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CONDITIONAL UP-REGULATION OF CYTOKININ STATUS INCREASES GROWTH AND SURVIVAL OF SUGARCANE IN WATER-LIMITED CONDITIONS

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ABSTRACT

Water deficit (water stress) is a major cause of yield loss across all crops. It is the single largest crop productivity constraint in sugarcane in most of sugarcane producing countries and is attracting considerable research interest. Several biological factors have been implicated to water stress responses in plants. Hormones, particularly abscisic acid (ABA), ethylene, cytokinins (CK) and gibberellins are emerging as key regulators of water stress responses. Here, I explored the effect of artificial elevation of CK levels on the response of young sugarcane plants to water stress. Focus of the research was on the onset of senescence and change in photosynthesis that typify water stress responses of plants. Understanding the mechanistic basis of responses of sugarcane to water stress may allow developing strategies for crop improvement and breeding. I studied the effects of modifying cytokinin (CK) levels in water-stressed sugarcane (*Saccharum officinarum* L.) via two strategies; exogenous supply of synthetic cytokinin N6-benzyladenine (BA) and conditional or constitutional up-regulation of CK biosynthesis in 113 independent transgenic sugarcane lines that were generated as part of this thesis research. Young plants were exposed to water stress in the glasshouse by maintaining soil moisture at 50% field capacity via daily irrigation. Exogenous application of CK occurred via foliar spray or a root drench, while conditional up-regulation of endogenous CK levels under water stress conditions was achieved by expressing the gene encoding CK biosynthesis regulatory enzyme, 2-isopentenyltransferase (IPT), via senescence-associated (*SAG12*) or abscisic acid-responsive (*RAB17*) promoters. Maize Ubi promoter was used for constitutive expression of IPT transgene. A popular Australian sugarcane variety $Q208^{A}$ was used for this study. All promotors elevated endogenous CK levels under water stress conditions in the majority of the transgenic lines created. Responses to water stress was studied by quantifying biomass, root/shoot allocation, leaf area, photosynthesis parameters, hormone profiles and gene expression to discern the effects of impaired water relations on carbon fixation and the basis of CK-induced growth improvement. Increased CK levels, via exogenous supply of BA or stress-induced expression of IPT, strongly improved sugarcane growth and survival under water stress. On average, plants with elevated CK-levels retained 30-40% more chlorophyll and 40-50% more green leaf area than wild-type plants at the end of 50 days of growth under water deficit conditions. Compared with control plants, CK-supplied and IPT-transgenic plants maintained higher photosynthetic rates and stomatal conductance, and achieved greater biomass under water stress. External supply of CK significantly increased growth under water stressed and non-stressed

conditions in wild type plants compared to those that did not receive CK. Transgenic line RAB25 showed the greatest improvement in biomass production. Some transgenic lines, including RAB25, also showed significant growth improvement in well-watered condition. This is surprising as CK production in transgenic lines should occur only under stress conditions as the transgene is driven by senescence or abscisic acid-induced promoters. However, gene expression data indicated transgene activity in non-stressed conditions as RAB25 plants showed up-regulation of *IPT* transgene. This suggests that even in the well-watered treatment plants may have been experiencing intermittent water deficit during mid-day, triggering *IPT* expression. Greater availability of CK (obtained via exogenous supply or by CK production in response to water stress) significantly reduced stress-induced senescence and increased stomatal conductance which in turn facilitated higher CO_2 uptake and net photosynthesis, and increased biomass production. Taken together, this research demonstrates a key role for CK in integrating the environmental and intrinsic cues of sugarcane to adapt and grow under water stress conditions. This knowledge may offer opportunities for biotechnological approaches to crop improvement.

DECLARATION BY AUTHOR

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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CONTRIBUTIONS BY OTHERS TO THE THESIS

Dr Prakash Lakshmanan, Prof Susanne Schmidt, Dr Nicole Robinson and Dr Anthony O'Connell contributed to the conception, data presentation, discussion and editing of this thesis.

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Dr Chuong Nguyen Ngo provided assistance with the gene expression study and primer design at SRA.

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STATEMENT OF PARTS OF THE THESIS SUBMITTED TO QUALIFY FOR THE AWARD OF ANOTHER DEGREE

None

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KEYWORDS

sugarcane, water stress, cytokinin, isopentenyltransferase, plant responses, stomatal conductance, leaf senescence, photosynthesis, gene expression, hormonal regulation

AUSTRALIAN AND NEW ZEALAND STANDARD RESEARCH CLASSIFICATIONS (ANZSRC)

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LIST OF ABBREVIATIONS

2, 4-D	2,4 dichlorophenoxyacetic acid
ABA	Abscisic acid
ACC	1-aminocyclopropane-1-carboxylic acid
ACO	*ACC oxidase*
ACS	*ACC synthase*
ADF	*Actin depolymerizing factor*
BA	N6-Benzyladenine
bp	Base pair
BR	Brassinosteroids
CCI	Chlorophyll content index
cDNA	complementary deoxyribonucleic acid
CK	Cytokinin
CKX	Cytokinin oxidase
CO_2	Carbon Dioxide
cv	cultivar
cZ	*cis*-Zeatin
dH_2O	Distilled water
DHZ	Dihydrozeatin
DMAPP	Dimethylallyl diphosphate
DNA	Deoxyribonucleic acid
EST	Expressed sequence tag
FBPase	Fructose-1, 6-bisphospatase
FC	Field capacity
g	gram
GA	Gibberellin
GA20ox	*GA 20-oxidases*
GA2ox	*GA 2-oxidases*
GA3ox	*GA 3-oxidases*
gDNA	genomic deoxyribonucleic acid
g_s	Stomatol conductance
h	hour
ha	hectare
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
iP	isopentenyl adenine
iPR	isopentenyladenine ribotides
IPT	*isopentenyltransferases*
kb	kilo base pairs
L	litre
LER	Leaf extension rate
m	metre
M	molar
mg	milligram
min	minute
mL	millilitre

mm	millimetre
Mpa	megapascal
mRNA	messenger ribonucleic acid
n	number
NCED	nine-cis-epoxycarotenoid dioxygenase
ng	nanogram
nM	nanomoles
NTC	No template controls
ºC	Degrees Celcius
PCR	polymerase chain reaction
PEG	Polyethylene glycol
P_N	Photosynthetic rate
qRT-PCR	reverse transcriptase polymerase chain reaction
RAB17	*ABA-responsive gene 17*
RLD	Root length density
RNA	Ribonucleic acid
RSWC	Relative soil water content
RuBisCo	Ribulose Bisphosphate Carboxylase
s	second
SAG12	*Senescence associated gene 12*
SE	Standard error
TDZ	Thidiazuron
tZ	*trans*-Zeatin
Ubi	*Ubiquitin gene*
Vp14	*Viviparous14*
W_D	Dry weight
WS	Water stress
W_T	Target weight
WT	wild-type
WW	Well-watered
W_W	Wet weight
Z	Zeatin
ZR	*trans*-zeatin 9-riboside
ZRMP	*trans*-zeatin 9-riboside-5'monophosphate
µg	microgram
µL	microliter
µM	micromoles
µmol	micromoles
Ψ_{leaf}	Leaf water potential
Ψ_{soil}	Soil water potential

CHAPTER 1: GENERAL INTRODUCTION

Plants are a significant component of the economy of countries in the tropics and subtropics with ever-increasing need for food, energy and biomaterials (El Bassam, 2010, Kole et al., 2012). Sugarcane is the most important sugar crop and a major source of bioenergy. It is cultivated on 24 million hectares, approximately 0.5% of the global agricultural land area (FAOSTAT, 2013). Sugarcane is one of the most efficient crops converting solar energy into chemical energy harvestable as sucrose and biomass (Moore et al., 2014, Botha and Moore, 2014, Lakshmanan et al., 2005, Petrasovits et al., 2007). In addition to the long-standing focus on sugar, biomass has become an important target for the sugarcane industry to produce ethanol, electricity and high-value products (Waclawovsky et al., 2010).

In Australia 95% of sugarcane (~350,000 ha) is produced in Queensland, with nearly half the production located in rain-fed areas. Water is limiting production in rain-fed areas due to recurring drought (Yang et al., 2011). Although irrigation can minimise water deficit and drought (collectively referred to as 'water stress' hereafter), increasing regulation and cost of water and water delivery limit accessibility (Canegrowers, 2011). The value of lost production associated with water stress is estimated to be ≈ $260 million per annum in the Australian sugarcane industry (Inman-Bamber et al., 2012).

