University of New England

Characterisation of Brassinosteroid Effects and Brassinosteroid-Responsive Genes in Cotton for Growth and Stress Tolerance Enhancement against Abiotic and Biotic Stresses

Submitted by:
Anahid A Essa Al-Amery

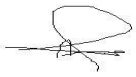
Submitted for the degree of Doctor of Philosophy

April 2020

Declaration by Author

This dissertation contains my original work and has no material previously published or written by another person except where due reference has been made. I have clearly stated the contribution of others in joint authorship and collective works in this dissertation including experimental procedures, data production and analysis, statistical assistance, professional editorial advice, and any other original research work reported in my dissertation. The content of my dissertation is the result of the work I have executed since the beginning of my PhD journey and does not include any work that has been submitted for any award in any tertiary institution.

I acknowledge that an electronic copy of my dissertation must be lodged in the University Library to be made available for research and study in accordance with the Copyright Act 1968 unless an embargo period has been approved by the Dean of the Graduate School and subject to the policy and procedures of The University of New England. I acknowledge that the copyright of all material contained in my thesis resides with the copyright holder(s) of that material. Where appropriate, I have obtained copyright permission from the copyright holder to reproduce material in this dissertation.



Signature

22 April 2020

Date

Acknowledgements

My four years of PhD journey has been very challenging, and I would never have come this far without the support of many individuals. First and foremost, I thank the Cotton Research and Development Corporation (CRDC) through the University of New England for providing me with scholarships and financial resources during my PhD. I also want to thank the Australian government for giving me the opportunity to go for a PhD as a domestic student.

I would like to thank my principal supervisor Dr. Heather Nonhebel for providing a stimulating learning environment, guidance and patience that helped me to stay on track and achieve my milestones. I thank my co-supervisors Dr. David Backhouse and Dr. Nigel Warwick who are guiding me with my experimental works. Additionally, I thank the Schenk Lab group, especially Professor Peer Schenk for approachability, personal help and smiles.

My family and friends have been the greatest support during my PhD. Special thanks must go to my Mom for her understanding, personal care and support during my PhD roller coaster ride. I wish to express my heartfelt gratitude to my aunties and siblings for having faith in me and showering me with unconditional love. I thank my friend Ainnatul Adawiyah Ahmad Termizi for encouraging me throughout this process. I thank my friend Hala Alzubeidi who inspired me to do my PhD in the first place.

My PhD would not have been possible without the support of these people and also others who indirectly helped me to achieve my PhD goals. I am deeply indebted to them; I say a big thank you for your wonderful presence in my PhD chapter.

Abstract

Brassinosteroids (BRs) are plant steroid hormones that not only play vital roles in plant growth and development, but also in mediating stress responses. A group of calmodulin-binding proteins, known as CBP60s are also involved in mediating the response of plants to stress. The aims of the present study were: (1) to investigate the effect of exogenous 24-epibrassinolide (EBR) on the phenotype of cotton (*Gossypium hirsutum)* seedlings under mild to moderate biotic and abiotic stresses, (2) to find and characterise cotton CBP60-encoding genes, orthologous to Arabidopsis CBP60s with known involvement in stress responses, and to investigate whether EBR may act by modulating the expression of *GhCBP60* genes in cotton leaf tissue under salt stress. Experiments were designed to demonstrate the effects of EBR application from 0.1 to 2 µM on the phenotypic responses of cotton seedlings to mild/moderate salt, drought and pathogen (*Verticillium dahliae*) stresses. Results show that the exogenous application of EBR at low concentrations of 0.1 and 0.2 µM had no positive effect on seedling growth under all stresses. In addition, EBR at a higher concentration (0.5 µM) or with the surfactant Tween 20 caused toxic effects. Bioinformatics approaches revealed the presence of GhCBP60 orthologues of AtCBP60. Phylogenetic analysis indicated that *CBP60a, CBP60g,* and *SARD1* from Arabidopsis each have four co-orthologues in cotton. *AtCBP60f* has two co-orthologues, whereas *CBP60b/c/d* have nine co-orthologues. Multiple amino acid sequence alignments indicate that the DNA-binding and CaM-binding domains of AtCBP60 are highly conserved in GhCBP60, suggesting similar protein structures to AtCBP60. Prediction of subcellular localisation suggested that all GhCBP60 proteins contain a nuclear localisation signal. This, together with the highly conserved putative DNA binding region, suggests that all GhCBP60 are transcription factors. The results of qRT-PCR demonstrated that EBR treatment of cotton up-regulated the expression of *GhCBP60a/f/g*. On the other hand, salt down-regulated the expression of *GhCBP60a* but up-regulated the expression of *GhCBP60f/g*. Interestingly, treatment with EBR in the absence of salt restored the expression of *GhCBP60a* to levels similar to the control tissue. Analysis of promoters of *GhCBP60* genes for putative BR-related transcription factor binding motifs indicated the presence of CANNTG and GGTCC elements. However, these were not significantly enriched in stress-regulated genes. Furthermore, higher stringency BR-signalling-related elements: BRRE (CGTGTG/CGTGCG), G-box (CACGTG) and transcription factors TGA 1/TGA4 (TGACG) sense strands were absent in stress-responsive genes *GhCBP60a/f/g* and *GhSARD1* as compared to other groups. In the light of

these results, I concluded that brassinosteroids (BRs) positively regulates the expression of novel *GhCBP60* genes suggesting a possible connection between BR signalling and GhCBP60 transcription factors in mediating abiotic stress responses in cotton. However, the results from the cis-element search suggest that this connection is likely to be indirect rather than via a direct interaction with the BR signal transduction pathway.

Contents

List of Tables

List of Figures

List of Abbreviations

ABA	abscisic Acid
APX	ascorbate peroxidase
BAK	BRI1-Associated Kinase
BES	BRI1-ems-suppressor
BES	BRI ems suppressor
BIN2	brassinosteroid insensitive
BKI	BRI1 Kinase Inhibitor
BL	brassinolide
BR	Brassinosteroid
BRI	Brassinosteroid Insensitive
BRP	brassinopride
BRZ	brassinazole
BSK	BR-signalling kinase
BSU	BRI1–suppressor
BZR	brassinazole-resistant
CAMTAs	calmodulin-binding transcription activators
CAT	catalase
Cd	cadmium
CMV	cucumber mosaic virus
CPD	constitutive photomorgenesis and dwarfism
DET	deetiolated
EBR	24-epibrassinolide
FHB	fusarium head blight
GA	gibberellin
GR	glutathione reductase
HBR	homobrassinolide
HS	Heat stress
HSPs	heat shock proteins
IAA	indole acetic acid
JA	jasmonic Acid
LRR	leucine-rich repeat
MT-sHSP	mitochondrial small heat shock protein
NPR	nonexpressor of pathogenesis-related genes
PAMPs	pathogen-associated molecular patterns
POX	peroxidase
RGA	repressor of ga1-3
RLK	receptor-like kinase protein
SOD	superoxide dismutase activity

Chapter 1. The Involvement of Brassinosteroid and Calmodulin-Binding Proteins in Abiotic and Biotic Stresses in Cotton *G. hirsutum*

1.1 Introduction

1.1.1 Introduction to the cotton industry and research

This chapter focuses on the industry's view of Australian cotton production relative to the existing challenges of drought, salinity and disease. This dissertation is a key reference tool to Australian cotton growers and was fully funded by the Cotton Research and Development Corporation (CRDC) under a crop protection strategy program.

1.1.2 Cotton: The world's most important fibre crop

The world's most popular natural fibre is cotton, with over 90% of annual global cotton production dominated by one species, *Gossypium hirsutum*, also known as Upland cotton (Wendel & Cronn, 2003). The unique characteristics of cotton fibre were discovered by ancient human cultures in both the Old and New Worlds, leading to the widespread domestication of cotton especially for textiles (Wendel & Cronn, 2003). The domestication process involved four species, two from Africa–Asia, namely *Gossypium herbaceum* (*G. herbaceum*) and *Gossypium arboreum* (*G. arboreum*) and the other two from the Americas, *Gossypium barbadense* (*G. barbadense*) and *Gossypium hirsutum* (*G. hirsutum*) (Wendel & Cronn, 2003). Cotton is harvested as 'seed cotton' which is later 'ginned' to separate the seed from the lint fibre. This is then spun to produce yarn that is woven into fabrics (Office of the Gene Technology Regulator, 2002). From 100kg seed cotton, 35kg of fibre can be extracted (Bremen Cotton Exchange, 2015). Worldwide, the cotton genus (*Gossypium*) has 51 species that are spread from arid to semi-arid regions of the tropic and subtropics. Out of these 51 species, 46 species are diploid (2n = 2x = 26) and five species are allopolyploids (2n = 4x = 52) (Fryxell,1992). Gossypium allopolyploids are the result of hybridisation between two diploid species (Cronn et al., 2002; Seelanan et al., 1997; Wendel 1989). For instance, *G. raimondii* and *G. arboretum* are the putative ancestral species of *G. hirsutum* (Li et al., 2015) and the sources of the D and A genomes, respectively. *G. hirsutum* was introduced to Australia from

its domesticated origin, Mexico (Brubaker, Bourland, & Wendel, 1999) and constitutes the majority of the cotton planted in Australia (99%), mostly in northern New South Wales and Queensland (Office of the Gene Technology Regulator, 2002). The timing of Australia's cotton cultivation lasts for about six months, starting between August to November (soil preparation) and ending between March to June (picking), depending on the region and climate (Cotton Australia, 2018). In 1996, genetically modified (GM) insect-resistant cotton varieties were introduced and 16 years later, GM varieties now represent almost 100% of cotton grown across Australia (Agricultural Biotechnology Council of Australia, 2012).

1.2 Australian cotton: Challenges and stresses

For many years, China had been the world's biggest producer, consumer, and stockholder of cotton (Baffes, 2004). However, a recent statistic shows that India has replaced China as the world's biggest cotton producer with production amounting to 6.21 million metric tons (MMT) in the 2017/2018 crop year, followed by China at 5.99 MMT and then United States at 4.56 MMT (Statistica Research Department, 2018). Meanwhile, during the same period, Australia came in sixth place producing 1.05 MMT of cotton. Interestingly, Australian cotton production per hectare is more than double the productivity in India or China. Cotton in Australia yields more than three times the global average due to a successful world-class plant breeding program and improvements in water, crop, pest and post-harvest management (Cotton Australia, 2018; CSIRO, 2015). According to the Department of Agriculture and Water Resources (2019), Australia is one of the world's biggest exporters of raw cotton, worth about $2 billion annually with over 90% of cotton produced in Australia, exported predominantly to Asian spinning mill customers such as China, Indonesia, and Thailand.

Notwithstanding the high quality of Australian cotton with 91.4% at or exceeding the base grade (Cotton Australia, 2018), the Australian cotton industry is not exempt from the impact of climate change. In 2018, the production of cotton nationwide has been halved due to drought and low-to-no water allocation for most cotton growers (ABC News, 2019). This is not the first-time drought has affected the industry. In fact, the drought in Australia during the 2007/2008 crop year resulted in the smallest cotton production in over 30 years span with only 601,810 bales as compared to 5,300,000 bales in the most productive year of 2011/2012 (Cotton Australia, 2018). Cotton Australia chief executive Adam Kay further emphasised the

expectation for the cotton production to be halved again for the next crop season as per his interview with the ABC News (2019) last year.

Moreover, the federal government declared that dryland salinity, which occurs when vast underground salt deposits rise to the surface with groundwater tables, could leave the productive farmlands, that make up more than half of the country, desolate and barren (Mochan & Gubana, 2018). Dryland and irrigation salinity also cost approximately $130 million in lost agricultural production (Mochan & Gubana, 2018). However, the full scale of the problem is unknown, so the exact value of the impact on Australia's annual $155 billion agriculture industry is unclear (Mochan & Gubana, 2018). Cotton seedlings are sensitive to salinity at relatively low levels until about 8-12 weeks after sowing (CottonInfo, 2015). However, cotton is placed in the moderately salt-tolerant group of plant species with a salinity threshold level 7.7 dS m^{-1}. Cotton growth and seed yield are severely reduced at high salinity levels and different salts affect the cotton growth to a variable extent (Ashraf, 2002).

Cotton crops in Australia are not only frequently susceptible to weather extremes and insect pests but can also be devastated by diseases such as seedling diseases and fungal wilt diseases (CSIRO, 2015; Office of the Gene Technology Regulator, 2002). Based on three decades of disease surveys in New South Wales, where about 66% of Australian cotton is produced, the four significant cotton diseases in Australia are *Verticillium* wilt, bacterial blight, black root rot and *Fusarium* wilt (Kirkby et al., 2013). For instance, *Vertillicum dahliae* (*V. dahliae)* is the soil-borne fungal pathogen that causes Verticillium wilt disease, with three strains of *V. dahliae* responsible for damaging Australian cotton with symptoms including leaf mottle and necrosis, defoliation, wilting, and even plant death. Failure to control this disease could lead to 10-64% yield losses (Holman et al., 2016). Furthermore, the existing cotton production practices in Australia and the frequent mobility of machinery, vehicles and people favour pathogen dispersal and survival. Besides, Australian cotton farmers have already been dealing with expensive input costs including electricity, diesel, water, skilled worker and high-value machinery (Cotton Australia, 2018). Thankfully, a better understanding of pathogen survival and transmission coupled with a better crop management strategies and plant genetics has alleviated the effects of the disease (Kirkby et al., 2013), although, more efforts are needed to sustain yields. One of the efforts to sustain the yield of cotton is by incorporating plant growth hormones in crop management, and thus lead to the present study.

1.2.1 Rationale of the study

Australia's cotton production was forecast to decline for the March 2020 harvest and estimated to range from 0.7 million to 1.3 million bales, which indicates a significant reduction in quantity, resulting from the effect of three years of drought (Seshadri, 2019). In the lint industry, the average lint yield realised in developing countries (India) and developed countries (Australia) is about 500 and 2,500 kg/ha, respectively, compared with the theoretical potential yield of 5,000 kg/ha (Nachimuthu & Webb, 2017). The yield gap is adversely affected by several biotic (viruses, fungi, parasites, insects and weeds) and abiotic (fluctuating temperature, intense sunlight, drought, flood, osmotic pressure and wind) stress factors (Nachimuthu & Webb, 2017). Different plants respond differently to several stress stimuli through various intercellular mechanisms geared towards ameliorating any adverse effects caused by extracellular biotic and abiotic stressors (Choudhury et al., 2013; Kissoudis et al., 2014; Šamajová et al., 2013; Zhou et al., 2014). Cotton (*Gossypium hirsutum*) is cultivated worldwide, yet its growth, development and productivity are frequently affected by biotic and abiotic stress factors (Nachimuthu & Webb, 2017). One of the plant mechanisms with the ability to modulate the action of adverse physiological and environmental stresses is plant growth regulators. Brassinosteroids (BRs) have been shown to promote seed germination, plant growth and plant development, while improving the plant's resistance to abiotic and biotic stresses (Deng et al., 2015; Fariduddin et al., 2014; Mir et al., 2015; Talaat et al., 2015). BRs can either promote or inhibit several stress responses independently (Kang et al., 2017; Vragović et al., 2015) or through cross-talk with other plant growth regulators (Chunget al., 2014; Kagale et al., 2007; Zhang et al., 2011; Zhou et al., 2014).

Further, many studies suggest the involvement of novel calmodulin binding proteins (CBP60s) in mediating stress tolerance against both biotic and abiotic stresses (Reddy et al., 2003; Kim, et al., 2013; Lu & Harrington, 1994; Qin et al., 2018; Truman et al. 2013; Zhang et al., 2010). The AtCP60a is a negative regulator of immunity, as a *cbp60a* reduced the growth of bacterial pathogen *Pseudomonas syringae* because of the higher production of SA in these mutants, compared with wild type (Truman et al., 2013). In their study, *cbp60a* plants are found to be more resistant to the pathogen due to the higher level of SA as well as of several SA-dependent and SA-independent pathogen-inducible genes in these mutant as compared to wild type plants. In contrast, the overexpression of AtCBP60g increased resistance to drought and abscisic acid, as compared with *cbp60g* plants (Wan et al., 2012). CBP60g is a DNA-binding domain that

binds specifically to the sequence 'GAAATTTGG' in the promoter of encoding isochorismate synthase gene ICS1 (Zhang et al., 2010). An independent study found that both CBP60g and SARD1 are key regulators for ICS1 induction and SA production. ICS1 is a key enzyme in SA synthesis. Both proteins are recruited to the ICS1 promoters, strongly suggesting the role of these proteins in pathogen infection (L. Wang et al., 2011; Zhang et al., 2010). The CaM-binding region of the protein is required for the activation of SA defence signalling during the microbe-associated molecular pattern (MAMP) response (Wang et al., 2009). Additionally, the production of two hypothetical proteins (CBP60c and CBP60d) in bean (*Phaseolus vulgaris*) plants increased in response to bacterial pathogen *Fusarium oxysporium* (Ali et al., 2003).. Given the ability of AtCBP60s to mediate biotic and abiotic stress, as well as the increase in the availability of sequenced plant genomes, many CBP60s have been identified at the whole genome level in several plant species, including Arabidopsis, tobacco and maize (Kim et al., 2013; Truman et al., 2013; Y. Zhang et al., 2010; Reddy et al., 1993; Lu & Harrington). There has previously been only one study on cotton CBP60b and no comprehensive research conducted on cotton CBP60s.

Convincing evidence for the positive effects of BRs on cotton fibre length has also been reported (Sun et al., 2005). However, there are limited studies on the effect of BRs on cotton growth and development in response to abiotic and biotic stresses. Existing studies on cotton used excessive stress levels of salt, which does not reflect field conditions (Surgun et al., 2015). Based on results indicating the significant roles of exogenous application of 24-Epibrassinloide (EBR) in plant growth, metabolism and plant tolerance to abiotic stresses, investigating the effect of exogenous EBR on cotton seedlings' growth in response to moderate levels of salt, drought and pathogen is necessary to ensure the sustainability of the cotton industry.

Arabidopsis CBP60s plays a crucial role in mediating stress response in plants (Qin et al., 2018; Truman et al., 2013; Wan et al., 2012; L. Wang et al., 2009; Zhang et al., 2010); therefore, the present study aimed to identify and characterise cotton CBP60s to understand its importance and characteristics. This study aimed to determine whether the exogenous application of EBR has the potential to modulate the expression of cotton CBP60s in leaf tissue in response to salt stress. As per previous studies, the objectives of this thesis were:

1. To study the effects of exogenous BR application on cotton plant growth and tolerance to drought, salt and pathogen stresses.

2. To identify genes and pathways most affected by BR under conditions where positive effects of BR are obtained.
3. To characterise in detail CBP60-related genes in cotton for gene structure and phylogenetic relationships.
4. To investigate the transcriptional response of the CBP60 gene family to exogenous BR.

Chapter 2. Literature Review

2.1 Introduction to Brassinosteroids and CBP60 proteins

Chapter 2 presents a review of the literature on BRs and a possible BR-regulated gene family, *CBP60.* These genes encode calmodulin (CAM)-binding transcription factors that play major roles in mediating stress in plants. BR biosynthesis, chemical structure, signalling pathway, hormonal interaction and physiological roles in abiotic and biotic stress responses are explained in this chapter. This literature review is important to understand the possible application of BRs to mediate stress in cotton crops.

2.2 BR structure and biosynthesis

In 1970, an organic extract called *brassin* that was first isolated from the pollen of *Brassica napus* was reported to have a novel growth-promoting effects in a group of treated young pinto bean plants (*Phaseolus vulgaris* L) (Mitchell et al., 1970). Later in 1979, the chemical structure of brassinolide (BL) (Figure 1) was determined after a purification process from bee-collected pollen (Grove et al., 1979). BL is the most active form of BRs, endogenous plant hormones that regulate aspects of plant growth and development such as seed germination, root development, cell elongation, cell differentiation, cell division, photomorphogenesis, senescence, vascular differentiation, and reproduction (Clouse, 2011; Mussig et al., 2003). BRs belong to the class of polyhydroxysteroids. Variation in BR structure is generated from the position of functional groups in rings A and B and the side chain. BL contains C-2α and C-3α hydroxyl groups in the A ring. However, a modification in the B ring results in the formation of 6-oxo (6-ketone) BRs, which are most abundant in plants. There are over 40 types of BRs that can be classified as C27, C28, or C29, depending on the alkyl-substitution pattern of the side chain. The C29 BRs have an ethyl group substituent and may be generated from sitosterol. The C29 BRs with a methylene group at C24 and an additional methyl group at C25 may come from 24-methylene-25-methyl cholesterol (Bajguz & Tretyn, 2003). Previous studies using gas chromatography-mass spectrometry (GC-MS) and feeding labelled isotopes to cell cultures of *Catharanthus roseus* (L.) were instrumental in identifying the BR biosynthesis pathways. In cultured *C. roseus* cells, it was proposed that BL could be synthesised by two alternative pathways; early C6 oxidation and late C6 oxidation (Figure 2-1). In the early C6 oxidation pathway, campestanol (CN) is used as the first intermediate, which is then converted to 6-

oxocampestanol (6-OxoCN), cathasterone (CT), teasterone (TE), 3-dehydroteasterone (3DT), typhasterol (TY) then castasterone (CS) via enzymatic oxidation (Fujioka et al., 1997; Fujioka & Sakurai, 1997).

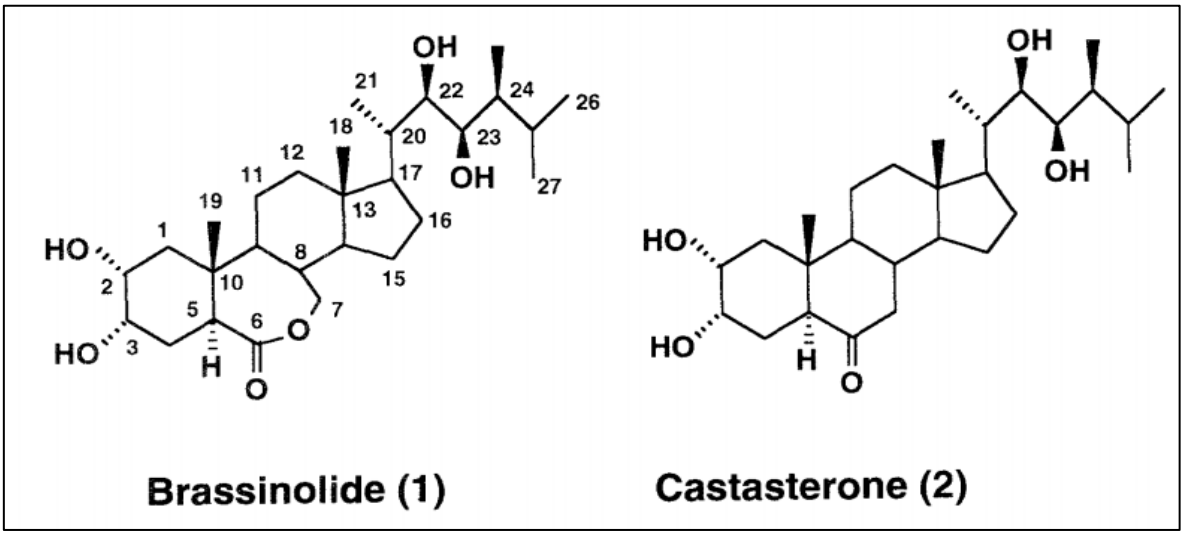


Figure Error! No text of specified style in document.**-1.** The chemical structure of the biologically most active BR. Figure adapted from Akira and Shozo (1997).

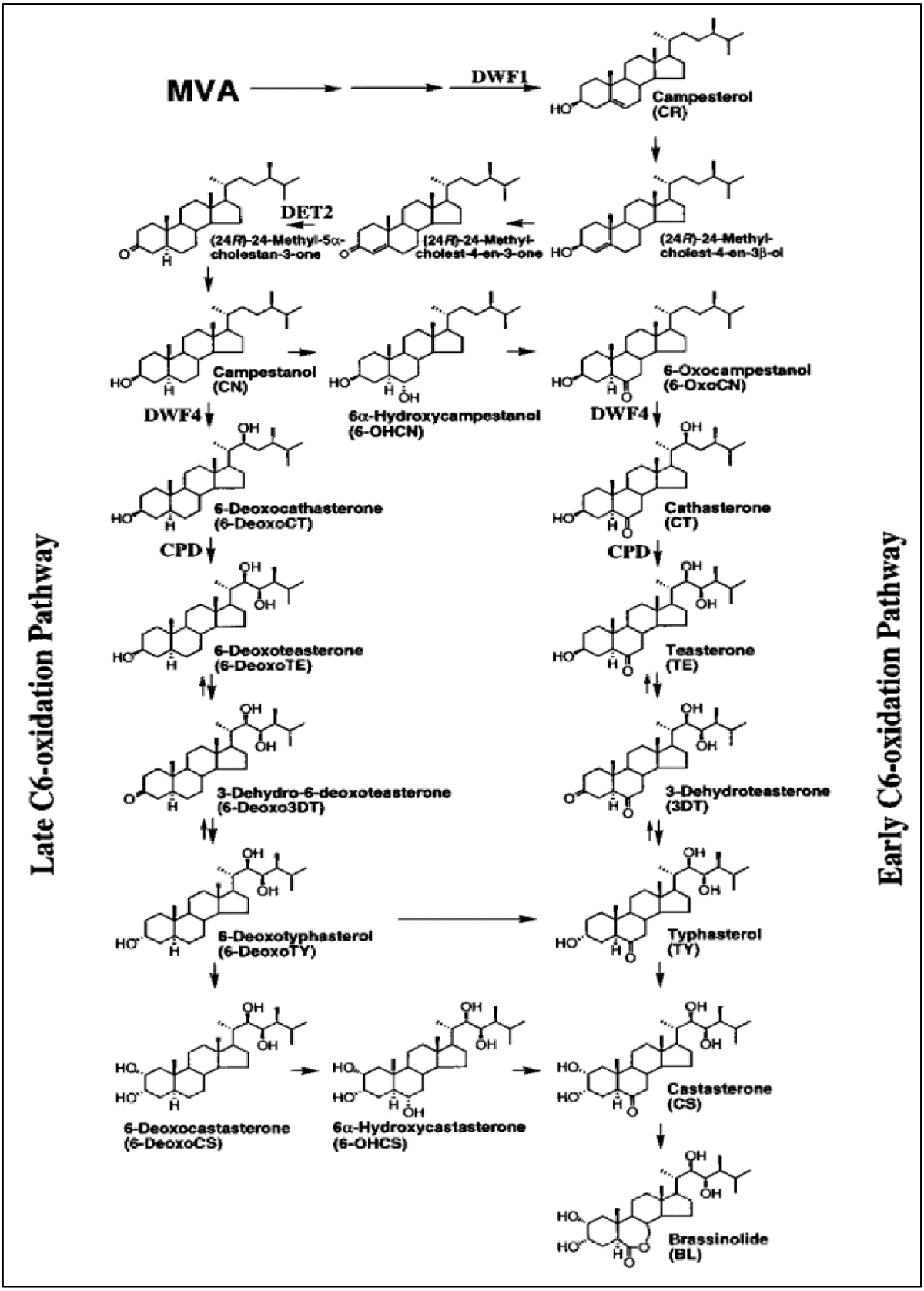


Figure Error! No text of specified style in document.**-2.** Biosynthetic pathways of BL in *A thaliana*. Figure adapted from Noguchi et al. (2000).

In the late C6 oxidation pathway, the synthetic pathway starts with the intermediate component CN, which is converted to 6-deoxocathasterone (6-DeoxoCT). The later intermediate is converted to 6-deoxoteasterone (TE), which goes through a series of oxidation steps to 6-hydroxycastasterone (6-DeoxoTE) and finally to CS, the immediate precursor to the synthesis of BL (Choi et al., 1996; Fujioka et al., 1997). While BL is the most active BR in Arabidopsis, CS is the most active BR in rice (Hong et al., 2005). BR-deficient mutants and metabolic studies demonstrated that the early and late C6 oxidation pathways are also functional pathways in Arabidopsis (Noguchi et al., 1999). An additional BR pathway, named as the early C-23 oxidation pathway, via a cytochrome P450–catalysed oxidative reaction has also been reported in Type-*A* Arabidopsis (Hong et al., 2005).

Any mutations in BR biosynthesis or signalling results in abnormal plant phenotypes. Many of the sterol biosynthetic enzymes in Arabidopsis have been identified through the molecular characterisation of BR-related mutants (Li et al., 1996). For example, the Arabidopsis DWF1 enzyme is involved in an early step of BR biosynthesis, the conversion of 24-methylenecholesterol to CR. The Arabidopsis mutant det2 was identified during the study of light-regulated development of plants. The DET2 gene encodes a steroid 5α-reductase, which catalyses the conversion of CR to CN or 6-DeoxoCT (Chory et al., 1991). Moreover, cytochrome P450 monooxygenases form the closely related CYP85 or CYP90 families that are essential for BR biosynthesis (Fujioka & Yokota, 2003). BR-related dwarf mutants of Arabidopsis, dwf4 and cpd, encode cytochrome P450 monooxygenases (CYP90) identical to the steroid hydroxylases and catalyse hydroxylation of the steroid side chain (C-22 and C-23) in both the early and late C-6 oxidation pathways of BR biosynthesis, respectively (Choe et al., 1998; Szekeres et al., 1996). Both BR-deficient mutants dwf4 and cpd exhibit cell elongation inhibition, de-etiolation, dwarfism, male sterility, hypocotyl shortening, cotyledon opening in the dark, lack of apical hook, and both depression of light-induced genes in the dark and activation of stress-regulated genes in the light, as compared to wild type. Phenotypes of BR-deficient mutants, det2, cpd, and dwf4, are reverted to the wild type through feeding with BL precursors and ectopic over-expression of wild-type genes, indicating a key role of BR in regulating plant development (Azpiroz et al., 1998; Chory et al., 1991; Noguchi et al., 1999; Szekeres et al., 1996).

Previous cotton studies demonstrated that application of a low concentration of EBR increased the fibre length of ovules as compared to untreated ovules (Ashcraft, 1996). Similarly, the

exposure to low concentrations of BL promoted fibre elongation, while treatment with BR biosynthesis inhibitor brassinazole (Brz) reduced fibre initiation, inhibited fibre elongation and fibre differentiation (Ashcraft, 1996; Sun et al., 2005). Fibre genes related to cell elongation in ovules treated with BL were upregulated, and down-regulation by Brz treatment suggests the involvement of BR in cotton fibre development (Sun et al., 2005). Transgenic cotton plants over-expressing a BR-responsive *xyloglucan transferase/hydrolase* (*XTH)* also had longer fibres (Allen et al., 2000). These studies indicate that BR and BR-responsive genes have important roles in cotton fibre development through direct modulation of BR signalling pathways. Aydin et al. (2006) examined BR effects on cotton regeneration through somatic embryogenesis. While BR treatment of cotton seedlings and hypocotyl decreased the fresh weight of callus as compared to controls, BR had a major role in the stimulation of somatic embryo maturation (Aydin et al., 2006). Earlier microarray analysis on cotton indicated high expression of BR biosynthesis genes DET2 and SMT1 during fibre development (Shi et al., 2006). The transcript levels of both DET2 and SMT1 genes increased from the day of anthesis to 10 days post-anthesis (DPA) and then decreased at 20 DPA, however, the mRNA levels significantly declined at 10 DPA in ovules of the fibreless mutant *fl* compared to wild types.

2.3 The signalling pathway for BR

BR signalling regulates plant growth, development and stress responses. The BR signalling pathway is now one of the best-understood plant hormone signalling pathways. BR is perceived by the plasma membrane-localised leucine-rich repeat (LRR) receptor-like kinase (RLK) BRI1 (Brassinosteroid Insensitive1) (Friedrichsen et al., 2000; Li & Chory, 1997). BR binding to BRI1 leads to auto-phosphorylation of BRI1 and its dissociation from BKI1 (BRI1 Kinase Inhibitor 1). BRI1 then activates another regulator, BAK1 (BRI1-Associated Kinase1) (Wang & Chory, 2006). The negative regulator of BR signalling BIN2 (Brassinosteroid Insensitive2) is located downstream of BRI1 and BAK1; in the absence of BR, BIN2 phosphorylates and thereby inactivates transcriptional regulators BES1 (BRI1-EMS-SUPPRESSOR1) and BZR1 (BRASSINAZOL E-RESISTANT1) (Kim et al., 2009; Choe et al., 2002; Li & Nam, 2002; Wang et al., 2002; He et al., 2002). Activated BRI1 phosphorylates BR-SIGNALLING KINASE (BSK1), which in turn activates the phosphatase BRI1–SUPPRESSOR (BSU1) (Kim et al., 2011). BSU1 dephosphorylates and inhibits BIN2, which leads to the accumulation of BES1 and BZR1 in the nucleus and activation of BR-mediated gene expression (Kim et al., 2009).

Several interactors of BES1 and BZR1 have been identified, which allow for the control of the broad-range of gene expression associated with BR's multiple activities (Wang et al., 2002). In summary, in the absence of BR, the following components are inactive: BRI, BAK1, BSK1, BSU1, BES1, and BZR1 (He et al., 2002; Yin et al., 2002), whereas BIN2 is active and phosphorylation of BES1 and BZR1 by BIN2 at multiple sites results in inhibition of their activities and proteasome-mediated degradation (Kim et al., 2009; Peng et al., 2008). In the presence of BR, BIN2 is inactivated and degraded, which results in the activation of BES1/BZR1, leading to BR-responsive gene expression (Nemhauser et al., 2003).

2.4 BR interaction with other plant hormones

The integration of signalling pathways involving BRs and other hormones is crucial for regulating developmental and stress-related processes in plants. Physiological interactions between BR and other plant growth hormones such as auxin promotes hypocotyl elongation and root development. Whereas some studies have indicated that BR and auxin can act independently, others have revealed that there is a significant overlap between BR and auxin (Goda et al., 2004; Goda et al., 2002; Zurek et al., 1994). The two hormones act synergistically in controlling hypocotyl elongation in different plant species (Mandava, 1988). Besides, the response of one hormone requires the function of the other hormone; for example, BR promotes auxin response resulting in a significant increase in hypocotyl elongation (Vert et al., 2008), while auxin regulates BR biosynthesis (Chung et al., 2011).

Abscisic acid (ABA), the major stress hormone in plants was found to inhibit BR signalling, as judged by the phosphorylation status of BES1, and the downstream BR-responsive gene expression (Zhang et al., 2009). In contrast, exogenous BR enhanced the levels of endogenous ABA and ABA-mediated gene expression (Divi et al., 2016). The latter observation indicates that BR-mediated stress tolerance, in part, occurs via enhancement of ABA signalling and ABA-mediated gene expression.

Gibberellins (GAs) have a vital physiological role in plant growth and development (Swain & Singh, 2005). Crosstalk between BR and GA signalling pathways is both synergistic and antagonistic at the transcriptional level (Bai et al., 2012, De Vleesschauwer et al., 2012). Microarray analysis of BR- and GA-treated rice seedlings revealed that both hormones promote growth by co-ordinately regulating the expression of specific genes (Yang et al., 2004). Recently, Li et al. (2012) have demonstrated that BZR1 interacts with a member of the DELLA

family called REPRESSOR OF GAL-3 (RGA) that inhibits the GA signalling pathway in Arabidopsis. Ectopic expression of DELLA proteins reduced the transcriptional activity of BZR1, indicating that DELLA proteins act as mediators between the BR and GA signalling pathways to control plant growth and development (Li et al., 2012).

Ethylene is another potent regulator of plant growth and development, including seed germination, root development, root nodulation, flower senescence and fruit ripening (Alba et al., 2006; Yang & Hoffman, 1984). BR upregulates ethylene biosynthesis genes and enhances ethylene biosynthesis. In cotton, high expression of the ethylene biosynthesis gene *Aminocyclopropane-1-Carboxylic Acid Oxidase1-3* (*ACO1-3*) during fibre growth stage is notable (Shi et al., 2006). In agreement with this observation, the ethylene biosynthetic inhibitor l-(2-aminoethoxyvinyl)-glycine (AVG) inhibited fibre elongation. Similarly, BR biosynthesis inhibitor brassinazole (Brz) also inhibited fibre elongation growth, which could be overcome by treatment with ethylene; however, the inhibitory effects of AVG on fibre growth were less controlled by BR (Shi et al., 2006). These results indicate that ethylene has a vital role in the stimulation of fibre growth and that BR stimulates fibre growth likely through enhancing ethylene biosynthesis.

Jasmonic acid (JA) is a signal molecule that regulates plant growth and development as well as biotic and abiotic stresses (Creelman & Mullet, 1995). There is a potential link between BR and JA synthesis and signalling (Divi et al., 2016; Sahni et al., 2016). Salicylic acid (SA) is a major signal molecule involved in plant defence against pathogens but also has roles in abiotic stress tolerance (Nazar et al., 2015; Kang et al., 2013; Ward et al., 1991). Backer et al. (2019) found that the NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1), which is the central regulator in SA-mediated defence, is an important component of BR-mediated abiotic stress tolerance.

2.5 Roles of BR in abiotic stress tolerance

Abiotic stress has detrimental effects on plant growth and development, reducing crop productivity. The ability of BR to confer resistance against environmental stresses depends on the ability to switch between growth activation and repression under unfavourable conditions (Bechtold & Field, 2018; Feng et al., 2016). Many studies have revealed that BR plays a role in mediating the response to abiotic stresses such as salinity and drought independently or by crosstalk with ABA pathways. BR signalling appears to mediate adaptation to stress via

alteration in the transcriptional activity of stress-responsive genes (Wang et al., 2018; Divi et al., 2010).

BRs and ABA perform mostly antagonistic regulation of stress-responsive genes at or after the BIN2 step in BR signalling pathways (Chung et al., 2014). This represses BR signalling, leading to the enhancement of ABA-mediated stress-responses by phosphorylating SnRK2 (SNF1-RELATED PROTEIN KINASE 2). This process leads to the expression of ABA-responsive genes (Chung et al., 2014). Another BR signalling pathway mediating salt tolerance in plants is via the regulation of ethylene biosynthesis. The exogenous application of BR leads to an increase in the production of ethylene, resulting from increasing the activity of 1-aminocyclopropane-1-carboxylate synthase (ACS), an ethylene synthesis enzyme (Zhu et al., 2016). The BR signalling here is mediated by BRI1 via the inhibition of *A. thaliana* ubiquitin-conjugating enzyme, UBC32, a stress-induced functional ubiquitin conjugation enzyme (E2) localised to the ER membrane. UBC32 increased the salt tolerance of both bri19 and bri15 mutants through the activation of BR signalling (Cui et al., 2012). On the other hand, the bin2 mutant was hypersensitive to salt because of the inhibition of salt-responsive genes (Zeng et al., 2010). These findings suggest that exogenous application of BR helps plants to adapt and survive under high salinity via the BR signal transduction pathway.

Exogenous application of EBR activates the plant's antioxidative defence system by regulating antioxidant gene expression, and EBR also acts as a signalling compound under salt stress, which leads to a decrease in oxidative stress and its consequences (Alam et al., 2019). Another mechanism that can be influenced by EBR is increasing the ratios of K+/Na+ and Ca2+/Na+ in the roots and leaves to alleviate Na+ toxicity (Dong et al., 2017). EBR increased K+ content and decreased Na+ in the shoot and root of a salt-stressed plant by modulating the expression of MhBZR1 and MhBZR2, which are the key transcription factors in BR signalling pathways. These transcription factors can directly bind to the E-box (CANNTG) promoter element of salt-responsive genes (MhSOS1 and MhNHX4-1), downregulating their expression, and leading to salt tolerance in apple *Malus hupehensis* Rehd (Su et al., 2020).

BRs not only can mediate stress responses against salt and drought but also heat and chilling. *B. napus* and tomato seedlings treated with EBR are more tolerant to heat stress as compared to control seedlings (Dhaubhadel et al., 1999). EBR-treated seedlings accumulated higher levels of heat shock proteins (HSPs) due to the maintenance of protein synthesis. The transcript levels of HSPs are higher in BR treated plants as compared to untreated plants (Dhaubhadel et

al., 1999). The study concluded that BR increases the level of expression of several translation initiation and elongation factors following thermal stress, resulting in an increase in cellular protein synthesis (Dhaubhadel et al., 2002). The treatment with EBR increased the activity of antioxidant enzymes such as polyphenol oxidase (PPO), superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APOX) in response to zinc metal stress in *Brassica juncea* (Arora et al., 2010) and tomato under stress conditions (Aghdam et al., 2012; Ahammed et al., 2012), thereby mitigating the detrimental effects of HS on plant growth. BR also increased carboxylation and photosynthetic efficiency in tomato leaves under HS (Ogweno et al., 2008; Singh & Shono, 2005). In Arabidopsis, EBR treatment reduced the bleaching effects of HS (Kagale et al., 2007).

Low temperature has a major effect on plant development and consequently on plant productivity (Hatfield & Prueger, 2015). EBR slightly improved the growth of mung beans exposed to chilling stress (Huang et al., 2006). Injection of EBR into primary leaves and cotyledons of rape seedlings helped overcome the cold-induced increase in membrane permeability (Janeczko et al., 2007). Treatment with EBR increased the pigment content in leaves exposed to low temperatures (2°C) as compared to the control. Arabidopsis and *B. napus* grown on a nutrient medium supplemented with EBR and subjected to low temperature, had increased expression of cold-responsive genes compared to the controls (Sahni et al., 2016). EBR increased the growth of cucumber seedlings exposed to cold stress by enhancing the activation of Rubisco and the expression of photosynthetic genes as compared to the control (Zhao et al., 2017).

BR can regulate the uptake of ions into plant cells, which can reduce and minimise the toxic effects of soil contaminated with heavy metals on the growth of *Brassica juncea* L. (Bhardwaj et al., 2008). Kinetic studies on cadmium (Cd) uptake in rape winter seedlings indicated a 14.7% decrease in Cd levels in EBR-treated cotyledons (Janeczko et al., 2005). In addition, BR plays a major role in cellular redox homeostasis, which is important for plant growth and tolerance to biotic and abiotic stresses. BR increased activities of catalase (CAT), superoxide dismutase activity (SOD), glutathione reductase (GR) and ascorbate peroxidase (APX) in rice seedlings subjected to salinity stress (Nunez et al., 2003; Özdemir et al., 2004), and in tomato leaf discs exposed to high temperatures (Mazorra et al., 2002). Similarly, BL treatment increased the antioxidant enzymatic activities of GR, CAT, and APX, as well as glutathione,

carotenoid and ascorbic acid contents in the microalga *Acutodesmus obliquus* (Talarek-Karwel et al., 2019).

2.6 Roles of BR in biotic stress

Chemical, physical, and inducible defence mechanisms in plants enable them to resist pathogens and act as mitigation strategies against the adverse effects of pathogen stress on plant growth. Complex signalling pathways induce plant defence mechanisms by employing phytohormones including ABA, ethylene (ETH), JA, salicylic acid (SA), and BR (Smith, 2017). For example, studies showed that BR activated disease resistance in both rice (*Oryza sativa*) and tobacco (*Nicotiana tabacum*) via a complex pathway that employed the BRI1 receptor and its co-receptor BAK1 (Chinchilla et al., 2009; Heese et al., 2007; Nakashita et al., 2003; Wang et al., 2012). Interestingly, pattern recognition receptors (PRRs) that are expressed in plant immune cells will give a signal to the plant of the incoming pathogen by recognising specific molecules of the microbes-associated molecular patterns (MAMPs). After identifying flg22 which is a class of this molecules, a receptor called FLAGELLIN-SENSING (FLS2) initiates flg22-signalling responses to avoid the spread of pathogen (Chinchilla et al., 2007; Segonzac & Zipfel, 2011). The binding of flg22 to its receptor FLS2 results in association and transphosphorylation of the co-receptor BAK1, which subsequently activates the FLS2 receptor. The activated FLS2 then phosphorylates a receptor-like cytoplasmic kinase called BIK1 (BOTRYTIS-INDUCED KINASE 1) that triggers plant immune responses (Chinchilla et al., 2007). These findings suggest the major role of the BAK1 receptor is to initiate the interchange between FLS2 and BR signalling responses.

Another independent study of Arabidopsis treated with BR and flg22 showed a reduction in immunity responses as compared to control. This study also shows that flg22 did not enhance or inhibit BR signalling. However, without the application of BR during flg22 treatment, the ROS and MAPKs (mitogen-activated protein kinases) stress markers were induced. This shows that BR inhibits FLS2-mediated immune signalling, without incorporating its co-receptor BAK1 and associated downstream phosphorylation. These findings suggest that BAK1 employed by the FL2S complex acts differently from BAK1 employed by BRI1 signalling (Albrecht et al., 2012).

Complex relationships between SA, JA, and BR phytohormones are also involved in the response to biotic stress. A previous study on the infestation of *Nilaparvata lugens*, also

commonly known as brown planthopper (BPH) in rice (*Oryza sativa*) showed a suppression of the BR signalling pathway. During BPH infestation in WT plants, BR treatment also reduced the production of SA and increased the production of JA, by downregulating ICS1 and PAL genes that are related to SA pathways and up-regulated MYC2, AOS2, and LOX1 genes that are related to JA pathways (Pan et al., 2018). This finding suggests that when BR suppressed SA signalling, the JA pathway was preferred. Therefore, researchers suggested the role of BR as a negative regulator of plants immune system (Campos et al., 2009; De Vleesschauwer et al., 2012; He et al., 2017).

on the attack by a moth (*Manduca sexta)* and a small insect known as onion thrips *(Thrips tabaci*) showed a positive genetic correlation upon the BR treatment in *Lotus japonicas* (*L. japonicas*). The *L. Japonicas* transgenic plants that over-expressed the Arabidopsis BIL1/BZR1 which is a BR master transcription factor, showed increased resistance to thrips feeding due to the increased amounts of JA in these plants (Miyaji et al., 2014). Hence, researchers concluded it was difficult to specify the role of BR in plant immunity and its relationship with JA and SA due to the different effects depending on the affected organ (shoot and/or roots) as well as different biotic stresses (microbial, biotrophic, necrotrophic, or insect).

BR induced disease resistance in tobacco and rice plants against *Pseudomonas syringae* and *Oidium* sp. (Nakashita et al., 2003). The authors suggested that BR-mediated disease resistance is distinct from systemic-acquired resistance (SAR) that is primarily mediated by salicylic acid (SA). However, Szekeres et al. (1996) argued that the low expression of SA-responsive *pathogen-related (PR*) genes in the Arabidopsis *cpd* mutant and the higher expression of *PR* genes in transgenic plants over-expressing *CPD,* suggests that BR mediates pathogen resistance by SA-mediated SAR. The role of BR in plant defence also includes regulation of *thionin* genes, which encode for antimicrobial peptides. The decline of thionin expression in rice coleoptiles was suppressed by co-treatment with GA and BR, which increased disease resistance (Kitanaga et al., 2006). In barley, EBR reduced the severe effects of *Fusarium* head blight (FHB) by 86% and reduced loss in grain weight by 33%. Gene expression studies in barley found that expression of *PR* and other genes related to photosynthesis, hormone signalling and chromatin remodelling were activated in treated plants (Ali et al., 2013).

2.7 Use of exogenous BRs for phenotypic response

Despite the positive effect of exogenous application of BRs on plant growth, these effects can vary greatly depending on plant species, application method and hormone concentration. A study by Nishikawa et al. (1994) suggested that the exogenous application of EBR can be taken up and transported from the roots and young and mature leaves in cucumber and wheat seedlings. However, another independent study by Symons and Reid (2004) suggested that BRs do not undergo long distance transport, yet can be transported at cellular level. BRs may exert long distance signalling by altering auxin transport (Symons et al., 2008). Different phenotypic effects have been observed from different applied EBR (soaking seeds, drenching and spaying seedlings) on the seeds, roots and shoots of wheat (*Triticum aestivum* L. cv. Cytra) using different concentrations of EBR (Janeczko & Swaczynová, 2010). These previous studies strongly suggest that, for an exogenous hormone application to have an observable phenotypic effect, it must enter the plant tissue and reach the appropriate cells to influence growth response. Further, the exogenous application of BRs must have a significant effect on the endogenous hormone concentration at the site of action and must present at a suitable concentration.

2.8 Involvement of calcium signalling, calmodulin and calmodulin-binding proteins (CBP60s) in stress

In order for plant cells to respond to developmental and environmental cues, numerous signalling networks are required including a sequence of receptors, transcription factors, enzymes and non-protein messengers (Sanders et al., 2002). One of the most important non-protein messengers is calcium as there is a significant change in cytosolic free calcium during the transduction of various abiotic and biotic signals (Rudd & Franklin-Tong, 2001; Sanders et al., 1999). Unlike the cell wall and organelles that have Ca^{2+} in the millimolar range, the cytosolic concentration of calcium $(Ca^{2+})_{cyt}$ is in the nanomolar range from 100 to 200 nm (Trewavas & Malhó, 1998). For instance, one of the first events during plant response to microbe and microbe-associated elicitors is a transient change in nuclear calcium $([Ca^{2+}]_{nuc})$ and/or cytosolic calcium $[Ca^{2+}]_{cyt.}$ (Lecourieux et al., 2006). The transient changes in free Ca^{2+} levels in response to developmental and stress signals are then perceived by Ca^{2+} sensors (Reddy et al., 2011; Trewavas & Malhó, 1998). The Ca^{2+} signal is transduced via Ca^{2+} binding proteins resulting in downstream regulation of transcription factors. This regulation of

transcription factors alters the expression of target genes (Kudla et al., 2010). In plants, there are at least four main families of Ca^{2+} sensors, calmodulin (CaM) and its isoforms, CaM–like proteins, Ca^{2+}-dependent protein kinases and Ca^{2+} binding proteins (Reddy, 2001; Snedden & Fromm, 2001). CaM is the main transducer of Ca^{2+} in eukaryotes (Reddy, 2001). On the other hand, Ca^{2+}-dependent protein kinases exist only in plants and protozoa (Reddy et al., 2002). The active form of CaM (Ca^{2+}-bound CaM) regulates the role of various CaM-binding proteins (CBPs) including transcription factors, metabolic enzymes, ion channels and pumps, and structural proteins (Reddy et al., 2011; Kim et al., 2009; Snedden & Fromm, 2001). One of the best well characterised CaM-binding transcription factors is CBP60. The CBP60 transcription factor family is specific to plants, with no homology to any other known proteins, and was first identified in maize (*Zea mays*; Reddy et al., 1993) followed by tobacco (Lu & Harrington, 1994), Arabidopsis (Reddy et al., 2002), and then bean (*Phaseolus vulgaris*) (Ali et al., 2003). Based on the initial study in Arabidopsis, seven members of the CBP60 family were identified *CBP60a/b/c/d/e/f/g*: At5g62570, At5g57580, At2g18750, At4g25800, At2g24300, At4g31000, and At5g26920) (Reddy et al., 2002; Wang et al., 2009). Later, an eighth family member, At1g73805 that is closely related to CBP60g was added to the group (Wang et al., 2011). This later member, known as SARD1 (systemic acquired resistance deficient 1) (Wang et al., 2011).

