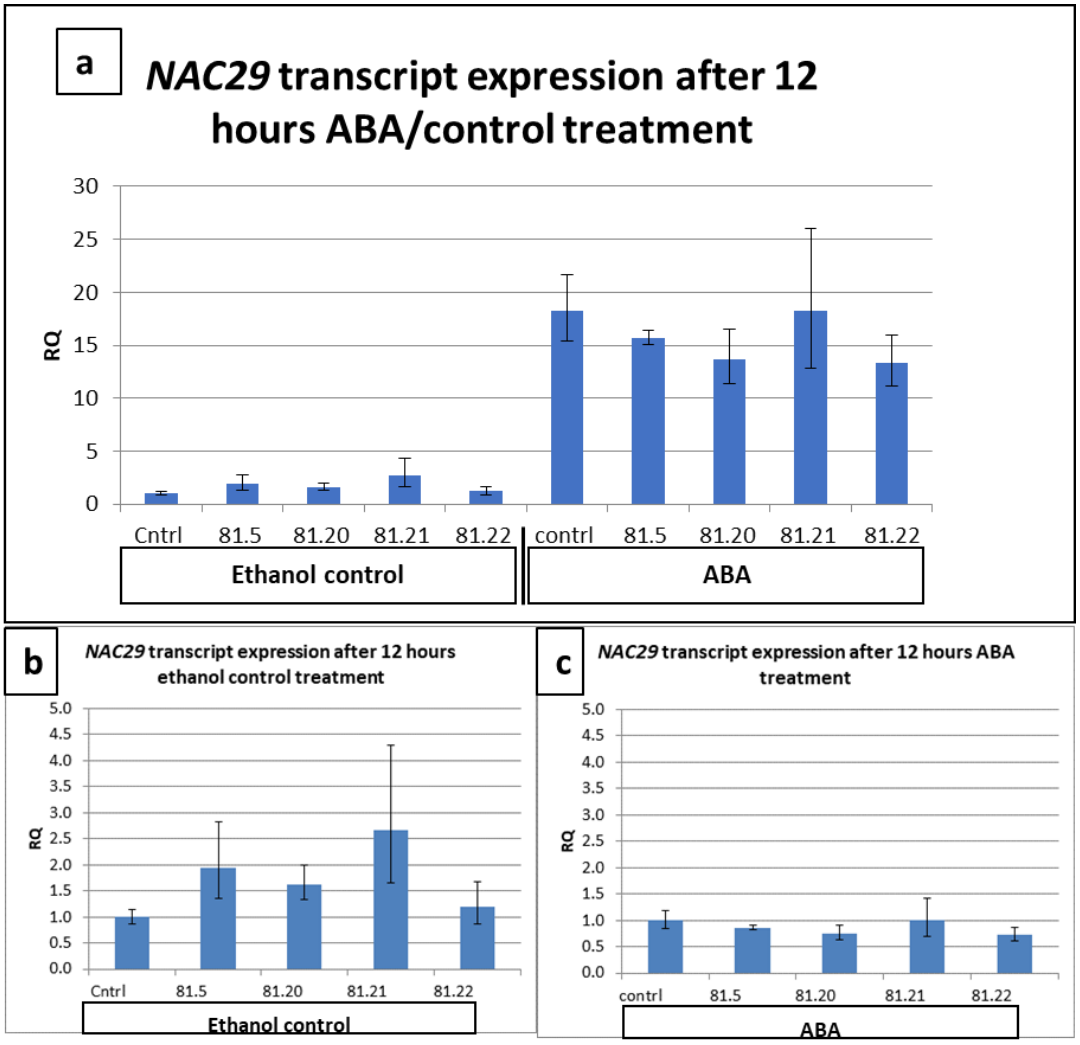


Figure 7 – (a) Real-time PCR *of TaNAC29* expression levels in wheat lines overexpressing *SbSDIR1* in comparison to untransformed control line in ethanol treatment, following 12 hours of ABA treatment with ethanol controls. (b) Magnification of data for expression of *TaNAC29* in ethanol treated lines. (c) Expression of *TaNAC29* in ABA treated lines, compared to ABA treated untransformed control line. The error bars show the RQ maximum and minimum values, representing the 95% confidence interval for Student's t test.

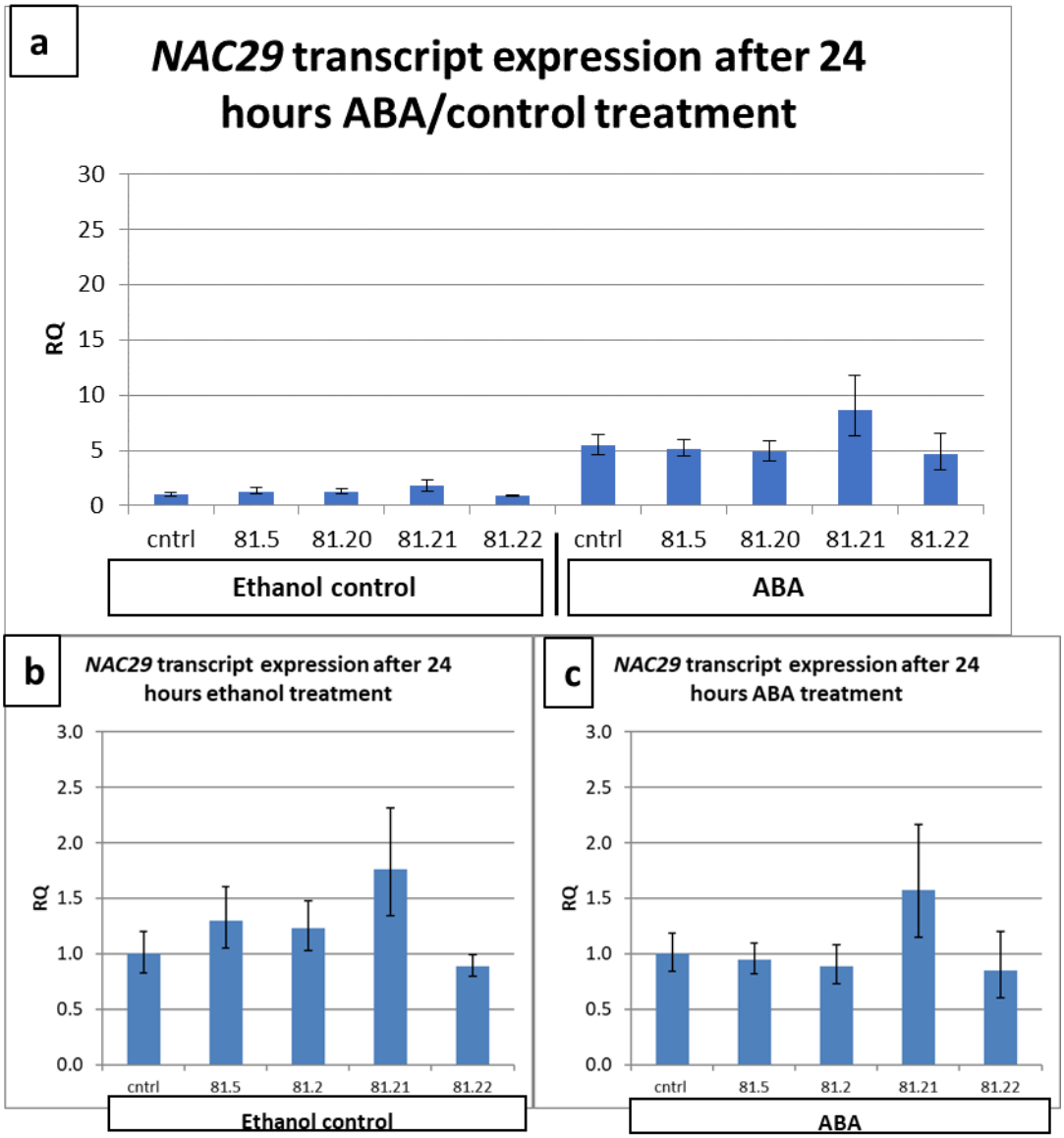


Figure 8 – (a) Real-time PCR *of TaNAC29* expression levels in wheat lines overexpressing *SbSDIR1* in comparison to untransformed control line in ethanol treatment, following 24 hours of ABA treatment with ethanol controls. (b) Magnification of data for expression of *TaNAC29* in ethanol treated lines. (c) Expression of *TaNAC29* in ABA treated lines, compared to ABA treated untransformed control line. The error bars show the RQ maximum and minimum values, representing the 95% confidence interval for Student's t test.

