Regulation of Cyanogenic Glucosides in *Sorghum bicolor*

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Abstract

Sorghum bicolor (L.) Moench is the fifth most important cereal crop grown worldwide, used for grain, animal forage, and biofuel. The C_4 crop's ability to withstand drought and heat cements its suitability for livestock forage in the dry tropics. However, a significant issue inherent in sorghum usage is its production of the cyanogenic glucoside dhurrin. Upon tissue disruption dhurrin is hydrolysed to release hydrogen cyanide gas, which is toxic to livestock when in high concentrations. Toxicity of the crop is often difficult to predict as dhurrin concentrations are regulated spatially and temporally, and in response to environmental factors such as drought and nitrogen fertilisation. In addition to its role in plant defence dhurrin may also be involved in nitrogen remobilisation and stress mitigation. Consequently, reducing or removing dhurrin from the plant may have detrimental effects on growth rate and stress tolerance.

The aim of this thesis is to explore the regulation of dhurrin concentration in the vegetative tissues of *Sorghum bicolor* from the molecular to physiological level. I first examined the interplay between the developmental and environmental regulation of dhurrin during drought in wild-type plants and a low-cyanogenic EMS-mutant *adult cyanide deficient class 1*. The effect of altered dhurrin concentration on stored nitrates was also analysed. I then investigated the potential epigenetic regulation of the dhurrin biosynthetic genes via genome-wide demethylation using the demethylating agent 5-Azacytidine. Finally, a yeast one-hybrid screen was used to identify candidate regulators of the key dhurrin biosynthetic gene, *CYP79A1*. A greater understanding of the regulation of dhurrin biosynthesis in sorghum aims to enable accurate toxicity predictions and the ability to manipulate dhurrin concentration in the crop in ways that do not compromise yield or stress tolerance.

General declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one original paper published in a peer reviewed journal and one submitted for publication. The core theme of the thesis is the regulation of the cyanogenic glucoside dhurrin in *Sorghum bicolor*. The ideas, development and writing of all chapters in this thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of Professor Ros Gleadow.

The inclusion of co-authors reflects that the work came from active collaboration between researchers and acknowledges input into team-based research.

In the case of chapters 2 and 3 my contribution to the work is as follows:

Thesis Chapter	**Publication Title**	**Status**	**Nature and % of student contribution**	**Co-authors**	**Co-authors, Monash student Y/N**
2	The interplay between water limitation, dhurrin and nitrate in the low-cyanogenic sorghum mutant *adult cyanide deficient class 1*	Submitted (*Frontiers in Plant Science*)	90%	Cecilia K. Blomstedt Birger Lindberg Møller Trevor Garnett Ros Gleadow	N
3	Investigation into the role of DNA methylation in cyanogenesis in sorghum (*Sorghum bicolor* L. Moench)	Published (*Plant Growth Regulation*)	80%	Alicia A. Quinn Samantha M. Fromhold Ros Gleadow Cecilia K. Blomstedt	N

This thesis also includes one chapter in preparation for submission to a peer-reviewed journal. In the case of chapter four my contribution to the work is as follows:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution	Co-authors, Monash student Y/N
4	The transcription factor *Sb*GATA22 as a regulator of *CYP79A1* in *Sorghum bicolor* (L.) Moench	In Preparation (*PLOS ONE*)	90%	Ros Gleadow Cecilia K. Blomstedt	N

I have re-numbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: **Date:**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor signature: **Date:**

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Conference presentations

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Chapter 1

Introduction

1.0 General introduction

In the presence of global climate change and a rapidly increasing population, investments in worldwide food security are not just a priority, but an urgent requirement. This is particularly pertinent in food insecure regions: parts of Africa, the Americas, and Southern Asia where, collectively, more than 800 million people are still undernourished (FAO et al., 2018). An important part of mitigating this is to increase the production of crops that are able to maintain a high yield in response to environmental challenges; principally drought and increased temperatures (Godfray et al., 2010, Zougmoré et al., 2016, Hadebe et al., 2017).

One such crop is sorghum (*Sorghum bicolor* (L.) Moench), which is highly adapted to semi-arid climates and suitable for growth on many of the world's continents. Sorghum, however, can be toxic when used as animal forage because of the production of the cyanogenic glucoside dhurrin. When plant cellular integrity is disrupted via mastication by herbivores, dhurrin is hydrolysed to release hydrogen cyanide gas (Kojima et al., 1979, Dunstan and Henry, 1902). Toxic concentrations of dhurrin arise in sorghum predominantly when plants are young or exposed to environmental factors such as chronic drought or high levels of nitrogen-based fertiliser (Wheeler et al., 1990, Busk and Møller, 2002). This review discusses the biosynthesis, catabolism, and turnover of cyanogenic glucosides; their regulation during development and in response to biotic and abiotic factors; and the molecular and epigenetic regulation of the cyanogenesis genes, all in the context of sorghum as a forage crop.

1.1 Sorghum

Sorghum is the fifth most important cereal crop grown worldwide; used for human consumption, animal fodder and forage, and more recently for biofuel (Srinivasa Rao et al., 2009). Originating in Africa, it is believed to have undergone domestication at two different sites on the continent more than six thousand years ago. Subsequently, sorghum became an agricultural crop in Asia spreading via trade routes before becoming established in the Americas and Australia (Kimber, 2000). Today sorghum is a staple food source across Africa, with the grain used for both human and animal consumption and the plants utilised for forage. Some of the largest producers of sorghum worldwide include Nigeria, USA, Mexico, Argentina, Australia, India and

Pakistan; while production for biofuel is on the rise in both the USA and Germany (http://www.fao.org/faostat/en accessed 26 January 2019). It is estimated that over 50% of sorghum acreage worldwide is used for animal feed (Etuk et al., 2012).

The widespread reliance on sorghum throughout many countries is a result of its adaptation for growth in semi-arid and sub-tropical environments, out-performing other major crop species (Dicko et al., 2006, Schittenhelm and Schroetter, 2014). Sorghum, as a C_4 plant sorghum uses a more efficient carbon fixation mechanism than other (C_3) plants, minimising photorespiration by separating the initial CO_2 fixation and the Calvin cycle into different tissues (Collatz et al., 1992). This results in reduced water loss and increased photosynthetic efficiency. In addition, sorghum has an extensive root system and a thick, waxy coating on the shoot tissue that further increases its ability to grow in semi-arid environments (Ludlow and Muchow, 1990). As well as its ability to tolerate hot, dry conditions, sorghum has a high nitrogen-use efficiency when nitrogen is limited and can therefore be grown on marginal lands that are unsuitable for wheat, rice, maize and barley (Massel et al., 2016).

1.2 Cyanogenesis

Despite many positive aspects of sorghum as a crop species, one significant issue which arises when it is used as animal forage is the production of dhurrin, a cyanogenic glucoside (Dunstan and Henry, 1902). Cyanogenesis, the release of hydrogen cyanide gas (HCN) by living organisms, has been documented in over 2,500 species of flowering plants, with approximately one fifth of crop species categorised as cyanogenic including barley, oats, rye, almonds, and apples (Jones, 1998). It is also known to occur in insects, bacteria, and non-flowering plants such as cycads. Cyanogenic glucosides are derived from amino acids and enable a cyanide group to be stably stored as a non-toxic compound via glycosylation of a cyanohydrin (Møller, 2010). As there are currently over 60 cyanogenic glucosides identified, the exact compound produced by each species often varies (due to different amino acids and sugars used in the biosynthetic pathways), although all can ultimately be broken down to release HCN (Takos et al., 2011)(Figure 1).

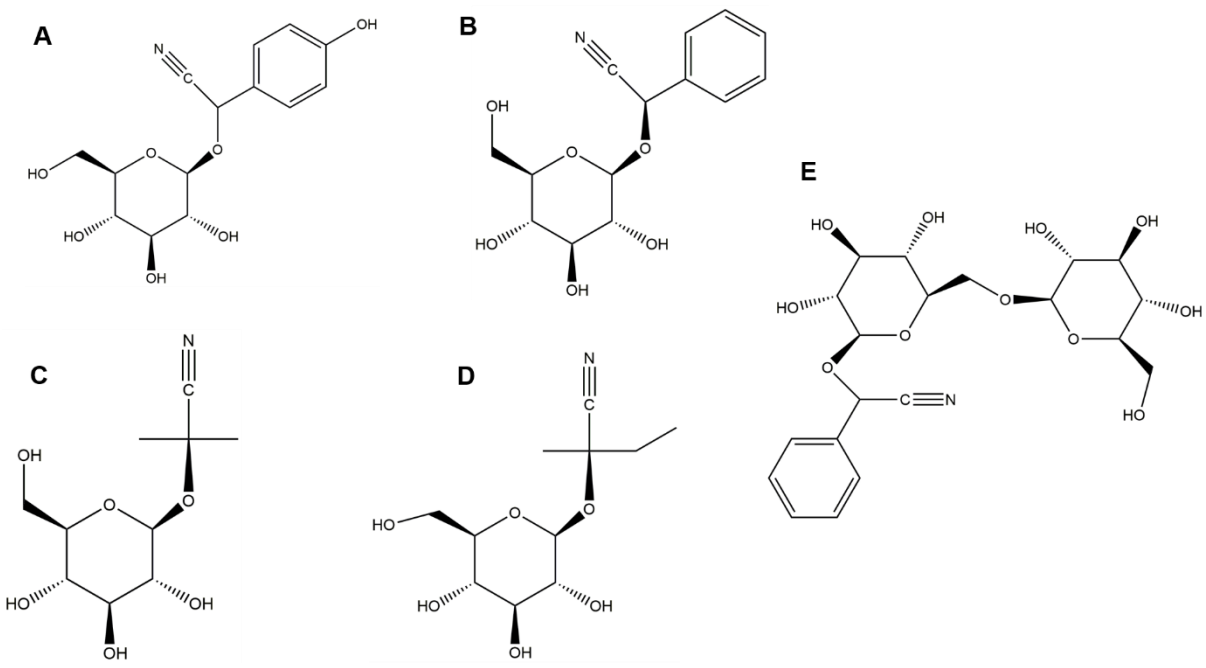

Figure 1: Structures of common cyanogenic glucosides **A)** Dhurrin derived from tyrosine and found in sorghum; **B)** Prunasin derived from phenylalanine and found in eucalypts; **C)** Linamarin derived from valine; and **D)** Lotaustralin derived from isoleucine with both C) and D) found in cassava; and **E)** Amygdalin: a diglucoside derived from prunasin and found in almonds (adapted from Gleadow and Møller, 2014)

The breakdown of cyanogenic glucosides to release HCN is a two-step process (Poulton, 1990). The first involves the cleavage of the sugar molecule producing an α-hydroxynitrile, which is then catabolised to produce HCN, and either a ketone or aldehyde (depending on whether the amino acid from which it is derived is aliphatic or aromatic). In high enough concentrations this will result in cyanide poisoning and even death in aerobic eukaryotes as HCN binds to cytochrome c oxidase in the mitochondria, inhibiting cellular respiration (Holland and Kozlowski, 1986). This process can deter generalist herbivores, although cyanogenic glucosides are not always effective as defence compounds with some animals immune to the effects of HCN, or preferring to ingest cyanogenic plants (Jones, 1998, Zagrobelny et al., 2004, Gleadow and Woodrow, 2002a).

Dhurrin, the cyanogenic glucoside synthesised by sorghum, is highly stable. Originally thought to be stored in the vacuole, new research using spectroscopic methods determined that in young seedlings it is stored within the cell walls and cytoplasm of the epidermal, cortical and vascular tissues (Heraud et al., 2018). To avoid unwanted breakdown and self-toxicity the catabolic enzyme (β-glucosidase) remains compartmentalised in the cytoplasm of the mesophyll cells (Kojima et al., 1979, Thayer and Conn, 1981). Upon tissue disruption (such as during mastication by herbivores) β-glucosidase comes into contact with dhurrin and HCN is released (Conn, 1980).

Livestock, particularly ruminants such as cattle and sheep, are particularly vulnerable to HCN poisoning as microbial fermentation of cyanogenic glucosides in the rumen will also cause HCN release (Vough and Cassel, 2006). Hydrogen cyanide potential (HCNp - the total amount of HCN evolved from hydrolysis of the entire content of endogenous cyanogenic glucosides) of 600ppm dry weight or higher is toxic to grazing animals, although the size of the animal and rate of ingestion can affect this (Stuart, 2002). Forage sorghum pastures have been recorded to have HCNp as high as 750ppm dry weight of HCN, making them unsuitable for grazing (Duncan, 1996). Sorghum hay is equally as toxic as forage and can pose an even greater risk of poisoning, as livestock will ingest it more rapidly (Wheeler and Mulcahy, 1989).

1.3 Biosynthesis and metabolic channelling

Overall, cyanogenesis at the biochemical level is a well understood process with the biosynthesis, catabolism and detoxification pathways all having been characterised (Gleadow and Møller, 2014) (Figure 2). In sorghum, dhurrin biosynthesis begins from an L-tyrosine precursor and involves three biosynthetic enzymes: two cytochromes P450, CYP79A1 and CYP71E1, and one NADPH-dependent UDP-glucosyltransferase, UGT85B1 (Bak et al., 1998, Sibbesen et al., 1995, Kahn et al., 1997, Koch et al., 1995, Kahn et al., 1999). These are encoded by three structural genes which are clustered on chromosome 1 in sorghum (Takos et al., 2011). In the first step of biosynthesis, CYP79A1 N-hydroxylates L-tyrosine twice, where the oxime intermediate formed is (Z)-*p*-hydroxyphenylacetaldehyde oxime, before this is converted by CYP71E1 to *p*-hydroxymandelonitrile. The initial step involving CYP79A1 is considered the rate-limiting step in the pathway (Kahn et al., 1999). Following these reactions, the UDP-

glycosyltransferase then converts the *p*-hydroxymandelonitrile to dhurrin via glycosylation, creating a stable compound (Reay and Conn, 1974, Busk and Møller, 2002).

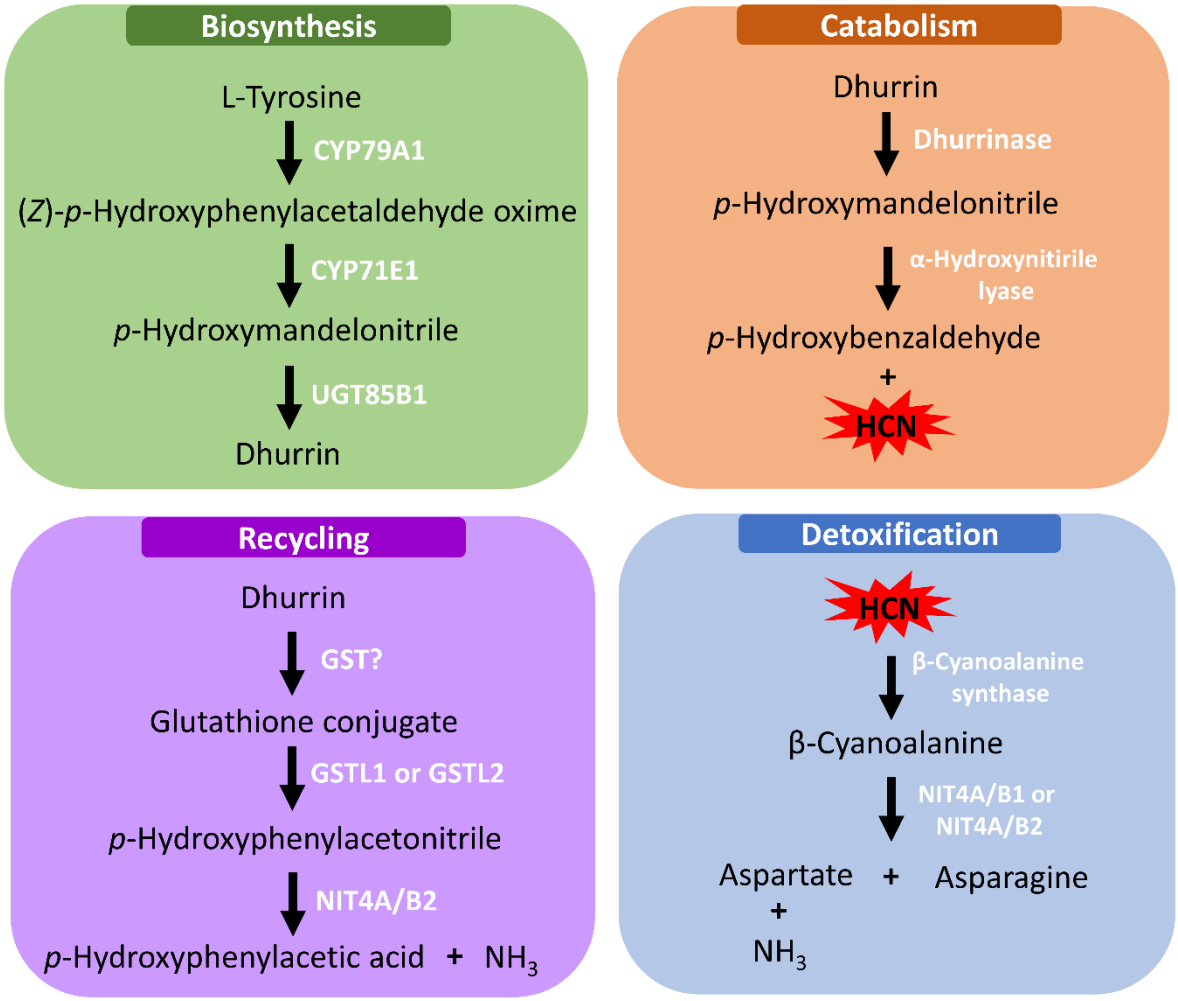

Figure 2: The dhurrin biosynthesis, catabolism, detoxification, and recycling pathway that occurs without the release of HCN (adapted from Pičmanová et al., 2015; Bjarnholt et al., 2018; Jenrich et al., 2007; and Gleadow and Møller, 2014). GST = glutathione transferase; GSTL = glutathione transferase of the lambda class (Bjarnholt et al., 2018); NIT = nitrilase (Jenrich et al., 2007).

In sorghum the three dhurrin biosynthetic enzymes form a metabolon allowing for improved metabolic efficiency (Nielsen et al., 2007, Laursen et al., 2016). A metabolon is a multi-enzyme complex, comprised of enzymes and structural components involved in a specific pathway. The metabolon in sorghum is anchored to the cytosolic surface of the endoplasmic reticulum membrane via the two cytochromes P450, and their redox partner the NADPH-dependent cytochrome P450 oxidoreductase (POR) which activates them (Laursen et al., 2016). Here, the metabolon works to increase the enzyme activity at the molecular level as the active sites of each of the enzymes are near to one another, facilitating metabolic channelling (Jørgensen et al., 2005b, Winkel, 2004). Furthermore, the metabolon prevents intermediate compounds from diffusing away from the enzymes and potentially undergoing degradation. This is the case in sorghum where the metabolon allows for UGT85B1 to rapidly convert the toxic (S)-*p*-hydroxymandelonitrile intermediate to dhurrin, preventing its potential breakdown to HCN which would result in self-damage to the plant (Jørgensen et al., 2005b).

Integrity of the metabolon tertiary structure is vital in sorghum, with even minute changes altering the synthesis of dhurrin (Nielsen et al., 2007). This has been observed in the binding of fluorescent proteins to the CYP79A1 and CYP71E1 enzymes, significantly lowering dhurrin production (Nielsen et al., 2007). The reduction in dhurrin production was thought to be due to the large size of the fluorescent proteins altering the tertiary structure of the metabolon and affecting the channelling of substrates between enzymes, rather than a direct effect on the active sites of the proteins.

1.4 Dhurrin catabolism, turnover, and transport

All plants produce cyanide as a by-product of ethylene biosynthesis, from which it is released in stoichiometrically equivalent amounts to ethylene (Peiser et al., 1984). To prevent self-toxicity HCN can be detoxified by nitrilase enzymes which convert the gas to asparagine (Jenrich et al., 2007, Kriechbaumer et al., 2007, Park et al., 2003) (Figure 2). In cyanogenic plants this pathway is also employed when HCN is released as a herbivore defence. However, dhurrin catabolism in sorghum is as high as 35% of the biosynthetic rate, greater than what would be expected if turnover were only occurring upon tissue disruption (Adewusi, 1990). Due to the high amount of dhurrin turnover in

sorghum it has been postulated that products resulting from dhurrin catabolism may be used to transport and release reduced nitrogen for primary metabolism.

Jenrich et al. (2007) proposed an endogenous turnover pathway in sorghum, allowing dhurrin to be catabolised without the release of HCN, thereby avoiding any potential self-toxicity. The pathway, which ultimately results in the release of 4-hydroxyphenylacetonitrile and ammonia, is achieved via nitrilase enzymes (Jenrich et al., 2007, Møller, 2010). Not only does this pathway allow for dhurrin to be turned over without the production of HCN, but the ammonia released can be accessed immediately for primary metabolism. More recently Pičmanová et al. (2015) investigated further the potential endogenous turnover pathways of cyanogenic glucosides. This involved searching for putative turnover products in the phylogenetically unrelated cyanogenic species: cassava (*Manihot esculenta*), almond (*Prunus dulcis*), and sorghum (*Sorghum bicolor*). From this, a pathway was proposed that results in the release of ammonia and carbon dioxide; a pathway different from that of Jenrich et al. (2007). This pathway allows for the recycling of both nitrogen and carbon. In addition, Bjarnholt et al. (2018) found that prior to the involvement of nitrilase enzymes the recycling of dhurrin requires glutathione transferases which cleave glutathione conjugates that are formed from dhurrin (Figure 2).

The function of cyanogenic glucosides in primary metabolism was observed in the cyanogenic species cassava, where the production of low cyanogenic and acyanogenic lines resulted in large reductions in growth unless the plants were grown in a high nitrogen environment (Jørgensen et al., 2005a). This indicated a significant role for cyanogenic glucosides in the primary metabolism of the species. The acyanogenic sorghum line *totally cyanide deficient 1* (*tcd1*) also exhibited a reduced growth rate during the early stages of growth in comparison to wild-type lines (Blomstedt et al., 2012, Blomstedt et al., 2018). These observations suggested that dhurrin was used for nitrogen (and possibly carbon) storage and remobilisation, thus not only balancing the costs of primary metabolism (plant growth) with secondary metabolism (plant defence), but also inextricably linking the two (Jenrich et al., 2007, Neilson et al., 2013, Gleadow and Møller, 2014).

Transport of dhurrin has not been confirmed in sorghum; however Selmar et al. (1996) determined that the diglucoside dhurrin-6'glucoside was present in leaf guttation droplets. Diglucosides allow for stable transport of HCN without the risk of self-toxicity in plants and therefore suggest the transport of dhurrin between tissues. Transport of cyanogenic glucosides is not uncommon among cyanogenic species. Clegg et al. (1979) found evidence that wild lima beans (*Phaseolus lunatus*) transport the cyanogenic glucoside linamarin intact throughout the plant. This was based on the finding that the concentrations within specific tissues changed throughout development, while the overall concentration of linamarin remained unaltered. Jørgensen et al. (2005a) established the production of cyanogenic glucosides in cassava as occurring predominantly in the leaves before being transported to the tuberous roots. Rubber trees (*Hevea brasiliensis*) utilise cyanogenic glucoside transport by converting the cyanogenic monoglucoside linamarin to the diglucoside linustatin, which moves from the endosperm to the seedling where it is cleaved and the resultant HCN reintegrated into non-cyanogenic compounds (Selmar et al., 1988). The transport of cyanogenic glucosides suggests that it is advantageous to have specific tissues designated to higher production of defence compounds, with differences between species being likely due to different evolutionary pathways.

Current research continues to find that cyanogenic glucosides appear to be used by cyanogenic species for physiological functions other than defence (Møller, 2010, Gleadow and Møller, 2014). These functions include roles in osmoprotection (Burke et al., 2015), ROS scavenging (Sendker and Nahrstedt, 2009), germination and bud burst (Barros et al., 2012, Richmond and Ghisalberti, 1994), and nitrogen storage, transport and turnover (Pičmanová et al., 2015, Bjarnholt et al., 2018). Dhurrin has also been linked to the stay-green phenotype in sorghum (Hayes et al., 2015b, Emendack et al., 2017). Additionally, the hydrogen cyanide released by cyanogenesis can act as a signalling molecule by affecting the post-translational modification of proteins (García et al., 2019).

1.5 Ontogenic and environmental regulation of cyanogenic glucosides

Concentrations of cyanogenic glucosides are highly dynamic in cyanogenic plant species. Production of the specialised metabolites change throughout development, often being highest when plants are young before decreasing with maturity, although the opposite pattern is also observed (Goodger et al., 2006). Hydrogen cyanide potential is also affected by biotic and abiotic factors, which will differentially affect the HCNp of above and below ground tissues (O'Donnell et al., 2013, Blomstedt et al., 2018, Miller et al., 2014).

1.5.1 Developmental regulation

Hydrogen cyanide potential is typically highest in young plants, or the young tissue of mature plants. This occurs across many species, including but not limited to sorghum, cassava, grapevines, flax, and some species of eucalyptus such as *Eucalyptus cladocalyx* (Gleadow and Woodrow, 2000, Jørgensen et al., 2005a, Franks et al., 2005, Krech and Fieldes, 2003, Gleadow et al., 2016). The high concentration of cyanogenic glucosides in young plants and tissues allows for increased protection against generalist herbivores when the plants are most vulnerable (Gleadow and Woodrow, 2002a). The subsequent reduction in cyanogenic potential upon maturity and establishment allows for more energy to be used directly for primary metabolism. However, there are species that do not follow this pattern including *Eucalyptus polyanthemos* and *Eucalyptus yarraensis* with seedlings having up to 70% lower cyanogenic potential than mature trees (Goodger et al., 2006).

In sorghum the mature seed is the only part of the plant that is completely acyanogenic (Akazawa et al., 1960, Loyd and Gray, 1970, Nielsen et al., 2016). Upon germination, the concentration of dhurrin rapidly increased in all vegetative tissues, peaking when the plant was approximately two to four days old at which stage the breakdown of dhurrin became equal to the rate of biosynthesis (Akazawa et al., 1960, Halkier and Møller, 1989, Busk and Møller, 2002, Adewusi, 1990). In sorghum, dhurrin concentrations are closely linked to ontogeny with dhurrin decreasing as leaf number increases (Gleadow et al., 2016, Blomstedt et al., 2018, Miller et al., 2014). Root tissue had significantly lower amounts of dhurrin than the above ground tissues; and despite an initial decrease

in HCNp in these tissues, dhurrin concentrations generally remained static in the roots as plants matured (O'Donnell et al., 2013, Blomstedt et al., 2018).

1.5.2 Environmental regulation

Drought

Water deficits are known to increase the concentration of secondary metabolites in many species and across taxa (Selmar and Kleinwächter, 2013) including dhurrin in sorghum (Neilson et al., 2015, Gleadow et al., 2016, Emendack et al., 2018). However, whether dhurrin increases or decreases may depend on whether water limitation is acute or chronic (Wheeler et al., 1990, Emendack et al., 2018). O'Donnell et al. (2013) addressed this question by growing sorghum at different levels of osmotic stress using polyethylene glycol (PEG). Plants grown at higher concentrations of PEG had significantly higher concentrations of dhurrin in the shoots, though this increase was not observed in the roots. Upon moving the plants to an environment lacking PEG, shoot dhurrin concentrations were found to decrease to a concentration comparable to non-stressed individuals (O'Donnell et al., 2013). Moaveni (2010) also observed an increase in dhurrin concentration in various lines of sorghum grown in the field under differing levels of irrigation. Furthermore, high dhurrin concentrations have been linked to post-flowering drought tolerance (Burke et al., 2013) and the stay-green phenotype (Hayes et al., 2015b) in sorghum.

Increases in cyanogenic glucosides in relation to drought have also been seen in cassava (Cliff et al., 2011, Brown et al., 2016). Vandegeer et al. (2013) found that droughted cassava displayed a 2.9- and 4-fold higher cyanogenic potential in the leaves and roots, respectively. *E. cladocalyx* also increased the concentration of cyanogenic glucosides present in mature leaves in water-limited environments by up to 70%; particularly when high levels of nitrogen were available (Gleadow and Woodrow, 2002b).

Despite phenotypic plasticity in the response to drought being well documented, the regulation of these changes in sorghum is not well understood. Increases in dhurrin concentration may be a primary response, where limited water availability causes an up-regulation of the biosynthetic pathway. Alternatively, it could be attributed to a concentration effect where growth rate is reduced in the presence of drought while

dhurrin production remains constant. O'Donnell et al. (2013) observed no difference in the total amount of dhurrin in osmotically stressed plants compared to the control treatment, attributing at least part of the HCNp to a concentration effect.

Nitrogen

Nitrogen applications are known to increase the cyanogenic potential of sorghum grown in the field (McBee and Miller, 1980, Wheeler et al., 1990, Wheeler et al., 1980). However, this response was only seen in older plants, with sorghum seedlings up to eight days old showing no change in dhurrin concentrations regardless of nitrogen application (Busk and Møller, 2002). This is likely due to expression of the biosynthetic genes already being at a maximum level during these early growth stages. At five weeks old, nitrogen fertilisation did elicit a response with a seven-fold increase in cyanide potential of whole plants compared to plants watered with potassium chloride or plain water (Busk and Møller, 2002). This increase was seen with the application of 25mM potassium nitrate when plants were grown in either soil or water and correlated with increased *CYP79A1* expression. Hayes et al. (2015a) used a genome-wide association study across 700 lines of sorghum to gain insight into the genetic control of dhurrin concentration. In nitrogen-fertilised environments dhurrin concentration was associated with transcript abundance levels representing dhurrin biosynthetic genes, also suggesting that nitrogen application increased the biosynthetic gene expression. Blomstedt et al. (2018) only observed an increase in leaf HCNp in response to high nitrogen when plants were close to eight weeks old, despite high nitrogen treatments being applied for up to five weeks prior to sampling. High nitrogen has also been found to exacerbate the effects of chronic drought stress in relation to HCNp (Neilson et al., 2015).

A study similar to that of Busk and Møller (2002) was performed in cassava, where plants supplied with 25mM potassium nitrate for seven days had a five-times higher cyanogenic potential than those watered with water or potassium chloride (Jørgensen et al., 2005a). *E. cladocalyx* also increased prunasin concentration in response to nitrogen (Gleadow et al., 1998), while barley grown in low nitrogen environments decreased cyanide potential by up to 75% in comparison to control plants (Forslund and Jonsson, 1997). The correlation between nitrogen fertilisation and increases in

cyanogenic glucoside concentration provides further evidence that these defence molecules are acting as nitrogen storage compounds and, when required, are catabolised to release reduced nitrogen for use in primary metabolism.

Herbivory and pathogens

The primary function of cyanogenic glucosides has long been thought to be defence, with their presence found to deter generalist herbivores (Tattersall et al., 2001, Dunstan and Henry, 1902). However, some specialist herbivores such as the burnet moth (*Zygaena filipendulae*) display a preference for cyanogenic plants (Zagrobelny and Møller, 2011). This is because the moth sequesters the cyanogenic glucosides linamarin and lotaustralin from their food plant, *Lotus corniculatus,* saving energy on endogenous production of the compounds which they use as a defence mechanism to deter predators. The sequestering of cyanogenic glucosides is predominantly seen in butterflies and moths (Erb and Robert, 2016), though some species have evolved the ability to synthesise them *de novo* (Zagrobelny et al., 2018).

Recognition of herbivore attack can commence as early as the mechano-stimulation of an insect landing on the leaves of a plant (Chehab et al., 2009), though commonly it is the mechanical damage via the chewing mouthparts of insects that is the first signal to be detected (Howe and Jander, 2008). This disruption of tissue breaks down the compartmentalisation of cells, which in cyanogenic species leads to release of toxic HCN gas, as well as initiating other defence responses. Defence pathways in plants are largely regulated by jasmonates, with specificity to particular herbivores possibly being achieved via the addition of other phytohormones, such as abscisic acid or salicylic acid (Erb et al., 2012).

Cyanogenic glucosides are classified as anticipins and are therefore expressed constitutively rather than being induced in response to herbivory (Gleadow and Møller, 2014). In the invasive English laurel (*Prunus laurocerasus*) leaf damage did not affect the concentration of cyanogenic compounds (Kautz et al., 2017), though some types of mechanical wounding in sorghum has been found to significantly increase dhurrin concentration (O'Donnell, 2012). Using cDNA microarray analysis Zhu-Salzman et al. (2004) investigated gene expression in sorghum in response to phloem feeding

greenbugs (*Schizaphis graminum*) and compared this to exogenous hormone treatment with methyl jasmonate or salicylic acid. The results identified five genes where expression was upregulated and three that were downregulated across all three treatments as suggested by transcript abundance. *CYP71E1* transcripts increased in abundance in response to greenbug attack at six- and twelve-hours following infestation, with methyl jasmonate increasing *CYP71E1* transcript abundance up to 48 hours post-infestation. Transcripts of the β-glucosidase, dhurrinase, did not increase in response to the greenbugs, though an increase (peaking at 48 hours) was seen when methyl jasmonate was applied. No increase was observed in transcript levels of *CYP79A1* (the rate-limiting enzyme in the dhurrin biosynthetic pathway) for any of the treatments. The lack of response may have been due to the use of young plants in the study and therefore the transcript abundance was already at a maximum. The study suggests that in sorghum the response to greenbug infestation may be mediated by the hormones methyl jasmonate and salicylic acid. This indicated that the dhurrin biosynthetic pathway can be upregulated following herbivore attacks even if the herbivores are sucking insects that do not disrupt tissue integrity. However, further research needs to be undertaken to address the molecular response of sorghum to herbivory by mammals and chewing insects.

In sorghum the relationship between cyanogenic potential and fungal pathogens differs from that of herbivore attack. Mizuno et al. (2012) performed global transcriptomic analyses of sorghum plants infected with the necrotrophic fungus *Bipolaris sorghicola*, finding that in infected tissues transcripts for all three of the biosynthetic genes, *CYP79A1*, *CYP71E1*, and *UGT85B1*, declined by up to 46%. A decrease in dhurrin degradation was also seen, with the transcripts for the dhurrinase genes declining, suggesting that both the biosynthetic and degradation pathways are reduced during fungal infection. This is may be due to the fact HCN inhibs antimicrobial phytoalexins from being produced, with the release of HCN potentially causing greater damage to the host plant than the pathogen (Mizuno et al., 2012). This has also been seen in other species of fungi that preferentially infect plants with higher cyanogenic potential due to their decreased concentrations of phytoalexins (Møller and Seigler, 1999). Lieberei (1988) observed this in rubber trees where clones with higher cyanogenic potential were more susceptible to infection by *Microcyclus ulei*. Ballhorn et al. (2010) observed

similar results in lima bean cultivars infected with *Colletotrichum gleosporioides*, with leaves of the three highly cyanogenic lines becoming significantly more damaged by the fungus than the three low cyanogenic lines. These results were attributed to a decrease in polyphenol oxidase (PPO) activity in the highly cyanogenic cultivars, with HCN likely inhibiting the activity of PPOs - compounds known to be involved in pathogen defence responses (Campos et al., 2004). Findings such as these indicate that there can be a trade-off between defence traits, with those that provide resistance to herbivory increasing the susceptibility of the plant to a fungal pathogen.

Light

Though yet to be investigated in sorghum, light intensity was determined to affect the regulation of cyanogenic glucosides in vegetative tissues of other species. Cassava exhibited diurnal variation of cyanogenic glucosides with levels in the leaves and stem tissues highest during the afternoon (Okolie and Obasi, 1993). This correlated with decreases in rhodanese, the enzyme that detoxifies HCN. In contrast to the leaves and stem, cassava tubers exhibited no diurnal variation in the concentration of cyanogenic glucosides or rhodanese. Schmidt et al. (2018) found that transcripts of the cyanogenic glucoside genes in cassava increased in abundance during the night and decreased during the day, reaching a minimum level by 9:00pm. Protein levels of the biosynthetic enzymes also increased during dark periods. The cyanogenic glucoside linamarin followed this pattern of increasing during the night and was also found to decrease during solar radiation spikes during the day. It was hypothesised that the turnover of linamarin during light periods was to provide reduced nitrogen to balance photosynthetic carbon fixation (Schmidt et al., 2018). Contrary to this, shade caused *Prunus turneriana* to redistribute cyanogenic glucosides to old, expanded and photosynthetically productive leaves (Miller et al., 2004). Shade also resulted in a decrease in the concentration of the cyanogenic glucoside prunasin in *E. cladocalyx* (Burns et al., 2002), though in black cherry (*Prunus serotina*) plants grown in low light had the highest root prunasin concentrations (Robakowski et al., 2016). It was postulated that plants grown in shaded conditions are disproportionately attacked by insects and nematodes, and therefore invest more resources into defence compounds (Robakowski et al., 2016, Karolewski et al., 2013).

The environmental factors that affect cyanogenic potential in sorghum, drought, nitrogen fertilisation, and wounding, are all stress factors to which field-grown sorghum is regularly exposed. This is particularly relevant as sorghum is largely produced in areas prone to inconsistent rainfall and drought, specifically because of its ability to withstand such climates. If water stress occurs or fertiliser is applied, often farmers are unable to accurately predict the toxicity levels of their crops and, consequently, their safe use as forage. This is confounded by variation in the concentration of cyanogenic glucosides between sorghum lines and individual plants, and the developmental plasticity of dhurrin synthesis (O'Donnell et al., 2013, Emendack et al., 2018, Hayes et al., 2015a). Testing of cyanogenic potential is largely unavailable in countries where sorghum is a staple food source; and because of the increase in dhurrin often being transient, results once obtained are frequently no longer relevant. Importantly, understanding how dhurrin synthesis is regulated at the molecular level is aimed at the ability to accurately predict the response of sorghum to the environment and therefore helps to alleviate livestock poisoning and pasture wastage.

1.6 Molecular regulation and manipulation of cyanogenesis

The publication of the annotated genome sequence of *Sorghum bicolor* (genotype BTx623) by Paterson et al. (2009) enabled the molecular regulation of cyanogenesis in this species to be explored. The clustering of all three dhurrin biosynthetic genes on chromosome one (1040kb-1170kb) was discovered, similar to other secondary metabolite clusters in cassava and *Lotus japonicus* (Takos et al., 2011)(Figure 3). There is still a lack of understanding of the underlying molecular mechanisms regulating these gene clusters, despite knowledge of the genomic sequence and an understanding of cyanogenesis at the biochemical and physiological level.

Busk and Møller (2002) determined that dhurrin synthesis was controlled at the transcriptional level in sorghum as the expression levels of the dhurrin biosynthetic genes reflect dhurrin concentrations during early development. This was further supported by Nielsen et al. (2016) who established that transcript levels of the dhurrin biosynthetic genes correlated with dhurrin concentrations in the developing grain of sorghum. This is consistent with specialised metabolites frequently being controlled at the transcriptional level in plants (Colinas and Goossens, 2018). In relation to

environmental regulation, Buchanan et al. (2005) found through transcriptome analysis that drought stress altered the transcript abundance of more than 1,000 genes in sorghum, many of which were involved in defence pathways. Dugas et al. (2011) also saw correlations between osmotic stress and expression of defence genes. However, the precise signalling pathways linking ontogeny and environmental factors to changes in HCNp remain unknown.

In sorghum, hydrogen cyanide potential of leaf, sheath, and root tissues does not always equate to the activity of the biosynthetic enzymes, with transport of dhurrin throughout the plants appearing to account for these anomalies (Halkier and Møller, 1989, Busk and Møller, 2002). From sectioning eight-centimetre-tall etiolated seedlings into one- and five-millimetre segments Halkier and Møller (1989) determined that the biosynthetic enzyme system is predominantly located in the upper section of the seedling and absent from the lower section and the root. In five-week old sorghum plants Busk and Møller (2002) failed to detect any CYP79A1 or CYP71E1 activity in the leaves, sheaths, or roots. As the leaves and stems of these plants had similar HCNp it suggested transport of dhurrin from the stem to the other tissues occurred and that a shift from synthesis in the leaves to the stems arose during development.