Five members of the gene family, AtCBP60a/b/c/d/e were found to bind CaM via a domain which is located at the C-terminal of the protein. (Reddy et al., 2002). AtCBP60g lacks the C-terminal CaM-binding domain but instead has a CaM-binding domain located on the N-terminal of the protein (Ali et al., 2003; Reddy et al., 2002). AtSARD1 does not bind CaM (Zhang et al., 2010). AtSARD1 proteins have an important role in defence responses and are involved in the production of SA (Wang et al., 2011). Although CBP60s are thought to be transcription factors, the DNA-binding domain has only been identified in CBP60g and SARD1 (Wang et al., 2011; Zhang et al., 2010). A previous study by Du and Poovaiah (2005) on another CaM-binding protein in Arabidopsis DWARF1 (At3g19820) suggested that this protein has a key role in an early step of BR biosynthesis, by converting 24-methylene cholesterol to campesterol. Unlike CBP60, DWF1 orthologues exist in both animals and plants but its C-terminal CaM-binding domain is only conserved in plants. To date, there is no literature on the role of CBP60 proteins in BR biosynthesis. As BR regulates plant growth and development, its possible interaction with members of CBP60 gene family in cotton in response

to developmental and stress signal is an interesting research topic to ensure necessary measures are taken to sustain the yield of Australian cotton struggling with abiotic and biotic stresses.

Abiotic and biotic stresses decrease crop yield nonetheless, application of EBR can alleviate the negative effect of these stressors. Literature shows that EBR application can improve plant growth under these stresses by playing a key role in plant metabolism. EBR can either act independently or by crosstalk with plant hormonal pathways. EBR can mediate adaptation to stress via alteration in the transcriptional activity of stress-responsive genes. EBR enhance the production of antioxidant enzymes. Little information is available regarding the role of EBR in regulating the expression of cotton CBP60s. Therefore, it is necessary to test the effect of the exogenous application of EBR on cotton seedlings under stress before focusing on identifying CBP60 gene family in cotton to further investigate the role of EBR in mediating stress responses in cotton.

Chapter 3. Effects of Brassinosteroids on Cotton *G. Hirsutum* Seeds and Seedlings under Abiotic and Biotic Stresses

3.1 Introduction to stress experiments in BR-treated cotton

Chapter 3 describes the first set of experiments for this dissertation that investigates the effect of exogenous application of BRs on seeds and seedlings of *G. hirsutum* in response to three different stresses drought, salt, and pathogen attack.

3.2 The effects of abiotic and biotic stresses on cotton

Cotton plants encounter multiple abiotic and biotic stresses such as salinity, drought, temperature, and pathogen attacks that limit growth and yield (Garber & Houston, 1966; Reinhardt & Rost, 1995; Wang et al., 2016). Salinity affects 30% of land area in Australia; whereby groundwater salinity and irrigation salinity, in particular, affect 16% of agricultural areas. Furthermore, new studies suggest that 67% of the agriculture area has the potential to develop transient salinity, a type of non-groundwater–associated salinity (Rengasamy, 2006). Cotton is considered a moderately salt-tolerant plant species with a salinity threshold level of 7.7 dS m−1. Cotton growth and seed yield are severely reduced at high salinity levels with different salts affecting the cotton growth to a variable extent (Ashraf, 2002).

Many studies have discussed the fact that salinity causes a reduction in the root growth of cotton seedlings *Gossypium hirsutum* (*G. hirsutum*) (Reinhardt & Rost, 1995; Silberbush & Ben-Asher, 1987; Zhong & Lauchi, 1993). Researchers further reported that a high level of salinity, between 150 mM and 225 mM, delays the growth of the primary root while a salinity of 75 mM inhibits the elongation of lateral roots (Reinhardt & Rost, 1995). Zhong and Lauchi (1993) reported that salinity reduced the elongation growth of the primary root when grown in a hydroponic solution by shortening the length of the growing zone as well as reducing the longitudinal rate. In contrast, other findings showed an increase in root growth under low salinity levels. For example, a study conducted by Jafri and Ahmad (1994) found that Niab-78 and Qalandri cultivars of cotton *G. hirsutum* exhibited promotion in the root growth under low concentration of salt at 42 mM. Moreover, root development of cultivar Niab-78 was promoted

552 even at a moderate level of salinity of 126 mM NaCl. Similar results were obtained from the
553 same genotypes which showed longer primary root growth when the plant was treated with 100
554 mM salinity as compared to the control (Leidi, 1994).

555 Like other plants, an increase in soil salinity also reduces shoot growth in cotton (Qadir &
556 Shams, 1997). For example, in that study, B-557 had a significant decrease of shoot fresh
557 weight as compared to MNH93, S-12, and NIAB-78 under salinity levels of 139 mM and 278
558 mM. Salinity also reduces the shoot/root ratio as shoots are more susceptible to salinity than
559 roots (Brugnoli & Björkman, 1992; Leidi et al., 1991). In another study, an experiment was
560 conducted by growing 15 cultivars of cotton in a hydroponic solution in the absence and
561 presence of NaCl at 137 mM. This level of salinity results in an osmotic potential of -0.7 MPa.
562 Results showed that salinity induced phytotoxicity, with stunting of leaf, leaf chlorosis, and
563 leaf margin and apex necrosis (Lira-Saldivar & Hernández-Rosales, 1988). However, there
564 were differences in sensitivity to salinity in the advanced lines: Roelca, 1656-52-36 and
565 Paymaster 404 being the most tolerant and Deltapine 80, C310 24, Q SU16-1, and Acala SJ-2
566 being the most sensitive to high salinity (Lira-Saldivar & Hernández-Rosales, 1988).

567 Another abiotic stress that negatively affects plant growth and development during the seedling
568 stage is water deficit (Boyer, 1982). Peter (2019) reported that drought had halved the crop
569 yield of the previous year in Australia. Drought is one of the significant challenges facing
570 cotton sustainability (Wang et al., 2016). Drought affects many aspects of cotton development
571 and growth, both functionally and physiologically (Loka et al., 2011). There are differential
572 growth responses of the shoots and roots of cotton seedlings of *G. hirsutum* in response to
573 drought stress whereby shoots are more susceptible to drought than roots. For example, a study
574 conducted by Pace et al. (1999) reported that cotton seedlings grown in clay-filled pots showed
575 increased in root length but no increase in its dry weight after 13 days of exposing the young
576 plants to drought followed by a recovery period of 10 days. The researchers further reported
577 that the shoot/root ratio was lower for drought-stressed plants as compared to the control. In
578 another independent study, young seedlings of cotton grown in the field or the growth chamber
579 experienced a reduction in root elongation after 6 days of drought stress followed by a 6-day
580 recovery period. Again, the leaf expansion was more susceptible to stress when compared to
581 the root elongation (Ball et al., 1994; Prior et al., 1995). Reductions in plant heights, and stem
582 and shoot dry weights were also reported by Pace et al. (1999) after 49 days of planting
583 followed by withholding water for 13 days and a recovery period of 10 days. Furthermore,

many studies have reported that drought stress reduced cell proliferation and the stem elongation leaf area index (Ball et al., 1994; Gerik et al., 1996; Jordan, 1970; McMichael & Hesketh, 1982; Turner et al., 1986). Growth rates for leaf, stem and root are very susceptible to water stress as they are dependent on water for cell expansion (Hearn, 1995). Photosynthesis is negatively affected by water deficit. Stomatal closure reduced leaf photosynthesis leading to lower CO_2 diffusion into the leaf and chloroplast dehydration (Matthews & Boyer, 1984). Plants have required a variety of mechanisms to adapt to drought that are related to molecular, morpho-physiological and biochemical processes which are regulated by different hormones signalling pathways such as ABA in order to survive (Boudsocq & Laurière, 2005; Riemann et al., 2015; Tan et al., 2012).

Plants are subject to constant attack by various microbial pathogens and pests including bacteria, viruses, fungi, parasites, and harmful insects, which are the major threats to plant growth and agricultural productivity (Glazebrook, 2005). Among these is the fungal disease caused by *Verticillium dahliae*. (*V. dahliae*) is a soil-borne fungus that infects plants throughout the growing season. The pathogen invades the root tips through root wounds and moves up until it reaches the water-conducting xylem vessels (Fradin & Thomma, 2006). Plant roots exhibit a variety of morphological changes such as root hair formation, branching and root diameter adjustment. This interferes with growth because roots are important organs to supply water and nutrients to the plants (Huisman, 1982). Many researchers have reported that, during the early stages of *V. dahliae*, the shoot remains symptomless due to the biotrophic behaviour of the fungi. However, in later stages of infection, plants become wilted and stunted, suffering from chlorosis because the fungi shift to necrotrophic interaction (Reusche et al., 2012). In cotton plants, the infection was reported to involve the direct penetration of the primary root and lateral roots (Garber & Houston, 1966).

BRs are a class of plant steroidal hormones that have been extensively studied due to their versatile role in modulating plant growth and development (Vardhini & Anjum, 2015; Wei & Li, 2016; Yusuf et al., 2017). In addition, many studies have revealed the involvement of BRs in mediating tolerance to abiotic and biotic stresses (Filova, 2014; Mahesh et al., 2013; Talaat et al., 2015), including salinity (Cui et al., 2012; Dalio et al., 2013; Mir et al., 2015); heat (Bajguz & Hayat, 2009; Fariduddin et al., 2014; Hayat et al., 2010), drought (Farooq et al., 2009) and heavy metals (Harpreet et al., 2014; Kanwar et al., 2013; Li et al., 2016; Sharma et al., 2012). BRs mediate salt stress tolerance through modulation of the antioxidant defence

system and up-regulation of transcription factors to enhance oxidative stress tolerance (Divi et al., 2010).

BL, 28-homobrassinolide (HBL) and EBR are the most active biological compounds that specifically modulate plant responses to abiotic stress (Vardhini et al., 2006). Seed priming with 3 µM EBR significantly increased the germination and chlorophyll content of seedlings grown with different concentrations of NaCl from 50 mM to 150 mM in cotton (*G. hirsutum*) (Surgun et al., 2015). Additionally, under excessive salt concentrations, in 21day-old cotton seedlings, salinity induced proline content to increase substantially because of superoxide dismutase (SOD) and glutathione peroxidase (GPX). However, the application of EBR significantly increased antioxidant enzyme activities and the proline level in salt-treated plants (Surgun et al., 2015). A similar study conducted by Rattan et al. (2014) revealed that the pre-treatment of maize seeds with different concentrations of EBR and HBL mediated morphological and physiological changes via the accumulation of glycine betaine, proline and mannitol under high concentration of salt in *Zea mays* plants. Researchers have suggested that BRs stimulate glycine betaine accumulation by increasing the catalytic activity of betaine aldehyde dehydrogenase (BADH), which results in the synthesis of glycine betaine from choline (Rattan et al., 2014). Contrary results have also been reported indicating that the application of EBR (0.0125 or 0.025 mg/L) has no significant effect on chlorophyll pigments, growth, water use efficiency and gas exchange in salt-tolerant wheat (*Triticum aestivum* L.) seedlings under 150 mM salt (Qayyum et al., 2007).

Brassinosteroid (BBR)-deficient mutant, *pag1* (*pagoda1*) in cotton plants exhibited shorter primary and lateral roots and increased sensitivity to drought stress. The deficiency is caused by increased inactivation of the active castastrone (CS) in the mutants as compared to control (Chen et al., 2019). In comparison, the hydroponically grown *pag1* mutant which was treated with EBR at a final concentration of 10 nM showed developmental enhancement as measured by four factors: root growth, stomata development, stomata aperture and photosynthesis (Chen et al., 2019). Increased plant stress tolerance was related to the expression of drought stress genes (Chen et al., 2019). Moreover, the application of 3 µM of each of 28-homobrassinolide and EBR has been shown to improve plant tolerance to drought in sorghum at the stages of both germination and seedling growth. The growth was linked to increased soluble proteins and free proline (Vardhini & Rao, 2003). EBR treatment also enhanced the activity of catalase and decreased both peroxidase and ascorbic acid oxidase activities (Vardhini & Rao, 2003).

Treatment with 0.01 and 1 µM EBR also mitigated the negative effect of drought on the growth of tomato seedlings after 3 and 5 days of withholding water grown in pots at the stage of four leaves. There was an increase in fresh and dry weights of roots and shoots of EBR treated seedlings in comparison with control plants. The researchers stated that the treatment with EBR has led to a decrease in malondialdehyde (MDA) and higher antioxidant enzyme activity (Damghan, 2009).

During pathogen infection, the primary plant response is the specific recognition of the pathogen and a rapid and localised cell death whereas the secondary response is to induce the defence system (Kuc, 1982; Ross, 196l). These responses are regulated by complex inter-connected signal transduction pathways in which plant hormones (BR, JA, ABA, ETH, and SA) play a fundamental role (Acharya & Assmann, 2009; S. Hu et al., 2017; Wu et al., 2017). The application of EBR to the heads of 'Lux' barley decreased the intensity of Fusarium head blight (FHB) originating from *Fusarium culmorum* by 86% and lessened the FHB-initiated loss of grain yield by 33%. Also, plants grown in soil amended with epiBL led to 28% and 35% reductions in Fusarium seedling blight (FSB) symptoms in 'Lux' and 'Akashinriki' barley varieties, respectively (Ali et al., 2013). Transcriptional profiling of these plants revealed differential gene expression. Genes involved in chromatin remodelling, hormonal signalling, photosynthesis, and pathogenesis were activated when grown in epiBL-amended soil (Ali et al., 2013).

However, exogenously applied BR showed no effect on inducing the resistance of wild-type Arabidopsis plants infected with the hemibiotrophic bacteria *Pseudomonas syringae*pv. Tomato (Pto) DC3000 or the necrotrophic fungus *Alternaria brassicicola* (Albrecht et al., 2012). In rice, instead of enhancing the plant's resistance, BRs were found to increase the susceptibility to the hemibiotrophic pathogens *Pythium graminicola* and *Meloidogyne graminicola* (Nahar et al., 2013; De Vleesschauwer et al., 2012). BR also induced the vulnerability of potato tuber tissues by triggering the growth in the mycelium, intensifying the spore production of *Phytophthora infenstans*, and weakening the plant tissues' immunity (Vasyukova et al., 1994).

These conflicting results suggest that further work on the involvement of BRs in alleviating the response of cotton to biotic and abiotic stress induced by salt, drought, and pathogens is required. The present study has therefore been conducted to investigate the effect of EBR on the early stages of cotton growth under drought, salt, and pathogen stresses by testing the effect

of exogenous application of EBR on germination and seedling growth under various stress conditions.

3.3 Hypotheses and aims

BRs enhance plant tolerance to various biotic and abiotic stresses. Studies on the manipulation of the genes involved in BR biosynthesis or signalling revealed the essential role of BR in plant development (Bishop & Yokota, 2001; Suzuki et al., 2003). Loss-of-function mutations of these genes usually lead to multiple developmental defects, male sterility, altering stomatal distribution, delayed flowering, and dwarfism (Clouse, 2011). The substantial role of exogenously applied EBR and its related antioxidant enzymes in mitigating various abiotic stresses such as salinity (Avalbaev et al., 2010) and drought (Li et al., 2012; Mahesh et al., 2013) have been studied extensively. Many reports have also indicated the role of BRs in defence response against biotic stresses via their interaction with different phytohormones (Albrecht et al., 2012; Campos et al., 2009; Segonzac & Zipfel, 2011). Based on these significant roles of exogenous application of EBR in plant growth, metabolism, and plant tolerance towards abiotic stresses, investigating the effect of exogenous EBR on cotton seedlings growth in response to salt, drought, and pathogen is necessary to ensure the sustainability of the cotton industry.

The hypotheses for this chapter are:

1. Exogenous application of EBR will improve tolerance of cotton seedlings to salt, drought, and pathogen stresses.

The objectives of this study are to:

1. Investigate the effect of exogenous EBR application on seed germination in the presence or absence of EBR with or without salt stress using a culture medium.
2. Investigate the effect of exogenous EBR application on cotton seedlings in the presence or absence of EBR with or without salt stress using a hydroponic system.
3. Investigate the effect of exogenous EBR application on seedling growth in the presence or absence of EBR using foliar spray under drought stress initiated by withholding water followed by re-watering using pot experiments.
4. Investigate the effect of exogenous EBR application on seedling growth in the presence or absence of EBR under pathogen stress using a hydroponic system.

3.4 Material and methods

3.4.1 Plant materials

Cotton seeds of genotype Sicot 730 were kindly provided by Cotton Seeds Distribution, the marketing arm of the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Australia. Sicot 730 is the conventional cotton variety currently used in Australia and has a higher yield and fibre length than other conventional varieties. This genotype is more resistant to Fusarium wilt than Sicot 71 (Cotton Seed Distribution Extension and Development Team, 2012).

3.4.2 EBR chemical treatment

Commercial EBR was purchased from Phyto Technology Laboratories. A total of 10 mg of EBR was dissolved in 2.1 mL of absolute ethanol and stored at -20ºC prior to use. Stock solutions of 2 mM were used to make up 0.1 µM and 1 µM EBR solutions; a 4 mM solution was used to make up 0.2 µM and 2 µM EBR solutions; and a 10 mM stock solution was used to make up 0.5 µM EBR solution. All stock solutions were diluted in absolute ethanol to make the required concentrations.

3.4.3 Seed germination and EBR treatment for salt stress (culture medium)

For the germination experiment, seeds were surface-sterilised by using 70% ethanol for 30-60s, rinsing 3-5 times with sterile water, soaking with 10% hydrogen peroxide (H_2O_2) for 1-2hours followed by washing three times with sterile water. Seeds were then incubated at 10ºC for three days in the dark to improve germination. Cotton seeds were germinated for 7 days in (150 mm x 25 mm) Petri plates containing half-strength Murashige and Skoog (MS) medium (Phyto Technology Laboratories) containing 0.8% agar (Sigma, Life Science) and 1% sucrose, pH 5.7. Four different concentrations of EBR 0, 0.1 µM, 0.2 µM, 0.5 µM were mixed with four correspondent concentration of salt 0, 100 mM, 150 mM, and 200 mM NaCl prior to their addition to MS medium. All salt concentrations were prepared from a 3 M NaCl stock solution dissolved in water. Control plants (not treated with EBR) were treated with an equivalent solution containing ethanol. Three replicates per treatment were used; each replicate consisted

of one Petri dish with 12 seeds. Petri dishes were incubated under 16h light, 8h darkness, 26^{o}C and light intensity of 150 µmol/s).

3.4.4 Seed germination and EBR treatment for salt stress (hydroponic)

For the hydroponic experiment, cotton seeds were soaked with deionised water overnight. Seeds were then placed on moist filter paper in Petri dishes and kept in the dark at 28 °C for 48 hours. Germinated seeds were transferred to sand and kept in a glasshouse under natural light conditions; the temperature was between 20-28^{o}C. Uniform seedlings with fully expanded cotyledons were transferred to half-strength Hoagland's solution. Plant roots were surface-sterilised with 10% bleach prior to immersing them in the hydroponic solution. The hydroponic solution was completely refilled twice a week and replenished daily by deionised water throughout the experiment to maintain constant nutrient and salt concentrations. Plants were grown individually in jars containing hydroponic solution until full expansion of the first two primary leaves under controlled growth condition at a 28°C (light) and 20°C (dark) cycle. Hormone treatment was initiated by providing the plants with half-strength Hoagland's solution containing 0.1 µM, 0.2 µM, or 0.5 µM EBR. Salt stress treatment of 100 mM NaCl was initiated after 24 h of hormone treatment. To avoid osmotic shock, salt concentrations were increased daily by 50 mM NaCl until reaching a final concentration of either 100 mM or 150 mM NaCl. A stock solution of 3 M NaCl was prepared by dissolving 175.32 g of NaCl in 1 L of distilled water. Plants were cultured in the nutrient solution in the presence and absence of EBR for three weeks before harvesting. Six biological replicates per treatment were used where each plant was considered as one replicate.

3.4.5 Seedling preparation using pot system and drought treatment

Seeds were soaked in 0, 1, and 2 µM EBR for 6 hours to investigate whether seed priming with EBR has an effect on seed germination and increase seedling growth tolerance to drought stress. Seeds were then sown on filter papers and incubated at 28 °C for 48 h to improve germination. Three germinated seeds were sown in cylindrical pots (30 cm height and 5 cm diameter). The seedlings were then transferred to a glasshouse and kept under controlled conditions using a 16-hour light/8-hour dark cycle. After that, seedlings were thinned to one plant per pot. Pots were filled with 850 g of soil (from Kirby SMART farm, University of New

England). The soil had a pH of 5.5 and field capacity of (-10 kPa) 19% gravimetric water content.

A total of thirty-six pots with six pots per treatment were used. The soil was initially fertilised with a solution containing 1.53 g urea, 0.65 g K_2SO_4, and 0.78 g KH_2PO_4 per litre of water before transferring germinated seeds. The pots were maintained in a glasshouse under controlled conditions of temperatures of 28°C (light) and 20°C (dark). Drought stress conditions were previously optimised and reported (Chakma, 2016). Seedlings were well watered on alternate days with 100 ml water per pot for three weeks until the start of drought. Two weeks old seedlings were sprayed with 1µM and 2µM EBR solution on alternative days for a total of four times, while control plants without EBR were sprayed with an equivalent solution containing ethanol. Plants were exposed to drought by withholding water after the last day of EBR treatment. Non-stressed plant (control) watering was maintained at the same level on alternate days until the end of the experiment. Stressed plants were subjected to drought for 14 days; then rewatered after displaying symptoms of wilting and drying leaves. Plants were allowed to recover for five days. Surviving plants were counted and harvested for further analysis, whereas permanently wilted plants (dead) were eliminated. This experiment was repeated using slightly different growth conditions such as using a different batch of Kirby soil due to the unavailability of the previously used batch. In the second experiment, the surfactant Tween 20, 0.05%, was added to the spray containing the EBR treatment. In the second experiment, forty-eight pots of plants (8 plants per treatment) were exposed to drought stress by withholding water for eight days, followed by re-watering for five days.

3.4.6 Fungal isolates and inoculum production

The highly virulent defoliating *V. dahliae* strain accession number DAR 31890, isolated from infected tomato plants, was kindly provided by NSW Plant Pathology and Mycology Herbarium, Orange Agricultural Institute, NSW Department of Primary Industries. The fungal isolate was cultured on potato dextrose agar (PDA) for 3 weeks at 26°C. Petri plates (9 cm diameter) with sporulating cultures were flooded with10mL of sterilised distilled water and shaken for a few minutes. The resulting suspension was filtered through muslin cloth and then through double layers of cheesecloth. The number of conidia was counted using a double Neubauer ruled haemocytometer. The spore suspension was adjusted to 10^6 spores per mL using sterile distilled water.

3.4.7 Pathogen treatment using hydroponic system

For the first experiment, the roots of two weeks old seedlings were treated with three different concentrations of EBR 0 µM, 0.1 µM and 0.2 µM for 24 h prior to pathogen infection. Seedlings were inoculated by dipping into a suspension of 1×10^6 conidia per mL of *V. dahlia* for 0.5h. Control plants were dipped into deionised water for 0.5 h. For the second experiment, the above condition was repeated with the addition of 0.5% Tween 20 surfactant to the Hoagland's solution and the dipping time in the conidial suspension was extended to 1 h. In both experiments, plants were harvested after three weeks post-inoculation with *V. dahliae*. Plant growth conditions were mentioned in section 3.4.4.

3.4.8 Plant growth measurement and data collection

In order to assess the germination rate of cotton seed in tissue culture under salt, seed germination was checked every three days. Seeds with 2 mm long radicals were considered as germinated. Root length was measured using a ruler. For the glasshouse experiments, several measurements were taken to assess the effect of EBR on plant growth under salt, drought and pathogen stresses.

The chlorophyll content of the first primary leaf was measured at 0, 4 8, 12, 16, and 20 days from the start of all experiments using a SPAD-502 meter (Konica–Minolta, Inc., Japan). The SPAD index was calculated by taking the average of three different readings per leaf due to variability of SPAD reading values. Data for plant heights from the cotyledonary node to the highest leaf tip were collected after 21 days of plant treatment for all experiments with salt, drought and pathogen. Root and shoot dry weights were obtained after oven drying at 60°C for 48 hours.

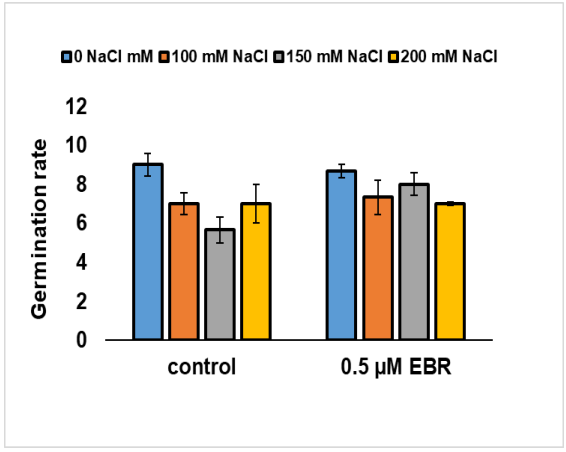
Two-way analysis of variance (ANOVA) was conducted using the Balanced ANOVA within the statistical program Minitab V18 to evaluate the significance between treatments and the interaction effect between EBR and salt, drought and pathogen stresses on plant growth.

3.5 Results

3.5.1 Effect of EBR on cotton seed germination and seedling growth under salt stress using a culture medium (MS)

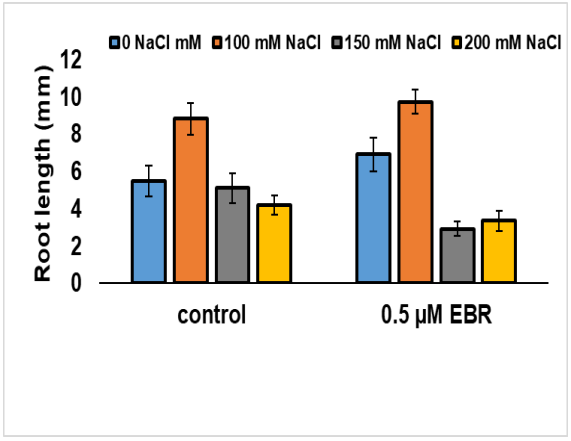
An experiment was conducted to investigate the effect of 0.5 µM EBR on seed germination and seedling growth using a culture medium in the presence and absence of salt (0, 100 mM, 150 mM and 200 mM NaCl). Figure 3-1 shows that the germination rate and root length were significantly reduced in response to salt ($P \leq 0.02$ and $P \leq 0.001$), respectively. There was no significant interaction effect between EBR and salt on germination. However, there was a significant interaction effect between EBR and salt on root length with a possible positive effect of BR at 0 and 100 mM and a negative effect at 150 and 200 mM of salt. Further experiments were carried out to further investigate the effect of EBR on germination and plant growth. Different concentrations of EBR at 0.1 µM, 0.2 µM, 0.5 µM, 1 µM, and 2 µM EBR were used in the presence and absence of moderate (100 mM) and high salt concentrations (150 mM and 200 mM). However, the results of these experiments were consistent where salt significantly reduced germination rate and root length but there was no positive interaction effect between EBR and salt on germination or plant health (data not shown), indicating that the previous positive effect of EBR in the salt experiment was just a variation. Other observations were poor plant growth on the MS medium and that plant variability was too great to assess the effects of EBR.

A: Germination rate



Source	P
EBR	0.219
NaCl	0.024
EBR*NaCl	0.203

B: Root length (mm)



Source	P
EBR	0.735
NaCl	0.001
EBR*NaCl	0.05

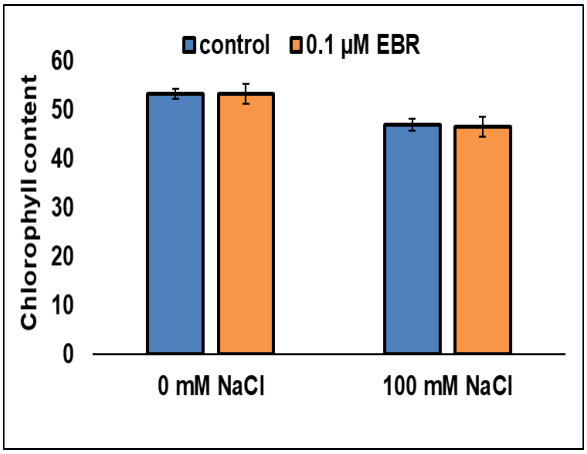
Figure Error! No text of specified style in document.**-3.** The effects of EBR on seed germination number and root length under salt stress using a culture medium. The figure shows the effect of two different concentrations of EBR (0 µM and 0.5 µM) on the growth of seedlings in the presence of four different concentrations of salt (0 mM, 100 mM, 150 mM and 200 mM) using a culture medium. Control plants without EBR or salt were treated with an equivalent solution containing ethanol or water, respectively. Data for germination and root length were collected after 7 days of treatments. The bar graphs represent the mean +/-standard error from three replicates (petri dish) per treatment. Data was analysed using two-way ANOVA statistical software Minitab version 18 to indicate differences between treated and untreated plants.

3.5.2 Effect of EBR on seedling growth under salt stress using hydroponic system

Variation in plant sizes obtained from the previous experiment resulted in difficulty in evaluating the effect of EBR on plant growth. Therefore, three independent experiments were conducted using the hydroponic system to further investigate the effect of EBR on the growth of healthy and uniform plants. In the preliminary experiment, three different concentrations of EBR were used (0, 0.1 µM and 0.5 µM) with and without NaCl (0 mM NaCl, 100 mM NaCl and 150 mM NaCl) to determine the optimal concentration of EBR under salt stress. The plant growth parameters of plant height, chlorophyll content, and shoot and root dry weights were measured to investigate the effects of EBR and salt on seedling growth. The results of my

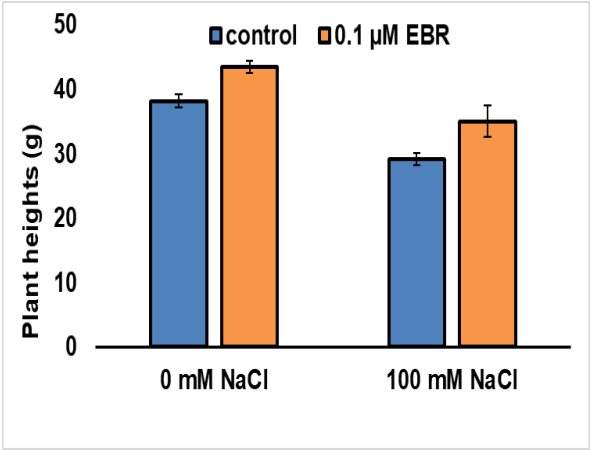
preliminary experiment indicated that plants treated with 0.5 µM EBR showed leaf epinasty and a reduction in growth as compared to control plants, indicating hormonal toxicity at this concentration. The concentration of 150 mM NaCl was too high as plants stopped growing and showed extensive leaf damage, thus both these concentrations were excluded from the next experiments. A second experiment was conducted using 0.1 µM EBR and 100 mM NaCl. Figure 3-2 shows salinity significantly decreased all growth parameters ($P \leq 0.001$). The concentration of 0.1µ MEBR had a possible positive effect on plant height only. In addition, there was no interaction effect between 0.1 µM EBR and 100 mM NaCl on plant growth parameters.

A: Chlorophyll content



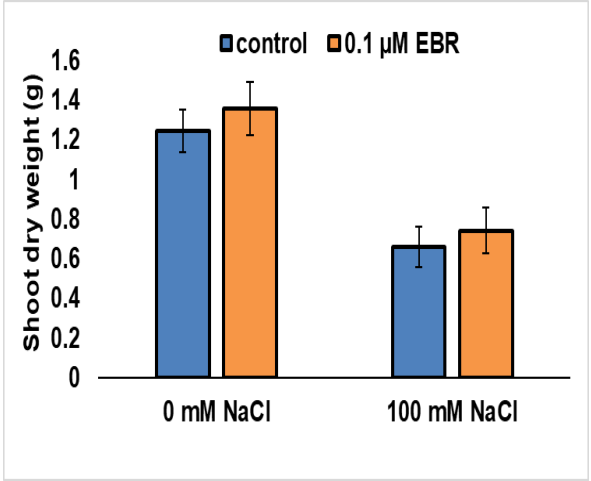
Source	P
EBR	0.85
NaCl	0.001
EBR*NaCl	0.85

B: Plant Height (mm)



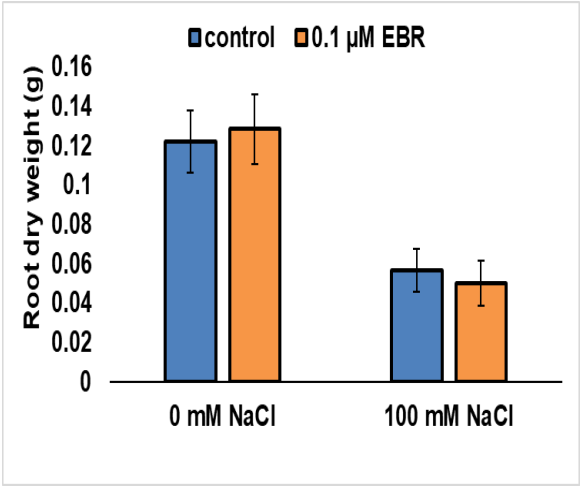
Source	P
EBR	0.001
NaCl	0.001
EBR*NaCl	0.869

C: Shoot dry weight (g)



Source	P
EBR	0.406
NaCl	0.001
EBR*NaCl	0.898

D: Root dry weight (g)

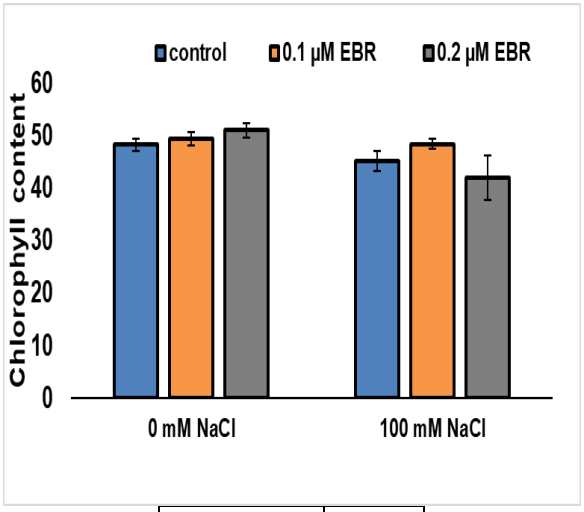


Source	P
EBR	1
NaCl	0.001
EBR*NaCl	0.653

Figure Error! No text of specified style in document.**-4.** Effect of EBR and salt stress on seedling growth without Tween 20 using hydroponic system. This figure shows the effect of 0.1 µM EBR on plant growth under 100 mM salt. Control plants without EBR or salt were treated with an equivalent solution containing ethanol or water, respectively. Bar graphs represent the mean +/- the standard error from six replicate plants per treatment. Data were collected after 16 days of treatments. Data were analysed using two-way ANOVA statistical analysis using Minitab version 18 to indicate significant differences between treated and untreated plants.

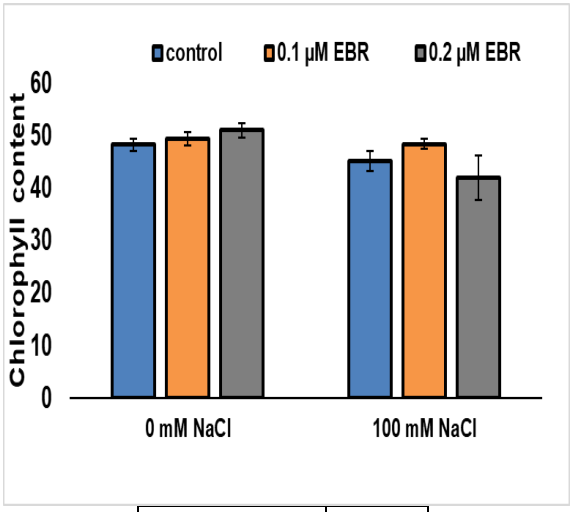
To confirm whether EBR had any effect on plant growth, further experiments were undertaken using 0.2 µM EBR and inclusion of Tween 20 (0.05%) to increase the EBR uptake. The results in Figure 3-3 show that the treatment with salt significantly reduced chlorophyll content and shoot dry weight (both $P \leq 0.01$) as well as plant heights and root dry weight (both $P \leq 0.01$) under the same stress condition. The results also showed that EBR had negative effects on plant heights and root dry weight (both $P \leq 0.01$) under non-saline condition. The results of these experiments were consistent in that the treatment with EBR has no effect on the chlorophyll content, plant heights and shoot dry weight under salt stress. However, in the second experiment when Tween 20 was used, EBR appeared to have a significant negative effect on root dry weight ($P \leq 0.02$) under no salt stress as compared to the control.

A: Chlorophyll content



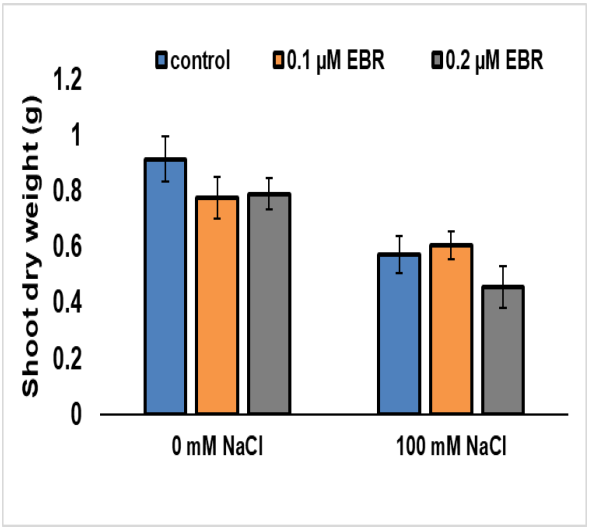
Source	P
EBR	0.469
NaCl	0.019
EBR*NaCl	0.173

B: Plant Height (mm)



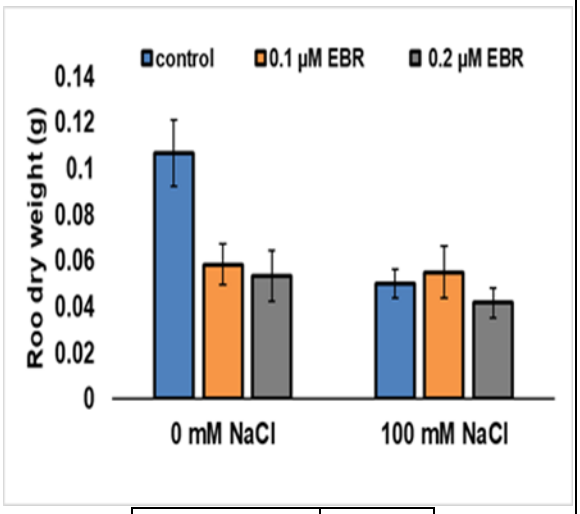
Source	P
EBR	0.012
NaCl	0.001
EBR*NaCl	0.082

C: Shoot dry weight (g)



Source	P
EBR	0.218
NaCl	0.01
EBR*NaCl	0.365

D: Root dry weight (g)



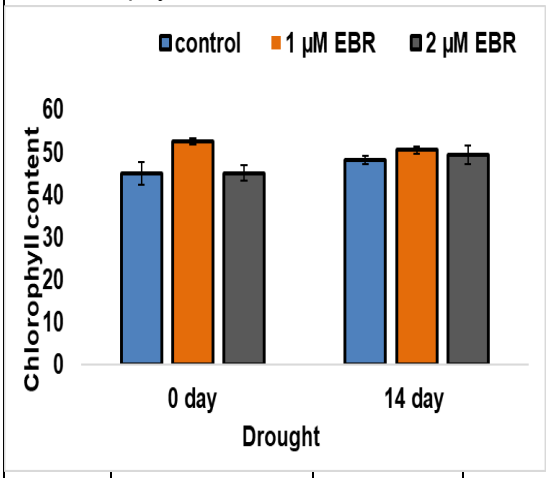
Source	P
EBR	0.014
NaCl	0.001
EBR*NaCl	0.027

Figure Error! No text of specified style in document.**-5.** The effect of EBR and salt stress on seedling growth with Tween 20 using a hydroponic system. The figure shows the effect of (0 µM, 0.1 µM and 0.2 µM) EBR on seedling growth under 100 mM salt. Control plants without EBR or salt were treated with an equivalent solution containing ethanol or water, respectively. Data were collected after 14 days of treatments. Bar graphs represent the mean +/- the standard error from six replicate plants per treatment. Data was analysed using two-way ANOVA statistical analysis using Minitab version 18 to indicate significant differences between treated and untreated plants.

3.5.3 Effect of EBR and drought on seedling growth using a pot trial

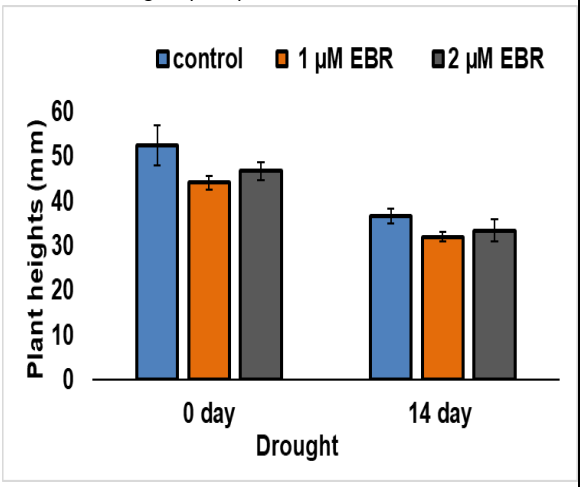
Two different experiments were conducted to investigate the effect of (0µM, 1 µM, and 2 µM) EBR on seedling growth in response to drought. EBR was applied both via seed priming (by incubating in a solution for 72 h) and via foliar spray of the seedlings. In the first experiment, 2 week- old plants were foliar sprayed with EBR solution four times before seedlings were subjected to drought by withholding water for fourteen days. Plants were then allowed to recover by re-watering for five days. The results in Figure 3-4 indicate that plant heights and shoot dry weight were significantly reduced by drought (both $P \leq 0.001$) and root dry weight was also inhibited ($P \leq 0.03$). However, no significant negative effect of drought on plant survival after plants re-watering was observed. There was no significant interaction effect between EBR and drought on all plant growth parameters. Under non-stress conditions, the treatment with 1 µM EBR led to a possible increase in chlorophyll content ($P \leq 0.01$) but significantly decreased plant heights ($P \leq 0.03$). There was no positive effect of EBR on the shoot and root dry weights under the same conditions.

A: Chlorophyll content



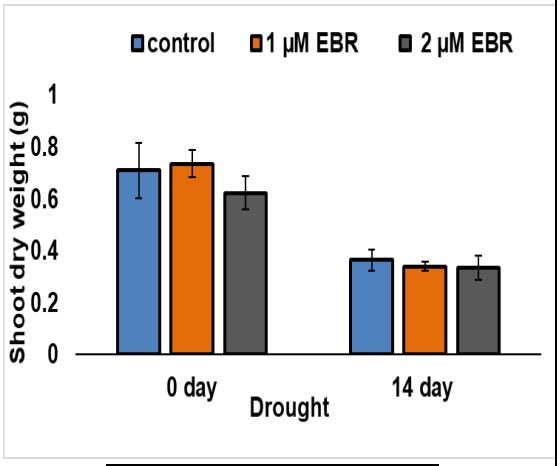
Source	P
EBR	0.012
drought	0.219
EBR*drought	0.16

B: Plant Height (mm)



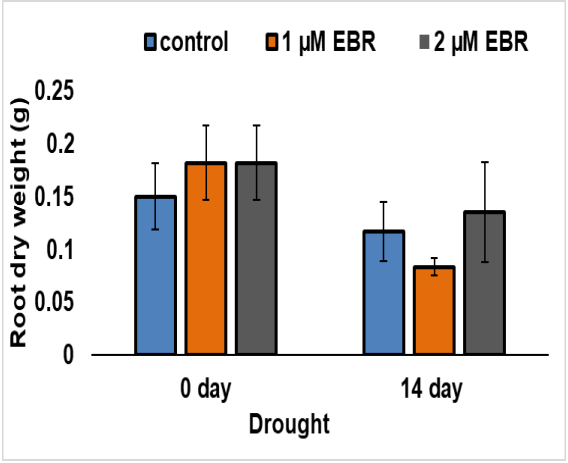
Source	P
EBR	0.037
drought	0.001
EBR*drought	0.75

C: Shoot dry weight (g)



Source	P
EBR	0.551
drought	0.001
EBR*drought	0.682

D: Root dry weight (g)



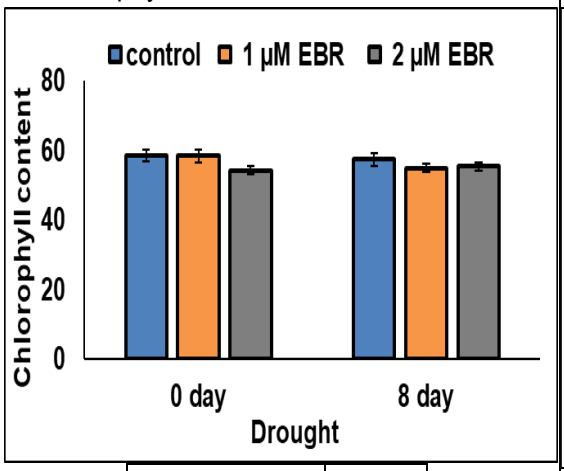
Source	P
EBR	0.676
drought	0.035
EBR*drought	0.587

Figure Error! No text of specified style in document.**-6.** The effect of EBR and drought on seedling growth without Tween 20 using a pot trial. The figure represents the effect of three different concentrations of EBR (0 µM, 1 µM and 2 µM) on seedlings growth in response to drought. Control plants without EBR treated with an equivalent solution containing ethanol. Data were collected after 14 days of subjecting seedlings to drought by withholding water followed by a recovery for 5 days after re-watering. Bar graphs represent the mean+/- the standard error from six replicate plants per treatment. Data was analysed using two-way ANOVA statistical analysis using Minitab version 18 to indicate significant differences between treated and untreated plants.

In the second experiment, the surfactant Tween 20 (0.05%) was added to the foliar spray and seed priming to enhance the penetration and the uptake of EBR by the plant tissue. Plants were exposed to drought stress by withholding water for 8 days and re-watering for 5 days. Figure 3-5 shows the findings for the second experiment. The plant heights and shoot dry weight were significantly reduced by drought (both $P \leq 0.001$). There was no significant interaction effect between EBR and drought on plant growth. Overall, the treatment with 0.1 µM EBR significantly decreased plant heights ($P = 0.016$) under both stress and non-stress conditions as compared to the control. Root dry weight measurement was excluded from the results of the second experiment due to the difficulty of separating the root from the soil.

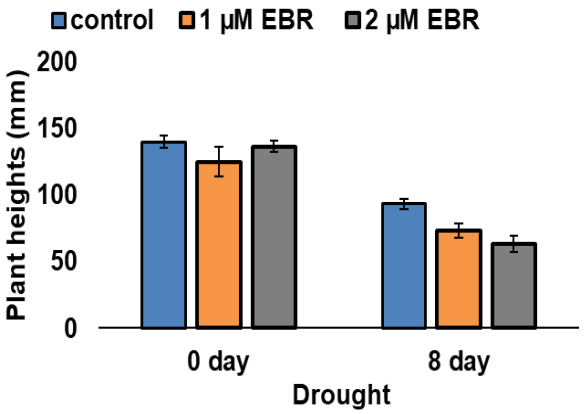
The results of these experiments were consistent in that the drought stress led to a reduction in plant growth and no positive interaction effect between EBR and drought on plant growth was observed.

A: Chlorophyll content



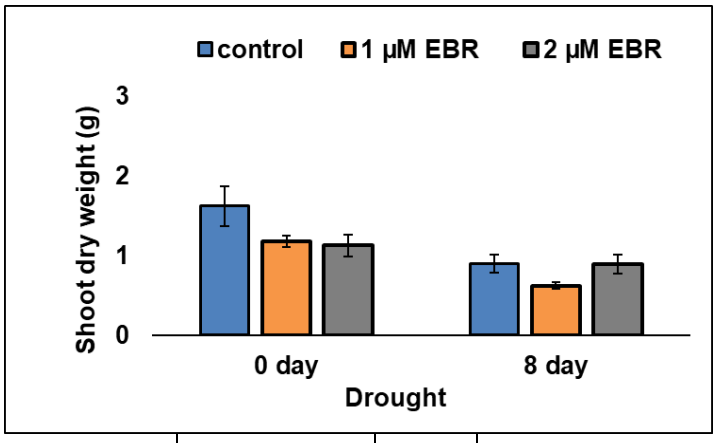
Source	P
EBR	0.11
drought	0.315
EBR*drought	0.301

B: Plant Height (mm)



Source	P
EBR	0.016
drought	0.001
EBR*drought	0.103

C: Shoot dry weight (g)



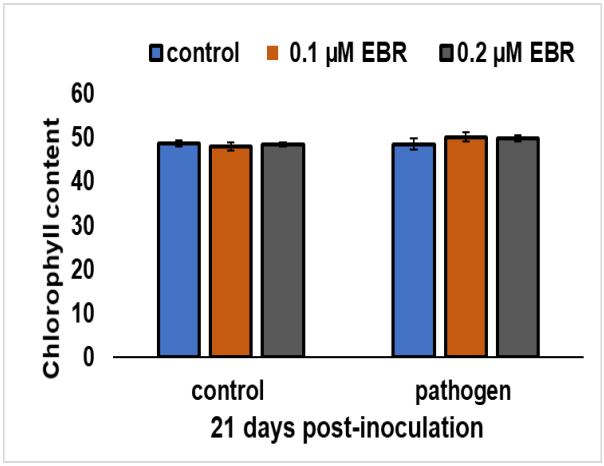
Source	P
EBR	0.067
drought	0.001
EBR*drought	0.285

Figure Error! No text of specified style in document.**-7.** The effect of EBR and drought on seedling growth with Tween 20 using a pot trial. The figure shows the effect of three different concentrations of EBR (0 µM, 1 µM and 2 µM) on seedling growth in response to drought**.** Control plants without EBR treated with an equivalent solution containing ethanol. Data were collected after 8 days of subjecting seedlings to drought by withholding water followed by a recovery for 5 days after re-watering. Bar graphs represent the mean +/- the standard error from six replicate plants per treatment. Data was analysed using two-way ANOVA statistical analysis using Minitab version 18 to indicate significant differences between treated and untreated plants.

3.5.4 Effect of EBR and *V. dahliae* on seedling growth in hydroponic system

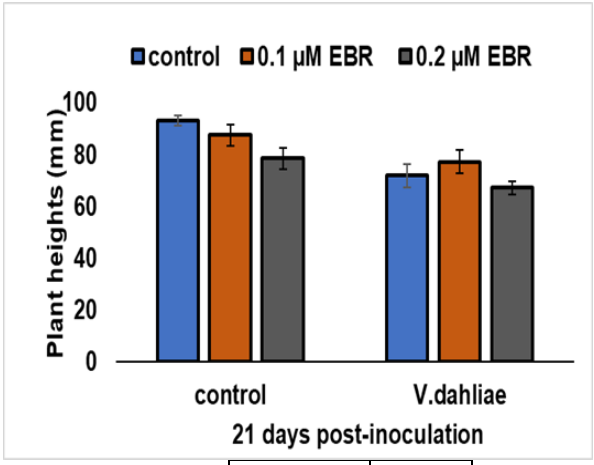
One of the study objectives was to investigate whether EBR application has the potential to increase seedling resistance to the fungal pathogen *V. dahliae.* The roots of young seedlings grown in hydroponic solution were firstly treated with EBR for 24h before being inoculated with a suspension of (1×10^6 conidia per mL of *V.dahliae*) for 0.5h. The same previously mentioned plant growth parameters were monitored in this experiment. Two experiments were conducted in this study. The results of the first experiment showed that the treatment with *V. dahliae* significantly reduced root dry weight only (data not shown), however, no significant negative effect of the pathogen on other growth parameters was observed. Therefore, a second experiment was conducted, extending the root inoculation period with the pathogen for 1h, to ensure that the pathogen infected and colonised the root successfully. In this experiment, Tween 20 (0.05%) was added to improve the uptake of EBR. The results in Figure 3-6 show that the pathogen significantly reduced plant heights and shoot and root dry weight (all $P \leq$ 0.001), respectively, as compared to the control. However, no significant interaction effect between EBR and the pathogen on plant growth was observed. The treatment with 0.1 µM and 0.2 µM EBR significantly reduced plant height ($P \leq 0.02$) and root dry weight ($P \leq 0.001$) under a non-stress condition as compared to the control.