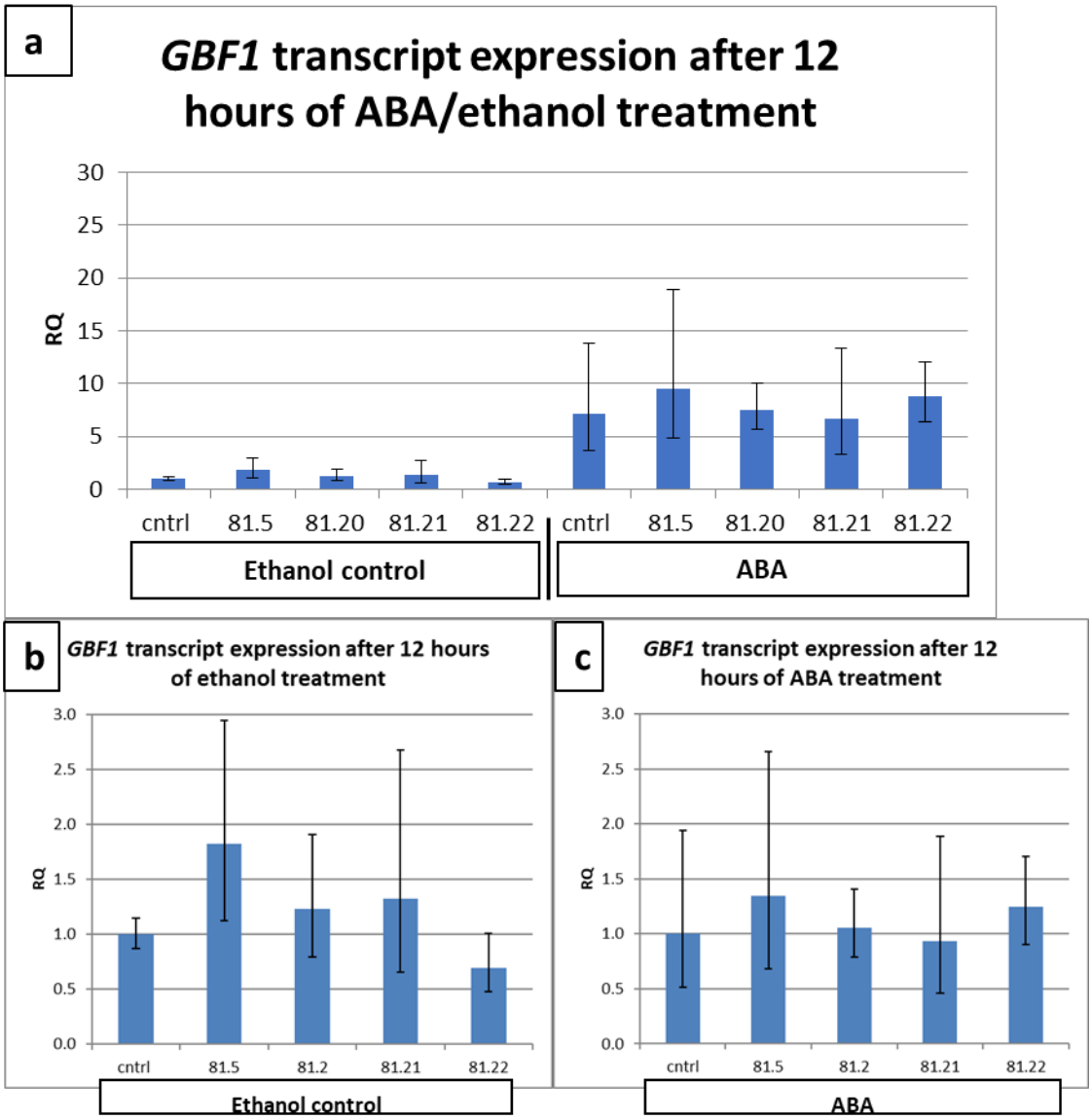


Figure 9 – (a) Real-time PCR *of TaGBF1* expression levels in wheat lines overexpressing *SbSDIR1* in comparison to untransformed control lines in ethanol treatment, following 12 hours of ABA treatment with ethanol controls. (b) Magnification of data for expression of *TaGBF1* in ethanol treated lines. (c) Expression of *TaGBF1* in ABA treated lines, compared to ABA treated untransformed control line. The error bars show the RQ maximum and minimum values, representing the 95% confidence interval for Student's t test.

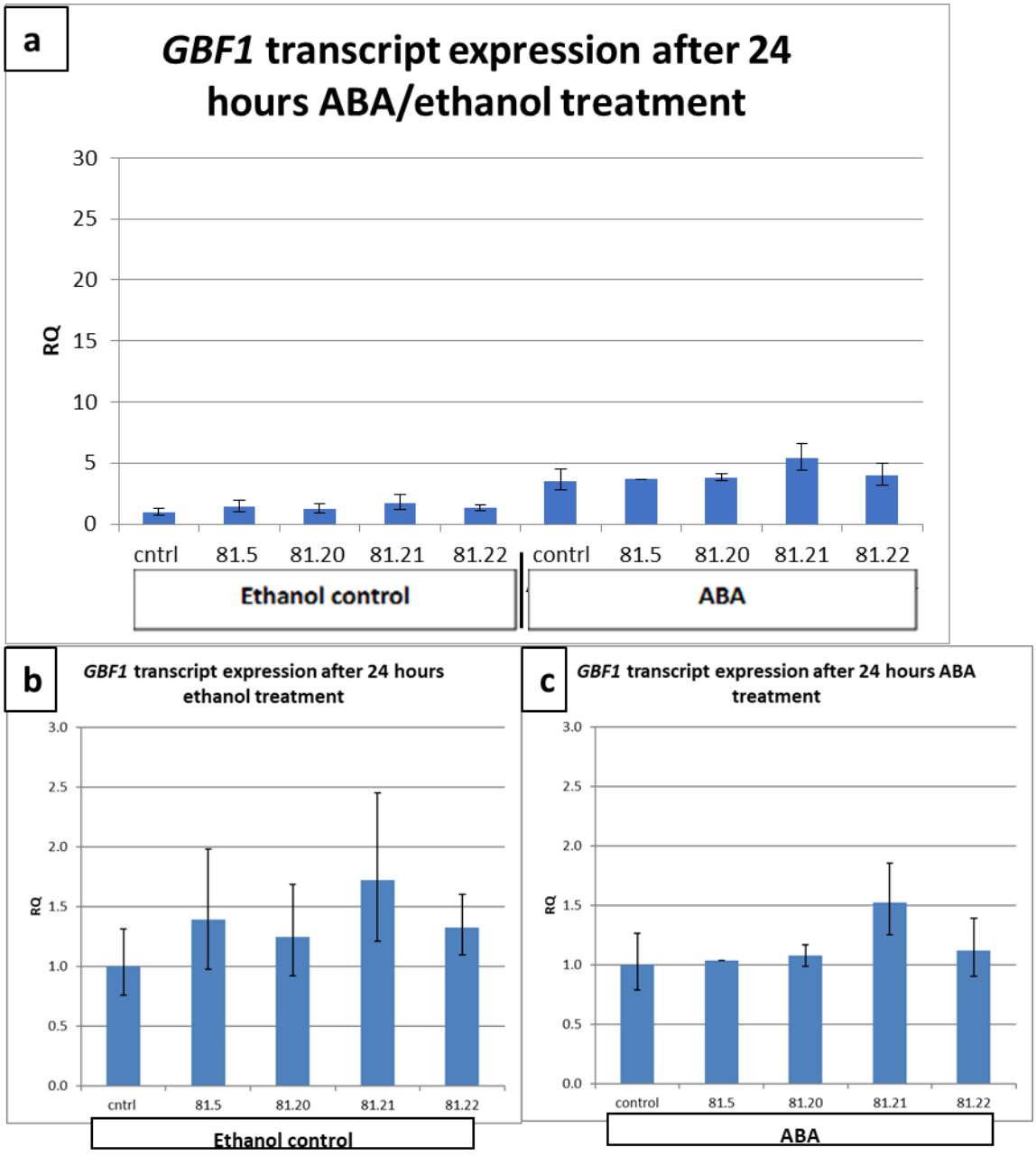


Figure 10 – **(a)** Real-time PCR *of TaGBF1* expression levels in wheat lines overexpressing *SbSDIR1* in comparison to untransformed control line in ethanol treatment, following 24 hours of ABA treatment with ethanol controls. (b) Magnification of data for expression of *TaGBF1* in ethanol treated lines. (c) Expression of *TaGBF1* in ABA treated lines, compared to ABA treated untransformed control line. The error bars show the RQ maximum and minimum values, representing the 95% confidence interval for Student's t test.

4.2.5 PEG-induced osmotic stress senescence assay

Previous papers have reported that overexpression of *SDIR1* and its homologs in various plant species has improved drought tolerance, and reduced the progression of senescence as a response to stress (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). Considering *SbSDIR1* is expressed significantly higher in sorghum stay green line B35 compared to R16, it may have a role in stay green-related drought tolerance mechanisms (Johnson et al., 2015b), possibly contributing to processes that allow the plant to delay senescence under water-limited conditions. To investigate stay green-like characteristics in the wheat *SbSDIR1* overexpression line, leaf sections from 25 day old plants were exposed to polyethylene glycol (PEG)-induced osmotic stress conditions by floatation on solutions of 15% (w/v) and 25% (w/v) PEG, compared to 0%. PEG solutions are high molecular weight osmotica that can impose low -water potential stress that is comparable to conditions of gradual soil drying, in contrast to low-molecular weight mannitol which can be taken up by plant cells and cause additional toxic effects as well as severe osmotic stress (Verslues et al., 2006). Sorghum *SbSDIR1* expression has previously been shown to be induced by growth in 10% PEG, but not mannitol (Johnson, 2015a). Visual symptoms of senescence in the drought tolerance assay were documented photographically, and evidence of senescence progression after 5 days is shown in figure 11. Section from leaves of the same developmental stage were used across all lines in order to account for age-related senescence variation.

Visible evidence of chlorophyll loss was first noticeable after 6 days (see figure 11). Surprisingly, variation in chlorosis between 0%, 15% and 25% (w/v) PEG within each line (including the control) was not clearly visible, despite 25% PEG being shown to be sufficient for initiating senescence-related drought responses in wheat (Konigshofer and Loppert 2015; Liu et al., 2015a and Tian et al., 2013). However, at all concentrations of PEG there appears to be greater visible chlorophyll loss in all the transgenic lines compared to the control line, which remains much greener at this stage. Therefore, compared to the control the transgenic lines appear to have display premature senescence possibly as a protective response to stress conditions unrelated to PEG. Visual observation of senescence would need to be confirmed by quantitative chlorophyll content analysis in the future.