As one of the fastest-growing high biomass crops globally, sugarcane requires a large quantity of water to sustain growth. In response to water stress, sugarcane displays pronounced morphological and growth responses that include a reduction in stalk and leaf elongation, and accelerated leaf senescence. Together, these responses result in a decrease in biomass production and sucrose yield due to reduced rates of photosynthesis and crop growth as well as altered partitioning of assimilates between roots, leaves, structural stem material and stored sucrose (Singels et al., 2000, Singels and Inman-Bamber, 2002, Inman-Bamber, 2004, Singels et al., 2010).

With water availability becoming a significant driver of crop productivity, it is imperative to improve water use efficiency (WUE) and water stress tolerance of sugarcane, and considerable effort is now directed towards that objective in the Australian sugarcane industry as well as globally (Inman-Bamber et al., 2012). To develop water use efficient and drought tolerant sugarcane for

irrigated and dry-land farming, the Australian sugarcane industry initiated the project "*More Crop Per Drop*" in 2006 (Basnayake et al., 2012). The project aimed to advance understanding of the morphological and physiological responses of sugarcane under water-limited growth conditions to identify traits that improve crop productivity under well-watered and water-deficit conditions. To achieve this outcome, mechanistic understanding of the processes underlying water stress-induced growth reduction and other important phenotypic changes is fundamental (Skirycz et al., 2010, Lembke et al., 2012).

Hormones are key chemical messengers that are central to plant function and an integral component to the regulation of plant responses to water limitation, exerting pronounced physiological and growth effects (Pospisilova, 2003). Hormone biosynthesis and its regulation has been a research focus of plant water relations. From cellular and whole plant perspectives, water stress elicits local and systemic hormonal changes that involve biosynthetic and signaling components of hormones abscisic acid (ABA), ethylene, cytokinin (CK), auxin, gibberellins (GAs), jasmonic acid (JA) and brassinosteroids (BR) (Kohli et al., 2013). Currently considerable research effort is directed to the interactions of environmental and internal factors to understand plant adaptation to both biotic and abiotic stresses, including water stress (Inman-Bamber et al., 2012, Wilkinson et al., 2012).

Past experiments show that it is possible to regulate water stress responses in crops *via* manipulation of hormones. The work reported here is investigating the role of cytokinin on responses of sugarcane to water stress. Experiments were conducted to investigate the impact of exogenously supplied CK as well as modification of plant endogenous CK levels *via* transgenic manipulation of CK pathway. Both approaches have enabled improved water stress tolerance and are discussed in this thesis. To the best of our knowledge, our study is the first to investigate the relationship between CK and water stress in sugarcane.

1.1 Plant responses to water stress

Water stress is one of the major abiotic stresses limiting growth and development of crops globally, including sugarcane (da Silva et al., 2012). Water stress alters plant-water relations *via* stomatal activity, carbon fixation and other plant metabolism which result in changes in growth and development (Figure 1-1). These physiological traits are directly or indirectly associated with crop growth and yields (Silva et al., 2007, Inman-Bamber et al., 2012, Wilkinson et al., 2012). There are

reports that there is genetic variation for water stress responses and yield among sugarcane varieties (Basnayake et al., 2012). Information on drought response of new cultivars is generally obtained from commercial production experiences, and indeed only a few genotypes have been evaluated extensively for drought response (Inman-Bamber et al., 2005). The physiological responses to water stress at the whole plant level are highly complex and involve both deleterious and adaptive changes. This complexity is due to the diverse responses elicited by different plant species to different levels of water stress (Kramer and Boyer, 1995). The net effect of water deficit is largely determined by the dynamics, duration and intensity of soil water depletion, atmospheric water demand, other prevailing environmental stresses, as well as the stage of plant growth and phenology (Grant, 2012).

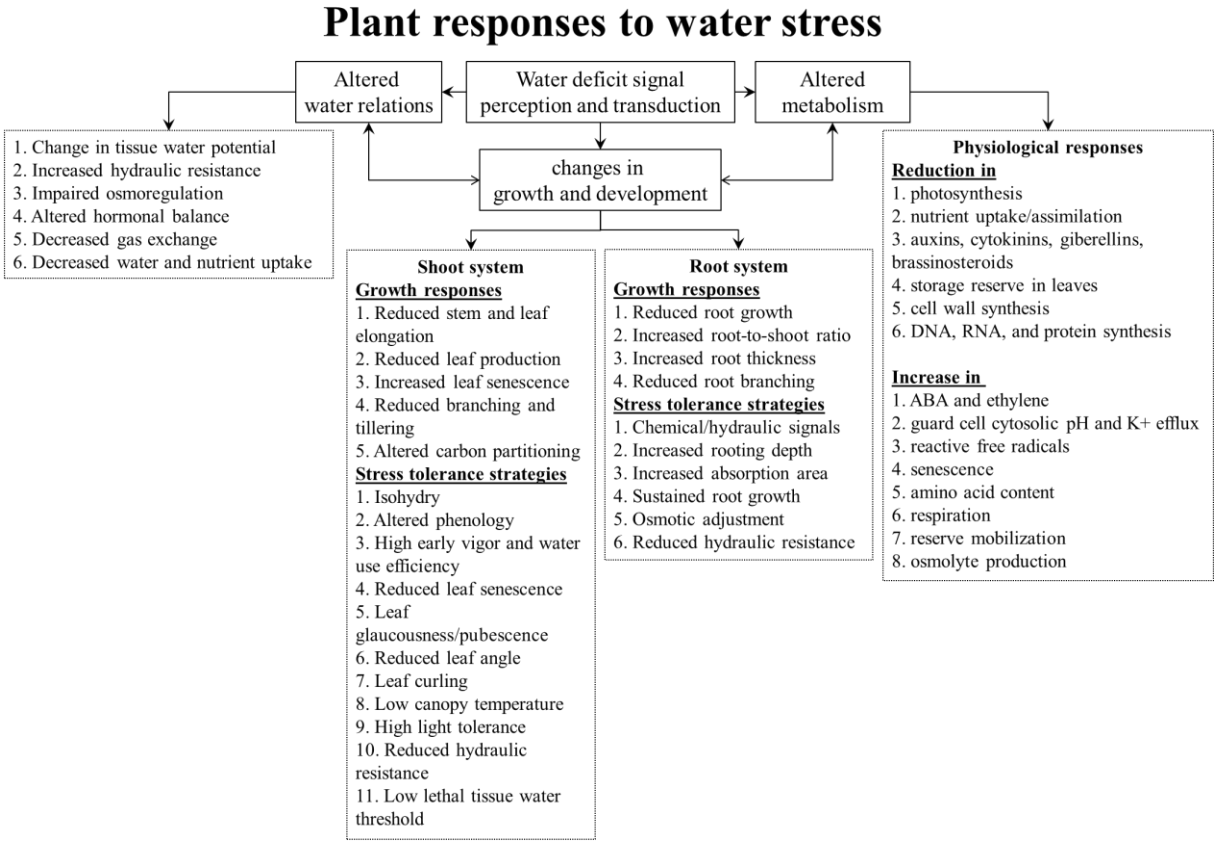

Figure 1-1: Schematic representation of plant responses and stress adaptation strategies. Note that the magnitude of these responses varies greatly between species and varieties within the same crop (Lakshmanan and Robinson, 2014).

1.1.1 Water stress and plant growth and development

Water stress is an important limiting factor during the initial phase of growth, especially crop establishment. It reduces cell division and cell expansion, resulting in substantial growth reduction (Hsiao, 1973, Chaves et al., 2003, Shao et al., 2008, Jaleel et al., 2009). Inhibition of stem and leaf development negatively affects plant height and leaf area (Table 1-1) and consequently reduces photosynthesis, transpiration (Shao et al., 2008) and crop productivity (Singels et al., 2010). Specific information on water stress effects on sugarcane growth and development are given below.