A: Chlorophyll content



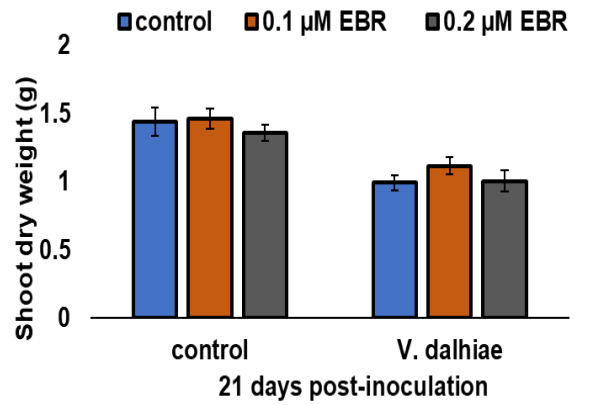
Source	P
EBR	0.784
VD	0.121
EBR*VD	0.411

B: Plant Height (mm)



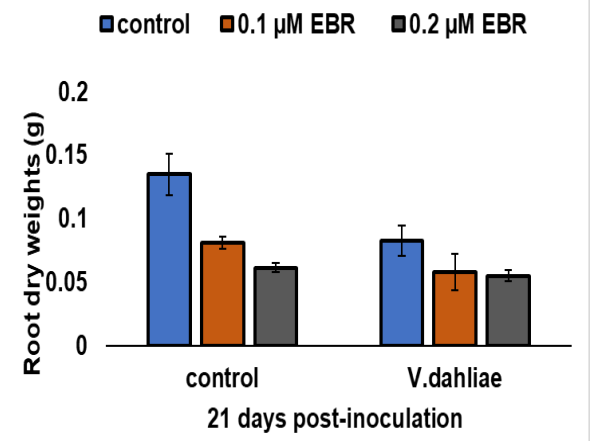
Source	P
EBR	0.025
VD	0.001
EBR*VD	0.317

C: Shoot dry weight (g)



Source	P
EBR	0.323
VD	0.001
EBR*VD	0.74

D: Root dry weight (g)



Source	P
EBR	0.001
VD	0.001
EBR*VD	0.097

Figure Error! No text of specified style in document.**-8.** The effect of EBR and *V. dahliae* on seedling growth with Tween 20 using a hydroponic system. The figure above shows the effect of three different concentrations of EBR (0 µM, 0.1 µM and 0.2 µM) on seedling growth in response to *V. dahliae.* Control plants without EBR treated with an equivalent solution containing ethanol. Data were collected after 21 days of root dip-inoculation with *V. dahliae* suspension for 1h. Bar graphs represent the mean+/- the standard error from six replicate plants per treatment. Data was analysed using two-way ANOVA statistical analysis using Minitab version 17 to indicate significant differences between treated and untreated plants.

3.6 Discussion

3.6.1 Effects of EBR on cotton seed and seedling growth under abiotic and biotic stresses

Plant hormones are known to regulate many processes in plant growth and development, as well as responses to environmental stresses (Yang et al., 2019). EBR is one of the plant hormones that can balance plant growth and resistance to different abiotic and biotic stresses independently or via crosstalk with other plant hormones (Lima & Lobato, 2017; Zou et al., 2018). There is significant evidence from previous studies in various plant species that exogenous BR application can enhance seed yield and stress tolerance (Hayat et al., 2000; Hayat et al., 2010; Thussagunpanit et al., 2015). A better understanding of the regulation and effect of EBR in cotton seedlings has the potential to improve the growth and quality of cotton crops, especially in this current uncertain time of abiotic and biotic stresses. Based on the previous evidence, I hypothesised that the application of EBR has the potential to improve cotton seedlings' tolerance to salinity, drought and pathogen. Therefore, this section aimed to establish a suitable system for testing the effects of EBR on plants' responses to salt stress.

Four different parameters were used to assess plant health under salt, drought and pathogen stresses: chlorophyll content, plant height, and shoot and root dry weight. In the salt experiment, MS medium was used to investigate the effect of EBR and salt on seed germination and seedling growth. However, plants grew poorly on MS medium and plant variability was too great to enable assessment of EBR effects. Therefore, a hydroponic system was used to assess seedling growth under salt stress. Exogenous EBR was applied to the cotton plant 24 hours before salt was added to the hydroponic solution. The hydroponic system was chosen mostly for the above-mentioned reasons and because it is difficult to impose defined salt concentrations/osmotic stress on plants grown in soil. Three different concentrations of salt were used to investigate cotton seedling responses to salt: 100, 150 and 200 mM NaCl. The results showed that 150 and 200 mM NaCl had a severe toxic effect on plant growth, as plants stopped growing and showed extensive leaf damage. The treatment with 100 mM NaCl caused a significant reduction in plant growth and was considered a more meaningful system for testing the possible effects of EBR on the response of cotton seedlings to salt stress.

To test the effect of EBR on drought-stressed plants, plants were grown in pots where three different concentrations of EBR (0, 1 and 2 µM) were applied as a foliar spray. Plants were

also exposed to drought stress by withholding water for 14 days (without Tween 20) or eight days (with Tween 20), followed by re-watering for five days. In this experiment, I observed a significant effect of drought on plant growth, but all drought-stressed plants were able to recover (survived) following re-watering.

In the pathogen experiment, the hydroponic system made it possible to treat the seedlings with a reproducible level of inoculum of *V. dahliae*. The results of the first experiment showed that the 30-minute treatment with the pathogen was ineffective, as no reduction in plant growth in response to the pathogen was observed. Therefore, there was a need to extend the exposure time to one hour with pathogen spores to attain infected seedlings.

Our results showed that the exogenous application of EBR had no positive effect on plant growth under mild yet effective salt, drought and *V. dahliae* stresses (Figures 3-1, 3-2 and 3-4). Further, the results showed that, under a non-stress condition, there was a negative effect of EBR on plant growth when Tween 20 was used (Figures 3-3, 3-5 and 3-6). Our findings disagree with many previous studies and are inconsistent with our hypotheses. A study by Shu et al. (2017) suggested that the application of EBR alleviates the negative effect of a high concentration of 200 mM NaCl. Their data showed that the concentration of 0.1 mM EBR increased the expression of differently expressed genes (DEGs) in the leaves and roots of salt-stressed plants (Shu et al., 2015). In their experiment, they investigated the effects of high concentrations of 0.1 mM EBR and 200 mM NaCl, which is unlikely to be encountered in the field. In my experiment, I investigated the effects of 0.5 µM EBR on plant growth in response to moderate to high concentrations of 150 and 200 mM NaCl. In my experiment, I observed toxic effects at only 0.5 µM, indicated by leaf epinasty and reduction in plant growth, compared with the control. Further, my findings showed that plants ceased growing after the treatment with moderate concentration compared with the control plants. Despite the interesting results of Shu et al. (2017), it is essential to determine whether more physiologically realistic concentrations of EBR are able to improve the outcomes for plants grown in the field under more realistic salinity conditions.

My results indicated that there were no positive effects on plant growth when EBR was supplied by soaking seeds or spraying leaves or supplied via roots through hydroponic solution. A study by Janeczkoa and Swaczynová (2010) investigated the effect of different levels of EBR on the uptake and content of endogenous BRs in wheat seedlings using different delivery methods. Researchers have reported positive effects of EBR on plant growth when EBR is

applied by soaking seeds and drenching plants, as compared with EBR applied by spraying seedlings. A higher content of endogenous EBR in the leaves of plants treated with 2 µM EBR was observed. However, there were positive effects of 0.1 µM EBR on plant growth only when EBR was applied by drenching (Janeczkoa & Swaczynová, 2010). Contrary to these previous results, my results indicated a negative effect of 2 µM EBR on seed germination when EBR was applied by soaking seeds (data not shown). Similar to the previous results, my results also showed that the application of 0.5 µM EBR using cultured medium significantly increased the root length of seedlings and there was a possible interaction effect between 0.5 µM EBR and 100 mM NaCl on root length under stress and EBR treated plants, as compared with the control. My results also indicated no positive or negative effect of 0.1, 0.2, 1 or 2 µM EBR (with tween) on plant growth when EBR was applied by soaking seeds, spraying seedlings and hydroponic solution.

In addition, another study by Chakma (2016) showed that the application of 1 and 2 µM EBR applied via soaking seed for six hours and foliar spraying of two-week-old seedlings had positive effects on plant survival under drought stresses in cotton plants. However, a critical observation of the data revealed that plants were subjected to severe drought stress, which eventually led to plant death. Therefore, it is difficult to determine whether plant survival was because of EBR or re-watering. However, in our experiment, I found that there was no positive effect of EBR on the growth of moderate drought-stressed plants where it was observed that all drought-stressed plants survived.

A previous study by Li et al. (2008) on the effects of EBR on *Robinia pseudoacacia* seedlings under three different watering regimes—normal water (17–18% soil moisture), mild water stress (12–13% soil moisture) and severe water stress (7–8% soil moisture)—also suggested that the response of the Robinia seedlings to EBR treatment varied depending on the EBR concentration and application delivery mothed. They further clarified that in pot experiment, soaking roots in 0.4 µM EBR followed by a foliar spraying application of 0.2 µM EBR increased the growth of seedling in response to drought stress as compared to control plants. Moreover, there is a need to quantify soil moisture to determine the severity of the drought in cotton in relation to the concentration for EBR uptake, given that Li et al. (2008) suggested that the optimal concentration for EBR uptake depends on the severity of the drought. They also found no significant effect of EBR under mild water stress, but a positive significant effect of EBR under severe water stress. Another independent study by Shu et al. (2015) found that the

root-applied BL through nutrient solution was able to eliminate the negative effect of high salt concentration of 200 mM NaCl on cotton growth. The treatment with BL increased the expression of the salt-responsive genes involved in various physiological responses, leading to growth recovery in plants.

A study by Nahar et al. (2013) suggested that BR mechanism to induce susceptibility or resistance to pathogens depends on the concentration and timing of EBR, along with the involvement of the activation or suppression of other hormone pathways. Their data showed that high EBR concentrations of 5 and 10 µM EBR sprayed on 15-day-old seedlings resulted in plant resistance to *M. graminicola*, while spraying the plants with low EBR concentrations (0.1 and 1 µM) promoted plant susceptibility. Further, either concentration of EBR in both BR-deficient d2 mutants and wild-type T65, an up- or down-regulation of BR biosynthesis was always antagonistic with a down- or upregulation of the JA pathway, respectively, confirming that, in rice roots, BR and JA mutually antagonise each other's signalling pathway (Nahar et al., 2013). These results point to the complexity of the exogenous application of BRs regarding the delivery method, hormone uptake by transport, optimal concentration, plant age, difficulty of penetration, specificity of site action and type of stress. These factors need to be explored to stimulate the plant nature for stress response.

Chapter 4. The Structure, Phylogeny and Prediction of Subcellular Localisation of Calmodulin-binding Protein 60 (CBP60) in Cotton *G. hirsutum*

4.1 Introduction to the discovery of CBP60 in cotton *G. hirsutum*

Chapter 4 describes the second set of experimental projects for this dissertation that investigates the CBP60 protein family in cotton and its relationship to the characterised CBP60 protein family in Arabidopsis. To date, there is no comprehensive study on CBP60 protein in cotton. This gap needs to be filled to understand the role of CBP60 proteins in response to biotic and abiotic stresses in cotton.

4.2 The structure of CBP60 in Arabidopsis

CBP60s are one of the best well-characterised CaM-binding transcription factors. They are specific to plants with no homology to any other known proteins (Reddy et al., 2000; Zhang et al., 2010). The eight members of the CBP60 proteins in Arabidopsis (AtCBP60a-g and AtSARD1) were characterised according to their sequence similarities with tobacco and maize homologs (Dash et al., 1997; Reddy et al., 1993). The phylogenetic analysis of AtCBP60a-g and AtSARD1 revealed that this family is comprised of two major groups. Group 1 contains three proteins AtCBP60a-g and AtSARD1 that are clustered together in one branch (Wang et al., 2011). Group 2 contains five proteins; within this, AtCBP60b/c/d are clustered together in one sub-branch, whereas AtCBP60e/f proteins form the other sub-branch (Wang et al., 2011). The AtCBP60 proteins contain two features common to transcription factors; a DNA-binding domain and regulatory domain. The amino acid sequences of the CaM-binding regulatory domain are quite divergent (Wang et al., 2009). Five family members, AtCBP60a/b/c/d/e/f contain a CaM-binding domain at the C-terminal end (L. Wang et al., 2009). AtCPB60g lacks this C-terminal domain, however it was found to bind to CaM through an N-terminal domain (Wang et al., 2009). Unlike its close homologue AtCBP60g, AtSARD1 which is also known as SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1 (SARD1), has no CaM-binding domain (Zhang et al., 2010). AtCBP60g and AtSARD1 contain conserved DNA-binding domains located in the middle region of the proteins (Zhang et al., 2010). The DNA-binding

region of AtCBP60g is located between amino acids (aa) 148 and 263. Similarly, the DNA-binding region of AtSARD1 protein is in the region 149-214 aa (Zhang et al., 2010). The subcellular localisation study showed that AtCBP60g protein is located in the nucleus (Qin et al., 2018). The DNA-binding regions of AtCBP60 a/b/c/d/e/f have not been located. CBP60 proteins appeared to mediate responses to biotic and abiotic stresses. At least four genes encoding *Phaseolus vulgaris*, green bean CBP60c/d were strongly induced in response to *Pseudomonas syringae* (Ali et al., 2003). In Arabidopsis, CBP60a was a negative regulator of immunity as the cbp60a mutant reduced the growth of bacterial pathogen *P. syringae* (Truman et al., 2013). Mutant proteins that lacked the CaM-binding domain failed to complement the Salicylic Acid (SA) and defence defects of AtCBP60a loss-of-function mutant. AtCBP60a was also found to bind calmodulin at the C-terminal end of the protein (Truman et al., 2013). Calmodulin-binding ability is required for the function of AtCBP60a to control the production of SA and defence (Truman et al., 2013). Two other CaM-binding proteins AtCBP60g and AtSARD1 were identified to have a role in the induction of plant defence responses and enhance the SA production (Wang et al., 2011). These proteins were found to bind DNA via a binding domain which is highly conserved, leading to the expression of specific genes (Wan et al., 2012). In another study, AtCBP60g was also implicated in disease resistance against *P. syringae*. The CaM-binding region of the protein is required for the activation of SA defence signalling during the microbe-associated molecular pattern (MAMP) response (Wang et al., 2009). In addition, the over-expression lines of AtCBP60g appeared to positively regulate the ABA-mediated pathway leading to improved drought tolerance as compared to control (Wan et al., 2012).

4.3 Hypotheses and aims

Based on the existing results, which indicate a significant role of the CBP60 gene family in mediating biotic and abiotic stress in bean and Arabidopsis, I aimed to identify members of the CBP60 gene family in cotton, *G. hirsutum*. The recent release of cotton genome sequences via the publicly available database COTTONGEN provides a useful tool to perform a comprehensive analysis of putative *CBP60* genes in cotton (Altschul et al., 1997; Li et al., 2015; Zhang et al., 2015). A phylogenetic tree using the Neighbour-Joining method can be used to find GhCBP60 orthologues of AtCBP60 (Saitou & Nei, 1987). Furthermore, multiple sequence analysis using Multalin tool (http://multalin.toulouse.inra.fr/multalin/) (version 5.4.1) (Corpet, 1988) can also be used to predict the presence and/or absence of DNA and

CaM-binding domains in cotton CBP60 (GhCBP60). In addition, Cello (http://cello.life.nctu.edu.tw/) and Bacello (http://gpcr.biocomp.unibo.it/bacello/) software can be used to predict the subcellular localisation of GhCBP60 proteins in the nucleus or other departments of the cell. The prediction of subcellular nuclear localisation is important to understand the function of the hypothetical proteins, because if these proteins are transcription factors like AtCBP60, then they will be localised to the nucleus when they control transcription. The prediction of subcellular nuclear localisation is important not only to understand the function of individual proteins but also for its important role in regulating the activity of transcription factors in response to environmental stimuli.

The hypotheses for this chapter are:

1. Cotton has a GhCBP60 gene family that has orthologues to the major groups of AtCBP60.
2. GhCBP60 proteins have CaM- and DNA-binding domains similar to Arabidopsis orthologues.
3. GhCBP60 genes encode transcription factors with nuclear localisation signal sequences.

The objectives of this study are therefore to:

1. Identify putative CBP60 orthologues in *G. hirsutum* using the publicly available COTTONGEN database and investigate the phylogenetic relationship between AtCBP60 and GhCBP60 proteins.
2. Characterise GhCBP60 proteins for the presence of conserved CaM- and DNA-binding regions by detecting evolutionarily conserved amino acids using a multiple sequence alignment software.
3. Use bioinformatics software to predict the subcellular localisation of GhCBP60 proteins; (http://gpcr.biocomp.unibo.it/bacello/).

4.4 Material and methods

4.4.1 Identification of the *AtCBP60* gene family in *G. hirsutum*

CBP60-related sequences of Arabidopsis CBP60 (AtCBP60) obtained from the Arabidopsis Information Resource (TAIR) online database (Rhee et al., 2003), were used as queries to

identify related sequences via BLASTP in the *Gossypium hirsutum* proteome accessed via Phytozome v10.3 (Goodstein et al., 2012) (Table 4-1). The entire protein sequences of GhCBP60 were downloaded following BLASTP search of cotton *G. hirsutum* NCBI database using COTTONGEN database (https://www.cottongen.org/tools/blast/blast) (Altschul et al., 1997). Six orthologous sequences of *Physcomitrella patens*, a moss species that signifies basal lineage of land plants were also obtained using Phytozome 1.3 (Goodstein et al., 2012).

4.4.2 Phylogenetic analysis of GhCBP60 proteins

A total of 23 putative GhCBP60 protein and eight AtCBP60 protein sequences were aligned using MUSCLE program (Edgar, 2004), and the phylogenetic tree was constructed using MEGA6 program (Tamura et al., 2013). The relationship between these proteins was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985). I chose only one of the six *P. patens* CBP60-related sequences, Phpat-002G082900 to root our phylogenetic tree.

4.4.3 Multiple sequence analysis of GhCBP60 proteins and secondary structure prediction

The amino acid sequence of each AtCBP60 protein was aligned with corresponding sequences of GhCBP60 using Multalin tool (http://multalin.toulouse.inra.fr/multalin/) (version 5.4.1) (Corpet, 1988) to identify conserved CaM- and DNA- binding motifs in the GhCBP60 protein sequences. For the online tool, all default parameters were kept except for maximum line length of amino acids (aa); this was adjusted from 130aa to 200aa. Comparative alignment analysis of the amino acid sequences of AtCBP60 proteins and their corresponding GhCBP60 proteins was carried out using the CLUSTAL OMEGA (ClustalO) tool by selecting the output format to Pearson/FASTA (https://www.ebi.ac.uk/) (Jenkinson et al., 2008). Secondary structure prediction performed using the JPRED method to compare the secondary structures of the CaM-binding domains of AtCBP60 proteins to the putative CaM-binding domains of GhCBP60 proteins (http://www.compbio.dundee.ac.uk/jpred4/index_up.html) (Drozdetskiy et al., 2015).

4.4.4 Prediction of subcellular localisation of GhCBP60

The protein sequences of 23 GhCBP60 were used to determine whether these proteins contain a predicted nuclear localisation signal using BaCello (http://gpcr.biocomp.unibo.it/bacello/) (Pierleoni et al., 2006) and Cello (http://cello.life.nctu.edu.tw/) (Yu et al., 2004). The results of subcellular localisation prediction analysis were supported by similar studies using *G. raimondii* and *G. arboretum*.

4.5 Results

4.5.1 Identification of *AtCBP60*-related gene family in *G. hirsutum*

Sequences of the eight-membered AtCBP60 were used to query *G. raimondii, G. arboretum, and G. hirsutum* genomes for related sequences via BLASTP. A total of 11, 9, and 23 of CBP60-related sequences were identified in *G. raimondii, G. arboretum, and G. hirsutum*, respectively (Table 4-1). I proposed names for GhCBP60 genes/proteins—refer to Table 4-2. Six related homologous to AtCBP60s proteins were identified in *Physcomitrella patens* (Table 4-2).

Table 4-1. Gene IDs of *AtCBP60*-related sequences in *G. raimondii*, *G. arboreum*, *G. hirsutum,* and *P. patens.*

Arabidopsis TAIR database	***G. raimondii* Phytozome version 10.3 database**	***G. arboreum* NCBI blast database**	***G. hirsutum* COTTONGEN database**	***P. patens* Phytozome version 10.3 database**
AT5G57580 (CBP60b) AT2G18750 (CBP60c) AT4G25800 (CBP60d) AT2G24300 (CBP60e) AT4G31000 (CBP60f) AT5G62570 (CBP60a) AT5G26920 (CBP60g) AT1G73805 (SARD1)	Gorai.011G022600 (CBP60b/c/d) Gorai.004G031000 (CBP60b/c/d) Gorai.009G173400 (CBP60b/c/d) Gorai.010G254300 (CBP60b/c/d) Gorai.004G291200 (CBP60e/f) Gorai.003G109800 (CBP60a) Gorai.008G297800 (CBP60a) Gorai.004G237500 (CBP60g) Gorai.013G128100(CBP60g) Gorai.006G059900 (SARD1) Gorai.008G287500 (SARD1)	KHG10046 (CBP60b/c/d) KHG17962 (CBP60b/c/d) KHG27572 (CBP60b/c/d) KHG24590 (CBP60b/c/d) KHG21283 (CBP60e/f) KHG14637 (CBP60a) KHG25212 (CBP60a) KHG14364 (CBP60g) KHG01964 (SARD1)	Gh_D05G1575 (CBP60b/c/d) Gh_A05G1410 (CBP60b/c/d) Gh_A06G1790 (CBP60b/c/d) Gh_D06G2188 (CBP60b/c/d) Gh_D08G0271(CBP60b/c/d) Gh_A08G0194 (CBP60b/c/d) Gh_A10G0202 (CBP60b/c/d) Gh_A13G2354 (CBP60b/c/d) Gh_D13G2214 (CBP60b/c/d) Gh_A08G2253 (CBP60e/f) Gh_D08G2619 (CBP60e/f) Gh_D03G0984 (CBP60a) Gh_A03G0544 (CBP60a) Gh_D12G2633 (CBP60a) Gh_A12G2506 (CBP60a) Gh_D08G2192 (CBP60g) Gh_A08G1834 (CBP60g) Gh_D13G1162 (CBP60g) Gh_A13G0918 (CBP60g) Gh_A12G2425 (SARD1-12A) Gh_A09G0482 (SARD1-9A) Gh_D12G2533(SARD1-12D) Gh_D09G0489(SARD1-9D)	Phpat.010G010700 Phpat.017G054600 Phpat.014G075500 Phpat.001G115400 Phpat.002G044700 Phpat.002G082900

4.5.2 Phylogenetic analysis of GhCBP60 proteins

The alignment of full-length protein sequences of both AtCBP60 and GhCBP60 revealed a tree with two major clades. Clade 1 contains AtCBP60a-g and AtSARD1. Each Arabidopsis CBP60 has four co-orthologous cotton proteins; with two derived from the A-genome and the other two derived from the D-genome. Clade 2 contains AtCBP60b/c/d/e/f with eleven homologues from cotton. AtCBP60b/c/d/ proteins are clustered in one sub-branch with nine homologues from cotton, five from A-genome and four from D-genome. AtCBP60e/f are clustered in another sub-branch with two homologous proteins in cotton, one derived from A-genome and the second derived from D-genome (Figure 4-1). High bootstrap values of major sub-branches CBP60a versus CBP60g-SARD1 indicate the relationships in Clade 1 are highly reliable. In Clade 2, the relationships between CBP60b/c/d are less reliable as some bootstrap values are lower, however, the bootstrap values of major sub-branches CBP60e/f versus CBP60b/c/d indicate high reliability. The tree was rooted with one of the *Physcomitrella patens* (Phpat-002G082900), as shown in Figure 4-1.

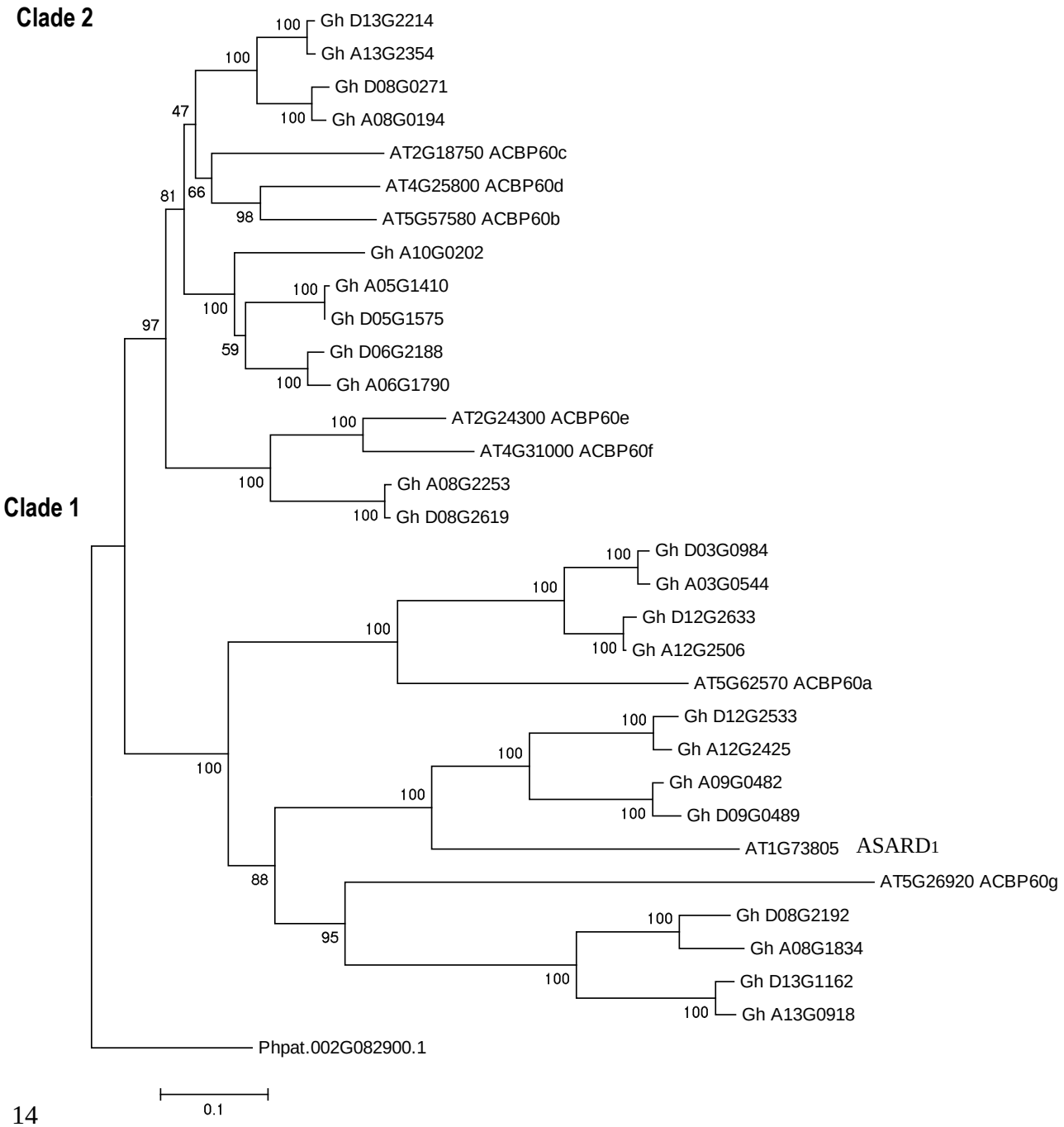


Figure 4-1. Phylogenetic analysis of the CBP60 protein family in Arabidopsis and cotton. The protein sequences of AtCBP60 were obtained from TAIR and of those of the GhCBP60 were obtained from COTTONGEN database. The phylogenetic tree was constructed by the Neighbour-Joining method using MEGA6, after alignment of the CBP60 sequences using MUSCLE. The tree was rooted with the moss homologue Phpat.002G082900. Bootstrap values from 500 replicates are shown at each node. Scale bar indicates 0.1 amino acid substitutions per site.

4.5.3 Evolutionary conservation of CaM- and DNA-binding domains in GhCBP60

The full-length protein sequence of each subgroup of AtCBP60 was aligned with its corresponding GhCBP60 sequences based on phylogeny using the Multalin tool to investigate whether previously identified functional domains in Arabidopsis proteins were conserved in cotton proteins.

The CaM-binding domain of AtCBP60a has been located between amino acids 555 -586 (Wang et al., 2009) and is shown in green (Figure 4-2). In the cotton orthologues of GhCBP60a, a total of 12 out of 31 amino acids (aa) in this domain are highly conserved (>90%), indicated in red. A further 10 out of 31 amino acids are partially conserved (>50%), shown in blue. The results of JPRED secondary structure prediction suggested the CaM-binding domain of AtCBP60a and GhCBP60a to be an alpha helix (Figures 4-4A and 4-4B). The results of ClustalO analysis in Figure 4-4C revealed that the predicted alpha-helical CaM-binding domain which is located between the RWTK motif and YVKL motif is highly conserved in GhCBP60a as shown by red hydrophobic residues. The DNA-binding region of AtCBP60a has not been studied. However, it is noticeable that the N-terminus and central regions between amino acids 1-400 are highly conserved. The total number of conserved amino acids in these regions are 230 out of 399 (>90%). To predict the DNA-binding domain of GhCBP60a, the protein sequences of GhCBP60a and their correspondent AtCBP60a are aligned with AtCBP60g. The DNA-binding domain of AtCBP60g has been located in the middle region of the protein sequence between amino acids 171-287 and is shown in blue (Zhang et al., 2010) (Figure 4-3). In the hypothetical DNA-binding domain of GhCBP60a, there is a total of 59 out of 115 amino acids that are highly conserved (90%) and a total of 28 out of 115 amino acids are partially conserved (50%). Among the cotton orthologues of GhCBP60a-3A/D, 12A/D, the predicted DNA-binding domain has a total of highly conserved amino acids of 85 out of 115.

The CaM-binding domain of AtCBP60g has been located at amino acids 27 to 52 (Wang et al., 2009) and is highlighted in yellow (Figure 4-5). The results of the secondary structure prediction of the AtCBP60g and GhCBP60g groups indicated that the N-terminal of proteins—where the predicted CaM-binding domain of AtCBP60a between RNLT and FMIQ motif and of AtCBP60g between RRAT and VLNL motif is located—is helix, but with gaps and insertions in its sequences (Figures 4-6A and 4-6B). The results of Multalin analysis and ClustalO analysis (Figures 4-5 and 4-6C) indicated that the N-terminal of the proteins contains a very low number (six) of highly conserved amino acids in Multalin, and only four amino acids of the CaM-binding domain of

GhCBP60g proteins were found to be similar to AtCBP60g. As a result of the similarities between the C-terminal region of AtCBP60a and GhCBP60g, I compared the C-terminal region of AtCBP60a with GhCBP60g. The results of the secondary structure prediction indicated that the CaM-binding domain of AtCBP60a between the RRAT and VLNL motif and the CaM-binding domain of GhCBP60g between the MGES and RRRL motif is predicted to be part of an alpha helix, while the other half is beta sheet for both AtCBP60a and GhCBP60g (Figures 4-6C and 4-6D). The results of ClustalO analysis in Figures (4-6E and 4-6F) showed that there are greater similarities between the C-terminal of the CaM-binding domain of AtCBP60a and the C-terminal of GhCBP60g than between the N-terminal of the CaM-binding domain of AtCBP60g and the C-terminal of GhCBP60s. The DNA-binding region of AtCBP60g has been located between amino acids 149-214 (Zhang et al., 2010) and is highlighted in purple in Figure 4-5. A total of 73 out of 115 amino acids are highly conserved (>90%). Among the cotton orthologues, GhCBP60g-8A/D, 13A/D, the total of highly conserved amino acids is 102 out of 115. The DNA-binding region is the most conserved region of the protein as compared to the region immediately upstream between 53 and 148, where 51 out of 95 amino acids are highly conserved (>90%). The region immediately downstream is also less conserved; between amino acids 265-400, 61 out of 135 amino acids are highly conserved (>90%). The DNA-binding domain appears to be more conserved than the CaM-binding domain.

The DNA-binding domain of AtSARD1 is also located in the central region of the protein between amino acids 149-214 (Zhang et al., 2010) and is highlighted in blue (Figure 4-7). The DNA-binding domain in the cotton orthologues, SARD1-9A/D, 12A/D, a total of 46 out of 65 amino acids are highly conserved (>90%). A further 14 out of 65 amino acids are partially conserved (>50%). The results also revealed that the hypothetical DNA-binding domain is highly conserved in GhCBP60g-8A/D, 13A/D than SARD1-9A/D, 12A/D. Our results also showed that GhSARD1 contains highly conserved DNA domains without CaM-binding domain similar to AtSARD1.

The CaM-binding domain of AtCBP60b/c/d is located at the C-terminus of the proteins between amino acids 641-669 (Wang et al., 2009) and is highlighted in red (Figure 4-8). The CaM-binding domains of GhCBP60b/c/d-5A/D,6A/D,8A/D,10A,13A/D have a total of 17 out of 24 amino acids that are completely conserved (>90%) and 6 out of 24 amino acids are partially conserved (>50%). The region immediately upstream of the CaM-binding domain between amino acids 400-640 is less conserved than the potential CaM-binding region as only 30 out of 240 amino acids are highly conserved (>90%).

The CaM-binding domains of AtCBP60e/f have been mapped to the region of the protein between amino acids 589-613 (Wang et al., 2009) and are highlighted in blue (Figure 4-9). A total of 22 out of 24 amino acids are highly conserved in the CaM-binding domain of GhCBP60f-8A/D (>90%). The region immediately upstream between amino acids 400-588 is less conserved than the potential CaM-binding region as only 59 out of 188 amino acids are highly conserved (>90%). However, the N-terminus and middle regions of the protein between amino acids 53-400 are highly conserved; 257 out of 347 amino acids are completely conserved (>90%).

Clade 1

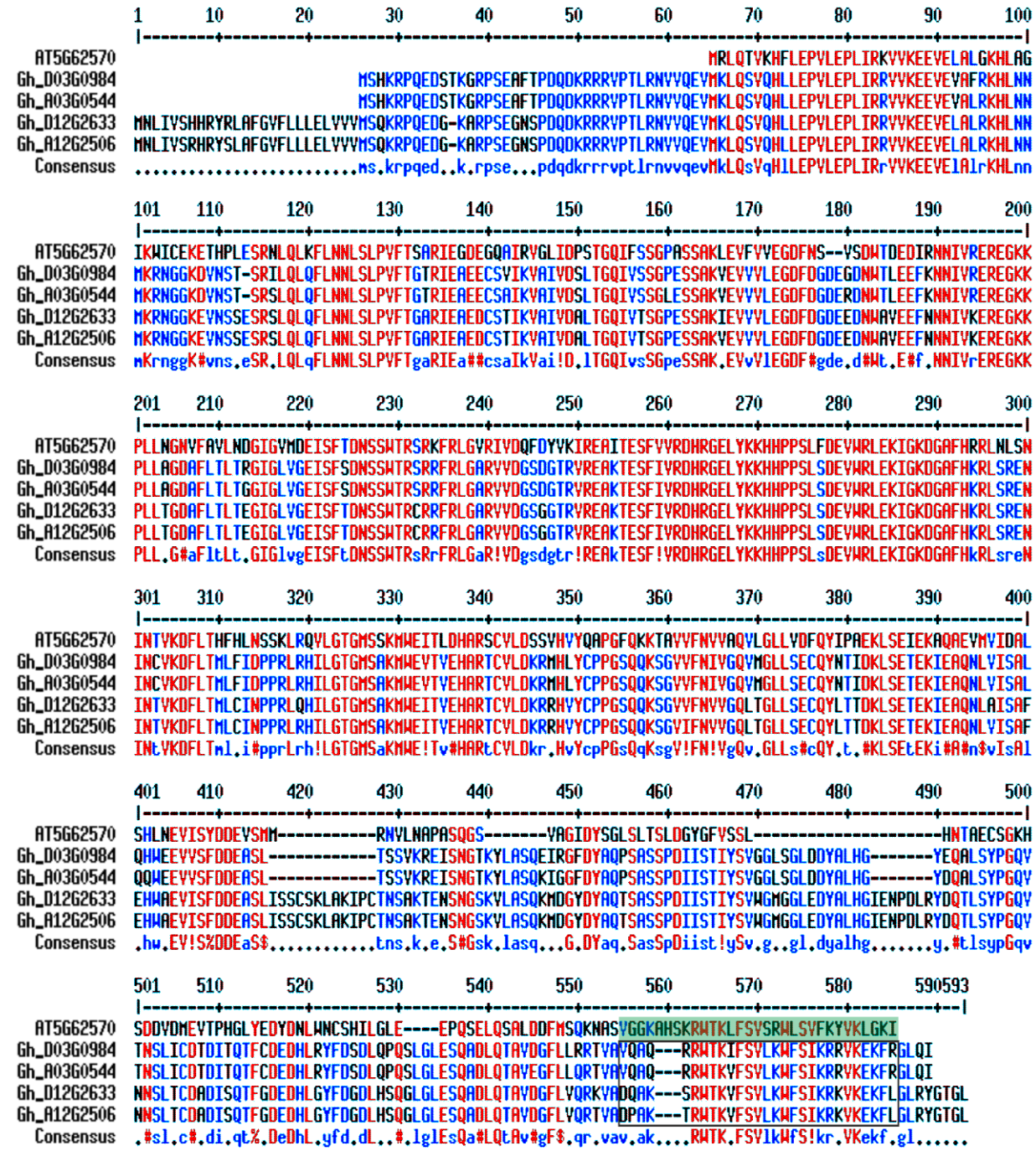


Figure 4-2. Multiple sequence alignment of AtCBP60a and GhCBP60a3A/D,12A/D. The CaM-binding region of AtCBP60a is located at C-terminus and is highlighted in green (Wang et al., 2009). The hypothetical CaM-binding domains of GhCBP60a are located at the C-terminus and are enclosed within the black box.

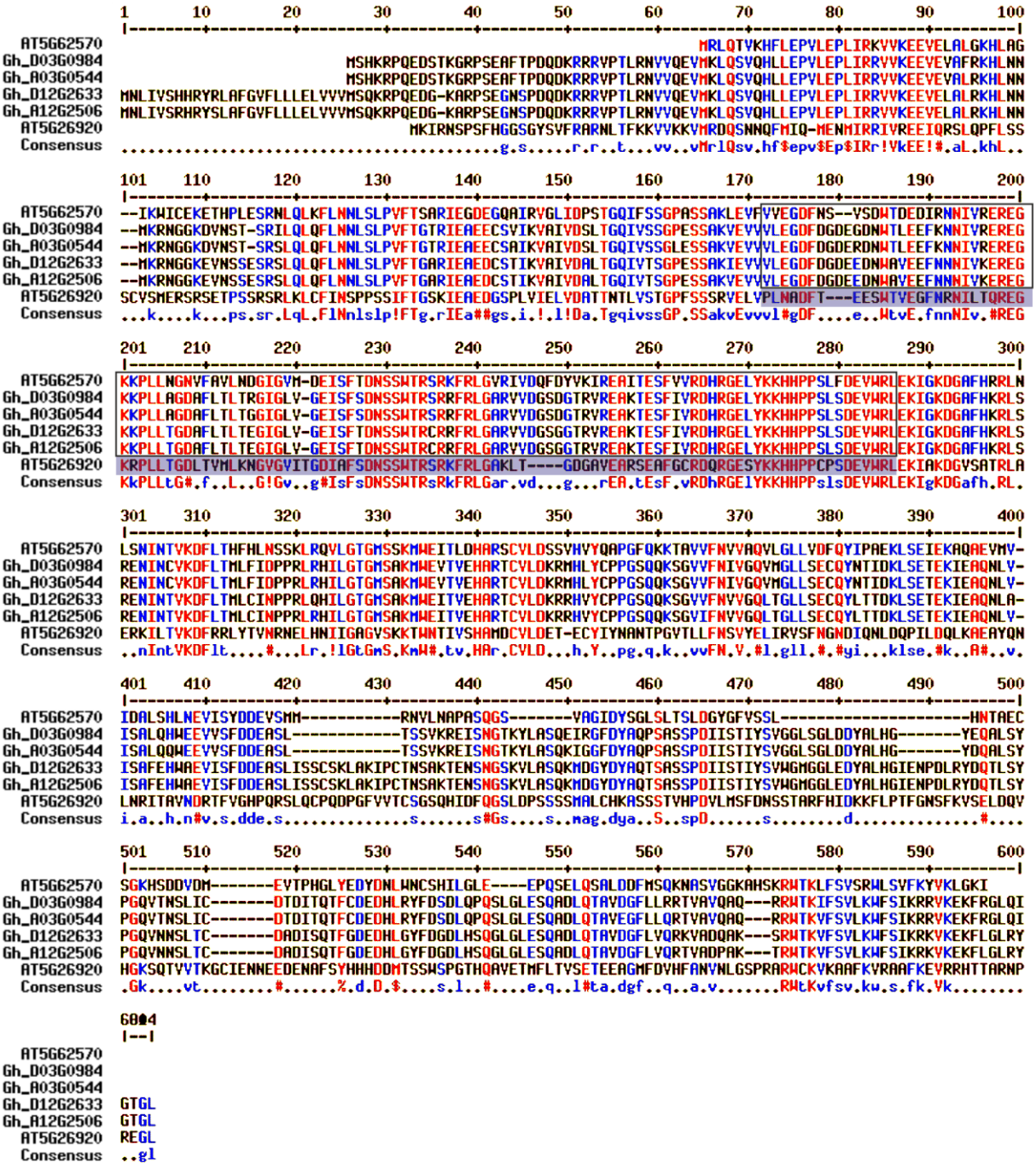


Figure 4-3. Multiple sequence alignment of AtCBP60a-g and GhCBP60a. The DNA-binding region of AtCBP60g is located in the middle region of the protein sequences and is highlighted in blue (Zhang et al., 2010). The hypothetical DNA-binding domains of GhCBP60a and AtCBP60a are located in the middle region of proteins and are enclosed within the black box.

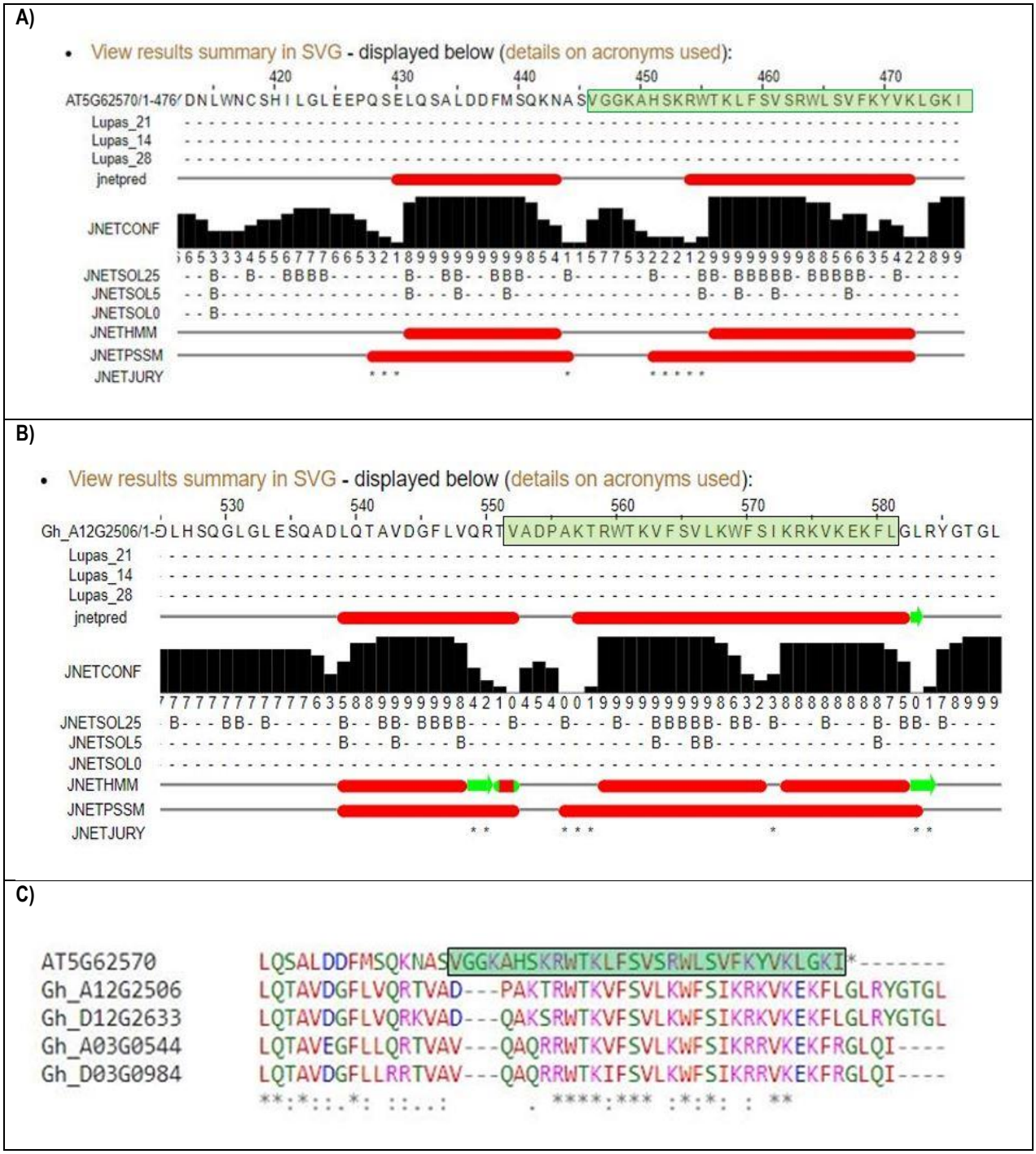


Figure 4-4. Prediction of secondary structure of the C-terminal of AtCBP60a and GhCBP60a showing the reported CaM-binding domain and is highlighted in green. C). ClustalO multiple sequence alignment of the C-terminal of AtCBP60a and GhCBP60a showing the CaM-binding domain.

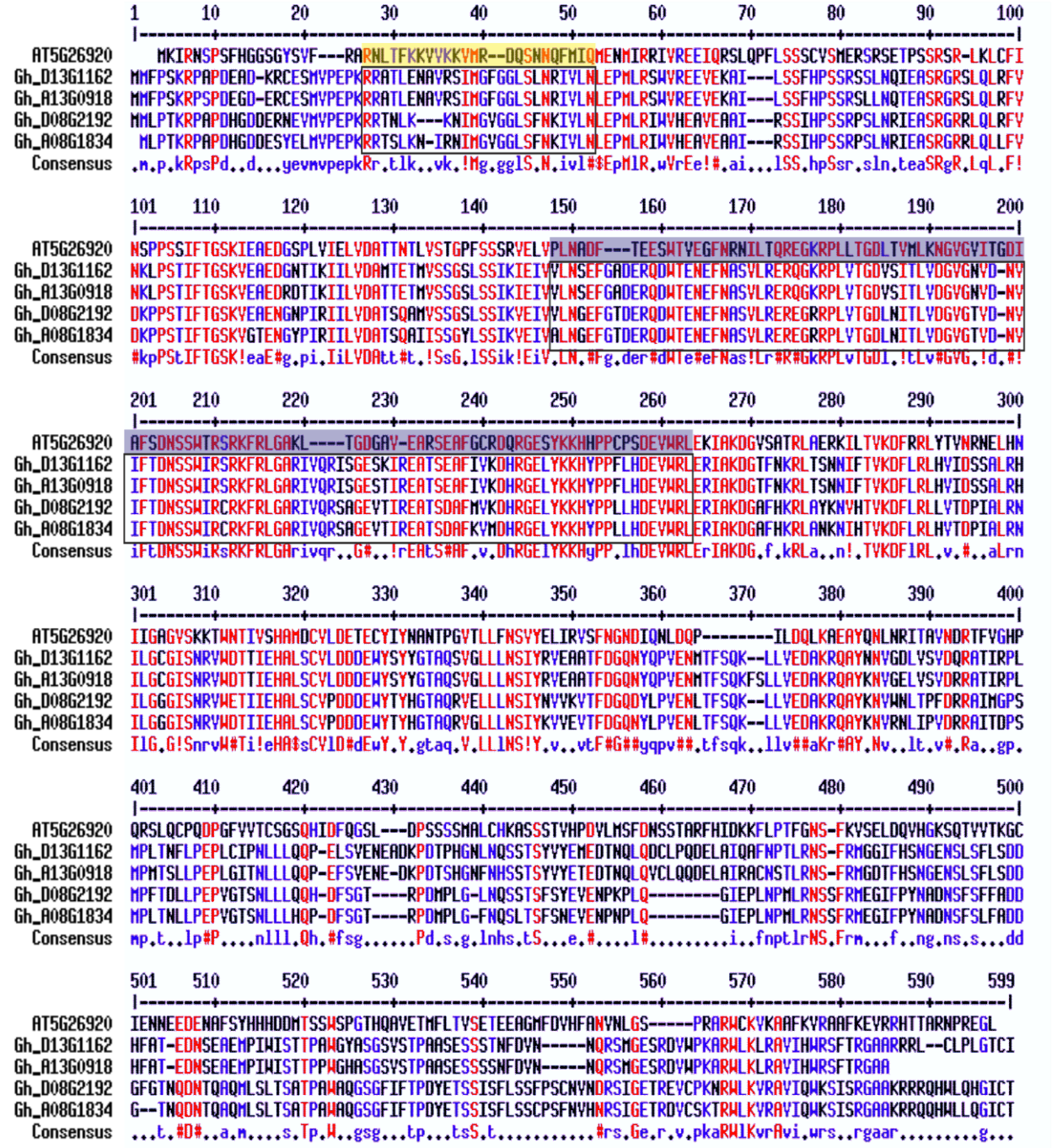
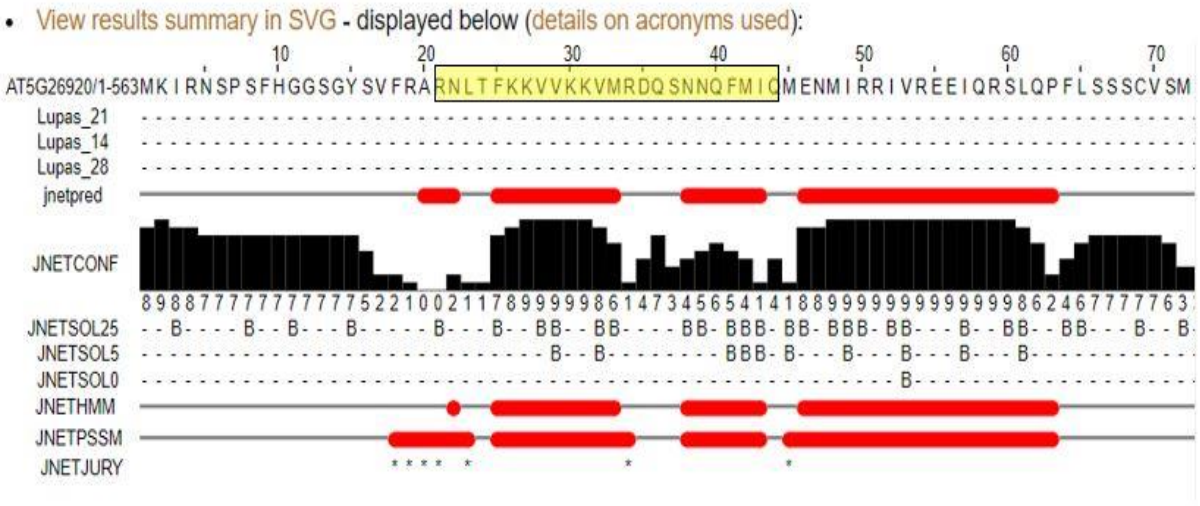
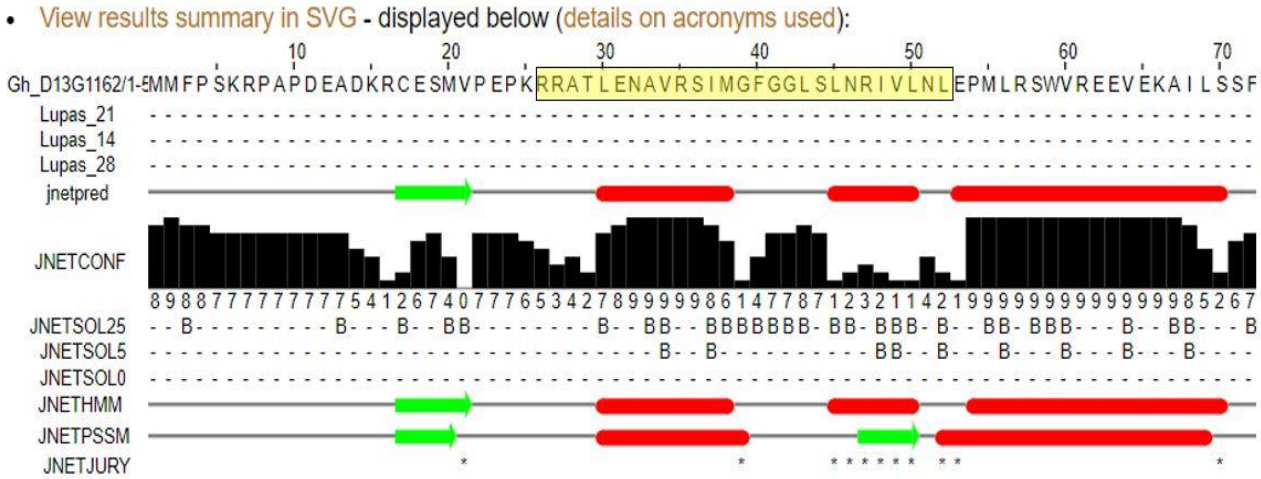


Figure 4-5. Multiple sequence alignment of AtCBP60g and GhCBP60g. The actual CaM-binding region of AtCBP60g is located at the N-terminus and is highlighted in yellow (Wang et al., 2009). The hypothetical CaM-binding domain of GhCBP60g is located at the N-terminus and is enclosed within the black box. The DNA-binding region of AtCBP60g is highlighted in purple. The hypothetical DNA-binding region of GhCBP60g resides in the middle region of the protein sequences and is enclosed within the black box.