To gain an insight into the molecular regulation of cyanogenesis, the biosynthesis and catabolism of cyanogenic glucosides were manipulated in a number of ways. Blomstedt et al. (2012) focussed on producing sorghum lines with low cyanogenic potential using ethyl methanesulfonate (EMS) treatments on seeds. Post-treatment, seeds were allowed to grow and self-fertilise before mutations in the M2 generation were identified through the reverse genetic technique of Targetted Induced Local Lesions IN Genomes (TILLING). This technique was previously successful in mapping mutants in other crop species such as barley and maize (Till et al., 2004, Talame et al., 2008). Several mutant lines displaying lowered cyanogenic potential were developed (Blomstedt et al., 2012). The *totally cyanide deficient 1* (*tcd1*) line contained a P414L mutation in *CYP79A1* causing a proline to be substituted for a leucine near the enzyme active site, resulting in a non-functional enzyme in homozygotes. The *totally cyanide deficient 2* (*tcd2*) line resulted from a mutation in the *UGT85B1* gene, producing a truncated gene product and no active enzyme (Blomstedt et al., 2016). The *adult cyanide deficient class 1-3* (*acdc1-3*)

lines were found to have wild-type HCNp in the seedlings though they became acyanogenic in the leaves once mature (Blomstedt et al., 2012). However, the cyanogenic potential of the roots in the *acdc1-3* plants remained equal to wild-type lines throughout development (Blomstedt et al., 2018). HCNp was found to increase in the *acdc* adult tissue in response to high nitrogen application (Blomstedt et al., 2018). The mutation responsible for this phenotype remains unknown, despite the coding regions of the three biosynthetic genes having been sequenced.

The cyanogenic pathway was also introduced into species that do not naturally produce cyanogenic glucosides. The three dhurrin biosynthetic genes were inserted into the chloroplasts of *Nicotiana tabacum*, with biosynthesis of dhurrin driven by electrons derived from photosynthesis (Gnanasekaran et al., 2016). Dhurrin reached 0.1-0.2% of the *N. tabacum* leaf tissue. Kristensen et al. (2005) produced transgenic *Arabidopsis thaliana* that expressed genes of the entire dhurrin biosynthetic pathway, *CYP71E1*, *CYP71A1* and *UGT85B1*, creating plants that produced dhurrin in concentrations up to 4% of their dry weight. The production of dhurrin in the transgenic *Arabidopsis* had minimal adverse effects on the metabolome or transcriptome of the plants, with morphology largely unaffected. Similar findings were documented by Tattersall et al. (2001) with transgenic *Arabidopsis* plants accumulating dhurrin in concentrations comparable to sorghum seedlings, with only minor reductions in plant growth rate.

The role of dhurrin in plant defence was also noted with flea beetles (*Phyllotreta nemorum*) feeding up to 80% more on wild-type plants when presented with both wild-type and cynanogenic *Arabidopsis* tissue (Tattersall et al., 2001). When only the cytochrome P450 genes (*CYP79A1* and *CYP71E1*) were inserted into *Arabidopsis*, plants were detrimentally affected; being underdeveloped, with changes in their transcriptomes and a build-up of molecules involved in the detoxification of intermediates of the dhurrin biosynthetic pathway. This was likely due to the loss of intermediate channelling through a metabolon missing *UGT85B1*, causing the toxic intermediates to accumulate (Nielsen et al., 2007, Laursen et al., 2016). This gives an insight into the importance of the three biosynthetic genes being clustered in sorghum and the necessity of their inheritance as a unit. However, it is hypothesised that there

are further reasons for the selection of gene clusters between generations as it facilitates the genes to be regulated together, such as via epigenetic mechanisms.

1.7 Epigenetics

The study of epigenetics, the heritable yet reversible modification of gene expression without changes to the underlying genetic sequence, is a rapidly expanding field. The modification of gene expression predominantly occurs via cytosine methylation and histone modifications. Current knowledge of cytosine methylation mechanisms has predominantly been obtained from four species: humans (*Homo sapiens*), mice (*Mus musculus*), *Arabidopsis thaliana*, and the red bread mould *Neurospora crassa* (Goll and Bestor, 2005). Methylation is utilised to silence transposons and repetitive sequences in all four species, suggesting methylation may have evolved first as a transposon defence (Zemach et al., 2010). However, our knowledge of the function of methylation across plant genomes has grown and it is now thought that the role of epigenetic changes expanded to include the control of gene expression during plant development and responses to environmental stressors (Lämke and Bäurle, 2017, Banerjee and Roychoudhury, 2017, Xu et al., 2016, Kim et al., 2015). Changes in cytosine methylation and chromatin remodelling allows a plant's response to environmental changes to be rapid without an alteration in DNA sequence and enables these changes to be reversed once the stress is removed.

1.7.1 Sorghum methylome

As shown by Olson et al. (2014) sorghum methylation follows the genome wide pattern of rice (also a member of the *Poaceae* family) and *Arabidopsis* (Zhang et al., 2006, Cokus et al., 2008). Euchromatin is sparsely methylated, corresponding to the transcription level of the genes, while regions rich in repetitive sequences are highly methylated (Olson et al., 2014). Transcription start sites and transcription end sites of highly expressed, high confidence gene models were generally found to be hypomethylated, while some classes of gene models displayed higher levels of CG methylation centrally within the gene. An important limitation to this study was that all tissues used for methylation came from the roots of the plants at a single developmental time-point.

As discussed previously, cyanogenesis in sorghum is developmentally regulated, with dhurrin concentrations and expression of the biosynthetic genes decreasing from approximately four days old (Halkier and Møller, 1989, Busk and Møller, 2002). Currently not much is known about the methylation state of the dhurrin biosynthetic genes. Although the methylome of sorghum has been published by Olson et al. (2014), this only analysed the methylation state of root tissue at a particular time-point, with any potential changes in relation to development being unknown. The *acdc1-3* lines can be considered developmental mutants as their leaves are acyanogenic when mature (Blomstedt et al., 2012). These tissue-specific differences in dhurrin concentration and changes in expression during development may be epigenetically regulated. This is supported by the fact no mutation is present in the coding regions of any of the biosynthetic genes in the *acdc1-3* mutants (Blomstedt et al., 2012), while the CΔT mutation in the *CYP79A1* promoter region is found to occur at a methylation site (Olson et al., 2014). Research into whether the methylation state of the dhurrin biosynthetic genes in sorghum is altered during development (and whether the methylation state of the *acdc1-3* mutants differs in comparison to wild-type plants) continues to be an area of interest.

Epigenetic regulation of cyanogenesis may also be transgenerational. Ballhorn et al. (2016) studied cyanogenic wild lima beans (*Phaseolus lunatus*) damaged by their natural herbivore: chrysomelid beetles (*Gynandrobrotica guerreroensis*). The first-generation progeny of the heavily damaged parents showed increased levels of β-glucosidase activity compared to control plants, although this was only evident in the first four weeks of growth. Although the mechanism behind this increase in β-glucosidase activity was not studied, Ballhorn et al. (2016) hypothesised that epigenetic changes were responsible in both methylation and histone modifications (Chinnusamy and Zhu, 2009, Boyko and Kovalchuk, 2008).

1.7.2 Gene clusters

Gene clusters are common in prokaryotes, with genes pertaining to functional pathways found in operons (Qian and Zhang, 2008). True operons are almost non-existent in eukaryotes, with the only known exceptions being the presence of operons in the nematode genome. However, clustering of non-homologous though functionally related

genes occurs in fungi and plants (Field et al., 2011). Clustering in plants is typically found to involve genes encoding secondary metabolites, such as cyclic hydroxamic acids in maize, the triterpene avenacin in *Arabidopsis* and diploid oats (*Avena strigosa*), and diterpenes in rice (Figure 3)(Frey et al., 1997, Qi et al., 2004, Qi et al., 2006, Chu et al., 2011, Nützmann et al., 2016, Nützmann and Osbourn, 2014). The evolutionary mechanisms that give rise to these clusters of non-homologous genes in eukaryotes are largely unknown.

Prokaryotic operons arise from horizontal gene transfer, though in plants they are thought to occur *de novo* or via gene duplication or translocation, before evolving new functions or subfunctions (Boycheva et al., 2014). Field et al. (2011) attempted to determine the events that lead to cluster formation of the triterpene (marneral and thalianol) clusters in *Arabidopsis*. It was concluded that they arose from different evolutionary events, though both events resulted in gene relocations as the clusters were located in regions containing genes from families prone to ectopic transposition (Field et al., 2011).

There is evidence that clustered genes are controlled epigenetically via histone modifications in plants (Nützmann et al., 2016, Chidambaranathan et al., 2016). Histones undergo a range of post-translational modifications of their amino-terminal tail regions including methylation, sumoylation, acetylation, ubiquitination, biotinylation and phosphorylation (Peterson & Laniel, 2004). Each histone has variant forms, which are encoded for by different genes (Chinnusamy & Zhu, 2009). The protein variants, positions of histones along the DNA strand, and post-translational modifications will all affect gene expression. While acetylation, phosphorylation and ubiquitination are generally considered to enhance gene expression, sumoylation and biotinylation are found to repress it (Cedar and Bergman, 2009). This is achieved by modifications altering the interaction between DNA and histones and therefore the accessibility of transcription factors and other regulatory elements. Histone variants and modifications often coincide with DNA methylation to silence or activate gene expression, although there is contention as to which change initiates the other (Henikoff and Smith, 2015, Jiang and Berger, 2017, Chinnusamy and Zhu, 2009).

In *Arabidopsis* the marneral and thalianol clusters have been determined to contain high levels of H3 lysine 27 trimethylation (H3K27me3), a histone modification that represses gene expression (Yu et al., 2016). Furthermore, while the clusters are associated with these histone modifications, flanking genes are not (Zhang et al., 2007). Aichinger et al. (2009) determined that the pickle (PKL) and pickle-related 2 (PKR2) chromatin remodelling proteins act as transcriptional activators to genes containing H3K27me3 modifications. PKL/PKR2 binding is known to be necessary for expression of at least three of the genes in the thalianol cluster, while the marneral cluster is partially dependent on them (Zhang et al., 2007).

Genes of the cyanogenic glucoside biosynthetic pathway have been found to be clustered in sorghum, cassava and *Lotus japonicus* (Figure 3)(Takos et al., 2011). There have been a number of proposed selective advantages to clustering: clustering enables the genes to be inherited as a functional unit; clustering of genes in pathways where toxic intermediates are involved ensures they are inherited together; and the clustered genes can be regulated synchronously, potentially by epigenetic mechanisms (Chu et al., 2011). In sorghum the cluster of cyanogenic glucoside biosynthetic genes also contains the dhurrin transporter *SbMATE2* gene that is co-expressed with the biosynthetic genes (Darbani et al., 2016).

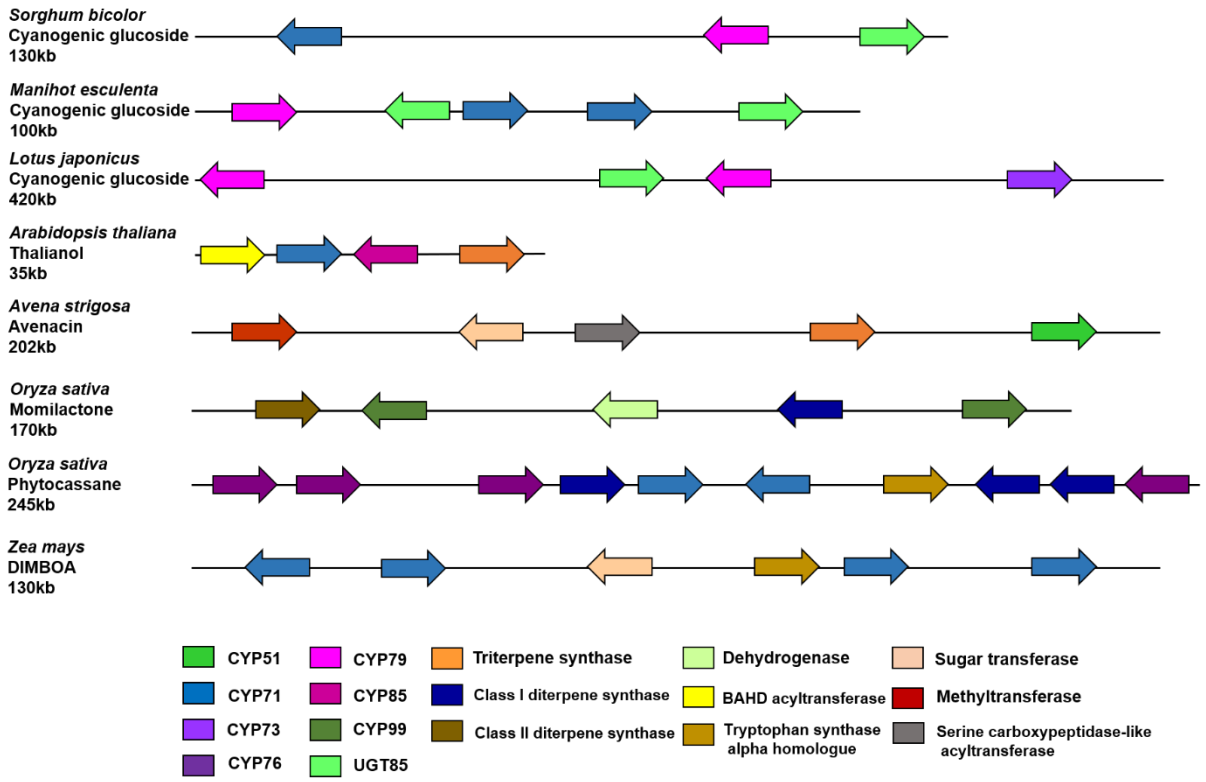

Figure 3: Examples of secondary metabolite gene clusters found in plants (adapted from Takos et al., 2011; Chu et al., 2011; and Nützmann et al., 2016).

There have been no studies pertaining to histone modifications associated with the cyanogenic glucoside biosynthetic gene cluster in sorghum. Based on the plasticity of dhurrin expression in sorghum and the fact the genes are clustered, research into whether gene expression of the biosynthetic genes is regulated via chromatic changes may provide more insight into how this trait is controlled at the molecular level. As epigenetic changes are meiotically heritable this allows for future generations to be primed for stress. If such priming alters the cyanogenic potential of sorghum crops, as seen in wild lima beans (Ballhorn et al., 2016), then it may be detrimental to its use as forage. An understanding of the epigenetic regulation of the dhurrin biosynthetic genes may facilitate the manipulation of this trait and an overall improvement of sorghum via epigenetic means rather than traditional changes to the DNA sequence.

1.8 This study: aims and structure

The overall aim of this thesis is to determine the molecular to physiological regulation of dhurrin content in *Sorghum bicolor*. Such an understanding should enable the toxicity of sorghum to be predicted more accurately than the current general guidelines allow. Moreover, it will point to possible ways to manipulate dhurrin concentration in the crop such that yield or stress tolerance is not compromised. Each chapter of original research in this thesis (Chapters 2 to 4) is written in the style of a journal article and presented in the correct format for the target journal. The thesis concludes with a general discussion and proposed future directions for the research.

Chapter 2: The interplay between drought stress, dhurrin and nitrate in the low-cyanogenic sorghum mutant *adult cyanide deficient class 1*

Chronic drought is known to increase dhurrin concentrations in sorghum to levels toxic to grazing animals (Wheeler et al., 1990, Stuart, 2002). However, reductions in hydrogen cyanide potential may be detrimental to plant growth and stress tolerance (Blomstedt et al., 2018, Jørgensen et al., 2005a). In this chapter I compared the drought response of wild-type plants to a low-cyanogenic EMS-mutant *adult cyanide deficient class 1*. Four successive harvests were undertaken, allowing for the interplay between developmental and environmental regulation of dhurrin to be investigated. Any resultant increases in nitrate, and the allocation of nitrogen to nitrate and dhurrin was examined for all vegetative tissues.

Chapter 3: Investigation into the role of DNA methylation in cyanogenesis in sorghum (*Sorghum bicolor* L. Moench)

The epigenetic regulation of the dhurrin biosynthetic genes in sorghum has not been investigated. Here, the effect of genome-wide demethylation on cyanogenesis was examined by germinating wild-type plants in the presence of the demethylating agent 5-Azacytidine; with the hypothesis that decreased methylation levels would correlate with higher *CYP79A1* expression and therefore dhurrin concentration. Expression levels of *CYP79A1* (the gene coding for the rate-limiting enzyme in the dhurrin biosynthetic pathway) and HCNp of leaf tissue were analysed. Furthermore, a determination was made regarding the methylation state of individual cytosines surrounding the *adult cyanide deficient class 1* mutation site over two stages of development (corresponding to

when dhurrin production was at a maximum and to when it had significantly decreased).

Chapter 4: The transcription factor *Sb*GATA22 as a regulator of *CYP79A1* in *Sorghum bicolor* (L.) Moench

To date no transcription factors that interact with the dhurrin biosynthetic gene promoters have been identified in sorghum. Yeast one-hybrid screens were used to obtain proteins that interact with the promoter region of *CYP79A1* – the key dhurrin biosynthetic gene in sorghum. The interaction of the candidate transcription factor *Sb*GATA22 with the *CYP79A1* promoter region was identified and confirmed *in vivo* using *Saccharomyces cerevisiae*. The expression of this transcription factor was analysed over fourteen days of sorghum development and in response to nitrate application in older plants.

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Chapter 2 Declaration

Monash University

The interplay between drought stress, dhurrin, and nitrate in the low-cyanogenic sorghum mutant *adult cyanide deficient class 1*

This thesis chapter is in the same format as the manuscript currently under review in the peer-reviewed journal *Frontiers in Plant Science*.

Declaration by candidate:

The nature and extent of my contribution to this work is as follows:

Nature of Contribution	Extent of contribution
Experimental design, execution of experimental work, analysis, manuscript writing and editing.	90%

The following co-authors contributed to the work:

Name	Nature of Contribution	Extent of contribution	Student at Monash University
Cecilia K. Blomstedt	Experimental design, execution of experimental work, and manuscript editing	2%	N
Birger Lindberg Møller	Experimental design and manuscript editing	1%	N
Trevor Garnett	Experimental design, execution of experimental work, and manuscript editing	2%	N
Ros Gleadow	Experimental design and manuscript editing	5%	N

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Chapter 2

The interplay between drought stress, dhurrin and nitrate in the low-cyanogenic sorghum mutant *adult cyanide deficient class 1*

2.1 Abstract

Sorghum bicolor (L.) Moench produces the nitrogen-containing natural product dhurrin that provides chemical defence against herbivores and pathogens via the release of toxic hydrogen cyanide gas. Drought can increase dhurrin in shoot tissues to concentrations toxic to livestock. As dhurrin is also a re-mobilizable store of reduced nitrogen and plays a role in stress mitigation, reductions in dhurrin may come at a cost to plant growth and stress tolerance. Here, we investigated the drought response in a unique EMS-mutant *adult cyanide deficient class 1* (*acdc1*) that has a low dhurrin content in the leaves of mature plants. A mutant sibling line was included to assess the impact of unknown background mutations. Plants were grown under three watering regimes using a gravimetric platform, with growth parameters and dhurrin and nitrate concentrations assessed over four successive harvests. Tissue-type was an important determinant of dhurrin and nitrate concentrations, with drought response differing between above and below ground tissues. Drought increased dhurrin concentration in the *acdc1* shoots to the same extent as in wild-type plants, and no growth advantage or disadvantage between the lines was observed. Lower dhurrin concentrations in the *acdc1* leaf tissue when fully watered correlated with an increase in nitrate content in the shoot and roots of the mutant. In targeted breeding efforts to down-regulate dhurrin concentration, parallel effects on the level of stored nitrates should be considered in all vegetative tissues of this important forage crop to avoid potential toxic effects.

2.2 Introduction

Cyanogenic glucosides are specialised secondary metabolites produced by over 2,500 species of plants and found in one-third of crop species (Gleadow and Møller, 2014). The role of cyanogenic glucosides in plant defence has long been established (Jones, 1998, Zagrobelny et al., 2004, Gleadow and Woodrow, 2002a), with defence theories assuming their production comes at a direct cost to primary metabolism when resources are limited (Cipollini et al., 2014, Herms and Mattson, 1992, Neilson et al., 2013).

The line between primary and secondary metabolism becomes blurred as cyanogenic glucosides are a re-mobilizable store of reduced nitrogen, transport compounds, and enhancers of stress tolerance as they mitigate oxidative stress (Bjarnholt et al., 2018, Burke et al., 2013, Møller, 2010, Pičmanová et al., 2015, Gleadow et al., 2016b, Schmidt et al., 2018). In stressed plants where photosynthetic rate is reduced, cyanogenic glucosides may also provide a ready source of nitrogen, remobilised when stress is alleviated (O'Donnell et al., 2013, Kongsawadworakul et al., 2009, Selmar et al., 1988, Bjarnholt et al., 2018, Schmidt et al., 2018). The cross-over of cyanogenic glucosides for use in primary and secondary metabolism is demonstrated by the negative effects on plant growth when they are reduced or removed, as seen in cassava (*Manihot esculenta* Crantz)(Jørgensen et al., 2005) and the acyanogenic sorghum line *totally cyanide deficient 1* (*tcd1*)(Blomstedt et al., 2012, Blomstedt et al., 2018).

Sorghum (*Sorghum bicolor* (L.) Moench) contains the cyanogenic glucoside dhurrin ((*S*)-4-hydroxymandelonitrile-β-*D*-glucopyranoside) in all main tissues except the mature grain (Bak et al., 1998, Kahn et al., 1997, Nielsen et al., 2016). Following tissue disruption, for example as a result of herbivore feeding, dhurrin is brought into contact with the endogenous β-glucosidase dhurrinase, resulting in hydrolysis of the glucoside and the release of hydrogen cyanide gas (HCN), also known as prussic acid (Cicek and Esen, 1998, Kahn et al., 1997). Dhurrin content varies with the ontogeny of the sorghum plant, increasing rapidly post-germination where it can reach up to 30% dry mass of the shoot tip before decreasing as the plant matures (Busk and Møller, 2002, Halkier and Møller, 1989, Adewusi, 1990). New growth also has high dhurrin concentrations presumably to ensure that such tissues, which are particularly vulnerable to herbivory

due to the softness of the tissue, are chemically defended (Gleadow and Woodrow, 2002a).

Developmental regulation of dhurrin formation in sorghum is confounded by environmental factors such as drought and nitrogen application that can induce higher dhurrin concentrations (Gleadow et al., 2016a, Emendack et al., 2018, Neilson et al., 2015a, Blomstedt et al., 2018, O'Donnell et al., 2013). This renders crop toxicity difficult to predict. Toxicity predictions are further complicated due to high variability between lines and individuals within lines (Hayes et al., 2015, Emendack et al., 2018). Moreover, the degree of HCN induction appears to differ depending on whether the stress is chronic or acute (Wheeler et al., 1990).

Sorghum also accumulates nitrate, particularly in the sheath tissue (Blomstedt et al., 2018, O'Donnell et al., 2013). Like dhurrin, nitrate concentrations are affected by the environment. Drought stress adds to the accumulation of nitrate as the ability to assimilate nitrate into protein is reduced (Sivaramakrishnan et al., 1988). There is conflicting evidence as to whether lower dhurrin concentrations are associated with higher nitrate levels in sorghum (O'Donnell et al., 2013, Blomstedt et al., 2018, Neilson et al., 2015a, Gleadow et al., 2016a). Furthermore, it remains unclear whether there is a stoichiometric trade-off in the allocation of nitrogen to dhurrin and nitrate.

In this study we used a sorghum mutant line with altered cyanogenic potential to investigate the effect of chronic drought stress on dhurrin and nitrate concentrations, as well as the allocation of nitrogen to both compounds. The *adult cyanide deficient class 1* (*acdc1*) is a sorghum EMS-mutant identified from a TILLING population (Blomstedt et al., 2012). This developmental mutant has wild-type concentrations of dhurrin in the leaf tissue when plants are young, before the leaves become acyanogenic at approximately three to four weeks of age. No sequence changes in the coding regions of the dhurrin biosynthetic genes (*CYP79A1*, *CYP71E1* and *UGT85B1*) are present in *acdc1*. However, a CΔT mutation (consistent with EMS treatment) that segregates with the phenotype has been identified ~1.1kb upstream of the *CYP79A1* transcription start site (Rosati et al., 2019). Sibling lines (Sibs) that lack the *acdc1* mutation were generated in parallel to account for any effect of background mutations generated from the EMS

treatment. The *acdc1* and Sibs were backcrossed three times to wild-type sorghum to reduce background mutations and appear phenotypically normal except for the altered cyanogenic status present in *acdc1*.

Simulating drought stress in greenhouse experiments equivalent to the chronic stress sorghum can experience in the field is known to be difficult (Tangpremsri et al., 1991, Sabadin et al., 2012, Flower et al., 1990, Passioura, 2006). In this study we used a gravimetric platform to allow for precise and reproducible water application and to ensure low soil water levels were maintained in a highly accurate manner throughout the course of the experiment. The platform allows the exact volume of water applied to each individual plant to be monitored. Plants were grown at either 15%, 30% or 100% field capacity of water. As sorghum is a highly drought tolerant C_4 crop with an extensive root system and thick, waxy cuticle on the leaves, the 15% and 30% field capacity of water treatments were selected to elicit a chronic stress response. The experiment was undertaken over a 35-day period, with a baseline harvest at 11 days post-germination (dpg) followed by three destructive harvests occurring every 8 days (19, 27, and 35dpg).

In this study we investigated the effects of chronic drought stress on a mutant line with altered cyanogenic potential in comparison to wild-type plants. This enabled us to assess how an altered hydrogen cyanide potential coupled with drought affect nitrate concentrations and nitrogen allocation in both above and below ground tissues. Successive harvests during early development also enabled the interplay between developmental and environmental regulation of dhurrin to be investigated.

2.3 Materials and Methods

Plant material and growth conditions

Seeds from the wild-type line BTx623, mutant line *adult cyanide deficient class 1* (*acdc1)*, and mutant sibling line (Sibs) (Blomstedt et al., 2012) were used to analyse the effects of drought on growth, hydrogen cyanide potential (HCNp), and nitrate concentrations. The plants were grown using a gravimetric platform (Phenospex Droughtspotter, Heerlen, The Netherlands) at the Australian Plant Phenomics Facility, Adelaide, South Australia, during January-February 2016. The plants received natural

light with the daily light integral (DLI) over the duration of the experiment being 10 mol m^{-2} d^{-1}. Temperatures ranged from 16°C at night to 26°C during the day with a daily average of 22°C. Relative humidity was 80% at night and 50% during the day.

Three seeds of each line were germinated in pots 20cm in diameter and 16.5cm in height containing 4.5 litres of soil 50% (v/v) University of California (UC) mixture (1:1 peat:sand) and 50% (v/v) cocopeat amended with Osmocote. At the 2-leaf stage plants were thinned to one per pot with a focus on overall plant uniformity. A baseline harvest of 6 plants from each line was undertaken at 11 days post-germination (dpg) before treatments commenced. Following this, plants from each line were randomly assigned to three treatment groups: 15%, 30%, or 100% field capacity of water. Pots were weighed every 10 min and water added when pots were 0.5% below target weight. Six replicates of each line for each treatment were destructively harvested every eight days for three additional harvests, resulting in harvests at 19, 27, and 35dpg. Harvest intervals were selected to cover the ontogenic reduction in HCNp seen in the *acdc1* (Blomstedt et al., 2012).

At each harvest plant tissues were separated into leaf blades (removed at the ligule), sheaths (comprising the rolled leaf sheaths and the shoot stem), and roots which were brushed free of soil, except for the 11dpg harvest where leaf and sheath tissues were harvested together due to the small size of the plants. Division of tissues allowed for chemical analyses to be undertaken for each tissue type. The fresh mass of each tissue was recorded, and the leaf blade area was measured using the LI-COR 3000 leaf area meter (LI-COR Lincoln, Nebraska, USA). Leaf, sheath, and root tissues were snap frozen in liquid nitrogen and stored at -80°C until freeze-dried. Freeze-dried tissue was weighed and ground to a fine powder using a MixerMill (MM 300, Retsch, Hann, Germany).

Growth indices

Growth parameters, relative growth rate (RGR), net assimilation rate (NAR), leaf area ratio (LAR), specific leaf area (SLA), and specific leaf nitrogen (SLN) were derived from the harvest data using the following equations, after Gleadow and Rowan (1982):

$$RGR\ d^{-1} = \frac{lnW_2 - lnW_1}{t_2 - t_1}$$

$$NAR\ (g\ m^{-2}\ d^{-1}) = \left(\frac{W_2 - W_1}{t_2 - t_1}\right)\left(\frac{lnA_2 - lnA_1}{t_2 - t_1}\right)$$

$$LAR\ (m^2\ g^{-1}) = \frac{A}{W\ total}$$

$$SLA\ (m^2 g^{-1}) = \frac{A}{W\ leaf}$$

$$SLN\ g\ g^{-1} = (leaf\ N)(\frac{W_L}{A_L})$$

Where W is total biomass; W_L is leaf biomass; A is leaf area; t is time; and N is leaf nitrogen.

Chemical analyses

Hydrogen cyanide potential (HCNp) was determined at all harvest time points using 10mg of finely ground leaf, sheath, or root tissue. HCNp is the total amount of HCN produced by hydrolysis of the entire content of endogenous cyanogenic glucosides as achieved by adding exogenous β-glucosidase (β-D-Glucoside glucohydrolase, G4511, Sigma-Aldrich, Sydney, Australia). The HCN produced was captured as NaCN in a 1M NaOH solution and measured via a colorimetric assay (Gleadow et al., 2012). The HCNp is used as a proxy for dhurrin, with each mg of HCN equivalent to 11.5mg of dhurrin in the plant tissue. Total nitrate concentration was measured for the final harvest time point via a colorimetric assay in 96-well microtiter plates using 15mg of finely-ground freeze dried leaf, sheath or root tissue (O'Donnell et al., 2013). Nitrates were extracted from the ground tissue in an Eppendorf tube with 300µl MilliQ water and one metal bead. Tubes were placed in a MixerMill for 4 minutes at 28 oscillations per second before being incubated for an hour at 45°C with occasional inverting by hand. These were then centrifuged at maximum speed for ten minutes and the supernatant

(containing the nitrates) collected. To determine nitrate concentration 5µl of sample was pipetted into a 96 well microtitre plate, with 20µl of 5% salicylic acid in concentrated sulphuric acid added and allowed to incubate for 20 minutes at room temperature. To increase the pH above 12, 300µl of 3M NaOH was added. A yellow colour was formed following the addition of the base, and the plate was then read in a microplate reader spectrophotometer at 410nm.

Total nitrogen of the leaf, sheath, and root samples for the final harvest was analysed using 5mg of tissue by the Environmental Analysis Laboratory (Lismore, NSW, Australia). Nitrogen, dhurrin, and nitrate per plant was converted to mg g^{-1} dry mass. In order to assess how nitrogen was partitioned, the proportion of nitrogen found as dhurrin or nitrate was calculated as a proportion of total elemental nitrogen.

Statistical analysis

The statistics package Sigmaplot v 13 (Systat Software) was used for statistical analyses by one- or two-way ANOVA. For all tests, a *P*-value of <0.05 was considered significant. Means that were significantly different were compared *post hoc* using Tukey's test. Data were log transformed if required to satisfy the assumptions of normality.

2.4 Results

Physiology and growth

EMS-mutants with low adult dhurrin levels (*adult cyanide deficient class 1*, *acdc1*), mutant sibling lines (Sibs, generated in parallel but lacking the mutation), and wild-type (WT) plants were grown for five weeks under well-watered or water-limited conditions (15%; 30%; and 100% field-capacity of water).

Water availability was the greatest determinant of plant growth at all harvest time-points for all lines. These differences were not proportional to water availability with few significant differences seen between the 15% H_2O and 30% H_2O treatments. This was congruent with the amount of water used over the course of the experiment, with no significant differences in total water use between the 15% H_2O and 30% H_2O treatments, while the 100% H_2O treatment resulted in an almost tripled amount of water consumed ($P<0.001$) (Figure 1A). Dry matter percentage (DM%) correlated to

water restriction levels, increasing as water availability decreased (Figure 1B). In relation to DM%, no differences between lines were observed.

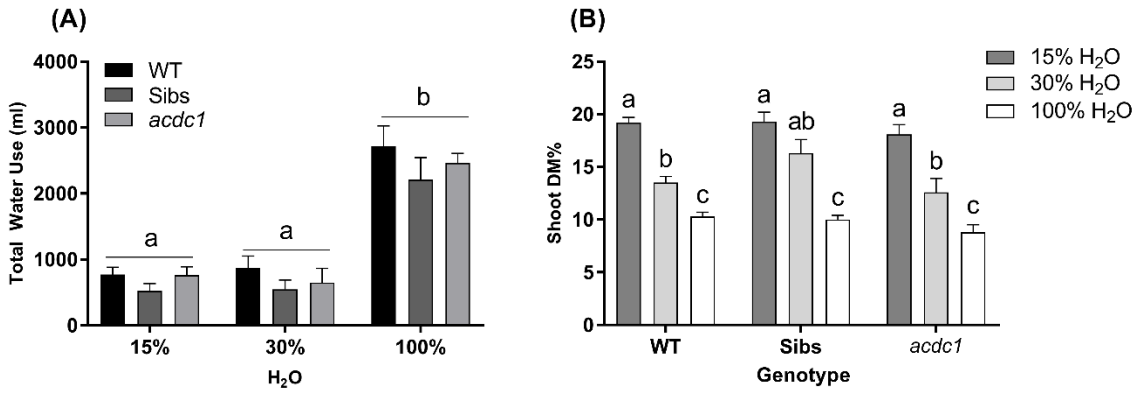

Figure 1: (A) total water use and **(B)** shoot dry matter percentage (DM%) of wild-type: WT; Siblings: Sibs; and *adult cyanide deficient class 1*: *acdc1* lines grown at 15%, 30% and 100% field capacity of water. Values denote means ± SEM (*n*=6), means with different letters are significantly different at P<0.05, analysed using ANOVA and Tukey's test. Data represent plants at 35 days post-germination (dpg), treatments commenced at 11dpg.

The first harvest showed few genotype effects, with the only significant difference between lines seen in the *acdc1* which had a greater specific leaf area (SLA) than the two other lines (P<0.001) (Table 1A). Once treatments commenced the *acdc1* line had significantly greater root mass (P<0.01) and R:S ratio (P<0.05) than wild-type plants at 100% H_2O (Table 1B). The main genotype effect at 27dpg occurred in the Sibs, which were significantly shorter than wild-type plants under both levels of water limitation (P<0.001) (Table 1C). By 35dpg the Sibs had reduced leaf area, leaf mass, and sheath mass at 15% H_2O and 30% H_2O than both the wild-type and *acdc1* lines (P<0.001) (Table 1D).

Water availability began to affect plant growth at 19dpg, when treatments had been applied for eight days. Height, leaf area and leaf dry mass were lower in the 15% H_2O and 30% H_2O treatment groups in comparison to fully watered plants across all

genotypes ($P<0.001$) (Table 1B) but no significant differences between the 15% H_2O and 30% H_2O treatments were observed. Both levels of water restriction resulted in an equivalent growth delay.

Height, leaf area, leaf dry mass, and sheath dry mass remained lower at 15% H_2O and 30% H_2O in comparison to fully watered plants for the final two harvests ($P<0.001$) (27dpg - Table 1C and 35dpg - Table 1D). This corresponded to a greater total biomass in the fully watered plants ($P<0.001$). No significant difference in total biomass was observed between the 15% H_2O and 30% H_2O treatments within lines. Higher SLN and RGR was seen at the final harvest (35dpg) at 100% H_2O in comparison to both levels of water limitation for all genotypes ($P<0.001$) (Table 1D).

Table 1: Growth parameters of three *Sorghum bicolor* genotypes grown at 15%, 30%, and 100% field capacity of water and harvested at **(A)** 11dpg - baseline harvest before treatments commenced; **(B)** 19dpg; **(C)** 27dpg; and **(D)** 35dpg

WT: wild-type; Sibs: siblings; *acdc1*: *adult cyanide deficient class 1* mutants. Values are the mean of 6 replicates (± SEM). Means with different letters are significantly different at P<0.05 analysed using ANOVA and Tukey's test. Abbreviations: DM – Dry mass; LAR - leaf area ratio; NAR - net assimilation rate; R:S ratio - root:shoot ratio; SLA - specific leaf area; SLN - specific leaf nitrogen; RGR - relative growth rate. **P*<0.05; ***P*<0.01; ****P*<0.001; ns - not significant.