1.1.1.1 Water stress and sugarcane shoot growth

Sugarcane yield is largely determined by the amount of cane harvested and the sugar content of the harvested cane. Stalk (shoot) height, stalk thickness and stalk number are important yield components of sugarcane. Among these three variables stalk height is considered to be the most important yield determinant (Karno, 2007). During water stress, tissue moisture content decreases, causing cells to lose turgor pressure and inhibiting cell expansion (Hsiao, 1973, Nable et al., 1999, Van Volkenburgh, 1999, Jaleel et al., 2009, Arve et al., 2011, Kim and van Iersel, 2011). In sugarcane, the reduction in stalk elongation (Figure 1-2) is one of the first and most pronounced morphological responses to water stress. However, stalk elongation resumes upon resupply of water (Robertson et al., 1999). There is large genetic variation for response of stem elongation growth to water deficit in sugarcane germplasm (*More Crop Per Drop* BSS305 Project Final Report. Sugar Research and Development Corporation, Australia), and sugarcane stem elongation is extremely sensitive to water stress (Nable et al., 1999). Under severe stress with leaf water potential reaching -1.5 MPa stem elongation ceases, but photosynthesis continues, although at very low levels. This perhaps explains why sucrose accumulation continues even when sugarcane crops are severely water stressed (Inman-Bamber et al., 2008).

Figure 1-2: Sugarcane stems showing compressed internodes that are produced during water stress periods. Stem elongation recovery occurred following irrigation. Image from the "*More Crop per Drop*" field trial in North QLD (Home Hill, Burdekin).

1.1.1.2 Water stress and sugarcane root growth

The development of the root system depends strongly on environmental conditions and soil water content is particularly important. Insufficient soil moisture inhibits cell division in the root meristem and results in poor root development, and crop establishment (Lakshmanan and Robinson, 2014). Root growth of sugarcane was substantially inhibited at comparatively mild water deficit (Ψ_{soil} to -0.5 MPa) (Singh and Srivastava, 1974). At Ψ_{soil} -0.07 MPa, root hydraulic conductance was reduced by up to 80% (Saliendra and Meinzer, 1992). Da Silva et al. (2011) reported development of deeper root systems in sugarcane experiencing water deficit. A side-by-side comparative field study of whole root system development in rainfed and fully irrigated sugarcane showed that greater root length density (RLD) in deeper soil layers is more beneficial in water-limited conditions (Laclau and Laclau, 2009). Overall, there is little knowledge of root responses in sugarcane to water stress.

1.1.1.3 Water stress and sugarcane leaf area retention

Development of optimal leaf area is a pre-requisite for maximising carbon fixation and biomass production. However, the reduction of leaf area is an effective strategy for controlling growth aimed at conserving water (Anami et al., 2009). At limiting water supply, leaf area expansion decreases, largely due to a decrease in leaf area production (Inman-Bamber and De Jager, 1986, Singels et al., 2000, Inman-Bamber and Smith, 2005). Leaf area index is highly responsive to water deficit in sugarcane, and any water stress in the active growth stage will have major impact on crop yield (Robertson et al., 1999). In addition to the reduction of leaf area due to reduced expansive growth, accelerated leaf senescence contributes to the long-term adaptation to water deficit (Chaves et al., 2009). Daily leaf extension rate (LER) decreased during the early onset of water stress imposed on South African cultivars NCo376 and N11 in pot culture. LER was about 40 mm day^{-1} with a leaf water potential (Ψ_{leaf}) at midday of -0.5 MPa and reduced to near 0 mm day^{-1} at -1.3 MPa (Inman-Bamber and De Jager, 1986). Moreover, processes such as sprouting of sugarcane buds and subsequent growth were best with a soil water potential (Ψ_{soil}) close to zero, while bud and root growth ceased at Ψ_{soil} -2.0 MPa (Inman-Bamber and Smith, 2005).

Another mechanism that reduces leaf area is leaf rolling. Young sugarcane leaves roll and therefore reduce the projected leaf area and resultant radiation load. For example, the youngest unfurled leaves of cultivar NCo376 and N11 commenced rolling at midday Ψ_{leaf} < -1.0 MPa, and were tightly

rolled at Ψ_{leaf} < -2.0 MPa, resulting in a projected width of less than 20% of actual leaf width (Inman-Bamber and De Jager, 1986). The propensity for leaf rolling varies considerably amongst sugarcane genotypes, with rolling associated with drought tolerance (Inman-Bamber and Smith, 2005). Some sugarcane growers interpret leaf rolling as an indication of susceptibility to water stress, but the opposite may be the case as drought tolerant cultivars tend to avoid water stress rather than endure it (Inman-Bamber and Smith, 2005).

These examples illustrate that the plant's leaf area is an important adaptation to water stress, in the short term *via* leaf rolling or in the longer term *via* reduced leaf expansion and canopy development as well as early leaf senescence. Interestingly, Antwerpen (1999) reported that a good correlation exists between leaf area and root length, which suggests that leaf area is a potential trait for screening of drought tolerant cultivars.

1.1.2 Stomatal activity and plant water relations

One of the first responses of plants to water deficit is the preservation of water potential through stomatal closure (Taiz and Zeiger, 2010, Xoconostle-Cazares et al., 2010, Mackova et al., 2013). Stomata are formed by a pair of epidermal cells, called guard cells, which control the entry of CO_2 and loss of water vapor from leaf, which is measured as "stomatal conductance (g_s)". Stomatal conductance plays a critical role in plant adaptation to water-limited conditions. It has been suggested that stomata may respond to drought-induced changes in hydraulic resistance of xylem and the consequent decreased water and nutrient uptake (Cochard et al., 2002, Lakshmanan and Robinson, 2014).

Sugarcane is an isohydric plant (Meinzer and Grantz, 1990), which means that nearly constant leaf water potential is maintained over a wide range of environmental conditions and plant sizes (McDowell et al., 2008, Sade et al., 2009, Pinheiro and Chaves, 2011). Sugarcane is highly sensitive to water stress-induced root signals and controls the loss of water from the canopy by stomatal closure (Franks et al., 2007). Smith et al. (1999) found a 30% decrease in stomatal conductance (g_s) of sugarcane in response to soil drying which provides evidence for root control of g_s, possibly through chemical and hydraulic signals. Meinzer and Grantz (1990) were able to link g_s in sugarcane with increased ABA levels in leaf tissue under drought conditions. In sugarcane, similar to other plant species, ABA in the xylem stream could serve as root-derived signal controlling leaf gas exchange upon mild drought conditions (Meinzer and Grantz, 1990). Evidence

that ABA regulates plant water relations by stomatal closure, resulting in a lowering of water loss *via* transpiration and photosynthetic gas exchange (Schroeder et al., 2001, Hartung et al., 2002, Davies et al., 2005, Kim et al., 2010). Though such a strategy improves overall plant water relations, it comes with a significant trade-off of reduced photosynthesis caused by decreased CO_2 uptake and associated growth penalty (Haworth et al., 2011). However, leaf water use efficiency may improve, if transpiration is decreased to a greater extent than photosynthesis (Gago et al., 2014).

1.1.3 Water stress induces leaf senescence

Water stress-induced leaf senescence occurs gradually (Figure 1-3) and is characterised by leaf yellowing (i.e. chlorophyll degradation) due to changes in gene expression, metabolism (e.g. protein degradation, lipid peroxidation) and cell ultrastructure (e.g. chromatin condensation, thylakoid swelling, plastoglobuli accumulation), which result in a decrease in size of functional canopy (Munne-Bosch and Alegre, 2004, Rivero et al., 2007).

Figure 1-3: Leaf senescence in water stressed sugarcane in the early growth stage (right) and mature stage (left) of Home Hill field trial (Basnayake et al., 2012).

In water stressed plants, senescence of mature leaves contributes to nutrient re-mobilisation to growing parts of the plant (i.e. youngest leaves, flowers, and fruits) (Nishiyama et al., 2011). In addition, water stress-induced leaf senescence reduces leaf area and thus water loss, thereby contributing to the maintenance of a favorable water balance at whole plant level (Munne-Bosch and Alegre, 2004). However, from a crop production perspective, these responses negatively impact crop yield (Rivero et al., 2009). In field studies of sugarcane conducted in Australia, the reduction in green leaf retention was the most pronounced morphological response to water stressed

sugarcane throughout the annual growth cycle. Green leaf number was reduced by approximately 42% at harvest after a 12-month growth period (Table 1-1), demonstrating that green leaf retention is sensitive to water stress across all growth stages (Figure 1-3).