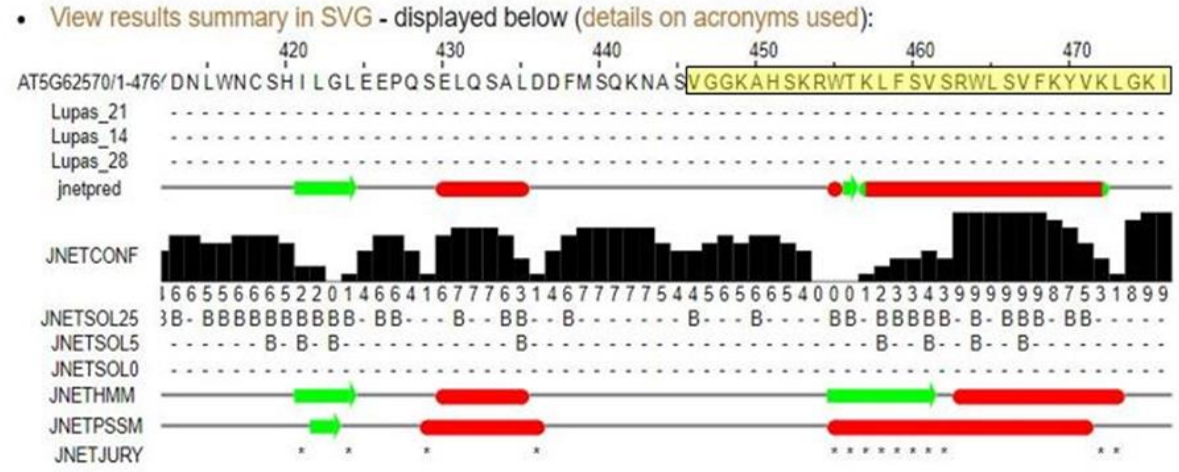
A)


View results summary in SVG - displayed below (details on acronyms used):
AT5G26920/1-563MK I RN SP SF HGGSGY SV FRA RNLT FKKVVKKVMRDQ SNNQ FM I Q MENM I RR I VREE I QRSLQP FL SSSCV SM
Lupas_21
Lupas_14
Lupas_28
jnetpred
JNETCONF
JNETSOL25
JNETSOL5
JNETSOL0
JNETHMM
JNETPSSM
JNETJURY

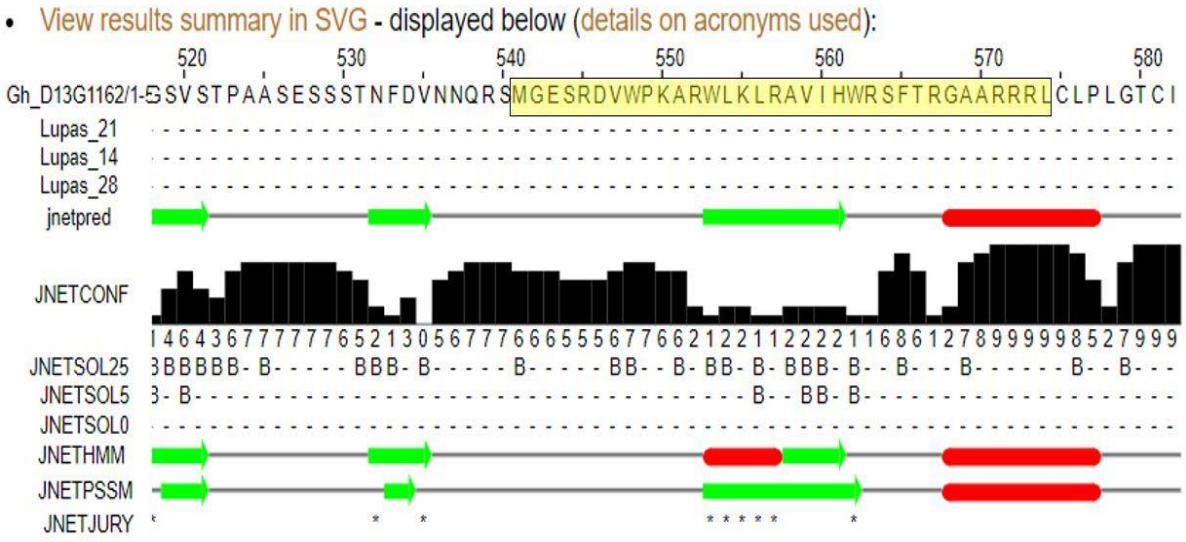
B)


View results summary in SVG - displayed below (details on acronyms used):
Gh_D13G1162/1-5MM FP SKRPAP DEADKRCESMVP EPK RRATLENAVRSIMGFGGLSLNRIVLNL EPMLRSWVREEVEKAILSSF
Lupas_21
Lupas_14
Lupas_28
jnetpred
JNETCONF
JNETSOL25
JNETSOL5
JNETSOL0
JNETHMM
JNETPSSM
JNETJURY

C)


View results summary in SVG - displayed below (details on acronyms used):
AT5G62570/1-476 DNLWNCSHILGLEEPQSELQSALDDFMSQKNAS VGGKAHSKRWTKLFSVSRWLSVFKYVKLGKI
Lupas_21
Lupas_14
Lupas_28
jnetpred
JNETCONF
JNETSOL25
JNETSOL5
JNETSOL0
JNETHMM
JNETPSSM
JNETJURY

D)



E)

F)

Figure 4-6. Prediction of the secondary structure of the CaM-binding domain of AtCBP60a, AtCBP60g and GhCBP60g groups using JPRED. A and B) JPRED secondary structure prediction analysis for the N-terminal of AtCBP60g and GhCBP60 respectively, and C and D)) JPRED secondary structure prediction analysis the C-terminal of AtCBP60a and GhCBP60g proteins respectively, showing the possible conserved CaM-binding domain and is enclosed within yellow box. E and F) ClustalO multiple sequence alignment for the N-terminal of AtCBP60g and GhCBP60g, and AtCBP60a and GhCBP60g proteins respectively, showing the possible conserved CaM-binding domain and is also highlighted within yellow box. The cotton protein (Gh_A13G0918) was excluded from the analysis because it is missing the C-terminal part and the recent RNA-seq from cotton (Zhu et al., 2017) also indicated that this gene is not expressed.

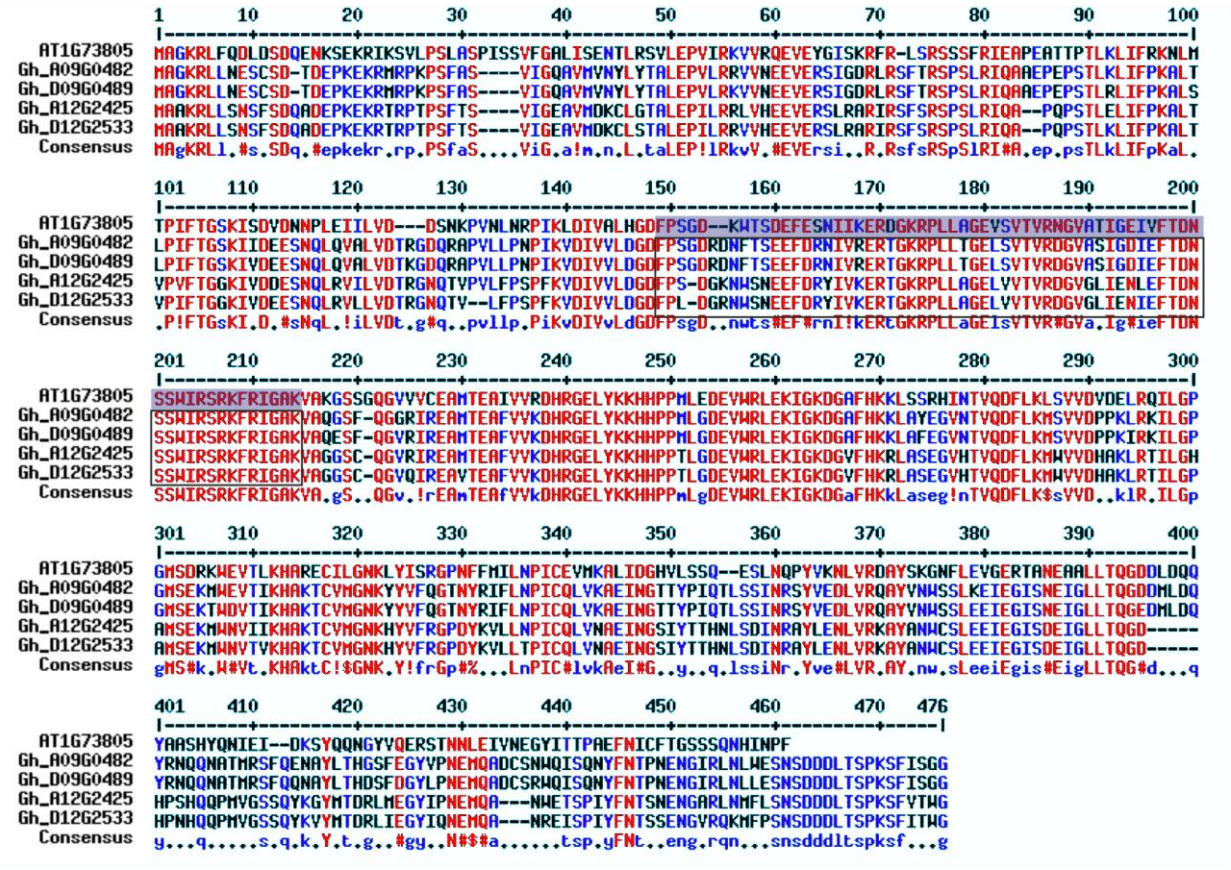


Figure Error! No text of specified style in document.**-7.** Multiple sequence alignment of AtSARD1 and GhSARD1. The DNA-binding region of AtSARD1 is highlighted in blue (Zhang et al., 2010). The hypothetical-DNA binding region of GhSARD1 resides in the middle region of the protein sequences and enclosed with the black box.

Clade 2

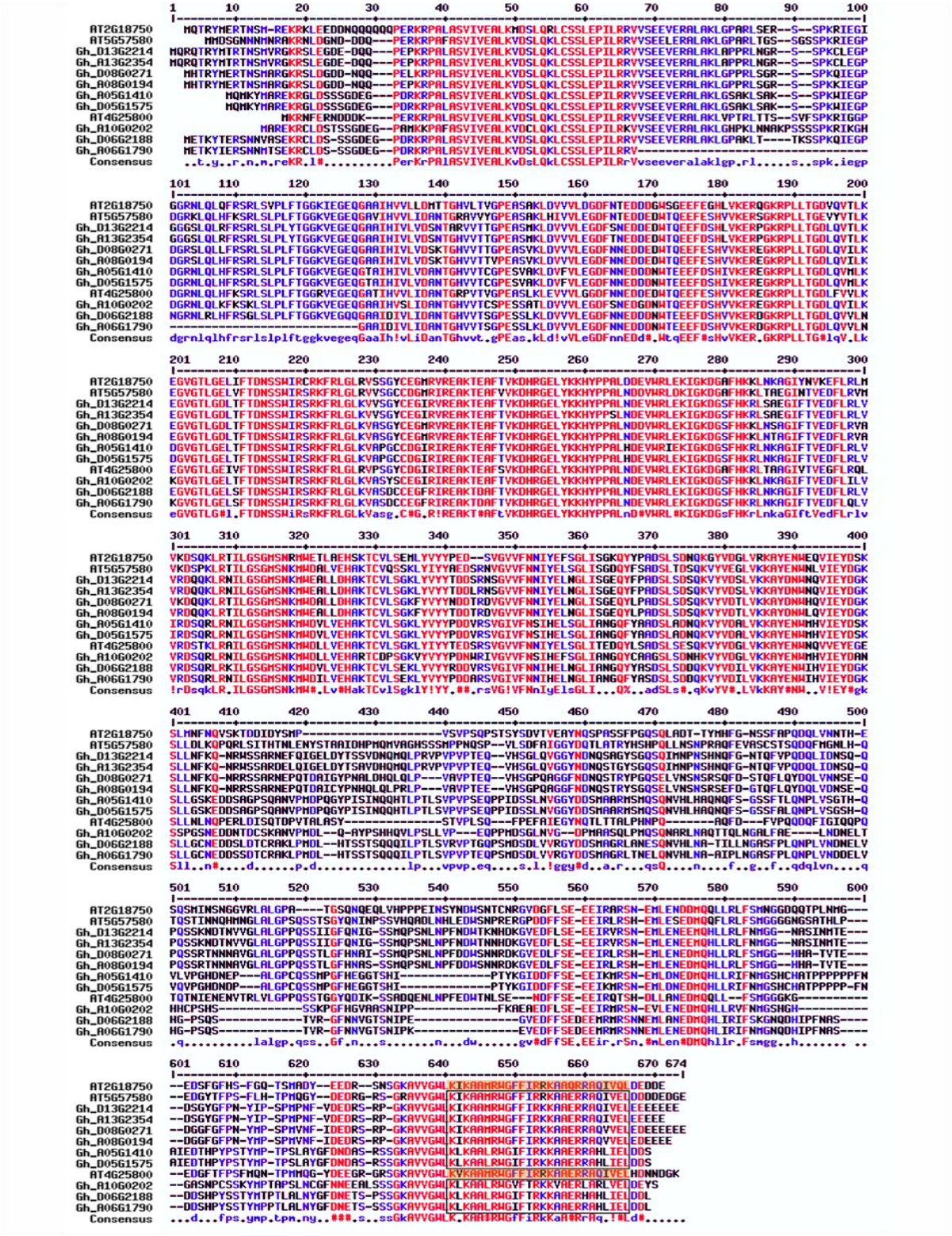


Figure 4-8. Multiple sequence alignment of AtCBP60b/c/d and GhCBP60b/c/d. The CaM-binding region of AtCBP60b/c/d are highlighted in orange (Wang et al., 2009). The hypothetical CaM-binding region of GhCBP60b/c/d is enclosed within the black box.

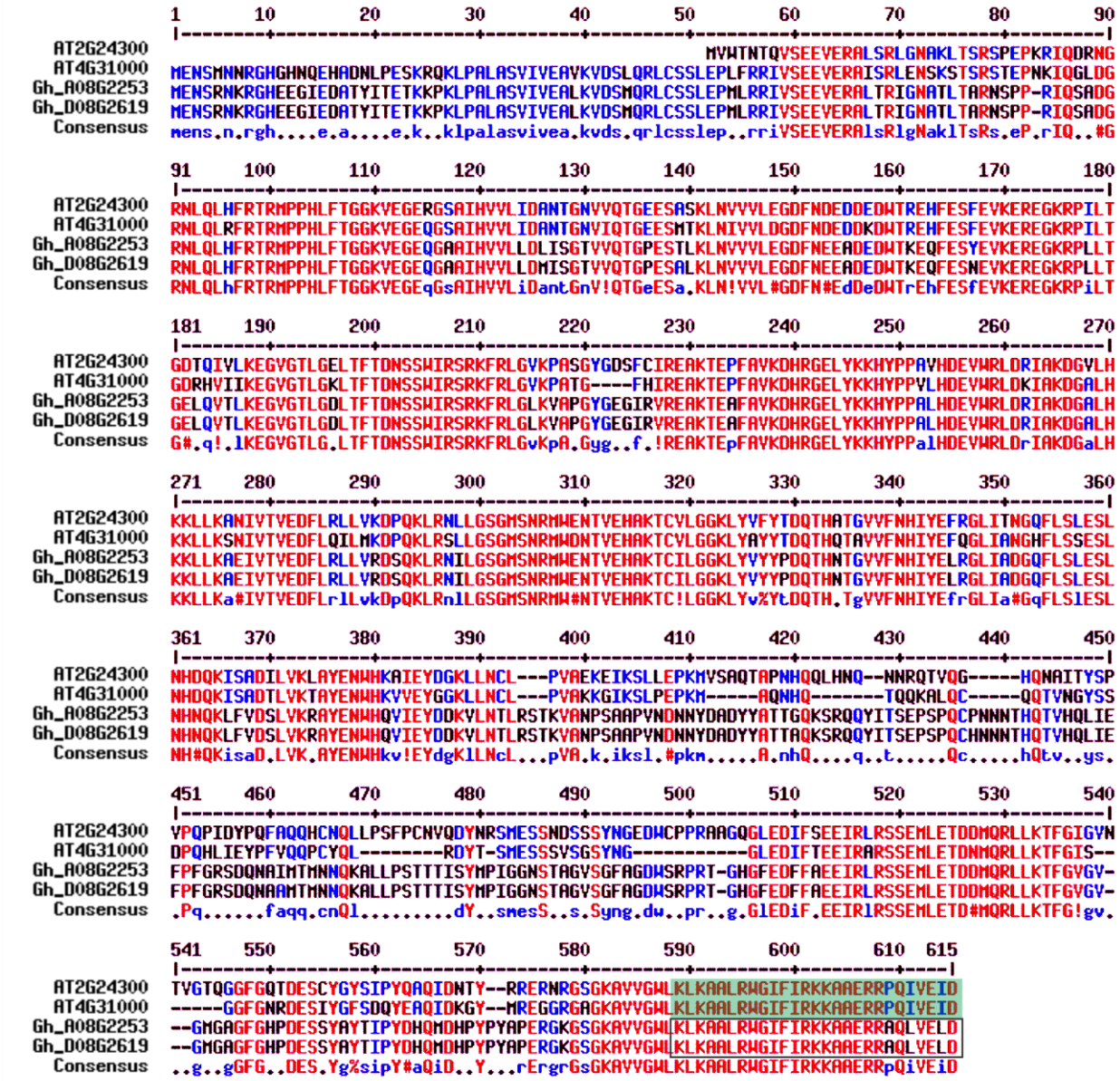


Figure 4-9. Multiple sequence alignment of AtCBP60e/f and GhCBP60e/f. The CaM-binding region of AtCBP60f is highlighted in green (Wang et al., 2009). The hypothetical CaM-binding region of GhCBP60f is enclosed within the black box.

4.5.4 Prediction of subcellular localisation of GhCBP60

To predict the subcellular localisation of GhCBP60 from nucleotide sequences, a computational tool was used namely BaCello database (http://gpcr.biocomp.unibo.it/bacello/). The results of prediction analysis indicated that all GhCBP60a-g and GhSARD1 except one of GhCBP60a are likely to be located in the nucleus. The GhCBP60a-12D protein was predicted to be secreted; however, the alternative subcellular prediction program Cello predicted this to be a nuclear protein (http://cello.life.nctu.edu.tw/cgi/main.cgi) (Table 4-2). The results of prediction analysis of BaCello

program showed that all *G. raimondii* and *G. arboreum* proteins are likely to be located in the nucleus except for GrCBP60a (Gorai.008G297800) and GaCBP60a (KHG14637) that were predicted to be extracellular and chloroplast proteins, respectively (Table 4-2). In contrast, the alternative program Cello predicted these to be nuclear proteins with prediction accuracy scores of 2.724* and 1.929*, respectively.

Table 4-2. Summary of subcellular localization prediction for CBP60 in *G. hirsutum* and its ancestral species; *G. raimondii* and *G. arboreum* using Bacello and Cello software

GhCBP60 group	*G. hirsutum* locus ID	Prediction of subcellular localisation by BaCeLLO	Prediction and reliability of subcellular localisation by Cello software	*G. raimondii* or *arboreum* locus ID	Prediction of subcellular localisation of GaCBP60 & GrCBP60 by BaCeLLO	Prediction and reliability of subcellular localisation by Cello software
GhCBP60b/c/d-5A	Gh_A05G1410	Nucleus	Nucleus 3.139*	Gorai.009G173400	Nucleus	Nucleus 3.246*
GhCBP60 b/c/d-5D	Gh_D05G1575	Nucleus	Nucleus 3.254*	KHG24590	Nucleus	Nucleus 3.246*
GhCBP60 b/c/d-6A	Gh_A06G1790	Nucleus	Nucleus 3.999*			
GhCBP60 b/c/d-6D	Gh_D06G2188	Nucleus	Nucleus 3.571*	Gorai.010G254300	Nucleus	Nucleus 3.302*
GhCBP60 b/c/d-8A	Gh_A08G0194	Nucleus	Nucleus 3.023*	KHG10046	Nucleus	Nucleus 3.100*
GhCBP60 b/c/d-8D	Gh_D08G0271	Nucleus	Nucleus 2.652*	Gorai.004G031000	Nucleus	Nucleus 2.730*
GhCBP60b/c/d-10A	Gh_A10G0202	Nucleus	Nucleus 2.867*	Gorai.011G022600 KHG17962	Nucleus Nucleus	Nucleus 2.488* Nucleus 2.710*
GhCBP60 b/c/d-13A	Gh_A13G2354	Nucleus	Nucleus 3.228*	KHG27572	Secretory	Nucleus 3.266*
GhCBP60b/c/d-13D	Gh_D13G2214	Nucleus	Nucleus 3.322*	Gorai.013G246400	Nucleus	Nucleus 3.483*
GhCBP60f-8A	Gh_A08G2253	Nucleus	Cytoplasmic 1.565*	KHG21283	Nucleus	Mitochondrial 1.475*
GhCBP60f-8D	Gh_D08G2619	Nucleus	Mitochondrial 1.534 *	Gorai.004G291200	Nucleus	Mitochondrial 1.563*
GhCBP60a-3A	Gh_A03G0544	Nucleus		KHG25212	Nucleus	Nucleus 2.248*
GhCBP60a-3D	Gh_D03G0984	Nucleus	Nucleus 3.192*	Gorai.003G109800	Nucleus	Nucleus 2.667*
GhCBP60a-12A	Gh_A12G2506	Nucleus	Nucleus 2.430*	KHG14637	Chloroplast	Nucleus 1.929*
GhCBP60a-12D	Gh_D12G2633	Secretory	Nucleus 2.833*	Gorai.008G297800	Secretory	Nucleus 2.724*
GhCBP60g-8A	Gh_A08G1834	Nucleus	Nucleus 1.546*	KHG14364	Nucleus	Nucleus 1.454*
GhCBP60g-8D	Gh_D08G2192	Nucleus	Nucleus 1.430*	Gorai.004G237500	Nucleus	Chloroplast 1.566 *
GhCBP60g-13A	Gh_A13G0918	Nucleus	Nucleus 2.576*			
GhCBP60g-13D	Gh_D13G1162	Nucleus	Nucleus 2.035*	Gorai.013G128100	Nucleus	Nucleus 1.952*
GhSARD1-9A	Gh_A09G0482	Nucleus	Nucleus 2.006*			
GhSARD1-9D	Gh_D09G0489	Nucleus	Nucleus 2.347 *	Gorai.006G059900	Nucleus	Nucleus 2.093*
GhSARD1-12A	Gh_A12G2425	Nucleus	Cytoplasmic 1.934*	KHG01964	Nucleus	Cytoplasmic 2.266 *

GhSARD1-12D	Gh_D12G2533	Nucleus	Cytoplasmic 1.593*	Gorai.008G287500	Nucleus	Cytoplasmic 1.906*

4.6 Discussion

4.6.1 Identification of a novel *CBP60* gene family in cotton

Plant specific CBP60s have been previously shown to have a role in mediating stress tolerance in response to abiotic and biotic stresses (Wan et al., 2012; Wang et al., 2011; Zhang et al., 2010). To date, there have been no comprehensive studies on the CBP60 gene family in cotton *G. hirsutum*. Hence, this gap of knowledge leads to the purpose of this study. Hopefully, a better understanding of this gene family will help us to understand its role in mediating stress tolerance. In this study, I hypothesised that cotton *G. hirsutum* has CBP60 proteins (GhCBP60) orthologous to CBP60 from Arabidopsis (AtCBP60). I further hypothesised that GhCBP60 have conserved CaM and DNA regulatory binding domains similar to AtCBP60 orthologues. I also hypothesised that GhCBP60s are transcription factors with nuclear localisation signal sequences. Therefore, this chapter aims to identify CBP60 gene family members in cotton; to determine whether GhCBP60s have conserved CaM- and DNA-binding domains and to predict the subcellular localisation signal of GhCBP60. Our results showed that a total of 23 genes were successfully identified in cotton *G. hirsutum*. This is due to the fact that *G. hirsutum* is an allotetraploid (AADD, 2n = 4x = 52), as the result of hybridisation between two diploid species (2n = 26) *G. arboretum* (AA) and *G. raimondii* (DD) (Endrizzi et al., 1985; Skovsted, 1937; Skovsted, 1934). Thus, allotetraploid cotton contains duplicated but slightly divergent copies of most genes (Paterson & Wendel, 2015). Out of 48 gene pairs, Senchina et al. (2003) found an average of about 3-4% sequence divergence. Another study of the *G. hirsutum* genome sequence also revealed that out of 76,943 annotated gene models, 93.76% or 72,142 were evenly distributed along chromosomes, with A sub-genomes containing 35,056 genes and D genomes 37,086 genes (Li et al., 2015). Our analysis revealed that 13 out of 23 GhCBP60 proteins are from the A-genome and 10 proteins are from the D-genome, with 12 GhCBP60s proteins clustered in Clade 1 and 11 proteins clustered in Clade 2.

Regarding the phylogenetic relationship between GhCBP60 and AtCBP60, Mega 6 software showed two clades of AtCBP60. Clade 1 contains three proteins AtCBP60a-g and AtSARD1 that are clustered in one branch with high reliability followed by Clade 2 that contains five proteins including AtCBP60b/c/d that are clustered together in one sub-branch and AtCBP60e/f proteins that form another sub-branch, similar to the previous study by Wang et al. (2011). The results in Figure 4-1 revealed that in Clade 1; cotton orthologues of AT5G62570 (AtCBP60a), have 2 different copies of D and A genes located on two different chromosomes; *GhCBP60a-3A/D* and *GhCBP60a-*

12A/D. While cotton orthologues of AT5G26920 (*CBP60g*) also have two different copies of the same gene located on two different chromosomes; *GhCBP60g-8A/D* and *GhCBP60g13A/D*. The cotton genome also revealed that cotton orthologues of AT1G73805 (*SARD1*) also have two copies located on two different chromosomes; *SARD1-9A/D* and *SARD1-12A/D*.

Unlike Clade 1, Clade 2 cotton genome has two different sub-groups; GhCBP60b/c/d and GhCBP60f. The first sub-group contains nine cotton orthologues ofAtCBP60b/c/d, while the lower second sub-group contains two orthologues of AtCBP60e/f. The upper sub-group which contains AtCBP60b/c/d is also divided into two distinct groups. The upper group of the cotton genome has two pairs of orthologues of AtCBP60b/c/d, each pair has two different copies of A and D genes located on two different chromosomes GhCBP60b/c/d-8A/D and GhCBP60b/c/d-8A/D. However, the lower group of cotton genome also has two pairs of orthologues of AtCBP60b/c/d located on two different chromosomes GhCBP60b/c/d-5A/D and GhCBP60b/c/d-6A/D and one copy from A gene *GhCBP60b/c/d-10A* without its D gene pair.

The second sub-group of this Clade 2 contains two clear orthologues, one member from A-gene (GhCBP60f-8A) and another member from D gene (GhCBP60f-8D). Unlike the first sub-group, the bootstrap values of major sub-branches of AtCBP60f versus AtCBP60b/c/d indicate high reliability suggesting similar functions for these proteins in cotton. I referred to the GhCBP60e as GhCBP60f because the amino acid sequence of GhCBP60e is more similar to GhCBP60f protein than GhCBP60e and also the N-terminal of AtCBP60e is missing.

4.6.2 Evolutionary conservation of CaM- and DNA-binding domains of Clade 1 in GhCBP60

The calmodulin-binding proteins AtCBP60a-g and AtSARD1 play a critical role in regulating plant growth and mediating plant response to abiotic and biotic stresses (Truman et al., 2013; Wang et al. 2009; Zou et al., 2017). The phylogenetic analysis indicated that cotton has four co-orthologues of each of AtCBP60a-g and AtSARD1. The Multalin, JPRED secondary structure and ClustalO bioinformatic tools were then employed to characterise each GhCBP60 group for the presence of possible conserved CaM- and DNA-binding domains. My results support our hypothesis that GhCBP60a-g orthologues have conserved CaM-binding domains located at the C-terminus similar to AtCBP60a. The DNA-binding domain of AtCBP60a has not been studied yet. Therefore, the highly conserved middle region of GhCBP60a between amino acids 1-400, was compared to the actual DNA-binding domain of AtCBP60g. The results showed that this region of GhCBP60a has

a high similarity to the DNA-binding domain of AtCBP60g suggesting that it is also likely to bind DNA. Therefore, I suggest that GhCBP60a also regulates gene transcription through their DNA-binding domains.

Unlike all the other CBP60 proteins, CBP60g is reported to have a CaM binding domain at the N-terminus and not at the C-terminus. Then the results of Multalin, JPRED and ClustalO indicated that there are very little sequence similarities between GhCBP60g and the CaM-binding domain of AtCBP60g at the N-terminus of the proteins. The results also show high sequence similarity between C-terminus of GhCBP60g and AtCBP60a; however, the results suggest that it is unclear where the conserved CaM-binding domain is located in both Arabidopsis and cotton. Given the significant role of CBP60b (CBP60g) in modulating plant immunity and given that the CaM-binding domain of CBP60g is required for VdSCP41 targeting (Qin et al., 2018), the CaM-binding domain of this protein requires experimental investigation. The Multalin results showed the C-terminal domain of SARD1 proteins is missing, therefore it lacks the ability to bind calmodulin. These results are consistent with the findings of Zhang et al. (2010) study in which they showed that the N-terminal of SARD1 is not conserved and therefore GhSARD1 is not able to bind CaM-binding domain (Figure 4-7). The DNA-binding domain is located in the middle region of the protein's sequences similar to the corresponding AtCBP60g protein. Due to the high conservation of the DNA-binding domain, I suggest that this putative domain is critical for the function of the GhCBP60g protein. A recent study conducted by Qin et al. (2018) revealed the involvement of the CaM-binding region of *CBP60g* in mediating gene activity against *V. dahliae* in Arabidopsis. They found that the secretory protein effector VdSCP41 that enhances *V. dahliae* virulence binds to the CaM region of AtCBP60g to inhibit plants' resistance to the pathogen, the study suggests the crucial role of the transcription factors CBP60g, SARD1, and GhCBP60b (GhCBP60g) in regulating plant responses to *V. dahliae*.

The DNA-binding domain of GhSARD1 proteins is also highly similar to the corresponding AtSARD1. The high conservation of the DNA-binding domain located at the most conserved region of the proteins indicates that AtSARD1 functions through this domain. The AtSARD1 was found not to bind CaM (Zhang et al., 2010) and the close homology between SARD1 proteins and AtSARD1 suggests that GhSARD1 is also unlikely to bind CaM.

Overall, the results showed that the two proteins GhCBP60a-g have highly conserved putative DNA-binding domains and partially conserved CaM-binding domains. While SARD1 has highly conserved DNA-binding domains. Therefore, I suggest that GhCBP60a-g and GhSARD1 have a

conserved function to protect the plant from biotic and abiotic stresses, similar to CBP60a-g and SARD1 proteins in other plants.

4.6.3 Evolutionary conservation of CaM- and DNA-binding domains of Clade 2 inGhCBP60

Multalin, JPRED secondary structure prediction and ClustalO tools were used to characterise GhCBP60a-g for the presence and absence of CaM-binding domains and DNA-binding domains. My results support my hypothesis in that GhCBP60a/b/c/d/f have highly conserved CaM-binding domains located at the C-terminus of the proteins similar to their corresponding AtCBP60c/b/c/d/f. The N-terminus and middle region of the proteins are also highly conserved. The CaM-binding domain of these proteins is highly conserved compared to the CaM–binding region of GhCBP60a-g suggesting its distinctive functional role in regulating these proteins in response to environmental stress. The results also show that the middle regions of all GhCBP60 appear to be highly conserved with potential DNA-binding regions like AtCBP60. The proteins also appear to have conserved subcellular nuclear signals indicating that all these proteins are transcription factors with functional properties in cotton similar to other plants.

Overall, this bioinformatics chapter has successfully identified AtCBP60 orthologues in *G. hirsutum*, namely GhCBP60. Therefore, due to the structural similarities between AtCBP60 and GhCBP60, I proved that GhCBP60a, GhCBP60g, and GhSARD1 could be DNA-targeting portions while ChCBP60f with Ca2+/CaM targeting proteins have a potential role in plant growth and development in response to environmental stimuli. I further proved that all 23 members of GhCBP60 contain nuclear localisation signals. The next question might be are all co-orthologues of each group expressed? The other question will be are these genes expressed in response to abiotic stress? This new information will provide us with a better understanding of biotic and abiotic stress tolerance mechanisms in cotton.

Chapter 5. Expression Profiling of *GhCBP60* in Cotton Seedlings Treated with Brassinosteroid and Salt and Analysis of Cis-acting Regulatory Elements

5.1 Introduction to *CBP60* gene expression in cotton

Chapter 5 describes the third and last set of experimental projects for this dissertation. Orthologues of Arabidopsis *CBP60* genes were identified in the cotton genome in the early stages of the project. Due to its genetic structure, cotton has multiple co-orthologues of CBP60s previously shown in the literature to be associated with stress responses. The previous results also suggest that GhCBP60 proteins have highly conserved CaM- and DNA-binding regulatory domains and contain nuclear localisation signals suggesting a similar function property to AtCBP60 in other plants. The knowledge of the genetic structure of cotton *GhCBP60* genes will be utilised to test their expression under abiotic and biotic stresses.

5.2 The expression of *CBP60* under abiotic and biotic stresses

CBP60s belong to a plant-specific calmodulin-binding proteins family with no homology in other organisms (Bouché et al., 2005; Reddy et al., 2002). Many studies have revealed the involvement of CBP60 in abiotic and biotic tolerance. Two different *CBPs* genes were identified in maize (*Zea mays*); *CBP1* and *CBP5*. The transcript level of *CBP5* gene increased in the root of wind-treated plants as compared to control, however, wind did not affect the expression of CBP1 gene (Reddy et al., 1993). In tobacco (*Nicotiana tabacum*, L), the transcript level of TCBP60 was down-regulated by heat shock treatment than control (Lu & Harrington, 1994).

The expression profile of calmodulin-binding proteins (CBPs) was also tested to find their involvement in defence responses in bean leaves (*Phaseolus vulgaris*) inoculated with compatible, incompatible and non-pathogenic *Pseudomonas syringae* strains (Ali et al., 2003). They found that out of eight CBP genes in *P. vulgaris* tested for expression in response to these bacterial pathogens, three genes were up-regulated including *PvCBP60-C* and *PvCBP60-D*. However, the expression of *PvCBP60-A* and *PvCBP60-B* were unchanged in response to the bacterial strains.

Three calmodulin-binding proteins *CBP60a/g* and *SARD1* are also involved in plant immunity in *Arabidopsis thaliana* (Kim et al., 2013; Truman et al., 2013; Zhang et al., 2010). The bacterial

growth of *P. syringae* reduced in Arabidopsis *cbp60a* deficient mutants as compared to the wild-type plants suggesting its role as a negative regulator of plant immunity (Truman et al., 2013).

The other two closely related proteins CBP60g and SARD1 were found to be positive regulators in plant immunity (Zhang et al., 2010). The transcript level of *Isochorismate Synthase 1*(*ICS1*) which encodes an enzyme that is responsible for the production of SA, was up-regulated in wild-type plants than in *cbp60g* and *SARD1* infected mutants with *P. syringae* (Zhang et al., 2010). The bacterial growth of *P. syringae* reduced in *sard1* deficient mutants as compared to wild-type plants. In response to abiotic stress, the expression of *AtCBP60g* and *AtSARD1* were also up-regulated after 3 weeks of plant exposure to cold as compared to the control (Kim et al., 2013). In another independent study conducted by Wan et al. (2012), the concentration of SA was strongly increased in *AtCBP60g* over-expressing lines compared to the control. The increased production of SA in these lines have led to increase plant resistance to the bacteria pathogen *P. syringae*. The transcription level of other defence genes such as ENHANCED DISEASE SUSCEPTIBILITY 5 (*EDS5*) was also up-regulated in these lines after post-infection with *P. syringae* (Wan et al., 2012). Furthermore, the study also suggested the involvement of AtCBP60g in abiotic stress. The expression of *ICS1* and EDS5 was up-regulated following abscisic acid (ABA) treatment and drought stress in *AtCBP60g* over-expressing lines as compared to control plants (Wan et al., 2012). *ICS1* produced more isochorismate synthase in the CBP60g over-expression lines as compared to wild-type control plants. Increased expression of these two genes enhanced plant resistance to drought stress, indicating that CBP60g acts as a positive link between ABA- and SA-mediated pathways in Arabidopsis (Wan et al., 2012).

To date, there is only one unpublished study on the effect of BR on the CBP60 gene expression. This study, conducted by Pallegar (2014) on two members of CBP60 family; CBP60f and CBP60g from the model plant Arabidopsis, revealed that both genes are salt- and BR-responsive. Promoter sequence analysis of these AtCBP60f/g genes revealed that the promoter region contains E-box elements (CANNTG) (Pallegar, 2014). CANNTG is the binding site for the transcription factor BRI1-EMS-SUPPRESSOR1 (BES1) also named as BZR2 (Kim et al., 2009). BES1 can directly activate the expression of many BR responsive genes that are involved in diverse signalling pathways of phytohormones and stress (Wang et al., 2012). BES1 accumulates in the nucleus in response to BR to regulate target gene expression (Yin et al., 2002). As BR is involved in stress responses in plants (Chung et al., 2014; Nolan et al., 2017; Nolan et al., 2020), it is possible that the *CBP60* gene family is regulated by BES1 and indirectly by BR if the promoter sequences of these

genes have the binding site for BES1. Recently, BES1 is also found to bind to two additional regulatory cis-elements BRRE (CGTGTG and CGTGCG) and G-box (CACGTG) which are overrepresented in the promoters of BR-biosynthetic genes. The result of binding inhibits targeted gene expression (Martínez et al., 2018). However, accumulation of the phytohormone-interacting transcription factor 4 (PIF4) competes for BES1 homodimer formation, resulting in up-regulation of BR biosynthesis at dawn and response to warmth (Martínez et al., 2018).

Another recent study conducted by Sun et al. (2018) on two members of the CBP60 family; *AtCBP60g* and *AtSARD1* found that these two genes are the direct target of other transcription factors; TGACG-binding factor 1 (TGA1) and TGACG-binding factor 4 (TGA4). Both TGA1/TGA4 are needed for full induction of *AtCBP60g* and *AtSARD1* in plant defence against pathogen. A significant reduction in the transcript level of A*tCBP60g*, *AtSARD1* and the production level of both SA was observed in *tga1*/*tga4* deficient mutants than the wild type (Sun et al., 2018). Both pathogens associated molecular pattern (PAMP)-induced pathogen resistance and systemic acquired resistance (SAR) were also reduced in *tga1*/*tga4* in Arabidopsis mutant plants.

5.3 Hypotheses and aims

Various studies have shown that AtCBP60a, AtCBP60g and AtSARD1 play a crucial role in mediating stress tolerance against biotic and abiotic stresses (Kim et al., 2013; Truman et al., 2013; Wang et al., 2011; Zhang et al., 2010). In Chapter 2, I successfully identified cotton CBP60 proteins with highly conserved CaM- and DNA-binding regulatory domains, as well as nuclear localisation signals, suggesting a similar function property to AtCBP60 in other plants. It has been previously demonstrated that the exogenous application of EBR might be involved in the upregulation of AtCBP60f/g gene expression under salt stress (Pallegar, 2014). Therefore, I hypothesised that BR upregulates the expression of GhCBP60 under salt stress. To determine whether the exogenous application of EBR may act by modulating the expression of GhCBP60 genes in cotton in leaf tissues under salt stress, I searched the public Plant Expression Database PLEXdb (http://www.plexdb.org/modules/tools/plexdb_blast.php) to obtain preliminary information on the transcriptional response of cotton CBP60 to abiotic stress (Dash et al., 2011). This analysis was used to identify promising genes for experimental investigation. Datasets of three experiments using Affymetrix cotton leaf and root tissue were used to determine tissue-specific expression patterns of cotton GhCBP60 genes in response to abiotic stress. The GO1 experiment investigated the global gene expression of cotton *G. hirsutum* in root after four hours and in leaf tissues after 24 hours

under waterlogging and non-waterlogging conditions, with two biological replicates (Christianson et al., 2010). GO5 examined the gene expression using a leaf microarray of the drought-sensitive and tolerant genotypes under drought stress in *G. herbaceum L.*, with three biological replicates (Ranjan et al., 2012). The GO7 experiment was used to measure the gene expression in leaf tissue of *G. hirsutum* under drought stress at the peak of the flowering stage, with three replicates (Padmalatha et al., 2012). The results from these experiments showed that GhCBP60s are stress-responsive genes. I hypothesised that BR upregulates the expression of GhCBP60 under salt stress. The response to EBR and salt stress of the most stress-responsive genes, GhCBP60a/f/g and GhSARD1, from microarray data was tested using qRT-PCR.

A previous study by Pallegar (2014) revealed the presence of E-box elements (CANNTG), which is the binding site for the transcription factor BRI1-EMS-SUPPRESSOR1 (BES1) in the putative promoter regions of AtCBP60g and AtCBP60f, suggesting the direct regulation of these two genes by BR. The transcription factors BES1 and BZR1 also bind to additional cis-regulatory elements, BRRE (CGTGTG and CGTGCG) and G-box (CACGTG), which are overrepresented in the promoters of BR-biosynthetic genes, resulting in the up/down-regulation of these genes in response to high temperature stress (Martínez et al., 2018). Further, the transcription factors TGA1 and TGA4 regulate Pip and SA biosynthesis by regulating the expression of SARD1 and CBP60g (Sun et al., 2018). The binding of TGA1 to the promoter region of SARD1 indicating that SARD1 is a direct target gene of TGA1(Sun et al., 2018). Therefore, I hypothesised the presence and overrepresentation of these motifs in the promoter sequences of GhCBP60 transcription factors that mediate BR and stress responses in plants. To determine whether the binding sites for BES1 (E-box [CANNTG], BRRE [CGTGTG and CGTGCG], G-box [CACGTG], TGACG-binding factor 1 [TGA1] and TGACG-binding factor 4 [TGA4]) are critical for promoting the expression of GhCBP60f/g and GhSARD1 genes under EBR treatment during abiotic and biotic stresses, a promoter sequence analysis of GhCBP60 was conducted to predict the likelihood of these elements using a manual search.

The hypotheses for this chapter are:

1. Expression of GhCBP60a, GhCBP60f, GhCBP60g and GhSARD1 genes is responsive to EBR, biotic and abiotic stress, similar to their Arabidopsis orthologues.
2. The promoter sequences of stress-responsive GhCBP60 are enriched in cis-regulatory elements, such as E-box, BRRE, G-box (CACGTG), TGA1 and TGA4.

Thus, the objectives of this study are to:

1. investigate the transcriptional response of GhCBP60 to EBR and abiotic and abiotic stress using a combination analysis of previous microarray data and quantitative RT-PCR
2. investigate the presence and significant enrichment of cis-regulatory elements E-box (CANNTG), BRRE (CGTGTG and CGTGCG), G-box (CACGTG), GGTCC motif and TGA1 and TGA4 (TGACG) in the promoter regions of GhCBP60 genes.

5.4 Materials and methods

5.4.1 In silico expression analysis using PLEXdb database

Thirteen probe sets matching most GhCBP60 genes were found via Blast search of cotton probe sets using the publicly available Plant Expression Database PLEXdb (Dash et al., 2011) (Table 5-1). RMA-normalised expression data for each probe-set was retrieved for cotton experiments GO1 (Christianson et al., 2010), GO5 (Ranjan et al., 2012) and GO7 (Padmalatha et al., 2012) in PLEXdb. These experiments were selected to investigate the differential expression of GhCBP60 genes under waterlogging and drought stress conditions. Data of only the sensitive genotype RAHS-14 was used in the present analysis as curators note indicated data from the tolerant genotype was of low reliability. Two-way ANOVA statistical analysis was used to evaluate differences between treated and untreated plants in GO1. Student's t. test was used to evaluate the s difference between treated and treated plants in GO5 and GO7.

Table Error! No text of specified style in document.**-1.** Affymetrix Probe-set IDs matching *GhCBP60* genes obtained from the publicly available cotton database PLEXdb

***G. hirsutum* gene ID**	**Proposed gene name**	**Probe set ID**
Gh_A05G1410	GhCBP60bcd-5A	No probe
Gh_D05G1575	GhCBP60bcd-5D	GraAffx.1560.1.S1_s_at
Gh_A06G1790	GhCBP60bcd-6A	No probe
Gh_D06G2188	GhCBP60bcd-6D	No probe
Gh_A08G0194	GhCBP60bcd-8A	GhiAffx.50075.2.S1_at
Gh_D08G0271	GhCBP60bcd-8D	GhiAffx.12675.1.S1_at
Gh_A10G0202	GhCBP60bcd-10A	GhiAffx.22900.1.A1_at GraAffx.13851.1.A1_at
Gh_A13G2354	GhCBP60bcd-13A	No probe
Gh_D13G2214	GhCBP60bcd-13D	GraAffx.15002.1.S1_s_at
Gh_A08G2253	GhCBP60f-8A	GhiAffx.31330.1.S1_at
Gh_D08G2619	GhCBP60f-8D	Ghi.8200.1.S1_at
Gh_A03G0544	GhCBP60a-3A	No probe
Gh_D03G0984	GhCBP60a-3D	GraAffx.33631.1.A1_s_at Ghi.4110.1.S1_s_at
Gh_A12G2506	GhCBP60a-12A	No probe
Gh_D12G2633	GhCBP60a-12D	Ghi.905.1.A1_at Ghi.905.2.S1_at
Gh_A08G1834	GhCBP60g-8A	Ghi.10344.1.S1_s_at
Gh_D08G2192	GhCBP60g-8D	GraAffx.34255.1.A1_s_at
Gh_A13G0918	GhCBP60g-13A	No probe
Gh_D13G1162	GhCBP60g-13D	GhiAffx.12571.1.S1_at
Gh_A09G0482	GhSARD1-9A	No probe
Gh_D09G0489	GhSARD1-9D	No probe
Gh_A12G2425	GhSARD1-12A	Ghi.4791.2.A1_at Ghi.4791.2.S1_at
Gh_D12G2533	GhSARD1-12D	No probe

5.4.2 Plant materials, growth conditions, treatments and harvesting of tissue

Cotton seeds of genotype Sicot 730 were surface sterilised by using 70% ethanol for 30-60s, rinsing 3-5 times with sterile water, soaking with 10% hydrogen peroxide (H_2O_2) for 1-2h followed by washing 3 times with sterile water. Seeds were then soaked in distilled water overnight at room temperature to improve germination. Germinated seeds were then grown on sand until the full establishment of cotyledons under controlled conditions at 28°C under 16-hour light / 8-hour dark condition with a light intensity of 40 µmol m^{-2} s^{-1}. Plants were then transferred to five 10L plastic basins containing half-strength Hoagland's solution under the same above-mentioned conditions. Each container was attached to a small air pump to improve the growth condition.

In order to investigate the expression of genes GhCB60a/f/g and GhSARD1 after 24h short-term treatment with BR and salt stress. The first two primary leaves of three-week-old seedlings were detached and floated on distilled water containing either (0 µM, 0.1 µM) EBR or (0 mM, 100mM) salt for a short-term treatment of 24h to allow direct and rapid entry of treatment chemicals to excised leaves, following a previously-published method (Lannoo et al., 2007). Three biological replicates per treatment were used, with each treatment consisting of the three petri dishes. Each petri dish constituted a replicate and contained three leaves from separate plants. The leaves from each dish were then snap-frozen in liquid nitrogen and stored at -80°C for further experiments.