(A) 11dpg

	WT	**Sibs**	***acdc1***	**ANOVA**
Leaf Area (cm^2)	4.6 (0.4)	4.7 (0.4)	4.9 (0.5)	ns
Shoot DM (g)	0.018 (0.002)	0.016 (0.001)	0.016 (0.002)	ns
Root DM (g)	0.017 (0.003)	0.017 (0.002)	0.015 (0.002)	ns
Biomass Total (g)	0.035 (0.003)	0.033 (0.003)	0.031 (0.004)	ns
R:S	1.0 (0.2)	1.1 (0.1)	1.0 (0.1)	ns
LAR ($cm^2\ g^{-1}$)	130 (20)	130 (10)	160 (10)	ns
SLA ($cm^2\ g^{-1}$)	250^a (10)	270^a (5)	310^b (10)	***

(B) 19dpg

	WT			Sibs			*acdc1*			ANOVA		
	15% H_2O	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**L**	**T**	**LxT**
Leaf Area (cm^2)	11^{a} (2)	15^{a} (2)	38^{b} (3)	20^{ac} (4)	10^{a} (2)	29^{bc}(3)	17^{a} (3)	22^{a} (4)	40^{b} (5)	ns	***	ns
Height (cm)	4.5^{ac} (0.4)	4.9^{a} (0.3)	8.8^{b} (0.3)	4.3^{ac} (0.7)	3.4^{c} (0.6)	5.9^{d} (0.2)	4.6^{ac} (0.5)	4.9^{a} (0.5)	7.5^{bd} (0.6)	**	***	ns
Leaf DM (g)	0.036^{ab} (0.003)	0.042^{ab} (0.005)	0.080^{c} (0.009)	0.043^{abd} (0.009)	0.028^{a} (0.003)	0.060^{cd} (0.008)	0.042^{ab} (0.010)	0.058^{b} (0.007)	0.087^{c} (0.018)	ns	***	ns
Sheath DM (g)	0.021^{a} (0.003)	0.025 (0.003)	0.044^{b} (0.004)	0.022 (0.004)	0.023 (0.006)	0.028 (0.003)	0.029 (0.004)	0.031 (0.006)	0.043 (0.008)	ns	**	ns
Root DM (g)	0.047 (0.015)	0.054 (0.012)	0.049^{a} (0.005)	0.059 (0.008)	0.060 (0.011)	0.066 (0.013)	0.068 (0.010)	0.086 (0.019)	0.113^{b} (0.027)	**	ns	ns
Biomass Total (g)	0.10^{a} (0.02)	0.12 (0.02)	0.17^{b} (0.01)	0.12 (0.02)	0.11 (0.02)	0.15 (0.02)	0.14 (0.02)	0.17 (0.02)	0.24 (0.05)	**	**	ns
R:S	0.8 (0.2)	0.8 (0.1)	0.4^{a} (0.1)	1.0 (0.2)	1.2 (0.2)	0.7 (0.1)	1.1 (0.2)	1.2 (0.4)	0.9^{b} (0.1)	*	*	ns
LAR ($m^2\ g^{-1}$)	110^{a} (20)	130^{a} (10)	220^{b} (10)	140^{ac} (18)	90^{a} (10)	200^{bc} (30)	120^{a} (10)	130^{ab} (30)	180^{ab} (20)	ns	***	ns
SLA ($m^2\ g^{-1}$)	300^{a} (50)	370^{ac} (20)	490^{b} (40)	430 (40)	360^{bc} (60)	500 (50)	440^{bc} (50)	360 (30)	510 (50)	ns	***	ns
NAR ($g\ m^{-2}\ day^{-1}$)	0.001^{a} (0.001)	0.002^{a} (0.001)	0.005^{b} (0.0004)	0.002 (0.001)	0.001^{a} (0.001)	0.004^{b} (0.001)	0.002^{ac} (0.001)	0.003^{b} (0.001)	0.008^{bc} (0.002)	**	***	ns
RGR ($g\ g^{-1}\ day^{-1}$)	0.13 (0.03)	0.15 (0.03)	0.22 (0.01)	0.16 (0.02)	0.14 (0.02)	0.19 (0.03)	0.10^{a} (0.06)	0.17 (0.02)	0.25^{b} (0.03)	*	ns	ns

(C) 27dpg

	WT			Sibs			*acdc1*			ANOVA		
	15% H_2O	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**L**	**T**	**LxT**
Leaf Area (cm^2)	62^{a} (9)	75^{a} (8)	275^{b} (32)	45^{ac} (12)	38^{c} (11)	273^{b} (36)	70^{a} (14)	81^{a} (15)	336^{b} (54)	*	***	ns
Height (cm)	8.3^{a} (0.2)	9.6^{a} (1.9)	16.8^{b} (0.3)	5.3^{c} (0.8)	4.7^{c} (0.7)	15.1^{b} (0.7)	6.7^{ac} (0.7)	6.8^{ac} (0.7)	16.0^{b} (0.6)	***	***	ns
Leaf DM (g)	0.16^{a} (0.02)	0.17^{a} (0.02)	0.63^{b} (0.11)	0.11^{a} (0.03)	0.13^{a} (0.04)	0.69^{b} (0.11)	0.17^{a} (0.03)	0.21^{a} (0.04)	0.78^{b} (0.23)	ns	***	ns
Sheath DM (g)	0.09^{ac} (0.01)	0.09^{ac} (0.01)	0.32^{b} (0.04)	0.06^{ac} (0.02)	0.05^{c} (0.01)	0.33^{b} (0.06)	0.09^{a} (0.02)	0.10^{a} (0.02)	0.45^{b} (0.10)	*	***	ns
Root DM (g)	0.65^{a} (0.08)	0.54^{a} (0.19)	1.06 (0.18)	0.85 (0.30)	0.70 (0.28)	1.71^{b} (0.34)	0.62^{a} (0.34)	0.31^{a} (0.09)	1.97^{b} (0.49)	ns	***	ns
Biomass Total (g)	0.91^{a} (0.09)	0.80^{a} (0.21)	2.00^{b} (0.26)	1.02^{a} (0.34)	0.88^{a} (0.32)	2.73^{b} (0.44)	0.88^{a} (0.34)	0.62^{a} (0.14)	3.20^{b} (0.69)	ns	***	ns
R:S	3.0 (0.5)	2.0 (0.6)	1.2 (0.2)	4.4^{a} (1.0)	3.7 (1.0)	1.8 (0.3)	2.8 (1.3)	0.9^{b} (0.1)	2.0 (0.5)	ns	ns	ns
LAR ($m^2\ g^{-1}$)	70^{ac} (10)	120^{bc} (30)	140^{b} (10)	80 (30)	60^{a} (10)	110^{b} (10)	130 (40)	140 (10)	120 (20)	**	*	ns
SLA ($m^2\ g^{-1}$)	380 (10)	440^{a} (10)	460 (30)	390 (30)	320^{b} (30)	410^{a} (20)	380 (30)	380 (10)	390 (50)	*	ns	ns
NAR ($g\ m^{-2}\ day^{-1}$)	0.02 (0.001)	0.02 (0.01)	0.06 (0.01)	0.03 (0.02)	0.03^{a} (0.01)	0.1^{b} (0.02)	0.02 (0.01)	0.01^{a} 0.004)	0.1^{b} (0.03)	ns	***	ns
RGR ($g\ g^{-1}\ day^{-1}$)	0.27 (0.03)	0.22 (0.03)	0.30 (0.02)	0.21^{a} (0.07)	0.22 (0.05)	0.36^{b} (0.04)	0.14 (0.08)	0.15 (0.03)	0.32 (0.03)	ns	*	ns

(D) 35dpg

	WT			Sibs			*acdc1*			ANOVA		
	15% H_2O	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**L**	**T**	**LxT**
Leaf Area (cm^2)	110^{a} (10)	280^{b} (40)	880^{c} (110)	60^{d} (10)	120^{e} (20)	660^{c} (60)	160^{a} (30)	350^{b} (50)	1050^{c} (130)	***	***	ns
Height (cm)	10.5^{a} (0.8)	14.7^{b} (1.1)	23.0^{c} (0.4)	7.4^{de} (0.4)	7.9^{d} (0.8)	18.0^{c} (1.7)	8.5^{ae} (0.7)	13.2^{b} (1.1)	22.8^{c} (0.9)	***	***	ns
Leaf DM (g)	0.34^{a} (0.05)	0.68^{b} (0.12)	2.42^{c} (0.41)	0.18^{d} (0.02)	0.28^{a} (0.04)	1.87^{c} (0.25)	0.45^{a} (0.09)	0.83^{b} (0.20)	2.92^{c} (0.80)	***	***	ns
Sheath DM (g)	0.19^{a} (0.03)	0.32^{b} (0.06)	1.28^{ce} (0.22)	0.09^{d} (0.01)	0.13^{d} (0.02)	0.90^{c} (0.14)	0.45^{ab} (0.24)	0.42^{ab} (0.08)	1.50^{e} (0.25)	***	***	ns
Root DM (g)	0.67^{a} (0.12)	1.21^{a} (0.27)	4.68^{b} (1.75)	0.56^{a} (0.13)	0.88^{a} (0.24)	2.11^{b} (0.46)	0.45^{a} (0.08)	1.24^{a} (0.47)	3.83^{b} (1.18)	ns	***	ns
Biomass Total	1.19^{a} (0.15)	2.21^{a} (0.43)	8.37^{b} (2.37)	0.83^{a} (0.15)	1.31^{a} (0.30)	4.88^{b} (0.81)	1.35^{a} (0.31)	2.49^{a} (0.71)	8.25^{b} (2.09)	*	***	ns
R:S	1.4^{a} (0.3)	1.2 (0.1)	1.1 (0.3)	2.1^{a} (0.4)	2.0^{a} (0.5)	0.7^{b} (0.1)	0.6^{b} (0.1)	0.9^{b} (0.2)	0.9 (0.2)	***	ns	*
LAR ($m^2\ g^{-1}$)	100 (10)	130 (10)	130 (20)	80^{a} (10)	100 (30)	140^{b} (10)	140 (20)	160^{b} (20)	150 (20)	*	*	ns
SLA ($m^2\ g^{-1}$)	350 (20)	430 (20)	380 (30)	350 (20)	410 (30)	360 (20)	370 (30)	490 (100)	410 (70)	ns	*	ns
SLN ($g\ g^{-1}$)	0.4^{a} (0.1)	0.7^{a} (0.20)	3.2^{b} (1.0)	0.2^{a} (0.02)	0.3^{a} (0.04)	2.2^{b} (0.4)	0.6^{a} (0.1)	0.9^{a} (0.3)	3.9^{b} (1.3)	ns	***	ns
NAR ($g\ m^{-2}\ day^{-1}$)	0.01^{a} (0.002)	0.03 (0.01)	0.1^{b} (0.06)	0.01 (0.002)	0.02 (0.006)	0.05 (0.02)	0.02 (0.01)	0.05 (0.02)	0.09 (0.03)	*	*	ns
RGR ($g\ g^{-1}\ day^{-1}$)	0.15^{a} (0.004)	0.17^{a} (0.01)	0.22^{b} (0.01)	0.13^{a} (0.01)	0.15^{a} (0.01)	0.21^{b} (0.01)	0.15^{a} (0.01)	0.18^{a} (0.01)	0.23^{b} (0.02)	*	***	ns

Plant Composition: HCNp, nitrate concentration, and total N of well-watered and droughted plants

HCNp analysis

Hydrogen cyanide potential (HCNp; mg HCN g^{-1} dry mass) was analysed in three tissue types for each line at each harvest (Figure 2; Supplementary Table 1). HCNp was highly dependent on developmental stage and tissue type, with varying levels of water availability not resulting in significant differences in HCNp until the later harvests (Figure 2). HCNp decreased markedly in both the leaf (Figure 2A-C) and sheath tissue (Figure 2D-F) between 11dpg and 27dpg in all lines and treatments. This decrease was more pronounced at 100% H_2O, and by 27dpg HCNp in the shoot was 0.46±0.08 mg HCN g^{-1} DM on average for all lines, compared with 0.92±0.19 and 1.01±0.19 mg HCN g^{-1} DM in the 15% H_2O and 30% H_2O treatments, respectively. Root tissue had approximately 80% lower HCNp than the shoot tissue at the baseline harvest (Figure 2G-I). HCNp in the root tissue displayed a similar pattern to the shoot tissue, with a general decrease in HCNp occurring from 19dpg to 27dpg (except in the *acdc1*), before an increase across all treatments at 35dpg (Figure 2G-I; Supplementary Table 1).

There were genotype differences in HCNp of the leaves and roots in plants grown at 100% H_2O. For example, in the leaves *acdc1* had significantly lower HCNp (0.29±0.05 mg HCN g^{-1} DM) at 27dpg than the wild-type (0.78±0.27 mg HCN g^{-1} DM) or Sibs (0.67±0.06 mg HCN g^{-1} DM) (Figure 2; $P<0.05$). The *acdc1* leaf tissue continued to have significantly lower HCNp through to the final harvest compared to both the other lines, with an average HCNp of 0.28±0.02 mg HCN g^{-1} DM compared to 0.65±0.06 and 0.60±0.03 mg HCN g^{-1} DM for the wild-type and Sibs lines, respectively. There was no significant difference in sheath HCNp between lines.

In the roots, genotype differences were observed at 27dpg. In contrast to the leaf tissue, *acdc1* had a HCNp at 100% H_2O of 0.24±0.03 mg HCN g^{-1} DM, approximately twice as high as HCNp in the roots of wild-type plants (0.12±0.02 mg HCN g^{-1} DM) (Figure 2; Supplementary Table 1). At 35dpg, *acdc1* roots still had a significantly higher HCNp, although the difference was not as great as in the earlier harvests.

Water limitation affected the HCNp differently in the above and below ground tissues (Figure 2; Supplementary Table 1). Leaf and sheath HCNp was significantly higher in plants grown at 15% H_2O and 30% H_2O compared to plants grown at 100% H_2O across all genotypes ($P<0.001$) (Supplementary Table 1A,B). For example, at 35dpg average leaf HCNp for the three lines was only 0.51±0.04 mg HCN g^{-1} DM at 100% H_2O compared to 2.43±0.38 mg HCN g^{-1} DM and 1.92±0.43 mg HCN g^{-1} DM in plants grown at 15% H_2O and 30% H_2O, respectively. In contrast to the shoot tissue, the roots had significantly higher HCNp at 100% H_2O compared to plants grown at 15% H_2O for all genotypes from the 27dpg harvest onwards (Supplementary Table 1C).

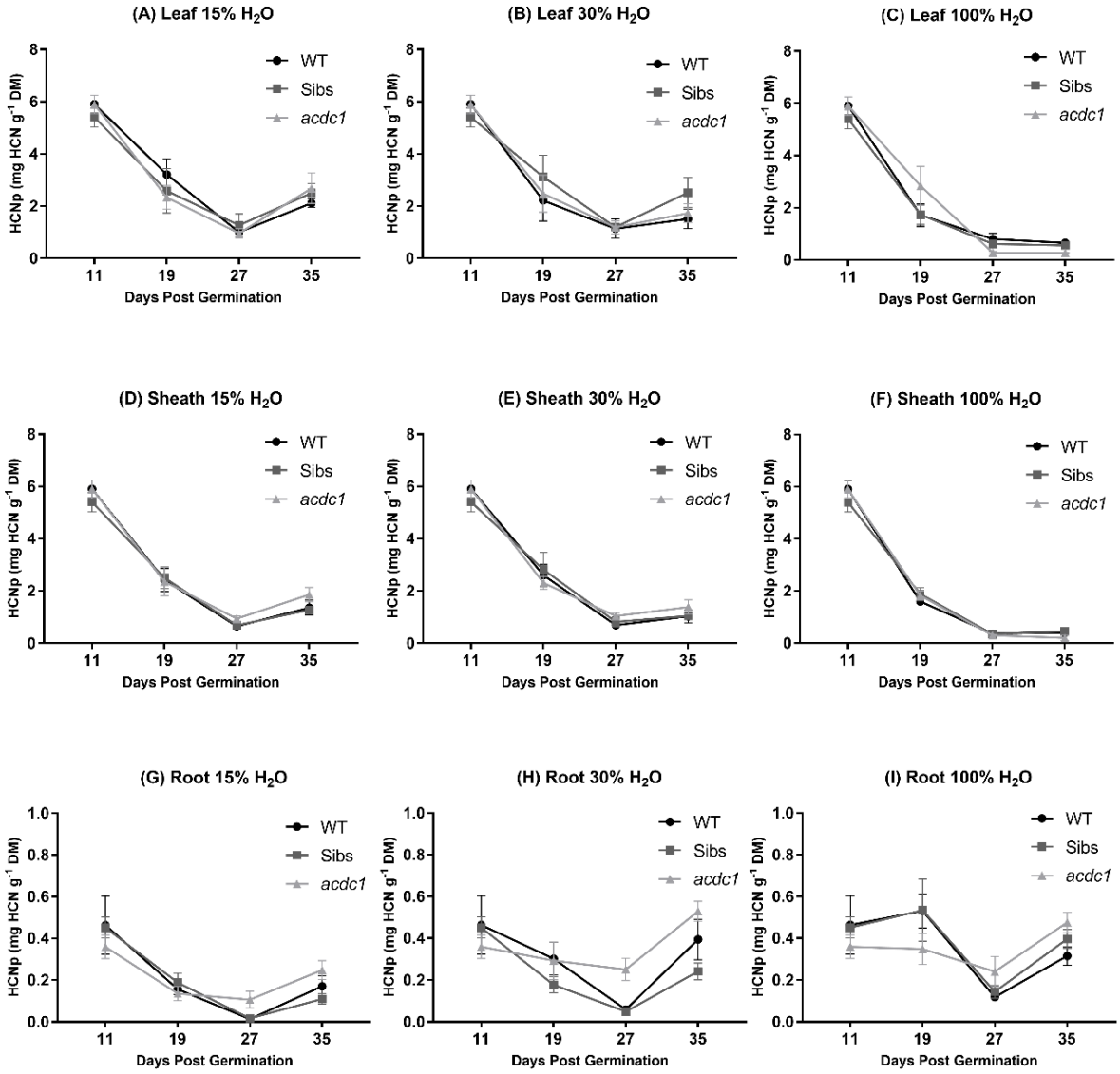

Figure 2: Hydrogen cyanide potential (HCNp; mg HCN g^{-1} dry mass) in the leaves **(A-C)**; sheaths **(D-F)**; and roots **(G-I)** of WT: wild-type; Sibs: siblings; and *acdc1*: *adult cyanide deficient class 1* sorghum lines grown at 15%, 30%, and 100% field capacity of water. A baseline harvest (prior to water limitation) was performed at 11 days post-germination (dpg), followed by harvests at 19dpg, 27dpg, and 35dpg. Values denote means ± SEM (*n*=6). Significance is listed in Supplementary Table 1, analysed using ANOVA and Tukey's test.

Nitrate concentration

Genotype, tissue type, and water availability all affected nitrate concentration. Leaf and root nitrate concentrations were higher in *acdc1* than both other lines when water was limited (Figure 3D-F). In the *acdc1*, nitrate concentrations were also higher than in wild-type plants in all tissues at 100% H_2O ($P<0.01$) (Figure 3E) and higher in the roots than wild-type plants for all treatments ($P<0.001$) (Figure 3F). Nitrate concentrations were tissue dependent across all lines, with nitrates highly concentrated in the sheath tissue compared to the leaf tissue (figure 3E). When water was limited root nitrate concentration increased across all lines (Figure 3F).

Total nitrogen concentration

Total elemental N concentration in each tissue followed similar patterns to nitrate concentration (Figure 3G-I). The *acdc1* leaves had higher N concentrations than the wild-type and Sibs under both levels of water limitation, but not in the well-watered plants ($P<0.001$) (Figure 3G). In the sheath and root tissues *acdc1* had higher N concentrations than the wild-type and Sibs for all treatments. ($P<0.001$) (Figure 3H,I). Nitrogen concentration was lower in the roots in comparison to leaf and sheath tissues which were comparable. No significant differences were observed in carbon concentration between treatment groups or genotypes (Figure 3J-L).

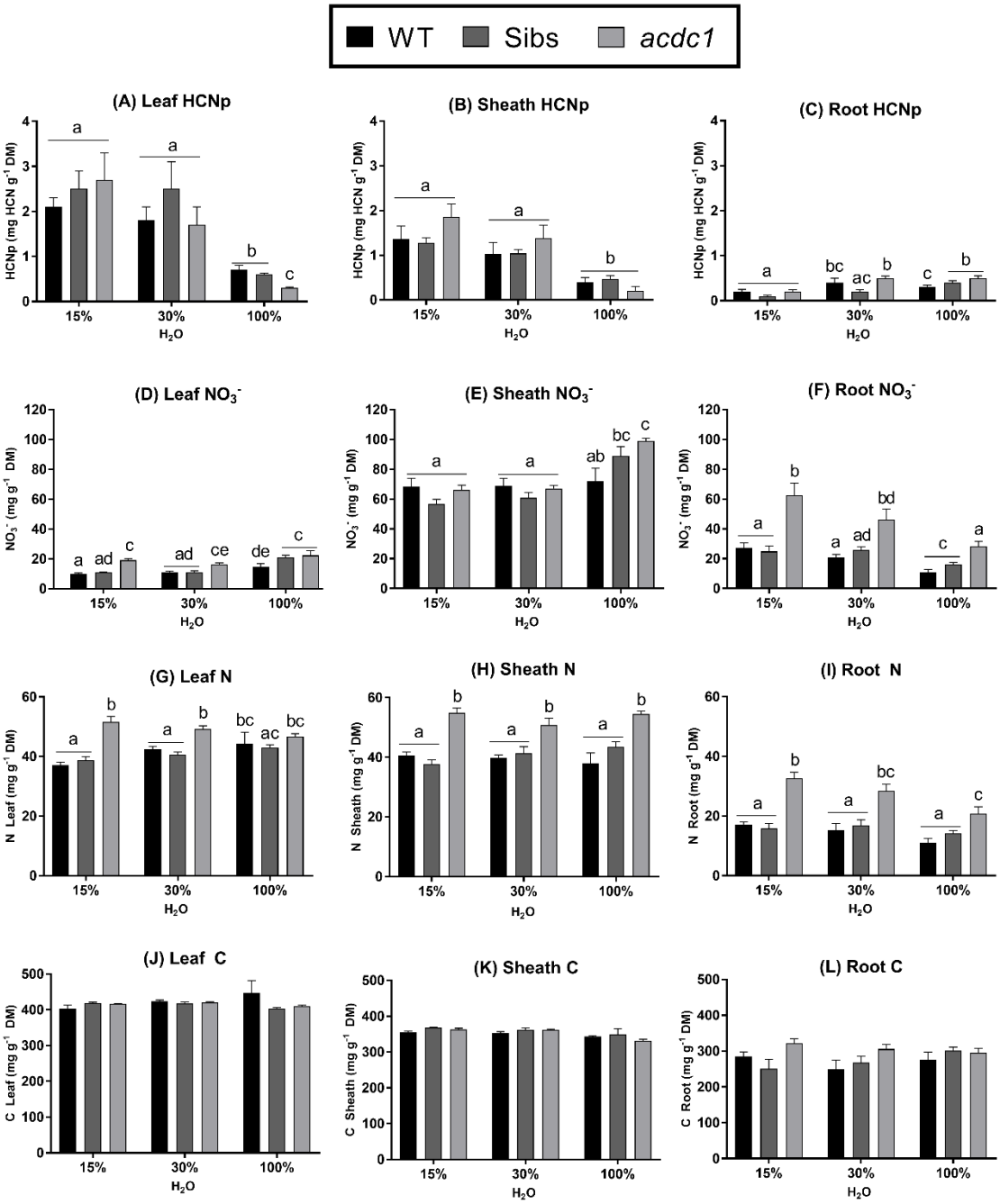

Figure 3: Hydrogen cyanide potential (HCNp; mg HCN g^{-1} dry mass) **(A-C)**; nitrate concentration **(D-F)**; nitrogen **(G-I);** and carbon **(J-L)** in the leaves, sheaths, and roots of WT: wild-type; Sibs: siblings; and *acdc1*: *adult cyanide deficient class 1* sorghum lines grown at 15%, 30% and 100% field capacity of water at 35 days post-germination. Values denote means ± SEM (n=6), means with different letters are significantly different at P<0.05, analysed using ANOVA and Tukey's test.

Nitrogen allocation

In order to analyse nitrogen allocation, we calculated the total amount of dhurrin and nitrate per plant (by multiplying the concentration in each tissue by the biomass of that tissue) before calculating expression as a percentage of total N. When water was replete, wild-type and Sibs allocated more N to dhurrin in the leaf compared to *acdc1* ($P<0.05$) (Figure 4A). This genotypic difference was not seen when water was limited, with more N allocated to dhurrin in both leaf and sheath tissues compared to the 100% H_2O treatment across all lines (Figure 4A-B). Root tissue displayed an opposite pattern to the shoots with less N allocated to dhurrin under fully watered conditions in comparison to 15% H_2O for all lines ($P<0.001$) (Figure 4C).

The proportion of nitrogen found in nitrate was tissue dependent more than water dependent, with up to six times more nitrate allocated to N in the sheath and root tissues than the leaf tissue across all lines (Figure 4D-F). There were also significant genotype effects. For example, the proportion of N allocated to nitrate was significantly higher in *acdc1* leaves than wild-type leaves when water was both replete and severely limited (15% H_2O) ($P<0.05$). Less N was allocated to nitrate in the *acdc1* sheath than in the wild-type sheath at both 15% H_2O and 30% H_2O ($P<0.001$), possibly due to the higher percentage of N in the *acdc1* sheath. In the root tissue more N was allocated to nitrate in the *acdc1* roots than the wild-type line at 100% H_2O ($P<0.05$).

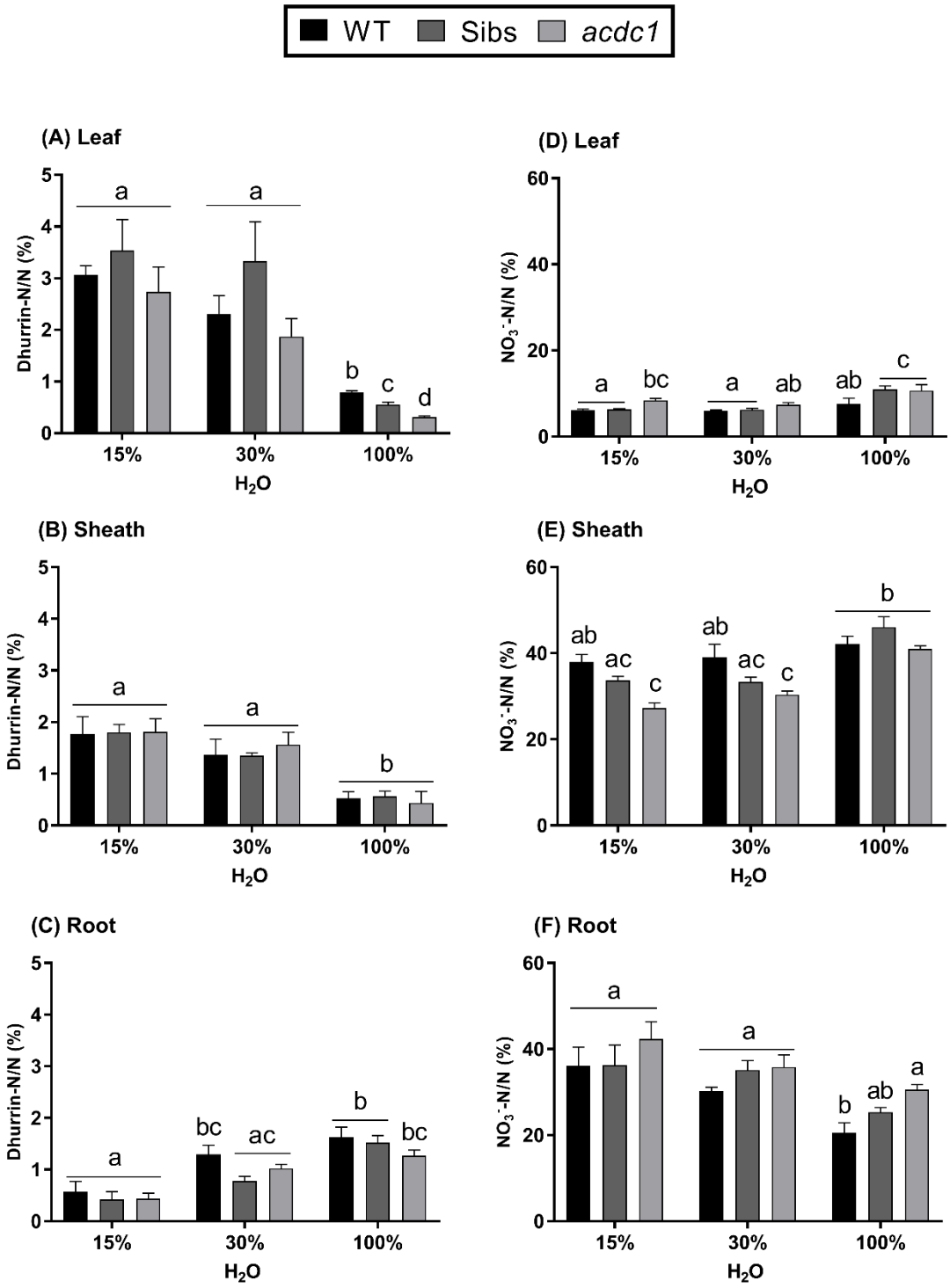

Figure 4: Proportion of nitrogen allocated to dhurrin **(A-C)**; and nitrate **(D-E)** in the leaves, sheaths, and roots of WT: wild-type; Sibs: siblings; and *acdc1*: *adult cyanide deficient class 1* sorghum lines grown at 15%, 30% and 100% field capacity of water at 35 days post-germination. Values denote means ± SEM (n=6), means with different letters are significantly different at P<0.05, analysed using ANOVA and Tukey's test.

2.5 Discussion

Drought is known to affect concentrations of cyanogenic glucosides in many species, generally affecting an increase in concentration in both field and controlled environments as reported in cassava (Brown et al., 2016), eucalypt (*Eucalyptus cladocalyx*)(Gleadow and Woodrow, 2002b), white clover (*Trifolium repens*)(Hayden and Parker, 2002), and lima bean (*Phaseolus lunatus*)(Ballhorn et al., 2011). In sorghum, the concentration of dhurrin may increase or decrease depending on tissue-type and the length and severity of stress (O'Donnell et al., 2013, Wheeler et al., 1990, Gleadow et al., 2016a, Emendack et al., 2018). Increases in dhurrin appear to be associated with chronic stress (Wheeler et al., 1990), though reproducing chronic levels of drought stress under controlled conditions are challenging (Tangpremsri et al., 1991, Sabadin et al., 2012, Flower et al., 1990, Passioura, 2006). In the past, experiments of this nature have been conducted with manual watering to weight which, due to logistical constraints, cannot be done regularly enough and leads to considerable variation in water stress. In our current study a gravimetric platform was used, enabling low levels of water to be accurately maintained over the course of the experiment.

Cyanogenic glucosides, in addition to their known role in herbivore defence (Gleadow and Woodrow, 2002a), may help mitigate drought stress such that less cyanogenic plants would have reduced growth under chronic drought conditions than those with higher concentrations (Gleadow and Møller, 2014). We compared the growth and chemical composition at three different levels of water using the publicly available sorghum breeding line BTx623 and a mutant line with low dhurrin concentration in adult leaf tissue (Blomstedt et al., 2012). As cyanogenic glucosides also play important roles in nitrogen metabolism, resulting in an interplay between HCNp and nitrate (Gleadow and Møller, 2014, Selmar et al., 1988, Blomstedt et al., 2018, Bjarnholt et al., 2018), we also determined nitrate concentration and nitrogen allocation in the different genotypes.

Overall, the *acdc1* sorghum mutant displayed neither a growth advantage nor disadvantage under drought stress in comparison to wild-type sorghum plants. Plants grown at 15% and 30% field capacity of water showed an equivalent reduction in water use (Figure 1A), leading to a reduction in biomass and an overall increase in shoot dry

matter content proportional to the level of water limitation in all lines tested (Figure 1B). Both levels of water limitation reduced biomass to the same degree across lines (Table 1). This may be due to plants experiencing the same level of stress despite the differences in water availability, with plants reducing water use and growth rate to the same extent in order to maintain a constant water potential. The overall relative growth rate (RGR) followed the same pattern, with slower plant growth when water was limited, but with no significant differences in RGR seen between the 15% H_2O and 30% H_2O treatments (Table 1D).

Water limitation overrides the developmental decrease of dhurrin in *acdc1*

We observed that HCNp during early growth was predominantly dependent on developmental stage and tissue type, while the effects of water availability and genotype became significant at the later harvests. These findings are consistent with other studies (Vanderlip, 1972, Miller et al., 2014, Gleadow et al., 2016a). Developmental regulation of dhurrin content, which causes a rapid decrease in HCNp from 4dpg onwards (Halkier and Møller, 1989, Busk and Møller, 2002), was the driving factor of HCNp during the early stages of plant growth with a decrease in dhurrin concentration in the leaf and sheath tissue from 11dpg to 27dpg observed across all lines and treatments (Figure 2).

In the *acdc1*, HCNp decreased more rapidly than both other lines when well-watered, consistent with earlier generations of the mutant (Blomstedt et al., 2012, Blomstedt et al., 2018). Drought stress appeared to override this developmental regulation with no significant difference in HCNp between *acdc1* and wild-type plants when water was limited. The difference in HCNp between watered and drought-stressed plants was therefore greatest in the *acdc1*, corresponding to either a slower decrease in endogenous remobilisation of dhurrin, or heightened induction of dhurrin synthesis when drought-stressed in comparison to wild-type plants. Production of dhurrin under the level of drought imposed may indicate that this is a direct stress response. However, as no plants maintained low-cyanogenic potential under water limitation, it was not possible to directly determine whether dhurrin plays a role in the mitigation of drought stress.

The higher HCNp in all lines when water was limited may be due to increased *de novo* biosynthesis of dhurrin, decreased remobilisation, or a concentration effect due to reduced plant growth. Total dhurrin content also increased above previous levels in the 100% H_2O treatment (Supplementary Figure 1) and although this did not correspond to higher HCNp due to a greater increase in the biomass of these plants, it does document on-going *de novo* synthesis. The total dhurrin content in the roots and shoots of drought-stressed plants was not higher than the 100% H_2O treatment at any harvest time-point; therefore the higher concentrations observed were attributable to a reduced total biomass. O'Donnell et al. (2013) found that hydroponically-grown sorghum exposed to 20% PEG contained the same amount of dhurrin on a whole plant basis as non-stressed plants, also attributing the HCNp increase at least partially to a concentration effect.

Very few studies report the effect of experimental treatments on root HCNp in sorghum. This is partly due to the misconception that sorghum roots are not cyanogenic which may stem from a misinterpretation of the study by Akazawa et al. (1960) where no free HCN was found in the seed or root of sorghum, a finding that was later misconstrued as signifying an absence of dhurrin in these tissues. Here, we showed clearly that sorghum roots are cyanogenic. Hydrogen cyanide potential of the root tissue was generally lower under drought, consistent with results for osmotic stress reported by O'Donnell et al. (2013) and in contrast to the increase observed in the shoots. There was also a significant increase in both root HCNp and total root dhurrin content in plants harvested at 35dpg compared with 27dpg for all treatments (Figure 2G-I; Supplementary Figure 1). This suggests that either dhurrin had been synthesised in the root tissues or transported from the shoots to the roots.

Evidently the regulatory mechanisms governing the deployment of dhurrin in the roots are different to what is occurring in the shoots. The cyanogenic status of roots differs between cyanogenic species; for example, roots of eucalypts and white clover have been reported to lack cyanogenic glucosides (Gleadow and Woodrow, 2002a, Hughes, 1991), while sorghum roots and cassava tubers contain them (O'Donnell et al., 2013, Jørgensen et al., 2005). In cassava the cyanogenic glucosides linamarin and lotaustralin are synthesised in the leaf tissue and transported to the tuber (Jørgensen et al., 2005). In

sorghum it is not confirmed whether the roots synthesise dhurrin *de novo*, or whether transport between tissues occurs. Busk and Møller (2002) found that in five-week old sorghum plants the rate-limiting dhurrin biosynthetic enzyme, CYP79A1 was only active in the stem, with no activity present in the leaves, leaf sheaths, or roots. From this it was deduced that in sorghum dhurrin is transported from the stem to the leaves. Selmar et al. (1996) found further evidence that dhurrin transportation may occur, with the diglucoside dhurrin-6'glucoside present in leaf guttation droplets. Diglucosides can be stably transported within plants, as seen in rubber trees (*Hevea brasiliensis*) which convert the cyanogenic monoglucoside linamarin to the diglucoside linustatin for transport from the endosperm to the seedling (Selmar et al., 1988).

In this study the lower HCNp present in the roots, compared to the higher dhurrin concentration seen in the shoot tissue under drought, may be due to less dhurrin being transported to the roots and more to the leaves and sheaths during these periods, rather than dhurrin synthesis in each individual tissue changing in response to water limitation. As few studies have analysed the HCNp of root tissues in older plants, this is an area that would benefit from further investigation both in sorghum and cyanogenic plants with edible underground storage organs, such as cassava and taro.

Decreases in plant dhurrin may result in higher nitrate concentrations

Overall, *acdc1* had higher concentrations of nitrate at the final harvest (Figure 3). This was particularly pronounced in the roots, which had more than double the concentration of nitrate compared to the wild-type plants. This supports the hypothesis that when less nitrogen is allocated to dhurrin there will be a resultant increase in stored nitrate. Previous studies have found conflicting results. In osmotically stressed sorghum, high dhurrin concentrations also correlated with lower nitrate concentrations (O'Donnell et al., 2013). Conversely, Gleadow et al. (2016a) and Neilson et al. (2015b) found that drought stress increased both dhurrin and nitrate concentrations in sorghum shoot tissue (root tissue was not analysed in these studies).

Nitrate concentrations are dependent on the rate of nitrate uptake from the soil and nitrate reduction via nitrate reductase. Though the activation state of nitrate reductase does not usually change in response to variations in nitrate supply (Diouf et al., 2004,

Kaiser and Huber, 2001) drought stress is associated with a decrease in nitrate reductase activity, with long term drought leading to the inactivation and degradation of the enzyme (Foyer et al., 1998, Fresneau et al., 2007, Kaiser and Huber, 2001). Re-allocation of nitrates to root tissues is found to occur under osmotic stress (Chen et al., 2012, Smirnoff and Stewart, 1985). This was observed in both the *acdc1* and wild-type plants in this study, with root nitrate concentration increasing under drought (Figure 3). Nitrate retention in roots may also be due to the reduction of nitrate transporters, with root nitrate retention in turn acting as a stress signal and activating osmotic stress related genes, as thought to occur in *Arabidopsis* (Chen et al., 2012).

In this study total nitrogen was also higher, on average, in the *acdc1* sheath and roots for all treatments compared to the wild-type and Sibs (Figure 3H,I). Though the higher nitrate concentration seen in the *acdc1* may account for the higher total N found in the roots, the *acdc1* sheaths at 15% H_2O and 30% H_2O did not have higher nitrate concentrations than other lines yet still had significantly greater amounts of N. The proportion of N allocated to dhurrin was lower in the *acdc1* leaf tissue at 100% H_2O but increased under water limitation where it was equivalent to the other lines (Figure 4A-C). This lower allocation of N to dhurrin in the *acdc1* leaves equated to a higher proportion of N allocated to nitrate compared to the wild-type line (Figure 4). In agreement with this, the sorghum EMS-mutant *totally cyanide deficient 1*, that does not produce dhurrin at any stage of development, has been found to allocate more nitrogen to nitrate in the leaf tissue than wild-type plants at later stages of development (Blomstedt et al., 2018). It is difficult to state whether there is a direct trade-off between dhurrin and nitrate occurring in sorghum, particularly as the differences are tissue dependent. Here, the results are further confounded by the *acdc1* having significantly higher levels of nitrogen in all tissues for all treatments.

Conclusions

In this study plant age and water limitation were found to be the most important determinants of dhurrin concentration in sorghum. The *acdc1* had lower dhurrin concentrations in the leaf tissue under fully-watered conditions, though this difference was not seen when water was limited. Despite HCNp decreasing as the plants matured

when water was replete, synthesis of dhurrin continued to occur, with total plant dhurrin content increasing until the final harvest.

The driving factor of nitrate concentrations was genotypic differences, with the *acdc1* storing higher concentrations of nitrates in the leaves and roots than wild-type plants for all treatments. Nitrate concentrations were affected by drought treatments, though less so than dhurrin, where they showed an opposite trend: decreasing in leaf tissues as water availability decreased, while increasing in the root tissues. Trade-offs between nitrogen and dhurrin may occur, with lower dhurrin concentration in the *acdc1* leaf tissue corresponding to higher nitrate concentration compared to the wild-type line at 100% H_2O. Growth indices in the *acdc1* were not affected by differences in dhurrin or nitrate concentrations in comparison to wild-type plants either under drought conditions or when fully watered.

This study demonstrates that dhurrin and nitrate concentrations in sorghum are highly dynamic, with regulation differing between above and below ground tissues. Changes in cyanogenic glucoside concentrations, both developmentally and in response to environmental factors, need to be considered with respect to their effect on stored nitrates for all tissues, as influencing concentrations in one tissue may affect another, particularly if transport of cyanogenic glucosides and nitrate is occurring between tissues.

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2.7 Supplementary Material

Supplementary Table 1: Hydrogen cyanide potential (HCNp - mg HCN g^{-1} dry mass) of three *Sorghum bicolor* genotypes grown at 15%, 30%, and 100% field capacity of water and harvested at 11 days post-germination (dpg), 19dpg, 27dpg, and 35dpg for **A)** Leaf; **B)** Sheath; and **C)** Root tissues. WT: wild-type; Sibs: Siblings; *acdc1*: adult cyanide deficient mutants. Values denote means ± SEM (*n*=6), means with different letters are significantly different at P<0.05 analysed using ANOVA and Tukey's test.