Table 1-1: Agronomic and yield observations of sugarcane grown in irrigated and water deficit conditions at Home Hill, Burdekin QLD in 2007-8 (Basnayake et al., 2012). The crop was harvested at 12 months after planting. Note: Commercial Cane Sugar (CCS), a measure of recoverable sugar in the cane.

Agronomic characters	Irrigated	Drought	Reduction (%)
No. of stalks/m row	16.8	16.4	2.4
Stalk length (cm)	348	287	17.7
Stalk diameter (cm)	2.5	2.4	4.0
No. of node stalk	24.1	20.8	13.7
No. of green leaf	9.5	5.5	42.5
Tons of cane per hectare (TCH)	91.3	68.6	24.8
Commercial cane sugar (CCS)	9.2	6.9	24.8
Tons of sugarcane cane per hectare (TSH)	8.7	5.2	39.8
Total dry mass (tons/ha)	24.7	16.5	33.3
Harvest index (yield/total biomass)	0.35	0.32	9.5

Considerable research has been directed to unravel the role of plant hormones on the regulation of water stress-induced leaf senescence in field conditions. Water stress affects synthesis and/or signaling pathways of hormones and triggers expression of stress-responsive genes, which in turn appear to affect leaf senescence (Shao et al., 2009, Ma et al., 2011). ABA (Wang et al., 2008, Gan, 2010), ethylene (Grbic and Bleecker, 1995), jasmonates (He et al., 2002), and salicylic acid (Morris et al., 2000) promote leaf senescence, whereas CK (Rubia et al., 2014), gibberellins (Mutui et al., 2006) and auxins (Mueller-Roeber and Balazadeh, 2014) delay senescence. Furthermore, carbohydrate accumulation and high carbon-to-low nitrogen ratios are other factors that regulate leaf senescence (Wingler et al., 2006).

1.1.4 Water stress and photosynthesis

Photosynthesis occurs in chloroplasts contained in the cells of green leaves and other green tissues. This process is directly dependent on the supply of water, carbon dioxide, and light. Limiting any one of these factors (as well as soil-derived nutrients and temperature) generally reduces the rates of photosynthesis (Tezara et al., 1999, Chaves et al., 2009, Taiz and Zeiger, 2010). Photosynthesis of the whole canopy is lowered with reduced g_s and green leaf area. With stomatal closure during water stress, the flux of CO_2 into the leaf is lowered and photosynthesis declines (Meinzer and

Grantz, 1990, Calvet, 2000, Lawlor and Cornic, 2002, Chernyad'ev, 2005, Flexas et al., 2006). Besides the low flux of CO_2 due to stomatal closure, water stress-induced reduction in photosynthesis may be caused by reduced biochemical activity in the mesophyll and bundle sheath cells. Under conditions of water stress, a decrease in Ribulose bisphosphate carboxylase (RuBisCo) activity is well documented and evident in all plants studied so far (Chernyad'ev, 2005, Yan et al., 2012).

In sugarcane under severe water stress, the reduction in photosynthesis is associated with reduced activity of phosphoenolpyruvate carboxylase (PEPCase), RuBisCo, malic enzyme (NADP-ME), fructose-1, 6-bisphospatase (FBPase) and pyruvate orthophosphate dikinase (PPDK). Despite photosynthesis being the key process underpinning sugar production, our knowledge of the impact of water stress on the biochemical network of photosynthesis is very limited (Saliendra et al., 1996, Du et al., 1998, Sage et al., 2013).

1.2 Plant hormones in responses to water stress

The involvement of hormones in plant water relations is an active area of research (Kohli et al., 2013). Many physiological, metabolic and developmental processes that ultimately impact crop performance and yield are controlled by hormones (Wilkinson et al., 2012, Kohli et al., 2013). Hormone response under water deficit is triggered by signals derived from roots or shoots in response to changing root or shoot environments (Wilkinson and Davies, 2002, Hartung et al., 2005, Alvarez et al., 2008). The main plant hormones up-regulated in response to water stress are abscisic acid (ABA) and ethylene, while others, notably gibberellins (GAs), auxin and cytokinin (CK) are down-regulated.

1.2.1 Abscisic acid (ABA)

ABA is a primary endogenous signal to initiate adaptive responses in plants challenged by water stress. Endogenous ABA levels in leaves increase rapidly and induce rapid stomatal closure when plants are subjected to water stress as well as other abiotic stresses (Kudoyarova et al., 2007, Ehlert et al., 2009, Wilkinson and Davies, 2010). ABA is also recognised for its role in modulating leaf and canopy expansion under water deficit conditions (Bower and Birch, 1992, Wilkinson and Davies, 2002). In a study by Tardieu et al. (2010), ABA has mainly three effects on growth: (i)

ABA tends to buffer the day-night alternations of leaf growth rate and the negative effect of evaporative demand by regulating stomatal movement and transpiration rate, (ii) ABA may improve leaf growth by improving by tissue and whole plant hydraulic conductivity and (iii) it may have a modest non-hydraulic growth promotive effect when supplied externally in well watered plants that are deficient in ABA (Tardieu et al., 2010). However, externally applied ABA may not have any growth effect at all depending on the plant species (Tardieu et al., 2010).

ABA is synthesised from an oxidative cleavage of the epoxy-carotenoids 9-cis neoxanthin and 9-cis violaxanthin, to produce xanthoxin that is subsequently converted to ABA. The epoxy-carotenoid cleavage is the first committed step in the pathway, and is believed to be the key regulatory step in the ABA biosynthetic pathway (Xiong and Zhu, 2003, Nambara and Marion-Poll, 2005). In maize, the cleavage is catalysed by a specific novel epoxy-carotenoid dioxygenase, *Viviparous14*, *Vp14* (Tan et al., 1997). A family of *Vp14-related* genes controls the first committed step of ABA biosynthesis. Maize *Vp14* is localized in plastids including chloroplasts (Tan et al., 2001). In maize, *Vp14* is highly stress-induced and accounts for roughly 35% of stress-induced ABA synthesis in leaves; it is also strongly expressed in roots and embryos (Tan et al., 1997). These genes are likely to play a key role in the developmental and environmental control of ABA synthesis in plants (Tan et al., 1997, Tan et al., 2001, Schwartz et al., 2003, Nambara and Marion-Poll, 2005). ABA catabolism is largely categorized into two types of reactions, hydroxylation and conjugation. The hydroxylation at C-8 position is commonly thought to be the predominant ABA catabolic pathway (Nambara and Marion-Poll, 2005). When dehydrated plants are subsequently rehydrated, a concomitant increase in the hydroxylated catabolites and the ABA level decreases are observed in *Arabidopsis* (Kushiro et al., 2004).

1.2.2 Ethylene

Ethylene regulates many aspects of growth and development, particularly under abiotic stresses. When up-regulated under stress-inducing growth conditions such as drought, ethylene accelerates leaf senescence and abscission (Costa et al., 2005, Christensen and Feldmann, 2007). Also there is evidence that water stress-induced increase in ethylene causes inhibition of root growth and development, and, like ABA, reduces leaf expansion (Pierik et al., 2006, Zhang et al., 2011). There is evidence that ethylene causes yield reduction in seed crops under abiotic stresses, and that the

prevention of ethylene production or perception is a desirable genetic or management target to improve stress tolerance (Wilkinson et al., 2012).

Ethylene is a gaseous hormone that is synthesised in almost all plant tissues in the presence of oxygen (Lin et al., 2010). Methionine is the starting point in the ethylene biosynthetic pathway (Figure 1-4), and it is converted into S-adenosylmethionine (SAM) by methionine adenosyltransferases (Yang and Hoffman, 1984). ACC synthase (ACS) catalyses SAM into 1-aminocyclopropane-1-carboxylic acid (ACC), the first committed step of ethylene biosynthesis. ACC is then converted by ACC oxidase (ACO) into ethylene with ACC oxidase activity largely considered as constitutive in plants (Yang and Hoffman, 1984).

In plants, ACC synthase and ACC oxidase are considered as the rate-limiting steps for ethylene biosynthesis which are largely induced by abiotic/biotic stresses (e.g. drought, wounding, pathogen) (Yang and Hoffman, 1984) and during plant development (e.g. senescence, ripening) (Zhang et al., 2009). A study in sugarcane demonstrated that ACC oxidase was strongly induced by treatment with auxin, cytokinin, and ethylene-forming compound called ethephon and cold stress (Wang et al., 2006). A study in maize (Habben et al., 2014) indicated that down-regulation of *ACC synthases* gene had significantly decreased ethylene and increased grain yield after a period of drought stress. In tomato, ABA induced the expression of both *ACS* and *ACO* genes, and promoted ethylene synthesis, fruit ripening and senescence (Zhang et al., 2009). In addition, ethylene is catabolised to CO_2, ethylene oxide, and ethylene glycol and its glycosylates. However, ethylene can be released from the plant as free ethylene (Botha et al., 2014).