5.4.3 RNA isolation and real-time quantitative qRT-PCR

Frozen plant samples were mechanically disrupted using the laboratory Mixer Mill (Retsch) in the presence of liquid nitrogen. RNA was extracted from 100 mg leaves tissue using Maxwell®16 LEV Plant RNA Kit (Promega Corporation, Madison, USA) according to the manufacturer's protocol. Total RNA of (3 µg) was reversed transcribed using Tetro cDNA synthesis kit (Bioline Inc., Taunton, MA, United States). Then cDNA samples were first standardised to the concentration of the 2.5 ng/µl before use in any qPCR reaction.

For quantitative gene expression analysis, primer pairs were designed to amplify the most stress-responsive genes GhCBP60a-12D, GhCBP60f-8A/D, GhCBP60g-8A/D, and GhSARD1-9A using Primer3 software (Table 5-1). One PCR primer was designed to amplify the most highly responsive gene of group GhCBP60a-12D. Due to the high similarity between A gene and D gene, a pair of primers were designed to amplify two genes of each group of both genes GhCBP60f-8A/D and

GhCBP60g-8A/D. Meanwhile, two different pair of primers were designed to amplify GhSARD1 group, one pair of primers was designed to amplify the two genes GhSARD1-9A/D. Due to the key role of AtSARD1 in biotic stress (Truman et al., 2103; L. Wang et al., 2011; Y. Zhang et al., 2010) and due to unavailability of expression data for the other two genes GhSARD1-12A/D, a pair of primers were designed to match these two genes, as shown in Table 5-2. Two housekeeping genes Gh-ubiquitin7 and Gh-actin14 were selected as reference genes based on their expression level and stability under abiotic stress in cotton *G. hirsutum* (M. Wang, Wang, & Zhang, 2013) (Table -2).

Each reaction mixture (10 µl) contained 4 µL of the standardised concentration of cDNA, SYBR green, 4 µL master mix, and 1 µl of each primer at (10 µM) of stock concentration. However, 4 µL of water (RNA in RNAse-free water) was added to the non-template control. RT- PCR (qPCR) reaction was carried out using the Real-Time PCR System (C1000 Touch Thermal Cycler) apparatus. In the qRT-PCR experiment, a non-reverse transcriptase control (no Rt) and no template control (NTC) were used. The thermal cycling for the program included 95°C for 2 min, 95°C for 0.05 sec, 55°C for 0.15 sec and 95°C for 0.5 sec. The RNA from three different treatments, each with three biological replicates, was used for each reaction. In addition, three technical replicates were included for each sample–primer set combination. The gene expression was calculated relative to two reference genes, Gh-ubiquitin7 and Gh-actin14 (Wang et al., 2013). Expression data were presented as the average and standard error of the biological replicates.

Table Error! No text of specified style in document.**-2.** List of primer sequences for selected stress-responsive GhCBP60a/f/g and GhSARD1 and reference genes

Gene(s) amplified	Primer name	Primer seq	Tm	Product length
Gh_A08G2253 Gh_D08G2619	GhCBP60f_F	TGCACCGGTAAACGATAACA	54.3	160bp
	GhCBP60f_R	CAGACCTTCCAAAGGGAAAT	52.5	
Gh_D12G2633	GhCBP60a_F	TCACTGGAGCACGAATTGAG	54.9	105bp
	GhCBP60a_R	TGTCCTCCTCATCACCATCA	55.3	
Gh_A08G1834 Gh_D08G2192	GhCBP60g_F	GAGGCATCAAGAGGACGAAG	55.3	232bp
	GhCBP60g_R	CGTTTTCGGTCCAATCTTGT	53.4	
Gh_A09G0482 Gh_D09G0489	GhSARD1-9_F	GGAGAAACGGATGAGACCTA	53.4	259bp
	GhSARD1-9_R	GATCTTGCTTCCGGTAAAGA	52.2	
Gh_A12G2425 Gh_D12G2533	GhSARD1-12_F	CGGCTTCTTAGCAACTCATT	53.0	172bp
	GhSARD1-12-R	GACTACGCTCCACTTCTTCG	55.1	
Gohir.A11G106600.1	Gh-ubiquitin7_F	AGAGGTCGAGTCTTCGGACA	63.4	101pb
	Gh-ubiquitin7_R	ACTCAATCCCCACCAGCCTTCTGG	62.9	
Gohir.A11G106600.1	Gh-actin14_F	CTGGAGACTGCCAAGAGCAGCT	61.4	97bp
	Gh-actin14_R	CCGGGCAACGGAATCTCTCAGC	62.5	

5.4.4 Identification of promoter sequences and transcription factor binding sites

The nucleotide sequences 1500 bp upstream of the transcriptional start sites for GhCBP60 retrieved using COTTONGEN database (https://www.cottongen.org/retrieve/sequences) (Yu et al., 2013). Promoter sequence analysis was carried out manually to identify putative transcription factor binding sites CANNTG and TGACG within the promoter sequence.

5.5 Results

5.5.1 In silico expression analysis of *GhCBP60* genes from datasets in PLEXdb

Data sets of three different experiments; GO1 (Christianson et al., 2010), GO5 (Ranjan et al., 2012) and GO7 (Padmalatha et al., 2012) from the Plant Expression Database PLEX were used for a preliminary investigation of the expression of GhCBP60 genes. Figure 5-1 compares the expression of 13 genes within GhCBP60a-SARD1 in root and leaf tissues of *G herbaceum* and *G. hirsutum* in response to flooding and drought stresses. Expression data were available for two out of four genes for GhCBP60a group; GhCBP60a-3D/12D. The results suggested that GhCBP60a-12D was more highly expressed and appeared to be drought stress-responsive in GO5 and GO7. However, this gene was down-regulated in the leaf in GO5 but up-regulated in the same tissue in GO7. The expression level of GhCBP60a-3D gene was low in all experiments, however, it appeared to be up-regulated in response to drought in GO7.

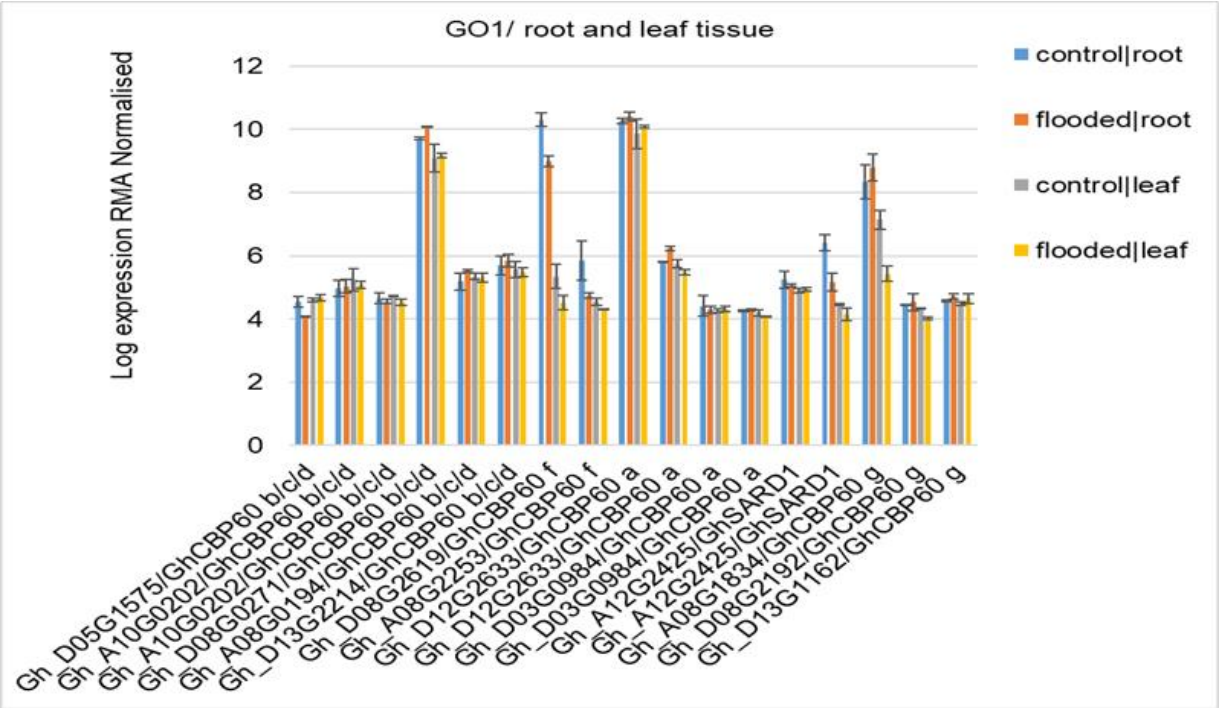
Expression data were available for three out of four genes in the GhCBP60g group; GhCBP60g-8A, GhCBP60g-8D, and GhCBP60g-13D. The results in Figure 5-1 showed that GhCBP60g-8A is stress-responsive and has higher expression than other genes in this group. However, the responses to stress were inconsistent. This gene appeared to be up-regulated in the root but down-regulated in the leaf tissue in response to waterlogging in GO1. In GO5, this gene down-regulated in the leaf tissue in response to drought. However, this gene was up-regulated in GO7. The results also indicated that the GhCBP60g-8D gene was also a stress-responsive gene in GO5 and GO7. This gene was down-regulated in leaf in GO5 but up-regulated in the same tissue in GO7. However, there was no response to waterlogging in GO1. The other gene GhCBP60g-13D also appeared to be drought-responsive and up-regulated in the leaf tissue in GO7. No response was shown for this gene to waterlogging stress in GO1 and drought in GO5. The expression of both GhCBP60g-8D and GhCBP60g-13D genes was low.

Expression data were available for only one out of four genes in the GhSARD1 group; GhSARD1-12A. The results suggested that GhSARD1-12A was stress-responsive in all experiments GO1, GO5 and GO7. This gene was down-regulated in the root in response to waterlogging in GO1 and also down-regulated in the leaf tissue in response to drought in GO5. However, this gene was up-regulated in leaf tissue in response to drought in GO7. A second probe set for SARD1-12A (Ghi.4791.2.A1) did not show any stress response.

Expression data were available for five out of nine genes for *GhCBP60bcd* group; GhCBP60b/c/d-5D, GhCBP60b/c/d-8A, GhCBP60b/c/d-8D, GhCBP60b/c/d-10A, and GhCBP60bcd-13D. None of these genes is responsive to waterlogging in GO1. Only one gene from this group GhCBP60b/c/d-8A appeared to be drought-responsive and up-regulated in the leaf tissue in GO5. However, GO7 results suggested that two other genes GhCBP60b/c/d-5D and GhCBP60b/c/d-10A were down-regulated in the leaf tissue in response to drought. GO7 results also showed that Gh_D13G2214 was up-regulated in the leaf tissue in response to drought. Expression data were available for both genes in the GhCBP60f group; GhCBP60f-8A and GhCBP60f-8D. Both genes were stress-responsive in GO1 and GO7. However, the response was inconsistent with both genes being down-regulated by stress in GO1 but up-regulated in GO7.

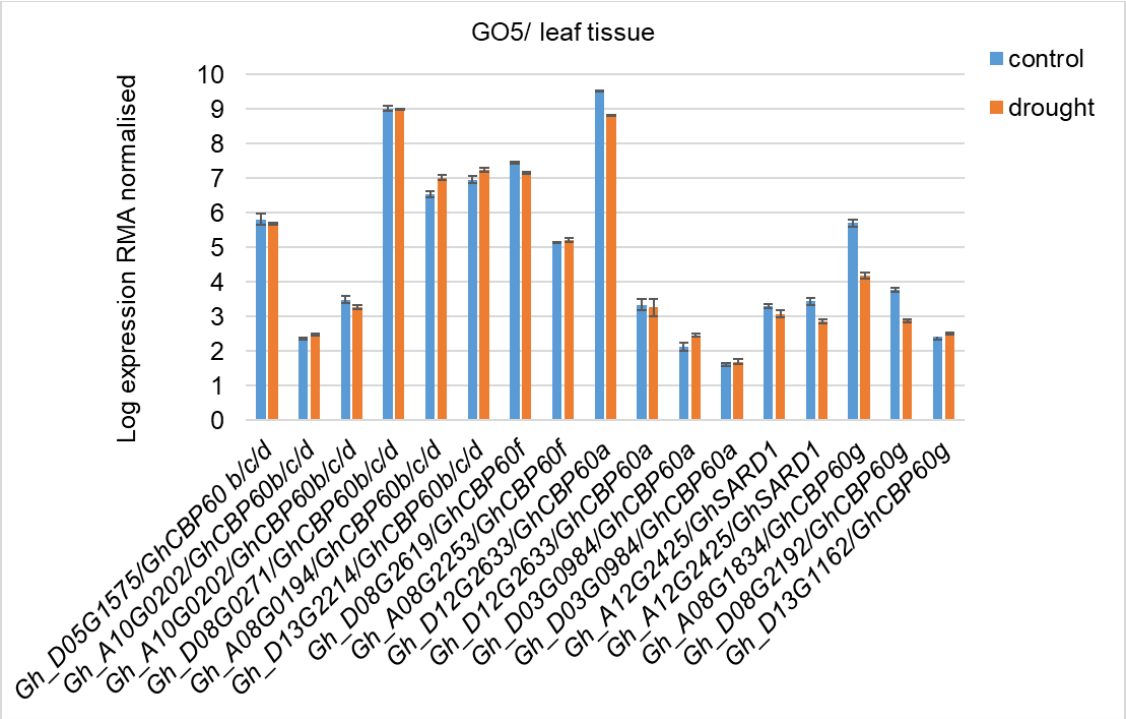
The overall findings from the microarray meta-analysis suggested that the following genes were most likely to be involved in stress response: GhCBP60a-12D, GhCBP60g-8A, GhCBP60f-8A, and GhCBP60f-8D. The expression of these genes was therefore investigated further by quantitative RT-PCR analysis in response to salt stress and BR application. As microarray data was only available for one GhSARD1-12A/D out of four genes, GhSARD1-9A/D were also included in the experimental investigation.

367 **A)**



368

369 **B)**



370

371

C)

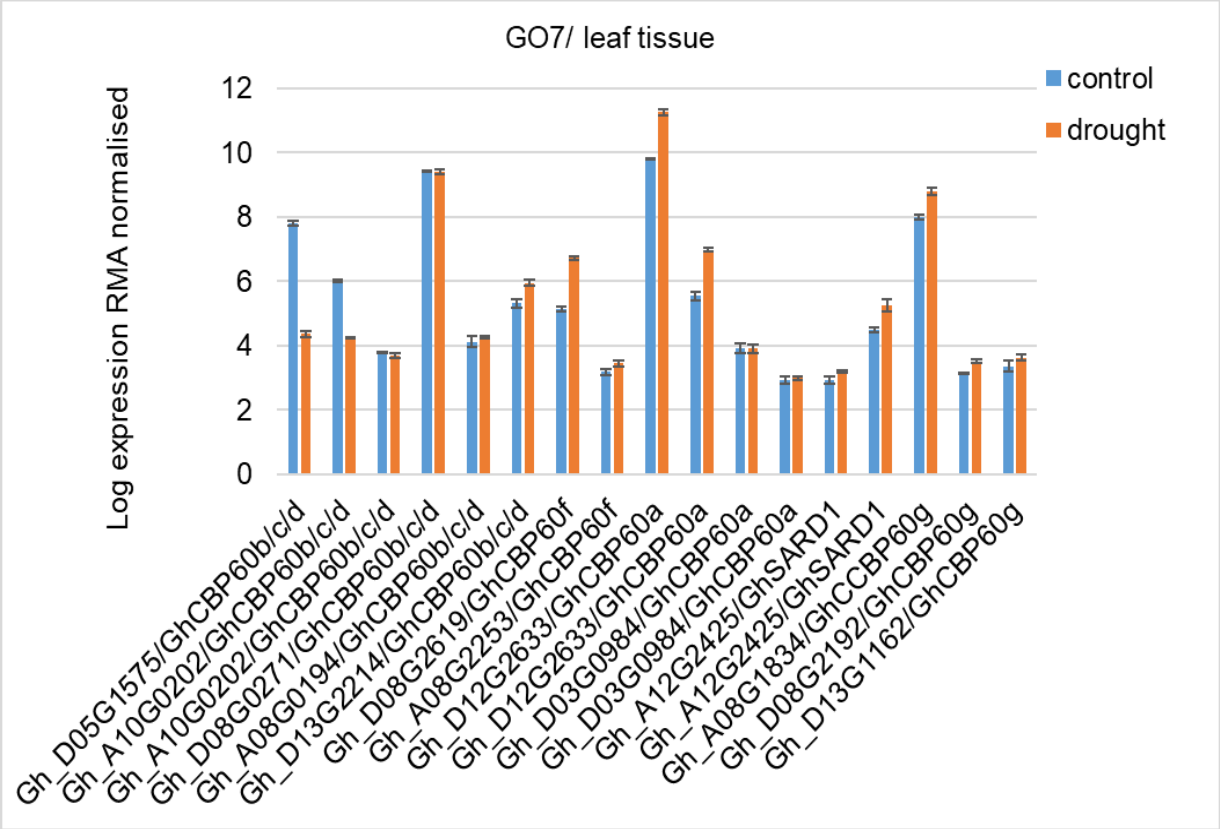


Figure 5-1. Comparison of gene expression analysis for seventeen probes representing the *GhCBP60a-g* and *GhSARD1* in response to waterlogging and drought stresses using RMA normalised expression data from Dash *et al*., 2011 "PLEXdb: gene expression resources for plants and plant pathogens" accessible as accession GO1 (Christianson et al., 2010), GO5 (Ranjan et al., 2012) and GO7 (Padmalatha et al., 2012) in PLEXdb. Data represent the mean of A) two samples and B and C three samples +/ standard error.

5.5.2 Expression profiling of GhCBP60a/f/g and GhSARD1 in response to EBR and salt using qRT-PCR

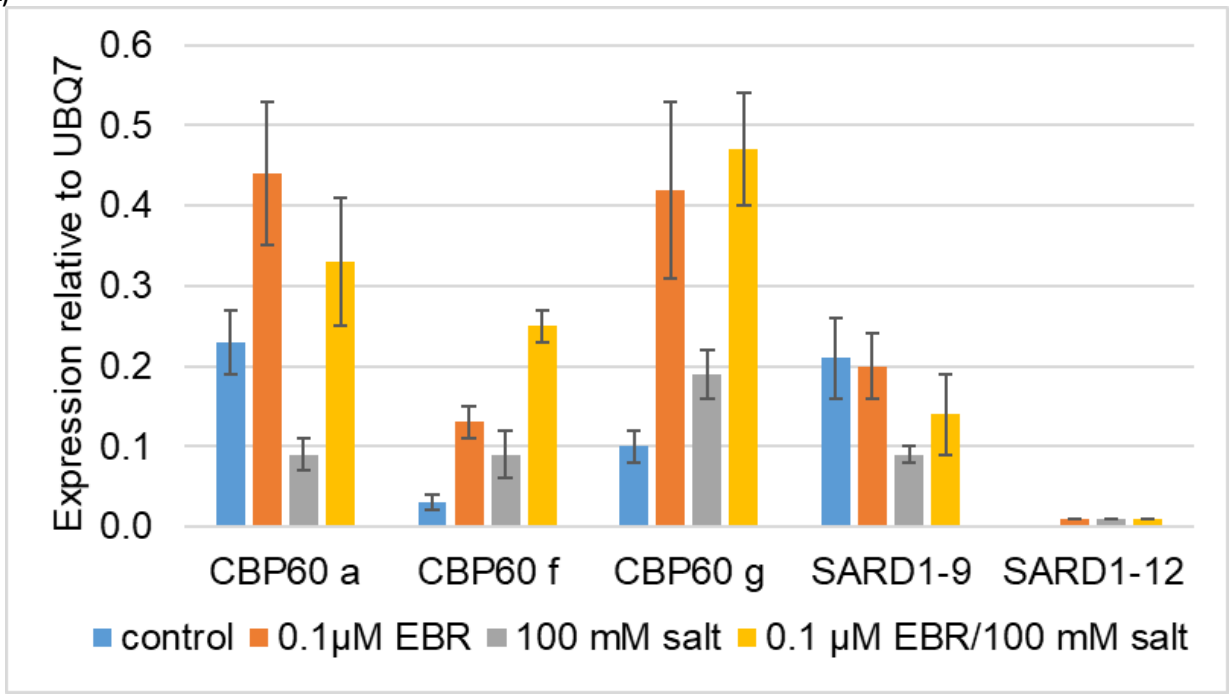
In order to investigate the effect of EBR and salt on GhCBP60 gene expression, the leaves from cotton seedlings were floated on distilled water containing 0 µM or 0.1 µM EBR with either 0 mM or 100 mM salt. Five genes/gene pairs were investigated (GhCBP60a-12D, GhCBP60f-8A/D, GhCBP60g-8A/D, GhSARD1-9A/D, and GhSARD1-12A/D). The gene expression level was calculated relative to the two reference genes Gh-ubiquitin7 and Gh-Actin14. The expression of three genes or gene pairs were significantly responsive to stress: GhCBP60a-12D, GhCBP60f-8A/D, GhCBP60g-8A/D as seen in Figure 5-2. GhCBP60a-12D (Gh_D12G2633) was significantly down-regulated by salt (both $P \leq 0.02$) to 0.44 of expression in controls in the absence of EBR. In contrast, this gene was significantly up-regulated by EBR treatment ($P \leq 0.01$ and $P \leq 0.04$), in

comparison to the two reference genes Gh-ubiquitin7 and Gh-Actin14 respectively. There were no significant interaction effects between EBR and salt on the expression of GhCBP60a gene (Gh_D12G2633).

Expression of GhCBP60f-8A/D (Gh_D08G2619 and Gh_A08G2253) was up-regulated by salt ($P \leq 0.003$ (Gh-ubiquitin7); $P \leq 0.0001$ (Gh-Actin14)) and EBR ($P \leq 0.002$ (Gh-ubiquitin7); $P \leq 0.01$ *(Gh-Actin14))* by 3.3-fold and 2.4-fold increase respectively; with the highest expression seen in the leaf tissue treated with both salt and EBR (>7-fold increase). A similar effect was observed with GhCBP60g-8A/D; (Gh_A08G1834, Gh_D08G2192). The expression of this gene pair was up-regulated by both EBR ($P \leq 0.001$ (Gh-ubiquitin7); $P \leq 0.0001$ (Gh-Actin14)) and salt treatment ($P \leq 0.0001$) with 2.4-fold and 2-fold increase respectively, only on the expression of GhCBP60g-8A/D relative to Gh-actin14. There was no significant interaction between effects of salt and EBR on the expression of GhCBP60g-8A/D relative to Gh-actin14. Similarly, significant interaction effects were observed between salt and EBR only on the expression of GhCBP60f-8A/D relative to Gh-actin14.

There was a possible effect of salt on the expression of SARD1-9A/D - Gh_A09G0482 and Gh_D09G04899 by $P \leq 0.03$ (Gh-ubiquitin7) and $P \leq 0.04$ (Gh-Actin14) respectively, but only in the absence of EBR as compared to the control. The expression of these two genes appeared to be down-regulated (0.77-fold decrease) in comparison to the control. There was no significant effect of EBR on the expression of this gene pair. Expression of GhSARD1-12A/D (Gh_A12G2425 and Gh_D12G2533) was very low and not responsive to salt or EBR.

A)



GhCBP60a	P
EBR	0.01
Salt	0.23
EBR*Salt	0.63

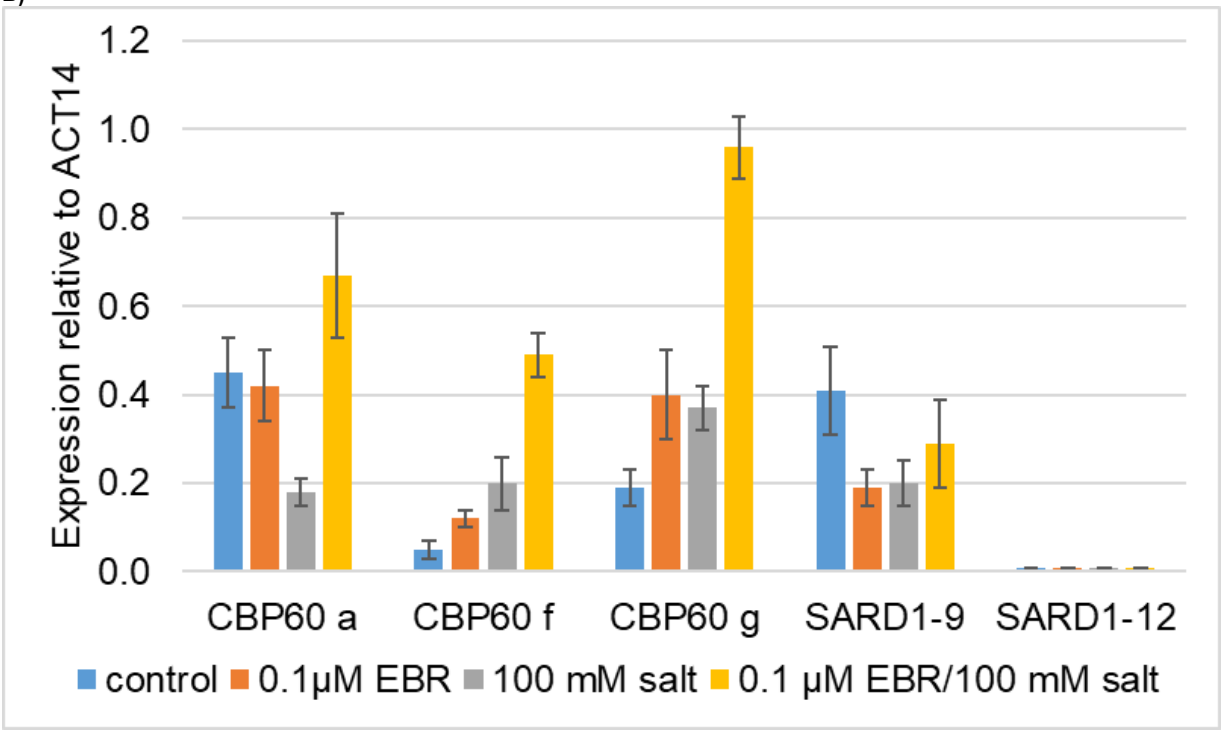
GhCBP60f	P
EBR	0.002
Salt	0.003
EBR*Salt	0.45

GhCBP60g	P
EBR	0.001
Salt	0.07
EBR*Salt	0.72

GhSARD1-9	P
EBR	0.68
Salt	0.08
EBR*Salt	0.32

GhSARD1-12	P
EBR	0.34
Salt	0.04
EBR*Salt	0.22

B)



GhCBP60a	P
EBR	0.04
Salt	0.82
EBR*Salt	0.07

GhCBP60f	P
EBR	0.01
Salt	0.001
EBR*Salt	0.13

GhCBP60g	P
EBR	0.0001
Salt	0.0001
EBR*Salt	0.04

GhSARD1-9	P
EBR	0.41
Salt	0.30
EBR*Salt	0.08

GhSARD1-12	P
EBR	0.65
Salt	0.01
EBR*Salt	0.78

Figure 5-9. Comparison of gene expression analysis of five members of GhCBP60 gene family in response to EBR and salt after short-term treatment of 24 hours. A) Bar graphs represent the mean of relative expression of GhCBP60a-12D, GhCBP60f-8A/D, GhCBP60g-8A/D, GhSARD1-9A/D and GhSARD1-12A/D to Gh-ubiquitin7 reference gene. B) Bar graphs represent the mean of relative expression of GhCBP60a-12D, GhCBP60f-8A/D, GhCBP60g-8A/D, GhSARD1-9A/D and GhSARD1-12A/D to Gh-Actin14 reference gene. Error bars represent the standard error of the mean for three biological replicates. Significance of effects of treatments on gene expression and the interaction between them was evaluated using a two-way ANOVA. Significance of effects of salt (in the absence of EBR) on gene expression was examined using a one-way ANOVA.

5.5.3 DNA sequencing analysis to determine whether one or both genes in A and D genomes are expressed

Due to the high similarity between A and D genes and the difficulty in designing gene-specific primers, I designed primers that matched gene pairs GhCBP60g-8A/D (Gh_A08G1834,

Gh_D08G2192), GhCBP60f-8A/D (Gh_D08G2619 and Gh_A08G2253), GhSARD1-9A/D (Gh_A09G0482 and Gh_D09G04899) and GhSARD1-12A/D (Gh_A12G2425 and Gh_D12G2533), refer to Table 2-5. Following amplification, the PCR products were sequenced to determine whether one or two genes were expressed. The results shown in Figure 5-3 indicated that in each case, both members of each gene pair are expressed.

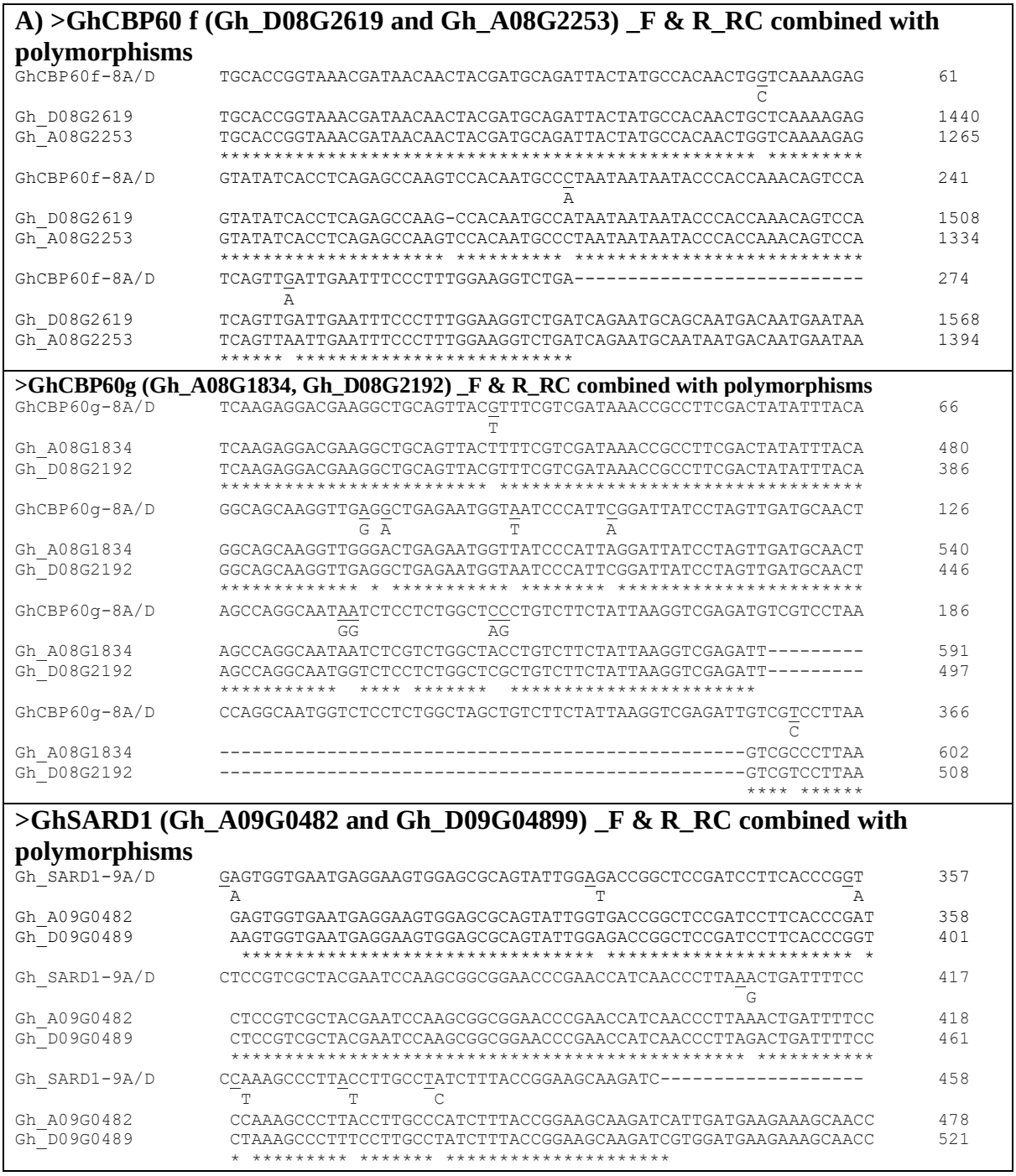


Figure 5-10. Multiple sequences alignment of the gene pairs sequence of *GhCBP60f-8A/D*, *GhCBP60g-8A/D* and *GhSARD1-9A/D* and their related amplified and sequenced sections using ClustalO tool.

5.5.4 The analysis of promoter sequence in GhCBP60

The promoter sequences of 1500 bp upstream from the transcriptional start sites of *GhCBP60* were searched manually to detect the presence of previously identified cis-regulatory elements CANNTG, BRRE (CGTGTG/CGTGCG), G-box (CATGTG), E-box (GGTCC) and TGACG either strands. Table 5-3 shows a summary of the stress responsiveness of transcription factors

GhCBP60a-g and GhSARD1 in both qRT-PCR and microarray experiments and the number of each regulatory element. The 23 promoters of GhCBP60 contain higher numbers of conserved CANNTG cis-elements as compared to TGACG motif (Table 5-3). The table shows the frequency of cis-elements in each group of genes, and stress-responsive versus non-stress responsive genes is shown in Figure 5-4. Student's t-test was used to evaluate the statistical significance of enrichment of each element. CANNTG is known to be a very low stringency element, found in 97% of genes, therefore its presence is not informative. However, the number of CANNTG was fewer in the GhCBP60b/c/d group (both $P<0.01$) than the other groups of genes. There was no difference between the numbers of CANNTG in the stress- responsive GhCBP60a/f/g and GhSARD1 and non-stress responsive GhCBP60a/f/g and GhSARD1 groups (P= 0.3).

BRREs (CGTGTG/CGTGCG) have been found to be enriched in the BZR1 binding regions associated with BR-induced and repressed target in Arabidopsis (Sun et al., 2010). I found that the number of BRRE (CGTGTG/CGTGCG) cis-elements was significantly higher in GhCBP60b/c/d group (both $P\leq0.01$) as compared to other groups. On the other hand, no GhCBP60a/f/g/ and GhSARD1 genes had the BRRE site within their promoters.

An additional cis-element enriched in the promoter sequences of Arabidopsis BZR1 is the G-box (CATGTG) which is a more stringent version of CANNTG and it contains two inverted repeats of the BRRE core sequences, CGTG and is also a type of E-box (Sun et al., 2010). The results in Table 5.3 showed the presence of CATGTG cis-elements in both GhCBP60b/c/d and non-stress responsive GhCBP60a/f/g and GhSARD1 with an average of 0.33 and 0.28 respectively. However, the results revealed that the promoter sequences of the stress-responsive GhCBP60a/f/g and GhSARD1 group do not have this binding site.

GGTCC is another binding site enriched in the promoter sequences of BZR1-induced and repressed Arabidopsis genes (Sun et al., 2010). There was a significantly higher number of GGTCC in the stress-responsive GhCBP60a/f/g and GhSARD1 group ($P\leq0.002$) than the GhCBP60b/c/d group. However, there was no significant difference in the frequency of GGTCC elements in stress-responsive versus non-stress responsive genes (both $P\leq0.34$) from the combined GhCBP60a/f/g and GhSARD1 groups.

TGACG is another binding site which is found in the promoter region of AtCBP60g and AtSARD1, both genes are found to be direct targets of TGA1 and TGA4 (Sun et al., 2018). Furthermore, these binding factors are found to regulate pipecolic acid (Pip) and SA biosynthesis by modulating the

expression of AtCBP60g and AtSARD1(Sun et al., 2018). However, this binding site is found in more than half of all genes. The GhCBP60b/c/d group had a significantly higher number of TGACG cis-elements ($P \leq 0.02$) than the combined GhCBP60a/f/g and GhSARD1 groups. However, there was no significant difference in frequency of TGACG elements ($P \leq 0.11$) in stress-responsive versus non-stress responsive genes from the combined GhCBP60a/f/g and GhSARD1 groups.

The results of this analysis indicated that there was no overrepresentation of cis-regulatory elements CANNTG, BRRE (CGTGTG/CGTGCG), G-box (CATGTG), E-box (GGTCC), and TGACG either strands in the promoters of stress-responsive GhCBP60a/f/g and GhSARD1 genes (Figure 5-4). Nevertheless, there are some interesting differences between GhCBP60b/c/d and GhCBP60a/f/g stress and non-stress responsive gene groups.

Table 5-3. Summary of GhCBP60 ID, GhCBP60(a-g and SARD1) group, stress-responsive transcription factors, number of stress-responsive transcription factors on sense and antisense strand, GhCBP60 stress signal from qRT-PCR and microarray data (Dash et al., 2011) and RNA-seq data from cotton (Zhu et al., 2017).

Gene (ID)	Group	Stress response	CANNTG	BRRE CGTGTG/CGTGCG	G-box CACGTG	GGTCC	TGACG sense	TGACG antisense	TGACG/CGTCA either
Gh-A05G1410	B/C/D	possible microarray/RNA-seq	1	1	0	0	0	0	0
Gh-D05G1575	B/C/D	possible microarray/RNA-seq	1	1	0	0	0	0	0
Gh-A06G1790	B/C/D	Not expressed	3	0	1	0	0	1	1
Gh-D06G2188	B/C/D	Not expressed	4	0	1	0	0	2	2
Gh-A08G0194	B/C/D	possible microarray/RNA-seq	5	1	0	0	5	2	7
Gh-D08G0271	B/C/D	possible microarray/RNA-seq	2	1	0	0	4	3	7
Gh-D13G2214	B/C/D	possible microarray/RNA-seq	3	0	0	0	1	1	2
GhA13G2354	B/C/D	possible microarray/RNA-seq	6	0	0	0	2	1	3
Gh-A10G0202	B/C/D	possible microarray/RNA-seq	4	1	1	1	1	0	1
Gh-D08G2619	F	Up-regulated	7	0	0	0	0	0	0
Gh-A08G2253	F	Up-regulated	5	0	0	0	0	0	0
Gh-A03G0544	A	Not expressed	4	0	0	1	0	1	1
Gh-D03G0984	A	No response	7	0	0	2	0	0	0
Gh-D12G2633	A	Down-regulated	3	0	0	1	0	0	0
Gh-A12G2506	A	Not expressed	2	0	0	1	0		0
Gh-D08G2192	G	Up-regulated	5	0	0	2	0	0	0
Gh-A08G1834	G	Up-regulated	5	0	0	2	0	1	1
Gh-A13G0918	G	Not expressed	7	0	0	0	1	0	1
Gh-D13G1162	G	No response	10	0	2	0	2	1	3
Gh_D09G0489	SARD1	Down-regulated	7	0	0	1	0	2	2
Gh_A09G0482	SARD1	Down-regulated	7	0	0	1	0	1	1
Gh_D12G2533	SARD1	No response	7	0	0	0	0	1	1
Gh_A12G2425	SARD1	No response	6	0	0	1	1	1	2

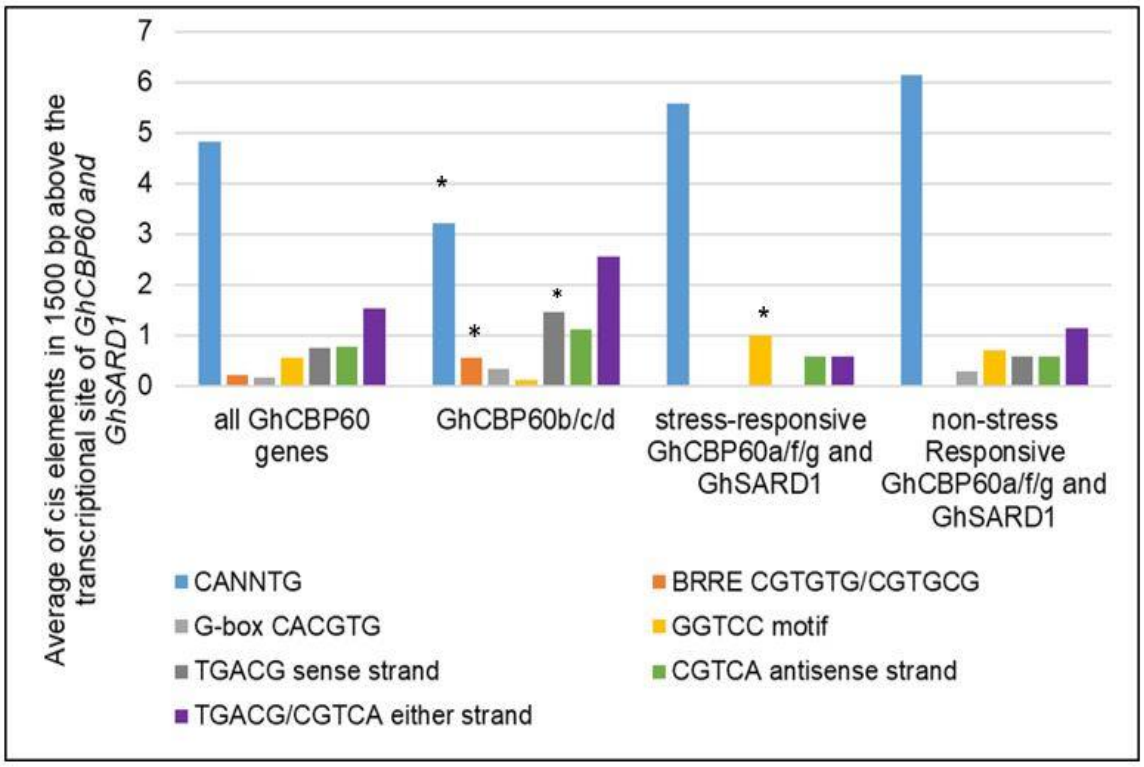


Figure Error! No text of specified style in document.**-11.** Comparison of the frequency of each cis-element in all GhCBP60, GhCBP60b/c/d, stress-responsive genes GhCBP60a/f/g and GhSARD1, and non-stress responsive genes GhCBP60a/f/g and GhSARD1. Bar graphs represent the mean of CANNTG, BRRE (GGTGTG/CGTGCG), E-box, GGTCC motif, TGACG sense, CGTCA and TGACG either strand. Significance of the frequency of each cis-element between GhCBP60b/c/d, and stress-responsive GhCBP60a/f/g and GhSARD1 gene groups; GhCBP60b/c/d, and non-stress responsive GhCBP60a/f/g and GhSARD1 gene groups or stress-responsive versus non-stress responsive GhCBP60a/f/g and GhSARD1 gene groups was performed by Student t-test, asterisks (*) represent the significance values of $P \geq 0.05$ of the frequency.

5.6 Discussion

5.6.1 *GhCBP60s* show similar stress responsiveness to *CBP60* genes from other plants

The previous results in Chapter 4 characterised the CBP60 gene family in cotton GhCBP60. They also identified cotton orthologues of AtCBP60a-g and AtSARD1 groups. I hypothesised that these genes play an important role in BR-mediated salt stress response in cotton and thus sought to determine whether the previously identified GhCBP60a-g and GhSARD1 were responsive to abiotic stress, similar to AtCBP60 genes. Given the large number of CBP60 genes in cotton, an initial screening to identify the most promising genes for the experimental study was undertaken using the publicly available PLEXdp datasets from a collaborative microarray project based on

Affymetrix arrays (Dash et al., 2011). Three different experiments—GO1, GO5 and GO7—were used to investigate the differential expression of GhCBP60 in response to waterlogging and drought. The results of *in silico* analysis suggested that GhCBP60a-12D, GhCBP60f-8A/D and GhCBP60g-8A/D may be stress-responsive. However, the data in regard to the up- or down-regulation in response to abiotic stresses were inconsistent and contradictory. In addition, there were no data for three of four GhSARD1 genes because of a lack of probe sets; therefore, I investigated the effect of salt and EBR on the expression of these genes by qRT-PCR.

Our quantitative gene expression results showed that GhCBP60a-12D and possibly GhSARD1-9/A were down-regulated in the leaf tissue in response to salt treatment, but only in the absence of EBR. In contrast, GhCBP60g-8A/D were significantly up-regulated by salt. A previous study revealed the contrasting roles of AtCBP60a as negative (Truman et al., 2013) and ATCBP60g and AtSARD1 as positive regulators of plant immunity (Wang et al., 2011; Zhang et al., 2010). The roles of each of *CBP60a*, *CBP60g* and *SARD1* appear to be unique. Truman et al. (2013) tested the effects of *cbp60a*, *cbp60g* and *sard1* on the growth of *Pseudomonas syringae* pv *maculicola* strain ES4326 (Psm ES4326). Their study revealed that bacterial growth was increased in *cbp60g* plants only when *CBP60a* existed, while the increase of bacterial growth in sard1 plants was independent of *CBP60a*, suggesting that the main role of *CBP60g* may be to counter the repressive effect of *CBP60a* (Truman et al., 2013). The contrasting role between the down-regulation of *GhCBP60a* and up-regulation of *GhCBP60g* in response to salt stress might be due to their antagonistic role in plant stress (Truman et al., 2013). However, there is no explanation for why *GhSARD1* was down-regulated under salt stress, considering that both CBP60g and SARD1 hypothetically act as positive regulators of plant immunity, opposite to *GhCBP60a*. A study by Wan et al. (2012) indicated another role of *AtCBP60g* in mediating stress, where the over-expression of *CBP60g* improved plant tolerance to drought stress and abscisic acid, while *cbp60g* increased plants' sensitivity to drought. In another independent study, the transcript levels of *CBP60g* were up-regulated in leaf tissue after three weeks of exposure to cold stress (Kim et al., 2013). The expression level of ICS1 was up-regulated after two weeks of leaf exposure to low temperature, compared with control results, in increased the freezing tolerance of Arabidopsis (Kim et al., 2013). A recent study by Qin et al. (2018) found that Arabidopsis *CBP60g*, *SARD1* and cotton *CBP60b* (*CBP60g-8D*) are direct targets of VdSCP41 protein to inhibit plants' immunity. They also revealed that the CaM-binding domain of AtCBP60b (AtCBP60g-8D) is required for VdSCP41 targeting. They further stated that both *cbp60g* and *sard1* were more susceptible to *V. dahliae*. Further, their study of virus-induced silencing of *GhCBP60b* decreased plant resistance to the pathogen, suggesting the key role of

transcription factors GhCBP60b, SARD1 and VdSCP41 in regulating plant immunity. It is worth noting that the cotton CBP60b gene, which was named CBP60b in Qin et al. (2018), is one orthologue out of four cotton orthologues of the CBP60g group (CBP60g-8D)—refer to Chapter 4.

Our results in Figure 5-2 indicated that the two genes from the GhCBP60f group (GhCBP60f-8A/D) and two genes from the GhCBP60g group (GhCBP60g-8A/D) were up-regulated in the leaf by salt. My results are similar to those of Pallegar (2014) in that the transcript level of AtCBP60f was up-regulated in the leaf tissue in response to salt treatment, as compared with the control. In addition, over-expressing lines of *AtCBP60f* showed increased tolerance to salt, as compared with the wild-type and *cbp60f* (Pallegar, 2014). The authors suggested that, under stress conditions, the elevated level of Ca^{2+} leads to the activation of CBP60s directly by Ca^{2+}/CaM cascade or via BR signalling pathways, where BR activates the transcription factor BES1, which binds to the promoter sequence of CBP60 to facilitate their expression. Induced CBP60s may act as transcriptional factors by binding to the specific DNA sequences on stress-related target genes. In contrast, another study by Truman et al. (2013) found that there was no effect of *cbp60f* on the growth of Psm ES4326, suggesting no role for *cbp60f* in plant immunity.

5.6.2 The expression of GhCBP60s in response to EBR and salt treatments

To date, there have been no comprehensive studies on the relationship between BRs and *CBP60* on the response of cotton plants to biotic and abiotic stresses. Therefore, I set out to test whether *GhCBP60* gene expression is responsive to EBR. The aim of the work reported here was to identify salt-responsive genes among the *GhCBP60* gene family that are also responsive to BR treatment. Our results revealed that the expression of *GhCBP60a-12D, GhCBP60f-8A/D,* and *GhCBP60g-8A/D* were down-regulated in the leaf tissue by EBR treatment. Here, in this study, I report an exclusive identification of novel EBR-responsive candidate genes *GhCBP60a-12D, GhCBP60f-8A/D,* and *GhCBP60g-8A/D* from cotton.

5.6.3 The stress response of *GhCBP60* gene has no relationship with cis-regulatory elements

I have shown that some gene members of *GhCBP60* (*GhCBP60a-12/D, GhCBP60f-8/A/D, GhCBP60g-8A/D)* were up-regulated by EBR treatment. These results raise many questions that remain to be addressed, are these genes directly regulated as part of BR signal transduction

pathways? And if so, do BES1 and BZR1 bind directly to the promoter of *GhCBP60* and facilitate their expression? If yes, I expected the over-representation of these cis-elements in the promoter sequences of *GhCBP60*.

In plant biotechnology, the knowledge on promoters is of major interest that will offer the chance to control gene expression in various areas (Lescot et al., 2002). Gene promoters refer to DNA sequences that are located upstream of gene coding regions and comprise several cis-acting elements, which are specific binding sites for proteins involved in the initiation and regulation of transcription (Hernandez-Garcia & Finer, 2014). These cis-acting elements control the regulation of gene expression at the promoter level. Regulation of gene expression and cell development in both animals and plants also require steroids hormones. The plant steroid hormone BR differs to animal steroid hormones that bind directly to nuclear receptor transcription factors. Instead, BR binds to a transmembrane receptor kinase, BRASSINOSTEROID INSENSITIVE1 (BRI1) (Wang et al., 2001) that contain a leucine-rich repeat (LRR) extracellular domain similar to the metazoans toll receptors (Wang et al., 2001). BRI1 signalling activates a plant-specific transcription factor BRASSINAZOLE RESISTANT1 (BZR1) via a phosphorylation-mediated signal transduction pathway (Clouse, 2011; Kim & Wang, 2010). In order to program genome expression and cell growth, BZR1 which has DNA binding domain that recognizes BR response element (BRRE, CGTG (T/C) G) (He et al., 2005) activates and represses different target genes. Similar to numerous plant transcription factors, BZR1 acts as a transcriptional repressor for some promoters but an activator for others. Previous studies on genome-wide identification of BZR1 direct binding sites and transcriptome profiling demonstrated that BZR1 binds to promoters of both BR-induced and repressed genes (He et al., 2005; Sun et al., 2010). Promoter cis-elements and trans-factors relatively determine BZR1 transcriptional activity. The genome-wide data from Arabidopsis plants showed that the promoters of BR-repressed gene is rich with BRRE whilst the promoters of BR-activated genes are rich with E-box motif (CANNTG) (Sun et al., 2010). I have shown above that *GhCBP60f* and *GhCBP60g* are up-regulated by BR. It is possible that BR may regulate *GhCBP60* gene expression via the BZR1 transcription factor that is enriched with binding sites of two regulatory cis-elements BRRE (CGTGTG and CGTGCG), (CACGTG) a type of G-box, GGTCC motif and TGACG-binding factor 1 (TGA1) and TGACG-binding factor 4 (TGA4). Our results revealed that the gene promoters of the *GhCBP60a/f/g* and *GhSARD1* group contain higher numbers of CANNTG cis-elements as compared to *GhCBP60b/c/d* group with an average of 5.5 to 3.2 cis-elements, respectively. However, CANNTG is less stringent and found to be overrepresented in the promoters of BR biosynthetic genes (Kim et al., 2009). The results also showed that the stress-

responsive *GhCBP60a/f/g* and *GhSARD1* had more GGTCC cis-elements as compared to the *GhCBP60b/c/d* group with an average of 1 to 0.1, respectively. In contrast to Sun et al. (2010), our result showed that the *GhCBP60a/f* and *GhCBP60g* genes that are up-regulated in EBR treatment have less (CANNTG) and GGTCC cis-elements, respectively than *GhSARD1* that was possibly down-regulated under salt stress.