A) Leaf

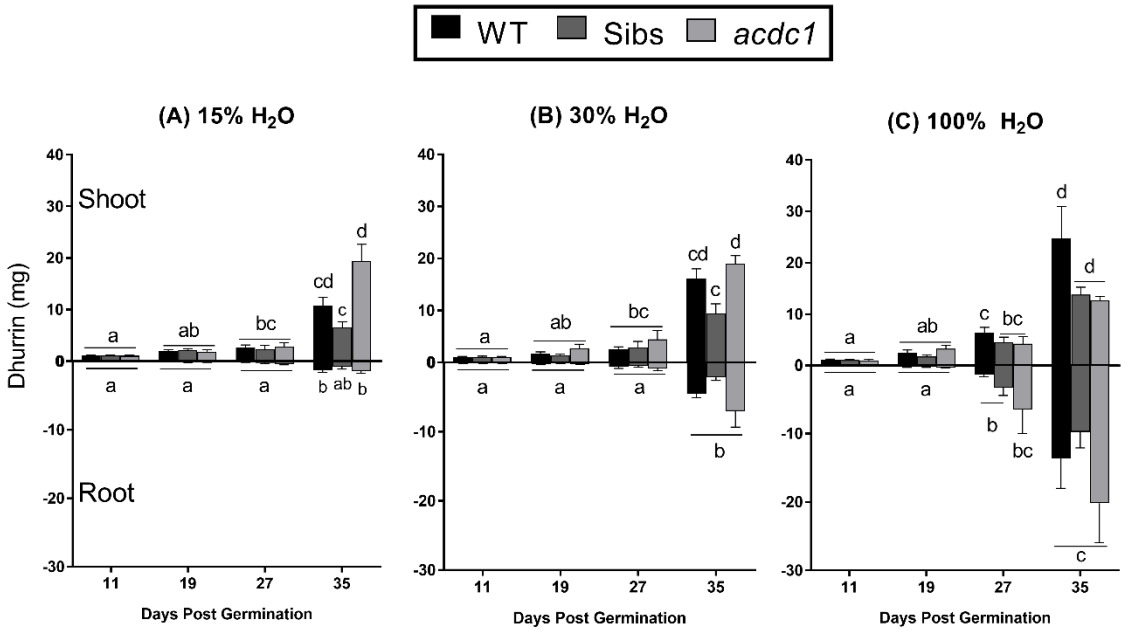
	WT			**Sibs**			***acdc1***			**ANOVA**		
	15%	**30%**	**100%**	**15%**	**30%**	**100%**	**15%**	**30%**	**100%**	**L**	**T**	**LxT**
11dpg			5.90 (0.34)			5.40 (0.37)			5.90 (0.35)	ns		
19dpg	3.21 (0.60)	2.21 (0.78)	1.72 (0.44)	2.59 (0.37)	3.12 (0.82)	1.73 (0.37)	2.33 (0.46)	2.48 (0.71)	2.84 (0.74)	ns	ns	ns
27dpg	1.00^{a} (0.17)	1.14^{a} (0.37)	0.78^{a} (0.27)	1.27^{a} (0.44)	1.20^{ab} (0.09)	0.67^{b} (0.06)	0.96^{a} (0.19)	1.19^{a} (0.25)	0.29^{c} (0.05)	*	***	ns
35dpg	2.11^{a} (0.17)	1.51^{a} (0.37)	0.65^{b} (0.06)	2.50^{a} (0.37)	2.52^{a} (0.57)	0.60^{b} (0.03)	2.68^{a} (0.59)	1.73^{a} (0.36)	0.28^{c} (0.02)	*	***	**

B) Sheath

	WT			**Sibs**			***acdc1***			**ANOVA**		
	15% H_2O	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**15% H_2O**	**30% H_2O**	**100% H_2O**	**L**	**T**	**LxT**
11dpg			5.90 (0.34)			5.40 (0.37)			5.90 (0.35)	ns		
19dpg	2.42 (0.44)	2.60 (0.42)	1.60 (0.14)	2.50 (0.42)	2.81 (0.66)	1.89 (0.25)	2.35 (0.54)	2.30 (0.25)	1.82 (0.18)	ns	ns	ns
27dpg	0.65^{a} (0.09)	0.69^{a} (0.10)	0.36^{b} (0.04)	0.68^{a} (0.12)	0.81^{a} (0.19)	0.35^{b} (0.05)	0.93^{a} (0.10)	1.04^{a} (0.11)	0.30^{b} (0.03)	ns	***	ns
35dpg	1.36^{a} (0.29)	1.03^{a} (0.25)	0.40^{b} (0.10)	1.27^{a} (0.12)	1.04^{a} (0.09)	0.46^{b} (0.08)	1.86^{a} (0.28)	1.39^{a} (0.28)	0.20^{c} (0.10)	*	***	*

C) Root

	WT			Sibs			*acdc1*			ANOVA		
	15% H_2O	30% H_2O	100% H_2O	15% H_2O	30% H_2O	100% H_2O	15% H_2O	30% H_2O	100% H_2O	L	T	LxT
11dpg			0.46 (0.14)			0.45 (0.05)			0.36 (0.06)	ns		
19dpg	0.16^{a} (0.02)	0.30 (0.08)	0.53^{b} (0.08)	0.19 (0.04)	0.18 (0.04)	0.53 (0.15)	0.14^{a} (0.03)	0.29 (0.09)	0.35^{b} (0.07)	ns	***	ns
27dpg	0.01^{a} (0.003)	0.06^{bc} (0.02)	0.12^{b} (0.02)	0.02^{a} (0.01)	0.05^{ac} (0.02)	0.14^{be} (0.03)	0.11^{b} (0.04)	0.25^{d} (0.05)	0.24^{de} (0.07)	***	***	ns
35dpg	0.17^{a} (0.05)	0.39^{bc} (0.10)	0.32^{cd} (0.04)	0.11^{a} (0.03)	0.24^{ac} (0.04)	0.40^{bd} (0.04)	0.25^{a} (0.05)	0.53^{b} (0.05)	0.48^{b} (0.05)	***	***	ns



Supplementary Figure 1: Total dhurrin content (mg) of shoot and root tissues of WT: wild-type; Sibs: siblings; and *acdc1*: *adult cyanide deficient class 1* sorghum lines grown at **(A)** 15% H_2O; **(B)** 30% H_2O; and **(C)** 100% H_2O. Values denote means ± SEM (n = 6), means with different letters are significantly different at P<0.05, analysed using ANOVA and Tukey's test.

Chapter 3 Declaration

Monash University

Investigation into the role of DNA methylation in cyanogenesis in sorghum (*Sorghum bicolor* L. Moench)

This thesis chapter is in the same format as the manuscript published in the peer-reviewed journal *Plant Growth Regulation*.

Declaration by candidate:

The nature and extent of my contribution to this work is as follows:

Nature of Contribution	Extent of contribution
Experimental design, execution of experimental work, analysis, manuscript writing, editing and revisions.	80%

The following co-authors contributed to the work:

Name	Nature of Contribution	Extent of contribution	Student at Monash University
Alicia A. Quinn	Execution of experimental work	4%	N
Samantha M. Fromhold	Execution of experimental work	2%	N
Ros Gleadow	Experimental design, manuscript editing	2%	N
Cecilia K. Blomstedt	Experimental design, execution of experimental work, analysis, manuscript writing and editing	12%	N

Student signature: **Date:**

Main Supervisor signature: **Date:**

Chapter 3

Investigation into the role of DNA methylation in cyanogenesis in sorghum (*Sorghum bicolor* L. Moench)

3.1 Abstract

Sorghum bicolor produces the cyanogenic glucoside dhurrin, a secondary metabolite integral to plant defence and stress responses. Dhurrin production is both developmentally and environmentally regulated in *S. bicolor*, with high levels of variation within and between lines. Such phenotypic variation may result from polymorphic differences or epigenetic modifications in genes associated with cyanogenesis. In this study the chemical 5-Azacytidine was used to assess *S. bicolor's* response to genome-wide demethylation, which had not previously been investigated in the context of cyanogenic glucoside regulation. Morphological changes, the expression levels of key genes involved in dhurrin synthesis and turnover, and the cyanogenic potential (HCNp) of leaf tissues were analysed. Treatment resulted in alterations in dhurrin synthesis, gene expression, and dhurrin levels, suggesting that DNA methylation is involved in the regulation of HCNp in the initial stages of *S. bicolor* development. Previously identified EMS mutants from the *adult cyanide deficient class* (*acdc*) have been found to exhibit altered dhurrin concentrations during development. This study shows that *acdc* mutants possess a CΔT change in the promoter of *CYP79A1,* a key gene in dhurrin synthesis, and that this mutation is stably inherited and associated with the *acdc* phenotype. To further investigate the role of epigenesis in dhurrin production, we determine the methylation status of the 250bp region surrounding the CΔT mutation site in wild-type and mutant plants over two stages of development.

3.2 Introduction

Plant secondary metabolites play important roles in both defence and stress responses. One such class of metabolites are the cyanogenic glucosides (CNglcs), produced by over 2,600 species of higher plants and one-fifth of crop species (Gleadow and Møller, 2014, Jones, 1998). The first CNglc biosynthesis pathway to be identified was in *Sorghum bicolor* (L.) Moench, which produces the CNglc dhurrin ((S)-4-hydroxymandelonitrile-β-D-glucopyranoside)(Bak et al., 1998, Kahn et al., 1997). Upon tissue disruption dhurrin, normally compartmentalised in the vacuole, becomes susceptible to the degradative β-glucosidase, dhurrinase, hydrolysing the compound to release toxic hydrogen cyanide gas (HCN) in a process known as cyanogenesis. Dhurrin biosynthesis is catalysed by three enzymes, coded for by three structural genes: *CYP79A1*, *CYP71E1* and *UGT85B1* (Kahn et al., 1997*)* (Figure 1a). Following their identification in *S. bicolor*, these genes were used to find the cyanogenic glucoside biosynthesis genes in other crop species, including cassava (*Manihot esculenta* Crantz), although pathways between species are not identical (Andersen et al., 1999, Jørgensen et al., 2005, Kannangara et al., 2011). For example, in cassava two paralogues for each of the three biosynthesis genes are present, resulting in the production of two CNglcs: lotaustralin and linamarin. Despite these differences, the first and key enzymes in the production of CNglcs across species have been shown to be the CYP79s, which are considered signature enzymes of cyanogenesis pathways (Gleadow and Møller, 2014).

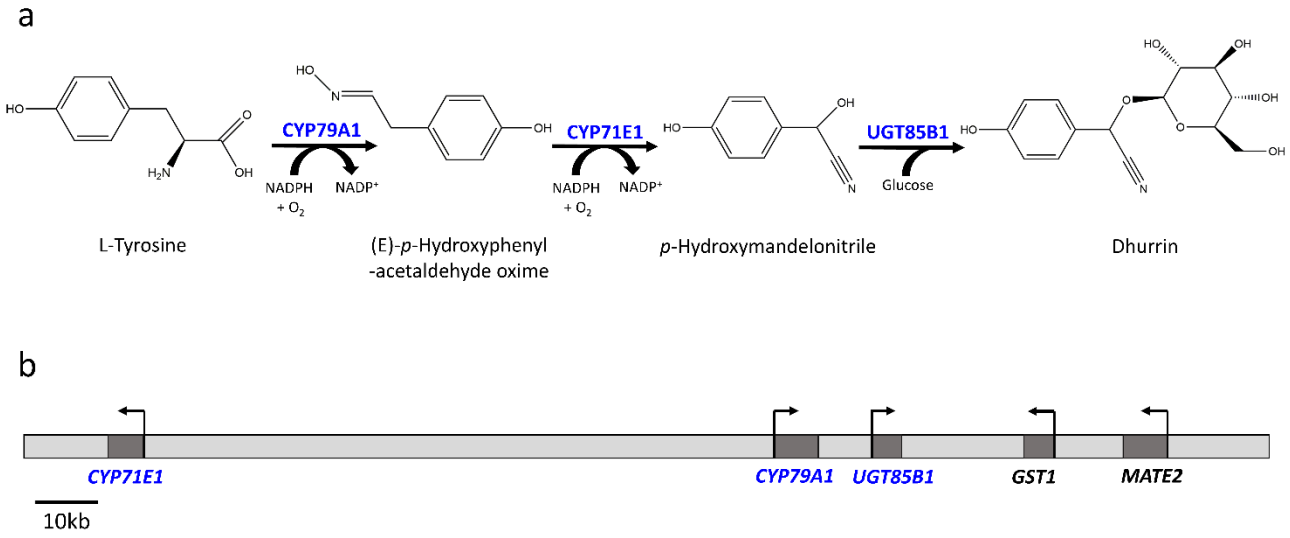


Figure 1: (a) Schematic illustrating the steps and enzymes involved in the biosynthesis of dhurrin. Dhurrin biosynthesis begins with an L-tyrosine precursor and is driven by the cytochromes P450 CYP79A1 and CYP71E1 that catalyse the first two steps in the pathway. The UDP-glycosyltransferase UGT85B1 stabilises the *p*-hydroxymandelonitrile via glycosylation to produce dhurrin. CYP79A1 is the rate-limiting and key enzyme in this pathway (Bak et al., 1998, Halkier and Møller, 1989, Kahn et al., 1997) **(b)** The dhurrin biosynthesis gene cluster on chromosome 1 in *Sorghum bicolor*, which has also been shown to include the MATE2 transporter and glutathione-S-transferase 1 (GST1)(Darbani et al., 2016). *CYP71E1*, Sb01g001180; *CYP79A1*, Sb01g001200; *UGT85B1*, Sb01g001220; *GST1*, Sb01g001230; *MATE2*, Sb01g001240.

Dhurrin concentrations are both developmentally and environmentally regulated in *S. bicolor* (Gleadow and Møller, 2014). The mature seed is the only part of the plant that is completely acyanogenic, although dhurrin concentrations rise rapidly following germination, reaching up to 30% of the dry weight of the seedling tip, before decreasing across all tissues as the plant matures (Busk and Møller, 2002, Halkier and Møller, 1989). Abiotic cues, including drought and high levels of nitrogen application, will also increase dhurrin concentrations to different extents between tissues, albeit only after

maximum production rates of the compound have dropped (Blomstedt et al., 2018, Burke et al., 2015, Busk and Møller, 2002, Neilson et al., 2015a). In addition, hydrogen cyanide potential (HCNp, the total amount of cyanide released per mass) can differ markedly both between and within *S. bicolor* lines, with individuals showing high levels of plasticity (Gleadow and Møller, 2014). Cyanogenic glucosides in other species are also spatially and temporally expressed. In cassava CNglcs are found in all tissues, but synthesis occurs primarily in the shoot before the compounds are transported to the root (Jørgensen et al., 2011). It is postulated that dhurrin in *S. bicolor* is primarily synthesised at the base of the stem and is then transported to the leaves (Busk and Møller, 2002), however, further evidence of this is needed.

Despite the biosynthetic pathway of dhurrin being well-defined biochemically, the molecular regulation of the biosynthetic genes is largely unknown. Utilisation of *S. bicolor* for forage is increasing due to its high drought and heat tolerance, as well as its increased suitability for marginal soils in comparison to maize (Getachew et al., 2016). Consequently, elucidation of the molecular regulation of the dhurrin pathway becomes pertinent to enable phenotypic predictions and to prevent livestock poisoning and pasture loss. Busk and Møller (2002) found dhurrin synthesis to be transcriptionally regulated, although this finding has been difficult to replicate. However, it has been shown that the biosynthetic genes, which are clustered on chromosome one in *S. bicolor* (Figure 1b), are co-expressed (Darbani et al., 2016, Takos et al., 2011). Similar gene clusters have been identified in cassava and the cyanogenic species *Lotus japonicus* (Takos et al., 2011). Gene clusters are thought to have evolved by gene duplication followed by acquisition of new functions and typically contain 3-6 genes key to the biosynthetic pathway of the specific metabolite (Nützmann and Osbourn, 2014). Though knowledge of the molecular regulation of these clusters is also lacking, clustering of the cyanogenesis genes may also have arisen to allow for synchronous regulation via epigenetic mechanisms, such as DNA methylation.

The role of epigenetic marks, including DNA cytosine methylation, in development and stress responses across plant species continues to be a focus of research (Baulcombe and Dean, 2014, Kellenberger et al., 2016, Pandey et al., 2016). Epialleles, alleles variably expressed due to differences in methylation rather than nucleotide mutations

in the gene, have been found to be involved in heritable phenotypes, including traits of agronomic importance (Amoah et al., 2012, Sharma et al., 2017, Smith et al., 2010). Plants methylate cytosines in three sequence contexts: CG, CHG, and CHH (H = A, C or T) (Gruenbaum et al., 1981) and while many studies have investigated changes in methylation during plant development, research into the epigenetic regulation of secondary metabolites in cereal crops has predominantly been undertaken in non-cyanogenic species or has not closely considered cyanogenic genes (Joel, 2013, Kou et al., 2011, Wang et al., 2011). Genome-wide methylation studies in *S. bicolor* have examined differences between tissues at single developmental time points (Turco et al., 2017) or within tissues between different treatments (Olson et al., 2014). Therefore, any methylation changes in the cyanogenesis genes within and between tissues over developmental time, or in response to environmental factors, have not been observed.

Dhurrin concentration is dependent on plant age, and in this study we investigate whether methylation changes during development. Wild-type *S. bicolor* seeds were germinated in the presence of the demethylating agent 5-Azacytidine (5-azaC). 5-azaC is a chemical analogue of cytosine which is incorporated into the DNA as a substitute for cytosine, while also inhibiting the action of DNA methyltransferases resulting in genome-wide hypomethylation (Christman, 2002). To test the hypothesis that developmental regulation of dhurrin is in part controlled by DNA cytosine methylation in the early stages of plant growth, the following were measured over a three-week period: genomic DNA methylation levels, the expression of genes involved in dhurrin synthesis and turnover, and the HCNp of *S. bicolor* seedlings.

In addition, we investigated the inheritance mechanisms and the regulation of dhurrin synthesis through the use of EMS mutants generated by TILLING (Blomstedt et al., 2012). In order to analyse the stable inheritance of the *adult cyanide deficient class* (*acdc*) mutation and its association with the low dhurrin phenotype, three independent mutant lines with a similar phenotype classified as *adult cyanide deficient class 1-3 (acdc1, acdc2, acdc3)* and the *totally cyanide deficient 1 (tcd1)* mutant were crossed and the progeny phenotyped. We also addressed whether the methylation state of the region surrounding the *acdc* mutation is important for the temporal and spatial control of dhurrin synthesis.

3.3 Materials and Methods

Genome-wide demethylation using 5-Azacytidine

Sorghum bicolor (L.) Moench line BTx623 was used to analyse the effects of the demethylating agent 5-Azacytidine (5-azaC, A1287, Sigma-Aldrich, Sydney, Australia) on plant growth, leaf dhurrin concentrations, and the expression of key genes involved in the dhurrin synthesis and turnover pathways. An initial germination dose-response gradient of 5-azaC at 0μM, 25μM, 50μM, 100μM, and 200μM in ½ strength Murashige and Skoog (MS) media was performed (data not shown). Based on this, the selected concentrations with which to proceed were 50μM and 100μM of 5-azaC. For each treatment 120 BTx623 seeds were sterilised then germinated in petri dishes containing either ½ MS media (controls), ½ MS media with 50μM 5-azaC, or ½ MS media with 100μM 5-azaC. Petri dishes were placed in a constant temperature room at 28°C under a cycle of 16-h light and 8-h darkness.

Seed germination percentage was determined before seeds were moved to new petri dishes containing fresh solution daily until 4 days post-germination (dpg), at which stage 70 seedlings of each treatment were randomly selected, washed thoroughly with MilliQ H_2O, and transferred to individual pots and grown in greenhouse facilities at Monash University according to Blomstedt et al. (2018). Plants were randomised spatially, rotated, and watered to saturation every second day to ensure water was never limited. Plants were harvested at 7dpg, 14dpg, and 21dpg, with 20 plants of each treatment randomly selected and harvested at the first two time-points, while 20 replicates of the control treatment and 23 replicates of the 50μM and 100μM 5-azaC treatments were harvested at the final time-point. Both height and leaf number were measured, with three leaf-discs (6mm diameter) taken from the youngest unfurled leaf for HCNp analysis, with HCNp assayed as per Gleadow et al. (2012). HCNp is the total amount of HCN evolved from hydrolysis of the entire content of endogenous cyanogenic glucosides and is used as a proxy for dhurrin, such that each mg of HCN is equivalent to 11.5 mg of dhurrin. The remainder of the tissue from the same leaf was snap frozen in liquid nitrogen and stored at -80°C until required for further analysis. Frozen tissue was pooled into three replicates for each treatment and harvest time-point, with the pooled tissue then divided into two for DNA and RNA analysis.

Genomic DNA was extracted using a modified CTAB method (Allen et al., 2006). The 5-methylcytosine level of the extracted gDNA was quantified using the 5-mC DNA ELISA Kit (Zymo Research, California, USA) using standards of known methylation levels as per the manufacturer's instructions. Three biological replicates and standards were run in technical duplicates with the colour allowed to develop for one hour before the plate was read on a spectrophotometer at 410nm (BMG FLUOstar Galaxy, MTX Lab systems, USA).

RNA extraction and quantitative PCR

RNA was extracted from the frozen leaf tissue using a Sigma Spectrum Plant Total RNA kit (Sigma-Aldrich, Sydney, Australia). On-column DNase digestion was carried out for all samples according to Appendix 1 of the manufacturer's instructions. cDNA was synthesised from the total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Melbourne, Australia) and an oligo dT primer. Transcript levels of *CYP79A1* (Sb01g001200) and *NIT4B2* (Sb04g026940) normalised to *Ubiquitin* (*UBT*, Sb01g030340) and *Actin* (Sb01g010030) (Paolacci et al., 2009) were determined by quantitative PCR (qPCR) using a Rotor-Gene 6000 (Qiagen, Melbourne, Australia). Forward and reverse primers (Sigma, Sydney, Australia) specific for a region in the 3' end of each gene sequence were used (Supplementary Table 1). SensiMix SYBR Green No ROX kit (Bioline, Sydney, Australia) was used for the qPCR as per the manufacturer's instructions. Each biological replicate was run in triplicate along with a set of standards specific for the gene being analysed. Each RNA preparation (the RNA used to create the cDNA) was also checked for DNA contamination by using crude RNA as the template and determining if amplification occurred. Relative expression was calculated as Mean Normalised Expression (MNE) as per Simon (2003).

Plant growth and phenotyping

To characterise the low cyanogenic mutants, crosses were made within near isogenic lines of the mutants (*acdc1* × *acdc2; acdc1* × *acdc3; acdc2* × *acdc3*; *tcd1* × *acdc1*) and between the mutants and the wild-type line (*Sorghum bicolor* (L.) Moench line BTx623). Seeds from individual crosses were collected and F_1 plants grown and allowed to self-fertilise with flower heads bagged to prevent cross-pollination. F_2 seeds were collected and sown in individual trays containing Debco seed raising mix and perlite (2:1, v/v).

All plants were grown at Monash University greenhouse facilities according to Blomstedt et al. (2018). Three leaf-discs (6mm diameter) from the youngest unfurled leaf were taken from F_1 and F_2 plants at the 7-leaf stage of development for HCNp analysis, with HCNp assayed as per Gleadow et al. (2012). From the F_2 populations, individuals were selected that were homozygous for the mutation for use in future experiments. In addition, sibling lines that segregate in the F_2 but no longer carry the mutation were also selected and used as controls. Mutant and sibling lines have previously been backcrossed to wild-type BTx623 *S. bicolor* to remove background mutations, with no phenotypic differences seen in the mutant plants other than the altered cyanogenic status.

Amplification and sequencing of the promoter region to confirm presence/absence of mutation

Genomic DNA was extracted from frozen leaf tissue of homozygous *acdc1, acdc2* and *acdc3* mutants, and BTx623 plants as per the modified CTAB protocol (Allen et al., 2006). Based on sequence data from the *S. bicolor* genome sequence (Phytozome http://phytozome.jgi.doe.gov/) a ~1.4kb region upstream of *CYP79A1,* including the putative site of the *acdc* mutation, was amplified by PCR. Primers specific for this region were designed using PerlPrimer (Marshall, 2004) and supplied by Sigma-Aldrich (Sydney, Australia) (Supplementary Table 2). PCR reactions were set up using the proofreading *Pfu* DNA polymerase (Promega, Sydney, Australia) according to the manufacturer's instructions, and amplified on a Mastercycler thermocycler (Eppendorf, Sydney, Australia) as follows: initial denaturation: 2 minutes, 95°C; denaturation: 30 seconds, 95°C; annealing: 30 seconds, 54°C; extension: 2.5 minutes, 72°C, for 35 cycles; final extension: 5 minutes at 72°C. Reactions were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, Sydney, Australia). Purified fragments were sequenced in both forward and reverse directions using the BigDye Terminator v3.1 Cycle Sequencing kit according to the manufacturer's instructions (Applied Biosystems 3730 Genetic Analyzer; Melbourne, Australia). Sequence analysis was performed with Chromas (Technelysium, Brisbane, Australia) and BioEdit (Ibis Biosciences, California, USA).

Assessment of methylation status using bisulfite conversion

Seeds of the wild-type line BTx623, mutant line *acdc1*, and mutant sibling line *ACDC1* were sterilised before being germinated in petri dishes on filter paper wetted with MilliQ H_2O. Petri dishes were placed in a constant temperature room at 28°C in complete darkness until 3dpg where root and shoot tissues were separated, randomly pooled into 0.200g of each tissue and snap frozen in liquid N for later analysis. Additional seeds of the three lines were placed in individual pots and grown in the glasshouse (Blomstedt et al., 2018) for 5 weeks, at which point tissue was taken from the roots and the youngest unfurled leaf and snap frozen in liquid N. Bisulfite conversion was completed with the EZ DNA Methylation Kit (Zymo Research, California, USA) as per the manufacturer's instructions using 500ng of genomic DNA (CTAB extraction as above). A region of the *CYP79A1* 5′ regulatory region for both converted and unconverted DNA was amplified (Supplementary Table 3a). An unmethylated region of the *ASA1* gene from *Arabidopsis* was used as a control for complete bisulfite conversion (Supplementary Table 3b). PCR reactions were set up using KAPA2G Robust DNA Polymerase (Sigma-Aldrich, Sydney, Australia) according to the manufacturer's instructions and amplified on a Mastercycler thermocycler (Eppendorf, Sydney, Australia) as follows: initial denaturation: 3 minutes, 95°C; denaturation: 15 seconds, 95°C; annealing: 15 seconds, 56°C; extension: 15 seconds, 72°C, for 40 cycles; final extension: 30 seconds at 72°C. Amplified PCR fragments were gel purified and sequenced as described in section 2.2. Sequence analysis was undertaken using KISMETH software (http://katahdin.mssm.edu/kismeth/revpage.pl).

Statistical analysis

The statistics package *SigmaPlot* Version 12.2 (Systat Software, California, USA) was used for statistical analyses by two-way ANOVA and also unpaired *t*-tests in GraphPad Prism (California, USA). For all tests, a *p*-value of <0.05 was considered significant. In the presence of an interaction, post-hoc comparisons were undertaken using Tukey's test or Dunn's test.

3.4 Results

Growth effects of the demethylating agent 5-Azacytidine treatment on *Sorghum bicolor* seedlings

An examination of the effects of 5-azaC at 0μM, 25μM, 50μM, 100μM, and 200μM on the growth of *S. bicolor* line BTx623 was conducted. The dose-response gradient of 5-azaC showed that both roots and shoots decreased in length as 5-azaC concentrations increased but no signs of phytotoxicity could be seen below 200μM, a result consistent with previous sorghum studies (Turco et al., 2017). Further experiments were conducted using concentrations of 50μM and 100μM 5-azaC. No differences in the percentage of germinated seeds between treatments were seen at either 24, 48, 72, 96, or 120 hours post-imbibition, with over 85% of seeds having germinated across all treatments by 120 hours (Supplementary Figure 1). Plant height and leaf numbers were measured at 7 days post-germination (dpg), 14dpg, and 21dpg (Table 1). The principal observation was that plants grown under 5-azaC were significantly shorter than the control lines at all harvest time-points ($P<0.001$; Figure 2). There was no significant difference in the level of height reduction between the 50μM and 100μM 5-azaC treatments (Table 1). Treatment with 5-azaC resulted in a slight delay in development; while there was no difference in the number of furled leaves between treatments at any harvest, both 5-azaC treatments resulted in significantly fewer unfurled leaves at 7dpg. However, this developmental delay was brief and by 14 and 21dpg, plants grown under 50μM 5-azaC had the same number of unfurled leaves as the control plants. Plants grown under 100μM 5-azaC had significantly higher numbers of unfurled leaves than both the controls and 50μM 5-azaC treated plants ($P<0.05$) at the final two harvests. No other morphological differences were seen between treatments (Figure 2).

Table 1: Height and leaf number of control and 5-azaC treated *S. bicolor* plants.

	Control	**50µM 5-azaC**	**100µM 5-azaC**
H1 - **7dpg**			
Unfurled leaf no.	1.9 ± 0.08[a]	1.0 ± 0.0[b]	1.0 ± 0.0[b]
Furled leaf no.	1.2 ± 0.08	1.0 ± 0.0	1.0 ± 0.0
Height (cm)	4.2 ± 0.2[a]	1.7 ± 0.06[b]	1.2 ± 0.06[b]
H2 - **14dpg**			
Unfurled leaf no.	3.0 ± 0.00[a]	3.0 ± 0.00[a]	3.2 ± 0.0[b]
Furled leaf no.	2.0 ± 0.00	2.0 ± 0.09	1.9 ± 0.1
Height (cm)	10.4 ± 0.13[a]	5.8 ± 0.40[b]	4.8 ± 0.6[b]
H3 - **21dpg**			
Unfurled leaf no.	5.0 ± 0.00[a]	4.9 ± 0.07[a]	5.3 ± 0.09[b]
Furled leaf no.	1.8 ± 0.10	1.52 ± 0.1	1.52 ± 0.1
Height (cm)	17.4 ± 0.25[a]	11.8 ± 0.7[b]	11.4 ± 0.7[b]

Data denotes mean ± SE (H1 and H2 n=20; H3 control n=20, 5-azaC treatments n=23). Rows marked with identical letters are not significantly different at P<0.05, analysed using ANOVA and Tukey's test

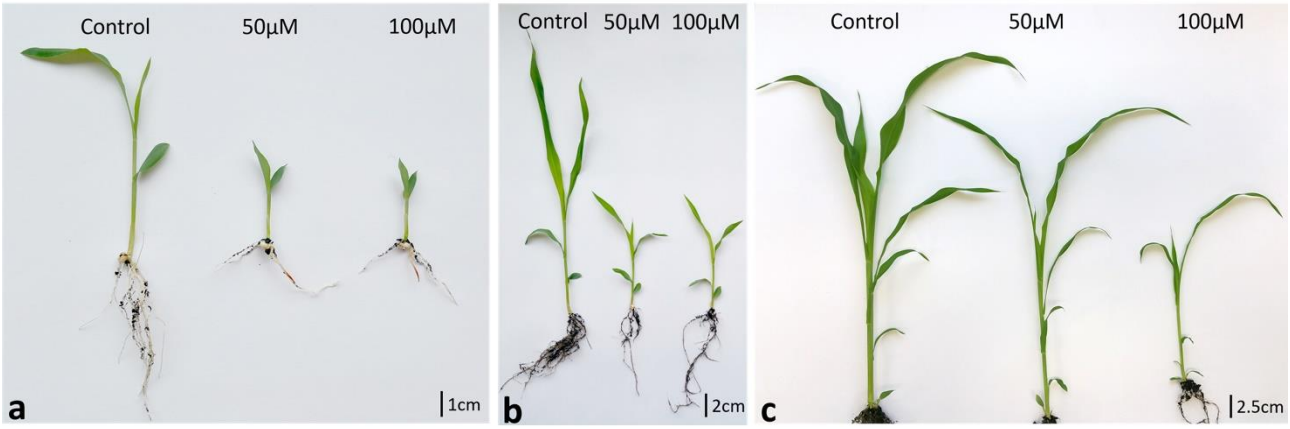


Figure 2: Representative examples of growth differences between wild-type *Sorghum bicolor* BTx623 control plants and plants treated with either 50µM or 100µM 5-azaC at **(a)** Harvest 1 (7 days post-germination - dpg), **(b)** Harvest 2 (14dpg), and **(c)** Harvest 3 (21dpg). Treatment with 5-azaC had the greatest effect on plant height. Leaf development was delayed by both concentrations of 5-azaC at Harvest 1, although this delay was brief with leaf numbers becoming consistent across treatments by the second harvest.

Genome-wide methylation levels of 5-azaC treated *Sorghum bicolor* leaf tissue

To confirm that the 5-azaC treatments had caused hypomethylation of the genome, total cytosine methylation (5-methylcytosine, 5mC) levels of control and 5-azaC treated *Sorghum bicolor* plants, line BTx623, were quantified via an ELISA. The first two harvests (7dpg and 14dpg) saw significantly lower percentages of methylation in plants treated with either concentration of 5-azaC (P<0.001), compared with control plants (Figure 3). The difference in methylation levels between the 50µM and 100µM 5-azaC treatments was not significant. By 21dpg the difference in average methylation levels between plants treated with 5-azaC had reduced and the difference between the control and 50µM 5-azaC treatments was no longer significant, although the 100µM 5-azaC treatment still had significantly lower levels of methylation than the controls.

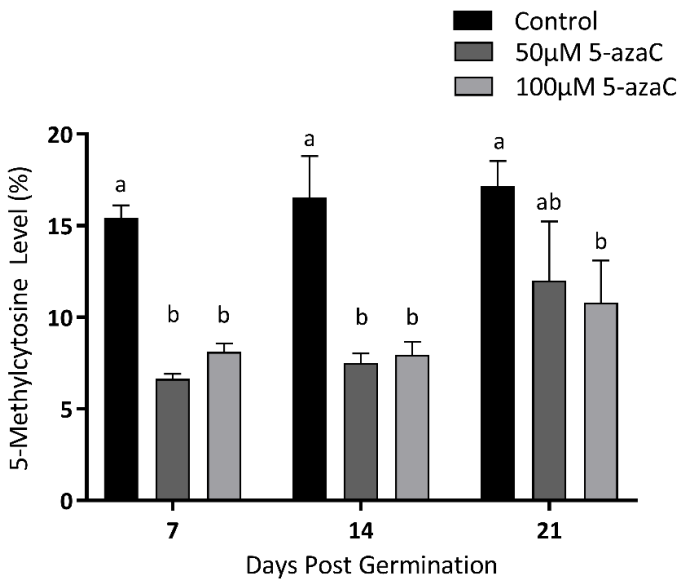


Figure 3: Genome-wide 5-methylcytosine levels (%) of leaf tissue in *Sorghum bicolor* (BTx623) controls in comparison to plants treated with 50µM or 100µM 5-azaC. Both concentrations of 5-azaC significantly decreased the percentage of genome-wide 5-methylcytosine until 14 days post-germination (dpg). By 21dpg the 50µM treatment was not significantly different to the controls, though the 100µM treatment continued to have lower 5-methylcytosine levels. Data denotes mean ± SE (n=3). Columns marked with identical letters are not significantly different at P<0.05, analysed using ANOVA and Tukey's test.

Effect of demethylation on HCNp in *Sorghum bicolor* leaf tissue

Cyanide assays were undertaken for all treatments at all time-points to correlate DNA methylation levels with HCNp. The greatest difference occurred at 7dpg where the 5-azaC treated plants had 30% higher HCNp than control plants (Figure 4). By 14dpg HCNp had decreased across all treatments although plants grown under 50µM 5-azaC had a significantly higher HCNp than both other treatments (P<0.001), while those grown under 100µM 5-azaC had significantly lower HCNp than both other treatments (P<0.001). By the final harvest (21dpg) HCNp had decreased in both the control and 50µM 5-azaC treated plants to equal the HCNp of plants treated with 100µM 5-azaC, with no significant differences in HCNp seen between any treatment.

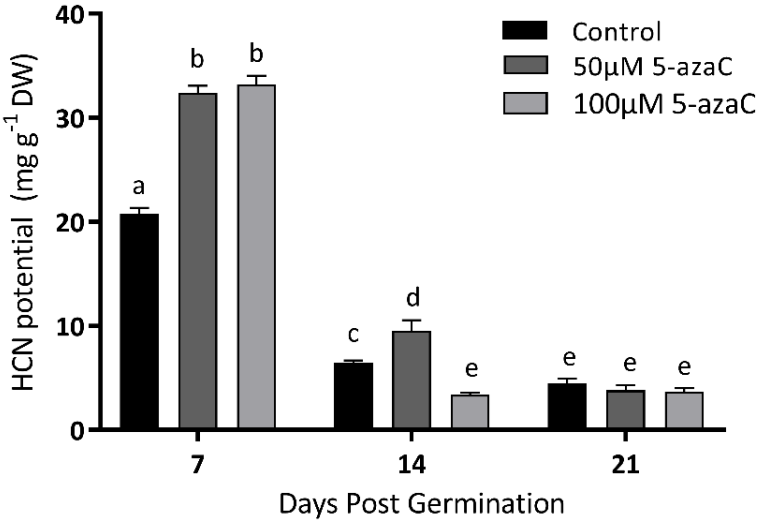


Figure 4: Leaf hydrogen cyanide (HCN) potential (mg g^{-1} dry weight) of *Sorghum bicolor* (BTx623) controls in comparison to plants treated with 50µM or 100µM 5-azaC. Data denotes mean ± SE (H1 and H2 n=20; H3 control n=20 and 5-azaC treatments n=23). Columns marked with identical letters are not significantly different at P<0.05, analysed using ANOVA and Tukey's test.

Transcript expression levels of key genes governing dhurrin synthesis and nitrogen turnover

To determine whether the expression of two genes involved in the dhurrin synthesis and turnover pathways were altered in the leaf tissue by 5-azaC treatments, relative transcript levels of *CYP79A1* (the gene encoding the rate-limiting enzyme in the dhurrin biosynthetic pathway (Busk and Møller, 2002)) and *NIT4B2* (involved in both the dhurrin detoxification and endogenous turnover pathways (Jenrich et al., 2007)) were measured and normalised to *Ubiquitin* and *Actin*. At 7 and 14dpg plants treated with 5-azaC had significantly greater levels of *CYP79A1* expression (Figure 5a). However, by 21dpg there were no significant differences between treatments. No significant differences in *NIT4B2* expression levels were seen at any harvest time point or between treatments (Figure 5b).

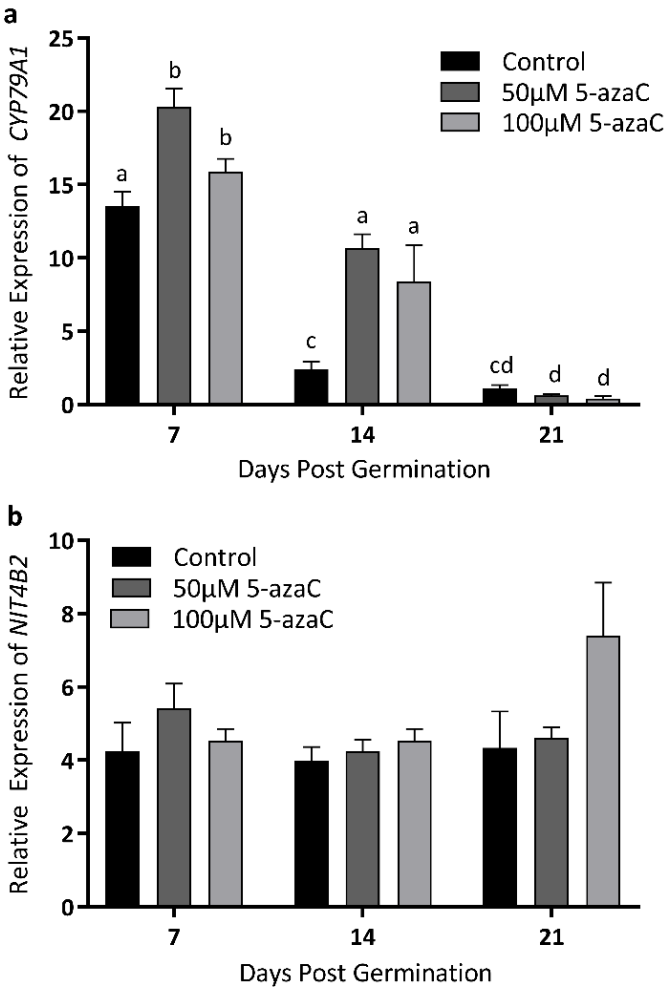


Figure 5: Relative transcript levels of **(a)** *CYP79A1* (Sb01g001200) and **(b)** *NIT4B2* (Sb04g026940) normalised to *Ubiquitin* (Sb01g030340) and *Actin* (Sb01g010030) in leaf tissue of *Sorghum bicolor* (BTx623) controls in comparison to plants treated with 50µM or 100µM 5-azaC. Data denotes mean ± SE (*n*=3). Columns marked with identical letters are not significantly different at $P<0.05$, analysed using ANOVA and Tukey's test. No significant differences were seen between *NIT4B2* expression levels.

Phenotyping of the *acdc* mutation and association of genotype with phenotype

Three independent mutant lines with a similar phenotype classified as *adult cyanide deficient class 1-3 (acdc1, acdc2, acdc3)* and the *totally cyanide deficient 1 (tcd1)* mutant were used to investigate the low cyanogenic phenotype. The *acdc1-3* lines are thought to be developmentally regulated mutants, in that the shoot tissue is only cyanogenic when the plant tissue is young (Blomstedt et al., 2012, Blomstedt et al., 2018).

Expression of *CYP79A1* in the *acdc* lines is equivalent to wild-type plants upon germination before rapidly decreasing to below wild-type levels (Supplementary Figure 2). No mutations have previously been found in the coding regions of the dhurrin biosynthetic genes in the *acdc1-3* mutants. However, in this study sequence analysis identified a CΔT change (typical of EMS treatment) 1188bp upstream of the ATG start codon in the *CYP79A1* promoter region (Supplementary Figure 3). The identical mutation found in the promoter region of the *acdc1-3* lines together with their low cyanide phenotype suggests that this region is involved in the developmental regulation of *CYP79A1*. The *tcd1* mutation is also a CΔT though it is located in the coding region of *CYP79A1*, resulting in a proline to leucine (P414L) change rendering the enzyme non-functional (Blomstedt et al., 2012).

To determine if there was an association between the mutation identified in the *acdc S. bicolor* lines and the low cyanogenic status of these plants, the HCNp was determined for the F_1 progeny of crosses between BTx623 and each of the mutants, and from crosses between the mutants (*tcd1* × *acdc* and *acdc* × *acdc*). The F_1 individuals from the crosses between the mutants and BTx623 are all heterozygous for the identified mutation and all have relatively high HCNp (Figure 6a). The HCNp of the progeny from the crosses between the mutants were significantly lower than the HCNp of the progeny from crosses with BTx623 (Figure 6a). The low HCNp of the individuals from *tcd1* × *acdc1* indicates that these lines are not complementary, confirming the hypothesis that the acyanogenic phenotype of *acdc* lines is due, or strongly linked, to the CΔT mutation in the promoter region. To confirm that the CΔT mutation co-segregates with the *acdc1-3* phenotype, the HCNp was compared with the genotype at the mutation site for a sample of *acdc* × BTx623 plants in the F_2 generation (n=45). The HCNp of plants heterozygous for the CΔT mutation was not significantly lower than that of homozygous wild-type plants (P =0.209), potentially due to the large variability in HCNp for these plants (Figure 6b). However, homozygous mutants were found to have significantly lower HCNp than both homozygous wild-type and heterozygous plants (P<0.0001) (Figure 6b). This suggests that the CΔT mutation itself, or a closely linked regulatory region, lowers the expression of *CYP79A1* substantially.