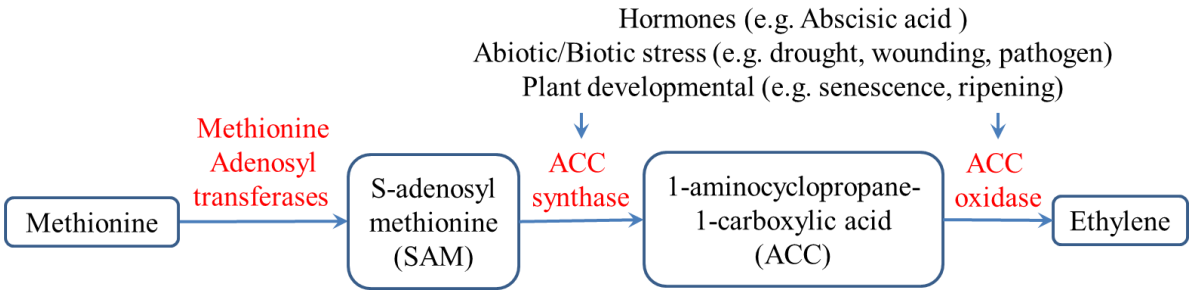

Figure 1-4: Ethylene biosynthetic and catabolic pathways in plants.

1.2.3 Gibberellins (GAs)

Gibberellins (GAs) are involved in several important biochemical and morphogenetic responses which include elongation of stem, leaves, and reproductive organs (Colebrook et al., 2014). Compared to ABA and ethylene, relatively little is known about the effect of water deficit on GA metabolism. Water deficit was shown to reduce GA content in maize leaves (Wang et al., 2008, Colebrook et al., 2014). Deficiencies in bioactive GA levels or signaling causes reduction in plant growth, particularly elongation growth (Peng et al., 1999, Spielmeyer et al., 2002, Sarkar et al., 2004, Colebrook et al., 2014), while GA overdose can cause excessive plant growth and increased sterility (Fleet and Sun, 2005). Most evidence points to the genes encoding *GA 20-oxidases*, *GA 3-oxidase* and *GA 2-oxidases* as the main sites of regulation of the GA biosynthetic pathway by developmental and environmental signals (Yamaguchi, 2008).

Gibberellin metabolism pathway is controlled by enzymes belonging to small multigenic families, with each member having a specific pattern of expression (Figure 1-5). In *Arabidopsis*, GA biosynthesis is tightly regulated through the modulation of the expression of members of two gene families encoding GA 20-oxidases (GA20ox) and GA 3-oxidases (GA3ox) that catalyse consecutive reactions that convert GA intermediates to the bioactive forms (Chiang et al., 1995, Phillips et al., 1995). Expression of *GA20ox* and *GA3ox* genes occurs in growing plant organs, which suggests bioactive GA synthesis occurs at their site of action (Kaneko et al., 2003). Through a series of oxidation steps, these enzymes lead to the formation of the major bioactive GAs, including GA_1, GA_3, GA_4, and GA_7 (Yamaguchi, 2008).

Homeostasis of GA also depends on GA deactivation pathways. Hence, the major route known to catalyse the conversion of bioactive GAs to inactive catabolites is the 2β-hydroxylation, catalysed by the GA 2-oxidases (GA2ox). Overexpression of the *GA2ox* gene in transgenic plants leads to bioactive GA-deficiency and various levels of dwarfism (Thomas et al., 1999, Sakamoto et al., 2001, Loucas and Clark, 2003, Schomburg et al., 2003, Rieu et al., 2008, Weston et al., 2008, O'Neill et al., 2010).

Gibberellin action and resultant plant growth are regulated by a family of proteins called DELLA proteins (Zentella et al., 2007, Achard and Genschik, 2009). They are strong repressors of several GA responses and characterized by the conserved DELLA domain which mediates the susceptibility of the protein to proteolytic degradation (Claeys et al., 2014). In response to

environmental and developmental inductions, this repression is relieved through the production of biologically active GAs, which rapidly promotes DELLA degradation (Figure 1-5). DELLA contributes to growth inhibition by various abiotic and biotic stresses (Colebrook et al., 2014). DELLAs are relatively stable, but when bioactive GAs levels are high, DELLA proteins are modified by attaching ubiquitin molecules and rapidly degraded by the 26S proteasome (Achard and Genschik, 2009, Claeys et al., 2012). Thus, DELLA protein abundance is inversely related to the amount of bioactive GAs.

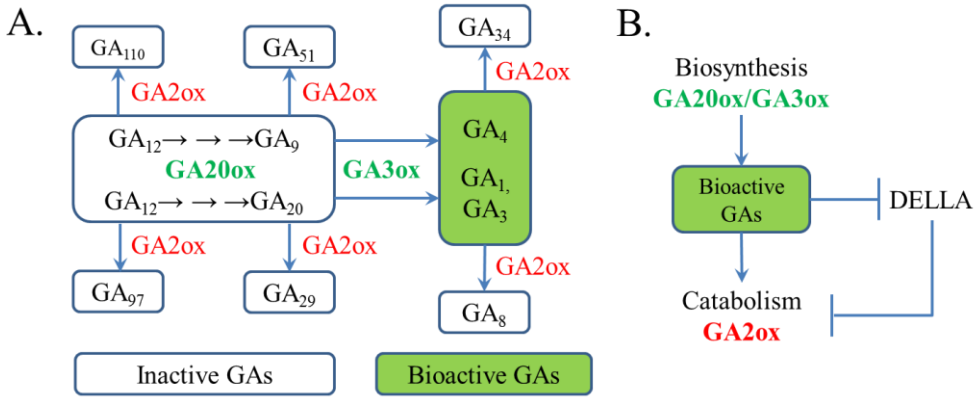

Figure 1-5: Gibberellin biosynthetic and catabolic pathways in plants. (A) Levels of biologically active GAs are under the control of GA biosynthetic enzymes (GA20ox [GA 20-oxidases] and GA3ox [GA 3-oxidases]) and GA deactivating enzymes (GA2ox [GA 2-oxidases]). (B) Expression levels of GA biosynthesis and catabolism genes (Achard and Genschik, 2009).

1.2.4 Auxin

Auxin regulates embryo development, stem cell maintenance, root and shoot architecture, and tropic growth responses (Davies, 2010a) and reaction to environmental changes (Benkova et al., 2003). The effect is stronger if GAs is also present. Auxin also stimulates cell division if CK are present. For example, when auxin and CK are applied to callus, rooting can be generated if the auxin concentration is higher than CK concentration. Moreover, xylem tissues can be generated when auxin and CK concentration is equal (Benkova et al., 2003, Woodward and Bartel, 2005).

At the molecular level, all auxins are compounds with an aromatic ring and a carboxylic acid group. The four naturally occurring (endogenous) auxins are indole-3-acetic acid (IAA), 4-chloroindole-3-acetic acid (4-CI-IAA), phenyl acetic acid (PAA) and indole-3-butyric acid (IBA); only these four were found to be synthesised by plants (Simon and Petrášek, 2011). The most important member of the auxin family is IAA (Woodward and Bartel, 2005). IAA generates the majority of auxin effects in intact plants, and is the most potent native auxin (Woodward and Bartel, 2005, Peer et al., 2011).

However, all conjugated or bound auxins found in higher plants are considered hormonally inactive and thus conjugation serves as a mechanism for inactivating or removing free IAA (Ludwig-Muller, 2011).

1.2.5 Cytokinin (CK)

Cytokinin homeostasis and CK signaling are rapidly altered under water-deficit conditions. CK content and transport are reduced by drought stress, either through reduced biosynthesis or by accelerated degradation (Shani et al., 2006, Kudoyarova et al., 2007, Barazesh and McSteen, 2008, Argueso et al., 2009, Nishiyama et al., 2011). It has been proposed that a reduction in CK amplifies leaf responses such as increased ABA synthesis and stomatal closure during stress (Alvarez et al., 2008, Schachtman and Goodger, 2008, Kravtsov et al., 2011, Wilkinson et al., 2012).