Our results showed that only *GhCBP60 b/c/d* group had the BRRE (GTGTG/CGTGCG) within their promoters as compared to the stress and non-stress-responsive *GhCBP60a/f/g* and *GhSARD1* groups. In contrast to Sun et al. (2010) and our hypothesis, the stress-responsive *GhCBP60a/f/g* and *GhSARD1 groups* that were up-regulated by EBR treatment, they were not up-regulated by BRRE (CGTGTG/CGTGCG) cis-elements suggesting that the up-regulation of these genes mediated through other motifs.

I also hypothesise that the oligonucleotide sequence TGACG binds to the promoter sequences of *GhCBP60a/f/g* and *GhSARD1* and this binding site frequently occurs in the promoters of *GhCBP60a/f/g* and *GhSARD1*. Therefore, it is likely that the core binding site TGACG is a direct target for up/down-regulation of *GhCBP60* in response to different signalling pathways and abiotic and biotic stresses. The results showed that the promoter region of the stress-responsive *GhCBP60a/f/g* and *GhSARD1* group has a smaller number of TGATC cis-elements than other groups. In contrast to our hypothesis, the stress-responsive *GhCBP60a/f/*g groups that were up-regulated by EBR treatment have a smaller number of TGACG cis-elements than *GhSARD1* that was possibly down-regulated by salt.

The results suggested that there was no positive correlation between cis-regulatory elements CANNTG, BRRE (GTGTG/CGTGCG), GGTCC motif and TGA1 (TGACG) and TGA4 (TGATC) strands and GhCBP60 stress responses. Overall, the discovery of cis-elements in the *GhCBP60* gene family will provide a foundation for the gene-editing technology in cotton.

Chapter 6. General Discussion

Brassinosteroids (BRs) are a class of plant steroidal hormones that play a versatile role in modulating plant growth and development. They are also known for their involvement in mediating tolerance to abiotic and biotic stresses. In this study, I investigated the effect of 24-epibrassinlide (EBR) on the phenotypic responses of cotton seedlings under salt, drought and *Verticillium dahliae*. Plant-specific calmodulin-binding proteins (CBP60s) are also involved in plant growth and stress response. Bioinformatics tools were used to find and characterise GhCBP60 proteins orthologous to AtCBP60s, and to predict the subcellular localisation of GhCBP60. *In silico* expression analysis was used to investigate the differential expression of *GhCBP60* under waterlogging and drought stress conditions to identify the most stress-responsive *GhCBP60* using PLEXdp database. Furthermore, a qRT-PCR experiment was conducted to investigate the expression of stress-related transcription factor-encoding cotton genes *GhCBP60a-12A*, *GhCBP60f-8A/D, GhSARD1/9, 12A/D* in the leaf tissue in response to EBR and salt stress. Finally, I searched for the presence and overrepresentation of previously studied cis-regulatory elements in the promoter regions of *GhCBP60* genes to investigate the direct or indirect regulation of these genes by BZR1 and BES1/BZR2 which are key transcription factors that mediate BR responsive gene expression in response to growth and stress in plants.

Interestingly, the present study has shown that there was no positive response of cotton seedlings under stress to a low concentration of 0.2 µM EBR and there was even a toxic effect on plant growth when a high concentration of 0.5 µM EBR was used. There are several possible reasons for the observed lack of effect of EBR on cotton plant growth under stress. As observed for other studies, these may include (1) poor uptake of EBR by plants (Symons & Reid, 2004), (2) non-optimal concentration (Hu et al., 2016) and (3) less extreme stress as compared to other studies (Li et al., 2008; Shu et al., 2015). It was hypothesised that the exogenous application of EBR may alleviate some of the biotic and abiotic stress symptoms in cotton plants. However, it can be concluded from the present study that the agrochemical application of EBR is unlikely to be the ideal way to mitigate these stresses or even to determine whether there is a potential effect of EBR on cotton growth in response to biotic and abiotic stresses. Indeed, most exogenous hormonal applications do not achieve the desired effect in plants and may lead to undesirable phenotype and possibly yield losses. Concentration, timing, tissue and organ location within the plant are critical when endogenous plant hormones are produced and transported to cells. For example, exogenous defence hormone applications (e.g. salicylic acid) may slow down growth and can lead to early senescence or cell

death (Brown & Saa, 2015; Ghazijahani et al., 2014; Janda et al., 2017). On the other hand, a recent study conducted by Chen et al. (2019) found that cotton brassinosteroid (BR)-deficient mutant (*pag1*) plants were more sensitive to drought as compared to wild-type plants, indicating a clear role of BR for plant stress responses in cotton. This suggests that genetic studies (forward and reverse genetic approaches) may have the potential to identify the functional role of BRs in mediating stress responses during biotic and abiotic stresses in cotton. For example, the modulation of regulatory and biosynthetic genes in the BR pathway can be achieved via GM plants by over-expressing genes or by CRISPR/Cas9-mediated gene-editing technology. To identify suitable candidates for this approach, this study aims to find cotton*CBP60* genes and to identify the most stress-responsive genes to BR, abiotic and abiotic stresses in plants.

Overall, the bioinformatics section of this study has successfully identified *AtCBP60* orthologues in *G. hirsutum*, namely from the *GhCBP60* gene family, which has been shown to be closely related to *AtCBP60* based on conserved amino acid sequence homology. In this study, I give all the cotton CBP60 systematic names (Table 6-1) to avoid confusing or misleading gene nomenclature as in Qin et al. (2018). In this paper, the authors had referred to the *GhCBP60g-8/D* gene as *GhCBP60b,* although it is orthologous to *CBP60g,* not *CBP60b*. The phylogenetic analysis of AtCBP60 and GhCBP60 proteins revealed the conservation of the two major clades in cotton similar to Arabidopsis. It has also been shown that each protein of *AtCBP60* had been expanded in the *GhCBP60* gene family because *G. hirsutum* is an allotetraploid. Clade 1 contains AtCBP60a-g and GhSARD1 proteins; each gene in Arabidopsis has four co-orthologues in cotton: GhCBP60a-3,12/A/D, GhCBP60g-8,13A/D and *Gh*SARD1-9,12A/D. Clade 2 contains five Arabidopsis proteins including AtCBP60b/c/d with nine co-orthologues in cotton that are clustered together in one sub-branch GhCBP60bcd-5A/D, GhCBP60bcd-6A/D, GhCBP60bcd-8A/D, GhCBP60bcd-10A, GhCBP60bcd-13A/D. A second sub-branch has AtCBP60e and AtCBP60f as well as two co-orthologues in cotton, GhCBP60f-8A/D.

The results of JPRED secondary structure prediction have shown that the predicted CaM-binding domain to be an alfa helix in both AtCBP60a and GhCBP60a. The results of ClustalO tool also show greater sequence similarities between the C-terminus of GhCBP60 and the CaM-binding domain of AtCBP60 indicated by the conservation of hydrophobic residues suggesting the high conservation of CaM-binding domain of GhCBP60a in cotton. The results of Multalin reveal that the DNA-binding domains of all GhCBP60s except GhSARD1 which has only the DNA-binding domain similar to AtSARD1. Furthermore, the conservation of nuclear localisation signals of

GhCBP60 also suggests a potential role of GhCBP60s as transcription factors in mediating stress response in cotton similar to other plant species.

Table 6-1. Arabidopsis *CBP60* gene family members and their proposed gene names in *G. hirsutum*

Arabidopsis-*CBP60*	**Proposed gene names for *CBP60* in *G. hirsutum***
AtCBP60b/c/d	*GhCBP60bcd-5A* *GhCBP60bcd-5D* *GhCBP60bcd-6A* *GhCBP60bcd-6D* *GhCBP60bcd-8A* *GhCBP60bcd-8D* *GhCBP60bcd-10A* *GhCBP60bcd-13A* *GhCBP60bcd-13D*
AtCBP60f	*GhCBP60f-8A* *GhCBP60f-8D*
AtCBP60a	*GhCBP60a-3A* *GhCBP60a-3D* *GhCBP60a-12A* *GhCBP60a-12D*
AtCBP60g	*GhCBP60g-8A* *GhCBP60g-8D* *GhCBP60g-13A* *GhCBP60g-13D*
AtSARD1	*GhSARD1-9A* *GhSARD1-9D* *GhSARD1-12A* *GhSARD1-12D*

Several studies have previously revealed the involvement of transcription factors *AtCBP60a*, *AtCBP60g*, and *AtSARD1* in mediating stress response to biotic and abiotic stresses. A study conducted by Truman et al. (2013) revealed the role of *AtCBP60g* and *AtSARD1* as positive and *AtCBP60a* as negative regulators of plant immunity. The functional analysis of these genes showed that the bacterial growth of *Pseudomonas syringae* pv. *maculicola* ES4326 (*P. syringae*) increased in *atcbp60g and atsard1* mutant plants but decreased in *atcbp60a* mutants. In particular, the CaM-binding activity of AtCBP60a represses the function of the proteins in plant immunity. This was demonstrated in a study where mutants of *atcbp60a* that lack the ability to bind CaM failed to complement the enhanced disease susceptibility phenotype of the mutants (Truman et al., 2013). Whereas, the CaM-binding activity of AtCBP60g is also required for the production of SA and the

function of AtCBP60g in defence signalling. Indeed, mutations in *atcbp60g* that abolish the CaM-binding activity of the protein failed in activating the plant immune response which is detrimental to the defence mechanisms of plants due to the low levels of SA in mutants (Wang et al., 2009).

Many questions were raised on whether the newly identified *GhCBP60* orthologues genes are also stress-responsive in cotton. If so, under what conditions? I searched the publicly available database from the Plant Expression Database (PLEXdp) to investigate the transcriptional responses of the various *GhCBP60* genes under abiotic stress (Dash et al., 2011). The results of microarray data showed that one gene of the *GhCBP60a* group (*GhCBP60a-12D*), one gene from the *GhCBP60g* group (*GhCBP60g-8A*) and the two genes of the *GhCBP60f* group (*GhCBP60f-8A/D*) appeared to be stress-responsive in cotton. The results of the microarray and a recent RNA-seq data analysis also suggest responsiveness of *GhCBP60b/c/d-5A/D, 6A/D, 8A/D, 10A, 13A/D* to abiotic stress (Zhu et al., 2017).

To investigate the putative roles of *GhCBP60* genes in BR- and abiotic stress signalling experimentally, I examined the transcriptional response of three newly identified genes or gene pairs (*GhCBP60a-12D, GhCBP60g-8A/D,* and *GhCBP60f-8A/D*) to salt stress using qRT-PCR. The results revealed the down-regulation of *GhCBP60a-12D* in the leaf tissue in response to salt in the absence of EBR. It also showed the up-regulation of *GhCBP60g-8A/D* under salt stress and possible down-regulation of *SARD1-9A/D* in the same tissue under salt stress but only in the absence of EBR. As mentioned above, *AtCBP60a,* and *AtCBP60g* and *AtSARD1* genes have antagonistic roles in regulating the growth of bacterial growth of *P. syringae* (Truman et al., 2013). They found that the bacterial growth of the pathogen increased in *atcbp60g* only in the presence of *atcbp60a*. However, the increase in the growth of the bacteria in *atsard1* was independent of *atcbp60a,* suggesting the key role of *AtCBP60g* in repressing the negative effect of *AtCBP60a* on plant immunity. A recent study conducted by Qin et al. (2018) confirmed the involvement of the CaM-binding domain of (*GhCBP60g-8D*) in regulating plant immunity in Arabidopsis. The authors suggested that the effector protein VdSCP41 binds to the CaM-binding domain of CBP60g to inhibit its activity, decreasing plant resistance to the pathogen *V. dahliae*. The CaM-binding domain of AtCBP60g is required for VdSCP41 targeting. Mutations in the master immune regulators of plant immunity *atcbp60g* and *atsard1*, partially impaired virulence mediated by VdSCP41 and compromised plant resistance against *V. dahliae*. The authors also reported that virus-induced silencing of *GhCBP60g-8D* decreased plant resistance to *V. dahliae* suggesting the involvement of this gene in mediating disease resistance in cotton. Another independent study recently carried out by Cai et al. (2019)

revealed the involvement of a different calmodulin-binding protein (*GauCBP1*) in disease resistance response against the same pathogen in the Australian native cotton relative, *Gossypium australe*. An earlier study by Wang et al. (2009) also revealed the significant up-regulation of *AtCBP60g* gene in response to infection by *Psm* ES4326. SARD1was identified as the eighth family member of CBP60 proteins (Zhang et al., 2010). Although both *AtCBP60g/ and atsard1* act as positive regulators of plant immunity, AtSARD1 does not bind calmodulin, unlike A*tCBP60g*. It was further revealed that amino acid Val-29 is needed for the binding of AtCBP60g to CaM, and this residue is not conserved in AtSARD1 (Zhang et al., 2010). Furthermore, a study conducted by Wan et al. (2012) also presented evidence for a similar role of AtCBP60g in mediating abiotic stress, in which they found that the over-expression of *AtCBP60g* improved tolerance to drought stress and abscisic acid, whereas a*tcbp60g* plants showed increased sensitivity to these stresses. The antagonistic relationship between *AtCBP60a* and other *AtCBP60* genes have been reported in response to plant immunity. My results suggest that a similar antagonistic relationship may occur between *GhCBP60a-12D,* and *GhCBP60g-8A/D* in response to salt stress in cotton. However, there is no clear indication of why *GhSARD1* was down-regulated while its closely related orthologue *GhCBP60g* was up-regulated in the leaf tissue of cotton in response to salt. In the present study, I report the novel role of *GhCBP60a-12D, GhCBP60g-A/D* and a possible down-regulation of *GhSARD1-9/A/D* in the absence of EBR as salt stress-responsive genes with evolutionary highly conserved CaM/DNA-binding domains suggesting a potential functional role in cotton CBP60 as in other plant species

Very little work has been done on the involvement of CBP60 proteins in Clade 2 in stress responses. However, a previous study by Truman et al. (2013) investigated the roles of *AtCBP60b/c/d/f* in biotic stress responses by testing the effects of mutations in these genes on the growth of Psm ES4326. Mutations in *atcbp60f* had no effect on the bacterial growth of Psm ES4326 indicating the non-stress responsiveness of this gene in plant immunity. Similarly, the small increase in the bacterial growth of the pathogen in *atcbp60c* and *atcbp60d* suggest the non-stress responsiveness of these genes in plants (Truman et al., 2013). In another independent study conducted by Pallager (2014), the functional role of *AtCBP60f* as a BR-responsive gene in mediating salt stress tolerance in plants was revealed. The results of knockout mutants of a*tcbp60f* show increased sensitivity to salt, While the over-expression lines of *AtCBP60f* led to improved salt tolerance of plants as compared to wild type. In this study, I report the novel role of *GhCBP60f-8A/D* and possible up/down-regulation of *GhCBP60bcd-5A/D, GhCBP60bcd-6A/D, GhCBP60bcd-8A/D, GhCBP60bcd-10A, GhCBP60bcd-13A/D* as salt stress-responsive genes with functional conserved

CaM/DNA-binding domains suggesting regulatory functions for these genes in cotton similar to their roles in different species

To date, there is only one study conducted by Pallegar (2014), that reveals the role of *CBP60* in BR mediated stress tolerance in Arabidopsis plants. The authors proposed the possibility of gene regulation of *AtCBP60g and AtCBP60f* by BR. Therefore, I further investigated the transcriptional response of the most stress-responsive genes from microarray data *GhCBP60a-12D, GhCBP60g-8A*, *GhCBP60f-8A/D*, as well as *GhSARD1-9A/D* and *GhSARD1-12A/D* to EBR treatment. The results of qRT-PCR indicate the up-regulation of all genes except the *GhSARD1* following 24 h of floating leaf tissue on EBR solution indicating the ability of the hormone to move directly to the leaf cells. The results of this experiment support my suggestion that the inability of EBR to travel long distance could be the main reason limiting the whole plant phenotypic response to EBR. Similar to my results, the up-regulation *AtCBP60g* and *AtCBP60f* in the leaf tissue following short-term treatment of 24 h by 0.1 µM BL and long-term treatment of two weeks of 150 mM NaCl have been reported by Pallegar (2014). The authors further state that both *AtCBP60g* and *AtCBP60f* genes are BR responsive genes in Arabidopsis. The functional analysis of the *atcbp60f* mutant revealed the sensitivity of this mutant to salt stress as compared to wild-type plants, however, *AtCBP60f* over-expressing lines showed increased salt tolerance indicating their essential role in conferring salinity stress tolerance in plants. Collectively, these results suggest potential molecular links between BR signalling pathways and GhCBP60 transcription factors and stress tolerance in cotton.

Pallegar (2014), have also reported the presence of E-box elements (CANNTG), which is the binding site for the transcription factor BRI1-EMS-SUPPRESSOR1 (BES1) in promoters of *AtCBP60g and AtCBP60f*, suggesting the possible direct regulation of *AtCBP60g and AtCBP60f* genes by BR. The transcription factors BES1 and BZR1 also bind to additional cis-regulatory elements, BRRE (CGTGTG and CGTGCG) and G-box (CACGTG) which are overrepresented in the promoters of BR-biosynthetic genes, resulting in the up/down-regulation of these genes in response to high-temperature stress (Martínez et al., 2018). Furthermore, the transcription factors TGA1 and TGA4 are required for the full induction of *AtCBP60g*and *AtSARD1* and in plant defence against pathogen attack (Sun et al., 2018). To further determine whether *CBP60* genes in cotton could be directly regulated by BR-signalling pathways, I conducted a search of the promoter region, 1500 bp upstream of the transcription start site, of *GhCBP60* genes to investigate the presence and over-representation of cis-regulatory elements: E-box (CANNTG), BRRE (CGTGTG and CGTGCG) and G-box (CACGTG), GGTCC motif and TGA1 and TGA4 (TGACG).

Promoter sequence analyses revealed the presence of CANNTG cis-elements and GGTCC motif in the promoter sequences of stress-responsive *GhCBP60a-g* and *GhSARD1*. However, there was no over-representation of either element in stress-responsive genes as compared to the non-stress-responsive genes of the *GhCBP60a/f/g* and *GhSARD1* group.

On the other hand, the BRRE (CGTGTG and CGTGCG) and G-box (CACGTG) cis-regulatory elements were absent from the promoter sequences of the stress-responsive *GhCBP60a-g* and *GhSARD1*group. These results are in conflict with my hypothesis that the stress-responsive genes are directly up-regulated by EBR and suggest that the up/down-regulation of these genes is controlled by other motifs.

The results also showed that TGACG cis-elements were not consistently present within the promoter sequences of stress-responsive *GhCBP60a-g* and *GhSARD1*. In fact, contrary to my hypothesis, the stress-responsive genes that were up-regulated by EBR *GhCBP60a, GhCBP60f* and *GhCBP60g* had a smaller number of TGACG than genes with no response to stress.

Thus, the cis-element analyses revealed that there was no positive correlation between BR-related cis-regulatory elements and stress responsiveness in *GhCBP60,* suggesting that any regulation of *GhCBP60a, GhCBP60f, GhCBP60g* by BR, the signalling pathway is likely to be indirect.

The promoter analysis of the *GhCBP60b/c/d* group revealed significantly lower frequencies of CANNTG and GGTCC motifs, and the enrichment of BRRE (CGTGTG and CGTGCG) and TGACG compared to all CBP60 genes and stress- responsive *GhCBP60a/f/g* and *GhSARD1* genes.

Collectively, these results suggest the stress responsiveness of *GhCBP60b/c/d* and possible direct regulation of these genes by BR signalling.

In conclusion, my results provide evidence of a possible connection between BR signalling and GhCBP60 transcription factors in mediating abiotic stress responses in cotton indicated by the indirect up-regulation of *GhCBP60f* and *GhCBP60g* by BR signalling in response to salt stress. Moreover, the possible direct up/down-regulation of *GhCBP60b/c/d* by BR signalling requires further investigation.

6.1 Future directions

Previous studies on the effects of BRs on plant response to abiotic and biotic stress have been conducted using both exogenous BRs and genetic studies (over-expressing lines, mutant, and

knockout gene plants). As I had difficulties to determine a positive effect of exogenous application of EBR on the growth of cotton seeds and seedlings under stress, I suggest that genetic studies that can be utilised instead may further reveal the role of BR signalling in regulating stress adaptation in cotton. There are several directions to extend this work. The transcriptional response of *GhCBP60a-12D*, *GhCBP60f-8A/D,* and *GhCBP60g-8A/D* under different stress conditions such as drought, cold, and pathogens should be investigated. If positive effects of BR on seedling growth and development are obtained, a transcriptome analysis using RNA-seq should be carried out using BR-treated seedlings under both stressed and non-stressed conditions to identify BR pathways and BR receptor genes mostly affected by EBR under normal conditions. Further information is necessary to determine whether BR signalling pathways play a key role in mediating salt-stress tolerance in cotton.

More importantly, a functional equivalence test of *GhCBP60a-12D*, *GhCBP60f-8/A/D* and *GhCBP60g-8/A/D* genes in Arabidopsis through expression in respective Arabidopsis knockout mutants should be undertaken. In addition, generation of Arabidopsis lines over-expressing cotton *GhCBP60f-8/A/D* and *GhCBP60g-8/A/D* may be used to examine their regulatory role in response to abiotic and biotic stresses. The outcomes of future experiments will further confirm the involvement of novel *GhCBP60a-12D, GhCBP60f-8A/D* and *GhCBP60g-8A/D* in cotton growth and development in response to environmental stimuli. The discovery of these genes can be used as a molecular tool for breeding which will produce breakthroughs in the understanding of stress signalling mechanisms and adaptation in cotton.

Another important point for future research is the possible involvement in stress and BR response of *GhCBP60b/c/d* genes. Thus, the transcriptional response of *GhCBP60b/c/d* in both leaf and root tissue to BR and abiotic and biotic stressors should be investigated. This new information will provide a clear indication the role of *GhCBP60b/c/d* in regulating cotton responses under normal and stressed conditions. Even though many questions remain to be answered, the new insights obtained will be considered as a foundation for future studies to illustrate the mechanism of GhCBP60 proteins and their relation to BR signal transduction pathways in cotton.

References

ABC News. (2019). *Australia's cotton production halved as drought and low to no water allocation takes its toll.* https://www.abc.net.au/news/rural/2019-06-04/drought-and-low-water-allocation-impacts-cotton-harvest/11172966

Acharya, B. R., & Assmann, S. M. (2009). Hormone interactions in stomatal function. *Plant Molecular Biology*, *69*(4), 451–462.

Aghdam, M. S., Asghari, M., Farmani, B., Mohayeji, M., & Moradbeygi, H. (2012). Impact of postharvest brassinosteroids treatment on PAL activity in tomato fruit in response to chilling stress. *Scientia Horticulturae, 144*(0304-4238), 116–120.

Agricultural Biotechnology Council of Australia. (2012). *GM cotton in Australia: A resource guide.* https://www.abca.com.au/wp-content/uploads/2012/09/ABCA_Resource_Guide_3_v2.pdf

Ahammed, J., Zhang, S., Shi, K., Zhou, Y. H., & Yu, J. Q. (2012). Brassinosteroid improves seed germination and early development of tomato seedling under phenanthrene stress. *Plant Growth Regulation, 68*(1), 87–96.

Akira, S., & Shozo, F. (1997). Studies on biosynthesis of brassinosteroids. *Bioscience, Biotechnology, and Biochemistry, 61*(5), 757–762.

Alam, P., Albalawi, T. H., Altalayan, F. H., Bakht, M. A., Ahanger, M. A., Raja, V. & Ahmad, P. (2019). 24-Epibrassinolide (EBR) confers tolerance against NaCl stress in soybean plants by up-regulating antioxidant system, ascorbate-glutathione cycle, and glyoxalase system. *Biomolecules*, *9*(11), 640.

Alba, R., Payton, P., Fei, Z., McQuinn, R., Debbie, P., Martin, G. B. & Giovannoni, J. J. (2005). Transcriptome and selected metabolite analyses reveal multiple points of ethylene control during tomato fruit development. *The Plant Cell*, *17*(11), 2954-2965.

Albrecht, C., Boutrot, F., Segonzac, C., Schwessinger, B., Gimenez-Ibanez, S., Chinchilla, D., & Zipfel, C. (2012). Brassinosteroids inhibit pathogen-associated molecular pattern–triggered immune signalling independent of the receptor kinase BAK1. *Proceedings of the National Academy of Sciences, 109*(1), 303–308.

Ali, G. S., Reddy, V. S., Lindgren, P. B., Jakobek, J. L., & Reddy, A. S. N. (2003). Differential expression of genes encoding calmodulin-binding proteins in response to bacterial pathogens and inducers of defence responses. *Plant Molecular Biology*, *51*(6), 803–815.

Ali, S. S., Kumar, G. S., Khan, M., & Doohan, F. M. (2013). Brassinosteroid enhances resistance to fusarium diseases of barley. *Phytopathology, 103*(12), 1260–1267.

Allen, R. D., Burns, T. M., Light, G., & Fokar, M. (2000). Investigating the role of xyloglucan endotransglycosylase in cotton fibre quality. In: C. Benedict & G. Jividen (eds.), *Genetic control of cotton fibre and seed quality* (pp. 166–174). Cotton Incorporated. Cary, NC, USA.

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Research, 25*(17), 3389–3402.

Arora, P., Bhardwaj, R., & Kanwar, M. K. (2010). 24-epibrassinolide induced antioxidative defence system of *Brassica juncea* L. under Zn metal stress. *Physiology and Molecular Biology of Plants, 16*(3), 285–293.

Ashcraft, C. W. (1996). *The effect of brassinolide on cotton fibre development* (Doctoral dissertation). Texas Tech University, USA. http://hdl.handle.net/2346/13868

Ashraf, M. (2002). Salt tolerance of cotton: some new advances. *Critical Reviews in Plant Sciences, 21*(1), 1–30.

Avalbaev, A. M., Yuldashev, R. A., Fatkhutdinova, R. A., Urusov, F. A., Safutdinova, Y. V., & Shakirova, F. M. (2010). The influence of 24-epibrassidinolide on the hormonal status of

wheat plants under sodium chloride. *Applied Biochemistry and Microbiology, 46*(1), 99–102.
Aydin, Y., Talas-Ogres, T., Ipekçi-Altas, Z., & Gözükirmizi, N. (2006). Effects of brassinosteroid on cotton regeneration via somatic embryogenesis. *Biologia, 61*(3), 289–293.
Azpiroz, R., Wu, Y., LoCascio, J. C., & Feldmann, K. A. (1998). An Arabidopsis brassinosteroid-dependent mutant is blocked in cell elongation. *The Plant Cell*, 10(2), 219-230.
Backer, R., Naidoo, S., & Van den Berg, N. (2019). The nonexpressor of pathogenesis-related genes 1 (NPR1) and related family: Mechanistic insights in plant disease resistance. *Frontiers in Plant Science, 10*(664-462X), 102.
Baffes, J., Dow, B., English, P., Estur, G., Gohou, G., Haniotis, T., & Newfarmer, R. (2004). Cotton Market Setting, Trade Policies, and Issues." Policy Research Working Paper Number 3218. The World Bank: Washington, D. C. see www. worldbank. org/research Bangwe, LB. In Zambia." Centre for Development Studies Working Paper. University of Bath.
Bai, M. Y., Shang, J. X., Oh, E., Fan, M., Bai, Y., Zentella, R. & Wang, Z. Y. (2012). Brassinosteroid, gibberellin and phytochrome impinge on a common transcription module in Arabidopsis. *Nature cell biology, 14*(8), 810-817.
Bajguz, A., & Hayat, S. (2009). Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiology and Biochemistry, 47*(1), 1–8.
Bajguz, A., & Tretyn, A. (2003). The chemical characteristic and distribution of brassinosteroids in plants. *Phytochemistry, 62*(7), 1027–1046.
Ball, R. A., Oosterhuis, D. M., & Mauromoustakos, A. (1994). Growth dynamics of the cotton plant during water-deficit stress. *Agronomy Journal, 86*(5), 788–795.
Bechtold, U., & Field, B. (2018). Molecular mechanisms controlling plant growth during abiotic stress. s. *J. Exp. Bot.* 69(11), 2753–2758.
Bhardwaj, R., Sharma, P., Arora, H. K., & Arora, N. (2008). 28-Homobrassinolide regulated Mn-uptake and growth of *Brassica juncea*. L. *Canadian Journal of Pure and Applied Sciences, 2*(1), 149–154.
Bishop, G. J., & Yokota, T. (2001). Plants steroid hormones, brassinosteroids: Current highlights of molecular aspects on their synthesis/metabolism, transport, perception and response. *Plant and Cell Physiology, 42*(2), 114–120.
Bouché, N., Yellin, A., Snedden, W. A., & Fromm, H. (2005). Plant-specific calmodulin-binding proteins. *Annual Review of Plant Biology, 56*(1), 435–466.
Boudsocq, M., & Laurière, C. (2005). Osmotic signalling in plants: Multiple pathways mediated by emerging kinase families. *Plant Physiology, 138*(3), 1185–1194.
Boyer, J. S. (1982). Plant productivity and environment. *Science, 218*(4571), 443–448.
Bremen Cotton Exchange. (2015). *Cotton*. https://baumwollboerse.de/en/informationen/baumwolle/
Brown, P., & Saa, S. (2015). Biostimulants in agriculture. *Frontiers in Plant Science, 6*(1664-462X), 671.
Brubaker, C. L., Bourland, F. M., & Wendel, J. E. (1999). Chapter 1.1: The origin and domestication of cotton. In: C. W. Smith & J. T. Cothren (eds.), *Cotton: Origin, history, technology, and production* (pp. 3–31). New York: John Wiley and Sons. https://www.wiley.com/en-au/9780471180456
Brugnoli, E., & Björkman, O. (1992). Growth of cotton under continuous salinity stress: Influence on allocation pattern, stomatal and non-stomatal components of photosynthesis and dissipation of excess light energy. *Planta, 187*(3), 335–347.
Cai, Y., Cai, X., Wang, Q., Wang, P., Zhang, Y., Cai, C. & Liu, F. (2020). Genome sequencing of the Australian wild diploid species *Gossypium australe* highlights disease resistance and delayed gland morphogenesis. *Plant biotechnology journal, 18*(3), 814-828.

Campos, M. L., De Almeida, M., Rossi, M. L., Martinelli, A. P., Litholdo Junior, C. G., Figueira, A., & Pereira Peres, L. E. (2009). Brassinosteroids interact negatively with jasmonates in the formation of anti-herbivory traits in tomato. *Journal of Experimental Botany, 60*(15), 4347–4361.

Chakma, S. P. (2016). *Effects of brassinosteroid on salt and drought stress responses in cotton seedlings* (Unpublished master's thesis). University of New England, Armidale, Australia.

Chen, E., Zhang, X., Yang, Z., Zhang, C., Wang, X., Ge, X., & Li, F. (2019). BR deficiency causes increased sensitivity to drought and yield penalty in cotton. *BMC Plant Biology, 19*(1), 220.

Chen, E., Zhang, X., Yang, Z., Zhang, C., Wang, X., Ge, X., & Li, F. (2019). BR deficiency causes increased sensitivity to drought and yield penalty in cotton. *BMC Plant Biology, 19*(1), 220.

Chinchilla, D., Shan, L., He, P., de Vries, S., & Kemmerling, B. (2009). One for all: The receptor-associated kinase BAK1. *Trends in Plant Science, 14*(10), 535–541.

Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Nürnberger, T., Jones, J. D., & Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defence. *Nature, 448*(7152), 497.

Choe, S., Dilkes, B. P., Fujioka, S., Takatsuto, S., Sakurai, A., & Feldmann, K. A. (1998). The *DWF4* gene of Arabidopsis encodes a cytochrome P450 that mediates multiple 22α-hydroxylation steps in brassinosteroid biosynthesis. *The Plant Cell, 10*(2), 231–243.

Choe, S., Schmitz, R. J., Fujioka, S., Takatsuto, S., Lee, M. O., Yoshida, S., & Tax, F. E. (2002). Arabidopsis brassinosteroid-insensitive dwarf12 mutants are semidominant and defective in a glycogen synthase kinase 3β-like kinase. *Plant Physiology, 130*(3), 1506–1515.

Choi, Y. H., Fujioka, S., Harada, A., Yokota, T., Takatsuto, S., & Sakurai, A. (1996). A brassinolide biosynthetic pathway via 6-deoxocastasterone. *Phytochemistry, 43*(3), 593–596.

Chory, J., Nagpal, P., & Peto, C. A. (1991). Phenotypic and genetic analysis of det2, a new mutant that affects light-regulated seedling development in Arabidopsis. *The Plant Cell, 3*(5), 445–459.

Choudhury, S., Panda, P., Sahoo, L., & Panda, S. K. (2013). Reactive oxygen species signaling in plants under abiotic stress. *Plant Signaling & Behaviour, 8*(4), e23681.

Christianson, J. A., Llewellyn, D. J., Dennis, E. S., & Wilson, I. W. (2010). Global gene expression responses to waterlogging in roots and leaves of cotton (*Gossypium hirsutum* L.). *Plant and Cell Physiology, 51*(1), 21–37.

Chung, Y., Kwon, S. I., & Choe, S. (2014). Antagonistic regulation of Arabidopsis growth by brassinosteroids and abiotic stresses. *Molecules and Cells, 37*(11), 795.

Chung, Y., Maharjan, P. M., Lee, O., Fujioka, S., Jang, S., Kim, B., & Park, T. (2011). Auxin stimulates DWARF4 expression and brassinosteroid biosynthesis in Arabidopsis. *The Plant Journal, 66*(4), 564–578.

Clouse, S. D. (2011). Brassinosteroid signal transduction: From receptor kinase activation to transcriptional networks regulating plant development. *Plant Cell, 23*(4), 1219–1230.

Corpet, F. (1988). Multiple sequence alignment with hierarchical clustering. *Nucleic Acids Research, 16*(22), 10881–10890.

Cotton Australia. (2018). *Cotton annual 2018—Australian cotton industry statistic*. https://cottonaustralia.com.au/statistics

Cotton Seed Distribution Extension and Development Team. (2012). *New varieties—Sicot 730 & Siokra* [Fact sheet]. Retrieved October 15, 2018 from https://www.csd.net.au/

CottonInfo. (2015). *Water & soil*. https://www.cottoninfo.com.au/sites/default/files/documents/Water%20and%20Soil%20Quality_3.pdf

Creelman, R. A., & Mullet, J. E. (1995). Jasmonic acid distribution and action in plants: Regulation during development and response to biotic and abiotic stress. *Proceedings of the National Academy of Sciences USA, 92*(10), 4114–4119.

Cronn, R. C., Small, R. L., Haselkorn, T., & Wendel, J. F. (2002). Rapid diversification of the cotton genus (Gossypium: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. *American Journal of Botany, 89*(4), 707–725.

CSIRO. (2015). *Case study of impact.* https://www.csiro.au/~/media/About/Files/Impact-case-studies/2015/ICS-COTTON

Cui, F., Liu, L., Zhao, Q., Zhang, Z., Li, Q., Lin, B., & Xie, Q. (2012). Arabidopsis ubiquitin conjugase UBC32 is an ERAD component that functions in brassinosteroid-mediated salt stress tolerance. *The Plant Cell, 24*(1), 233–244.

Dalio, R. J. D., Pinheiro, H. P., Sodek, L., & Haddad, C. R. B. (2013). 24-epibrassinolide restores nitrogen metabolism of pigeon pea under saline stress. *Botanical Studies, 54*(1), 9.

Damghan, I. R. (2009). Exogenous application of brassinosteroid alleviates drought-induced oxidative stress in *Lycopersicon esculentum* L. *General and Applied Plant Physiology, 35*(1–2), 22–34.

Dash, S., Niemaczura, W., & Harrington, H. M. (1997). Characterization of the basic amphiphilic α-helix calmodulin-binding domain of a 61.5 kDa tobacco calmodulin-binding protein. *Biochemistry, 36*(8), 2025–2029.

Dash, S., Van Hemert, J., Hong, L., Wise, R. P., & Dickerson, J. A. (2011). PLEXdb: Gene expression resources for plants and plant pathogens. *Nucleic Acids Research, 40*(D1), D1194–D1201.

De Vleesschauwer, D., Van Buyten, E., Satoh, K., Balidion, J., Mauleon, R., Choi, I. R., & Höfte, M. (2012). Brassinosteroids antagonize gibberellin-and salicylate-mediated root immunity in rice. *Plant Physiology, 158*(4), 1833–1846.

Department of Agriculture and Water Resources. (2019). *Cotton*. https://www.agriculture.gov.au/ag-farm-food/crops/cotton

Dhaubhadel, S., Browning, K. S., Gallie, D. R., & Krishna, P. (2002). Brassinosteroid functions to protect the translational machinery and heat-shock protein synthesis following thermal stress. *The Plant Journal, 29*(6), 681–691.

Dhaubhadel, S., Chaudhary, S., Dobinson, K. F., & Krishna, P. (1999). Treatment with 24-epibrassinolide, a brassinosteroid, increases the basic thermotolerance of *Brassica napus* and tomato seedlings. *Plant Molecular Biology, 40*(2), 333–342.

Divi, U. K., Rahman, T., & Krishna, P. (2010). Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. *BMC plant biology, 10*(1), 151.

Divi, U. K., Rahman, T., & Krishna, P. (2016). Gene expression and functional analyses in brassinosteroid-mediated stress tolerance. *Plant Biotechnology Journal, 14*(1), 419–432.

Dong, Y., Wang, W., Hu, G., Chen, W., Zhuge, Y., Wang, Z., & He, M. R. (2017). Role of exogenous 24-epibrassinolide in enhancing the salt tolerance of wheat seedlings. *Journal of Soil Science and Plant Nutrition, 17*(3), 554–569.

Drozdetskiy, A., Cole, C., Procter, J., & Barton, G. J. (2015). JPred4: A protein secondary structure prediction server. *Nucleic Acids Research, 43*(W1), W389–W394.

Du, L., & Poovaiah, B. W. (2005). Ca2+ / calmodulin is critical for brassinosteroid biosynthesis and plant growth. *Nature, 437*(29), 741.

Edgar, R. C. (2004). MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*(5), 1792–1797.

Endrizzi, J. E., Turcotte, E. L., & Kohel, R. J. (1985). Genetics, cytology, and evolution of Gossypium. *Advances in genetics, 23*(0065-2660), 271-375.

Fariduddin, Q., Yusuf, M., Ahmad, I., & Ahmad, A. (2014). Brassinosteroids and their role in response of plants to abiotic stresses. *Biologia Plantarum, 58*(1), 9–17.

Farooq, M., Wahid, A., & Basra, S. M. A. (2009). Improving water relations and gas exchange with brassinosteroids in rice under drought stress. *Journal of Agronomy and Crop Science, 195*(4), 262–269.

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, 39(4), 783-791.

Feng, W., Lindner, H., Robbins, N. E., & Dinneny, J. R. (2016). Growing out of stress: The role of cell-and organ-scale growth control in plant water-stress responses. *The Plant Cell, 28*(8), 1769–1782.

Filova, A. (2014). The responses of *helianthus annuus* l. To foliar application of 28 homobrassinolide. *Research Journal of Agricultural Science*, *46*(1), 226-235.

Fradin, E. F., & Thomma, B. P. (2006). Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology, 7*(2), 71–86.

Friedrichsen, D. M., Joazeiro, C. A., Li, J., Hunter, T., & Chory, J. (2000). Brassinosteroid-insensitive-1 is a ubiquitously expressed leucine-rich repeat receptor serine/threonine kinase. *Plant Physiology, 123*(4), 1247–1256.

Fryxell, P. A. (1992). A revised taxonomic interpretation of Gossypium L (Malvaceae). *Rheedea, 2*, 108–116. https://ci.nii.ac.jp/naid/10016730878/en/

Fujioka, S., & Sakurai, A. (1997). Brassinosteroids. *Natural Product Reports, 14*(1), 1–10.

Fujioka, S., & Yokota, T. (2003). Biosynthesis and metabolism of brassinosteroids. *Annual Review of Plant Biology, 54*(1), 137–164.

Fujioka, S., Li, J., Choi, Y. H., Seto, H., Takatsuto, S., Noguchi, T., & Sakurai, A. (1997). The Arabidopsis deetiolated2 mutant is blocked early in brassinosteroid biosynthesis. *The Plant Cell, 9*(11), 1951–1962.

Garber, R. H., & Houston, B. R. (1966). Penetration and development *Verticillium albo-atrum* in the cotton plant. *Phytopathology, 56*, 1121–1126. https://ci.nii.ac.jp/naid/20001309885/en/

Gerik, T. J., Faver, K. L., Thaxton, P. M., & El-Zik, K. M. (1996). Late season water stress in cotton: I. Plant growth, water use and yield. *Crop Science, 36*(4), 914–921.

Ghazijahani, N., Hadavi, E., & Jeong, B. R. (2014). Foliar sprays of citric acid and salicylic acid alter the pattern of root acquisition of some minerals in sweet basil (*Ocimum basilicum* L.). *Frontiers in Plant Science, 5*(1664-462X), 573.

Glazebrook, J. (2005). Contrasting mechanisms of defence against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology, 43*(1), 205–227.

Goda, H., Sawa, S., Asami, T., Fujioka, S., Shimada, Y., & Yoshida, S. (2004). Comprehensive comparison of auxin-regulated and brassinosteroid-regulated genes in Arabidopsis. *Plant Physiology*, *134*(4), 1555–1573.

Goda, H., Shimada, Y., Asami, T., Fujioka, S., & Yoshida, S. (2002). Microarray analysis of brassinosteroid-regulated genes in Arabidopsis. *Plant Physiology, 130*(3), 1319–1334.

Goodstein, D. M., Shu, S., Howson, R., Neupane, R., Hayes, R. D., Fazo, J., & Putnam, N. (2012). Phytozome: A comparative platform for green plant genomics. *Nucleic Acids Research, 40*(D1), D1178–D1186.

Grove, M. D., Spencer, G. F., Rohwedder, W. K., Mandava, N., Worley, J. F., Warthen, J. D., & Cook, J. C. (1979). Brassinolide, a plant growth-promoting steroid isolated from *Brassica napus* pollen. *Nature*, 281(5728), 216-217.

Harpreet, K., Geetika, S., Renu, B., Poonam, S., & Mir, M. (2014). 28-homobrassinolide modulate antenna complexes and carbon skeleton of *Brassica juncea* L. under temperature stress. *Journal of Stress Physiology & Biochemistry*, *10*(10), 186-196.

Hatfield, J. L., & Prueger, J. H. (2015). Temperature extremes: Effect on plant growth and development. *Weather and Climate Extremes, 10*(2212-0947), 4–10.

Hayat, S., Ahmad, A., Mobin, M., Hussain, A., & Fariduddin, Q. (2000). Photosynthetic rate, growth, and yield of mustard plants sprayed with 28-homobrassinolide. *Photosynthetica, 38*(3), 469–471.

Hayat, S., Hasan, S. A., Yusuf, M., Hayat, Q., & Ahmad, A. (2010). Effect of 28-homobrassinolide on photosynthesis, fluorescence and antioxidant system in the presence or absence of salinity and temperature in *Vigna radiata*. *Environmental and Experimental Botany, 69*(2), 105–112.

He, J. X., Gendron, J. M., Sun, Y., Gampala, S. S., Gendron, N., Sun, C. Q., & Wang, Z. Y. (2005). BZR1 is a transcriptional repressor with dual roles in brassinosteroid homeostasis and growth responses. *Science, 307*(5715), 1634–1638.

He, J. X., Gendron, J. M., Yang, Y., Li, J., & Wang, Z. Y. (2002). The GSK3-like kinase BIN2 phosphorylates and destabilizes BZR1, a positive regulator of the brassinosteroid signalling pathway in Arabidopsis. *Proceedings of the National Academy of Sciences, 99*(15), 10185–10190.

He, Y., Zhang, H., Sun, Z., Li, J., Hong, G., Zhu, Q., & Chen, J. (2017). Jasmonic acid-mediated defence suppresses brassinosteroid-mediated susceptibility to rice black streaked dwarf virus infection in rice. *New Phytologist, 214*(1), 388–399.

Hearn, A. B. (1995). The principles of cotton water relations and their application in management. In: G. A. Constable & N. W. Forrester (eds.), *Challenging the future: Procedures of the World Cotton Conference* 1(pp. 66–92) (Brisbane: February 14-17, 1994) (Melbourne: CSIRO Publishing). http://hdl.handle.net/102.100.100/233430?index=1

Heese, A., Hann, D. R., Gimenez-Ibanez, S., Jones, A. M., He, K., Li, J., & Rathjen, J. P. (2007). The receptor-like kinase SERK3/BAK1 is a central regulator of innate immunity in plants. *Proceedings of the National Academy of Sciences, 104*(29), 12217–12222.

Hernandez-Garcia, C. M., & Finer, J. J. (2014). Identification and validation of promoters and cis-acting regulatory elements. *Plant Science, 217*(0168-9452), 109–119.

Holman, S., Kirby K., Smith L., & Hartnett H. (2016). *Vert update: The latest in vert research*. https://www.cottoninfo.com.au/sites/default/files/documents/Vert%20update%20%28long%29%20-%20August%202016%20v3.pdf

Hong, Z., Ueguchi-Tanaka, M., Fujioka, S., Takatsuto, S., Yoshida, S., Hasegawa, Y., & Matsuoka, M. (2005). The rice brassinosteroid-deficient *dwarf2* mutant, defective in the rice homolog of Arabidopsis *DIMINUTO/DWARF1*, is rescued by the endogenously accumulated alternative bioactive brassinosteroid, dolichosterone. *The Plant Cell, 17*(8), 2243–2254.

Hu, S., Wang, C., Sanchez, D. L., Lipka, A. E., Liu, P., Yin, Y., & Lübberstedt, T. (2017). Gibberellins promote brassinosteroids action and both increase heterosis for plant height in maize (*Zea mays* L.). *Frontiers in Plant Science, 8*(1664-462X), 1039.

Hu, Y., Xia, S., Su, Y., Wang, H., Luo, W., Su, S., & Xiao, L. (2016). Brassinolide increases potato root growth in vitro in a dose-dependent way and alleviates salinity stress. *BioMed Research International Int. 2016*, 1–11. https://doi.org/10.1155/2016/8231873

Huang, B., Chu, C. H., Chen, S. L., Juan, H. F., & Chen, Y. M. (2006). A proteomics study of the mung bean epicotyl regulated by brassinosteroids under conditions of chilling stress. *Cellular & Molecular Biology Letters, 11*(2), 264.

Huisman, O. C. (1982). Interrelations of root growth dynamics to epidemiology of root-invading fungi. *Annual Review of Phytopathology, 20*(1), 303–327.

Jafri, A. Z., & Ahmad, R. A. F. I. Q. (1994). Plant growth and ionic distribution in cotton (*Gossypium hirsutum* L.) under saline environment. *Pakistan Journal of Botany, 26*(1), 105-114.

Janda, T., Pál, M., Darkó, É., & Szalai, G. (2017). Use of salicylic acid and related compounds to improve the abiotic stress tolerance of plants: Practical aspects. In: Nazar, R., N. Iqbal and

N. A. Khan (eds.), *Salicylic acid: A multifaceted hormone* (pp. 35–46). Singapore: Springer. https://doi.org/10.1007/978-981-10-6068-7_3

Janeczko, A., & Swaczynová, J. (2010). Endogenous brassinosteroids in wheat treated with 24-epibrassinolide. *Biologia Plantarum, 54*(3), 477–482.

Janeczko, A., Gullner, G., Skoczowski, A., Dubert, F., & Barna, B. (2007). Effects of brassinosteroid infiltration prior to cold treatment on ion leakage and pigment contents in rape leaves. *Biologia Plantarum, 51*(2), 355–358.

Janeczko, A., Koscielniak, J., Pilipowicz, M., Szarek-Lukaszewska, G., & Skoczowski, A. (2005). Protection of winter rape photosystem 2 by 24-epibrassinolide under cadmium stress. *Photosynthetica, 43*(2), 293–298.

Jenkinson, A. M., Albrecht, M., Birney, E., Blankenburg, H., Down, T., Finn, R. D., & Kähäri, A. (2008). Integrating biological data—The distributed annotation system. *BMC Bioinformatics, 9*(8), 1–7.

Jordan, W. R. (1970). Growth of cotton seedlings in relation to maximum daily plant-water potential. *Agronomy Journal, 62*(6), 699–701.

Kagale, S., Divi, U. K., Krochko, J. E., Keller, W. A., & Krishna, P. (2007). Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta, 225*(2), 353–364.

Kang, G. Z., Li, G. Z., Liu, G. Q., Xu, W., Peng, X. Q., Wang, C. Y. & Guo, T. C. (2013). Exogenous salicylic acid enhances wheat drought tolerance by influence on the expression of genes related to ascorbate-glutathione cycle. *Biologia Plantarum*, *57*(4), 718-724.

Kang, Y. H., Breda, A., & Hardtke, C. S. (2017). Brassinosteroid signaling directs formative cell divisions and protophloem differentiation in Arabidopsis root meristems. *Development, 144*(2), 272–280.

Kanwar, M. K., Bhardwaj, R., Chowdhary, S. P., Arora, P., Sharma, P., & Kumar, S. (2013). Isolation and characterization of 24-epibrassinolide from *Brassica juncea* L. and its effects on growth, Ni ion uptake, antioxidant defence of Brassica plants and in vitro cytotoxicity. *Acta Physiologiae Plantarum, 35*(4), 1351–1362.

Khripach, V., Zhabinskii, V., & de Groot, A. (2000). Twenty years of brassinosteroids: Steroidal plant hormones warrant better crops for the XXI century. *Annals of Botany, 86*(3), 441–447.

Kim, T. W., & Wang, Z. Y. (2010). Brassinosteroid signal transduction from receptor kinases to transcription factors. *Annual Review of Plant Biology, 61*(1545-2123), 681–704.

Kim, T. W., Guan, S., Burlingame, A. L., & Wang, Z. Y. (2011). The CDG1 kinase mediates brassinosteroid signal transduction from BRI1 receptor kinase to BSU1 phosphatase and GSK3-like kinase BIN2. *Molecular Cell, 43*(4), 561–571.

Kim, T. W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J. X., & Wang, Z. Y. (2009). Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. *Nature Cell Biology, 11*(10), 1254.

Kim, Y., Park, S., Gilmour, S. J., & Thomashow, M. F. (2013). Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of Arabidopsis. *The Plant Journal, 75*(3), 364–376.

Kirkby, K. A., Lonergan, P. A., & Allen, S. J. (2013). Three decades of cotton disease surveys in NSW, Australia. *Crop & Pasture Science, 64*(1836-0947), 774–779.

Kissoudis, C., van de Wiel, C., Visser, R. G., & van der Linden, G. (2014). Enhancing crop resilience to combined abiotic and biotic stress through the dissection of physiological and molecular crosstalk. *Frontiers in Plant Science, 5*(1664-462X), 207.

Kitanaga, Y., Jian, C., Hasegawa, M., Yazaki, J., Kishimoto, N., Kikuchi, S., & Yamaguchi, I. (2006). Sequential regulation of gibberellin, brassinosteroid, and jasmonic acid biosynthesis occurs in rice coleoptiles to control the transcript levels of anti-microbial thionin genes. *Bioscience, Biotechnology, and Biochemistry, 70*(10), 2410–2419.