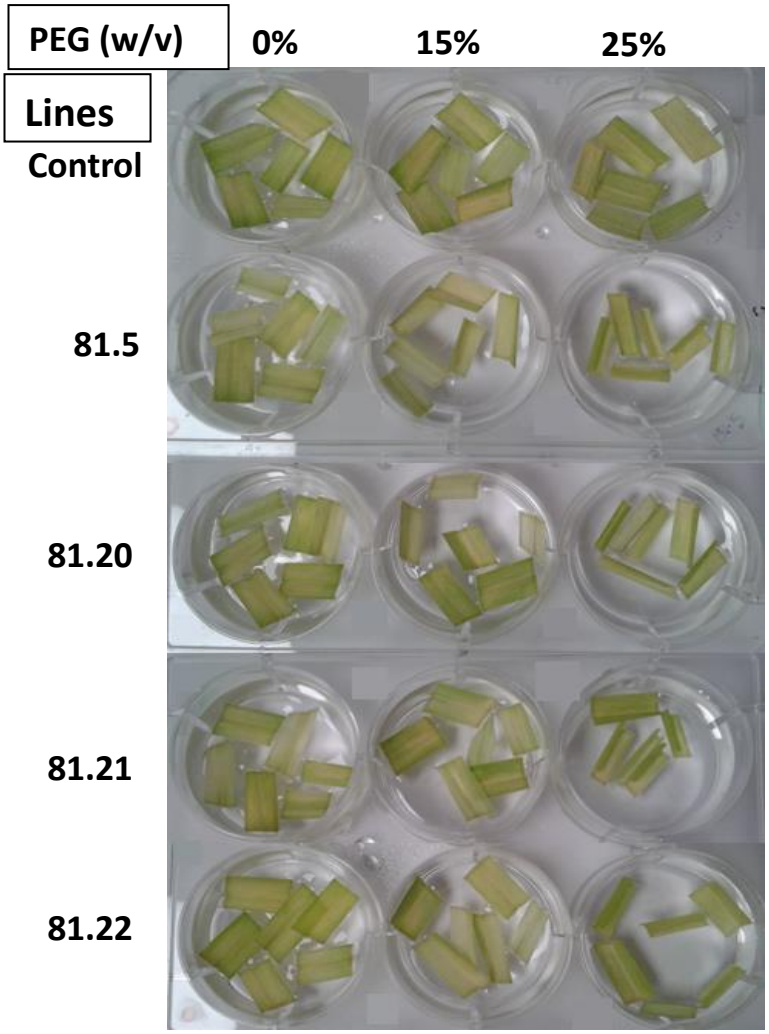


Figure 11 – Leaf section assay for tolerance to various PEG concentrations, photographed 6 days after treatment. Wheat plants were grown for 25 days under normal growth conditions. Leaf 4 was excised and cut into sections roughly 1cm in length, and floated adaxial side up in 7ml of 0, 15 and 25% solutions of PEG (w/v) in 6-well plates, incubated at 20°C with a 16hr photoperiod.

4.2.6 Light and dark induced senescence assay

In addition to delayed drought-induced senescence, stay green sorghum lines also display delayed developmental senescence (Thomas and Howarth 2000). Analysis of developmental senescence is problematic due to the lack of coordination in the development of individual cells within a leaf, particularly between the proximal and distal ends, resulting in asynchronous senescence progression (Buchanan-Wollaston et al., 2005). To investigate whether the wheat transgenic lines had acquired the stay green characteristic of delayed developmental senescence, an assay for dark-induced senescence in excised leaves was used. This method is frequently used as a model for age-triggered senescence, and produces synchronised senescence across the leaf to better facilitate comparison, as the leaf responds to the removal of light and subsequent carbon starvation (Keech et al., 2007; Song et al., 2014). The paper by van der Graaf et al., 2006 suggests dark-induced senescence shares many common pathways and processes to developmental senescence.

Leaves of the same developmental stage were excised from 24 days-old wheat plants, grown under normal conditions. These were incubated at 20 °C in petri dishes on wettened filter paper covered in tin foil, with uncovered plates used as a control. Visible progression of senescence was then documented photographically. Interestingly, light exposed leaves showed a greater rate of senescence than covered leaves, with significant chlorophyll loss observed in all lines after 5 days (figure 12), and extensive senescence after 7 days (figure 13). The rapid and synchronized senescence across the entire leaf is characteristic of stress-responsive senescence, possibly as a result of photodamage and oxidative stress (Buchanan- Wallaston 2005). There is possible evidence of more advanced senescence after 5 days in all transgenic lines compared to the control line (figure 12), but this minute variation requires confirmation by actual chlorophyll content measurement. In the covered plates, there is some initial evidence of senescence in the transgenic lines after five days but not the control (figure 12), but after 7 days the faster progression of senescence within the overexpression lines compared to the control is much more visible (figure 13). It is possible that the lowest *SbSDIR1* expressing line 81.5 shows the lowest chlorophyll loss out of all the transgenic lines after 7 days (figure 13) but confirmation by chlorophyll content analysis would again be required to confirm this. The progression of dark-induced chlorosis

occurs in a gradient across the leaves, which is more characteristic of developmental senescence rather than stress-responsive (Song et al., 2014).

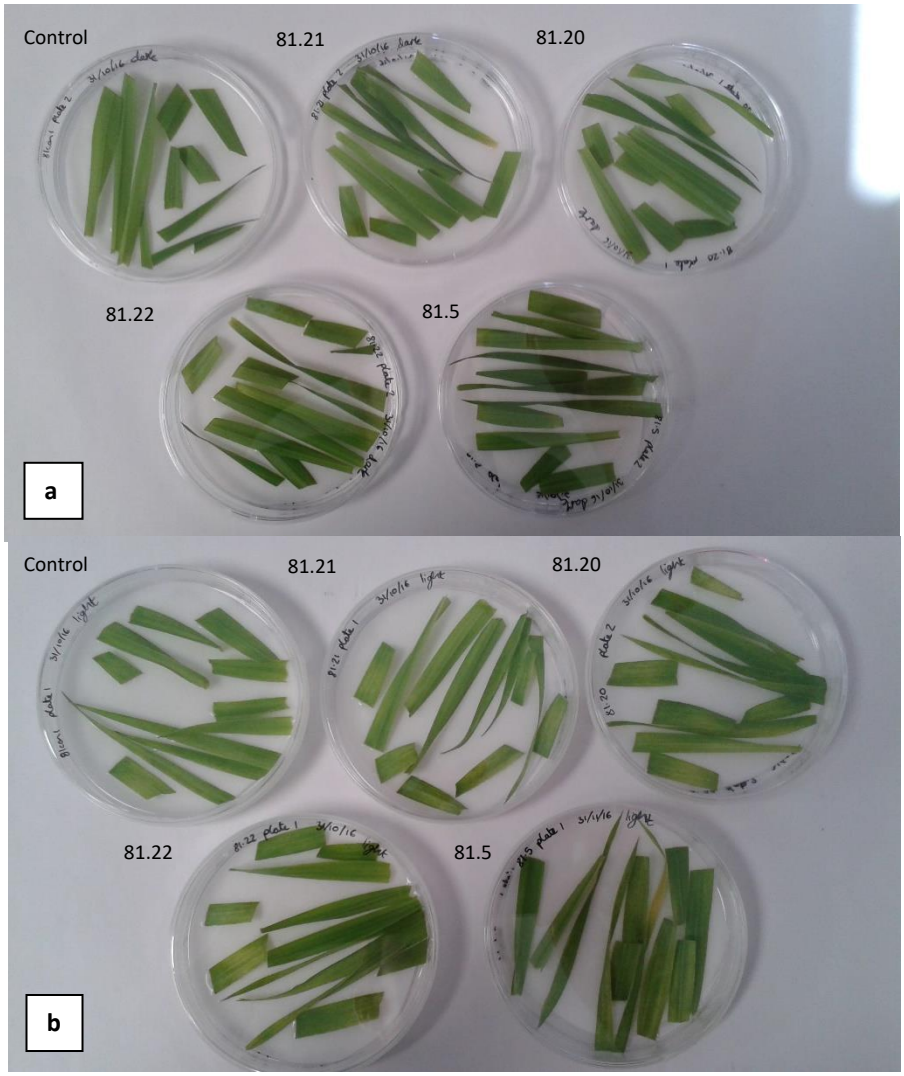


Figure 12 – *Photograph of senescence assay showing phenotypic differences after 5 days. Plants were grown for 24 days under normal conditions, and the 4th leaf was excised and cut into 4 sections. Sections were incubated on wet filter paper in petri dishes covered in tin foil (a) or light exposed (b). See Section 2.3.3 for growth conditions and light regime.*

Figure 13 – *Continuation of senescence assay described in figure 12, photographed after 7 days. Image of covered plates (a) and uncovered plates (b).*

4.3 Discussion

The sorghum gene *SbSDIR1* is an E3 ubiquitin ligase previously selected for further analysis following its identification as an upregulated gene in B35 stay green sorghum, in comparison to the R16 senescent line (Johnson, 2015a; Johnson et al., 2015b). Homologs of this gene are found in several other crop species, and overexpression of *SDIR1* has been shown to improve drought tolerance, reduce transpiration rates in excised leaves, and produce ABA-associated phenotypes (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). By overexpressing *SbSDIR1* in wheat the function

of this gene within a monocot plant system can be investigated, and conservation of its function compared to that of homologs in other species can be assessed. In this chapter, wheat transgenic lines overexpressing *SbSDIR1* were analysed for the acquisition of stay green and drought tolerance characteristics, including delayed senescence, reduced transpiration rate and ABA hypersensitivity.