Moreover, alterations in the rate of export of CK and ABA from roots in drying soil generate a root-to-shoot signal that causes growth inhibition through various processes including reduction of meristem size and activity (Shani et al., 2006, Barazesh and McSteen, 2008, Spichal, 2012). There is evidence that changes in CK content in xylem sap modify water use in pea during water stress (Dodd et al., 2004, Dodd, 2003).

Cytokinins play an important role in controlling senescence. CK counteracts ABA and ethylene as exogenous application of CK delays senescence (Lara et al., 2004). Studies on CK and primary metabolism indicate a direct link between CK-induced delay of senescence and a phloem-unloading pathway (Lara et al., 2004, Hwang et al., 2012).

The physiological basis of altered CK homeostasis under water stress remains largely unclear. However, it is known that CK are antagonistic to ABA responses, especially stomata closure, senescence, and inhibition of photosynthesis. The focus of this thesis is the role that CK play in the responses of sugarcane to water stress. Hence, current knowledge of CK, with focus on water relations and water stress responses, are briefly outlined below.

1.3 Cytokinin (CK), plant growth regulator

1.3.1 Chemical structure of cytokinin

Cytokinins have a chemical structure similar to adenine, promote cell division and have functions similar to kinetin (Kakimoto, 2001, Amasino, 2005, Sakakibara, 2006, Sakakibara, 2010, Spichal, 2012). Kinetin (Kin) was the first CK discovered and so named because it promotes cytokinesis (Miller et al., 1955). The most common form of naturally occurring CK in plants is zeatin (Sakakibara, 2006), abundant in monocot species (Ha et al., 2012) and first isolated from corn (*Zea mays*) (Miller, 1961). Zeatin represents the major CK transported in the xylem (Davey and Vanstaden, 1976, Hirose et al., 2008). However, more recent studies indicate that CK metabolism itself can influence the CK status in leaf tissue under drying conditions (Neuman et al., 1990, Kudoyarova et al., 2007). It is therefore important to consider that CK concentrations in leaves depend on the balance between CK input from xylem sap delivery and putative CK synthesis in leaves, and CK loss *via* catabolism and phloem export.

CK concentrations are highest in meristematic regions and areas of continuous growth such as roots, young leaves, developing fruits, and seeds (Shani et al., 2006, Taiz and Zeiger, 2010). It has been suggested that CK functions as regulatory signal of local and long-distance coordination of plant development *via* the vascular system (Hirose et al., 2008, Kudo et al., 2010) although this has not been conclusively demonstrated.

Based on the side chain configuration, naturally occurring CK are classified as either isoprenoid or aromatic forms (Kakimoto, 2001, Amasino, 2005, Sakakibara, 2006, Spichal, 2012). Isoprenoid type CK is the most abundant class of CK found in nature and includes isopentenyl adenine (iP), containing an isopentenyl at the N6-side chain, or zeatin-type CK, containing a hydroxylated isopentenyl at the N6-side chain which occurs in either cis or trans pattern, depending on which of the two methyl groups is hydroxylated. The trans-form is usually more active in plants. Reduction of the double bond in the zeatin side chain leads to dihydrozeatin (DHZ).

Aromatic-type CK have an aromatic benzyl group at N6, are less abundant, and fewer are known. However, aromatic CK is often used in plant tissue culture, for example 6-benzyladenine (BA). In addition, CK can be present in plants as ribosides, *trans*-zeatin 9-riboside (ZR) which has a ribose

sugar attached to the N9 of the adenine base structure, or ribotides, *trans*-zeatin 9-riboside-5'monophosphate (ZRMP) with a ribose sugar side chain that contains a phosphate group.

Numerous chemical compounds have been synthesised and tested for CK activity of which almost all are N6-substituted aminopurines. However, there are some synthetic CK compounds that elicit similar responses as CK although they differ in structure, one of which is thidiazuron (TDZ) (Sakakibara, 2006, Spichal, 2012).

1.3.2 Cytokinin biosynthesis

CK are present and synthesised in roots and shoots and can be translocated *via* the xylem (Spichal, 2012). CK biosynthesis occurs through biochemical modification of dimethylallyl diphosphate (DMAPP) as shown below (Figure 1-6). (1) The first step is the transfer of an isopentenyl moiety from dimethylallyl diphosphate (DMAPP) to the N6 position of ATP/ADP, catalysed by isopentenyl transferases (*IPT*) (Kakimoto, 2001, Argueso et al., 2009). The direct products of the *IPT* reaction are (2) isopentenyladenine ribotides (iPR), the isoprene side chain of which is subsequently trans-hydroxylated by the specific cytochrome P450 monooxygenases (CYP735A) to yield (3) zeatin ribotides (ZRDP, ZRTP and ZRMP). Cytokinin nucleotides can be converted to their most active free base forms *via* (4) dephosphorylation to form zeatin 9-riboside (ZR) and then (5) deribosylation to form Zeatin (Z) (Sakakibara, 2006, Taiz and Zeiger, 2010, Sakakibara, 2010).

1.4 Cytokinin application for enhanced drought tolerance

Several reports have demonstrated that manipulation of CK production and action can be an effective strategy for the development of drought tolerant crops. The discussion centres on CK as a key hormone involved in the regulation of growth and development during water stress. The level of active CK in particular cell types is the summation of the *de novo* biosynthesis/degradation and transport into those cells. Thus, optimal level of bioactive CK could promote plant growth and development or greater tolerance to water stress (Pospisilova et al., 2005, Nishiyama et al., 2011, Reguera et al., 2013, Rubia et al., 2014). Application of *trans*-zeatin (Z), *trans*-zeatin riboside (ZR), 6-benzyladenine (BA) and other CK to the leaf epidermis can reverse ABA-induced stomatal closure and retard senescence (Pospíšilová, 2003). The three approaches used to study the interactive effects of CK and water stress on plant growth and development are (i) analysis of CK

mutants, (ii) external application of exogenous CK, and (iii) manipulation of endogenous CK production by transgenesis.

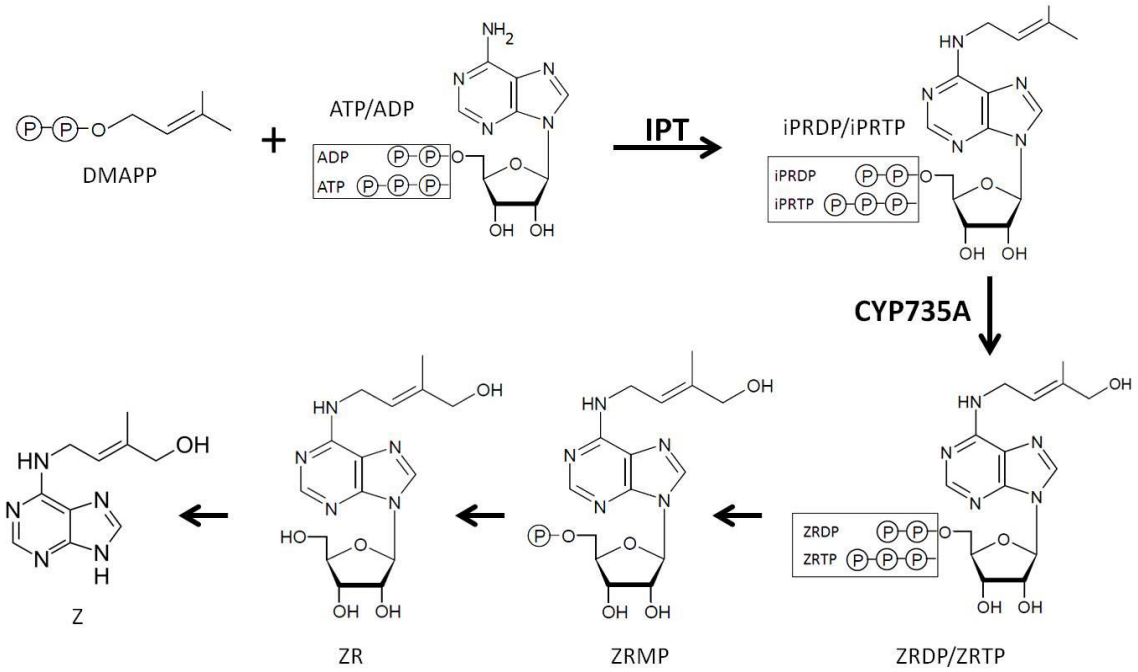

Figure 1-6: Cytokinin biosynthetic and catabolic pathways in plants.