716 Kuc, J. (1982). Induced immunity to plant disease. *Bioscience, 32*(11), 854–860.
717 Kudla, J., Batistič, O., & Hashimoto, K. (2010). Calcium signals: The lead currency of plant
718 information processing. *The Plant Cell, 22*(3), 541–563.
719 Lannoo, N., Vandenborre, G., Miersch, O., Smagghe, G., Wasternack, C., Peumans, W. J., & Van
720 Damme, E. J. (2007). The jasmonate-induced expression of the Nicotiana tabacum leaf
721 lectin. *Plant and Cell Physiology, 48*(8), 1207–1218.
722 Lecourieux, D., Ranjeva, R., & Pugin, A. (2006). Calcium in plant defence-signalling pathways.
723 *New Phytologist, 171*(2), 249–269.
724 Leidi, E. (1994) Genotypic variation of cotton in response to stress by NaCl or PEG. In: Peeters
725 MC (ed) Cotton biotechnology, *REUR technical series*, 32, (pp. 67–73), FAO, Rome (Italy).
726 https://agris.fao.org/agris_ods/
727 Leidi, E., Nogales, R., & Lips, S. (1991). Effect of salinity on cotton plants grown under nitrate or
728 ammonium nutrition at different calcium levels. *Field Crops Research, 26*(1), 35–44.
729 Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., & Rombauts, S. (2002).
730 PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in
731 silico analysis of promoter sequences. *Nucleic Acids Research, 30*(1), 325–327.
732 Li, F., Fan, G., Lu, C., Xiao, G., Zou, C., Kohel, R. J., & Liang, X. (2015). Genome sequence of
733 cultivated upland cotton (*Gossypium hirsutum* TM-1) provides insights into genome
734 evolution. *Nature Biotechnology, 33*(5), 524.
735 Li, J., & Chory, J. (1997). A putative leucine-rich repeat receptor kinase involved in brassinosteroid
736 signal transduction. *Cell, 90*(5), 929–938.
737 Li, J., & Nam, K. H. (2002). Regulation of brassinosteroid signalling by a GSK3/SHAGGY-like
738 kinase. *Science, 295*(5558), 1299–1301.
739 Li, J., Nagpal, P., Vitart, V., McMorris, T. C., & Chory, J. (1996). A role for brassinosteroids in
740 light-dependent development of Arabidopsis. *Science, 272*(5260), 398–401.
741 Li, K. R., Wang, H. H., Han, G., Wang, Q. J., & Fan, J. (2008). Effects of brassinolide on the
742 survival, growth and drought resistance of *Robinia pseudoacacia* seedlings under water-
743 stress. *New Forests, 35*(3), 255–266.
744 Li, L., Ye, H., Guo, H., & Yin, Y. (2010). Arabidopsis *IWS1* interacts with transcription factor
745 BES1 and is involved in plant steroid hormone brassinosteroid regulated gene expression.
746 *Proceedings of the National Academy of Sciences, 107*(8), 3918–3923.
747 Li, M., Ahammed, G. J., Li, C., Bao, X., Yu, J., Huang, C., & Zhou, J. (2016). Brassinosteroid
748 ameliorates zinc oxide nanoparticles-induced oxidative stress by improving antioxidant
749 potential and redox homeostasis in tomato seedling. *Frontiers in Plant Science, 7*(1664-
750 462X), 615.
751 Li, Q. F., Wang, C., Jiang, L., Li, S., Sun, S. S., & He, J. X. (2012). An interaction between BZR1
752 and DELLAs mediates direct signalling crosstalk between brassinosteroids and gibberellins
753 in Arabidopsis. *Sci. Signal*. 5(244), ra72.
754 Lima, J. V., & Lobato, A. K. S. (2017). Brassinosteroids improve photosystem II efficiency, gas
755 exchange, antioxidant enzymes and growth of cowpea plants exposed to water deficit.
756 *Physiology and Molecular Biology of Plants, 23*(1), 59–72.
757 Lira-Saldivar, R.H., Hernandez-Rosales, S.P. (1988). Characterization of salt tolerance in genotypes
758 of cotton. *Informes de Invest*. PRONAPA, 299–317.
759 Loka, D.M., Derrick, M., Oosterhuis, D.M. and Ritchie, G.L. (2011) Water-deficit stress in cotton.
760 In: Oosterhuis, D.M (eds.), *Stress Physiology in Cotton*. 7(pp. 37–72). Number Seven The
761 Cotton Foundation Book Series. National Cotton Council of America.
762 Lu, Y., & Harrington, H. M. (1994). Isolation of tobacco cDNA clones encoding calmodulin-
763 binding proteins and characterization of a known calmodulin-binding domain. *Plant*
764 *Physiology and Biochemistry, 32*(3), 413–422.

765 Mahesh, K., Balaraju, P., Ramakrishna, B., & Rao, S. S. R. (2013). Effect of brassinosteroids on
766 germination and seedling growth of radish (*Raphanus sativus* L.) under PEG-6000 induced
767 water stress. *American Journal of Plant Sciences, 4*(12), 2305.
768 Mandava, N. B. (1988). Plant growth-promoting brassinosteroids. *Annual Review of Plant*
769 *Physiology and Plant Molecular Biology, 39*(1), 23–52.
770 Martínez, C., Espinosa-Ruíz, A., de Lucas, M., Bernardo-García, S., Franco-Zorrilla, J. M., & Prat,
771 S. (2018). PIF4-induced BR synthesis is critical to diurnal and thermomorphogenic growth.
772 *The EMBO Journal, 37*(23), e99552.
773 Matthews, M. A., & Boyer, J. S. (1984). Acclimation of photosynthesis to low leaf water potentials.
774 *Plant physiology*, *74*(1), 161-166.
775 Mazorra, L. M., Nunez, M., Hechavarria, M., Coll, F., & Sánchez-Blanco, M. J. (2002). Influence
776 of brassinosteroids on antioxidant enzymes activity in tomato under different temperatures.
777 *Biologia Plantarum, 45*(4), 593–596.
778 McMichael, B. L., & Hesketh, J. D. (1982). Field investigations of the response of cotton to water
779 deficits. *Field Crops Research, 5*(0378-4290), 319–333.
780 Mir, B. A., Khan, T. A., & Fariduddin, Q. (2015). 24-epibrassinolide and spermidine modulate
781 photosynthesis and antioxidant systems in *Vigna radiata* under salt and zinc stress.
782 *International Journal of Advanced Research, 3*(5), 592–608.
783 Mitchell, J. W., Mandava, N., Worley, J. F., Plimmer, J. R., & Smith, M. V. (1970). Brassins—A
784 new family of plant hormones from rape pollen. *Nature, 225*(5237), 1065–1066.
785 Miyaji, T., Yamagami, A., Kume, N., Sakuta, M., Osada, H., Asami, T., & Nakano, T. (2014).
786 Brassinosteroid-related transcription factor BIL1/BZR1 increases plant resistance to insect
787 feeding. *Bioscience, Biotechnology, and Biochemistry, 78*(6), 960–968.
788 Mochan, K., & Gubana B. (2018). *Salinity crisis destroying Australia's farmland, but farmers hope*
789 *to stop it.* https://www.abc.net.au/news/rural/2018-06-02/salinity-crisis-for-australias-
790 farmland-but-farmers-fight-back/9826834
791 Mussig, C., Shin, G. H., & Altmann, T. (2003). Brassinosteroids promote root growth in
792 Arabidopsis. *Plant Physiology, 133*(3), 1261–1271.
793 Nachimuthu, G., & Webb, A. A. (2017). Closing the biotic and abiotic stress-mediated yield gap in
794 cotton by improving soil management and agronomic practices. In: Senthil- Kumar, M.
795 (Ed.), *Plant tolerance to individual and concurrent stresses* (pp. 17–31). New Delhi:
796 Springer. https://doi.org/10.1007/978-81-322-3706-8_2
797 Nahar, K., Kyndt, T., Hause, B., Höfte, M., & Gheysen, G. (2013). Brassinosteroids suppress rice
798 defence against root-knot nematodes through antagonism with the jasmonate pathway.
799 *Molecular Plant-Microbe Interactions, 26*(1), 106–115.
800 Nakashita, H., Yasuda, M., Nitta, T., Asami, T., Fujioka, S., Arai, Y., & Yoshida, S. (2003).
801 Brassinosteroid functions in a broad range of disease resistance in tobacco and rice. *The*
802 *Plant Journal, 33*(5), 887–898.
803 Nazar, R., Umar, S., & Khan, N. A. (2015). Exogenous salicylic acid improves photosynthesis and
804 growth through increase in ascorbate-glutathione metabolism and S assimilation in
805 mustard under salt stress. *Plant signaling & behavior*, *10*(3), e1003751.
806 Nemhauser, J. L., Maloof, J. N., & Chory, J. (2003). Building integrated models of plant growth
807 and development. *Plant physiology*, *132*(2), 436-439.
808 Nishikawa, N., Toyama, S., Shida, A., & Futatsuya, F. (1994). The uptake and the transport of 14
809 C-labeled epibrassinolide in intact seedlings of cucumber and wheat. *Journal of Plant*
810 *Research, 107*(2), 125–130.
811 Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Tax, F. E., Yoshida, S., & Feldmann, K. A. (2000).
812 Biosynthetic pathways of brassinolide in Arabidopsis. *Plant Physiology, 124*(1), 201–210.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Yoshida, S., Yuan, H., & Tax, F. E. (1999). Brassinosteroid-insensitive dwarf mutants of Arabidopsis accumulate brassinosteroids. *Plant Physiology, 121*(3), 743–752.

Noguchi, T., Fujioka, S., Takatsuto, S., Sakurai, A., Yoshida, S., Li, J., & Chory, J. (1999). Arabidopsis det2 is defective in the conversion of (24R)-24-methylcholest-4-en-3-one to (24R)-24-methyl-5α-cholestan-3-one in brassinosteroid biosynthesis. *Plant Physiology, 120*(3), 833–840.

Nolan, T. M., Brennan, B., Yang, M., Chen, J., Zhang, M., Li, Z., & Yin, Y. (2017). Selective autophagy of BES1 mediated by DSK2 balances plant growth and survival. *Developmental Cell, 41*(1), 33–46.

Nolan, T. M., Vukašinović, N., Liu, D., Russinova, E., & Yin, Y. (2020). Brassinosteroids: Multidimensional regulators of plant growth, development, and stress responses. *The Plant Cell, 32*(2), 295–318.

Nunez, M., Mazzafera, P., Mazorra, L. M., Siqueira, W. J., & Zullo, M. A. T. (2003). Influence of a brassinosteroid analogue on antioxidant enzymes in rice grown in culture medium with NaCl. *Biologia Plantarum, 47*(1), 67–70.

Office of the Gene Technology Regulator. (2002). *The biology and ecology of cotton (Gossypium hirsutum) in Australia*. https://www.yumpu.com/en/document/read/30540888/the-biology-and-ecology-of-cotton-gossypium-hirsutum-in-australia

Ogweno, J. O., Song, X. S., Shi, K., Hu, W. H., Mao, W. H., Zhou, Y. H., & Nogués, S. (2008). Brassinosteroids alleviate heat-induced inhibition of photosynthesis by increasing carboxylation efficiency and enhancing antioxidant systems *in Lycopersicon esculentum*. *Journal of Plant Growth Regulation, 27*(1), 49–57.

Özdemir, F., Bor, M., Demiral, T., & Türkan, İ. (2004). Effects of 24-epibrassinolide on seed germination, seedling growth, lipid peroxidation, proline content and antioxidative system of rice (*Oryza sativa* L.) under salinity stress. *Plant Growth Regulation, 42*(3), 203–211.

Pace, P. F., Cralle, H. T., El-Halawany, S. H., Cothren, J. T., & Senseman, S. A. (1999). Drought-induced changes in shoot and root growth of young cotton plants. *Journal of Cotton Science, 3*(4), 183–187.

Padmalatha, K. V., Dhandapani, G., Kanakachari, M., Kumar, S., Dass, A., Patil, D. P., & Leelavathi, S. (2012). Genome-wide transcriptomic analysis of cotton under drought stress reveal significant down-regulation of genes and pathways involved in fibre elongation and up-regulation of defence responsive genes. *Plant Molecular Biology, 78*(3), 223–246.

Pallegar, P. (2014). *Functional analysis of two brassinosteroid responsive, putative calmodulin-binding proteins 60 (CBP60S) in Arabidopsis Thaliana* (master's dissertation). The University of Western Ontario, Canada. https://www.semanticscholar.org/paper/Functional-Analysis-of-TwoBrassinosteroid-Putative-Pallegar/1901eafbec6b558304cfb5e19b4928a60b8668bc

Pan, G., Liu, Y., Ji, L., Zhang, X., He, J., Huang, J., & Liu, L. (2018). Brassinosteroids mediate susceptibility to brown planthopper by integrating with the salicylic acid and jasmonic acid pathways in rice. *Journal of Experimental Botany, 69*(18), 4433–4442.

Paterson, A. H., & Wendel, J. F. (2015). Unravelling the fabric of polyploidy. *Nature Biotechnology, 33*(5), 491–493.

Peng, P., Yan, Z., Zhu, Y., & Li, J. (2008). Regulation of the Arabidopsis GSK3-like kinase BRASSINOSTEROID-INSENSITIVE 2 through proteasome-mediated protein degradation. *Molecular Plant, 1*(2), 338–346.

Peter, H. (2019, March 15). Cotton growers defend growing 1.2m bales in midst of drought. The *The Weekly Times.* https://www.weeklytimesnow.com.au/subscribe/news/1/?sourceCode=WTWEB_WRE170_a_GGL&dest=https%3A%2F%2Fwww.weeklytimesnow.com.au%2Fnews%2Fnational

%2Fcotton-water-lower-darling-dries-amid-claims-crop-uses-2000gl%2Fnews-story%2F858990120b447286cf6365c114ab958c&memtype=anonymous&mode=premium
Pierleoni, A., Martelli, P. L., Fariselli, P., & Casadio, R. (2006). BaCelLo: A balanced subcellular localization predictor. *Bioinformatics, 22*(14), e408–e416
Prior, S. A., Rogers, H. H., Runion, G. B., Kimball, B. A., Mauney, J. R., Lewin, K. F., & Hendrey, G. R. (1995). Free-air carbon dioxide enrichment of cotton: Root morphological characteristics. *Journal of Environmental Quality, 24*(4), 678–683.
Proceedings, Beijing: Atlantis Press, p. 68-75. (Series: Advances in Engineering Research).
Qadir, M., & Shams, M. (1997). Some agronomic and physiological aspects of salt tolerance in cotton (*Gossypium hirsutum* L.). *Journal of Agronomy and Crop Science, 179*(2), 101–106.
Qayyum, B., Shahbaz, M., & Akram, N. A. (2007). Interactive effect of foliar application of 24-epibrassinolide and root zone salinity on morpho-physiological attributes of wheat (*Triticum aestivum* L.). *International Journal of Agriculture & Biology, 9*(4), 584–589.
Qin, J., Wang, K., Sun, L., Xing, H., Wang, S., Li, L., & Zhang, J. (2018). The plant-specific transcription factors CBP60g and SARD1 are targeted by a Verticillium secretory protein VdSCP41 to modulate immunity. *Elife, 7*(2050-084X), e34902.
Ranjan, A., Nigam, D., Asif, M. H., Singh, R., Ranjan, S., Mantri, S., & Koul, B. (2012). Genome wide expression profiling of two accession of *G. herbaceum* L. in response to drought. *BMC Genomics, 13*(1), 94.
Rattan, A., Kapoor, D., Kapoor, N., & Bhardwaj, R. (2014). Application of brassinosteroids reverses the inhibitory effect of salt stress on growth and photosynthetic activity of *Zea mays* plants. *International Journal of Theoretical and Applied Sciences, 6*(2), 13.
Reddy, A. S. (2001). Calcium: Silver bullet in signalling. *Plant Science, 160*(3), 381–404.
Reddy, A. S. N., Takezawa, D., Fromm, H., & Poovaiah, B. W. (1993). Isolation and characterization of two cDNAs that encode for calmodulin-binding proteins from corn root tips. *Plant Science, 94*(1–2), 109–117.
Reddy, A. S., Ali, G. S., Celesnik, H., & Day, I. S. (2011). Coping with stresses: roles of calcium- and calcium/calmodulin-regulated gene expression. *The Plant Cell, 23*(6), 2010-2032.
Reddy, A. S., Day, I. S., Narasimhulu, S. B., Safadi, F., Reddy, V. S., Golovkin, M., & Harnly, M. J. (2002). Isolation and characterization of a novel calmodulin-binding protein from potato. *Journal of Biological Chemistry, 277*(6), 4206–4214.
Reddy, A., Reddy, V. S., & Golovkin, M. (2000). A calmodulin binding protein from Arabidopsis is induced by ethylene and contains a DNA-binding motif. *Biochemical and Biophysical Research Communications, 279*(3), 762–769.
Reinhardt, D., & Rost, T. (1995). Primary and lateral root development of dark-and light-grown cotton seedlings under salinity stress. *Plant Biology, 108*(5), 457–465.
Rengasamy, P. (2006). World salinization with emphasis on Australia. *Journal of Experimental Botany, 57*(5), 1017–1023.
Reusche, M., Thole, K., Janz, D., Truskina, J., Rindfleisch, S., Drübert, C., & Teichmann, T. (2012). Verticillium infection triggers VASCULAR-RELATED NAC DOMAIN7–dependent de novo xylem formation and enhances drought tolerance in Arabidopsis. *The Plant Cell, 24*(9), 3823-3837.
Rhee, S. Y., Beavis, W., Berardini, T. Z., Chen, G., Dixon, D., Doyle, A., & Montoya, M. (2003). The Arabidopsis Information Resource (TAIR): A model organism database providing a centralized, curated gateway to Arabidopsis biology, research materials and community. *Nucleic Acids Research, 31*(1), 224–228.
Riemann, M., Dhakarey, R., Hazman, M., Miro, B., Kohli, A., & Nick, P. (2015). Exploring jasmonates in the hormonal network of drought and salinity responses. *Frontiers in Plant Science, 6*(1664-462X), 1077.

912 Ross, A. F. (1961). Systemic acquired resistance induced by localised virus infections in plants.
913 *Virology, 14*(3), 340–358.
914 Rudd, J. J., & Franklin-Tong, V. E. (2001). Unravelling response-specificity in Ca2+ signalling
915 pathways in plant cells. *New Phytologist, 151*(1), 7-33.
916 Sahni, S., Prasad, B. D., Liu, Q., Grbic, V., Sharpe, A., Singh, S. P., & Krishna, P. (2016).
917 Overexpression of the brassinosteroid biosynthetic gene DWF4 in *Brassica napus*
918 simultaneously increases seed yield and stress tolerance. *Scientific Reports, 6*(2045-2322),
919 28298.
920 Saitou, N., & Nei, M. (1987). The neighbor-joining method: A new method for reconstructing
921 phylogenetic trees. *Molecular Biology and Evolution, 4*(4), 406–425.
922 Šamajová, O., Plíhal, O., Al-Yousif, M., Hirt, H., & Šamaj, J. (2013). Improvement of stress
923 tolerance in plants by genetic manipulation of mitogen-activated protein kinases.
924 *Biotechnology Advances, 31*(1), 118–128.
925 Sanders, D., Brownlee, C., & Harper, J. F. (1999). Communicating with calcium. *The Plant Cell,*
926 *11*(4), 691-706.
927 Sanders, D., Pelloux, J., Brownlee, C., & Harper, J. F. (2002). Calcium at the crossroads of
928 signaling. *The Plant Cell, 14*(suppl 1), S401–S417.
929 Seelanan, T., Schnabel, A., & Wendel, J. F. (1997). Congruence and consensus in the cotton tribe
930 (Malvaceae). *Systematic Botany, 22*(2), 259–290.
931 Segonzac, C., & Zipfel, C. (2011). Activation of plant pattern-recognition receptors by bacteria.
932 *Current Opinion in Microbiology, 14*(1), 54–61.
933 Senchina, D. S., Alvarez, I., Cronn, R. C., Liu, B., Rong, J., Noyes, R. D., & Wendel, J. F. (2003).
934 Rate variation among nuclear genes and the age of polyploidy in Gossypium. *Molecular*
935 *Biology and Evolution, 20*(4), 633–643.
936 Seshadri, R. (2019). *A current look at the Australian cotton industry*.
937 https://www.cottongrower.com/cotton-production/a-current-look-at-the-australian-cotton-
938 industry/
939 Sharma, I., Bhardwaj, R., & Pati, P. K. (2012). Mitigation of adverse effects of chlorpyrifos by 24-
940 epibrassinolide and analysis of stress markers in a rice variety Pusa Basmati-1.
941 *Ecotoxicology and Environmental Safety, 85*(0147-6513), 72–81.
942 Shi, Y. H., Zhu, S. W., Mao, X. Z., Feng, J. X., Qin, Y. M., Zhang, L., & Zhu, Y. X. (2006).
943 Transcriptome profiling, molecular biological, and physiological studies reveal a major role
944 for ethylene in cotton fibre cell elongation. *The Plant Cell, 18*(3), 651–664.
945 Shu, H. M., Guo, S. Q., Gong, Y. Y., Jiang, L., Zhu, J. W., & Ni, W. C. (2017). RNA-seq analysis
946 reveals a key role of brassinolide-regulated pathways in NaCl-stressed cotton. *Biologia*
947 *Plantarum, 61*(4), 667–674.
948 Shu, H., Ni, W., Guo, S., Gong, Y., Shen, X., Zhang, X., & Guo, Q. (2015). Root-applied
949 brassinolide can alleviate the NaCl injuries on cotton. *Acta Physiologiae Plantarum, 37*(4),
950 75.
951 Silberbush, M., & Ben-Asher, J. (1987). The effect of salinity on parameters of potassium and
952 nitrate uptake of cotton. *Communications in Soil Science and Plant Analysis, 18*(1), 65–81.
953 Singh, I., & Shono, M. (2005). Physiological and molecular effects of 24-epibrassinolide, a
954 brassinosteroid on thermotolerance of tomato. *Plant Growth Regulation, 47*(2–3), 111.
955 Skovsted, A. (1934). Cytological studies in cotton. II. Two interspecific hybrids between Asiatic
956 and New World cottons. *J. Genet, 28*(3), 407-424.
957 Skovsted, A. (1937). Cytological studies in cotton. *Journal of Genetics, 34*(1), 97-134.
958 Smith, S.M., Li, C., Li, J., 2017. Hormone function in plants. In: Li, J., Li, C., Smith, S.M.

Snedden, W. A., & Fromm, H. (2001). Calmodulin as a versatile calcium signal transducer in plants. *New Phytologist, 151*(1), 35–66.

Statistica Research Department. (2018). *Cotton production by country worldwide in 2017/2018 (in 1,000 metric tons).* https://www.statista.com/statistics/595561/distribution-of-global-cotton-production-by-country/

Su, Q., Zheng, X., Tian, Y., & Wang, C. (2020). Exogenous brassinolide alleviates salt stress in *Malus hupehensis* Rehd. by regulating the transcription of NHX-Type Na+ (K+)/H+ antiporters. *Frontiers in Plant Science, 11*(1664-462X), 38.

Sun, T., Busta, L., Zhang, Q., Ding, P., Jetter, R., & Zhang, Y. (2018). TGACG-BINDING FACTOR 1 (TGA1) and TGA4 regulate salicylic acid and pipecolic acid biosynthesis by modulating the expression of systemic acquired resistance deficient 1 (*SARD1*) and calmodulin binding protein 60g (*CBP60g*). *New Phytologist, 217*(1), 344–354.

Sun, Y., Fan, X. Y., Cao, D. M., Tang, W., He, K., Zhu, J. Y., & Patil, S. (2010). Integration of brassinosteroid signal transduction with the transcription network for plant growth regulation in Arabidopsis. *Developmental Cell, 19*(5), 765–777.

Sun, Y., Veerabomma, S., Abdel-Mageed, H. A., Fokar, M., Asami, T., Yoshida, S., & Allen, R. D. (2005). Brassinosteroid regulates fibre development on cultured cotton ovules. *Plant and Cell Physiology, 46*(8), 1384–1391.

Surgun, Y., Altunlu, H., Turkekul, S., Burun, B., & Yokas, İ. (2015). Effects of 24-Epibrassinolide on growth and some antioxidant enzymes of cotton (*Gossypium hirsutum* L.) cultivars under NaCl stress. *Journal of Applied Biological Sciences, 9*(3), 09-17.

Suzuki, Y., Saso, K., Fujioka, S., Yoshida, S., Nitasaka, E., Nagata, S., & Yamaguchi, I. (2003). A dwarf mutant strain of Pharbitis nil, Uzukobito (kobito), has defective brassinosteroid biosynthesis. *The Plant Journal, 36*(3), 401–410.

Swain, S. M., & Singh, D. P. (2005). Tall tales from sly dwarves: Novel functions of gibberellins in plant development. *Trends in Plant Science, 10*(3), 123–129.

Symons, G. M., & Reid, J. B. (2004). Brassinosteroids do not undergo long-distance transport in pea: Implications for the regulation of endogenous brassinosteroid levels. *Plant Physiology, 135*(4), 2196–2206.

Symons, G. M., Ross, J. J., Jager, C. E., & Reid, J. B. (2008). Brassinosteroid transport. *Journal of Experimental Botany, 59*(1), 17–24.

Szekeres, M., Németh, K., Koncz-Kálmán, Z., Mathur, J., Kauschmann, A., Altmann, T., & Koncz, C. (1996). Brassinosteroids rescue the deficiency of CYP90, a cytochrome P450, controlling cell elongation and de-etiolation in Arabidopsis. *Cell, 85*(2), 171–182.

Talaat, N. B., Shawky, B. T., & Ibrahim, A. S. (2015). Alleviation of drought-induced oxidative stress in maize (*Zea mays* L.) plants by dual application of 24-epibrassinolide and spermine. *Environmental and Experimental Botany, 113*(0098-8472), 47–58.

Talarek-Karwel, M., Bajguz, A., & Piotrowska-Niczyporuk, A. (2019). 24-Epibrassinolide modulates primary metabolites, antioxidants, and phytochelatins in *Acutodesmus obliquus* exposed to lead stress. *Journal of Applied Phycology*, 32, 263–276. https://doi.org/10.1007/S10811-019-01966-8

Tamura, K., Stecher, G., Peterson, D., Filipski, A., & Kumar, S. (2013). MEGA6: Molecular evolutionary genetics analysis version 6.0. *Molecular Biology and Evolution, 30*(12), 2725–2729.

Tan, J., Tu, L., Deng, F., Wu, R., & Zhang, X. (2012). Exogenous jasmonic acid inhibits cotton fibre elongation. *Journal of Plant Growth Regulation, 31*(4), 599–605.

Thussagunpanit, J., Jutamanee, K., Sonjaroon, W., Kaveeta, L., Chai-Arree, W., Pankean, P., & Suksamrarn, A. (2015). Effects of brassinosteroid and brassinosteroid mimic on photosynthetic efficiency and rice yield under heat stress. *Photosynthetica, 53*(2), 312–320.

Trewavas, A. J., & Malhó, R. (1998). Ca2+ signalling in plant cells: The big network! *Current Opinion in Plant Biology, 1*(5), 428–433.

Truman, W., Sreekanta, S., Lu, Y., Bethke, G., Tsuda, K., Katagiri, F., & Glazebrook, J. (2013). The calmodulin binding protein 60 family includes both negative and positive regulators of plant immunity. *Plant Physiology, 163*(4)*:* 1741–1751.

Turner, N. C., Hearn, A. B., Begg, J. E., & Constable, G. A. (1986). Cotton (*Gossypium hirsutum* L.) physiological and morphological responses to water deficits and their relationship to yield. *Field Crops Research, 14*(0378-4290), 153–170.

Vardhini, B. V., & Anjum, N. A. (2015). Brassinosteroids make plant life easier under abiotic stresses mainly by modulating major components of antioxidant defence system. *Frontiers in Environmental Science, 2*(2296-665X), 67.

Vardhini, B. V., & Rao, S. S. R. (2003). Amelioration of osmotic stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum. *Plant Growth Regulation, 41*(1), 25–31.

Vardhini, B. V., Anuradha, S., & Rao, S. S. R. (2006). Brassinosteroids-New class of plant hormone with potential to improve crop productivity. *Indian Journal of Plant Physiology*, *11*(1), 1.

Vasyukova, N. I., Chalenko, G. I., Kaneva, I. M., Khripach, V. A., & Ozeretskovskaya, O. L. (1994). Brassinosteroids and potato late blight. *Prikladnaia Biokhimiia I Mikrobiologiia, 30*(3), 464–470.

Vert, G., & Chory, J. (2006). Downstream nuclear events in brassinosteroid signalling. *Nature, 441*(7089), 96.

Vert, G., Walcher, C. L., Chory, J., & Nemhauser, J. L. (2008). Integration of auxin and brassinosteroid pathways by Auxin Response Factor 2. *Proceedings of the National Academy of Sciences, 105*(28), 9829–9834.

Vragović, K., Sela, A., Friedlander-Shani, L., Fridman, Y., Hacham, Y., Holland, N., & Savaldi-Goldstein, S. (2015). Translatome analyses capture of opposing tissue-specific brassinosteroid signals orchestrating root meristem differentiation. *Proceedings of the National Academy of Sciences, 112*(3), 923–928.

Wan, D., Li, R., Zou, B., Zhang, X., Cong, J., Wang, R., & Li, G. (2012). Calmodulin-binding protein CBP60g is a positive regulator of both disease resistance and drought tolerance in Arabidopsis. *Plant Cell Reports, 31*(7), 1269–1281.

Wang, H., Tang, J., Liu, J., Hu, J., Liu, J., Chen, Y. & Wang, X. (2018). Abscisic acid signaling inhibits brassinosteroid signaling through dampening the dephosphorylation of BIN2 by ABI1 and ABI2. *Molecular plant, 11*(2), 315-325.

Wang, L., Tsuda, K., Sato, M., Cohen, J. D., Katagiri, F., & Glazebrook, J. (2009). Arabidopsis CaM binding protein CBP60g contributes to MAMP-induced SA accumulation and is involved in disease resistance against *Pseudomonas syringae*. *PLoS Pathogens, 5*(2), e1000301.

Wang, L., Tsuda, K., Truman, W., Sato, M., Nguyen, L. V., Katagiri, F., & Glazebrook, J. (2011). CBP60g and SARD1 play partially redundant critical roles in salicylic acid signalling. *The Plant Journal, 67*(6), 1029–1041.

Wang, M., Wang, Q., & Zhang, B. (2013). Evaluation and selection of reliable reference genes for gene expression under abiotic stress in cotton (*Gossypium hirsutum* L.). *Gene, 530*(1), 44–50.

Wang, R., Gao, M., Ji, S., Wang, S., Meng, Y., & Zhou, Z. (2016). Carbon allocation, osmotic adjustment, antioxidant capacity and growth in cotton under long-term soil drought during flowering and boll-forming period. *Plant Physiology and Biochemistry, 107*(0981-9428), 137–146.

Wang, X., & Chory, J. (2006). Brassinosteroids regulate dissociation of BKI1, a negative regulator of BRI1 signalling, from the plasma membrane. *Science, 313*(5790), 1118–1122.

Wang, Z. Y., Bai, M. Y., Oh, E., & Zhu, J. Y. (2012). Brassinosteroid signalling network and regulation of photomorphogenesis. *Annual Review of Genetics, 46*(1545-2948), 701–724.

Wang, Z. Y., Nakano, T., Gendron, J., He, J., Chen, M., Vafeados, D., & Chory, J. (2002). Nuclear-localized BZR1 mediates brassinosteroid-induced growth and feedback suppression of brassinosteroid biosynthesis. *Developmental Cell, 2*(4), 505–513.

Wang, Z. Y., Seto, H., Fujioka, S., Yoshida, S., & Chory, J. (2001). BRI1 is a critical component of a plasma-membrane receptor for plant steroids. *Nature, 410*(6826), 380–383.

Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. S., Wiederhold, D. L., Alexander, D. C. & Ryals, J. A. (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. *The Plant Cell*, *3*(10), 1085-1094.

Wei, Z., & Li, J. (2016). Brassinosteroids regulate root growth, development, and symbiosis. *Molecular Plant, 9*(1), 86–100.

Wendel, J. F. (1989). New World tetraploid cottons contain Old World cytoplasm. *Proceedings of the National Academy of Sciences, 86*(11), 4132–4136.

Wendel, J. F., & Cronn, R. C. (2003). Polyploidy and the evolutionary history of cotton. *Advances in Agronomy, 78*(2572-679X), 139–186.

Wu, W., Zhang, Q., Ervin, E., Yang, Z., & Zhang, X. (2017). Physiological mechanism of enhancing salt stress tolerance of perennial ryegrass by 24-epibrassinolide. *Frontiers in Plant Science, 8*(1664-462X), 1017.

Yang, G. X., Jan, A., Shen, S. H., Yazaki, J., Ishikawa, M., Shimatani, Z., & Komatsu, S. (2004). Microarray analysis of brassinosteroids-and gibberellin-regulated gene expression in rice seedlings. *Molecular Genetics and Genomics, 271*(4), 468–478.

Yang, J., Duan, G., Li, C., Liu, L., Han, G., Zhang, Y., & Wang, C. (2019). The crosstalks between jasmonic acid and other plant hormone signaling highlight the involvement of jasmonic acid as a core component in plant response to biotic and abiotic stresses. *Frontiers in Plant Science, 10*(1664-462X), 1349.

Yang, S. F., & Hoffman, N. E. (1984). Ethylene biosynthesis and its regulation in higher plants. *Annual review of plant physiology*, *35*(1), 155-189.

Yin, Y., Wang, Z. Y., Mora-Garcia, S., Li, J., Yoshida, S., Asami, T., & Chory, J. (2002). BES1 accumulates in the nucleus in response to brassinosteroids to regulate gene expression and promote stem elongation. *Cell, 109*(2), 181–191.

Yu, C. S., Lin, C. J., & Hwang, J. K. (2004). Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein Science, 13*(5), 1402–1406.

Yu, C. S., Lin, C. J., & Hwang, J. K. (2004). Predicting subcellular localization of proteins for Gram-negative bacteria by support vector machines based on n-peptide compositions. *Protein science*, *13*(5), 1402-1406.

Yu, J., Jung, S., Cheng, C. H., Ficklin, S. P., Lee, T., Zheng, P., & Main, D. (2013). Cotton gen: A genomics, genetics and breeding database for cotton research. *Nucleic Acids Research, 42*(D1), D1229–D1236.

Yusuf, M., Khan, T. A., and Fariduddin, Q. (2017). "Brassinosteroids: physiological roles and its signalling in plants," In: M. Sarwat, A. Ahmad, M. Z. Abdin, and M. M. Ibrahim (eds.), *Stress Signaling in Plants: Genomics and Proteomics Perspective* (pp. 241–260). Berlin: Springer International Publishing. https://doi.org/10.1007/978-3-319-42183-4_10

Zeng, H., Tang, Q., & Hua, X. (2010). Arabidopsis brassinosteroid mutants *det2-1* and *bin2-1* display altered salt tolerance. *Journal of Plant Growth Regulation, 29*(1), 44–52.

Zhang, A., Zhang, J., Zhang, J., Ye, N., Zhang, H., Tan, M., & Jiang, M. (2011). Nitric oxide mediates brassinosteroid-induced ABA biosynthesis involved in oxidative stress tolerance in maize leaves. *Plant and Cell Physiology, 52*(1), 181–192.

Zhang, S., Cai, Z., & Wang, X. (2009). The primary signalling outputs of brassinosteroids are regulated by abscisic acid signalling. *Proceedings of the National Academy of Sciences, 106*(11), 4543–4548.

Zhang, T., Hu, Y., Jiang, W., Fang, L., Guan, X., Chen, J., & Hulse-Kemp, A. M. (2015). Sequencing of allotetraploid cotton (*Gossypium hirsutum* L. acc. TM-1) provides a resource for fibre improvement. *Nature Biotechnology, 33*(5), 531.

Zhang, Y., Xu, S., Ding, P., Wang, D., Cheng, Y. T., He, J., ... & Zhang, Y. (2010). Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. *Proceedings of the National Academy of Sciences*, *107*(42), 18220-18225.

Zhao, G., Xu, H., Zhang, P., Su, X., & Zhao, H. (2017). Effects of 2, 4-epibrassinolide on photosynthesis and Rubisco activase gene expression in Triticum aestivum L. seedlings under a combination of drought and heat stress. *Plant Growth Regulation*, *81*(3), 377-384.

Zhong, H., & Lauchli, A. (1993). Spatial and temporal aspects of growth in the primary root of cotton seedlings: Effects of NaCl and CaCl2. *Journal of Experimental Botany*, *44*(4), 763–771.

Zhou, J., Wang, J., Li, X., Xia, X. J., Zhou, Y. H., Shi, K., & Yu, J. Q. (2014). H2O2 mediates the crosstalk of brassinosteroid and abscisic acid in tomato responses to heat and oxidative stresses. *Journal of Experimental Botany, 65*(15), 4371–4383.

Zhu, T., Deng, X., Zhou, X., Zhu, L., Zou, L., Li, P., & Lin, H. (2016). Ethylene and hydrogen peroxide are involved in brassinosteroid-induced salt tolerance in tomato. *Scientific reports*, *6*(1), 1-15.

Zhu, T., Liang, C., Meng, Z., Sun, G., Meng, Z., Guo, S., & Zhang, R. (2017). Cotton FGD: An integrated functional genomics database for cotton. *BMC Plant Biology, 17*(1), 101.

Zou, B., Wan, D., Li, R., Han, X., Li, G., & Wang, R. (2017). Calmodulin-binding protein CBP60g functions as a negative regulator in Arabidopsis anthocyanin accumulation. *PloSOne, 12*(3), e0173129.

Zou, L. J., Deng, X. G., Zhang, L. E., Zhu, T., Tan, W. R., Muhammad, A., & Lin, H. H. (2018). Nitric oxide as a signalling molecule in brassinosteroid-mediated virus resistance to Cucumber mosaic virus in *Arabidopsis thaliana*. *Physiologia Plantarum, 163*(2), 196–210.

Zurek, D. M., Rayle, D. L., McMorris, T. C., & Clouse, S. D. (1994). Investigation of gene expression, growth kinetics, and wall extensibility during brassinosteroid-regulated stem elongation. *Plant Physiology, 104*(2), 505–513.

Appendix

Promoter DNA sequences for GhCBP60b/c/d-5A

5′…TAATATTAACTATACTTTAAAATTTAAACTTAAGAAAAAATCCCTTAAGACTTCTAGCTTTTGATTTCAACCAGAATTTTACCTAACGTTTTA
AAATAATTTTTTCCGACATAATTGAAGTTTGTGAAAAGAATAGGTCTATCTGATCTGATAGTCATTATAATTTGGTTGCATGACTGATCCCGTCCC
GGGTGTTTTTCTGGTTGAGAATAAATGGACCAAAAATGCTGCCTTTTTTTAAAAAAAATAGAATAAAATCCGTCCGAGGTCTTTGATGAAATGATAG
AGGAAATGATGGTTATGTTTGGCCTCTGTTACCTTTTAACATAAAATTTTGCTTAAACCACTACAATGGATTGGAAGCTAGTTAACTAAGCCAGAC
AAAACAGCCCCCCTCATGATTAATACCACAAAACAGACCCCCCAATGATTAATACCTATGTGAAGTATTTGATTATGATTAGGTTAAAATTTGTCA
TAACCCTTGTACTATTTGAAAGTTAAAAATTTTCTCTTTGTATTTTTATTTTTAGAATTTTATTTTTTTATTTTTTAAATTTTAAAATTTGAGTCT
AGTTATTAAATTTTAAAAATTAAATTGAAGTTTATTATAACATATTATTTTGGTTACATAGTTATGAGGTGATTTTTTTTTATAAATTGTTATACC
AATAAAGTTAACAAAATAATTTAATAATATTAACAATTGGACCTGAAATTTAAAATTTGAAAAGTAAATGAATTAAATTTATAAAATTAAATATAA
AGAGACTAAATTATAAATTTTCAAAAGATAGAGGGGCTTATAACATATTTCAAATTTATGTTTATTATGTATGGATAATAGTAATACGGAAAATAA
ACCTGTATGCGAAATCGACAGAAATTCAAACAATTTCGTAAACGTAACAGTGCTGATCACGTACGTATACGAACCAGCAATCATTACTCCTAACTA
CGAAGTTGAAATTAACCGCAAATAATTACAAACAAGTACAGCGTAGAATCGAACGGTTCTGTAAAAGAAGGGAAAAAAAAAGAACCACGAAGATCA
TAGCTGAGCGGGGACGACCGTGCGAATTTTGTAAAGAGATTCCTAAACAGATTAGCCACAGTGCCAAAGCCATCACTGTGAGTAAACACAACATTG
TCCACAACGCAATAACGACAACGAAAGTTCGCCGACGTTTCCTTGAACCTTCTCTTCCTAAAATCCAATTCCCTCGCTTTTCACTTTACTCCTCCT
CTCTTTTTCTTTTTCAAAAAGGAAAAAAAAAAGGAGAGAGAGAAATTTTATTCTCTTTTTTTTTTTGGGTGGATAAAAATCGAATCACCTTTGAAG
AGAAAGAGAAGTGTTTTTCGAGTTTGGGGTTTTGTTTTTTTTGAGAAATGCAAAGTTCAAAATAAGATAGGAGAAGAGGGAAAAGAAAGAGAGAGA
GAGGTGTTTTGTTGTGTTAGTAGTTTCTGGGCAACCAAACAGGGCTGAGTTTGAAAAAAAA3′…

Promoter DNA sequences for GhCBP60b/c/d-5D

5′…ATATCTGAGTGATTCATTTAAAATTTAAACTTAAAAAGAAAAAAAAATCCCTTTTGATTTCAACCAGAATTTTTCCTAGCCTTTTTAAAATAA
TTTTTTTGCGACATAATTGAAGTTTGTGAAAAGAATAGGTTATCTGATTTGATAGTCATATAATGCGGTTGCATGACTGATCCCGTCCCGGGTGTT
TTTGTCTGGTTGAGAATAAATGGACCAAAAATGTTGCCTTTTTTTTAAAAAAATATAGAATAAAATCTGTCCGAAGTCTTGGATGAAATGATGGTTA
GGTTTGGCCTCTGTTTCCTTTGAACATAAAGTTTTGCTTAAACCACAACAATGGATTGGAAATTAGTTAACTAAGCCAGACAAAACAGATCCCCCC
CCCCCATGAGTAATGCCACAAAACAGATCCCCCCAATGATTAATACCTAAGGGAAGTATTTGATTATGATTAGGTTAAAATTTGTCATAACTCCTG
TACTATTTGAAAATTAAAAATTTTCTCCTTGTATTTTTATTTTTAGAATATTAATTTTTTATCTTTCAAATTTTAAATTTTAAGTCCAGTTATTAA
ATTTTAAAAATTAAATTGAAGTTCAATATAACATATTATTTTGGTTACATAGTTATGAGGTGAGTTTTTTTTAATTTTAAATTGATATACCAACAA
ATTTAACAAAATAATTTAATAATATTAACAACTGGACCTGAAATTTAAAATCTGAAAAGTAGATGAATTAAATTTCTAAAATTAAATATAAAGGAC
TAAATTCTAAATTTTCAAAAGATAGAGGGACTTATAACATATTTTAACTTTATGATTATTATGTATGGATATTAGTAATAAGGAAAATAAACCTGT
ATGCGAAATCGACAGAAATTCAAACAATTTCGTAAACGGAACAGTGCCGATCACGTACGTATACGAACCAACAATCATTACTCCTAACTACAAAGT
TGAAATTAAACGCAAATAATTACAAACAAGTAAAGCGTAGGAGAGATTCCGTAATCGAACGGCTCTGTAAAAGAAGGGAAAAAAAAAAGGACAACG
AAGATCATAGCTGAGCGGGGACGACCGTGCGAATTTTGTAAAGAGATTTCTAAACAGATTGGCCACAGTGCCAAAGTCATCACTGTGAGTAAACAC
AACATTGTCCACAACGCAATAACAACAACGAAAGTTCGCCGACGTTTCCTTGAACCTTCTCTTCCTAAAATCCAATTCCCTCGCTTTTCACTTTAC
TCCTCCTCTCTTTTTCTTTTTCAAAAAGGAAAAAAAAAAAGGAGAGAGAAATTTTATTCTCTTCTTTTTTGGGTGGATAAAAATCGAATCACCTTT
GAAGAGAAAGAGAAGTGTTTTTCGAGTTTGGGGTTGTGTTTTTTTTGAGAAATGCAAAGTTCAAAATAAGCTAGGAGAAGTGGGAAAAGAAAGAGAG
AGAGAGAAGTTTTGCTGTGTTAGTAGTTTCTGGGCAACCAAACAGGGCTGAGTTTGAAAAAAA3′…

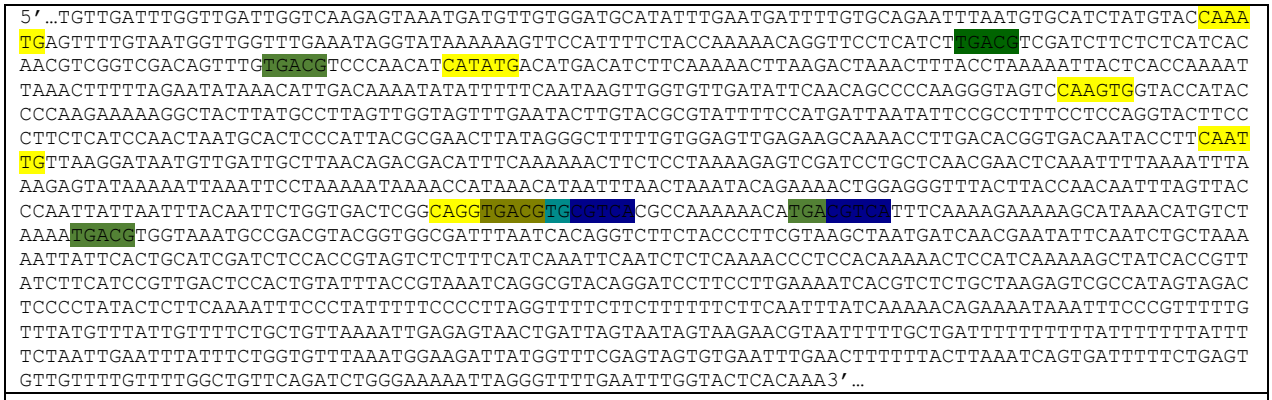
Promoter DNA sequences for GhCBP60b/c/d-6A

5′…TGGTGCAAACATAGAATGTTATATAACAACAGTTTTAACAACCCAACAACTTAAATGAAAATTTATGAATGGTTTAGTGACCATTTTGTAACT
TTTTGATGTTAAGTGACCAAAATATAAACATACTAATAACCGTAGATGACATTTTTTCAATGATACTAAAACAGCGCCTTGTTTGGCCAGAAAAAA
GGCAAATCAGTCAATTCCCAATTATTACGCTTTATAATTAAATAAAACAATAATAAGTTTAAAAAAACAAATGAGTACGGCCAACATAAGAATTAA
ATATAAAAATATAAAAGAGTACGGGCAAAAACTAAAAAAGAAGAAGTTAAATTACACTTAAGGTCACTTAACTATTAGTAATTCTACGTTTTGGTC
ACTTAATTTTAAAAAGTTACAAAATTATCACTAAATCATTTAAAAGTTTCCATTTAAGTCACGTGGTTAGGCTGTTAAAATTTCTACTGTATGGCC
TTCTTTGTTTGCACCACCTGCACTAATTGAAAACTTTCTTCCCCTTCTCTTTTATAGTTTAATTTCTTTTTCATGAAACAGTTTTGAAACATCACG
AATTTACAAACCAAAATTTCAGATAGTTTTCTTCTTCGATTTTCGATACTAACCGTCAGATCAACTTGGATATAAGGTATGTTGTTTTACTCATCA
ATTGGTATCCACGATACCAATCGTTGAATCATCACTTCGAGCTCGCTAGCTGAACTTTTTAATAAAAAAATTTTATTAGTTTAGTAACTTGAATAA
AAACTTTCAAATAGTTTAGTGTGGGATATTCAAACAGTCGATTCAGTTATTAACAGAACTGAATTAAAAAATATATTATATATAATATATTATTT
TAATTTGGTTAATTACTCGATTTCGAACCTAATTAACCGATAATCAAAATTTTAAGAAATCATTAATTGACTAAATTAAATCCGGCCACCGACCGA
TTAACTAAATTAAATCGATTCGATTGATTAATTCGATTTTAATTGAACTATTAACACCCTGATTTAATTATTTAAATGAACATATTTAAATAGCTT
AATATCTATTTTATAATATTTTAAAGTTAAATGGTTAAAATGTAAACTTCCTATAATTCTGATACAATGAGTGTAGTTTAACGAAAACATCCTACG
GCTCTGATCTGATCAGAGCTTGTGACTGAGAATTTAATTTATGCGAATCCGACCATTTAGCTAAAGTTACAGAAATTCGTTACTTCAAAACAAGAA
GGAAAATTCGCCGACTTTTCCTCGGTGCTTTTTAATTTACAAAAATCCAAATTTTCCGTCTTTTCGCTTTTCTATGATAAATGATAAAATACTATT
TTTTCCTGGAAATTTGATAGAGAGAGAAATTTGTTTTTATTTGTTTGTTTATTTATGTTGTTGAATAAATTTGATTCGCTGTTGAAGGGAAAGGGC
GAATTTTTAAAAGTTGCGTTTTTTTTTCTCGGGAACCAAACAGGTTTTTCAGTGTGCAGCAAA3′…

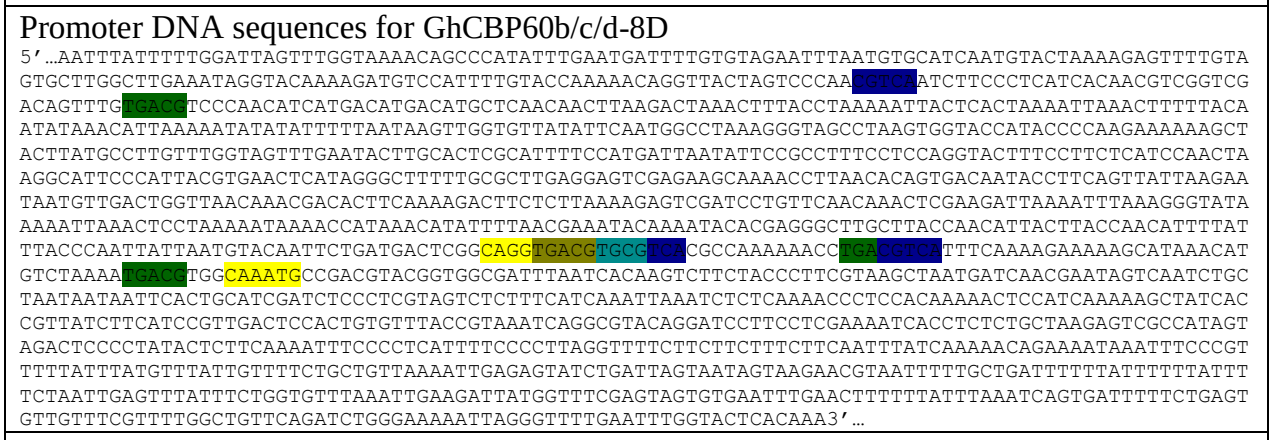
Promoter DNA sequences for GhCBP60b/c/d-6D