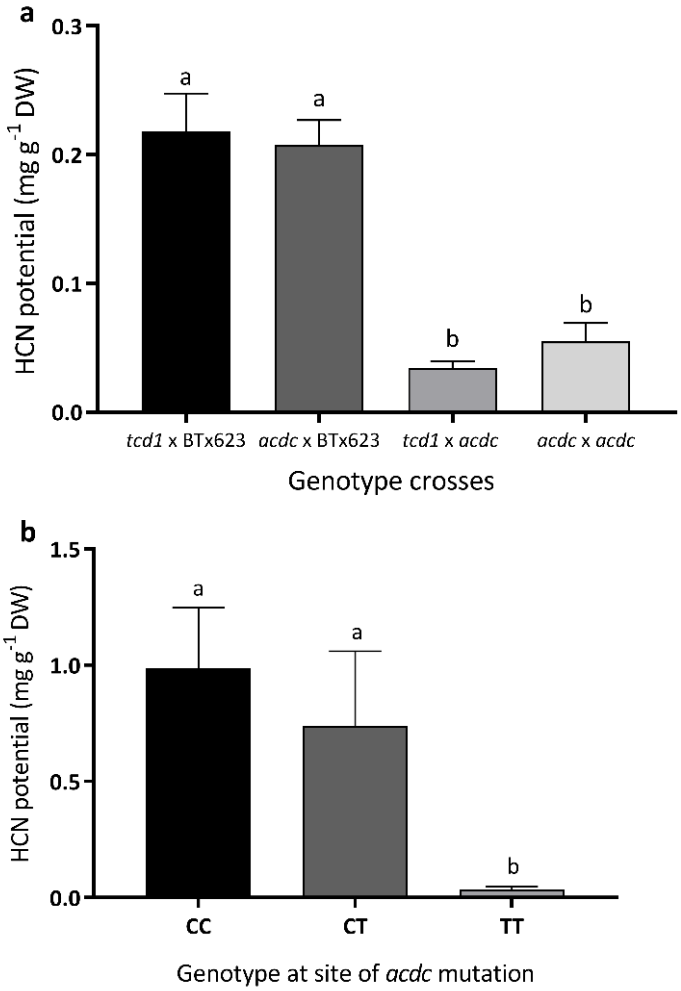


Figure 6: (a) Hydrogen cyanide (HCN) potential (mg g^{-1} dry weight) of F_1 progeny from crosses between sorghum BTx623 and the *totally cyanide deficient 1* (*tcd1*) and *adult cyanide deficient class* (*acdc*) mutants and between the two different mutant lines (*tcd1* × BTx623 *n*=20; *acdc* × BTx623 *n*=44; *tcd1* × *acdc* *n*=12; *acdc* × *acdc* *n*=14). **(b)** Hydrogen cyanide (HCN) potential (mg g^{-1} dry weight) of a total of 45 F2 individuals from a BTx623 × *acdc* cross showing the association between genotype at the *acdc* CΔT mutation and HCN potential. Homozygous mutant (TT; *n*=7) plants had much lower HCN potential than both homozygous (CC; *n*=29) wild-type and heterozygous (CT; *n*=9) plants. Data denotes mean ± SE; different letters indicate significant differences determined by unpaired *t*-test.

Analysis of methylation of *CYP79A1* promoter in *acdc* mutation region during development

Bisulfite sequencing of a 250bp region surrounding the *acdc1* CΔT mutation revealed that the BTx623 has a CHG methylation site where the *acdc* mutant CΔT EMS mutation has converted this methylated cytosine to a thymine (Figure 7). No further differences in the methylation pattern of shoot and root tissues between or within lines were found. Furthermore, no changes in methylation state were seen between tissues harvested at 3dpg, when HCNp was at its peak, or at 5-weeks post-germination when HCNp had decreased and the *acdc1* line had become acyanogenic in the leaf tissue. All CG and half of the CHG sites were found to be methylated, while all CHH sites were unmethylated. As no phenotypic or genotypic differences between the *acdc1-3* lines were detected, only the *acdc1* line was used for methylation analysis.

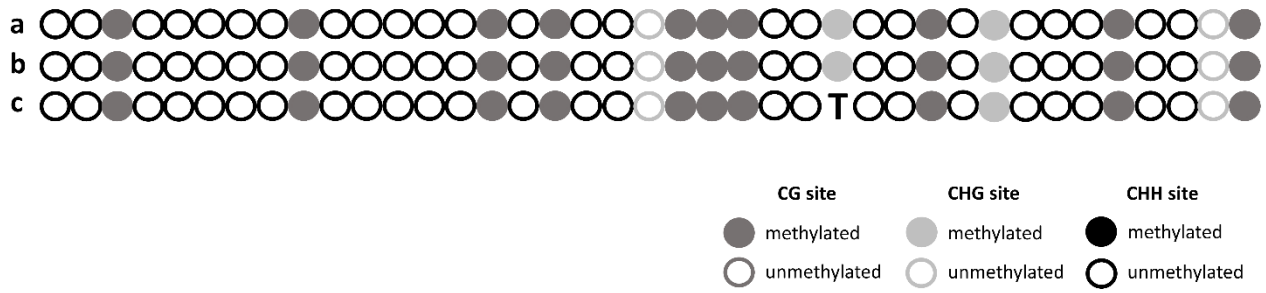


Figure 7: Schematic representation showing the methylation status of a 250bp region surrounding the *acdc* CΔT mutation in the *CYP79A1* putative 5′ regulatory strand in **(a)** wild-type (BTx623) **(b)** sibling (*ACDC1*); and **(c)** mutant (*adult cyanide deficient class 1 - acdc1*) root and shoot tissues. The mutant line lacks a CHG methylation site where the mutation has converted the cytosine to a thymine. No changes in methylation were seen between plants that were 3-days old or 5-weeks old, or between root and shoot tissues. All CG methylation sites were found to be methylated, while all CHH sites were unmethylated. CG methylation site = dark grey circles; CHG methylation site = light grey circles, CHH methylation site = black circles. Filled and empty circles denote methylated and unmethylated cytosines, respectively.

3.5 Discussion

Cyanogenic glucosides (CNglcs) are fundamental in the defence and stress responses of many plant species. However, concentrations of the compounds can differ widely between tissues and display high levels of plasticity throughout plant development and in response to environmental cues, even amongst genetically identical individuals (Gleadow and Møller, 2014). Despite the biosynthesis and degradation pathways being well defined biochemically, the molecular mechanisms underlying regulation of the CNglc dhurrin in the important cereal crop *Sorghum bicolor* remain largely unknown. Dhurrin production in *S. bicolor* is developmentally regulated, with high levels being produced during the seedling germination stage, while also being environmentally upregulated in adult plants by abiotic factors such as drought and nitrogen fertilisation (Blomstedt et al., 2018, Neilson et al., 2015b, O'Donnell et al., 2013). An understanding of the mechanisms regulating cyanogenesis would allow for improved phenotypic profiling and therefore toxicity predictions, both critically important in reducing livestock illness and pasture loss.

Expression of key cyanogenesis gene, *CYP79A1*, and HCNp altered by genome-wide demethylation in *S. bicolor*

This study examined the role of DNA methylation in *S. bicolor* development and the regulation of dhurrin biosynthesis with a focus on the key gene associated with dhurrin production, *CYP79A1*. It was demonstrated that non-specific DNA demethylation induced by two different levels of 5-azaC altered the growth of *S. bicolor* plants in the early stages of development, with significant differences in height between the control plants and those treated with 5-azaC. A slight delay in leaf production was also observed between treated and control plants in the initial stages of growth, though the reduction in unfurled leaves was transient and by 14dpg there was little difference in leaf number between any of the treatments. This reduction in height and differences in leaf development between treatments was consistent with findings undertaken in cereals, *Arabidopsis*, and other species such as the *Brassicaceae* oilseeds, where hypomethylation was seen to reduce plant growth, including inducing dwarfism in rice (*Oryza sativa*), while also altering phytomorphology and developmental timing (Fieldes et al., 2005, Finnegan et al., 1996, Kellenberger et al., 2016, Kim et al., 2008, Sano et al., 1990). The level of growth delay in plants treated with 50µM or 100µM 5-azaC was

similar, with the lack of height and growth differences between 5-azaC treatments being likely due to plant growth parameters having a sigmoidal dose-response relationship to 5-azaC hypomethylation (Amoah et al., 2012).

At 7dpg and 14dpg plants treated with 5-azaC had significantly greater levels of *CYP79A1* expression in leaf tissue compared to control plants. As DNA methylation predominantly represses gene expression (Finnegan et al., 2000), higher expression of the dhurrin biosynthetic gene would be expected in a hypomethylated genome if *CYP79A1* is in fact regulated via this mechanism. By the final harvest methylation levels of the 5-azaC treated plants had increased and *CYP79A1* expression was equal across all treatments. This increase in methylation by 21dpg may have resulted from the restoration of cytosine methylation patterns via METHYLTRANSFERASE1 (MET1) (Saze, 2008). As *S. bicolor* grows rapidly during the first month of development, rounds of mitotic replication are high, thus reducing the amount of 5-azaC maintained in the genome, eliminating methyltransferase inhibition, and allowing genome-wide methylation to be reinstated. Another possibility is that dhurrin acts in a negative feedback loop, with the higher concentration of the secondary metabolite seen in the treatment groups ultimately facilitating repression of the gene and resulting in transcript levels equivalent to the control group by the final harvest (Figure 8).

In sorghum, leaf number is used as a determination of ontogeny, where plants with the same leaf number will have comparable dhurrin levels (Miller et al., 2014). In general, dhurrin concentrations decrease as leaf numbers increase. Leaf development was slightly delayed in the initial growth of 5-azaC treated plants with a minor reduction in the number of unfurled leaves, though the dhurrin levels were substantially higher than control plants. Though leaf development was comparable in 5-azaC treated plants by the second harvest (14dpg), HCNp and *CYP79A1* expression remained significantly higher in the 5-azaC treatments than in the controls in both the first two harvests. This indicates that there is potential for DNA methylation to affect cyanogenesis in *S. bicolor* leading to an increase in dhurrin levels. However, due to demethylation occurring throughout the genome, the genes and signalling pathways that are being affected and potentially influencing the cyanogenesis genes and the upregulation of *CYP79A1* cannot be definitively stated.

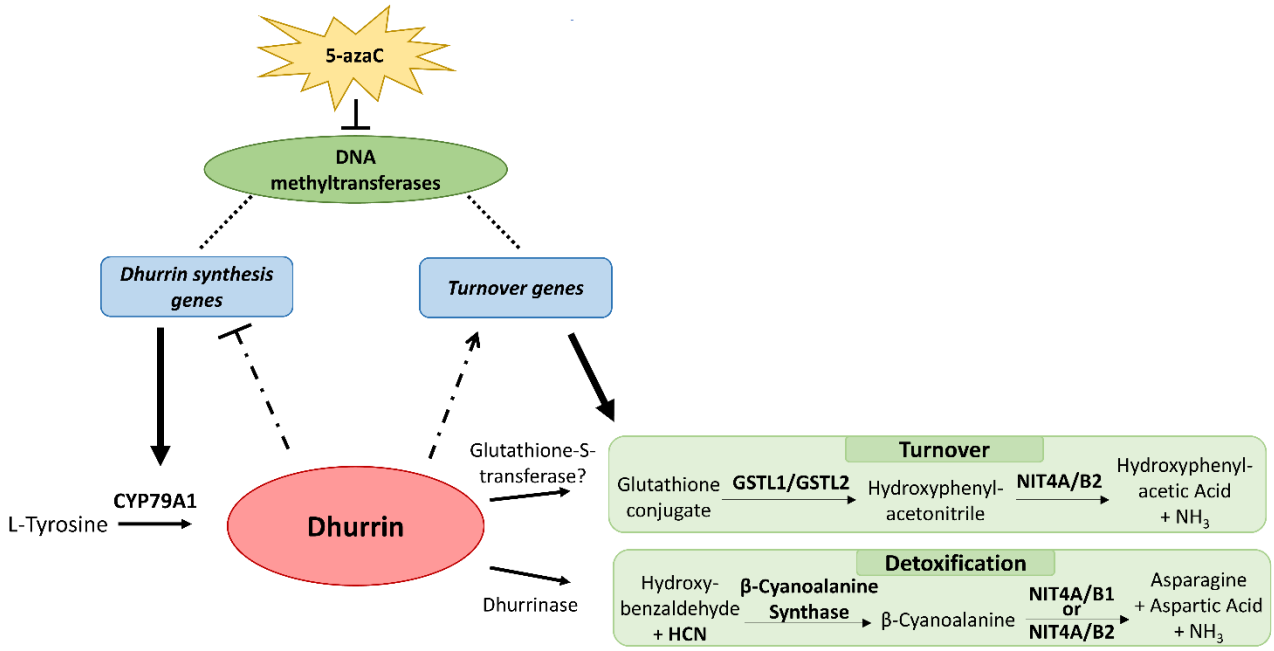


Figure 8: Representation of the potential effects of 5-azaC on cyanogenesis in *Sorghum bicolor*. 5-azaC inhibits DNA methyltransferases causing genome-wide hypomethylation, including in the dhurrin biosynthetic and turnover genes (dotted lines). This results in increased expression of *CYP79A1*, leading to higher dhurrin concentrations in young plants. Elevated dhurrin levels may then trigger a feedback loop resulting in down-regulation of the dhurrin biosynthetic genes and induction of the turnover genes in the endogenous turnover pathway (dashed lines), leading to less production and higher turnover of dhurrin. The rapid turnover of dhurrin would account for the lack of correlation between *CYP79A1* relative transcript levels and HCNp seen in the plants treated with 100µM 5-azaC at 14dpg. While *NIT4B2* was not found to change in expression during these stages of development or between treatment groups, it is possible that one or more of the other genes involved in the endogenous turnover pathway was affected. *NIT4B2* is also involved in the detoxification of HCN released within the plant as a result of the reaction between dhurrin and dhurrinase, and genes in this pathway may also be affected. Information on the genes involved in the turnover and detoxification pathway is based on Bjarnholt et al. (2018); Jenrich et al. (2007); Nielsen et al. (2016). Abbreviations: GSTL – Glutathione transferase of the lambda class (Bjarnholt et al. 2018).

At 14dpg higher *CYP79A1* expression in the 100 µM 5-azaC treatment did not result in higher dhurrin concentrations, with HCNp being significantly lower than the control. Specialised metabolites are often found to be controlled at the transcriptional level in plants (Colinas and Goossens, 2018). There is evidence that *CYP79A1* is regulated at the transcriptional level in *S. bicolor* (Busk and Møller, 2002). However, these results have been difficult to replicate, particularly in older plants when both dhurrin concentrations and *CYP79A1* expression are low. Therefore, it is possible that transcript levels of *CYP79A1* are not corresponding to HCNp leading to the discrepancy seen between the two in the 100µM 5-azaC treatment. An increase in dhurrin turnover rate would also account for the substantial decline in HCNp seen in the treatment groups despite higher *CYP79A1* expression. No significant differences in expression levels of the turnover gene *NIT4B2* (involved in multiple dhurrin turnover pathways) were seen at any harvest time-point or between treatments, suggesting that cytosine methylation does not play a role in the regulation of this gene. While *NIT4B2* expression was not seen to change in response to 5-azaC treatment, this may not be the case for the other genes involved in the dhurrin turnover pathway (Figure 8).

Stable inheritance of *acdc* phenotype and methylation state of the mutation region

The *adult cyanide deficient class* (*acdc*) mutants exhibit reduced dhurrin synthesis in mature plants. This is a valuable trait for animal forage production as cyanide can protect young seedlings from predation while adult plants have reduced toxicity. It is known that the mutation resulting in the *S. bicolor* acyanogenic *tcd1* line is due to a CΔT change within the coding region of *CYP79A1*, which renders the CYP79A1 enzyme non-functional (Blomstedt et al., 2012). The inability of the *acdc* × *tcd1* to restore wild-type function indicates that the *CYP79A1* allele is also affected in the *acdc* type mutants. Following germination, the *acdc1* mutant has reduced *CYP79A1* expression in comparison to wild-type lines (Supplementary Figure 2). Sequence analysis showed that the mutation associated with this *acdc* phenotype is an EMS generated CΔT mutation ~1.2kbp upstream of the *CYP79A1* transcription start site (Supplementary Figure 3), while there was no mutation in the *acdc CYP79A1* coding sequence. Through the analysis of F_1 and F_2 generations of different crosses we showed that this CΔT mutation is stably inherited and co-segregates with the adult cyanide deficient

phenotype. The presence of the same lesion in all three independent *acdc* mutants suggests that this region may be a hotspot for EMS mutation (Henry et al., 2014).

As the *acdc* mutation in the 5′ regulatory region of the *CYP79A1* gene causes an early decrease in *CYP79A1* expression and HCNp in the mutant lines during development, bisulfite sequencing was undertaken to investigate whether cytosine methylation is altered in this region. Besides the CΔT mutation site, no differences in methylation were seen between wild-type and *acdc1* plants in either root or shoot tissues, even though these tissues differ in dhurrin concentrations in comparison to each other and over the course of development. It is possible that a mutation, as yet unidentified, is tightly linked to the CΔT site and is influencing *CYP79A1* regulation, or that the CΔT mutation itself may reside in an important binding site for a transcription regulator that can influence regulatory protein binding and subsequent gene expression, leading to the reduced expression of *CYP79A1* and the sudden drop in HCNp seen in the mutant line. This information is currently being incorporated into experiments analysing the regulation of *CYP79A1*.

Genes of the CNglc biosynthetic pathway have been found to be clustered in *S. bicolor*, cassava and *Lotus japonicus* (Takos et al., 2011). While the mechanisms that form these clusters of non-homologous genes in eukaryotes are largely unknown, there have been several proposed selective advantages of clustering in cyanogenic species. Clustering enables the genes to be inherited as a functional unit, clustering of genes in pathways where toxic intermediates are involved ensures they are inherited together, and clustered genes can be regulated synchronously (Chu et al., 2011). Synchronicity of regulation is imperative in *S. bicolor* as the gene products function together as a metabolon to facilitate metabolic channelling of toxic intermediates (Laursen et al., 2016).

Groups of genes in clusters which are functionally related may reside in epigenetic control regions where they are co-ordinately regulated by DNA methylation. Such co-ordinated regulation mechanisms may be linked to environmentally induced epigenetic transgenerational inheritance. Stress priming related to cyanogenesis has been found to be heritable across generations. For example, cyanogenic wild lima beans (*Phaseolus*

lunatus) that are heavily damaged by herbivory, produce progeny with increased levels of β-glucosidase activity compared to control plants (Ballhorn et al., 2016). Although the mechanism for this inheritance was not studied, Ballhorn et al. (2016) hypothesised that epigenetic factors are at play. The results of the study presented here support the hypothesis that methylation levels are involved in the regulation of dhurrin production, although conclusions as to all genes and signalling pathways that are being altered cannot be definitively stated due to the stochastic nature of 5-azaC demethylation. Further studies are required to determine whether environmental factors influence the methylation state of the dhurrin biosynthetic genes.

Understanding the molecular regulation of cyanogenesis in *S. bicolor* will enable plant toxicity to be predicted more accurately and could reveal similarities in regulation between other cyanogenic species. This information may well allow for the manipulation of HCNp and overall improvement of *S. bicolor* and other cyanogenic crop species.

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We thank Dr Alan Neale for valuable comments on the manuscript and Dr Peter Stuart (Seed Tek Pty Ltd) for carrying out the crosses between the various sorghum lines analysed in this study. The project was supported by Australian Research Council grants LP100100434 and DP130101049. VCR is supported by an Australian Government Research Training Program Scholarship and AW Howard Memorial Trust Inc. Research Fellowship.

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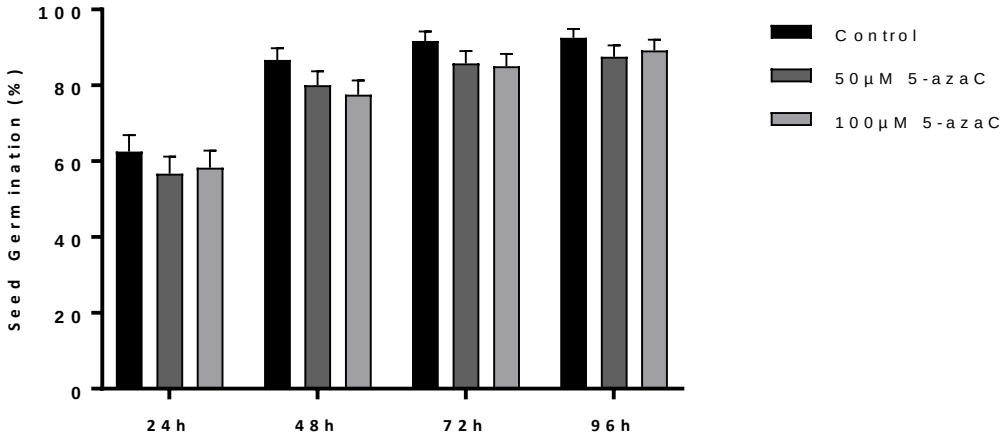
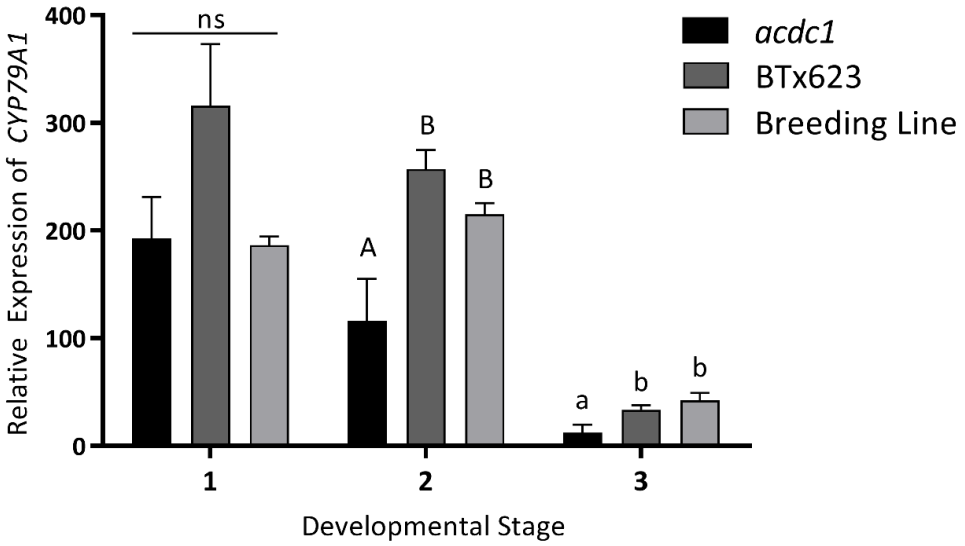
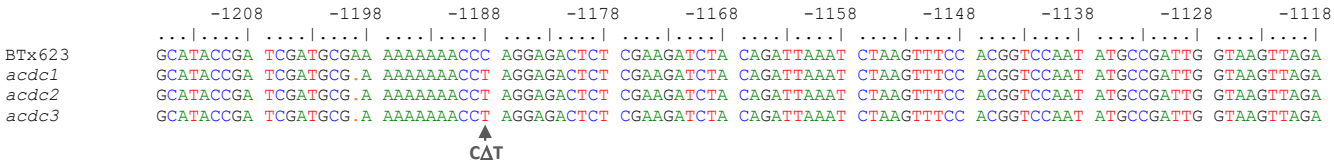
3.7 Supplementary Material



Supplementary Figure 1: Germination percentages of control and 5-Azacytidine treated seed post-imbibition, data denotes mean ± SE (*n*=120). No significant differences in seed germination were seen between treatments.



Supplementary Figure 2: Relative transcript levels of *CYP79A1* (Sb01g001200) normalised to *Ubiquitin* (Sb01g030340) and *Actin* (Sb01g010030) in leaf tissue of the EMS-mutant *adult cyanide deficient class 1* (*acdc1*) (homozygous T/T) in comparison to two wild-type *Sorghum bicolor* lines (both homozygous C/C): BTx623 and a commercial breeding line. Developmental stages are 1: seed with emerging radicle; 2: coleoptile tissue three days post-germination; and 3: 2-leaf stage 12 days post-germination. The *CYP79A1* relative transcript levels are equivalent in *acdc1* to both wild-type lines upon germination. *CYP79A1* expression then decreases in *acdc1* more rapidly than in both wild-type lines. Data denotes mean ± SE (*n*=3). Columns marked with identical letters are not significantly different at $P<0.05$, analysed using ANOVA and Tukey's test. ns = not significant.



Supplementary Figure 3: Sequence of the promoter region of *CYP79A1* in BTx623 and *acdc1-3* plants showing the CΔT mutation located 1188bp upstream of the *CYP79A1* ATG start codon.

Primers used for qPCR, PCR amplification and sequencing, and bisulfite sequencing

Supplementary Table 1: Primers used for qPCR analysis

Primer Name	Primer Sequence	Tm	Size (bp)
CYP79A1 Forward	5′ CATGCTCTTCGGAAGGCTCCT 3′	66	162
CYP79A1 Reverse	5′ ATGGAGATGGACGGGTAGAGGT 3′		
Nitrilase 4B2 Forward	5′ CGTTACAGCTGACCTCGACCTT 3′	63	152
Nitrilase 4B2 Reverse	5′ TCTCTCCATCGGCTGAAGCA 3′		
Ubiquitin Forward	5′ CGGAGGAGCTCTATGCCACA 3′	66	122
Ubiquitin Reverse	5′ AAGACGCTCCACTGCAGCAT 3′		
Actin Forward	5’ ATGGCTGACGCCGAGGATATCCA 3’	60	298
Actin Reverse	5’ GAGCCACACGGAGCTCGTTGTAG 3’		

Supplementary Table 2: Primers used for PCR and sequencing reactions of the promoter of *CYP79A1*

Primer Sequence	Direction	Tm	Position in relation to ATG of CYP79A1	Purpose
5′ GAACACATACCTTGGTTTGC 3′	Forward	54	-1332	amplification/sequencing
5′ CCCGGTAGGTATGGTTTCAA 3′	Reverse 2	60	-806	sequencing
5′ CAGGTACGAGAGCGTCAC 3′	Reverse 1	60	+111	amplification

Supplementary Table 3 Primers used for methylation analysis

a) Primers used for amplification of the *CYP79A1* 5′ regulatory region surrounding the *acdc1* mutation site for both converted and unconverted DNA

Primer Sequence	Direction	Tm	Size (bp)	Purpose
5′ TGGTTATTTGATTATAGTTAGAATTGAAT 3′ 5′ TAACTTACCAATCRACATATTAAACC 3′	Forward Reverse	54	333	Bisulfite converted DNA amplification/sequencing
5′ CGTTAGGTTGAGGCTTTGGA 3′ 5′ ATGTTGGTGTTGACGCAGAC 3′	Forward Reverse	54	558	Unconverted DNA amplification/sequencing

b) Primers used for amplification of an unmethylated region of the *Arabidopsis ASA1* gene for both converted and unconverted DNA

Primer Sequence	Direction	Tm	Size (bp)	Purpose
5′ GAAAATGATTGTTAAGAGTT 3′ 5′ TCCTTAATATCATCATCAATAAC 3′	Forward Reverse	54	306	Bisulfite converted DNA amplification/sequencing
5′ CACCAACCAAATCTCCTTCC 3′ 5′ TCATAATAGCAAGAACAATAGCA 3′	Forward Reverse	54	384	Unconverted DNA amplification/sequencing

Chapter 4 Declaration

Monash University

The transcription factor *Sb*GATA22 as a regulator of *CYP79A1* in *Sorghum bicolor*

This thesis chapter is in preparation for submission to *PLOS ONE*.

Declaration by candidate:

The nature and extent of my contribution to this work is as follows:

Nature of Contribution	Extent of contribution
Experimental design, execution of experimental work, analysis, chapter writing and editing	90%

The following co-authors contributed to the work:

Name	Nature of Contribution	Extent of contribution	Student at Monash University
Ros Gleadow	Experimental design, chapter editing	2%	N
Cecilia K. Blomstedt	Experimental design, analysis, chapter editing	8%	N

Student signature: **Date:**

Main Supervisor signature: **Date:**

Chapter 4

The transcription factor *Sb*GATA22 as a regulator of *CYP79A1* in *Sorghum bicolor* (L.) Moench

4.1 Abstract

Cyanogenic glucosides are specialised metabolites produced by over 2,600 species of higher plants from more than 110 families. Involved in plant defence and stress responses, cyanogenic glucosides are influenced by biotic and abiotic factors in addition to being developmentally regulated. However, there is limited knowledge regarding the molecular mechanisms that regulate their biosynthesis. The dhurrin biosynthetic pathway of the important cereal crop sorghum (*Sorghum bicolor* (L.) Moench) was the first to be elucidated, though to date no direct regulator of the dhurrin biosynthetic genes has been identified. To isolate regulatory proteins that bind to the promoter region of the key dhurrin biosynthetic gene of sorghum, *CYP79A1*, yeast one-hybrid screens were performed. A bait fragment containing 1177 base pairs of the *CYP79A1* 5' regulatory region was cloned upstream of a reporter gene and introduced into *Saccharomyces cerevisiae*. Subsequently, the yeast was transformed with library cDNA, representing RNA from two different sorghum developmental stages. From these screens we identified *Sb*GATA22, a long B-GATA transcription factor that binds specifically to the GATA transcription factor binding motifs in the *CYP79A1* promoter region. Expression of *SbGATA22*, in comparison to *CYP79A1* expression and dhurrin concentration, was analysed over 14 days of sorghum development and in response to nitrogen application. Collectively, these findings indicate that *Sb*GATA22 is a regulator of *CYP79A1* expression and provides a novel insight into the molecular regulation of dhurrin biosynthesis in sorghum.

4.2 Introduction

Cyanogenic glucosides (CNglcs) are plant specialised metabolites. Derived from amino acids, they enable a cyanide group to be stably stored as a non-toxic compound via glycosylation of a cyanohydrin. Over 2,600 species of higher plants produce CNglcs including important crop species, such as barley, sorghum, clover, cassava, apples, and almonds (Gleadow and Møller, 2014, Jones, 1998). Currently there are over 60 CNglcs identified and though all can be broken down to release hydrogen cyanide gas (HCN), the exact compound produced by each species differs due to different amino acids and sugars used in their biosynthesis (Gleadow and Møller, 2014). Cyanogenic glucosides were first characterised for their role in herbivore defence as the released HCN binds to cytochrome c oxidase in the mitochondria inhibiting cellular respiration (Holland and Kozlowski, 1986). More recently, alternate turnover pathways of CNglcs that release ammonia and carbon dioxide rather than HCN have also been identified (Jenrich et al., 2007, Pičmanová et al., 2015, Bjarnholt et al., 2018), establishing a role for CNglcs in primary (in addition to secondary) metabolism.

The concentration of CNglcs found in plant tissues varies between species, as does the site of synthesis (Gleadow and Møller, 2014). Cyanogenic glucoside concentrations are highly dynamic, generally decreasing as the plant matures; although the opposite pattern has been observed in some species (Goodger et al., 2006). Biotic and abiotic factors such as herbivory (Zagrobelny et al., 2004), pathogen attack (Ballhorn et al., 2010), light (Schmidt et al., 2018), drought (Emendack et al., 2018), and nitrogen application (Blomstedt et al., 2018) can also induce or repress CNglc biosynthesis in cyanogenic plants.

Despite their important roles in plant metabolism CNglcs may be problematic when cyanogenic plants are used for human and animal consumption. For example, the irreversible paralytic disease konzo may arise when cassava (*Manihot esculenta* Crantz) is consumed without being adequately processed (Kashala-Abotnes et al., 2018, Burns et al., 2010) and forage sorghum that is high in CNglcs can cause chronic or acute cyanide poisoning in ruminants such as sheep and cattle (Wheeler and Mulcahy, 1989). The plasticity of CNglcs makes toxicity of forage difficult to predict, particularly during periods of drought (Gleadow et al., 2016, Emendack et al., 2018). While removing the

capacity of these crops to produce CNglcs appears to be a solution, their putative roles in nitrogen remobilisation and stress tolerance may result in reduced growth rates and yields in acyanogenic lines (Jørgensen et al., 2005, Bjarnholt et al., 2018, Pičmanová et al., 2015).

The first CNglc biosynthetic pathway to be elucidated was the synthesis of dhurrin in sorghum (*Sorghum bicolor* (L.) Moench). Dhurrin is found in all vegetative tissues of sorghum but not in the mature grain (Nielsen et al., 2016). Dhurrin concentrations are highest when plants are young, peaking between three to four days post-germination before decreasing in all tissues (Halkier and Møller, 1989, Busk and Møller, 2002). The dhurrin biosynthetic pathway begins with an L-tyrosine precursor and involves three enzymes coded for by three structural genes: two cytochromes P450, CYP79A1 and CYP71E1; and the glycosyltransferase, UGT85B1 (Sibbesen et al., 1995, Koch et al., 1995, Bak et al., 1998). In sorghum, the three genes were found to be clustered on chromosome one, and once identified were used to locate the CNglc biosynthetic genes in other species including cassava and *Lotus japonicus* (Takos et al., 2011). CYP79s are signature enzymes of these pathways, with *CYP79A1* being the rate-limiting enzyme of dhurrin biosynthesis in sorghum (Sibbesen et al., 1995).

Currently, little is known about the molecular regulation of CNglc biosynthesis. Sorghum studies have focussed on dhurrin regulation at the physiological level or analysed gene expression without identifying the drivers of this expression (Busk and Møller, 2002, Nielsen et al., 2016, Buchanan et al., 2005). While dhurrin is believed to be regulated at the transcriptional level in sorghum (Busk and Møller, 2002, Nielsen et al., 2016), to date no direct regulator of the dhurrin biosynthetic genes has been reported. Glucosinolates, secondary metabolites found in *Brassicaceae*, share similar biosynthetic pathways to CNglcs and are primarily regulated by MYB transcription factors (Seo and Kim, 2017). It is possible that a specific transcription factor family is also responsible for CNglc regulation across cyanogenic species.

In this study, we used yeast one-hybrid screens to identify candidate regulators of the key dhurrin biosynthetic gene *CYP79A1*. Yeast one-hybrid (Y1H) screening is a valuable technique used for identifying physical interactions *in vivo* between a DNA sequence of

interest (known as the “bait” sequence) and a protein (the “prey”) (Ouwerkerk and Meijer, 2001). The bait sequence is placed upstream of a reporter gene, while protein coding sequences from a cDNA library are fused to a yeast GAL4 transcriptional activation domain. Positive interactions between the bait and prey are detected by reporter gene expression in the yeast *Saccharomyces cerevisiae*. This enables the identification of novel DNA-protein interactions and provides an advantage over methods such as ChIP-Seq as it does not require prior identification of the DNA-binding proteins. To our knowledge it is the first time this strategy has been applied to a gene involved in CNglc biosynthesis.

As *cis*-regulatory elements that may be important in *CYP79A1* regulation are largely unknown, a native promoter fragment of 1177bp was used as bait, rather than an artificial run of repeated binding motifs. cDNA libraries from two developmental stages were constructed. The first screen used seeds that had been imbibing for 24 and 48 hours, corresponding to the developmental stage prior to maximum dhurrin concentration. The second screen used coleoptile tissue from seedlings 3 days post-germination, corresponding to when dhurrin concentration is at a maximum. From these screens positive colonies were selected and their inserts characterised, resulting in the identification of a putative GATA transcription factor, *Sb*GATA22. The direct interaction of *Sb*GATA22 with putative GATA binding motifs in the *CYP79A1* promoter region is reported. The expression of *SbGATA22* during sorghum development and in response to nitrogen application is also detailed.

This study provides a novel insight into the transcriptional control of dhurrin biosynthesis in sorghum. Uncovering the molecular pathways regulating CNglc production may enable crop toxicity predictions that are more accurate than the current guidelines permit; and may also allow for CNglc production to be manipulated in ways that do not compromise yield or stress tolerance.

4.3 Materials and Methods

Yeast One-Hybrid Screen

Construction of the *CYP79A1* promoter-AbAi reporter (Bait) plasmids

The promoter sequence of *CYP79A1* (*Sb01g001200*) was obtained from Phytozome (Goodstein et al., 2011) and fragments of 1177bp (F1), 843bp (F2), and 460bp (F3) (Supplementary Figure 1) were amplified from *Sorghum bicolor* genomic DNA extracted via a modified CTAB protocol (Allen et al., 2006). Forward and reverse primers contained a HindIII and SalI cleavage site, respectively (Supplementary Table 1). Amplification was performed with proof-reading *Pfu* DNA polymerase (Promega) on a Mastercycler thermocycler (Eppendorf) with cycling parameters as follows: initial denaturation: 2 minutes, 95°C; denaturation: 30 seconds, 95°C; annealing: 30 seconds, 64°C; extension: 2.5 minutes, 72°C, for 30 cycles; final extension: 5 minutes at 72°C. The vector pAbAi containing the *AUR1-C* reporter gene (conferring resistance to the antibiotic Aureobasidin A) (Clontech) and the amplified bait fragments were digested with HindIII and SalI and purified using the *Accuprep* PCR/Gel Purification Kit (Bioneer). Fragments were ligated using a 1:3 vector:fragment ratio, producing the bait constructs pAbAi-F1, pAbAi-F2, and pAbAi-F3 for the 1177bp, 843bp, and 460bp promoter fragments, respectively (Supplementary Figure 2). Constructs were electroporated into DH5α *E. coli* cells and selected by ampicillin resistance (50μg ml^{-1}). The constructs were purified from *E. coli* using the *Accuprep* Plasmid Mini Extraction Kit (Bioneer) and sequenced to ensure no mutations were present.

The pAbAi-F1, pAbAi-F2, pAbAi-F3 vectors and the control vector p53-AbAi (Clontech) were linearised with BstBI and 1μg transformed into *S. cerevisiae* Y1H Gold (Clontech) using the small-scale yeast transformation method as described in the Matchmaker Yeast One-Hybrid Library Screening System manual. Cells were spread onto synthetic dropout media lacking uracil (SD/-Ura) and allowed to grow for 5 days at 30°C. Colonies were analysed by colony PCR using the Matchmaker Insert Check PCR Mix 1 (Clontech) according to the manufacturer's protocol to identify clones with correctly integrated vectors. Untransformed Y1H Gold colonies were used as a negative control.

Bait autoactivation test

The Y1H Gold AbAi-F1, AbAi-F2, and AbAi-F3 bait strains were tested for autoactivation of the *AUR1-C* reporter gene, with Y1H Gold p53-AbAi used as a control strain. The four yeast strains were spread onto SD/-Ura media containing the antibiotic Aureobasidin A (AbA) between 100-200ng ml^{-1} (SD/-Ura/AbA^{100}; SD/-Ura/AbA^{150}; SD/-Ura/AbA^{200}) and incubated for 5 days at 30°C to determine the concentration of AbA required to inhibit autoactivation of the AbA reporter gene by endogenous yeast transcription factors.

cDNA library construction

Plant material

Two cDNA libraries were constructed and screened using plant tissue from different developmental stages:

1. Seeds of *Sorghum bicolor* (L.) Moench line BTx623 were sterilised and germinated in petri dishes containing filter paper wetted with sterile MilliQ H_2O. Petri dishes were placed in the dark at 28°C and imbibed for either 24 or 48 hours at which point the seeds with emerging radicle (24h), or radicle and emerging coleoptile (48h) were snap frozen in liquid nitrogen and stored at -80°C.

2. Seeds underwent the same process as above but were germinated for three days at which point the tips of the coleoptiles (1cm in length) were cut off, the coleoptile tips pooled, snap frozen in liquid nitrogen and stored at -80°C. The remainder of the coleoptiles, seeds, and the radicles were discarded.