4.3.1 Morphological and developmental phenotype of wheat lines overexpressing *SbSDIR1* compared to the control

When grown under well-watered conditions, no obvious differences were observed between wheat *SbSDIR1* overexpression (OE) lines and the control line at germination, in agreement with previous reports in Arabidopsis (Zhang et al., 2007). However, previous studies on Arabidopsis and rice *SDIR1* overexpression lines have recorded a reduction in the size of the aerial parts at the seedling growth stage when grown on MS (Murashige and Skoog) media (Gao et al., 2011; Zhang et al., 2007). Considering *SDIR1* has previously been implicated in ABA signal promotion, it has been suggested that this stunted growth could reflect constitutive activation of ABA and ABA-mediated growth inhibition (Zhang et al., 2007). This would be consistent with findings of ABA-hypersensitivity at germination and early growth stages in overexpression lines (Zhang et al., 2007). No differences in the size of aerial parts were observed for the wheat transgenic lines in this report, but this may be due to differences in growth conditions, where germinated seeds were directly transferred to soil rather than grown on media.

At later growth stages, overexpression lines in Arabidopsis, tobacco and rice showed no obvious differences in height or leaf number (Gao et al., 2011; Xia et al., 2013; Zhang et al., 2007). However, in this report the wheat transgenic lines showed a significant tillering phenotype compared to the control, with a higher occurrence of primary tiller development in all transgenic lines compared to the control, within the 25-day growth period. There is some discrepancy between the two separate experiments for this observation which may be explained by slight and unintentional alteration in water regime. For example, it is possible that tillering could have been reduced overall in experiment 2 as a response to a slight reduction in water availability (Borell et al., 2014a). However, in both batches the line 81.21 consistently shows evidence of primary tiller emergence in the majority of the individuals. Considering the role of ABA in promoting tiller development

(Cai et al., 2014; Hall and McWha 1981; Lin et al., 2016; Quarrie and Jones 1977), this difference in tillering may be considered an ABA-associated phenotype.

Interestingly, an increase in tillering would contrast the growth phenotype associated with sorghum stay green (stg) QTLs, where stg near isogenic lines (NILs) have displayed reduced tillering and reduced upper leaf size at flowering, minimising canopy size (Borrell et al., 2014a; Borrell et al., 2014b; Kassahun et al., 2010). It has been suggested that this morphology enhances water conservation pre-flowering and increases water availability post-anthesis, contributing to increased grain yield under water limiting conditions (Borrell et al., 2014a; Borrell et al., 2014b). If early emergence of a primary tiller in wheat leads to an increased tillering phenotype in later in growth, these results may suggest that the overexpression of *SbSDRI1* in the wheat lines has produced the opposite of the stay green characteristic possibly due to the sorghum gene behaving differently in a different plant system, or *SbSDIR1* may be involved in other aspects of the stay green mechanism but not regulation of tiller development. Observation of tiller number at later growth stages closer to flowering stage (as has been done in previous studies such as Borrell et al., 2014a; Borrell et al., 2014b; Kassahun et al., 2010) may be more relevant for investigating stay green-associated phenotypes because the balance between supply and demand is likely to have more effect on water availability during grain filling at this stage. In general, repeats of this experiment using more replicates is required in order to carry out proper statistical analysis.

4.3.2 Excised-leaf water loss assay

The link between stay green and improved transpiration efficiency (TE) is well established in sorghum. By minimising water loss through transpiration, crop water use is more efficient and water is more readily available during grain filling, improving yield in drought conditions (Borrell et al., 2014a). Stay green QTLs are shown to be associated with differences in the balance of water usage pre- and post- anthesis (Borrell et al., 2014a; Borrell et al., 2014b; Vadez et al., 2011), and improved water retention has been observed at the leaf level in the B35 sorghum line (the same stay green source used in this study) compared to R16 (Johnson, 2015a). In addition to reducing canopy area, transpiration can also be minimized by stomatal control. Stomata in the epidermal leaf tissue control gas exchange and water evaporation through coordinated changes in aperture via ABA-controlled guard cells (Schroeder et al., 2001). Other E3 ubiquitin ligases have previously

been found to improve drought tolerance through ABA-induced stomatal closure when overexpressed, including *AtAIRP1* and *RHA2B* (Ryu et al. 2010; Li et al., 2011). Several studies have suggested that *SDIR1* also contributes to stay green and drought tolerance mechanism via stomatal regulation (Gao et al., 2011; Zhang et al., 2007; Zhang et al., 2008).

An excised-leaf water loss assay on Arabidopsis lines overexpressing sorghum *SbSDIR1* (35S::*SbSDIR1*) showed improved water retention, positively correlated with *SbSDIR1* transcript abundance, when grown in well-watered conditions suggesting that the sorghum gene contributes to drought tolerance by improving TE via stomatal regulation (Johnson, 2015a). These findings are consistent with the TE phenotypes observed when homologs of this gene are overexpressed in other species. Reduced leaf water loss is observed under normal conditions in tobacco, Arabidopsis and rice overexpression lines (Gao et al., 2011; Liu et al., 2013; Zhang et al., 2007; Zhang et al., 2008), and following drought treatment in tobacco OE lines (Liu et al., 2013; Xia et al., 2013). Reduced Stomatal aperture was also observed in these lines under well-watered and drought conditions (Gao et al., 2011; Liu et al., 2013; Xia et al., 2013; Zhang et al., 2007; Zhang et al., 2008). Interestingly, in Arabidopsis, ABA-treatment revealed a greater sensitivity in stomatal response in the transgenic lines than the control, suggesting *SDIR1* contributes to stomatal control via ABA-signalling pathways (Zhang et al. 2007). This agreement between studies suggests that the function of *SDIR1* and its role in the stay green mechanism is conserved across several plant species, including sorghum.

The wheat lines overexpressing *SbSDIR1* analysed in this report showed no statistically significant differences in the rate of water loss from excised leaves. However, the lines 81.5 and 81.20 displayed a statistically non-significant increase in the rate of water loss from isolated leaves compared to the untransformed line, suggesting a reduced ability to retain water. This appears to be in opposition to the stay green characteristics of sorghum (Johnson, 2015a), and to previous findings of improved water retention when homologs of *SDIR1* are overexpressed in other species (Gao et al., 2011; Liu et al., 2013; Zhang et al., 2007; Zhang et al., 2008). Considering Arabidopsis *SbSDIR1* OE lines discussed above display improved water retention and reduced stomatal aperture (Johnson, 2015a), it is possible that while the function of *SDIR1* in drought tolerance is conserved in the sorghum gene, it may not behave the same within the wheat plant system and genetic background. For example, post-transcriptional regulation that may be essential to the functioning of *SbSDIR1* may vary in wheat, or target proteins may differ in expression or structure.

However, the line 81.21 does show a non-statistically significant reduction in the rate of water loss compared to the control line which would be consistent with previous observations in of TE in Arabidopsis *SbSDIR1* OE lines (Johnson, 2015a). This line has potentially acquired the stay green-associated phenotype of improved water retention, which could be attributed to alteration in stomatal conductance.

Discrepancies between the three individual water loss experiments, and the large error bars in this data, may be explained by the low number of replicates in the experiment and the minute changes in weight under analysis. In order to confirm these findings, water loss from several leaves or the whole aerial tissue should be measured, which could make differences in transpiration rate easier to detect and identify in the plant as a whole rather than restricting the experiment to observations at just one leaf stage. Stomatal conductance and density measurements could reveal direct differences in stomatal control and transpiration efficiency at the leaf level, without relying on water loss experiments which follow very small changes in weight, which may be affected by inconsistencies in weighing and room temperature across the 5 hour period. Studying water retention and stomatal conductance in the wheat lines following drought treatment may also accentuate any true differences in TE and drought response of the OE lines which may not have been detectable under normal conditions (Liu et al., 2013; Xia et al., 2013). Stomatal conductance following ABA-treatment could also be analysed to determine whether *SbSDIR1* is involved in ABA-controlled stomatal closure directly, as has been done previously in the paper Zhang et al., 2007. Stomatal conductance measurements are also a more sensitive measurement for changes in stomatal aperture, and are more likely to detect differences between B35 and R16 if there are any.

Both the tillering phenotype and water retention measurements of the wheat OE lines appear to be the opposite of stay green-associated responses when compared to the control line, and contrast those phenotypes observed in *SDIR1* OE lines in several other species (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). This could be explained by the sorghum *SbSDIR1* gene acting differently in the wheat plant system, but this seems unlikely due to the conservation of *SbSDIR1* function in relation to stomatal aperture and transpirational efficiency observed in Arabidopsis *SbSDRI1* OE lines and homologs of other species (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). Alternatively, it could be hypothesised

that the transformation of wheat lines with the *SbSDIR1* overexpression construct has produced transgenics acting as pseudo-suppression lines due to co-suppression of endogenous wheat *SDIR1* by the homologous *SbSDIR1*. Three *SDIR1* homologs were found in wheat using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) with high identity at 80.3%, 80.9% and 80.3%, potentially sufficient for dominant negative activity with *SbSDRI1*.

4.3.3 PEG-induced osmotic stress senescence assay

The process of senescence constitutes a loss of photosynthetic activity as chlorophyll is degraded and nutrients are remobilized to younger leaves, characterized visibly by a loss of green leaf area (Distelfeld et al., 2014; Lim and Nam, 2007). Drought conditions reduce photosynthetic activity as stomatal closure limits carbon dioxide (CO_2) uptake and ROS accumulation interferes with photosynthetic machinery (Chaves, 1991). Premature senescence is a drought response mechanism that reduces transpiration water loss, allowing remobilization of nutrients to younger leaves (Lim and Nam 2007; Munne-Bosche and Aelgre 2004). The functional stay green phenotype found in sorghum delays stress responsive senescence, maintaining photosynthetic activity for longer and enhancing yields under drought conditions (Thomas and Howarth, 2000).