5′…GCAAATCAGTCAATTTCCAATAATAAATTTAAAAAACAAATGAGTACGGCCAACGTAAGAATTAAATATAAAAATATAAAAGAGTACGGTCTA
AAACTAAAAAAAAAGAGTTAAATTACACTAACTTTACGTTTTGGTCACTTAATTTTAAAAAGTTATAAAATGATCCCTGAATCATTCAAAAGCTTC
CATTTAAGCCACGTGGTTAGGCTGTTAAAATTTCTACTCTATAACCTTCTTTGTTTGCACCACCTGCACTAATTGAAAACTTTCTTTCCCTTCTCT
TTTATAGTTTAATTCCTTTTTCATGAAACAGTTTTGAAACGTCATGAATTTGCGAACCAAAATCTCAGATAGTTTTCTTCTCTGATTTTCGATATT
GACCGTCAGATCAACTTGGATATAAGGTATGTTGTTTTACTAGTCAATGGGTATCCACGATACCAATCGTTGAATCATCACTTCAAACTCGCTAGC
TGAATTTTTTAATAAAAAAAATTATTAGTTTAGTAATTTGAATAAAAACTTTCAAATCATTCAGTATGGGGGGTTCAAACGGTAGGTTCGATTATT
AACAGAACTGAATTACTATTAACCGAATTATCCAAAATGTAAAAATCTTTAACCGTTAACTGAACGGAATATTTTTTCAAATACATTAAGTGAACC
AAAACTGAATTAACTGAAATTTATATGTTTTTGTCTTTTGGTTAAATTAAGTATAAAACATATAAAAAAACAGATCACTATGTTCATTTTCTTTTT
TTTTTAATAGTTCAAATATATATTATATTATATATATTATTTATTTTGATTAATATAAAAAAATAGTTCAAAAAATACCTTGCTAATATAACAAAT
ATATTATATATAATATATTGTTTAATTCAGTTAATTTCTCAATTTTGAACCGAATTAACCAATAATTGAAATTTTAAAAAATTATTAATTGATTGA
ACTAAATTCGGCCACCCACTGATTAACGAAATTAAATCGATTCGGTTGGTTAATTCGATTTTAACCGAACTATGAACACTCGTGATTCAATGATTT
AAATGAACCTATTTATATAGTTTAATAACTATTTTGTAATTTTTTAAAGTTAAATGATTAAAATGTAAACTTGGTGATAATTCAGAGAGAATTGGT
GTAGTTTAGCGAAAAAATTCTACGGCTCTGATCTGATCAGAGCTTGTGACTGAGAATTTAATTTATGCGAATCCGACCATTTAGCTATAGTTACAG
AATTCGTTACTTGAAAACAAAAACGAAAATTCGCCGACTTTTCCTCAGTGCTTTTTAATTTACAAAAATCCAAAATTGCCGTCTTTTCGCTTTTCT
ATGAAAATGATAAAATACTATTTTTTCCTGGAAATTTGATAAGAGAGAGAAATTTGTTTTTATTTATTTGTTTATTTATGTTGTTGAATACATTTG
ATAAAAGTGGGTTTTTTTTTTCTCGTGAACCAAACAGGTTTTTCAGTGTGCAGCAAAATGGAA3′…

Promoter DNA sequences for GhCBP60b/c/d-8A

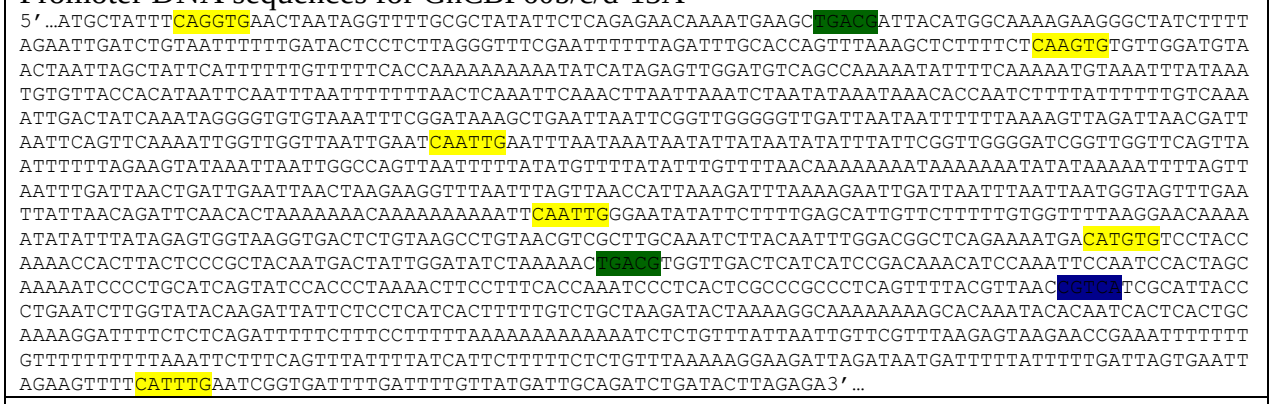


Promoter DNA sequences for GhCBP60b/c/d-8D



Promoter DNA sequences for GhCBP60b/c/d-13D

Promoter DNA sequences for GhCBP60b/c/d-13A



Promoter DNA sequences for GhCBP60b/c/d-10A

GCAAATATTATTCAATTTACGCTTTGGTAATTTAATTTTAAAAAAATTATAAAATAGTCATTGAATTATTTTAAAATTTTTATTTAAGTCATTAGA
TTATTTAAATTGTTGTTGTGTGATCTTCTCTATTTACACCTCTTGTACGTAGAGGTGAATCTAACGAGGGGTAAGTAGTGGCCTTCTAAAATTTAA
ATATTATAAATTAGTATAATGATAAAATTGTATTTTGGTCTCAAAAAATTTATAATTTAAAATTGCCCTCTAAAATGATCGAAATCTCTTGTTCCT
TTTCTCATTCACAAGTTTATTTATTTTTTCATGAAATAATTTTGAATGTCACGAATTTATGAACTAAAATACAAACAGCTTTATTATCTGATTTCT
AATACTAACTATTAAAATAATTTGGATCTAAGGTACGTTGTTTCACTTGTTGATAGGTACTAATCTATTATATCAATTGTCAAATAGTAGCTTAAT
TAGTTACACGTGTATTTACCCAAAATAAAATCAAAACAGTTCCAAACAAGCAAATACAAAGAAGGAAAATTCGTAAGCTTTACCACGAAGCTTCTT
TGCCCACAAAATTCCAATTCTCAATAAATTTCATTGACCCTTTTTCTTTTGTTTCCGTTACATTTTCCTTTTGAAAATAAATTGAAAAAAAAGAAC
AAATAGAGAAATTTGTTTCCCCTTCTTGTTGGATAAATAATTACGAATTTCCCTTTTGAAGAGTAGTGTTTTTCTTTTCCTTTTTCCCTTTTGGGA
GTTTGGGTTTTGTTCTTTTACGAAGGAATGTGAAGTTCAAAGTACGAAATGGAAGAAGGATTTTGATTTCAGTTTTTATTGCTTCTTTTTTTCTCG
GCAACCAAACAGGCTAAAGTTATTTGAAAAATGTAAAAGGGGTACGCGAAGAGGTCGAAAAAT3’…

Promoter DNA sequences for GhCBP60f-8D

5’…GTGTGCTTTATGCTGATCTACAATAACCAATTTTTAACTGTAGAAATAAGTTTTTGCCTTTGCTTCTTAATCTCCTCCTGCCACACATCGATT
CCAATCGTTAGAAAAAGCTGTTCTCGAACAGGTAGGTATGGTGGTCTCTTTATACGCAATTTCAAGTGATATTTTTAAACAAAAGAATTCATCTGCT
CTTTAATCTAATGTGCAGGGACTAATTTGACCATTTCTTTAATAGATGGAGCAAATTGTAATCTAACTCTTAATATAAAGCTTTTATGATACTTTT
ACCAAGTTGTTTTAATATGGCAATTAACTCTCACATCTGAATCCTAAAATAGTTAAGATCCACTTATATTTAAATACAATGCATGTTCCCCCACTT
CTAAATGGCATGGACCGGCAGAAAGAAAAAGCTTTTGTTTTTTCCTTTATCAAGTTAATATTAAGTTTATATTTATGTAAAGTTAGTATTGTATTT
AAAATACAAGAATAAAGTTTTAATTTTACTTTTAATAGTGTGTTTCTACATATCAGAACAAAAAATGGACTATGAATTTTGTCAAAACATCCATCA
TTTTCTTTTTTCCTTTTTTTCCTTCTTCCGAGTATCCAAACAAACAAAAGGTAAAACATCAAATTTAGCTCTTAATGCTTATATTTTTATCAATTA
AATCCAGCCTTCAATTTTTAAAAAGAGTCAAATTTAATCATTAATTTTTTGAAAAATAATTGAATTAATTCGTTTTAATGGAAATGTTAGCTAAAC
GGTTAAATTTTAAACATGAAAACCCACATGACAGCCTATATATACTAAATTGCAAATAGTATCATATCAACATAAAGTGTAAGTCGACGAGTTGCC
AAGTTAACATGTTAAAAGTTGAATATTTTAGTTTATATTTTTATTTAAAAAAATTATTTAACTTTTTTTTAAACCTTATTAAGTACCAAATTGATA
AAATATGATTCTAACATAAAAAAAACCAATAATGGTATGATGCATACATGGATCAAGCATTGACTTTCCTCAAGAGCCAAACACAAACAAAGAGTG
TTTTTAAACAGAAAAGCGCACATGCGTAGCATTTGAGTTAAAAAGACATGAAGTGAGATGAGATGCCTAAAAAAACGCGGTTTCAATACTTTCAAC
GTTCATGCACCCAGTCTAGTCTCAAAACACAGCTGTGTTTTCTTCTTCAATTATATATAAATTTTAAAAAAAATTATCAACATTATAATATTGATC
ACTAGACAGAAAGTAAAATATAATATTATAAATCTAGATCTACCAATAAACATATATTTTAGCCCTTTGTTAATTTTACACCTAGATCTATAATAA
CATTAACTTCATTTTCTAAAAACAACTAAAATAAAATGAGTCTAAGCTTTGGACCTTGGTTTAGGGAACTCAAAGTTTTGTTCTAAAAGTTTCTTC
TTTGAATCTTCATTGGGATATCTTGGGTTCTTATTATTTTTCTTTTATTTTCTTGTTATCTAA3’…

Promoter DNA sequences for GhCBP60f-A

5’…ATCTACAATGACCAGTTTTTAATTATAGAAATAGGTTTTTGCTTTGCTTCTTAATCTCCTCCTGCCACACGTCGATTCCAATCGTTAGAAAAA
GCTGTTCTCGAACAGGTAGGTATGGTGGTCTCCTTGTACACAATTTCAAGTGATATTTTTAACAAAAGAATTCATCTACTCTTTAATCTAATACAT
ATGAATTAATTCGACTATTTCTTTAATAGATGGAGCAAATTGTAATCTAACTTTTAATATAAAGCTATACTATTACCAAGTTGTTTTAATATGACA
ATTAACTCTCATATCTAAATCCTAAAATAGTTAAGATCCACTTATATTTAAATATAATCCATGTTCCCCCACTTCTAAATGGCATGGACCGACAGA
AGGAAAAATATTTTGTTTTTTCCTTTGTCTAAGTTAAATAAGTTTATATTTATGTAAAGATGGTATTGTATTTCAAATACAAGAATAAGGTTTTAA
TTTTACTTTTACTTTTAATAGTGTGTTTCTACATAACAGAACAAAAAATGGTGGAAAGGACTATGAATTTTTGTCAAAACATCCATCATTTTCTAT
TTTCCTTTTGTTTTTCTTCTTCCTACAAACAAAAGGTAAAATATCAAATTTAGCTATTAATATTTATATTTTTGTCAATTAAATCTAGTCATTTTA
ATTTTTAAAAAGAGTTAAATTTAATCATTAATTTTTTTAAAAAATAATTGAATTAATTCATTTTAATAGAAATGTTAGCTAAATGGTTAAATTTTA
AAATATGAAAACTTGCATAATAGTCTACGTATACTAAATTATAAATAATATCATATCAACATAAACTGTATGTGGACGGGTTACCAAGTTAACATG
TAAAAAGTTGAATATTTTAGTCTATATTTTTATCTAAAAAAATTATTTAACTCTTTTTAAAATATAATTAATTACTAAATTGATAAAATATGATTC
TAACATAAAAAAACCAATAATGGTATGATGCATTATGCATACATGGATCAAGCATTGACTTTCCTTAAGAGCCAAACACAAACAAAGAGTGTTTTC
AAACAGAAAAGCGCACATGCGTAGCATTTGAGTTAAAAAGACATGAAGTGAGATGAGATGCCTAAAAAAACGCGGTTTCAATACTTTCAACGTTCA
TGCACCCAGTCTAGTCTCAAAACACAGCTGTGTTTTCTTCTTCAATTATATATAAATTTTAAAAAAATTATCAACATTATAATATTGATCACTAGA
CAGAAAGTAAAATATAATATTATAAATCTAGATCTACCAATAAACATATATTTTAGCCCTTTGTTAATTTTACACCTAGATCTATAATAATTGTCA
TTAACTTCATTTTCTTAAAAAAAAACTAAAAACAAAATGAGTCTAAGGTTTGGACCTTGGTTTAGGGAACTCAAAGTTTTGTTCTAAAAGTTTCTT
CTTTGAATCTTCTTTGGGATATCTTGGGTTCTTATTATTTTTCTTTTATTTTCTTGTTATCTAA3’…

Promoter DNA sequences for GhCBP60a-3A

5’…CACACATTTCATATATCCTGTCGAGATGAGACTGTTACCTGAAAAACCTATCCTGTGAAAGTCAGGTTCCAAGGTGATGGTGTTTTATATGGT
CACGGGTCGAAGTCTACTAGAGGTTTCGGTCGATGACCGTTATGAGGTCATAGGTCGAAGTATAACTGAAGAGCCACTTAACGGTTATTCTGTGGG
CACGAGCTCAAACTTTTTGTACACCCTATGATTATGGCATCAATCTTATGTATGGTATATATAAGGCATCATTTGCAGGTATCCAAAAAGCTTGAA
CTCGTGACTGTATAACGACCGTCAATTAGCCCCTAGTTATACTCAAACCCTTGACCGCATAACGACCGCTGACTAGGACCTCCACTTAGATTTTGA
CCTATAATTGTATCAAGTATTGTCACATTGAAACCTGACTTCCATAAGATGAGTTTCTTAAGTAATGGCCCTATCCTGACATGACATATGAAGTGT
ATGTGTTTTGAGATACGTTCTTACATCCCTAGTCTTTCAAATCTACCTATAAACTTTCACGGTTTCACTCTCAAAACCTTAAACCAAATAAAAACC
CTAATTTTTAAACAAAAAAATCTAACCCTAATACCAACCTTAACCCTAAAAAATCATAAAAAATTGGTGGCTGAGGAGAGTGTGTGCAAGCGCATC
TAGCTAGGCGCGCTTTCACACACTGTCTTCAAAATCGAACTATTTTTTTAAATCATAAAAAAAGACCTATTTAATCAATTAAAAAACTACATATAT
TGCGTATTTAACCGATATACGAAATAGGAAGAATAAATAAACACGTAGTGTTAAGATAAAATCAATTATGTTAAACAAGCTAATAGAAAAAGAAAA
TAATTTTTTAAGTCTCGAGGCTAATTTAAAATTTAATAGATAATTTAGGAACATCTCAACCTCACTTAATACCAAATAATTACGTGTCTTTTTTTC
TTTTATTTCATTTGAAATTTCCTTTCTTCCAATAATTCACAAGTAAAAAGAGAAGAAAGGACAGCAAATCATATTTATAGATTTGTTTAGTTCCAA
AACGAGGTCAGTGGGGTCCCACATTCAGACAGATATCCTTTGGTTACATATAAACACACAACACAGAATCACAACCACCTACCCAAGCAGTTAAAG
TGTTAAACTCAGACAAGAGAGAGAGAGAGAGAAAGGGCCAATACAGGATCATGAAAGAGATTCAGGGTGAAGAAGCATCCCAGACACAGCCAAATT
CAGACAATGGGTAGGAAAGTGGGATGGGAAAAAGGGTTTTAACTCTTGCTTCTTCTTTTTTTCCACTTTTGGATTTACTCTTTGATTATCATTTAC
TGATGAATGAATAAATAAATAGTATCTCCTCCTGCTGGTTTCCTTTTCAATTGAAGTGCCGCATGTTGGGTTCTTTAATGTTTGTGAATGAACCTC
ATCACCATCCAAACAGCTTAGCTTCTGGGGGTGTTTTCATTCTTGGAGTTAATAGTTTTAACA3’…

Promoter DNA sequences for GhCBP60a-3D

5’…GACGTAGGAGGACTCTTCGGCGGGCAATGACTATTTCGATGGAGTAAAGGTGATGCTCTTTTTAGTTAGTAAAAAATCATTAATAACTACAAC
TGTTGTAGCACCGACTCAAGTTTGACTTTTACTCCACCTGAACATTCGTTTTTCGCTTGAGAGCCCTCTTGTGTCAACCTCGATGCCAACCCTCAG
ATAAGAATGAAAGGTTAAAGATATCAACTGCTTGGAAATAGAGAAAAAAATTACATTGATTACATCGAAAAGCCCTTCATTTTTTCCTATGATTAT
GGCATCAATCTTATGTATGGTATATATAAGGCATCATTTGCGGGTATCCAAAAAGCTTGAACTCGTGACTGCATAACGACCGCCAACTGACCCCCA
GTTATACTCAAACCTGTGACCGCATAACGACCGTTGACTAGGACCTCCACTTAGACTTCAACCCGTGATTGTATCAAGCATTGTCACCCTGAAAGC
TGACTTATATAAGATGAATTTCTTAGGTAATCGTCCTATCCTGACATGACATATGAAGTGTATGTGTTTTGAGATACGTTTTTGTATCCCCAATCT
TTCAAATCTACCTATAAACTTTCACAGTTTCACTCTCAAAACCTTAAACCCAATAAAAACCCTAATTCTTAAACAAAAAATCTAACCTTAATACC
AACCTTAACCCTAAAAAATCATAAAAAAAATTGATGGCTGAGGAGAGTGTGTGTAAACGTGTCTTGCTAGGCGCGCTTTCACAAACAGTCTTCAAA
ATTGACCTATTTTCCTAAATCTTAAAAAAACGACCTATTTAATCAATTAAAAAACCACATATATTGCCTATTTAACCTATATATGAAATAGGAAGA
ATAAATAAACAAATTAAAATCAATTATGTTAAACAAGCTAATAGAAAAAGAAAATAATTTTTTAAGTCTCGAGGCTAATTTAAAATTTAATAGATA
ATTTAGGAAAATCAAATTTCCTTTCTTCCAATAATTCCCTAGTAAAAAGAGAAAGGACAGCACATCATGTTTATAGATTTGTTTAGTTCCAAAACG
GGGTCAGTGGGGTCCCACATTCAGACGGATATCCTTTGGTTACATATAAACACACAACACAGAAACACAACCACCTACCCAAGCAGTTAAAGTGTT

AAACTCAGACGAGAGAGAGAGAGAGAGAGAGAAAGGGCCAATACAGGATCATGAAAGAGATTCAGGGTGAAGAAGCATCCCAGACACAGCCAAATT
CAGACAATGGGTAGGAAAGTGGGATGGGAAAAAGGGTTTTAACTCTTGCTTCTTCTTTTTTTCCACTTTTGGATTTACTCTTTGATTATCATTTAC
TGATGAATGAATAAATAAATAGTATCTCCTCCTGCTGGTTTCCTTTTCAATTGAAGTGCCGCATGTTGGGTTCTTTAGTGTTTGTGAATGAACCTC
ATCACCATCCAAACAGCTTAGCTTCTGGGGGTGTTTTCATTGTTGGAGTTAATAGTTTTAACA3′…

Promoter DNA sequences for GhCBP60a-12D

5′…GTAATGAAGGCTATGATATTTTGGATACAAATTCCGTGATTCCCATCATGTGTATATAATTTCCTAGATTTATTAAAATATAAAAAGATAAAT
ACTCTCAAAATAATAATCGTTATTTTGTAAAAGAGATGAGATTATAGTAATTTCACAATTGAATTGAAACTCCATTGATACATTACATCAATTTAG
TCACTTTTAATAATAATAATATATTATATATTATTATTAAATAATTATAAAAACACTATAAAAAATTATTTAAAACTATTAAAATTTTTGCAACTT
TTAATAATTTTTAAATAATTATCTATGTTTTTATAATTTTTTGAAAAATCATATCCAAGATGAATTAGAAAAATCATTTGTCTAGATTCTTCTTGG
ATGTAGTTTTTTAAATAATTATAATTATAACTATAACAAATATTTATTTTTAGGATACTTTAATTTTTAAAATATTATCACATCAACACTTAGTAC
TTAGTTTTTTTGTAAATCATATCAAAATTTAAGAGTAGATATAAATGGAAATTTTAATAGAAGATTAGTTTGCACTAATTTACCAATTTTGAGTTG
AAATGGAAAACTGCAATATGAATATTAATACTTTTTTTACCTAATGCTACTCACCATTGAATTTAATATCACTTACATAATAAACTTTAAAAATCT
AAATCATCATTTAACAACATATATGGATAGATTTTTTAACGAAAAAGTTAAATTACACTTTTTTTTAATATATAAAAATTAATTAATTTATTTATT
AATAAAGAAGATAAAATATAATCTAATTTTTAATATTGATATTTTTACTTGATTCAAACCTAAATATTATAATTTTTAATAACTCAATTTTACTAT
TTAAAATAAAATCATATTTAATTAGTATTATATCAAAATTAATAAATATATTATGTAAACTTTTTTACGTACGGGGTGTTGTATGTATAAAATGTA
AGAAATATTTTATCCTAAAATTAAAATTGAAATTAAAGTCAAACTCCATTACACGCTTAGATTAGATAGGATCGGAACGAAATGGAAGGAAAATGC
GTATTATTATGTGGACCCCACCCCTCAAATCTAGTCCAAAACGCGGTTTGTCGGGTCCCATGTTACGGACAAAAATCCTTGCTTGCAGACACAGCT
TATACAGCAACCTTTGGCATTGAAGTTAAACTCGAAGAAAGGAAATAGAGAAAAAATATAATAATAAAATGAAAAGAGTTTTGCGGCGAAGAAGCA
TCCCAGATTCCGCCAAATTCAGGCAATGGATAGGAAAGTGAGATTAAAGTGCTAGGTTGCTTTTGCCTTTACTTTTTATTTTTATTCTTTTGGATT
TTACTCATTTGATTAGCAGTTATTTATGAATGAGTAGCTTATATATGTATATGTGAACTCCCCCGGGCTGTTTCATGATTTCATTTTATTATTGAA
GTGGCGCATGGTTGGTTCTTTGGCTGTTATTGTTGTTGTTGTTGTCGTCTAGGTGTTTGTTAA3′…

Promoter DNA sequences for GhCBP60a-12A

5′…GTAATTTATGTTTACCTTTTAAGTGTAAATTAAATTAAAGATTACGCATGATTTGAATTTAAATATAAATTAAAATATAAAAAATTTGTTTGG
TTATGCAAAAATAAAGCCAAAATTAAATAAAACTAGATCTTAAAAAAATACATTTATAAAAAAAATAGAAATAGAAATAAAAAATGACTTTAATGGAA
ATGATAAAGTTAAATAGTGAAGGCTATGATATTTTGGATACAAATTTTGTGATTCCTATAATGTGTATATAATTTCCTAGATTTATTAAAGTATAA
AAAAATAAAAACGCTCAAAATAATAATTGTTATTTTGTAAAAGGGATGAGATTATGGTAATTTCACAATTGAATTAAAACTTCATTAATACATTAC
ATCAATTTAGTCATACTTTTAATAATAATAATATTATTATTATTATTATTTATAACTATAACAAATATTTATTTTTAGGTTACTTTAATTTTTAAA
ATATTATCACATCAACCACATCAAAATTTAAGAGTAAATATAAATGTAAATTTTAATAGATGATTAATTTGCACTAATTTACCCATTTTGAGTAGA
AATAGAAAAATGCAACATGAATCTTAATACTTTTTTTACCTAATGCTACTCATCATTGAATTTAATATTACTTACATAAGAAATTTTAAAAATTTA
AATCATCACTTAACAACATATATGGATAATTTTTTAACGAAAAAGTTAAATTACACTTTTTTCTAACATATAAAAATTAATTAATTTATTTTTAA
TAAAAAAGATAAAATATAATCTAACTTTTAATATTGATATTTTTACTTGATTCAAACCTAAATATTATAATTTTTAATAACTTAATTTTACCATTT
AAAATAAAATCATATTTAATTAGTATTATATCAAAATTAATAAATATATTATATAAACTTTTTTACGTACGGGGTGTTGTATGTATAAAATGTAAG
AAATATTTTATCGTAAAATTAAAATTGAAATTAAAGTCAAATTCCATTACACGCTTAGATTAGATTTAGATAGGATCGGAACGAAATGGAAGGAAA
ATGCGTATTATTATGTGGACCCCACCCCTCAAATCTAGTCCAAAACGCGGTTTGTCGGGTCCCATGTTACGGACAAAAATCCTTGCTTGCAGACAC
AGCATATACAGCAACCTTTGGCATTGCAGTTAAACTCGAAGAAAGGAAAGAGAGAAAAAAGATAATAATAAAATGAAAAGAGTTTTGCGGCGAAGA
AGCATCCCAGATTCCGCCAAATTCAGGCAATGGATAGGAAAGTGAGATTAAAGTGCTAGGTTGCTTTTGCCTTTACTTTTTATTTTTATTCTTTTG
GATTTTACTCATTTGATTAGCAGTGTTTATGAATGAGTAGCTTATATATGTATATGTTAACTCCCCCGGGCTGTTTCATAATTTCATTTTATTATT
GAAGTGGCGCATGGTGGGTTCTTTGGCTGTTGTTGTTGTTGTTGTCGCCTAGGTGTTTGTTAA3′…

Promoter DNA sequences for GhCBP60g-8D

5′…TTTTATAATTTTGTTAAAAGGGGTCACAATTTATTATTTCGCCTAGGGTCCCAAAAATATCAAGAACGGGTTGTTATTTGTTATGTTGTTAAA
GTAAGGTTGAATTGATGGGAAATGTATATAAAGTACATTAAAATTTTAAAAAATAAGACACATTACAAATTTATAATTCTATTTGCCAAGTTAAAA
CATGCGCTGTAGTATATCAAATACTCACCCATTAATATATAGTTTAATAAAACCTTTTATTGATTGTTTGTGTATATACATACTTTCACCAAACCA
ATTATAAATTTGTATAGTCAATTAAATTTTAACTCGATTGGTATTGTTACTGTTATTATAATTTAGAGAAGATGCATTAATGTCTTCCACTTTAGA
AATTGTATCAATATATATTGGTTAAATTCTGCAATTAGTCATTATATAATTTGGTCATTTTTAGTTCTATATTTTTGAATTAGTCAATTATTGTCC
TCGTACTTTTAAAATTTTAAAAATTTAATCCTAATCCAAACAACAACCGTTAAATCCATTTAGTTAAATTCAATAATTACTAGTCATGTACCATGC
GCACAATCGTAGATTCAATTCATATTCTCCAACTGGATTATTTTAAGTCTTTATATTTTTCGAATTTTAAAATTTTAATTTTGACACAAATAACAA
TAGTTAATACATTAATTAAAATTTTAAGGAATATTAGAATCTAAGCATTTTATTTTTCTAGAAAATTAAGTTAAGCGAGAAAGGGAATGCGATTTT
CAATACATGTCGCGCGATTCTCAGCAGTCAAAGCTAGACCCAACGCGGTATTTGTGGAAAAACAACACAATGCATATGCTTTTTGAGTTCTTTTCA
TGCACGCGTTTTAATTTAATGCAATCCAACCGTGTTTTTTTTCATATTTCTCTTGTCATAAAAACAACTACAATCCAACATTTTCTGTTTCATAAT
CCTCTAATATTACATTTAAAATCGAATAATATTTCAATGAATGACTCGTTTTCACAGTACATTTTATCGTTTTTCTTTATGTACTTTTCAAATTTC
AATGATGATAGCGCGTTGTTTGTTGTTCCACTTTCGTTAATCGTTAATGGTCCCAATGATTTTATTTGCAGTTTTTTTTTTAGTTAAAGTCTGGTTC
AGTGATGTGTACAACTTAAGTCTGAGGTTGATCCTTTTTAAAGTATATACTCACCAAAATTAGCATAAAATAGTGTTGTTTTCTTGTTTTAGGGTT
TTTCACCTTTTCTCCTTTCTTATCTTTTAAATACAATATTTCCTTGACTTTAATGACATTTACGTGTTTTTATACAAGTGGCTTCGAAGAGTTTCC
AGGTACCTTCAGTGAGTGACTTGAGTTTTCAAGTCAAGGAAAGCAACATTTGCTCTCTGATTATTTATATAGAACTAGCTGGATACAACTGGGTTT
AAGGGCTTGTTTGCTCACAGTGTTTTCTGCTTCTTTTTCTTTTTATATTTTGTTTCCGTTTTC3′…

Promoter DNA sequences for GhCBP60g-8A

5′…GTTTTTATTTTATTATATTACATTTTATAATTTTGTTAAAAGGGGTCTCAATTTATTGTTTCGCCTAGGGTCCCAAAAATATCAAGTACAGGT
CTGTTATTTTATAATTCTATTTGTGGAATTAAAAAATAGACCGTCAGTATATCAAATACTCACCCATTAATATATAGTTTAATAAAACCTTTTAAT
GATTGTTTGTGTATATACATACTTTCACCAAATCAATTATAAATTCGTATAGTCAATTAAATTTTAACTCGATTAGTATTGTTACTGTTATTATAA
TTTAGAGGAGTTGTCTTCCACCTTAGAAATTGTATCAATATATATCAACTCATATATATCAGTTAAATTCTGCTATAGTCATTATATAATTTGGTC
ATTTTTAGTTTTATATTTTTGAATTAGTCAATTATTGTCCCTGTACTTTTGAAATTTTAAAAATTTAATCCTAACCTAAACAACAACAGTTAAATC
CATTTAGTTAAATTCAATAATTATTAGTCATGTACCATATGTACCATGCGCATAATTGTAGATTCAATCCATATTCTCTAACGGGATCATTATAAC
TATTTATATTTTTTAAATTTTTAAATTTTAATCTTAATACAAATAATAATAATTAATACATTAATTAAAATTTTAAGATATAATTTATAAAATAAT
AAACAAACATAATATTTGATTAGAATTTAACCACTTTATTTATTTTTCTAGAAAATTAACTTAAGCGAAAAGAGGAATGCCATTTTCAATACATGT
CGCGCGATTCTCTAACAGTCAAAGCTAGACCCAACGCGGTATTTGTGGAAAAATAACACAATGCATATGCTTTTTGAGTTCTTTTCATGCACGCGT
TTTAATTTAATGCAATCCAACCGTGTTTTTTTTTTTTCATATTTCTCTTGTCATAAAAACAACTACAATCCAACATTTTCTTTTTCATAATCTTCTA
ATATGACATTTAAAATCGAATAATATTTTCAATGAATGACTCGTTTTCATAGTACATTTTATCGTTTTTCTTGATTTACTTTTCAAATTTCAATGA
TGATAGCGCGTTGTTTGTTGTTCCACTTTTGTTAATCGTTAATCGGTCCCAATGATTTTATTAGCAGTTTTTTTTTTTTAGTTAAAGTCTGGTTCAGT
GATGTGTACAATTTAAGTCTGAGGTTGATCCTTTTCAAAGTATATACTCACCAAAATTATCATAAAAATAATGTTCTTTTCTTGTTTTAGGGTTTTT
CACCTTTTCTCCTTTCTTATCTTTTAAATACAATATTTCCTTGACTTTAATAACATTTACGTGTTTTTATAGCAGTGGCTTTGAAGAGTTTCCAGG
TACCTTCAGTGGGTGACTTGAGTTTTCAAGTCAATGAAAGCAACATTTGCTCTCTGATTATTTATATGGAACCAGCTGGCTACAAGTGGGTTTAAG
GGCTTGTTTGCTCACAGTGTTTTCTGCTTCTTTTTCTTTTTATATTTTGTTTCCGTTTTCATG3′…

Promoter DNA sequences for GhCBP60g-13A

5′…AGTTTTATAATTTAAAAAAAAAATTAAATTATTTACATATTATATATAATTATTTACATTTTTATTCAATATAACATATATAATATTAAATAA
AAAAAAAAGAACATGTAGCAGGTTCTAATGGTGCCATGTGGATGGGCAATGGAGGTCAACTATAGTCAATGCCAATCAATAATGATCAAGAGTGAG
TTAAATCAAATATGTTTTTAATTTAATTTTAATATTTTTATATTAAATAAATTTAATTATCATGTGGCACATTGTGATTGGTTGGATGTGCCACAT
GGCACATTTTTAATTAGCAATATATGACGATCGATTTTTTAACATCATTACAAAAAAAAAGTTAAAATAATAATTTAAATACTAATATGAGAAAAA
ACAAAATTTGAGTACAAAGGAGGAAAACAGACAGAATTTGAGGGAAAACCCAAATAAAGAGCTGTAGAAAAAACTCCTTGAATTGAATTTTTTAGA
TTTACTTGGATAATTAAATTTATTTTTTAAAAAAATATCGAAAACATCCATCTTTAGTAATTAAAAATTCATAATAGTCGGGGGAAAAAAGCTCTT
TATTTCCAAATAAAGTTTGCCGAGGCTAATATTATCACAAATATATGAACAGGCGGAATTTTACTTGTTACTTAGGGACAAGGCAAAAATATACTA
AAGCAAAAGAGCAAGAATGGAGGTTTTGAAATTTACTGAAGAGACAAAACTAGGAATAATCATGGCAGTATATTGAATCTTTGTCAGAAAAACAAT
TATTTAGTTTAAGAAGACAGTGACATTTTCAAATCTAAAAAACTCAATCCCATCACAACGCGCTATTTGTAGAAAAACAAAAAAGTTTTCTCCTCC
ATGCACGCGTTTTAATCCACCGTGTTTTTTTGTCATAAGTTTCTTGTCCGAAGAACTACAATTAAGAAGAAAAAAAAAACTGTTTTCTTGGATTAC
TCGTTTTCATTGAACTATTTTTTGCCATTTTATGAACTATTCAATGATCATCACGCGTTTGTTCATTTTCCAGCTCATTGTACCCTTCCAAAATCC
AGTTTTTCCAAGTTTACTTGTCTGGTTTTTAAAGTCTAGTGATGTGTACAACAATTCAAGCAGTTGTTGATACTTTTGAAAGAAACTATATATATA
TAAAGAGTAGTTATACAAGCATATTTTAATAATGTTTTCAGTTGCTAGCTCTTTTCTTTTTGGGTTTTTCTTATTGCTTCAAATGTTTTAATTACA
ATATTTTATTGACTTTAGTAGCATTAAGTGTTTTGGGATTAGTGATATTCTTTAATGAGTTTCCAGGTATCTTCTATGACTGAGTTTAGTTCTAGT
TATTAGTCAACTAAAGCAATCTATATACTGCTAAGCATATCTATAAAGGGGACACCTGGAGAAGGTTTTGGCTACTGGGGTTTGAATATTTATGCT
CCCAAAGTGTTTGGGTTTTTGCCTTCATTAAATCACTGTCAAGTTCCAACTTTCATTTTTCCC3′…

Promoter DNA sequences for GhCBP60g-13D

5′…TTTCAAATTTTATATTTATATATTTTAGAGTTTTATAATTTTTTAAAAAAATTGAATTATTTAATATTATATATGATTTTTTTATATTTTTAA
TCAATATAATATGTATAATATTAAAAAAGGACACGTGGCACGTTCTAATTGTGCCATGTGGATGGGCAATTGAGGTCAACTGTGGTCAATGCCAAT
CAATGATGGTCAAGGGTGAGTTAAAATCAAATATGTTTTTAATTTAATTTAATATTTTTATTTTAAATAAATTTAATTATCATATGGCGC
ATTGTGATTGGTTAAATGTGCCACGTGACGATCAATTTTTTTAACATCAACACAAAAAAAATTGAAATAATAGTTTAAGTAGTAATCGAAGACAAA
ACAAAATTTGAGTACAAAGTAGGAAAACAGACAGAATTTGGGAGAAAACCCAAATAAAGAGCTGTAAAAAAAACTCCTTGAATTGAATTTTTTAGA
TTTACTTGGATAATTAATTTTATTTTTAAAAAAAATATAAAGCATTCATCTTTAGTAATTAAAAATTCATATTAGTCGGGGGAAAAAGCTCGTCAT
TTCCAAATAAAGTTTGCCGAGGCTAATATTATCCCAAATATATGAACAGTCGGAATTTTACTTGTTACTTAGGGACAAGGCAAAAATATATTTATA
ATTATATATGCAAAGCAAAAGAGAAAGAATGGAGGTTTTGAGCTTTACTAAAGAGACAAAACTAGGAATAATCATGGCAGTTATTGAATCTTTGTC
AGAAAAACAATTATTTAGTTTAAGAAGACAGTGACGTTTTCAAATCTAAAAAATCTCAATCCCAACAACGCGCTATTTGTAGAAAAACCAAAAAGT
TTTCTCCTCCATGCACGCGTTTTAATCCACCGTGTTTTTTTTGTCATAAGTTTCTTGTCCGAAGAACTACAATTAAGAAAAAAAAAAACTGTTTTC
TTGGATTACTCGTTTTCATTGTACTAGTTTTTGCCATTTTATGAACTATTCAATGATCATCACGCGTTTGTTCAATTTCCAGTTCATTGTATCCTT
CGAAAATCCAGTTTTTCAAGTTTACTTGTCTGGTTTTTAAAGTCCAGTGATGTGTACAACAATTCAAGCAGTTGTACTATATATGTATTTATAT
ATAAACAGTAGTTATACAAGCATATTTTAATAATTTTTCAGTTGCTAGCTCTTTTCTTTTTGGGTTTTTCTTATTGCTTCAAATGTTTTAATTACA
ATATTTTATTGACTTTAGTTGCATTTCGTGTTTTGGGATTAGTGATGTTCTTTAATGAGTTTCCAGGTATCTTCTATGACTGAGTTTAGTTCTAGT
TATTAGTCAACTAAAGCAATCTATTTACTGCTAAGCATATCTATAAAGGGGACACCTGGAGAAGGTTTTCGCTACTGGGGTTTGAATATTTATGCT
CTCAAAGTGTTTGGGTTTTTACCTTCATTAAATCACTGTCAAGTTCCAACTTTCATTTTTCCC3′…

Promoter DNA sequences for GhSARD1-9D

5′…AGAGGTAAGTATAAATTGGTGGTATGTGTCCGCCTAGGTACTATGGGTGTTAGGTTTAAATTGGTGAAGTGTTGCCATCAGAAATTACGGCTG
ATGAATGGACATATTGTGTTTGTAAGACTAAGTCTTACGAAATTATTTATAATTCTCATGAGTACTATTGCTTGTGGATTATCAGTGTTCTTGTAT
TCGTTTTGTAGCTATTTGGAACTTACTAAGTTCAAATGAACTTACTTCATTACTTTTCTTTCTCAGGCATTTTGTTTTAGAAGAAGATCAGTAGAA
GGGGACTATGCTAGAGCTGCGTCTGCGAGTGAGCCATTTGCCTATTTTTGTATCATGCATGGCTATGTGACAGACAATGTATACATATGTTTTGTA
ATTAACCTTTACAATTAACTACGTCTTCTGTATCGTTTTATCTTAAAAGAGAATTGTTCCTTCTTTGAAAGTGTACGTTGAACCCTCTTGAATTAC
TAATCATAAATTTTATGATTTGAATATAAACGTCATAATTAAGTAGTAGTTGAAAATATACTTTTCTTTTATAATTTCTATTATTTTCTCTAAAGA
TGCGTTATCTACTGAAAAAAAAAAAGAGATTTTTATGCTAAGGAAAATCTTCATAATTATTTTTCCCAAATTTTTAAGTTAAGTTGAATGGTTTAA
ATCCATTAATAATGGCTTAGTTCGTCGGACAATCTATGTGGTTGATTGATACATGCCACGTCTACCCAACAATGAACATGTACTTGAAAATTTTTT
AAAGCCGGAACTCGGCCCGGTCACGTAGAAATGTACCGTAATCCCAAGCAAAAAAGGATGATTATAAAACTATAATCTAATTGCAAGCAGGACAGT
TTTACTTGAAGAATGACACAAATATAATGACTAAAATAATGCAAAAAAGAAAACCTTTGAATTATCAAAAAAGAGAAGAAAAGAGTTGATGACTAT
GGCAAGGGCCTACACAAAAGCAGACACTTCTAAGAAATGAGGCATTTGTGGACTATGTGATCTTTTCCCAAACTTTTTTCCCTCTTAAAAGTAGTC
TAAACATAATAATATTATTTTATATATCTCTATTCATTTGTCACAAACTTTTGCTTTTTTCTCTTTTTATCAACATAATCCAAGTTCTACTTGTAT
TAACCGTTGGATCAATATCTAAGGATTGGAGATGTCGTTTTAACTCTTTTTTTATATATCAATGTAATAATAAATAGAAATAGTTGACTTAAAAGCA
GAGTTTAGATGTCCAAAAGTCATTCATAACATGAGACAAATACCTTGGTTGCTCCGTGGCTGTTACTCAGTTGTTTCCACCAACATTCCAAGAACC
TTCTTTTTATTATTATTGACATGTATTTAAACTCCCCCCTTACAACATCGTTTTAAGACCATGACATATTCTCAAAATCCCTATCTTTTTCTTCAG
TTATGATCTGTTAGGGGTTTTTTTTGTGTGTGTGTGTGTGTTTTGGTCCTTGAATCTTTGGAA3′…

Promoter DNA sequences for GhSARD1-9A

5′…TTGAAAATGTAGGTCCGAGGTTAGCACAATGATAGGTCACAAGTAAACTAACATGATTATGAGAGGTGAGTATAAGTTGGTGGTATGTATCCA
CCTGGGTACTATGGGTGTTATGTTTAAAATGGTGAAGTGTTGCCGTCAGAAATTGCGGCTGATGAATCGATGTATTGTGTTTGTAAGACTAAGACT
TATGAAATTATCTGTAATTCTCATGAGTACTGTTGCTTGTAGATTATCTGTGTTCTTGTATTCGTTTTGTGGCTATTGGAACTTACTAAGTTCAAA
TGAACTTACTTCATTACTTTTCTTTCTCAAGCATTTTGTTCTAGAAAAAGATCACTAGAAGGGGACTACACCAGAAGTCCGTCTGCGAGTGAGCCA
TTTGTCTATTTTTGTATCATGCATGACTATGTGATGAACAATGTATACACATGTTTTCTAATTAACCTTTGCAATTAACTACGTCGTTTGTATCGT
TTTATCTTAAAAGAGATTTGCTCCTTTGAAGGTGTACACTGAACCCTCTTGAATTGCTAATCATAAATTTTATGATTTTGATATAACCGCAATAAC
TAGGCAGTAGTTGAAAATAGATTTTTCTTTTATAATTTTTATTATATTCTCTGAAAATGCGTTATCTAATGAAAGAAGAAAATTAGTTTTTACGCT
AAGGAAAATCTTCATAATTTTTTTTTCCAAATTTTTAAGTTAAATGGTTTGAATCCATTAATAATGCCTCAGTTTGTCAGGCAATCTATCTAGTTG
ATTGATACAACCCAACAATAAACATGTAGTTGAAAATTTTTTAAAACCAGAACTCGGCCGGTCACGTTGAAATGTACCGTAATCCCAAGCAAAAAA
GGATGATTATATAATTGCAAGCAGGACAGTTTTACTTGAAGAATGACACAAATATAATAACTAAAATAATGCAAAAAAAGAAAACCGTTGAATTAT
CAAAAAAGAGAAAAGAAAAGATTTGATGACTAAGGCAAGGGCCTACACAAAAGCAGACACTTCTAAGAAATGAGGCATTTGTGGACTTGGTGATCT
TTTCCCAAACTATTTTCCCTCTTAAAAGTAGTCTAAACAAAATAATATTATTTTATATATCTCTATTCATTTGTCACAAACCTTTGTTTTTTTTCT
TTTTATCAACATAATCCAAGTTCTACTTGTATTAACCGTTGGATCAACATCTAAGGGCATAGATATGATTGGAGATGTCGTTTTAACTCATTTTTA
TATATCAATGTAATAATAAATAGAAATAGTTGACTTAAAAGCAGAGTTAGATGTCCAAAAGTCATAACATGAGACAAATACCTTGGTTGCTCCGGG
GCTGTTACTCAGTTGTTTCCACCAACATTCCAAGAACCTTCTTTTTATTATTATTGACATGTATTTATATTCTCAAAATCCCTATCTTTTTCTTCA
GTTATGATCTGTTAGGTTTTTTTTTGTGTGTGTGTGTGTGTTTTGGTCCTTGAATCTTTGAAA3′…

Promoter DNA sequences for GhSARD1-12D

5′…AAATTCGTATTATATATATTTTATGCAAGAAAAATGAAATGATAAGTAAACTTGCATCTGACATATTGTATAATTTATCATATTTAGCTGATA
TGGCGAGTTCCTTTATTTGAAGAAATTTGATTTCTAAAGAATACAACCTTAAGAAGATCTCTACTACTATATCACATATTAAAAACCTGATCAGAG
CTGATTTTAAGCAGGTAAAGTTTTATATTGATGGTAGGGGTTTATTATTTTATGCATTATGTACAAGTTTAAAAGCATTTATCTTGTTTGTGCAAG
GAATTCTATTTTATGAATAGGAACGAGGTTGCAGCTTGATGATTATGTTAAATTTAAATTACGGATTGTACAAATAAAATTATAGTGAACTATTCT
CTAGATAAAATTCCGCAGATCTACAAAGTTTTTGTCCATCTCTATCTTGTACTATTTAGTTAGTTAGTTAGTTAGCCTAGTTGCAATTGAATATGC
ATCTAAAAGGCAAATTTAACAAGCTATTATGAGACACGACGGCTTTTTAACCTGACTTGTGGAGTTAAAAAACTCCACTAATGTATTAAATAACAA

GATTTATATTATTATTTTATTACACCCTAAAACCAAATGATATCATTCTAGACGATTAAAGTACATGGGAGGTCCCTATACTATAGGGACTAGATA
AAATTAGTCCCTCTATTATTAAATAGATTAGTTTAATCTCTATACTAATAAAAAGAATCAATTAAGTCCGAATTGAAATAAAATTATTCAATTGTA
ATTAAATTTTAAACAAAAAAAATTCATTTATAGAACAATAAAAATTTTAAACATATTAACTCTATTTAAATCTACCTTATTTGATTATGTTTAATA
ATACTTTTGATAATAGAATGATTAATTTAATCAGGCCCCTATAATAGAAGACCTCTCGGAAACATTCACCTATTCTAGATACATTTGTAAAAAATT
TTACTCCATCGAAAATATACAATATAATATTTGTTTTTATAAAATAACATAATTTAGAGAACTATTCGTTATTTATTAACATACATTTTTGAGTTT
AAATTAAAAAATTATAAATCAAATAAACTATCTGTTGAATCGAATTTTTATCTCGATTTAACCAGTTCAAAATTATTCTTTAATTCAAACTAATGT
ATCGTTTTATTCACGATTCCAACCTATTAAATTGACTTGGTATCTACTATCATGGAATTAACCCTAAATAATAAAAAACAAATGATTGACTCAAAG
GTTAGTCGACGTCAAAGTCATTGCATTATTTTATACACATATATTAATTTACATAGGTTAGTACGTGGCTATTACACTGCTGTTTCCTCTAACGTT
CCAAGAACCTTCTTTTTTTATTATTAATTTTTTTGAACTCTCCTGTATTTAAACTCCATTTTCGCCTTTACACCATTCTTAAACCCATTTCCTCTCC
TCTCTCTCTCCTTTTTGTTGGGTTTCAAATATCTGGGAATTAATAAATACTCAGCTGAAAATA3′…

Promoter DNA sequences for GhSARD1-12A

5′…CATATTTAATTGGTATCTTTTTATCGTAAAACTTTGGACTTCTTTTAAAAATTGTATGAAATTTGAAGAAATTTGATTTCTAAAGAATACAAC
CTTAAGAAGATCTCTATTACTATATCCCATATTGAAAACATGACCAAAGCTGATTTTAAGTAGGTAAAGTTTTATATTGACTGTATTTTTGTTAAG
CCAATACATGTCTAAGAGGGGTTTACTGTTTTATGCATTATGTACAAGTTCAAAAGCATTTATCTTGTTTATGGAAGGAATTTTATTTTACAAATA
GAAACGAAGGTTGCAACTTGATGAATATGTTGAATTTAAATCATGGATTGTACAAATAAAATTATAATAAACTATTCTTTAGATAAAATTCCACAC
ACCTACGAAGTTTTTGTCCATCTCTGTCTCTATCTTGTACTATTTTGTACTCAATCTCGTTGCAATTGAATATGCATCTAAAAGGCAAAATTAACA
AGCTATTATGAGACACGGTGACTTTTTAACCCGGCTTATAGAGATAAAAAACTCTACTAATGTATTTTAAATAACAAGATTTATTTTATTATTTTA
TTACACCCTAAAACCAAATGTTATCATTCTAGACTATTGTGCTTGAGAGGTCCCTATATTATAGGGACTAAATAAAATTAGTCCCTCTATTATTAA
ATAAATTAATTTAGTCTCTATATTATTAAAAAGAATCAATTAAGTCCAAGTTGAAATAGAGTTATTCAATTGTAGTTTAATATCAAACAAAATTAA
TTCATTTAAAGCTACCTTATTTGATTCTGTTTAATAGTACATGAACTAAATTATTTTGTTTAATAGTAGAATGATTAATTTAATCAAGTCCCTATA
ATAGAAGACCTCTCAGAGACATTCATCTATTTCTAAATACGTTTGTAAAATTTTTTACTCTACCAAAAATATACAATATAATAATTTTTTTATAAA
ATAATATAATTTAGAGAACAAAGAGGTTAAAACTATTCGTTATTTATTAACATGTAATTTAGAGTTTAAATTAAAAAAATTGTAAATTAAATAAAA
TTATTTGCTCAATCAAAATTTTTTATCTCAACTCGACTAGTTCAAAATAAAATTTTAATTTAAACTAATGTATCATTTATTCACGGTTCAATTGAT
TCAACCTATTAATTTAACTTGATATCGATATCAACCACCATGGAATTAACCCTAAATAATAAAAAGCAAATGATTGACTCAAAGGTTTGTTGACGT
CAAAGTCATTGCATTATTTTATACATATATTTTAATTTACATAGGTTAGTACGTGGCTATTACACTGCTGTTTCCTCTAACGTTCCAAGAACCTTC
TTTTTTTATTATTAATTTTTTTAAACTCTCCTGTATTTAAACTCCATTTTCGCCTTTACACCTTCTTAAACCCATTTCCTCTCATCTCTCTCTCTC
TCCCCCTCTCCTTTTTGTTGGGTTTCAAATACCTGGGAACTAACAAATACTCAGCTGAAAAAA3′…

1142