RNA extraction

Seeds that had germinated for 24h and 48h were pooled and 500mg of the tissue ground to a fine powder using a mortar and pestle chilled on dry ice. The same procedure was undertaken for the coleoptile tissue. Total RNA from the ground tissue was extracted using the LiCl/phenol extraction method detailed by Verwoerd et al. (1989). RNA integrity was analysed using a 2100 Bioanalyzer (Agilent) and samples with an RNA integrity number (RIN) greater than 9 were used for mRNA extraction.

mRNA was extracted using the Dynabeads mRNA Purification Kit (Invitrogen) as per the manufacturer's instructions, then treated with RQ1 RNase-free DNase (Promega) for 30 minutes at 37°C. mRNA was extracted from the DNase mix with 1:1 phenol:chloroform, precipitated from the aqueous phase with 1/10 volume of 3M sodium acetate pH 5.2 and 2.5 volumes of ethanol, and re-suspended in 5µl of sterile MilliQ H_2O.

cDNA synthesis for library construction

First-strand SMART cDNA with flanking end sequences homologous to the vector pGADT7-Rec was synthesised from 1µg of mRNA using the Matchmaker Yeast One-Hybrid Library Screening System (Clontech). Double stranded cDNA was generated and amplified by long-distance PCR using the Advantage 2 PCR Kit (Clontech) with an oligo-dT primer as per the manufacturer's instructions. The concentration and size of the resultant double-stranded cDNA was analysed on a 1.2% agarose/EtBr gel alongside a 1kb ladder. Samples were purified using CHROMA SPIN +TE-400 Columns (Clontech), precipitated via an ethanol precipitation, and re-suspended in 20µl sterile MilliQ H_2O.

Yeast one-hybrid cDNA library construction and screening

The yeast one-hybrid cDNA library construction and screening was performed using the Matchmaker Yeast One-Hybrid Library Screening System (Clontech). The Y1H Gold AbAi-F1 bait strain was co-transformed with cDNA and pGADT7-Rec that had been linearised with SmaI (Clontech). This allowed for the cDNA inserts and vector to recombine in the yeast cells, placing the cDNA "prey" inserts downstream of a GAL4 activation domain. Co-transformation was undertaken using the large-scale yeast transformation method as described in the Matchmaker Yeast One-Hybrid Library Screening System manual. In order to calculate the number of clones screened, 100µl of the transformation reaction was diluted 1/10, 1/100, 1/1000, and 1/10,000 and spread on synthetic dropout media lacking leucine (SD/-Leu). The remainder of the transformation reaction (15ml) was spread onto SD/-Leu/AbA^{100} media with 90µl of the transformation reaction spread onto each agar plate (90mm in diameter). Plates were incubated at 30°C for 5 days.

After 5 days the number of clones screened was calculated as: $clones\ screened = (cfu/ml\ on\ SD/-Leu) \times (dilution\ factor) \times (resuspension\ volume\ (15mls))$.

Colonies on the SD/-Leu/AbA^{100} plates greater than 2mm in diameter were re-streaked onto fresh media and grown at 30°C for 5 days. Healthy colonies were analysed by yeast colony PCR using the Matchmaker Insert Check PCR Mix 2 (Clontech). Products were purified using the *Accuprep* PCR/Gel Purification Kit (Bioneer) and sequenced with a T7 primer. Based on the sequencing results, colonies with relevant inserts were streaked onto SD/-Leu/AbA^{200} media to determine whether the bait/prey interaction was strong enough to allow for growth using double the concentration of AbA. Vectors containing inserts of interest were rescued using the Easy Yeast Plasmid Isolation Kit (Clontech) as per the manufacturer's instructions. Rescued vectors were transformed into DH5α *E. coli* via electroporation and selected by ampicillin (50μg ml^{-1}). The construct was purified from *E. coli* using the *Accuprep* Plasmid Mini Extraction Kit (Bioneer) and sequenced with a T7 primer to confirm the cDNA insert sequence.

The above methods were completed for both cDNA libraries.

Confirmation of transcription factor binding

To confirm the binding of *Sb*GATA22 to the putative GATA transcription factor binding motifs in the *CYP79A1* promoter region the following was undertaken. The native *CYP79A1* (*Sb01g001200*) promoter region (1,204bp upstream of the transcription start site) and a mutant *CYP79A1* promoter region (with all putative GATA transcription factor binding sites mutated from GAT to GTA)(Supplementary Figure 3) were synthesised by GenScript (USA) and cloned into pUC57 (Supplementary Figure 4). The native and mutated promoter:pUC57 vectors were digested with KpnI and SalI and the resulting promoter sequences ligated into the vector pAbAi (also digested with KpnI and SalI) using a 1:1 vector:insert ratio. This produced the vectors pAbAi-Native and pAbAi-Mutant (Supplementary Figure 4). Constructs were electroporated into DH5α *E. coli* cells and selected by ampicillin resistance (50µg ml^{-1}). The constructs were purified from *E. coli* using the *Accuprep* Plasmid Mini Extraction Kit (Bioneer) and sequenced to ensure no mutations were present. Vectors were linearised with BstBI and 1µg transformed into *S. cerevisiae* Y1H Gold (Clontech) using the small-scale yeast transformation method as described in the Matchmaker Yeast One-Hybrid Library Screening System manual. Cells were spread onto synthetic dropout media lacking uracil (SD/-Ura) and allowed to grow for 5 days at 30°C. Colonies were analysed by

colony PCR using the Matchmaker Insert Check PCR Mix 1 (Clontech) to identify clones with correctly integrated vectors. Untransformed Y1H Gold colonies were used as a negative control. The pAbAi-Native and pAbAi-Mutant strain was tested for autoactivation of the bait sequence as described previously.

The Y1H Gold pAbAi-Native and pAbAi-Mutant strains were then transformed with 1µg of the pGADT7-Rec vector that contained the full-length *Sb*GATA22 insert from Screen 1 (Supplementary Figure 5) and selected on SD/-Leu media using the small-scale yeast transformation method as above. Colonies were analysed by yeast colony PCR using the Matchmaker Insert Check PCR Mix 2 (Clontech) to ensure the correct vector was present. Both strains were spread onto SD/-Leu/AbA^{100} media and the growth of the strain harbouring the native promoter (Y1H Gold Native-*Sb*GATA22) was compared to the strain harbouring the mutant promoter (Y1H Gold Mutant-*Sb*GATA22) after five days at 30°C.

Expression of *SbGATA22* and *AP2/B3-Like* during development

Expression of two candidate transcription factors was analysed over 14 consecutive days of sorghum development. *Sorghum bicolor* (L.) Moench line BTx623 was grown in the greenhouse complex at Monash University, Clayton, Vic., Australia in August 2018. The greenhouse received natural light, with temperatures ranging from 18°C to 28°C with an average of 26°C /20°C day/night. Seeds were germinated and grown in punnet trays on Debco seed raising substrate and perlite (2:1 v/v). Plants were watered to saturation every second day to ensure water was never limited. Plants were harvested daily from 1 day post-germination (dpg) to 14dpg. Harvesting commenced at 11am each day to avoid possible circadian differences. Tissues from each plant were separated into leaf, stem, and root tissue except for the first four days where shoot tissues (the coleoptile on days 1-2; leaf and stem on days 3-4) were harvested together due to the small size of the plants. For HCNp analysis approximately 20mg was taken from the coleoptile tip (days 1-2), first fully unfurled leaf (days 3-14), base of the stem (days 3-14), and the root tips (days 1-14) for 10 replicates. The remainder of the tissue was snap frozen in liquid N for six of the ten replicates and stored at -80°C for later analysis.

Gene expression in response to nitrogen application

Plants were grown as detailed above but in individual pots 20cm in diameter instead of punnet trays. At 5-weeks post-germination a baseline harvest was undertaken (Time 0). Following the Time 0 harvest plants were watered daily with 400mL of water (control) or 25mM KNO_3 (treatment). Further harvests were completed after two days (Time 1) and five days (Time 2) of KNO_3 application. At each time-point tissue was harvested from 10 plants for HCNp analysis by taking three leaf discs (4mm in diameter) from the youngest fully unfurled leaf, and approximately 20mg of tissue from the base of the stem and the root tips. For gene expression analysis 200mg of the same tissues from three plants was collected and snap frozen in liquid N, then stored at -80°C.

HCNp analysis

Hydrogen cyanide potential (HCNp) was determined using approximately 20mg of fresh leaf, stem, or root tissue. HCNp is the total amount of HCN produced by hydrolysis of the entire content of endogenous cyanogenic glucosides as achieved by adding exogenous β-glucosidase (β-D-Glucoside glucohydrolase, G4511, Sigma-Aldrich). The HCN produced was captured as NaCN in a 1M NaOH solution and measured via a colorimetric assay (Gleadow et al., 2012). Following the assay all tissue was dried and accurately weighed to calculate mg HCN g^{-1} dry mass.

Quantitative PCR (qPCR)

RNA was extracted from the frozen leaf, stem, and root tissues using a Sigma Spectrum Plant Total RNA kit (Sigma-Aldrich). On-column DNase digestion was carried out using RQ1 RNAse-Free DNase (Promega) for all samples according to Appendix 1 of the manufacturer's instructions. The concentration was determined using a NanoDrop spectrophotometer (ND-1000) and the quality checked by running the RNA on an 8% formaldehyde gel. cDNA was synthesised from the total RNA using SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and an oligo-dT primer. Transcript levels of *CYP79A1* (*Sb01g001200*)*, SbGATA22* (*Sb10g022580*), and *AP2/B3-Like* (*Sb07g000220*) normalised to *Ubiquitin* (*Sb01g030340*)(Paolacci et al. 2009) were determined by quantitative PCR (qPCR) for each tissue (leaf, stem, and root). Relative expression levels of *nitrate reductase* (*Sb07g022750*) were also measured for the nitrogen experiment. An Epmotion 5075 Robot (Eppendorf) was used to set up the 384

well plates and qPCR reactions were performed using a Light Cycler 480II (Roche) with cycling parameters as follows: initial denaturation: 10 minutes, 95°C; denaturation: 15 seconds, 95°C; annealing: 15 seconds, 66/63°C; extension: 15 seconds, 72°C (acquire at end of extension), for 40 cycles. Melt profile analysis was undertaken from 60-99°C rising by 1°C and waiting for 5s at each step. SensiMix SYBR Green No ROX kit (Bioline) was used for the qPCR as per the manufacturer's instructions, with forward and reverse primers specific to a region in the 3' end of each gene sequence used for amplification (Supplementary Table 2). Each biological replicate was run in triplicate along with a set of standards specific for the gene being analysed. Each RNA preparation (the RNA used to create the cDNA) was also checked for DNA contamination by using crude RNA as the template and determining if amplification occurred. Relative expression was calculated as Mean Normalised Expression (MNE) (Simon, 2003) .

Sequencing and bioinformatic analysis

Sanger sequencing was performed by the Micromon DNA sequencing facility at Monash University, Melbourne, Australia. Sequence data was analysed using Chromas (Technelysium) and databases on the National Centre for Biotechnology Information (NCBI) website. Homologues were identified using BLAST analysis. Protein sequences were aligned using MUSCLE (Edgar, 2004) and visualised using Jalview (Waterhouse et al., 2009). Identification of putative binding motifs present in promoter regions was performed using PlantPAN 2.0 (Chow et al., 2015) and the Plant Transcription Factor Database (Jin et al., 2016). Protein analysis was performed using ExPASy (Gasteiger et al., 2005).

Statistics

The statistics package Sigmaplot v 13 (Systat Software) was used for statistical analyses by one- or two-way ANOVA. For all tests, a *P*-value of <0.05 was considered significant. Means that were significantly different were compared *post hoc* using Tukey's test. Data were log transformed if required to satisfy the assumptions of normality.

4.4 Results

Bait autoactivation test

Three *CYP79A1* promotor bait fragments (F1 - 1177bp; F2 – 843bp; F3 – 460bp; Supplementary Figure 1) were cloned into pAbAi vectors upstream of the *AUR1-C* reporter gene and introduced into the *S. cerevisiae* strain Y1H Gold. This was also undertaken for the control vector p53-AbAi. The resultant yeast bait strains Y1H Gold AbAi-F1, AbAi-F2, AbAi-F3, and p53-AbAi were tested for their ability to grow on SD/-Ura media (Figure 1). Growth on SD/-Ura media indicated that the reporter sequences had stably integrated into the genome of Y1H Gold yeast strain, confirmed by colony PCR (Supplementary Figure 6).

To determine whether endogenous yeast proteins recognised the bait sequence, reporter strains were streaked onto SD/-Ura media containing either 100, 150, or 200ng ml^{-1} of the antibiotic Aureobasidin A (AbA). Growth of the reporter strains was completely supressed by a minimum concentration of 100ng ml^{-1} AbA (Figure 1). The reporter strain Y1H Gold AbAi-F1 was chosen for further analysis as it contained the longest *CYP79A1* promoter fragment (1177bp) and lacked background *AUR1-C* expression at the lowest concentration of AbA (100ng ml^{-1}).

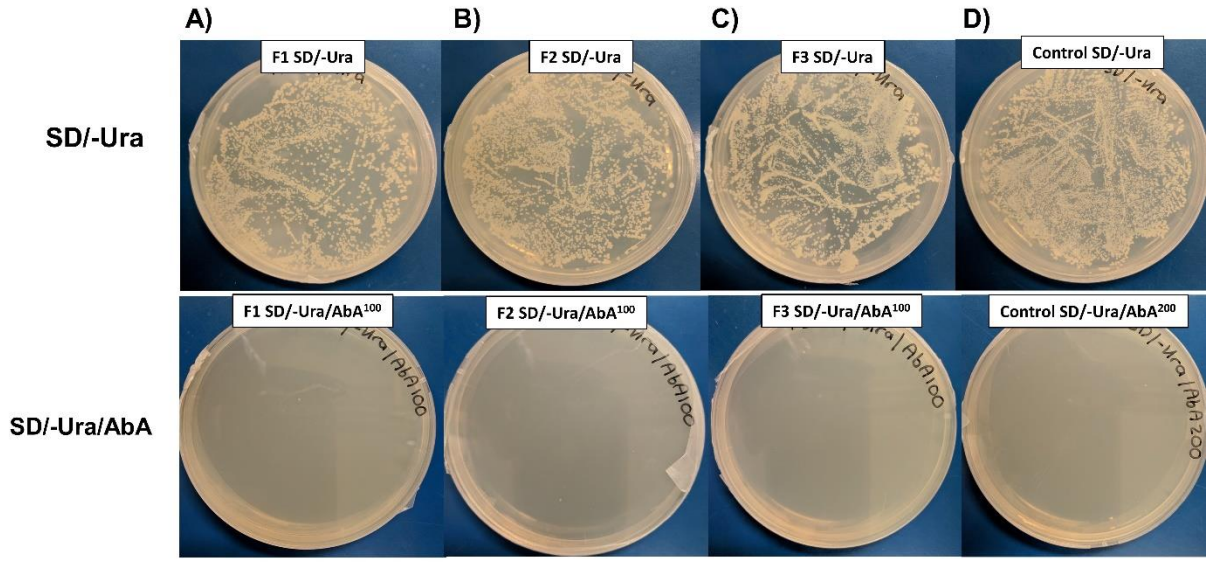


Figure 1: Representative examples of growth of the Y1H Gold bait strains **A)** AbAi-F1; **B)** AbAi-F2; **C)** AbAi-F3; and **D)** the control strain p53-AbAi (unable to grow on concentrations of AbA over 100ng ml^{-1}). All strains were able to grow on SD/-Ura media (upper panel). The promoter strains were repressed by a minimum concentration of AbA 100ng ml^{-1}, while the p53-AbAi control strain was repressed by AbA 200ng ml^{-1} (lower panel).

cDNA library screening

Two cDNA libraries were constructed and screened: Screen 1 - seeds, radicles, and emerging coleoptiles; Screen 2 - coleoptile tips. To ensure adequate coverage of a cDNA library it is essential to screen a minimum of 1 million clones. In this study Screen 1 and Screen 2 resulted in 1.6 million and 3.3 million clones screened, respectively. Colonies over 2mm in diameter after 5 days of incubation were re-streaked onto fresh SD/-Leu/AbA^{100} media and any healthy colonies that appeared after 3 days were treated as positive clones, as growth suggested a positive interaction between the prey protein coded for by the cDNA insert and the *CYP79A1* promoter bait fragment. Colony PCR was used to amplify the cDNA inserts ranging in size from approximately 230bp to 1400bp which were then sequenced (Supplementary Figure 7). Screen 1 resulted in 16 positive clones (Table 1A) and Screen 2 resulted in 11 positive clones (Table 1B).

Table 1: Results from a BLASTn analysis of cDNA library inserts that interacted with the *CYP79A1* promoter region and activated expression of the *AUR1-C* reporter gene. Approximate insert sizes and their matches to putative genes listed in the GenBank database are indicated for **A)** Screen 1 and **B)** Screen 2. Whether the open reading frame of the cDNA insert was in-frame to the GAL4 activation domain is indicated.

A)

~Insert Size	Gene	E value	GenBank Accession Number	In-Frame
1250	GATA transcription factor 22	0.0	XM_002437140.2	Y
870	AP2/B3-Like transcription factor	0.0	XM_021465842.1	Y
750	Glucuronosyltransferase	0.0	XM_002458737.2	N
230	Glutaredoxin-C7	$7x10^{-61}$	XM_002439376.2	Y
1400	Receptor-like serine/threonine-protein kinase ALE2	0.0	XM_021462690.1	Y
800	AP2/B3-Like transcription factor	0.0	XM_021465841.1	N
340	Uncharacterised	$2x10^{-118}$	XM_002438966.2	N
1050	Uncharacterised	0.0	XM_002445297.2	N
873	Uncharacterised	0.0	XM_002445297.2	Y
756	Peroxisome biogenesis protein 19	0.0	XM_002452754.2	Y
509	Alpha-galactosidase	0.0	XM_021451891.1	N
828	Peptidyl-prolyl cis-trans isomerase	0.0	XM_002452143.2	N
826	60S acidic ribosomal protein P2B	0.0	XM_021447306.1	N
655	Small subunit ribosomal RNA protein	0.0	MK050854.2	N
591	60S ribosomal protein L35a-1	0.0	XM_002452891.2	N
290	Uncharacterised	$7x10^{-94}$	XM_002468027.2	N

B)

~Insert Size	Gene	E value	Accession Number	In-Frame
930	Reduced wall acetylation 3	0.0	XM_002440281.2	N
771	Uncharacterised	0.0	XM_002458148.2	Y
813	L-ascorbate peroxidase 2	0.0	XM_002463406.2	N
526	Uncharacterised	0.0	XR_002454913.1	N
920	GATA transcription factor 22	0.0	XM_002437140.2	Y
1000	Vacuole membrane protein KMS1-like	0.0	XM_021456958.1	N
481	Zinc finger A20/AN1 domain-containing stress-associated protein 8	0.0	XM_021448944.1	N
324	Tubulin beta-4 chain	$6x10^{-160}$	XM_002456458.2	N
307	Defensin Tm-AMP-D1.2	$1x10^{-151}$	XM_002452439.2	N
910	PKHD-type hydroxylase	0.0	XM_021456766.1	N
700	AP2/B3-Like transcription factor	0.0	XM_021465841.1	N

From the cDNA inserts sequenced, the following were deemed relevant as they were fused in-frame to the GAL4 activation domain and coded for putative proteins that were predicted to have DNA binding affinity: the GATA transcription factor 22 (Screen 1 and 2) and AP2/B3-Like domain containing protein (Screen 1). Although AP2/B3-Like domain containing proteins appeared in both screens only XM_021465842.1 from Screen 1 was fused in-frame to the GAL4 activation domain. Though yeast may tolerate translational frame-shifts, it is likely that the out-of-frame inserts code for artificial gene products that exhibit binding affinity to the *CYP79A1* promoter region. For the proteins not typically localised in the nucleus, it was assumed that they were able to enter and bind to the bait fragment due to the SV40 nuclear localisation signal present at the N terminus of the GAL4AD-protein hybrid (Supplementary Figure 4). The zinc finger A20/AN1 domain-containing stress-associated protein 8 identified in Screen 2 is a transcription factor known to be involved in development and abiotic stress responses (Giri et al., 2013) but was not analysed further as it was out-of-frame to the GAL4 activation domain and the insert did not code for the entire DNA binding domain.

When tested on higher concentration of AbA the GATA transcription factor 22 and AP2/B3-Like transcription factor activated the *AUR1-C* reporter gene strongly, allowing for growth in the presence of 200ng ml^{-1} AbA (Figure 2).

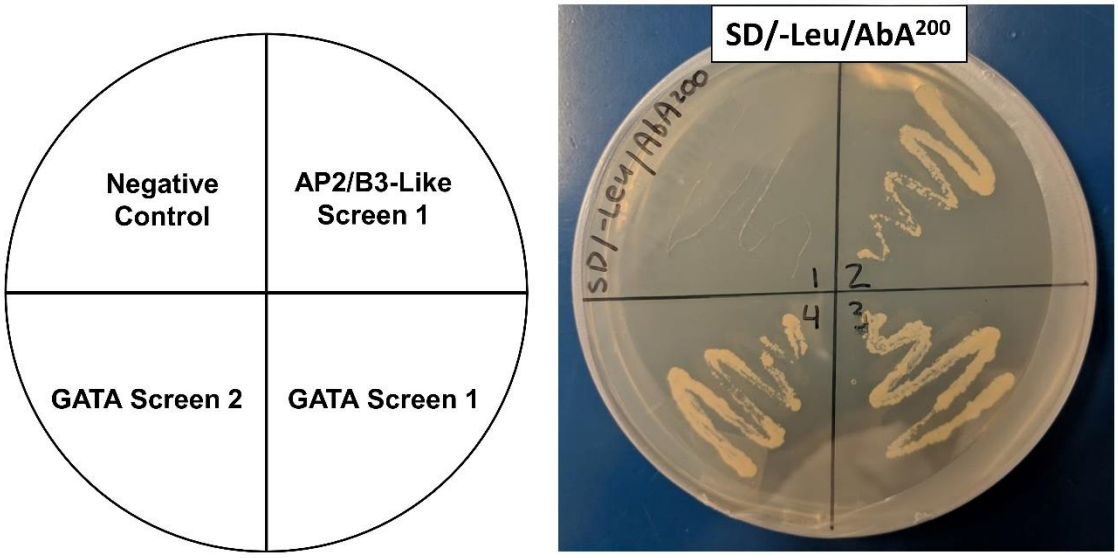


Figure 2: The yeast strains containing the putative AP2/B3-Like transcription factor cDNA insert from Screen 1, and putative GATA transcription factor 22 insert from Screen 1 and Screen 2 growing on media containing 200ng ml^{-1} of the antibiotic Aureobasidin A in comparison to a negative control.

Sequence analysis

The GATA transcription factor cDNA insert from Screen 1 is a full-length insert, 1435bp long, which includes 41bp of the 5' untranslated region and 234bp of the 3' untranslated region including the polyA tail (Supplementary Figure 8). The open reading frame (ORF) encodes a 386 amino acid polypeptide. The protein, hereafter named *Sb*GATA22, is an uncharacterised long B-GATA, with a conserved type IV zinc finger DNA binding domain (Ranftl et al., 2016). The polypeptide contains a run of 9 histidines at the N-terminal region, a putative bipartite nuclear localisation signal, and a conserved Leucine-Leucine-Methione (LLM) domain at the C-terminal region (Behringer et al., 2014)(Figure 3). GATA transcription factors preferentially bind to the motif 5' GATC 3'

in plants with the core AT more conserved than the flanking sequence (Weirauch et al., 2014). The *CYP79A1* promoter region analysed has 22 putative GATA binding sites on the forward strand and 11 on the reverse (Supplementary Figure 9).

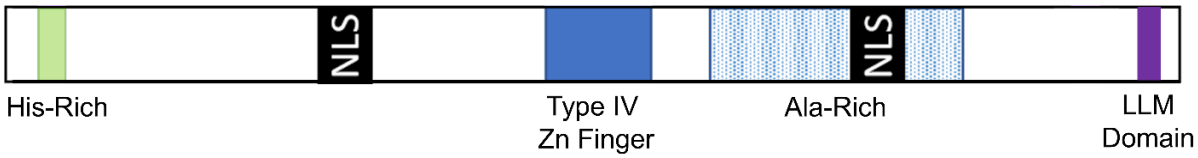


Figure 3: Schematic of the putative transcription factor *Sb*GATA22. The polypeptide consists of 386 amino acids, containing a run of 9 histidines at the N terminus, a putative bipartite nuclear localisation signal (NLS), and a signature Leucine-Leucine-Methione (LLM) domain at the C terminus. The DNA binding domain is a type IV zinc finger made up of 45 amino acids, followed by an alanine rich region.

The in-frame AP2/B3-Like transcription factor cDNA insert from Screen 1 consists of 870bp, though only 331bp corresponds to the protein coding sequence, with the remainder being part of the 3' untranslated region and polyA tail. The ORF encodes 110 amino acids of the B3 binding domain. This codes for a truncated protein that does not contain any sequence upstream of the B3 binding domain. The sequence shows 93% similarity to the B3-domain of the transcription factor *IRON DEFICIENCY FACTOR 1* (*IDEF1*) in *Zea mays* which recognises an RY binding motif of 5' CATGC 3' (Supplementary Figure 9). In sorghum there are two putative transcript variants of this gene with differences in the 60 base pairs at the 5' region. As the insert only contains the 3' region of the gene (corresponding to the C-terminus of the protein) it is not possible to determine which transcript variant was expressed during this developmental stage. The out-of-frame AP2/B3-Like transcription factor insert sequences from Screen 1 and 2 correspond to the midsection of the protein and do not code for the B3-binding domain. Due to this it was decided that *Sb*GATA22 would be analysed in greater detail, although expression of the *AP2/B3-Like* gene would be analysed in parallel.

Confirmation of *Sb*GATA22 binding to GATA transcription factor binding motifs

As with pAbAi-F1, 100ng ml^{-1} AbA suppressed growth of the pAbAi-Native and pAbAi-Mutant strains on SD/-Ura media, confirming that endogenous yeast transcription factors were not binding to the bait sequences (data not shown). The Native-*Sb*GATA22 strain (containing the native *CYP79A1* promoter bait region and expressing *Sb*GATA22 protein with a GAL4 activation domain) grew on both SD/-Leu media and SD/-Leu/AbA^{100} media, while the Mutant-*Sb*GATA22 strain was unable to grow in the presence of 100ng ml^{-1} AbA (Figure 4). This confirmed that *Sb*GATA22 binds to the core motif of GAT while being unable to bind to the mutated motif GTA.

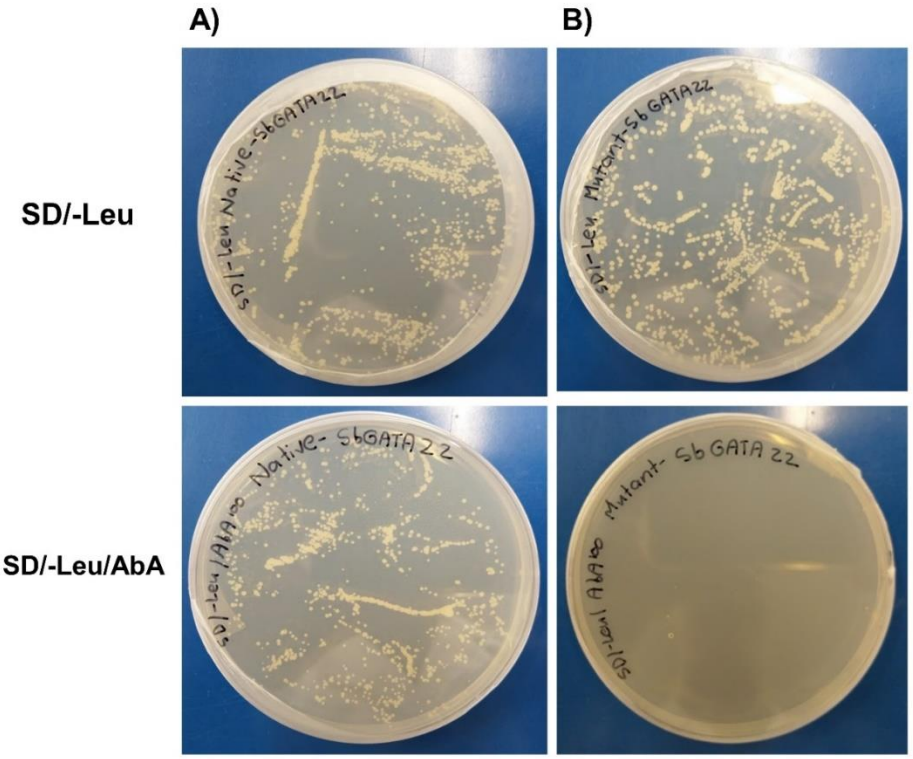


Figure 4: Growth of the Y1H Gold bait strains **A)** Native-*Sb*GATA22; and **B)** Mutant-*Sb*GATA22. Both strains were able to grow on SD/-Leu media (upper panel). Native-*Sb*GATA22 was also able to grow on AbA 100ng ml^{-1} media, while the growth of Mutant-*Sb*GATA22 was completely suppressed by the antibiotic (lower panel). This confirms that the *Sb*GATA22 protein is binding to the putative GATA transcription binding sites (Supplementary Figure 9) and that the interaction between the "prey" and "bait" is a genuine interaction.

Homology of *Sb*GATA22

The *Sb*GATA22 protein sequence is homologous to putative GATA transcription factors in both C_3 and C_4 grasses, including maize (*Zea mays*), barley (*Hordeum vulgare*), and rice (*Oryza sativa*) (Figure 5). The type IV zinc finger binding domain and the LLM-domain are highly conserved across these species. *Sb*GATA22 has only 59% sequence identity (query coverage 24%) to *Arabidopsis At*GATA21 (accession NP_200497.1) and no significant sequence similarity to GATA proteins in the cyanogenic species cassava (*Manihot esculenta* Crantz) and *Lotus japonicus* (data not shown).

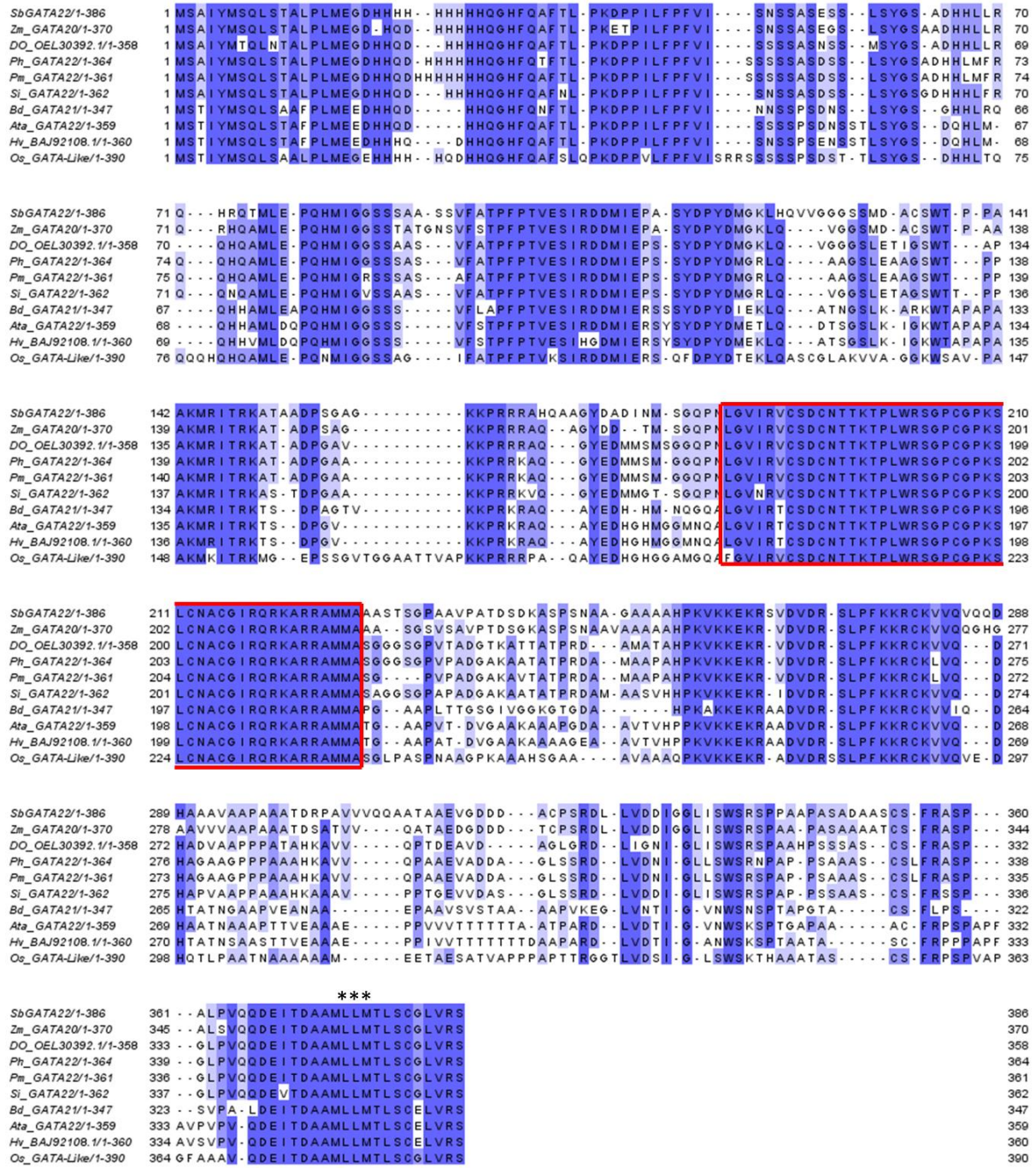


Figure 5: Alignment of the *Sb*GATA22 (accession XP_002437185.1) amino acid sequence with its closest homologues from the following species *Zea mays* (*Zm*, accession: ACG27673.1); *Dichanthelium oligosanthes* (*Do*, accession OEL30392.1); *Panicum hallii* (*Ph*, accession XP_025812836.1); *Panicum miliaceum* (*Pm*, accession RLM56087.1); *Setaria italica* (*Si*, accession XP_004966043.1); *Brachypodium distachyon* (*Bd*, XP_003563721.1); *Aegilops tauschii* (*Ata*, accession XP_020170490.1); *Hordeum vulgare* (*Hv*, accession BAJ92108.1); *Oryza sativa* Japonica (*Os*, accession XP_015641328.1). The zinc finger binding domain is indicated in red and the LLM-domain by asterisks.

To investigate the potential signalling pathways in which *Sb*GATA22 is involved, a 1kb region upstream of the *SbGATA22* transcription start site (TSS) was analysed for the presence of putative regulatory protein binding motifs (Table 2). The putative binding motifs were predicted to be recognised by transcription factors involved in drought, light, and defence pathways; as well as phytohormone signalling pathways including auxin, ethylene, and gibberellic acid.

Table 2: Putative transcription factor binding motifs located in the 1000bp region upstream of the *SbGATA22* transcription start site (TSS).

Putative binding motif	Signal Pathway	Core Sequence	Position from TSS	Strand
Auxin Response Factor (ARF)	Auxin	GTCGG	-178 -319 -451	-
Dof	Light Defence C metabolism Gibberellic acid Auxin	AAAAG	-279 -863	+
AP2/Ethylene response factor				
GCC-Box	Ethylene	GCCGCC	-166 -175 -386	+
Drought response element (DRE)	Drought	CCGAC	-198 -450 -546	
MIKC-Type MADS-Box	Floral organ identity	CC(A/T)$_6$GG	-740	+

Developmental expression of *SbGATA22* and *AP2/B3-Like*

Relative expression levels of *SbGATA22* and *AP2/B3-Like* were analysed in wild-type sorghum from 1 to 14 days post-germination (dpg) (Figure 6) and compared to plant HCNp.

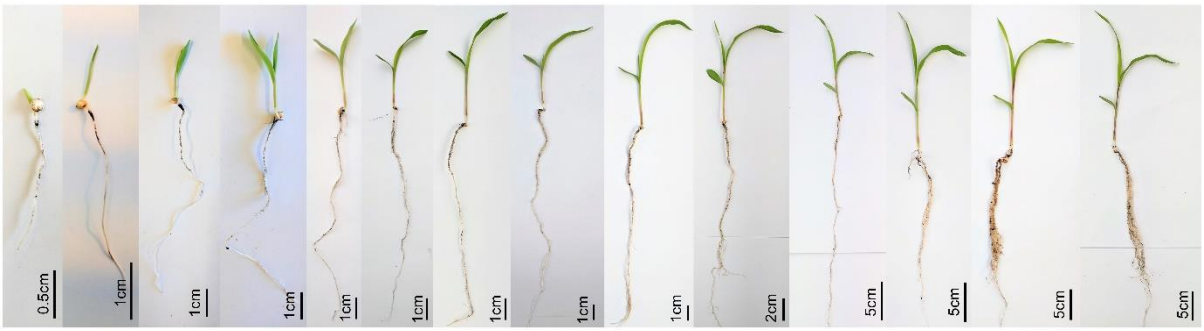


Figure 6: Representative examples of the growth of wild-type *Sorghum bicolor* (L.) Moench line BTx623 from 1 to 14 days post-germination.

Hydrogen cyanide potential of the leaf and stem tissue was highest following germination before immediately decreasing (Figure 7A,B). In the root tissue HCNp was significantly lower than the shoot tissue, though it was also highest directly following germination (Figure 7C). HCNp plateaued at 3dpg in the roots, unlike in the shoot tissue where dhurrin concentrations continued to decrease as the plants matured.

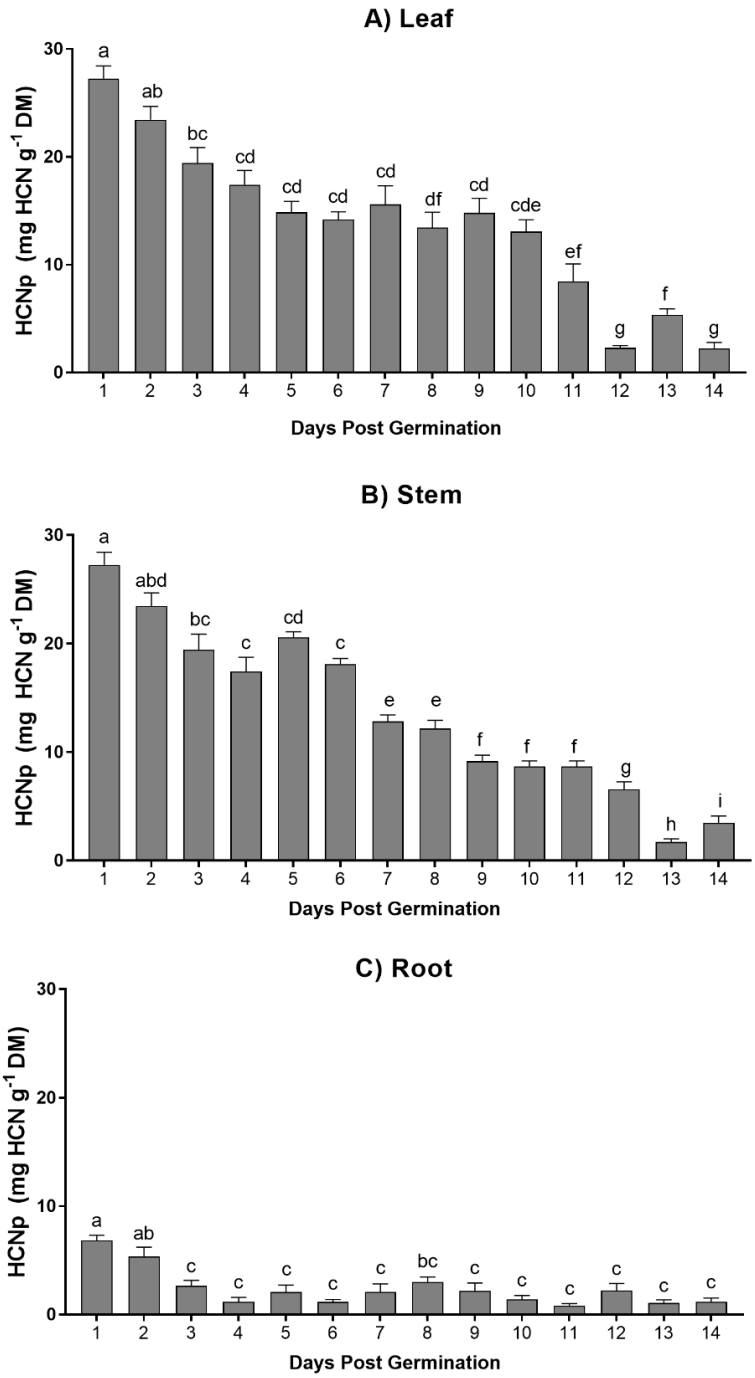


Figure 7: Hydrogen cyanide potential (mg HCN g^{-1} dry mass) for **A)** Leaf; **B)** Stem; and **C)** Root tissue of *Sorghum bicolor* line BTx623 from 1-day post-germination (dpg) to 14dpg. HCNp was highest directly following germination in the leaf and stem tissue before immediately decreasing. Data denote mean ± SE (n=10). Columns marked with identical letters are not significantly different at P<0.05, analysed using ANOVA and Tukey's test.