Overexpression of the grapevine *SDIR1* homolog in tobacco increased tolerance to PEG-induced osmotic stress, with significantly less visible senescence observed after 4 days of treatment with 10% (w/v) PEG compared to the wildtype (Tak and Mahtre, 2013). Overexpression lines in several other species have been shown to confer enhanced drought tolerance with reduced evidence of senescence and wilting following water withdrawal at the whole plant level, for example in Arabidopsis (Zhang et al., 2007) and rice (Gao et al., 2011; Zhang et al., 2008). Additionally, the sorghum *SbSDIR1* gene has previously been shown to be induced by 10% (w/v) PEG, suggesting this gene is specifically responsive to the drought stress type imposed by this osmotica (Johnson, 2015a). To determine whether the function of *SbSDIR1* in drought tolerance and senescence delay is conserved within sorghum, the PEG-induced senescence response of the wheat OE lines was analysed. Analysis of Arabidopsis *SbSDIR1* OE lines did not cover either developmental or stress-induced senescence progression (Johnson, 2015a), so the function of *SbSDIR1* in association with senescence timing has yet to be investigated.

There appeared to be no obvious visible difference in senescence within each wheat line (including the control) in relation to varying degree of PEG (0%, 15% and 25% (w/v)). This is surprisingly considering the visible effect of 20% (w/v) PEG concentration on wildtype wheat recorded in previous papers (Konigshofer and Loppert 2015; Liu et al., 2015a; Tian et al., 2013). However, there does appear to be some evidence of premature senescence within all transgenic lines (irrespective of PEG concentration) compared to the control, suggesting an earlier initiation of senescence in response to a PEG-unrelated trigger. This could be a response to photodamage of excised leaf segments, or disturbed osmotic balance as a result of floatation on aqueous solution. It is possible that equilibration on water prior to PEG treatment may have been required to remove any effect of PEG-unrelated osmotic shock that could interfere with the analysis. A major limitation of this analysis is that it is reliant on visual inspection of leaves rather than chlorophyll quantification. Due to time constraints of the project, quantitative analysis was not possible. Therefore, in the future actual chlorophyll content measurement or photochemical activity is required to confirm visual differences.

Despite the apparent lack of sensitivity to PEG concentration there does appear to be a phenotype of premature stress-responsive senescence within all wheat OE lines for this developmental stage and this type of drought stress induction. This is inconsistent with previous findings of delayed senescence in *SDIR1* overexpression lines, and is in opposition to observed stay green phenotypes in sorghum (Rosenow et al., 1983; Kebede et a., 2001; Borrell and Hammer et al., 2000). This could be because the sorghum *SbSDIR1* doesn't have a conserved function in drought tolerance and senescence delay, or that the gene functions differently within the wheat plant system and genetic background than it does in sorghum (see explanation 4.3.2). It could also be explained by the theory that *SbSDIR1* expression within the wheat lines has a dominant negative effect of wheat SDIR1, and suppressing the function of this gene (see section 4.3.2).

In future experiments it would be important to investigate the drought tolerance phenotype of these wheat lines at the whole plant level, as well as at the level of the leaf. The process of senescence constitutes a change in the source-sink relationship between the senescing leaf and the rest of the plant, and studying the senescent phenotype of leaves of different developmental ages in the context of the whole plant may reveal more about the drought response phenotype (Distelfeld et al., 2014). Whilst PEG is often used as

a proxy for gradual soil drying, there will be differences in the stress conditions imposed on leaf sections in aqueous solution (for example possible additional osmotic stress of floatation on solution) that doesn't reflect the conditions of soil-grown plants under limited water. Analysing the senescent phenotype of whole plants grown on soil during water limitation and following recovery may give a more representative impression of the wheat OE lines' ability to tolerate stress and recover from it.

4.3.4 Dark-induced senescence assay

In addition to delayed drought-responsive senescence, stay green sorghum also shows delayed developmental senescence under well-watered conditions (Thomas and Ougham, 2014; Johnson, 2015a; Xu et al., 2000a; Xu et al., 2000b). Dark-induced senescence assays are commonly used as a model for age-triggered developmental senescence (Keech et al., 2007; Song et al., 2014).

The wheat OE lines displayed accelerated dark-induced senescence compared to the control line, with visible chlorosis after 5 days. This suggests that overexpression of *SbSDIR1* in wheat has again produced a phenotype that is the opposite of stay green characteristics. The OE line 81.5 appeared to have the least advanced senescence within the transgenic lines. This line has the lowest *SbSDIR1* transcript levels of the OE lines, so it is possible that lower transcript abundance of the ubiquitin ligase produces an intermediate senescence phenotype. If the phenotype can be explained by co-suppression of the endogenous wheat *SDIR1*, it could be hypothesised that suppression is lower within this transgenic line. Due to time constraints this analysis is limited to visual inspection of leaves not quantitative analysis of chlorophyll levels. In the future, actual chlorophyll content analysis is essential to confirm these minute differences in senescence progression.

The senescent patterning in dark-induced senescence has previously been found to be synchronized across the whole of the leaf as a response to carbon starvation (Keech et al., 2007; Song et al., 2014). However, the covered wheat OE lines displayed a gradient of chlorosis more like that observed in ageing attached leaves, which was less easy to compare visibly. Although all excised leaves were at the same developmental stage, due to time constraints of the experiment they were not the active (currently growing leaf) and may be less responsive to carbon starvation, so using the active leaf in future experiments

may give a more uniform impression of senescence progression for this method. Covering attached leaves in foil and observing the progression of senescence could give a more representative impression of dark-induced senescence when the leaf remains a functional part of the whole plant, removing the additional stress effect of excision. Covering leaves of several developmental stages could remove age-related effects on senescence progression which could mask any phenotype specifically related to *SbSDIR1* expression. Ultimately, developmental senescence is best observed by allowing the plants to reach terminal senescence under normal conditions, such as the experiments in the paper by Thomas and Ougham in 2014, removing any additional stress effect from excision or coverage.

4.3.5 ABA-induced gene expression

ABA plays a vital role in mediating plant responses to common abiotic stress, contributing to the regulation of several developmental and physiological processes including seed dormancy, germination, vegetative growth and stomatal behaviour (Finkelstein et al., 2002; Leung and Giraudat 1998). Overexpression of *SDIR1* produces several ABA-related phenotypes, including altered stomatal aperture, exaggerated stomatal closure response to ABA, hypersensitivity at germination and seedling growth stage, salt hypersensitivity and shorter primary root length (Gao et al., 2011; Liu et al., 2013; Tak and Mahtre, 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007; Zhang et al., 2008). To investigate the ABA sensitivity of wheat *SbSDIR1* OE lines and the potential role of *SbSDIR1* in transcriptional regulation of ABA/stress responsive genes, the expression of known wheat ABA-inducible genes were analysed following ABA treatment. The genes *TaGBF1* and *TaNAC29* were used in this analysis (Sun et al., 2015; Xu et al., 2015).

The variation in expression of *TaGBF1* and *TaNAC29* particularly between the OE line 81.21 and the control suggests that there could be a difference in their ABA-controlled transcriptional regulation, which could be directly or indirectly related to *SbSDIR1*. Therefore, this could represent another ABA-related phenotype for this wheat OE line, in addition to altered senescence timing and transpiration efficiency.

For all wheat lines, the peak expression level of both *TaNAC29* and *TaGBF1* was reached at 12 hours following ABA treatment, and transcript abundance was reduced for both genes after 24 hours. Although non-statistically significant, the expression levels of both

TaNAC29 and *TaGBF1* remained higher than control levels for the OE line 81.21 after 24 hours of ABA treatment, which could suggest a longer duration of transcriptional response to ABA. Additionally, non-ABA treated 81.21 tissue displayed statistically significant upregulation of *TaNAC29* compared to the control line at both 12 and 24 hours after treatment, which could potentially indicate constitutive promotion of ABA signalling even in the absence of exogenous ABA treatment. This may be comparable to the ABA hypersensitive phenotype seen in Arabidopsis *SDIR1* overexpression lines (Zhang et al., 2007).

The wheat gene *TaGBF1* belongs to the G group in the bZIP transcription factor family (Sun et al., 2015). Arabidopsis *SDIR1* has previously been found to promote ABA signalling, functioning upstream of the bZIP transcription factors *ABI5, ABF3* and *ABF4*. (Sun et al., 2015; Zhang et al., 2007, Zhang et al., 2008). The ABA signalling component *ABI5* has been shown to heighten sensitivity to ABA when overexpressed, and is vital to ABA-mediated stress response pathways (Lopez-Molina et al., 2001; Sun et al., 2015). Overexpression of *TaGBF1* in wheat and Arabidopsis confers a salt and ABA hypersensitivity phenotype, but when *TaGBF1* is overexpressed in *abi5-1* mutant background this hypersensitivity is not observed, suggesting that *ABI5* is required for the *GBF1* mediated hypersensitive response (Sun et al., 2015). These findings, supported with the expression data in this report, could be consistent with a model in which *SDRI1* acts as a positive regulator of ABA signaling, functioning upstream of transcription factors such as *GBF1* (directly or indirectly controlling degradation rate or stability) which in turn function upstream of ABA signalling components such as *ABI5* to mediate ABA/stress response pathways.

The wheat gene *TaNAC29* encodes an NAC transcription factor (named after NAM, ATAF, CUC proteins), and has been shown to be induced by ABA, salt and drought stress. Overexpression in wheat produces enhanced salt stress tolerance, with reduced evidence of chlorosis when grown in high salt (Murashige and Skoog) MS medium and increased activity of antioxidant enzymes (Xu et al., 2015). Several studies in Arabidopsis examining the association between NAC transcription factors and senescence, have found evidence for both positive and negative regulators of senescence timing (Guo and Guan, 2006), whilst RNA interference (RNAi) lines for *TaNAM* genes in wheat exhibit delayed senescence phenotypes associated with the preservation of chloroplast ultrastructures (Checovich et al., 2016; Uauy et al., 2006). Altered expression level of *TaNAC29* in the *SbSDIR1* OE line

81.21 could be consistent with a model for *SbSDIR1* functioning in which the ubiquitin ligase acts upstream of NAC transcription factors such as *NAC29* to alter senescence timing.