1.4.1 Analysis of cytokinin mutants

Functional analysis of *Arabidopsis thaliana isopentenyltransferase* (*AtIPT*) mutants indicated that ATP/ADP *IPTs* are responsible for the synthesis of isopentenyladenine (iP)- and *trans*-zeatin (tZ)-type CK, whereas transfer RNA *IPTs* are required for the biosynthesis of *cis*-zeatin (cZ)-type CK (Miyawaki et al., 2006). Analysis of mutants showed that irreversible CK degradation is catalysed by cytokinin oxidase (CKX), which selectively cleaves unsaturated isoprenoid side chains, resulting in the formation of adenine/adenosine and the corresponding side chain aldehyde (Sakakibara, 2006, Werner et al., 2006, Werner et al., 2010, Bartrina et al., 2011, Nishiyama et al., 2011). Clearly, these enzymes are important players in regulating CK concentrations and thereby influencing plant growth and development.

Kang et al. (2012) showed that pre-treating *Arabidopsis* wild-type plants and *Arabidopsis histidine kinases* (ahk) single mutants with CK enhanced tolerance to dehydration and freezing (Jeon et al., 2010). Tran et al. (2007) reported a similar observation with the *Arabidopsis cre1* (cytokinin response 1) mutant exhibiting a salt-tolerant phenotype in the presence of exogenous CK. Sergiev et al. (2007) used 1-(4-chlorophenyl)-3-(pyridin-2ylmethyl)urea (2PU-3) and 3-benzyl-7-(4-

methylpiperazin-1-yl)-3H-[1,2,3]triazolo [4,5-d]pyrimidine (TP-5) to enhanced cytokinin degradation and significantly diminished the CK-induced delay of chlorophyll degradation in *Arabidopsis* wild-type plants. Dodd et al. (2004), using pea (*Pisum sativum* L.) mutants with decreased xylem-CK concentration and transpiration rate, suggested that changes in xylem-supplied CK modify plant water use.

1.4.2 Exogenous application of cytokinin

In many crop species, the application of exogenous CK increases stomatal aperture and transpiration rate (Pospisilova et al., 2005, Nishiyama et al., 2011) indicating that CK content and leaf gas exchange are functionally connected (Reeves et al., 2007). Moreover, the application of CK leads to an accumulation of chlorophyll and promotes the conversion of etioplasts to chloroplasts (Davies, 2010a) which is important for maintaining photosynthetically active green leaf area. Applications, concentrations and reported effects of exogenous CK are summarised in Table 1-2. Another method of increasing endogenous CK may be through the inoculation of plants with CK-producing bacteria that release CK in concentrations within the physiological range of plants (Arkhipova et al., 2005). Treatment of plants with CK-producing bacteria resulted in an increase in CK content in lettuce and wheat accompanied by faster growth in well-watered and water deficit conditions (Arkhipova et al., 2006). The responses of stomata to exogenous CK are species-specific and depend on CK concentration (Pospisilova, 2003, Wachowicz et al., 2006, Can, 2009).

1.4.3 Endogenous modification of cytokinin biosynthesis *via* transgenesis

Genetic engineering provides a new approach to study the regulatory roles of CK which has advantages over traditional methods as it can directly control CK metabolism and action at the gene level (Merewitz et al., 2011a). For many years, studies have explored transgenic plants carrying the CK biosynthetic transgene, *isopentenyltransferase* (*IPT*) gene from *Agrobacterium tumefaciens* (Blackwell and Horgan, 1993, Jordi et al., 2000, Miyawaki et al., 2004). *IPT* is the key regulatory enzyme of the *de novo* biosynthesis pathway of CK in higher plants (Barry et al., 1984, Kakimoto, 2001, Takei et al., 2001).

To manipulate CK biosynthesis by genetic modification, the *IPT* gene has been fused to various promoters and introduced into plants to elevate levels of endogenous CK. The strategies and promoters used to direct the expression of *IPT* in transgenic plants are summarised in Table 1-3.

The choice of promoter is largely dependent on the purpose of the study. However, CK content and composition were dependent not only on promoters but also on plant species and age (Pospisilova et al., 2000).

Several studies have reported that enhanced CK content delays senescence, increases chlorophyll content and enhances photosynthetic capacity in transgenic plants expressing the *IPT* gene, using a senescence-inducible promoter from the senescence-associated gene (*SAG12*). Species tested in this respect include *Arabidopsis* (Medford et al., 1989, Zhang et al., 2000), lettuce (McCabe et al., 2001), tobacco (Rivero et al., 2007, Rivero et al., 2009), petunia (Clark et al., 2004), tall fescue (Hu et al., 2005), and creeping bentgrass (Xu et al., 2009, Merewitz et al., 2010, Merewitz et al., 2011d). Other stress-inducible genes have been successfully developed for control of *IPT* transcription, such as light inducible promoters (Synkova et al., 1999), heat shock inducible promoters (Ghanem et al., 2011), chalcone synthase promoter (Wang et al., 1997) and others.

However, constitutive over-expression of *IPT* results in detrimental pleiotropic effects such as decreased root growth, altered flowering time, and poor tissue-water relations (Wilkinson et al., 2012). In transgenic tobacco and *Arabidopsis*, root-specific promoters resulted in over-production of CK oxidase, enhanced CK degradation, and increased root growth and plant survival under drought conditions (Werner et al., 2010). Transgenic hydroponically-grown tomato plants grafted to rootstocks constitutively expressing *IPT* had decreased root biomass under non-stress conditions but under salinity-stress conditions the transgenic plants yielded 30% more than the wild-type plants highlighting that benefits from over-expression of *IPT* during water stress conditions may be accompanied by traits with negative effects on plant development and crop performance in field environment (Ghanem et al., 2011). The recent study of Merewitz et al. (2010) demonstrated that the controlled modification of CK levels *via* a drought-inducible and senescence-inducible promoter (P_{SAG12}) in creeping bentgrass increased total root biomass, root length, and root to shoot ratio, where roots of transgenic plants had higher levels of bioactive CK. The improved root-to-shoot ratio is possibly a reflection of the combination of better hormonal balance and alleviation of stress effects arising from stress-induced up-regulation of CK production and the inherent plant response of increased root-to-shoot ratio under water deficit.

Table 1-2: External application of cytokinin, concentrations and reported effects on plants grown under water stress and non-stress conditions.

Application	Conc.	Frequency	Species	Reported effects	Reference
Detached shoot or leaf, incubated with CK solution	22 µM	Once	Barley (*Hordeum vulgare*)	Increased chlorophyll content	(Kravtsov et al., 2011)
	100 µM	Once	*Arabidopsis thaliana*	Delayed leaf senescence	(Sergiev et al., 2007)
	2.2 µM	Once	Apple (*Malus* × *domestica* Borkh.)	Changes in the chlorophyll content	(Dobránszki and Mendler-Drienyovszki, 2014)
	1 mM	Once	Banana (*Musa acuminata*)	Increased chlorophyll content	(Aghofack-Nguemezi and Manka'abiengwa, 2012)
	100 µM	Once	Broccoli (*Brassica oleracea* L.)	Delayed senescence	(Costa et al., 2005, Xu et al., 2012)
BA supply to roots *via* watering or spraying on leaves	100 µM	Once	Bean (*Phaseolus vulgari*)	Increased photosynthetic rate	(Metwally et al., 1997)
	100 µM	Once	*Arabidopsis thaliana*	Induced ARR transcription	(Taniguchi et al., 1998)
	1 and 100 µM	Once	Cucumber (*Cucumis sativus*)	Increased stomatal conductance	(Can, 2009)
	35.5 µM	Every 3 days	Ryegrass (*Lolium multifolorum*)	Enhances the above ground productivity	(Wang et al., 2012)
	25 µM	Every 15 days	Kentucky bluegrass (*Poa pratensis L.*)	Enhanced growth and physiological recovery of plants from drought stress	(Hu et al., 2012)
	1 to 100 µM	Once	sugar maple (*Acer saccharum*)	Increased stomatal conductance	(Reeves et al., 2007)
	25 µM	Once	*Arabidopsis thaliana*	Enhancing the germination	(Srivastava et al., 2007)
	66 µM	Once	*Arabidopsis thaliana*	Regulated de-etiolation	(Luo et al., 1998)
	5 and 10 µM	Once	Bean (*Phaseolus vulgaris L.*) Sugar beet (*Bata vulgaris* L.)	Increased stomatal conductance and photosynthetic rate	(Pospisilova et al., 2001)
	10 µM	Every 2 Days	Eggplants (*Solanum melongena*)	Increased chlorophyll content and stomatal conductance	(Wu et al., 2014)
	10 µM	Once	Kentucky bluegrass (*Poa pratensis L.*)	Increased stomatal conductance and photosynthetic rate	(Hu et al., 2013)
	10 µM	Once	*Arabidopsis thaliana*	Inhibit ABA, increased stomatal conductance	(Tanaka et al., 2006)
Added into medium *in vitro*	10 µM	Once	Pineapples (*Ananas comosus* L. Merr.)	Increased shoot growth	(Al-Saif et al., 2011)

Table 1-3: Strategies and promoters used to manipulate the *isopentenyltransferase* (*IPT*) expression in transgenic plants under stress and non-stress conditions.