The relative expression pattern of *CYP79A1* corresponded closely to HCNp in all tissues (Figure 8A-C). In the leaf and stem tissue *CYP79A1* expression was highest following germination, remaining high for the first three days, before it rapidly decreased. This decrease was greatest in the leaf tissue, and by 7dpg relative expression had decreased to below 0.2, from a peak of over 40. In the leaf and stem tissue relative expression of *SbGATA22* was low at 1dpg, before it increased and peaked at 3dpg (Figure 8D,E). No expression was observed in the root tissue, consistent with data found in gene expression databases. In the leaf tissue *AP2/B3-Like* expression was initially high before a sudden decrease at day 5, with relatively uniform expression from this point onwards. *AP2/B3-Like* relative expression also decreased in the stem tissue at 5dpg before it increased at 10dpg. A similar pattern to stem *AP2/B3-Like* expression was observed in the root tissue, though at a lower level of expression.

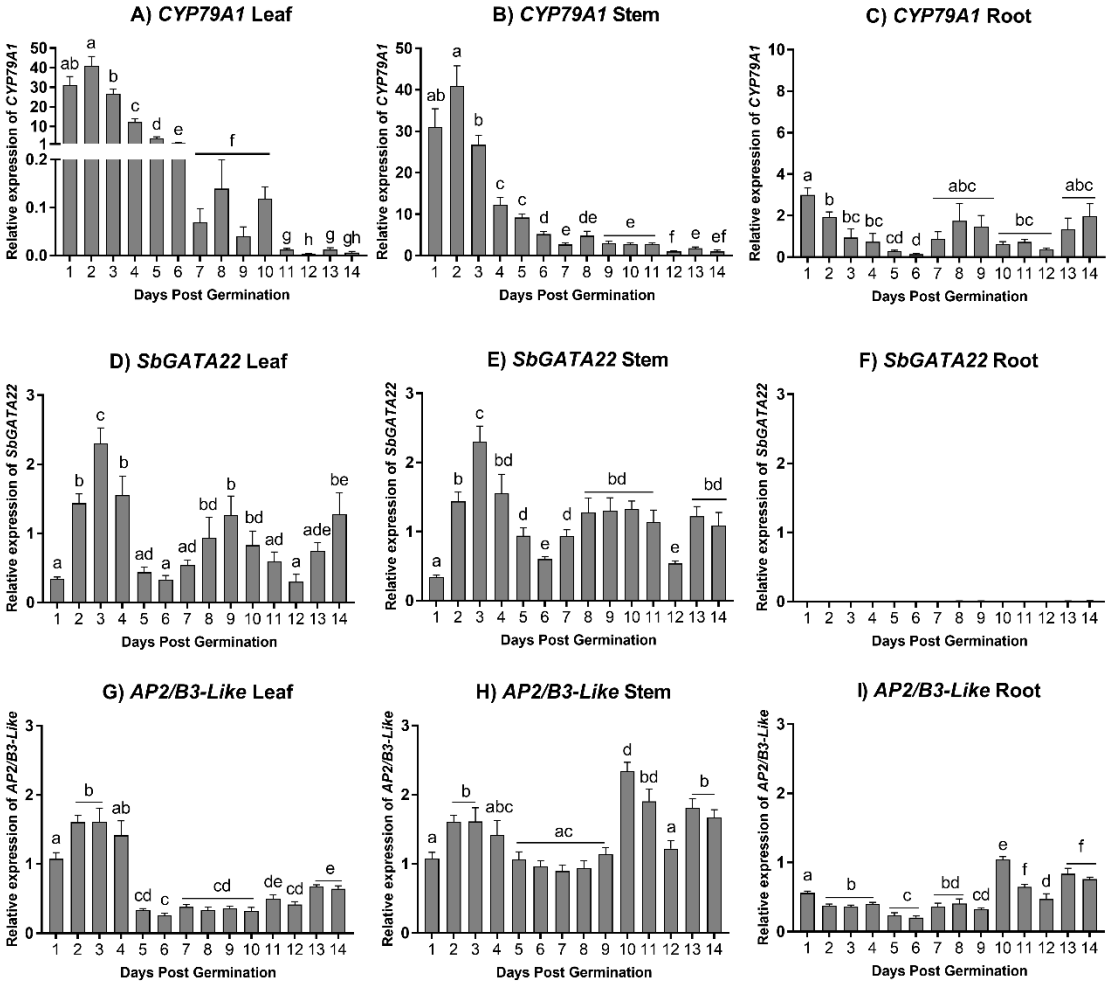


Figure 8: Relative expression levels of *CYP79A1*, *SbGATA22*, and *AP2/B3-Like* normalised to *Ubiquitin* in the leaf, stem, and root tissue of *Sorghum bicolor* line BTx623 from 1-day post-germination (dpg) to 14dpg. Data denote mean ± SE (n=6). Columns marked with identical letters are not significantly different at P<0.05, analysed using ANOVA and Tukey's test.

Expression of *SbGATA22* and *AP2/B3-Like* in response to nitrogen application

Hydrogen cyanide potential and relative expression of *CYP79A1*, *SbGATA22*, *AP2/B3-Like*, and *nitrate reductase* (*NR)* were analysed in 5-week old plants (T0) and following application of either H_2O or 25mM KNO_3 for 2 days (T1) and 5 days (T2). No induction of HCNp was observed in any tissue following KNO_3 application (Figure 9A-C). Despite this, relative expression of *CYP79A1* increased in response to KNO_3 in the leaf tissue at T2, and stem tissue at T1 and T2 (Figure 9D-F). *SbGATA22* expression was higher in the leaf tissue of plants treated with KNO_3 compared to control plants at T1 and T2, though no induction occurred in the stem or root tissues (Figure 9G-I). Relative expression of *AP2/B3-Like* did not change in response to KNO_3 in any tissue (Figure 9J-L). Relative expression of *nitrate reductase* increased in the leaf and root tissue at T1 and T2 in response to KNO_3 (Figure 9M-O).

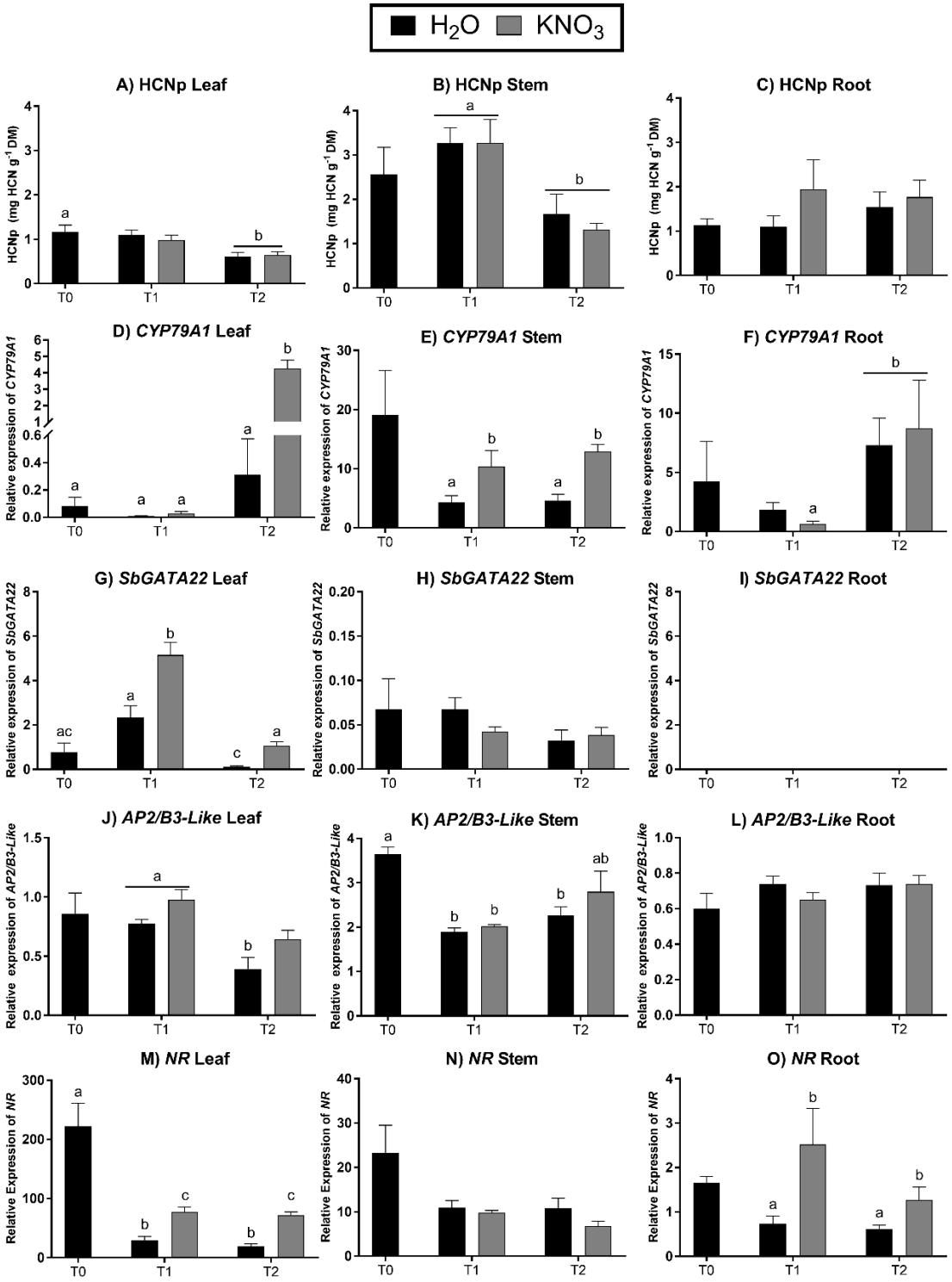


Figure 9: Hydrogen cyanide potential (mg HCN g^{-1} dry mass) and relative expression levels of *CYP79A1*, *SbGATA22*, *AP2/B3-Like*, and *nitrate reductase* (*NR*) normalised to *Ubiquitin* in the leaf, stem, and root tissue of *Sorghum bicolor* line BTx623 at 5-weeks old. A baseline harvest was performed (T0) before H_2O or KNO_3 was applied for either 2 days (T1) or 5 days (T2). Data denote mean ± SE (HCNp n=10; relative gene expression n=3). Columns marked with identical letters are not significantly different at P<0.05, analysed using ANOVA and Tukey's test.

4.5 Discussion

Cyanogenic glucosides (CNglcs) are found in one-third of crop species, with their biosynthesis influenced by ontogeny and environmental factors including light, water and nitrogen availability (Gleadow and Møller, 2014, Jones, 1998). Despite the CNglc biosynthetic pathways being well characterised and the structural genes for the pathways identified, the transcriptional regulation of these genes is unknown and no direct regulators have been identified. Here, we used yeast one-hybrid (Y1H) screens to detect candidate regulatory proteins of CNglc biosynthesis in the cereal crop sorghum.

In this study, developmental rather than environmental regulation of the dhurrin biosynthetic genes in sorghum was investigated. This was achieved by screening cDNA libraries made from mRNA that was extracted during the early stages of plant development. In sorghum, dhurrin concentrations peak at three to four days post-germination, with transcript levels of the cytochrome P450 genes involved in dhurrin biosynthesis (*CYP79A1*, and *CYP71E1*) highest in the short period following germination (Busk and Møller, 2002). Therefore, we aimed to identify regulatory proteins present in sorghum during the stages of maximum *CYP79A1* expression and dhurrin production. Screen 1 pooled seeds that had been germinating for 24 hours (emerging radicle) and 48 hours (radicle and emerging coleoptile); and Screen 2 used the tips of coleoptiles at 3-days post-germination, this being the tissue and time-point where dhurrin concentration is at a maximum.

As important *cis*-regulatory elements are not known, a native promoter fragment containing 1177bp of the *CYP79A1* promoter region (Supplementary Figure 1) was used as bait. While many Y1H screens use trimers of core binding motifs as the bait sequence, the use of large promoter fragments has also been successful although they also yield higher numbers of false positives (Gao et al., 2016, Gong et al., 2015). Over 5 million clones were screened in this study and two transcription factors that bound to the bait sequence were analysed in greater detail: AP2/B3-Like and *Sb*GATA22.

The role of AP2/B3-Like transcription factors in plant development

The AP2/B3-Like transcription factor identified in Screen 1 (Table 1) has the greatest level of sequence similarity to IRON DEFICIENCY FACTOR 1 (IDEF1) in maize (*Zea mays*). IDEF1 senses cellular iron status by binding directly to iron and divalent metals in the cell before activating downstream genes and is known to be constitutively expressed in the roots and leaves of species such as rice (*Oryza sativa*) (Kobayashi et al., 2012). There is no evidence linking dhurrin concentration to cellular iron status, however, cytochromes P450 are hemoproteins that require iron-containing heme as a co-factor (Bolwell et al., 1994, Vazquez-Albacete et al., 2017, Hasemann et al., 1995). It is therefore possible that a link between iron availability and expression of *CYP79A1* exists. However, as only the B3 binding domain was present in the Y1H positive clone it may also suggest that the *CYP79A1* promoter region is recognised by B3 binding domains. The binding domain of IDEF1 is homologous to the Abscisic Acid Insensitive 3/Viviparous 1 (ABI3/VP1) transcription factor family. These transcription factors are highly expressed during seed maturation and are integral in maintaining seed dormancy, including in *Sorghum bicolor* (Carrari et al., 2003).

Dhurrin increases in concentration during the early stage of seed development in sorghum, peaking at 25 days post-pollination (Nielsen et al., 2016). This increase correlates with the transcript levels of the three dhurrin biosynthetic genes *CYP79A1*, *CYP71E1*, and *UGT85B1* (Nielsen et al., 2016). When the grain has fully matured dhurrin is no longer present, presumably catabolised via endogenous turnover pathways (Nielsen et al., 2016, Halkier and Møller, 1989). This would suggest regulators of *CYP79A1* are expressed during seed development. Transcription factors homologous to ABI3/VP1 cannot be ruled out as regulators of *CYP79A1* expression during this developmental period, especially as they are known to regulate other secondary metabolites during seed maturation, namely anthocyanins (McCarty et al., 1989). Furthermore, these transcription factors are also involved in dehydration stress responses in vegetative plant tissues (Bedi et al., 2016), an abiotic factor known to affect dhurrin production in cyanogenic species (Neilson et al., 2015).

Here, the transcriptional profile of *AP2/B3-Like* revealed expression in all vegetative tissues from 1-14 days post-germination (Figure 8G-I) and in 5-week old plants (Figure

9J-L). Expression of *AP2/B3-Like* was not influenced by nitrogen application (Figure 9J-L). In the leaf tissue *AP2/B3-Like* expression was highest in the four days following germination which correlated to *CYP79A1* expression. The expression in the root tissue was also similar to the *CYP79A1* expression pattern, although *AP2/B3-Like* expression in the stem tissue did not correlate. The role of ABI3/VP1 in seed maturation and germination (developmental stages where dhurrin production is precisely controlled), and in dehydration stress responses (with drought stress being known to affect dhurrin concentration in sorghum) suggest that the binding of AP2/B3-Like may constitute a genuine interaction between the transcription factor B3 domain and the *CYP79A1* promoter region. This warrants further investigation, beginning with the isolation and binding analysis of full-length *AP2/B3-Like* clones.

GATA transcription factors in plant development and stress responses

Unlike AP2/B3-Like proteins that are specific to the plant kingdom, GATA transcription factors are found in all eukaryotes and are characterised by a type IV zinc finger binding domain (Lowry and Atchley, 2000, Behringer and Schwechheimer, 2015). So-called because of their recognition of GATA binding motifs, it was later determined that in plants GATA transcription factors preferentially bind to GATC motifs, with the core AT sequence most highly conserved (Weirauch et al., 2014, Xu et al., 2017). We confirmed *Sb*GATA22 recognises GAT motifs, as the mutation of these sites to GTA resulted in no expression of the *AUR1-C* reporter gene (Figure 4). GATA binding motifs were first found to be enriched in promoter regions of circadian clock and light-regulated genes (Argüello-Astorga and Herrera-Estrella, 1998) and in turn were characterised in *Arabidopsis* for their involvement in nitrogen signalling, germination control, greening, senescence, and flower development (Ranftl et al., 2016, Chiang et al., 2012).

*Sb*GATA22 is classified as a long B-GATA with a C-terminal Leucine-Leucine-Methionine (LLM) domain (Behringer et al., 2014). Long B-GATAs have between 74 to 230 amino acids N-terminal to the type IV zinc finger binding domain, as opposed to short B-GATAs that have between one and 66 amino acids at the N-terminus (Behringer et al., 2014). There are 11 B-GATA transcription factors in *Arabidopsis*; the first two characterised were the paralogous NITRATE-INDUCIBLE, CARBON METABOLISM-INVOLVED (GNC), and CYTOKININ-RESPONSIVE GATA FACTOR 1/GNC-LIKE (CGA1/GNL) (Chiang et al.,

2012, Hudson et al., 2011). GNC was originally found to respond to nitrate, while CGA1/GNL responded to cytokinin. However, it is now known that they are functionally redundant (Hudson et al., 2011).

In *Arabidopsis*, GNC and CGAI/GNL are involved in light signalling pathways and regulated by nitrogen availability and the phytohormones gibberellic acid, auxin, and cytokinin (Klermund et al., 2016, Behringer and Schwechheimer, 2015). They can act as either activators or repressors of downstream target genes. *Sb*GATA22 is homologous to GNC (*At*GATA21) and while GNC is well characterised in *Arabidopsis*, the same cannot be said for monocot homologues.

In monocots GATA transcription factors have predominantly been characterised in rice (*Oryza sativa*), where they are involved in similar pathways to *Arabidopsis* (Reyes et al., 2004). This includes their expression increasing in response to light, nitrogen, and cytokinin. In rice, GATA transcription factors have also been implicated in abiotic stress responses with gene expression changing in response to salinity, drought, and abscisic acid (a phytohormone with roles in drought and salt stress) (Gupta et al., 2017). Drought and salinity also result in alternative splicing of *CGAI/GNL* transcripts (Gupta et al., 2017). Despite knowledge of GATA transcription factor function for rice, functional conservation between monocots is largely unknown.

In this study *SbGATA22* was expressed at all developmental stages analysed, including directly following germination (Figure 8; Figure 9). Expression was tissue specific with no expression of *SbGATA22* in the roots. Lack of GATA transcription factor expression in root tissues is also observed in other species and consistent with their role in greening (Chiang et al., 2012, Hudson et al., 2013). However, as the seedlings used for RNA extraction in the Y1H screens were etiolated and harvested in the dark, light is not required for *SbGATA22* expression - evidence that in sorghum *Sb*GATA22 is likely involved in pathways other than greening. This is further supported by the presence of *cis*-regulatory elements in the *SbGATA22* promoter region that are not only involved in light signalling pathways, but also in defence, carbon metabolism, and drought stress (Table 2). It is significant to note that in cyanogenic species, cyanogenic glucosides are regulated in response to light, drought, and nitrogen availability (Gleadow and Møller,

2014, Schmidt et al., 2018); the same pathways in which GATA transcription factors are involved.

The lack of *SbGATA22* expression in sorghum root tissue correlates with dhurrin production being regulated differently between above and below ground tissues both during development and in response to environmental factors (O'Donnell et al., 2013, Blomstedt et al., 2018). The transcription profile of *Sb*GATA22 may suggest that it is a transcriptional repressor of *CYP79A1*, as shown by the leaf and stem tissue expression of *SbGATA22* increasing when *CYP79A1* was at a maximum (1-3dpg); in turn correlating to a decrease in *CYP79A1* expression (Figure 8). However, this cannot be definitively stated as it is likely that *Sb*GATA22 is also involved in other pathways and, due to this, expression in relation to *CYP79A1* may differ. Furthermore, it is not confirmed as to whether transcript abundance is relative to protein abundance. Interestingly, CGAI/GNL in rice has reduced expression during dark periods (Hudson et al., 2013), which is also when CNglcs have been found to increase in concentration in the cyanogenic species, cassava (Schmidt et al., 2018). If GATA transcription factors are involved in CNglc regulation, their reduced expression during dark periods may also correspond to them acting as repressors of CNglc biosynthetic genes.

In animals GATA transcription factors have binding partners known as FRIENDS OF GATA (FOG) (Fox et al., 1999, Robert et al., 2002). These transcriptional co-factors interact with GATA proteins via their own zinc finger domains and either enhance or repress GATA activity. No genes homologous to FOGs have been found in plant genomes. However, yeast 2-hybrid screens have identified some binding partners of plant GATAs including the transcriptional repressors SIN3-Like1, involved in the histone deacetylase complex, and TOPLESS, involved in jasmonate, auxin and defence responses (Causier et al., 2012, Bowen et al., 2010). Though the precise consequences of these interactions are not known, it suggests that the functions of GATA transcription factors in plants are also being modulated by co-regulators.

SbGATA22 expression in response to nitrogen application

As GATA transcription factors are linked to nitrogen signalling, KNO_3 was applied to 5-week old sorghum plants for either two (Time 1) or five (Time 2) days. This followed the protocol of Busk and Møller (2002) where a large induction in dhurrin concentration in leaves and stems was observed following KNO_3 application. Their study also confirmed that the induction in dhurrin was due to the NO_3^- and not potassium. Here, dhurrin was not induced at either harvest (Figure 9A-C). However, *nitrate reductase* (*NR*) expression was used as an indicator of plant response to nitrate application. *NR* expression was higher in both leaf and root tissues of the treatment group compared to the controls at both T1 and T2 (Figure 9J-L). Therefore, despite the lack of dhurrin induction, it appears the plants were responding to KNO_3 application. In the leaf, *CYP79A1* expression was over ten-fold higher in the treatment group than the control at Time 2; while in the stem it was twice as high in the treatment group at Time 1 and Time 2. As increased *CYP79A1* expression did not result in higher dhurrin concentration in either the leaves or stem, it is possible that dhurrin production was induced, but turned over at an equal rate via the endogenous turnover pathway (Bjarnholt et al., 2018, Pičmanová et al., 2015).

Expression of *SbGATA22* was higher in the leaf tissue when nitrate was applied at both Time 1 and Time 2, but no changes in expression were seen in the stem or the root (Figure 9G-1). As with the developmental expression data, *Sb*GATA22 fits the profile of a repressor more so than an activator, as once again high *SbGATA22* expression correlated to lower *CYP79A1* expression. As *CYP79A1* is expressed in the roots, while *SbGATA22* expression is absent, it can be deduced that *Sb*GATA22 is not the sole regulator of *CYP79A1* expression in sorghum.

Conclusions

To date, attempts to alter the cyanogenic status of plants have predominantly focussed on CNglc biosynthetic genes, producing acyanogenic individuals, which can affect plant growth rate and stress tolerance. Research into the molecular regulation of CNglc biosynthesis provides new targets for the manipulation of HCNp and may enable the reduction (rather than removal) of CNglcs in cyanogenic species. The ability to alter the

induction of CNglc biosynthetic genes in response to environmental factors would also prevent their toxicity when used for food and forage.

This study is the first to identify candidate regulators of sorghum's key dhurrin biosynthetic gene, *CYP79A1*. While it is not possible to definitively state whether AP2/B3-Like and *Sb*GATA22 are controlling *CYP79A1* expression *in planta*, a preliminary point for investigation into the transcription factors regulating CNglc production has been established.

4.6 References

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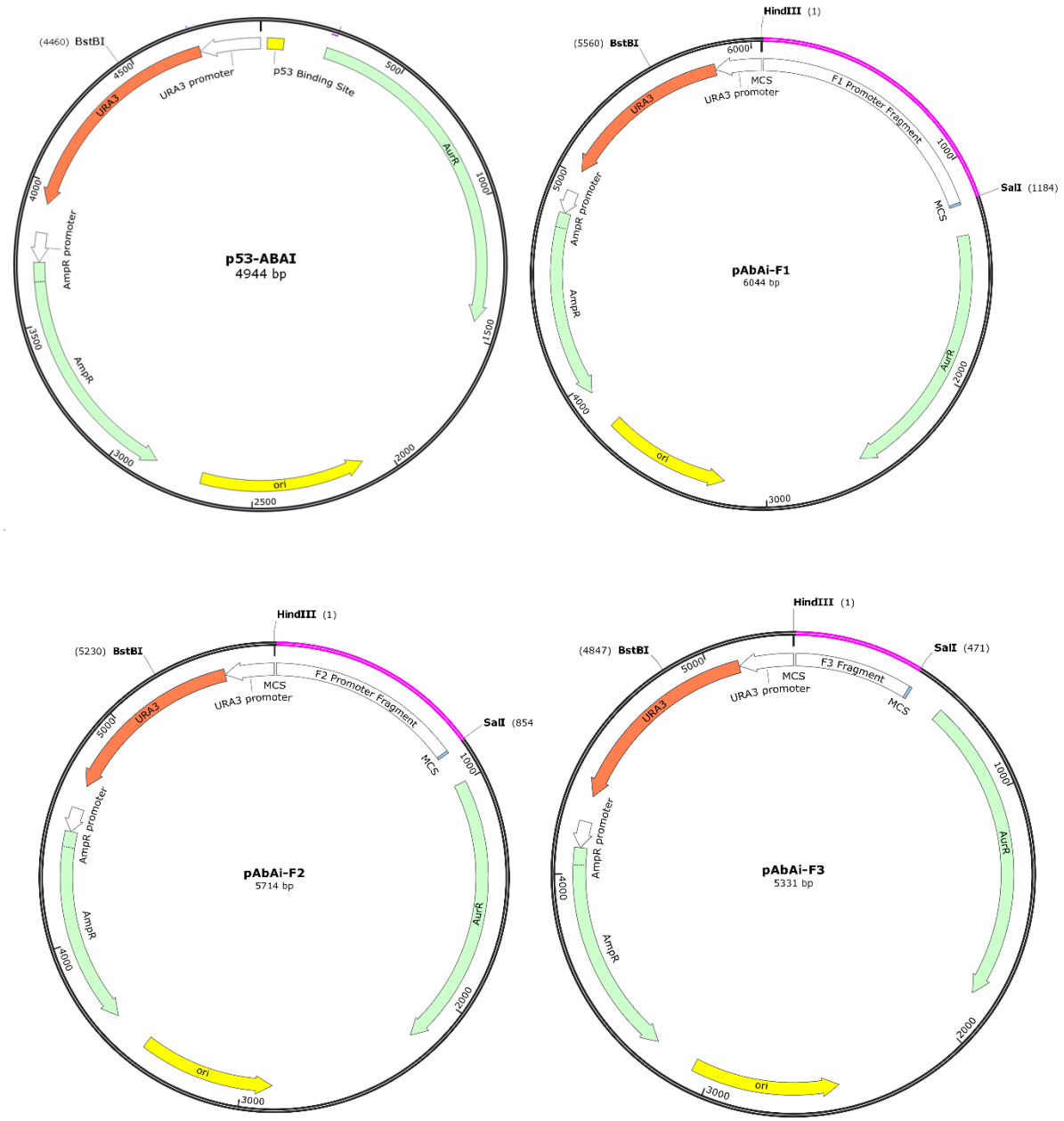
4.7 Supplementary Material

```
-1204  GCATACCGATCGATGCGAAAAAAAAACCCAGGAGACTCTCGAAGATCTACAGATTAAATC
-1144  TAAGTTTCCACGGTCCAATATGCCGATTGGTAAGTTAGAACGAAGTAGGGAGAGAGAAAA
-1084  AAAACCATAGGGAGGAGGAAGACAAGAAAAAAGACTCGGGCAGGAAGAGTGTATGCTGGT
-1024  TTAAAATAGGAGCTCGGTGTCTTAGATCATGGTGCCAAATTTGACGCCAATGGTAATCGC
-964   GTCGAGGTCTCTGCCACGTCAACGCCATGTCTGCGTCAACACCAACATGGCACAACGACC
-904   TTGGCGCCTAATTGGGCTGGCGCCGAGATGTGTGACCTTGGCGGCAATTACAACGGCGCT
-844   GAGCAGAGGGTCTAGATTTTGAAAGCATACCTACCGGGACAGATTTATGAAAATCTTTTA
-784   AAAAAAAGAAGCAAAAAAACAAAAAAAAATGCCAGACATGCGGTGGCTCTTTGGCACGCC
-724   TGCGTGACACCCCTCCCTTCATGCACGCCGACAAGCAGACAGAAAAAGCCTTGGTGAGAG
-664   AAAGGGGATGACATGGGCCCACATGTATGAGTGAGGGAGAGGGCAGTCGTTGCGGTATTT
-604   TGGGTAATGTGAAAATACAACAATCTGGTTCCCATCTTGTTTCAAAGGTAGAAAATGGCA
-544   TGGATCAAGTATAGAAACAATTGTATTGGCAAGTTTCCAAATACATGAATAGTAATAAGT
-484   GTGAAAATTATAATGGCATGGTTCAAATAAACCCCAAAAATATTGTTAGTATTGTAATAA
-424   TAAAGAAAACCAAAACGGTTCGGACTGGGCTTGTCCCCTCAACACTATCGTCATGGGTAG
-364   CGCACACAGCATGATGTGCTCGATCATTCTGAGCTCCCACATCATGTGCATGCTGCATGG
-304   TCGATGCGCTGCACACAGTTAGCTGTGGGACTGTGCATGCACGCGTGGTTGGTGGCGATC
-244   CAGCATCCCATAAATACCTGCGAACCGCCGTGTGTTCTGCCTCAAGCAGGAGCACATACA
-184   GCTAGCTAGCTCATCGGGTGATCGATCAGTGAGCTCTCTCTTTGGCCTAGCTAGCTGCTA
-124   GCAGTGCAGGTAGCCAATCAAAGCAGAAGAACTCGATCGATCGATCATCACGATCGCTGC
-64    TAGCTAGCTAGCTGCTCGCTCTCACACTAGCTACGTGTTTTTGTTAATTTGATATATATA
-4     TATAATG
```

Supplementary Figure 1: *CYP79A1* (*Sb01g001200*) promoter "bait" fragments cloned into pAbAi for stable integration into the yeast genome. F1 =1177bp; F2= 843bp; F3 = 460bp. The region immediately upstream from the *CYP79A1* transcription start site was not included in the bait sequence due to the AT rich region (highlighted in grey). F1 is 1177 nucleotides from -27 to -1204; F2 is 843 nucleotides from -27 to -870; and F3 is 406 nucleotides from -27 to -487.



Supplementary Figure 2: feature maps of p53-AbAi control vector and pAbAi vectors containing the F1, F2, and F3 *CYP79A1* (*Sb01g001200*) promoter "bait" fragments. Vectors were transformed into the *S. cerevisiae* Y1H Gold strain where they were stably integrated into the yeast genome allowing growth in the absence of uracil.

```
Native      GCATACCGATCGATGCGAAAAAAAAACCCAGGAGACTCTCGAAGATCTACAGATTAAATC    60
Mutant      GCATACCGTACGTAGCGAAAAAAAAACCCAGGAGACTCTCGAAGTACTACAGTATAAGCT    60
            ********  **  ******************************  ******  ***

Native      TAAGTTTCCACGGTCCAATATGCCGATTGGTAAGTTAGAACGAAGTAGGGAGAGAGAAAA    120
Mutant      TAAGTTTCCACGGTCCAATATGCCGTATGGTAAGTTAGAACGAAGTAGGGAGAGAGAAAA    120
            ************************  ********************************

Native      AAAACCATAGGGAGGAGGAAGACAAGAAAAAAGACTCGGGCAGGAAGAGTGTATGCTGGT    180
Mutant      AAAACCATAGGGAGGAGGAAGACAAGAAAAAAGACTCGGGCAGGAAGAGTGTATGCTGGT    180
            ************************************************************

Native      TTAAAATAGGAGCTCGGTGTCTTAGATCATGGTGCCAAATTTGACGCCAATGGTAATCGC    240
Mutant      TTAAAATAGGAGCTCGGTGTCTTAGTACATGGTGCCAAATTTGACGCCAATGGTAGCTGC    240
            *************************  ****************************   **

Native      GTCGAGGTCTCTGCCACGTCAACGCCATGTCTGCGTCAACACCAACATGGCACAACGACC    300
Mutant      GTCGAGGTCTCTGCCACGTCAACGCCATGTCTGCGTCAACACCAACATGGCACAACGACC    300
            ************************************************************

Native      TTGGCGCCTAATTGGGCTGGCGCCGAGATGTGTGACCTTGGCGGCAATTACAACGGCGCT    360
Mutant      TTGGCGCCTAATTGGGCTGGCGCCGAGTAGTGTGACCTTGGCGGCAATTACAACGGCGCT    360
            *************************  ******************************

Native      GAGCAGAGGGTCTAGATTTTGAAAGCATACCTACCGGGACAGATTTATGAAAATCTTTTA    420
Mutant      GAGCAGAGGGTCTAGTATTTGAAAGCATACCTACCGGGACAGTATTATGAAAGCTTTTTA    420
            ***************  ************************  ********   *****

Native      AAAAAAAGAAGCAAAAAAACAAAAAAAAAATGCCAGACATGCGGTGGCTCTTTGGCACGCC    480
Mutant      AAAAAAAGAAGCAAAAAAACAAAAAAAAAATGCCAGACATGCGGTGGCTCTTTGGCACGCC    480
            ************************************************************

Native      TGCGTGACACCCCTCCCTTCATGCACGCCGACAAGCAGACAGAAAAAGCCTTGGTGAGAG    540
Mutant      TGCGTGACACCCCTCCCTTCATGCACGCCGACAAGCAGACAGAAAAAGCCTTGGTGAGAG    540
            ************************************************************

Native      AAAGGGGATGACATGGGCCCACATGTATGAGTGAGGGAGAGGGCAGTCGTTGCGGTATTT    600
Mutant      AAAGGGGTAGACATGGGCCCACATGTATGAGTGAGGGAGAGGGCAGTCGTTGCGGTATTT    600
            *******  ***************************************************

Native      TGGGTAATGTGAAAATACAACAATCTGGTTCCCATCTTGTTTCAAAGGTAGAAAATGGCA    660
Mutant      TGGGTAATGTGAAAATACAACAGCTTGGTTCCCGCTTTGTTTCAAAGGTAGAAAATGGCA    660
            **********************   ********   ************************

Native      TGGATCAAGTATAGAAACAATTGTATTGGCAAGTTTCCAAATACATGAATAGTAATAAGT    720
Mutant      TGGTACAAGTATAGAAACAATTGTATTGGCAAGTTTCCAAATACATGAATAGTAATAAGT    720
            ***  ********************************************************

Native      GTGAAAATTATAATGGCATGGTTCAAATAAACCCCAAAAATATTGTTAGTATTGTAATAA    780
Mutant      GTGAAAATTATAATGGCATGGTTCAAATAAACCCCAAAAATATTGTTAGTATTGTAATAA    780
            ************************************************************

Native      TAAAGAAAACCAAAACGGTTCGGACTGGGCTTGTCCCCTCAACACTATCGTCATGGGTAG    840
Mutant      TAAAGAAAACCAAAACGGTTCGGACTGGGCTTGTCCCCTCAACACTGCTGTCATGGGTAG    840
            ********************************************   ************

Native      CGCACACAGCATGATGTGCTCGATCATTCTGAGCTCCCACATCATGTGCATGCTGCATGG    900
Mutant      CGCACACAGCATGTAGTGCTCGTACATTCTGAGCTCCCACGCTATGTGCATGCTGCATGG    900
            *************  *******  ****************   *****************

Native      TCGATGCGCTGCACACAGTTAGCTGTGGGACTGTGCATGCACGCGTGGTTGGTGGCGATC    960
Mutant      TCGTAGCGCTGCACACAGTTAGCTGTGGGACTGTGCATGCACGCGTGGTTGGTGGCGTAC    960
            ***  ************************************************  *

Native      CAGCATCCCATAAATACCTGCGAACCGCCGTGTGTTCTGCCTCAAGCAGGAGCACATACA    1020
Mutant      CAGCGCTCCATAAATACCTGCGAACCGCCGTGTGTTCTGCCTCAAGCAGGAGCACATACA    1020
            ****   ************************************************
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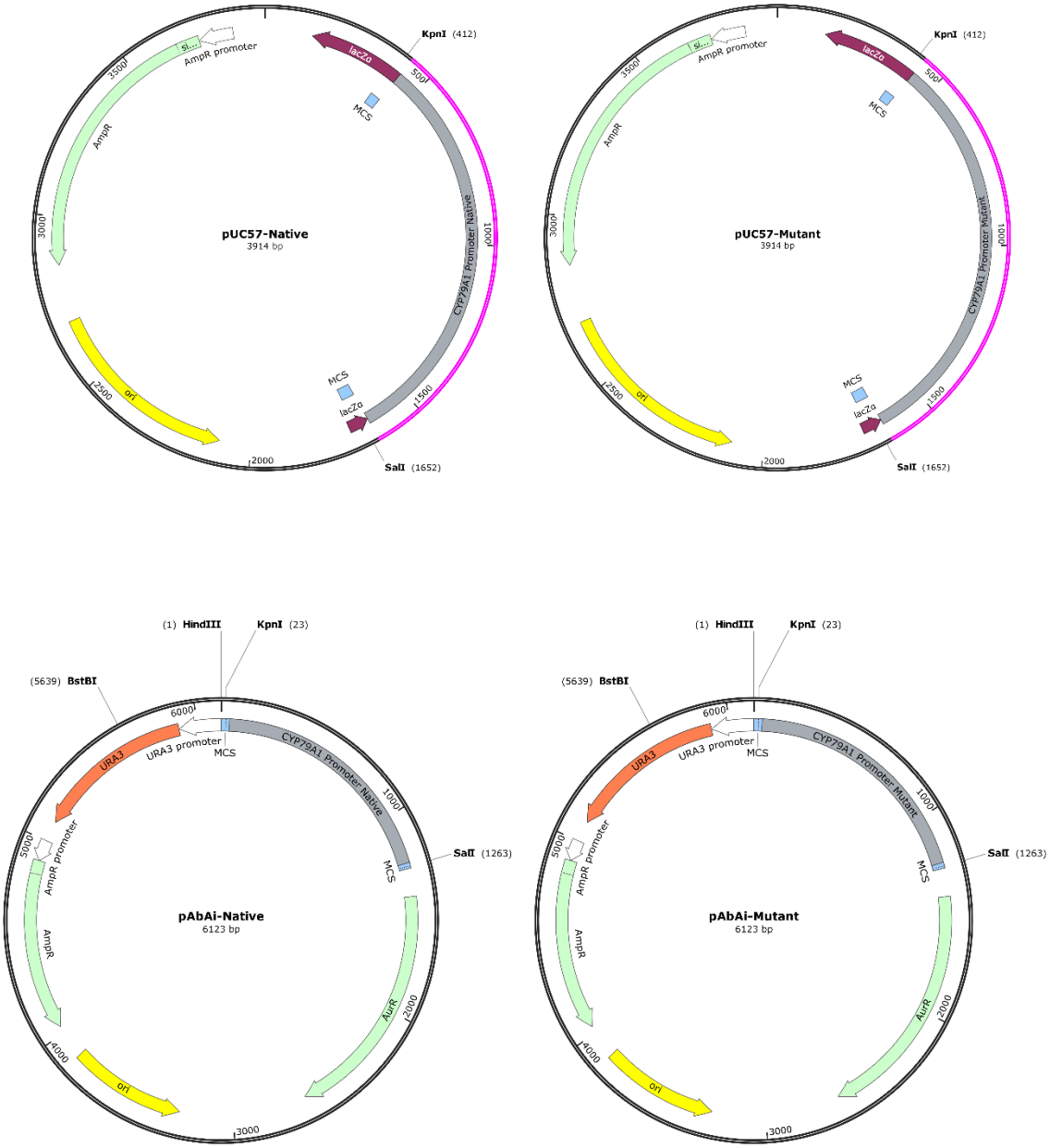
Native      GCTAGCTAGCTCATCGGGTGATCGATCAGTGAGCTCTCTCTTTGGCCTAGCTAGCTGCTA    1080
Mutant      GCTAGCTAGCTCGCTGGGTGTACGTACAGTGAGCTCTCTCTTTGGCCTAGCTAGCTGCTA    1080
            ************   *****  **  ********************************

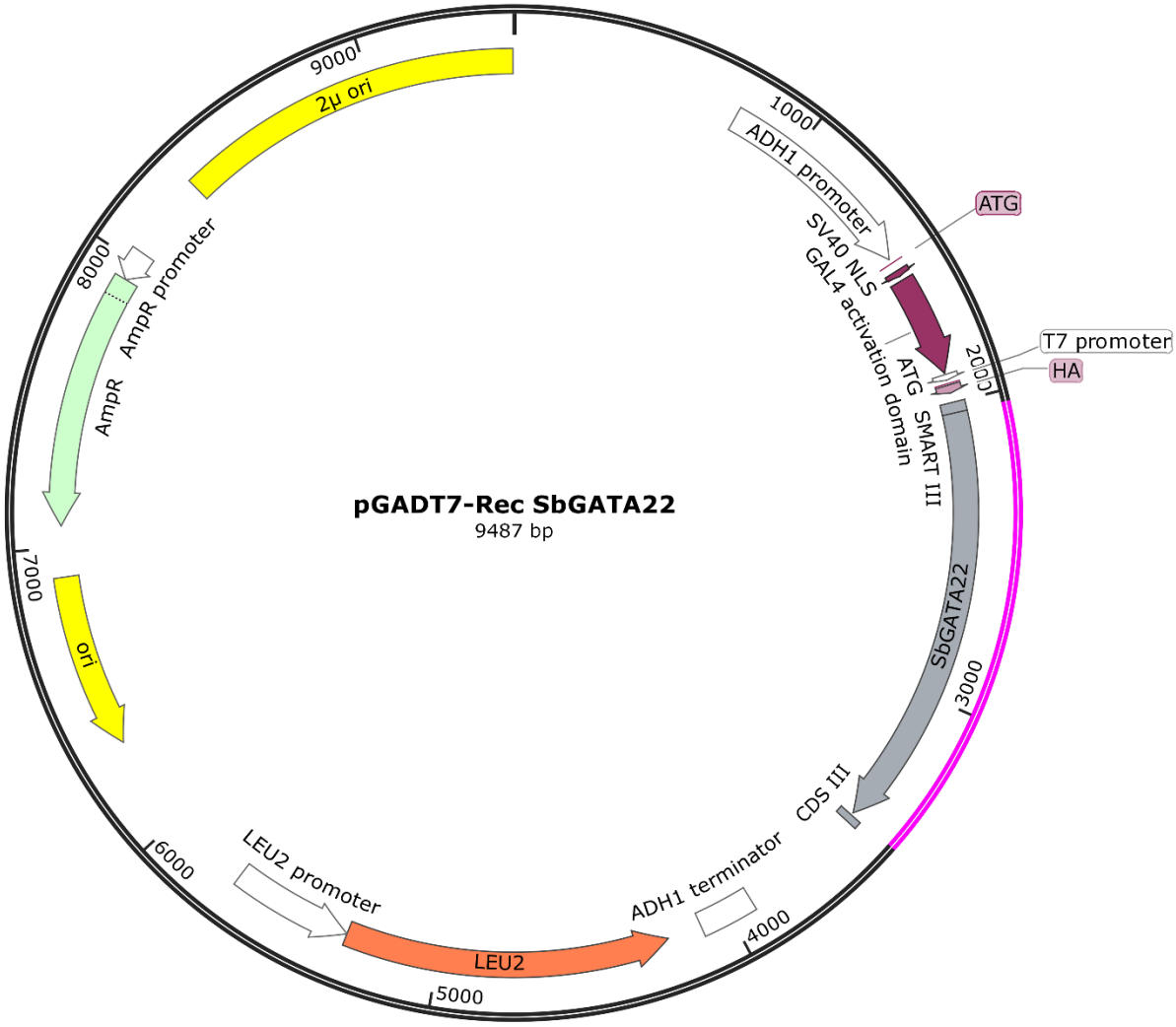
Native      GCAGTGCAGGTAGCCAATCAAAGCAGAAGAACTCGATCGATCGATCATCACGATCGCTGC    1140
Mutant      GCAGTGCAGGTAGCCAGCTAAAGCAGAAGAACTCGTACGTACGTACGCTACGTACGCTGC    1140
            ****************   ****************  **  **  *   ***  ******

Native      TAGCTAGCTAGCTGCTCGCTCTCACACTAGCTACGTGTTTTTGTTAATTTGATATATATA    1200
Mutant      TAGCTAGCTAGCTGCTCGCTCTCACACTAGCTACGTGTTTTTGTTAATTTGTAATATATA    1200
            ************************************************  *******
```