There is some disagreement regarding the *SbSDIR1* OE lines in terms of ABA response within this experiment. While 81.21 appears to show a similar peak in *TaNAC29* expression level to that of the control following 12 hours of ABA treatment, lines 81.5, 81.20 and 81.22 show a lower peak in expression at this time point (although not statistically significant) which could suggest an ABA insensitivity phenotype for these lines. Arabidopsis *sdir1-1* mutant plants were recorded to have a lower sensitivity to ABA treatment at the seedings stage (Zhang et al., 2007), which could be consistent with the wheat OE lines in this report acting as pseudo-suppression lines for *SDIR1*. Incongruence between lines could be due to differences in expression level, but line 81.21 was selected as an intermediate-level *SbSDIR1* expresser with very similar transcript abundance to that of the line 81.22 (see figure 3).

Future experiments could look at the change in expression of ABA signalling components such as *ABI5, ABF3* and *ABF4* (as has been done previously in Arabidopsis (Zhang et al,, 2007)) in order to investigate promotion/suppression of ABA signalling at the level of its core components rather than investigating ABA signalling indirectly through genes involved in ABA-response. ABA sensitivity at germination and early seedling growth (as has been observed in Arabidopsis (Zhang et al., 2007)) may also indicate more clearly if there are differences in ABA-responses at the whole plant level which may not be decipherable at the gene expression level. Analysing gene expression at more frequent time points would ensure that the point of peak expression was captured. ABA-mediated upregulation is short-lived in both genes (Sun et al., 2015; Xu et al., 2015), and the initial measurement following 12 hours may not represent the highest level of expression for all OE lines.

In several instances in this chapter the line 81.21 has shown contrasting morphological and physiological phenotypes in comparison to all other wheat OE lines and the control line. It displayed the most consistent evidence of primary tiller emergence within the transgenic lines, which is in opposition to stay green associated behaviour (Borrell et al., 2014a; Borrell et al., 2014b; Kassahun et al., 2010). However, it also shows higher capacity for water retention than the wildtype, and increased expression of ABA-regulated genes in the presence and absence of exogenous ABA, both of which observations could suggest the

acquisition of stay green-like traits (Borrell et al., 2014a; Borrell et al., 2014b; Vadez et al., 2011; Zhang et al., 2007). Although it is inconclusive whether this line has acquired the stay green mechanism, overexpression of *SbSDIR1* within this wheat line does appear to affect similar pathways and processes affected when homologs from other species are overexpressed (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). Further analysis of this line could improve understanding of the functioning of *SbSDIR1* in comparison to its homologs.

4.4 Conclusions

This chapter focusses on the functional characterisation of sorghum *SbSDIR1,* and investigates its potential contribution to drought tolerance and stay green mechanisms. Preliminary data identified this gene as being upregulated in B35 stay green sorghum compared to the R16 senescent line (Johnson et al., 2015b). Overexpression of *SbSDIR1* in Arabidopsis produced the stay green-like characteristics of reduced transpiration water loss, reduced stomatal conductance and shorter primary root length, suggesting that increased expression of this gene is sufficient to confer stay green and drought tolerance features in the transgenic line (Johnson, 2015a). By overexpressing *SbSDIR1* within wheat, the function of this monocot gene was investigated within a monocot system, and conservation of its function compared to that of homologs in other species was assessed.

Overexpression of *SbSDIR1* within wheat was sufficient to confer morphological and physiological changes within the transgenic lines. However, whether these changes support a putative connection between expression of this gene and acquisition of stay green-associated characteristics is unclear. The fact that the lines analysed were T1 generation and therefore not homozygous means that these results are preliminary, and lack of homozygosity could explain the variable phenotype. The promotion of tiller development, reduced ability to retain water, and accelerated senescence observed in the OE lines are the opposite of the characteristics associated with the stay green phenotype (Zhang et al., 2007; Gao et al., 2011). However, considering the stay green-associated phenotypes conferred in Arabidopsis lines overexpressing *SbSDIR1* (Johnson, 2015a)*,* it likely that the *SDIR1* drought tolerance function observed in homologs in other species is conserved in sorghum but that the gene either acts differently in the wheat system and genetic background, or the OE construct has produced a dominant negative effect. Several

differences between the wheat OE lines and the control lines are ABA-related, such as transpiration efficiency, tiller development and ABA-induced gene expression which is consistent with a role for *SbSDIR1* in ABA-mediated stress response (Zhang et al., 2007; Zhang et al., 2008), irrespective of whether the transgenic lines are acting as overexpression lines or pseudo-suppression lines.

Short term future experiments have already been outlined in the relevant sections. Overexpression lines of *SDIR1* homologs in other systems have revealed differences in primary root length, antioxidant gene expression, stress gene expression and stomatal aperture response to exogenous ABA (Gao et al., 2011; Liu et al., 2013; Tak and Mahtre 2012; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007; Zhang et al., 2008; Zhang et al., 2015). Investigation into these additional features within the wheat *SbSDIR1* OE lines could reveal more about the functioning of *SbSDIR1* in association with drought stress, within this plant system. It could also be useful to check the presence of the *SbSDIR1* overexpression construct using PCR to confirm the presence of the construct particularly in the lower and intermediate expressing lines (i.e. 81.5, 81.21 and 81.22). This was done by NIAB on the previous generation (see Appendix B), but not confirmed in the segregating T1 generation in the current results. Ultimately, work towards successful sorghum transformation is essential to investigate how *SbSDIR1* functions within sorghum itself, and the extent to which the function of the gene contributes to stay green and drought tolerance within this plant system. Investigating the expression and functioning of this gene within other stay green sorghum varieties could also determine whether a putative role of *SbSDIR1* in stay green mechanisms is specific to B35 or more universal. Additional long term experiments and future perspectives are outlines in chapter 5.

Chapter 5

Discussion and conclusions

5.1 Summary of experiments conducted

Abiotic stresses have been estimated to cause 50-70% of average yield losses to major crops (Ghosh and Xu, 2014; Mittler, 2006). Drought stress and water scarcity are considered major threats to food security, affecting not only yield production but also promoting soil erosion and land desertification (Fang and Xiong, 2015). In response to the threat of global climate change, several countries and international organizations have launched projects focussed on improving crop performance under drought conditions, including the Generation Challenge Programme (https://www.generationcp.org/) initiated by The Consultative Group on International Agricultural Research (CGIAR), and the 2005 project in European and African countries for Improving Water Use Efficiency in Mediterranean agriculture (WUEMED, http://www.distagenomics.unibo.it/wuemed/index.html).

Water limiting conditions can restrict photosynthesis and metabolic function (Farooq et al., 2009; Samarah et al., 2009), expose photosynthetic components to oxidative stress (Munne-Bosch and Penuelas 2003), promote chlorophyll degradation and senescence, alter carbohydrate metabolism and assimilate partitioning (Farooq et al., 2009), trigger stress response signalling, alter membrane lipid composition (Toumi et al., 2008), and reduce growth and yield (Anjum et a., 2011; Fracasso et al., 2016a; Fracasso et al., 2016b; Hussain et al., 2008). The cereal crop sorghum is well suited to drought-prone climates, having evolved features such as a waxy cuticle, a deep rooting system, C4 photosynthesis and stay green characteristics (Fracasso et al., 2016a; Fracasso et al., 2016b; Dugas et al., 2011). As a vital food crop in semi-arid African and Asian regions, Sorghum has received significant attention as a model crop for drought tolerance studies, and has a fully sequenced genome (Paterson et al., 2009), and vast genetic diversity across its multiple accessions (Mace et al., 2013).

This project focussed specifically on the stay green mechanism of drought tolerance observed in some sorghum varieties. The 'stay green' characteristic refers to the ability of some varieties to maintain leaf chlorophyll content and photosynthetic activity for a longer duration under drought stress conditions, contributing to higher grain yields under such stress (Borrell and Hammer 2000; Harris et al., 2007). This thesis aimed to investigate the underlying mechanisms and pathways involved in the stay green trait in Sorghum, in relation to its ability to tolerate higher levels of drought.

The transcriptome of the B35 stay green sorghum line and the R16 senescent line were previously compared under normal unstressed conditions, in the paper by Johnson et al., in 2015b. In the B35 line, 1038 genes were upregulated and 998 genes were downregulated compared to R16, showing clear differences between these two varieties at the gene expression level (Johnson et al., 2015b). As a continuation of this experiment, this report compared the full proteome of the same two sorghum varieties, grown in identical conditions to the same developmental stage. Variation in gene expression may not correlate directly to actual protein levels due to factors such as protein degradation and post-translational modification, and consequently the proteomic data provides further insight into the differences between the stay green and senescent line, and could reveal more about the candidate genes and processes contributing to the stay green phenotype (Bailey-Serres 1999; Jedmowski et al., 2014; Mustroph et al., 2009; Salekdeh et. al., 2002). The observed proteomic variation (see sections 3.2.1 and 3.3.2) suggests there are differences at the protein level as well as the transcript level. Future experiments can be used to identify the differentially expressed proteins between B35 and R16, and functional characterization could be conducted to investigate their potential role in contributing to the stay green trait. Analysing the extent of the correlation between transcriptome and proteome variation could be used to corroborate the identification of specific genes/proteins as putative contributors to stay green characteristics, but could also indicate the level at which the regulation of those candidate genes/processes differs between the two lines, i.e. pre- or post-transcription.

Chapter 4 in this thesis investigates the function of the Sorghum gene 'salt and drought dependent 1' *SDRI1* in relation to the stay green trait and drought stress response. This ubiquitin ligase was previously found to be upregulated in B35 stay green sorghum compared to senescent R16, and has consistently been implicated in drought stress

response within several plants species (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). Therefore, this gene was selected for further analysis as a putative contributor to the stay green trait and drought tolerance characteristics in sorghum. Sorghum *SbSDIR1* was overexpressed in Fielder wheat by Dr Stephanie Johnson (Durham University) in collaboration with NIAB (Huntington Road, Cambridge, CB3 0LE), and the transgenic lines were analysed for the acquisition of stay green-associated characteristics. Some variation in morphological and physiological features were observed, including variation in tiller development, rate of water loss by transpiration, rate of stress-induced senescence, and ABA-induced stress gene expression. Evidence of ABA-related phenotypes in connection with a change in *SDIR1* expression level corroborates several previous studies which have found this gene to function in an ABA-mediated manner (Zhang et al., 2008; Zhang et al., 2015). However, whether the overexpression of *SbSDIR1* within wheat has produced an overall phenotype with enhanced or reduced stay green-associated traits was inconclusive. Considering overexpression of *SbSDIR1* in Arabidopsis has previously been shown to confer stay green-associated traits such as improved water retention and lower stomatal conductance (Johnson, 2015a), it is possible that *SbSDIR1* is behaving differently in the wheat plant system and genetic background than it would in Arabidopsis and sorghum. For example, although we know that the *SbSDIR1* is being transcribed within the wheat lines (see figure 3 in section 4.2.1) post-transcriptional regulation (e.g. phosphorylation) that could be essential to its endogenous function may differ in wheat compared to sorghum. A yeast 2-hybrid screen of Arabidopsis SDIR1 previously found that the ubiquitin ligase interacts with SDIRIP1 (SDIR1-INTEREACTING PROTEIN1) and 38 other clones (Zhang et al., 2015), and it is possible that within the wheat plant system these target proteins may differ in expression or structure in comparison to Arabidopsis and sorghum, interfering with regular functioning. Further experiments have been suggested in section 4.3 to further dissect the phenotype of the wheat overexpression lines in relation to stay green and drought tolerance associated traits.

5.2 Experimental limitations and short-term future experiments

This thesis reports preliminary progress towards a proteomic comparison between B35 and R16, and functional characterization of *SbSDIR1* as a putative contributor to the stay

green state. However, there are several key limitations within the experimental approach which could be addressed in future experiments.

The proteomic data analysed in this report (chapter 3) was focussed only on plants that are 14 days old, and the acquisition of stay green traits in the wheat *SbSDIR1* overexpression lines (chapter 4) was measured only at the early seedling growth stage between 2 and 5 weeks old. Therefore, observations of molecular and physiological differences between varieties and transgenic lines is limited to those observed during the seedling growth stage, and characteristics that may be specific to earlier or later growth stages are not considered. Transcriptomic data comparing stay green and senescent varieties of sorghum 14 days and 50 days after sowing (DAS) found that some genes were differentially expressed only at one time point (Johnson et al., 2015b; Johnson, 2015a), suggesting a difference in gene expression across these two developmental stages for B35 and R16 which is not investigated at the protein level within this thesis. In the context of the *SDIR1* overexpression lines, previous studies have observed phenotypic differences at a variety of specific growth stages which are not analysed within the wheat lines, such as hypersensitivity to ABA observed at germination in Arabidopsis *SDIR1* overexpression lines (Zhang et al., 2007). The reduced tillering phenotype associated with sorghum stay green QTL near isogenic lines (NILs) has previously been observed at anthesis rather than the early seedling stage, at which stage water conservation prior to grain filling may be a greater priority (Borrell et al., 2014a; Borrell et al., 2014b; Kassahun et al., 2010). It appears that drought stress response and stay green mechanisms are complex, and pathways and processes involved may be specific to a particular developmental stage. Analysis at various different growth stages will give a fuller picture of the overall morphological and physiological differences between sorghum varieties, and between *SbSDIR1* overexpression lines compared to the wildtype.

Several studies including this report only compare or introgress a single stay green variety with one comparative senescent line (Jedmowski et al., 2014; Swami et al., 2011). This limits the investigation of the stay green trait to the genetic basis of that specific variety, ignoring the possible genetic and phenotypic variability within the trait across several accessions. Other B35-unrelated sorghum stay green sources include E36-1, SC56 and KS19 (Anami et al., 2015; Haussmann et al., 2002; Kebede et al., 2001). This study focussed solely on the stay green source of B35, which has been extensively characterized

in previous papers (Crasta et al., 1999; Johnson et al., 2015b; Vadez et al., 2011;). However, in the thesis by Johnson, in 2015a the transcriptome of the E36-1 was analysed alongside B35 and R16, finding 993 and 1406 differentially expressed genes to be unique to B35 and E36-1 respectively (Johnson, 2015a). A variable transcriptomic basis for the stay green phenotype in E36-1 corroborates earlier findings of minor stay green associated QTLs unique to this line, in addition to the four major QTLs consistent with B35 (Anami et al., 2015; Haussmann et al., 2002; Wu et al., 2016). It could also reflect the unique senescence phenotype observed in this line, where delayed senescence is only visible under stressed conditions, in contrast to B35 which additionally exhibits developmental senescence (Thomas and Howarth et al., 2000). The stay green line E36-1 could also be included in the proteomic analysis of this study, and the unique genes/proteins further characterized. By analysing multiple stay green lines simultaneously, correlations between proteomic or transcriptomic variation associated with the trait could identify universal processes and genes contributing to the stay green trait across multiple varieties, but could also have the potential to identify unique mechanisms specific to single varieties. It could also be interesting to investigate lines such as SPV475 which exhibit accelerated senescence compared to the intermediate senescing R16, and identify the genes and processes contributing to this extreme phenotype (Thomas and Howarth 2000).

Within the sorghum genome, several regions have been identified as quantitative trait loci (QTLs) that associate to the stay green trait (Sanchez et al., 2002). Genetic variation within these regions correlates to variation in stay green characteristics. Studies using B35 sorghum as a source of stay green consistently identify four major stay green QTLs (Kassahun et al. 2010; Sanchez et al., 2002; Xu et al., 2000b). The thesis by Stephanie Johnson (Johnson, 2015a) maps the transcriptomic changes between B35 and R16 to the known loci of stay green QTLs, and several differentially expressed genes map directly to QTLs, including *P5CS1* located within Stg1. It would be interesting to map the proteomic changes between B35 and R16 to known stay green QTLs. Variation in proteins directly genetically linked to QTLs may represent proteins and processes that directly underlie the trait. Other variable proteins may lie outside of QTLs and be regulated upstream by other QTL located genes (Johnson, 2015a).

This study analysed the variation in proteome between B35 and R16 only under well-watered unstressed conditions. Similarly, the transcriptome comparison between B35 and

R16 in the thesis by Johnson, 2015a is only characterized under well-watered conditions, whilst transcriptome comparison of heat, drought and combined stress have only been analysed in the R16 variety (Johnson et al., 2014). It would be interesting to analyse the difference in transcriptome and proteome under stress conditions between the stay green and senescent line, and identify the genes and processes contributing to the variation in adaptive response before, during and in recovery from the stress. The full proteome of drought tolerant and drought sensitive accessions in several other crop species have been compared under drought stress, including in maize (Benesova et al., 2012), rice (Zang and Komatsu 2007) and apple (Zhou et al., 2015), and have described protein level variation in relation to various processes including lipid metabolism, ROS scavenging, and the UPS. It could also be interesting to characterize the temporal change in protein and gene expression phenotype during developmental and stress-induced senescence, as previously analysed in sorghum leaves of a rapid senescing accession (Wu et al., 2016). This method could be used to identify variation in senescence associated genes/proteins between stay green and senescent lines, and potentially could help to dissect the distinct senescence phenotypes of E36-1 and B35, which display similar stress-induced senescence but divergent developmental senescence phenotypes (Anami et al., 2015; Haussmann et al., 2002; Johnson, 2015a; Thomas and Howarth 2000). Studies could look at changes in the whole transcriptome during senescence, or specific change in genes known to be involved in senescence regulation such as NAC transcription factors (Guo and Gan et al., 2006).

For the functional characterization of *SbSDIR1*, expression of the sorghum gene within wheat limits analysis to the behaviour of *SbSDIR1* specifically within the wheat plant system and genetic background, which may differ to its functioning within sorghum due to different post-transcriptional regulatory mechanisms in wheat, or different structure/expression of downstream target proteins. Transformation of sorghum with an *SbSDIR1* overexpression construct is essential to investigate its role and contribution to stay green within sorghum itself. The sorghum transformation method using particle bombardment is detailed in the paper Liu et al., 2014, and should be re-attempted with *SbSDIR1*, as well as with other candidate genes (Johnson, 2015a).