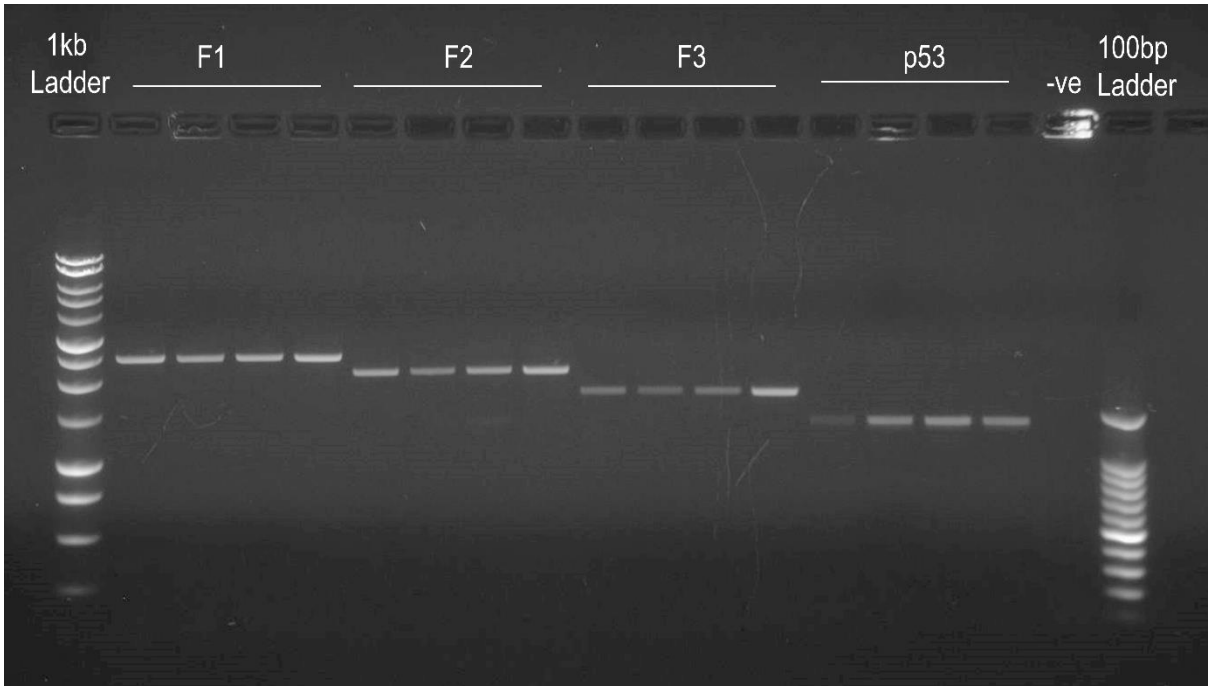
Supplementary Figure 3: Native and mutant promoter "bait" fragments of *CYP79A1* (*Sb01g001200*) cloned into pUC57 and then into pAbAi. The mutated promoter fragment had all putative core GATA transcription binding sites changed from GAT to GTA on both the forward and reverse strands. Putative binding sites were identified using PlantPAN 2.0 (Chow et al., 2015).



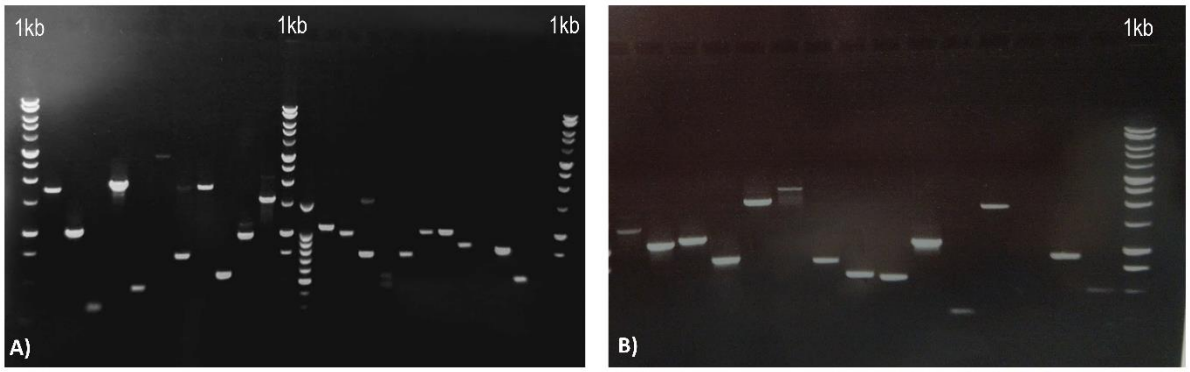
Supplementary Figure 4: feature maps of pUC57 vectors containing the Native and Mutant "bait" fragments synthesised by GenScript (USA). Mutant vectors had all 5' GAT 3' sites altered to 5' GTA 3' (Supplementary Data 2). pUC57 vectors were digested with KpnI and SalI and the promoter fragments inserted into pAbAi. pAbAi-Native and pAbAi-Mutant were transformed into the *S. cerevisiae* Y1H Gold strain where they were stably integrated into the yeast genome allowing growth in the absence of uracil.



Supplementary Figure 5: feature map of the pGADT7-Rec vector containing the *SbGATA22* "prey" cDNA insert downstream of the GAL4 activation domain. pGADT7-Rec *Sb*GATA22 was transformed into the Y1H Gold AbAi-Mutant and AbAi-Native strains to confirm the interaction between *Sb*GATA22 and the *CYP79A1* promoter region.



Supplementary Figure 6: Integration of the *CYP79A1* bait fragments confirmed by colony PCR. Four colonies of each bait strain were randomly selected for colony PCR. Gel shows the *CYP79A1* bait fragments F1, F2, F3, and the positive control p53 vector had stably integrated into the *S. cerevisiae* Y1H Gold genome. Expected band size equalled 1.4kb + bait fragment size (F1 = 1177bp; F2 = 843bp; F3 = 460bp) and 1.35kb for the control strain. An untransformed Y1H Gold colony was used as a negative control.



Supplementary Figure 7: Yeast colony PCR of strains displaying a positive interaction between the cDNA insert and the *CYP79A1* promoter bait fragment on SD/-Leu/AbA^{100} media for **A)** Screen 1 and **B)** Screen 2. Colonies were subjected to yeast colony PCR to observe variations in vector insert sizes. Each lane contains an amplified insert from a single yeast colony. The lanes labelled 1kb contain molecular weight markers.

```
                                                      M  S  A  I  Y  M
   1 GGCGCAAGCAAATTCCTGATCTCATCAGTCCCTATAGCTCCCATGTCTGCCATCTACATG

     S  Q  L  S  T  A  L  P  L  M  E  G  D  H  H  H  H  H  H  H
  61 AGCCAGCTCTCCACTGCTCTCCCTCTCATGGAGGGAGACCACCACCACCACCACCACCAC

     H  H  Q  G  H  F  Q  A  F  T  L  P  K  D  P  P  I  L  F  P
 121 CACCACCAAGGCCACTTCCAAGCCTTCACGCTGCCAAAGGATCCCCCAATCCTTTTCCCC

     F  V  I  S  N  S  S  A  S  E  S  S  L  S  Y  G  S  A  D  H
 181 TTTGTGATCAGCAATAGTAGCGCCAGCGAAAGCAGTCTGAGCTATGGATCGGCAGATCAT

     H  L  L  R  Q  H  R  Q  T  M  L  E  P  Q  H  M  I  G  G  S
 241 CACTTGTTGAGGCAGCATCGTCAAACTATGCTCGAGCCCCAACATATGATTGGTGGATCG

     S  S  A  A  S  S  V  F  A  T  P  F  P  T  V  E  S  I  R  D
 301 TCATCAGCTGCGAGTAGTGTCTTTGCGACGCCGTTCCCGACTGTGGAGAGCATCCGTGAC

     D  M  I  E  P  A  S  Y  D  P  Y  D  M  G  K  L  H  Q  V  V
 361 GACATGATCGAGCCTGCCTCGTACGATCCATACGATATGGGGAAGCTGCACCAGGTGGTC

     G  G  G  S  S  M  D  A  C  S  W  T  P  P  A  A  K  M  R  I
 421 GGTGGCGGGTCGTCGATGGATGCTTGCAGCTGGACGCCGCCGGCGGCCAAGATGAGGATC

     T  R  K  A  T  A  A  D  P  S  G  A  G  K  K  P  R  R  R  A
 481 ACGAGGAAGGCCACTGCCGCCGATCCCAGTGGTGCCGGGAAGAAGCCGAGGAGAAGGGCG

     H  Q  A  A  G  Y  D  A  D  I  N  M  S  G  Q  P  N  L  G  V
 541 CATCAGGCAGCAGGGTACGACGCCGACATCAACATGAGCGGCCAACCAAATTTGGGTGTT

     I  R  V  C  S  D  C  N  T  T  K  T  P  L  W  R  S  G  P  C
 601 ATTAGGGTGTGCTCCGACTGCAACACCACCAAGACGCCCTTGTGGAGGAGTGGTCCTTGC

     G  P  K  S  L  C  N  A  C  G  I  R  Q  R  K  A  R  R  A  M
 661 GGCCCCAAGTCGCTCTGCAACGCGTGCGGTATCAGGCAGCGGAAGGCACGGCGGGCGATG

     M  A  A  A  S  T  S  G  P  A  A  V  P  A  T  D  S  D  K  A
 721 ATGGCCGCCGCGTCTACCTCCGGGCCAGCAGCAGTGCCCGCCACCGACAGCGATAAGGCC

     S  P  S  N  A  A  G  A  A  A  A  H  P  K  V  K  K  E  K  R
 781 TCGCCGAGCAACGCCGCCGGGGCCGCAGCAGCACACCCCAAGGTGAAGAAGGAGAAGAGA

     S  V  D  V  D  R  S  L  P  F  K  K  R  C  K  V  V  Q  V  Q
 841 TCAGTGGACGTGGACCGGTCGCTGCCGTTCAAGAAACGGTGCAAGGTGGTCCAGGTCCAG

     Q  D  H  A  A  A  V  A  A  P  A  A  A  T  D  R  P  A  V  V
 901 CAGGATCACGCTGCGGCCGTCGCAGCTCCTGCCGCGGCCACTGACAGGCCGGCTGTCGTC

     V  Q  Q  A  A  T  A  A  E  V  G  D  D  D  A  C  P  S  R  D
 961 GTGCAGCAGGCGGCCACCGCCGCCGAGGTTGGTGACGACGACGCCTGTCCGAGCAGGGAC

     L  L  V  D  D  I  G  G  L  I  S  W  S  R  S  P  P  A  A  P
1021 CTGCTTGTCGACGACATTGGTGGGCTCATCAGCTGGAGCAGGAGTCCGCCGGCGGCTCCC

     A  S  A  D  A  A  S  C  S  F  R  A  S  P  A  L  P  V  Q  Q
1081 GCATCTGCAGATGCTGCCTCCTGCAGTTTCCGGGCGTCGCCGGCGTTGCCGGTGCAGCAG

     D  E  I  T  D  A  A  M  L  L  M  T  L  S  C  G  L  V  R  S
1141 GACGAGATCACGGACGCTGCCATGCTGCTCATGACGCTGTCCTGCGGGCTTGTCCGAAGC
```

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     *
1201 TGATCGACGATCGATCTACCTCTACAAAGGCCCCAGCACCATAGGCTTTTTGCAGCTAGC

1261 TACCTCTAGCTAGTGCCTTTGACTGTTAGTGTTACTGTTAGTGTTGTTACCATCGTCGTT

1321 TACCCTACGACCTGTAGTTTTTTTTATTTGGCTTCTTGTTCTTGTTGCCAGTGTAACCCG

1381 TGTCACTGTTCAAGTCATGTGATTCGTTTGAGAAAAAAAAAAAAAAAAAAAAAAA
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Supplementary Figure 8: Amino acid sequence of *Sb*GATA22 aligned to the nucleotide insert sequence from Screen 1. The nucleotide sequence is 1435bp long, which includes 41bp of the 5' untranslated region and 234bp of the 3' untranslated region including the polyA tail (*italics*). The open reading frame (ORF) encodes a 386 amino acid polypeptide, the putative stop codon is indicated (*).

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5′ GCATACCGATCGATGCGAAAAAAAAACCCAGGAGACTCTCGAAGATCTACAGATTAAATC      -1145
3′ CGTATGGCTAGCTACGCTTTTTTTTTGGGTCCTCTGAGAGCTTCTAGATGTCTAATTTAG

5′ TAAGTTTCCACGGTCCAATATGCCGATTGGTAAGTTAGAACGAAGTAGGGAGAGAGAAAA      -1085
3′ ATTCAAAGGTGCCAGGTTATACGGCTAACCATTCAATCTTGCTTCATCCCTCTCTCTTTT

5′ AAAACCATAGGGAGGAGGAAGACAAGAAAAAAGACTCGGGCAGGAAGAGTGTATGCTGGT      -1025
3′ TTTTGGTATCCCTCCTCCTTCTGTTCTTTTTTCTGAGCCCGTCCTTCTCACATACGACCA

5′ TTAAAATAGGAGCTCGGTGTCTTAGATCATGGTGCCAAATTTGACGCCAATGGTAATCGC      -965
3′ AATTTTATCCTCGAGCCACAGAATCTAGTACCACGGTTTAAACTGCGGTTACCATTAGCG

5′ GTCGAGGTCTCTGCCACGTCAACGCCATGTCTGCGTCAACACCAACATGGCACAACGACC      -905
3′ CAGCTCCAGAGACGGTGCAGTTGCGGTACAGACGCAGTTGTGGTTGTACCGTGTTGCTGG

5′ TTGGCGCCTAATTGGGCTGGCGCCGAGATGTGTGACCTTGGCGGCAATTACAACGGCGCT      -845
3′ AACCGCGGATTAACCCGACCGCGGCTCTACACACTGGAACCGCCGTTAATGTTGCCGCGA

5′ GAGCAGAGGGTCTAGATTTTGAAAGCATACCTACCGGGACAGATTATGAAAATCTTTTA      -785
3′ CTCGTCTCCCAGATCTAAAACTTTCGTATGGATGGCCCTGTCTAAATACTTTTAGAAAAT

5′ AAAAAAAGAAGCAAAAAAACAAAAAAAAATGCCAGACATGCGGTGGCTCTTTGGCACGCC      -725
3′ TTTTTTTCTTCGTTTTTTTGTTTTTTTTTACGGTCTGTACGCCACCGAGAAACCGTGCGG

5′ TGCGTGACACCCCTCCCTTCATGCACGCCGACAAGCAGACAGAAAAAGCCTTGGTGAGAG      -665
3′ ACGCACTGTGGGGAGGGAAGTACGTGCGGCTGTTCGTCTGTCTTTTTCGGAACCACTCTC

5′ AAAGGGGATGACATGGGCCCACATGTATGAGTGAGGGAGAGGGCAGTCGTTGCGGTATTT      -605
3′ TTTCCCCTACTGTACCCGGGTGTACATACTCACTCCCTCTCCCGTCAGCAACGCCATAAA

5′ TGGGTAATGTGAAAATACAACAATCTGGTTCCCATCTTGTTTCAAAGGTAGAAAATGGCA      -545
3′ ACCCATTACACTTTTATGTTGTTAGACCAAGGGTAGAACAAAGTTTCCATCTTTTACCGT

5′ TGGATCAAGTATAGAAACAATTGTATTGGCAAGTTTCCAAATACATGAATAGTAATAAGT      -485
3′ ACCTAGTTCATATCTTTGTTAACATAACCGTTCAAAGGTTTATGTACTTATCATTATTCA

5′ GTGAAAATTATAATGGCATGGTTCAAATAAACCCCAAAAATATTGTTAGTATTGTAATAA      -425
3′ CACTTTTAATATTACCGTACCAAGTTTATTTGGGGTTTTTATAACAATCATAACATTATT

5′ TAAAGAAAACCAAAACGGTTCGGACTGGGCTTGTCCCCTCAACACTATCGTCATGGGTAG      -365
3′ ATTTCTTTTGGTTTTGCCAAGCCTGACCCGAACAGGGGAGTTGTGATAGCAGTACCCATC

5′ CGCACACAGCATGATGTGCTCGATCATTCTGAGCTCCCACATCATGTGCATGCTGCATGG      -305
3′ GCGTGTGTCGTACTACACGAGCTAGTAAGACTCGAGGGTGTAGTACACGTACGACGTACC

5′ TCGATGCGCTGCACACAGTTAGCTGTGGGACTGTGCATGCACGCGTGGTTGGTGGCGATC      -245
3′ AGCTACGCGACGTGTGTCAATCGACACCCTGACACGTACGTGCGCACCAACCACCGCTAG

5′ CAGCATCCCATAAATACCTGCGAACCGCCGTGTGTTCTGCCTCAAGCAGGAGCACATACA      -185
3′ GTCGTAGGGTATTTATGGACGCTTGGCGGCACACAAGACGGAGTTCGTCCTCGTGTATGT

5′ GCTAGCTAGCTCATCGGGTGATCGATCAGTGAGCTCTCTCTTTGGCCTAGCTAGCTGCTA      -125
3′ CGATCGATCGAGTAGCCCACTAGCTAGTCACTCGAGAGAGAAACCGGATCGATCGACGAT

5′ GCAGTGCAGGTAGCCAATCAAAGCAGAAGAACTCGATCGATCGATCATCACGATCGCTGC      -65
3′ CGTCACGTCCATCGGTTAGTTTCGTCTTCTTGAGCTAGCTAGCTAGTAGTGCTAGCGACG

5′ TAGCTAGCTAGCTGCTCGCTCTCACACTAGCTACGTGTTTTTGTTAATTTGATATATATA      -5
3′ ATCGATCGATCGACGAGCGAGAGTGTGATCGATGCACAAAAACAATTAAACTATATATAT

5′ TATA                                                               -1
3′ ATAT
```

Supplementary Figure 9: Core GATA transcription binding motifs: 5’ GAT 3’ and core AP2/B3 transcription factor RY binding motifs: 5’ CATGC 3’ present on both forward and reverse strands of the *CYP79A1* promoter region identified using PlantPAN 2.0 (Chow et al., 2015).

Supplementary Table 1: Primers used to amplify *CYP79A1* "bait" fragments from *Sorghum bicolor* L. Moench line BTx623 genomic DNA. Forward and reverse primers contain a HindIII and SalI restriction site, respectively.

Primer Name	**Primer Sequence (5' – 3')**	**Tm**	**Size (bp)**
F1 Forward	TTACCCAAGCTTGCATACCGATCGATGCG	64	1177
F2 Forward	TTACCCAAGCTTACCTTGGCGGCAATTACA	64	843
F3 Forward	TTACCCAAGCTTAGTGTGAAAATTATAATGGCATGG	64	458
F1-3 Reverse	TTACGCGTCGACCACGTAGCTAGTGTGAGAGCG	64	

Supplementary Table 2: Primers used in qPCR

Primer Name	**Primer Sequence (5' – 3')**	**Tm**	**Size (bp)**
CYP79A1 F	CATGCTCTTCGGAAGGCTCCT	66	162
CYP79A1 R	ATGGAGATGGACGGGTAGAGGT		
SbGATA22 F	TGACGACGACGCCTGTCCGA	66	179
SbGATA22 R	TGAGCAGCATGGCAGCGTCC		
AP2/B3-Like F	TGCTGAGGCTGGTCTTCCACCAT	66	181
AP2/B3-Like R	ATCCCCTGCCTGAAGGCCATGT		
NR F	CCCATGTACCAGGTCATCCA	63	172
NR R	CCTGGTCGATCACGTACCAC		
Ubiquitin F	CGGAGGAGCTCTATGCCACA	66	122
Ubiquitin R	AAGACGCTCCACTGCAGCAT		

Chapter 5

Conclusions and future directions

5.0 General discussion

As a consequence of climate change and the rise in the global demand for food there is an urgent requirement to increase the resilience of crops to biotic and abiotic stresses, or alternatively, to increase the reliance on crop species that already harbour these traits (Zougmoré et al., 2016, Hadebe et al., 2017). *Sorghum bicolor* (L.) Moench is one alternative to grains such as maize due to its superior drought and heat tolerance, and its ability to grow on marginal lands (Getachew et al., 2016). Climate change also increases the risk of crop toxicity, particularly in cyanogenic species such as sorghum and cassava, where increases in cyanogenic glucoside concentrations can result in chronic or acute cyanide poisoning in people and animals (Kashala-Abotnes et al., 2018, Brown et al., 2016, Wheeler and Mulcahy, 1989).

The biochemical pathway of cyanogenic glucoside production is well defined (Gleadow and Møller, 2014). However, few studies have investigated the interplay between developmental and environmental regulation across all vegetative tissues, with even less being known about the molecular regulation of the underlying regulatory pathways. This thesis presents original discoveries regarding the environmental, developmental, and molecular regulation of the cyanogenic glucoside dhurrin in sorghum, with the aim that elucidation of dhurrin regulation may i) ultimately improve the ability to predict toxicity so that consumption can be reduced when risks of poisoning are high, or ii) allow for hydrogen cyanide potential to be manipulated in ways that do not reduce crop yield or stress tolerance.

5.1 Drought response of the low-cyanogenic mutant *acdc1*

Drought stress is known to affect dhurrin concentration in the shoot tissue of sorghum, although whether concentrations increase or decrease depends on the length and severity of the stress (Wheeler et al., 1990, Neilson et al., 2015, Gleadow et al., 2016, Emendack et al., 2018). The inability to accurately predict the hydrogen cyanide potential of sorghum forage following periods of low-rainfall is known to result in pasture wastage and cyanide poisoning in livestock (Stuart, 2002). Removing or reducing dhurrin would alleviate this problem, but since dhurrin is involved in nitrogen remobilisation and stress mitigation, it is possible that doing so may affect growth rate

and stress tolerance (Jørgensen et al., 2005, Bjarnholt et al., 2018, Selmar et al., 1988, Møller, 2010).

In chapter 2, the aim was to i) characterise the response of the low-cyanogenic sorghum mutant *adult cyanide deficient class 1* (*acdc1*) to drought; ii) investigate whether altering dhurrin concentration affects nitrate concentration; and iii) examine whether dhurrin regulation in response to drought differs between above and below ground tissues. Analysing hydrogen cyanide potential (HCNp) over four successive harvests also enabled the relationship between developmental and environmental regulation of dhurrin to be examined.

The experimental evidence presented in Chapter 2 demonstrated that ontogeny is the principal driver of HCNp in the early stages of sorghum development, as drought did not elicit an increase in dhurrin concentration until plants were over three weeks old. The inability of an abiotic factor to alter dhurrin concentration in young plants has also been observed in relation to nitrogen application (Busk and Møller, 2002, Blomstedt et al., 2018). This highlights the importance of longer-term sorghum studies, as the literature often reports research undertaken during the early stages of sorghum development, including on plants less than two weeks old (Bhargava and Paranjpe, 2004, Buchanan et al., 2005, Salzman et al., 2005). This may result in environmental responses going unnoticed.

The *acdc1* mutants display a desired phenotype as all tissues are cyanogenic when the plants are young, before the leaf tissue becomes acyanogenic later in development (Blomstedt et al., 2012, Blomstedt et al., 2018). This enables dhurrin to be used for nitrogen remobilisation and stress mitigation when growth rate is at its highest, before the leaf-tissue becomes non-toxic when plants are ready to be grazed. High nitrogen conditions are known to increase dhurrin concentrations in the *acdc1* to the same extent as wild-type plants (Blomstedt et al., 2018). Here, drought also increased HCNp in all tissues of the *acdc1* mutant to the same extent as wild-type plants; thereby showing that the mutation affecting the developmental regulation of HCNp in the *acdc1* mutant does not influence the environmental regulation of dhurrin, at least in relation to both nitrogen application and drought stress.

Environmental conditions that limit plant growth are associated with high HCNp in sorghum (O'Donnell et al., 2013, Neilson et al., 2015, Gleadow et al., 2016). This has been attributed to a concentration effect, where higher dhurrin concentrations are due to reduced plant growth rather than *de novo* production of the specialised metabolite. In this study it was found that drought stressed plants had a lower total dhurrin content compared to non-stressed plants, suggesting the higher HCNp observed at the final harvest is attributable to a concentration effect. Interestingly, overall dhurrin content was higher at the final harvest for all tissues and watering levels than previous harvests, demonstrating that *de novo* dhurrin production continues throughout development.

My research indicates that dhurrin is regulated independently between above and below ground tissues. While drought increased HCNp in the shoot tissue, the opposite pattern was observed in the root tissue, consistent with previous studies (O'Donnell et al., 2013). Few sorghum studies have analysed root tissues, and the only previous study that reports CYP79A1 activity found no active enzyme present in the roots (Busk and Møller, 2002); it is therefore possible that transport of dhurrin from the shoot to the roots is occurring and that this transport is altered by drought stress, as seen in cassava (Brown et al., 2016). Selmar et al. (1996) proposed that dhurrin transport is occurring in sorghum due to the presence of the diglucoside dhurrin-6'glucoside in the leaves, although root tissues were not mentioned in this study. The transport of dhurrin in sorghum is an area requiring further investigation, particularly as cyanogenic glucoside transport is known to occur in other species like *Hevea brasiliensis* (Selmar et al., 1988, Selmar et al., 1987) and may have implications for crops with edible underground storage organs, such as cassava and taro.

Few studies have analysed both nitrate and dhurrin concentrations in all vegetative tissues of sorghum. It was found that nitrate concentrations were higher in the *acdc1* mutant compared to wild-type plants when water was both limited and replete, suggesting that altering sorghum HCNp will, in turn, lead to an increase in nitrate concentration. For sorghum research it is imperative that nitrate concentration is analysed in parallel to dhurrin, as replacing dhurrin toxicity with nitrate toxicity is futile. Chapter 2 also demonstrated that mutant phenotypes which are desirable in the

absence of stress can become equally or more toxic than wild-type lines when stress is applied.

5.2 Epigenetic regulation of cyanogenic glucosides

The dhurrin biosynthetic genes are clustered on chromosome one in sorghum (Darbani et al., 2016, Takos et al., 2011), similar to the cyanogenic glucoside biosynthetic gene clusters in cassava and bird's foot trefoil (*Lotus japonicus*) (Takos et al. 2011). Gene clusters are thought to have evolved by gene duplication, followed by acquisition of new functions and typically contain 3-6 genes key to the biosynthetic pathway of the specific metabolite (Nützmann and Osbourn, 2014). Clustering opens up the possibility of co-regulation at the chromosomal level through DNA methylation or chromatin modification, though knowledge of the molecular regulation of the cyanogenic glucoside biosynthetic gene clusters is lacking. Epigenetic marks are known to be involved in the regulation of other secondary metabolites including the triterpenes thalianol in *Arabidopsis* and avenacin in oats (Nützmann and Osbourn, 2014).

In Chapter 3 experimental support was presented for cyanogenic glucosides being at least partially controlled via epigenetic mechanisms. Demethylation of the sorghum genome in germinating seeds resulted in significantly higher expression of *CYP79A1* in plants up to two weeks old, despite their developmental stage being equivalent to control plants. As methylation is associated with gene silencing (Cao and Jacobsen, 2002), increased *CYP79A1* expression would be expected in a demethylated genome if in fact expression was being regulated via this mechanism. This was in contrast to the *nitrate reductase* where expression did not change in response to genome-wide demethylation.

Epigenetics challenges the traditional idea of transcription factors and "master regulators" as the primary drivers of gene expression, and instead incorporates dynamic changes in epigenetic marks and chromatin structure into gene regulation. In cyanogenic plant species, cyanogenic glucoside concentration can change dramatically over the course of development, and also during much shorter time periods such as the course of the day, or in response to environmental factors (Gleadow and Møller, 2014,

Schmidt et al., 2018). Histone modifications and DNA methylation patterns are also known to change gradually during development or rapidly in response to biotic and abiotic stresses (Kim et al., 2015), and it is possible that these are the predominant molecular mechanisms underlying the regulation of cyanogenic glucoside biosynthesis in sorghum and other cyanogenic species.

In Chapter 3 the *acdc* mutation was characterised, where it was found that mutants possess a CΔT change in the *CYP79A1* promoter region, 1188bp upstream from the transcription start site. The CΔT mutation is stably inherited and segregates with the *acdc* phenotype. Crosses between *acdc1* and the acyanogenic mutant *totally cyanide deficient 1* (*tcd1*) that has a mutation in the *CYP79A1* coding region (Blomstedt et al., 2012) revealed that the lines are not complementary, and confirmed that the cyanogenic phenotype of the *acdc1* is due, or strongly linked, to the CΔT mutation in the promoter region.

Besides the removal of the methylation site seen in the *acdc1*, no other difference in methylation in the region surrounding this mutation was seen between lines, tissues, or developmental stages. It is possible that an unidentified mutation is tightly linked to the CΔT site and is influencing *CYP79A1* regulation, or that the CΔT mutation itself interrupts a *cis*-regulatory motif, altering the binding of a regulatory element and altering *CYP79A1* gene expression in the *acdc1* leaf tissue. This idea led to the research undertaken in the following chapter.

5.3 Candidate regulators of *CYP79A1* expression

Altering the cyanogenic glucoside potential of crop species has often focussed on mutating or silencing biosynthetic genes (Jørgensen et al., 2005, Blomstedt et al., 2012, Blomstedt et al., 2016). This has produced plants that are completely acyanogenic and pose no risk of cyanide toxicity such as the *totally cyanide deficient 1* and *totally cyanide deficient 2* mutants (Blomstedt et al., 2012, Blomstedt et al., 2016), and plants with reduced cyanogenic potential including the sorghum *adult cyanide deficient 1* (Blomstedt et al., 2012), and transgenic cassava (Jørgensen et al., 2005). However, this has resulted in undesirable effects including severe reductions in growth rate

(Jørgensen et al., 2005, Blomstedt et al., 2016) and high nitrate concentrations (Blomstedt et al., 2018). The *acdc1* mutant phenotype, though desirable when abiotic stresses are absent, has equivalent cyanogenic glucoside toxicity to wild-type lines under drought, and also higher nitrate toxicity in the leaf tissue (Chapter 2).

Identifying the molecular mechanisms regulating cyanogenic glucoside biosynthetic genes may provide novel pathways for the manipulation of plant hydrogen cyanide potential. For example, maintaining developmental patterns of dhurrin production, while repressing dhurrin induction in response to nitrogen application, would enable sorghum pastures to be fertilised without the risk of dhurrin increasing to toxic concentrations.

In Chapter 4 the first candidate regulators of *CYP79A1* expression were identified. This is the first report of transcription factors linked to the regulation of cyanogenic glucosides in any species. The utilisation of yeast one-hybrid screens allowed for the identification of proteins that bind to the *CYP79A1* promoter region without any prior knowledge of those proteins. This provides an advantage over methods such as ChIP-Seq. Both transcription factors identified, *Sb*GATA22 and AP2/B3-Like, are homologous to proteins that act in light and nitrogen signalling, and responses to drought stress in other species (Bedi et al., 2016, Gupta et al., 2017, Klermund et al., 2016, Ranftl et al., 2016). These are regulatory networks that are known to influence dhurrin regulation in sorghum.

The characterisation of GATA transcription factors in monocots, particularly C_4 grasses, requires a great amount of further research. The extent of GATA functional conservation between plant species is largely unknown. In monocots most research has been undertaken in rice (*Oryza sativa*); a non-cyanogenic, C_3 grass species (Gupta et al., 2017, He et al., 2018). My research is the first to suggest that GATA transcription factors play a role in cyanogenic glucoside regulation in sorghum, a function that may also be present in other cyanogenic species.

The research in Chapter 4 found that *Sb*GATA22 is recognising and binding to the putative GATA transcription factor *cis*-binding motifs in the *CYP79A1* promoter region

in vivo and is likely to be a genuine interaction *in planta*. It can be speculated that *Sb*GATA22 may act as a transcriptional repressor of *CYP79A1* in sorghum, as the transcription profile of *SbGATA22* during development and in response to KNO_3 is reflective of a repressor more so than an activator. However, this cannot be confirmed from my research. Furthermore, as *Sb*GATA22 is likely to be involved in more than one regulatory pathway, its expression profile in relation to *CYP79A1* would be confounded. GATA transcription factors interact with co-regulators in animals (Robert et al., 2002), and preliminary data suggests they also interact with other proteins in plants (Causier et al., 2012, Bowen et al., 2010). It is possible that the binding targets of *Sb*GATA22 change in sorghum depending on its binding partners, with co-regulators also affecting whether it activates or represses downstream target genes.

5.4 Conclusions and future directions

The research presented in this thesis opens new avenues for the exploration of cyanogenic glucoside regulation. Chapter 3 suggests that cytosine methylation is involved in the regulation of the dhurrin biosynthetic genes in sorghum. The methylome of *Sorghum bicolor* has been published (Olson et al., 2014), but this research only presents the genome-wide methylation pattern at one developmental time-point in a single tissue. Analysing cytosine methylation over developmental time and in relation to environmental factors would be highly informative. Olson et al. (2014) did not report any information on histone modifications. Given that histone modifications are involved in the regulation of other secondary metabolites (Nützmann and Osbourn, 2014), research into both histone modifications and chromatin structure of the dhurrin biosynthetic gene cluster, and how these change throughout the course of development and in response to environmental stress, warrants further investigation.

An important step to further the work presented here is to confirm the binding of *Sb*GATA22 to the *CYP79A1* promoter region *in planta*; and to classify *Sb*GATA22 as either an activator or repressor of *CYP79A1* gene expression. The characterisation of plant GATA transcription factors generally occurs via gene over-expression (Klermund et al., 2016, Ohnishi et al., 2018) as due to the redundancy of GATA genes, gene knock-outs often fail to produce a mutant phenotype. This is likely to occur in sorghum, as 40 putative GATA transcription factor genes have been identified in the species (Jin et al.,

2016). Transient expression of *SbGATA22* in species such as *Nicotiana* or *Arabidopsis* can be used to confirm binding *in planta*; however, these species are not cyanogenic and therefore expression of *SbGATA22* will not reveal whether cyanogenic glucoside production is increased or decreased.

Monocot transformations are still not as routinely successful as dicot transformations. This is true of sorghum, where efficient transformations have proven difficult. However, as this technology continues to rapidly improve (Che et al., 2018, Ahmed et al., 2018), over-expression of *SbGATA22* in *Sorghum bicolor* tissues may be feasible. The discrepancy between root and shoot hydrogen cyanide potential in sorghum, coupled with the fact that *SbGATA22* is typically only expressed in the shoots, presents a unique situation to study the function of this GATA transcription factor. If *Sb*GATA22 is acting as a repressor, shoot HCNp should decrease in response to *Sb*GATA22 over-expression, while if it is an activator, root expression of *CYP79A1* should increase.

Now that candidate regulators of *CYP79A1* have been identified, methods such as ChIP-Seq and RNA-Seq are obvious tools to determine where *Sb*GATA22 and AP2/B3-Like are binding throughout the sorghum genome, and how their expression correlates to other genes of interest. Utilising yeast two-hybrid screens to identify protein-protein interactions (Bartel and Fields, 1995) would also be beneficial in detecting potential binding partners of *Sb*GATA22.

The functions of cyanogenic glucosides in roles other than plant defence continue to emerge, as do the biotic and abiotic factors by which they are influenced. The relationship between development, environment and plant hydrogen cyanide potential are complex and likely to involve both active and passive regulation. The results from this thesis give an indication of the role of cytosine methylation in cyanogenic glucoside regulation and identify the first candidate regulators of *CYP79A1*. Further work in this area will continue to reveal the molecular mechanisms governing expression of the dhurrin biosynthetic genes in sorghum which, in turn, may help elucidate these mechanisms in other cyanogenic species. Though in its infancy, this research may enable new approaches for the manipulation of cyanogenesis in crop plants; approaches that do not rely on the direct mutation or silencing of the cyanogenic glucoside

biosynthetic genes. Ultimately this may allow for the production of non-toxic, but resilient, crops that can maintain yield in a hotter, drier climate.

5.5 References

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