5.3 Long term experiments and future perspectives

This study focussed on the functional characterization of *SbSDIR1*. This gene was selected for further investigation due to the differential expression of *SbSDIR1* in B35 sorghum

compared to R16 (Johnson, 2015a), and its previously described contribution to the stay green phenotype (Gao et al., 2011; Tak and Mahtre 2013; Xia et al., 2012; Xia et al., 2013; Zhang et al., 2007). However, several other genes have been identified as potential candidates for contribution to the stay green trait in sorghum. For example, several NAC genes have been found to be differentially regulated between B35 and R16, and *Sb10g027100* in particular was strongly upregulated in B35 (Johnson, 2015a). NAC transcription factors have been studied extensively in relation to abiotic stress tolerance, and several have been implicated in senescence regulation (Guo and Gan 2006; Yang et al., 2011; Lee et al., 2012; Sperotto et al., 2009). The Universal Stress Protein (USP) *Sb01g037580* was found to be expressed to higher levels in the StgB introgression line compared to the parent senescent line (Johnson, 2015a), suggesting that this gene may contribute to the stay green phenotype and the genetic variability underlying this QTL. Universal Stress Proteins are ubiquitous in plants and appear to play significant roles in promoting abiotic stress responses (Loukehaich et al., 2012; Nachin et al., 2005). Additionally, the sorghum gene 'Stomatal density and distribution 1' (*SDD1*) was found to be significantly upregulated in B35 compared to R16 (Johnson, 2015a). Overexpression of *ZmSDD1* in maize has been found to improve drought tolerance via reduced stomatal numbers (Liu et al., 2015c), and consequently it has been hypothesised that this sorghum gene could be contributing to the stay green trait, specifically the aspect of transpiration efficiency, through regulation of stomatal density (Johnson, 2015a). Functional characterization of additional candidate genes within sorghum, in comparison to homologues in other species, could broaden our understanding of the genetic basis for the stay green trait within this crop.

The drought tolerance of stay green varieties has partly been attributed to reduced transpirational water loss via stomatal regulation (Borrell et al., 2014a; Vadez et al. 2011; Zhang et al., 2007). Regulation of stomatal conductance as a drought avoidance strategy has been investigated previously in comparisons of stay green and non-stay green sorghum under well-watered conditions (Johnson, 2015a), and in the assessment of the *SbSDIR1* OE lines within this study. However, there are several other drought avoidance strategies in plants associated with plant-water relations and water transport, which could potentially be employed to varying degrees within sorghum varieties, in relation to the stay green trait. For example, enhanced root exploration to maximise root water uptake is a common drought avoidance strategy, and the general drought tolerance of sorghum is associated with its deep rooting system (Borrell et al., 2014a). An aberrant root phenotype has been

observed in *SDIR1* overexpression lines in rice and Arabidopsis (Gao et al., 2011; Zhang et al., 2007), and the loci of QTLs for root nodal angle within sorghum have been found to overlap with stay green QTLs (Borrell et al., 2014a; Mace et al., 2013). Transcriptomic comparison between R16 and B35 is so far restricted to leaf tissue (Johnson et al., 2015b). Future experiments should study the variation in physical root morphology and root tissue gene expression between R16 and B35, or in *SbSDIR1* OE lines, to investigate the potential role for root architecture in conferring drought tolerance in sorghum, in relation to the stay green trait.

Aquaporins are water and solute transport channels vital for water flux control throughout the plant, influencing stomatal conductivity, root hydraulic capacity, and transpiration (Afzal et al., 2016; Moshelion et al., 2015). A potential role for aquaporins in the stay green mechanism within sorghum was previously suggested following the transcriptomic comparison of B35 and R16, in which *PIP2B* isoforms were identified in association with the enriched GO category 'water transport' (Johnson, 2015a). Both plasma membrane intrinsic proteins (PIPs) and tonoplast intrinsic proteins (TIPs) appear to be critical to drought stress response, and multiple *PIP1* and *PIP2* genes in sorghum have been found to be induced by salt and osmotic stress (Hasan et al., 2017; Liu et al., 2014; Liu et al., 2015b). Overexpression of various aquaporin genes in different species has produced contrasting results, with some transgenic plants in Arabidopsis, sorghum, maize and tobacco exhibiting improved drought tolerance and enhanced water retention capabilities (Cui et al., 2008; Hasan et al., 2017; Zhou et al., 2012) whilst rapid water loss following overexpression of Arabidopsis *PIP1* and *PIP2* genes has proved detrimental to drought survival rates (Aharon et al., 2003). Analysis of aquaporin expression levels within B35 and R16, under stressed and non-stressed conditions, could investigate a potential role of these genes in the improved water use efficiency and drought survival within stay green lines.

The proteomic analysis in chapter 3 sought to complement earlier transcriptomic data by analysing actual differences in protein levels between B35 and R16. However, as well as looking at differences in proteins, it could also be interesting to look at the variation in metabolites such as sugars within the two varieties. During drought stress sugars have been shown to be involved in stomatal closure, ROS scavenging, membrane protection, osmotic adjustment, senescence initiation and expression of senescence associated genes (Sami et al., 2016). The stay green trait in sorghum is associated with accumulation of stem

sugar which has been attributed to the maintenance of photosynthetic activity during grain filling which removes the demand on stem assimilate stores (Borrell and Hammer, 2000; Duncan et al., 1981). It could be interesting to look at assimilate partitioning into different sugars in relation to the stay green phenotype. In particular, the gene trehalose-6-phosphate (*TPS*) is upregulated in B35 compared to R16 (Johnson, 2015a), and future experiments could investigate whether this transcript abundance correlates to an increase in trehalose, which has known roles as a compatible solute and in membrane stability (Goddijn and van Dun 1999). Previous overexpression studies for trehalose biosynthesis genes have recorded an improvement in drought tolerance but with only minimal accumulation of trehalose, and it has been suggested that this phenotype may be attributed to changes in signalling (possibly involved the intermediate trehalose-6-phosphate) and sugar-mediated gene expression changes rather than actual trehalose content (Garg et al., 2002; Karim et al., 2007).

5.4 Conclusions

This thesis reports the investigation of the pathways and processes that underlie the stay green trait in sorghum, a phenotype which improves grain yield under water limited conditions. Progress has been made toward conducting a full proteome comparison between the stay green sorghum line B35 and the senescent line R16, and the functional characterization of the sorghum gene *SbSDIR1*. Further investigation into proteomic and transcriptomic variation between these lines and other accessions when under stressed conditions, and functional characterization of additional candidate genes, will broaden our understanding of the mechanisms involved in this drought tolerance strategy. In the future, this knowledge could be utilized in the breeding programmes aimed at improving stress tolerance in important crops.

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Appendix A

Primers used for real time PCR

Primers for the ADP-ribosylation factor (ARF) gene Ta. 2291 used as an endogenous control for wheat was designed by Dr. Mark Skipsey (Durham University).

MS137	TaARFqPCR5'	gggttgtacgagggtcttga
MS138	TaARFqPCR3'	tccagcacgtttgtttcttg

Primers for analysing the transcript level of sorghum *SbSDIR1* in wheat overexpression lines were designed by Stephanie Johnson (Durham University) as described in Johnson et al., 2015b.

SbSDIR1 Fw	ccaaattcgttgctgcgtga
SbSDIR1 Rev	ccctgcatgaattcgcatgg

Primers for analysing the transcript level of *GBF1* in wheat *SbSDIR1* overexpression lines were designed by Sun et al., 2015.

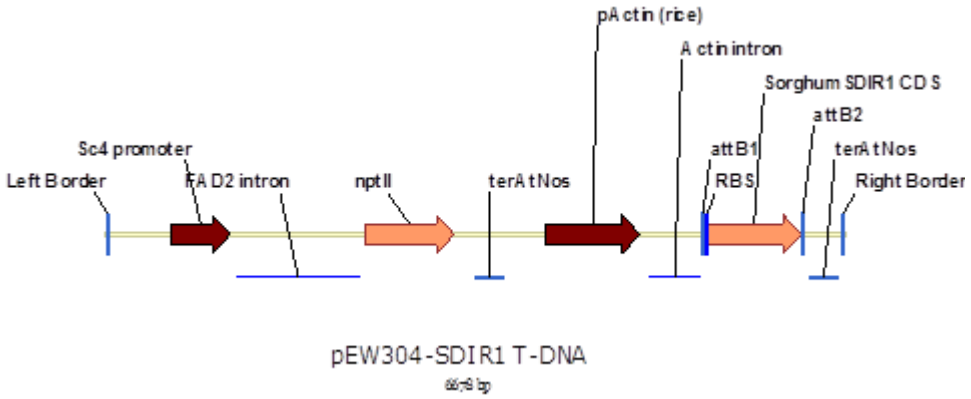
GBF1 Fw	tgagacagaggaattggccacac
GBF1 Rev	caactgctgatttgtccagaggc

Primers for analysing the transcript level of *NAC29* in wheat *SbSDIR1* overexpression lines were designed by Xu et al., 2015.

NAC29 Fw	gacgccggagcagactaccagc
NAC29 Rev	gatctcttcctctccatgccgtt

Appendix B

Stephanie Johnson (Durham University) produced a construct for overexpressing the sorghum gene *SbSDIR1* (Sb01g039740.1) using a rice actin promoter, see map below courtesy of Dr Emma Wallington, at NIAB (Huntington Road, Cambridge, CB3 OLE).



Dr Emma Wallington conducted agrobacterium-mediated transformation of Fielder wheat (*Triticum aestivum L.*) with the overexpression construct (pEW304-*SbSDIR1*). The table below lists the names and qPCR copy number of the 36 transformed lines, with the 14 lines used in chapter 4 highlighted in yellow. From these 14 lines, numbers 81.5, 81.20, 81.21 and 81.22 were selected for further analysis. The copy number data is based on the selectable marker nptII.

Plant number	QPCR copy number
81.1	4+
81.2	1
81.3	4+
81.4	3
81.5	2
81.6	4+
81.7	4+
81.8	4+
81.9	4
81.11	3

81.12	3
81.13	4+
81.14	4+
81.15	4+
81.16	4+
81.17	4+
81.18	1 or 2
81.19	1
81.20	1 or 2
81.21	3 or 4
81.22	2 or 4
81.23	4+
81.24	4+
81.26	4+
81.27	4
81.28	4+
81.29	4+
81.30	4+
81.31	4+
81.32	2
81.33	4+
81.con1	0
81.con2	0
81.con3	0
82.1	4+
82.2	4+
82.3	4+
82.6	2 or 3
82.7	4+
82.con1	0