Promoters	Plant species	Results	References
P_{SAG12}, senescence associated promoter	Creeping bentgrass (*Agrostis stolonifera*)	Increased photosynthesis rate, chlorophyll content, and water use efficiency (WUE)	(Merewitz et al., 2011a)
	Creeping bentgrass (*Agrostis stolonifera*)	Stimulated tiller formation and root production, and delayed leaf senescence	(Xu et al., 2009)
	Cassava (*Manihot esculenta*)	Increased chlorophyll content	(Zhang et al., 2010)
	Wheat (*Triticum aestivum*)	Increased chlorophyll content	(Sykorova et al., 2008)
	Tomato (*Lycopersicon esculentum*)	Delayed leaf senescence and increased fruit weight and fruit total soluble solids (TSS)	(Swartzberg et al., 2006)
	Tobacco (*Nicotiana tabacum* cv SR1)	Delayed leaf senescence	(Lara et al., 2004)
	Arabidopsis thaliana	Delayed leaf senescence	(Zhang et al., 2000)
	Tobacco (*Nicotiana tabacum*)	Delayed leaf senescence	(Gan and Amasino, 1995, Jordi et al., 2000)
	Petunia (*Petunia* x hybrida)	Delayed leaf senescence	(Clark et al., 2004)
	Lettuce (*Lactuca sativa L.* cv Evola)	Delayed leaf senescence	(McCabe et al., 2001)
P_{SSU}, light- inducible promoter from pea	Tobacco (*Nicotiana tabacum*)	Increased CK and chlorophyll content	(Synkova et al., 1999)
	Tobacco (*Nicotiana tabacum* cv. SR1)	Increased CK content when grown in low light	(Thomas et al., 1995)
	Tobacco (*Nicotiana tabacum*)	Increased photosynthetic rate, chlorophyll and carotenoid contents	(Pospisilova et al., 1998)
	Tobacco (*Nicotiana tabacum*)	Increased stomatal conductance	(Synkova et al., 1999)
P_{HSP70}, heat shock inducible promoter from maize	Tomato (*Solanum lycopersicum*)	Delayed leaf senescence and stomatal closure, and increased shoot growth	(Ghanem et al., 2011)
	Tobacco (*Nicotiana tabacum*) *Arabidopsis thaliana*	Increased CK levels	(Medford et al., 1989)
P_{HS6871}, heat shock inducible promoter from soybean	Tobacco (*Nicotiana tabacum*)	Delayed leaf senescence	(Smart et al., 1991)
$P_{HSP18.2}$, a heat shock promoter	Creeping bentgrass (*Agrostis stolonifera*)	Increased chlorophyll content, leaf relative water content, and root:shoot ratio	(Merewitz et al., 2010)
P_{SAUR}, auxin-inducible bidirectional promoter from soybean	Tobacco (*Nicotiana tabacum*)	Inducing stunting, loss of apical dominance, reduction in root initiation and growth, and delayed leaf senescence	(Li et al., 1992)
P_{CHS}, chalcone synthase promoter	Tobacco (*Nicotiana tabacum*)	Increased transpiration rates and stomatal conductance	(Wang et al., 1997)
P_{SARK}, senescence associated receptor protein kinase promoter	Rice (*Oryza sativa*)	Improved drought tolerance and increased yield	(Peleg et al., 2011)
	Peanut (*Arachis hypogaea* L.)	Improved drought tolerance and increased yield	(Qin et al., 2011)
	Cotton (*Gossypium hirsutum*)	Delayed senescence, increased biomass, increased chlorophyll content and photosynthetic rates	(Kuppu et al., 2013)

1.5 Thesis objective

Worldwide nearly 75% of sugarcane is grown in rain-fed conditions. Due to its year-long crop cycle rain-fed crops often experience long periods of moderate to severe water deficit. Such protracted stress periods cause substantial reduction in photosynthesis, green leaf area and yield. Inhibition of shoot growth, loss of green leaf area and photosynthesis are the most pronounced water stress-induced morphological effects observed in sugarcane (Inman-Bamber and Smith, 2005, Lakshmanan and Robinson, 2014). Sugarcane growth is very sensitive to water stress (Nable et al., 1999) and little is known about hormonal regulation of sugarcane growth and development, especially in relation to water availability.

As indicated earlier, ABA and ethylene play a significant role in plant responses to water stress (Kohli et al., 2013) and ABA and ethylene actions could be reversed by CK (Peleg and Blumwald, 2011, Ha et al., 2012, Wilkinson et al., 2012). Hence, this project investigates the effect of CK, the hormone directly involved in the regulation of leaf senescence (Hwang et al., 2012, Gan and Amasino, 1995) and photosynthesis (Chernyad'ev, 2009), on growth of sugarcane under water deficit. More specifically, it was aimed to determine whether water stress-induced loss of photosynthetic area, photosynthesis and growth inhibition in sugarcane can be reversed by up-regulation of CK production (Figure 1-7). It was hypothesised that controlled up-regulation of CK may lead to improved CO_2 fixation and growth under drought conditions by 1) increasing stomatal conductance, possibly by negating ABA action, to increase CO_2 uptake and fixation, and 2) delaying leaf senescence and accompanying improved photosynthetic activity. It is therefore likely that plants with higher CK levels in stress conditions show increased growth and yield under mild to moderate stress conditions.

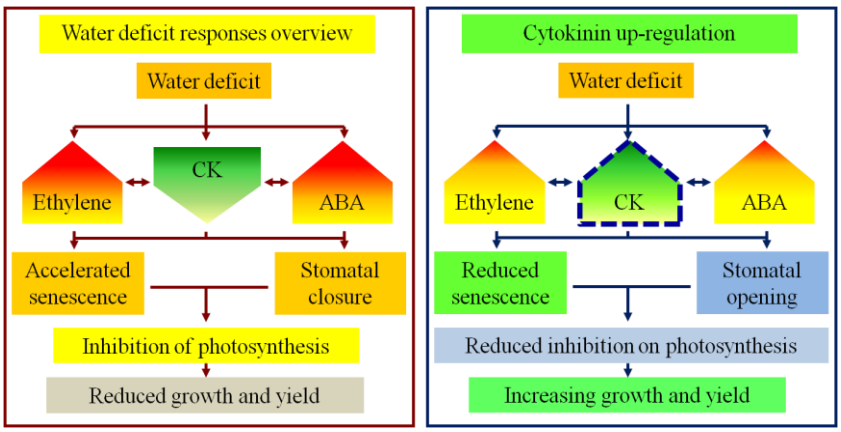

Figure 1-7: Conceptual framework for testing the role cytokinin in water stressed sugarcane.

In this thesis, two experimental approaches were used for modifying CK content, (1) exogenous application of CK, and (2) introduction of the *IPT* transgene into sugarcane under the control of stress-induced promoters. Both approaches aimed to test whether stomatal opening and leaf senescence in sugarcane is modulated in the presence of higher levels of CK. Hence, this research project investigated empirically:

1. The responses of sugarcane to exogenously supplied CK on stomatal function, leaf senescence, and biomass production.
2. The development of transgenic sugarcane with conditional up-regulation of CK production using *IPT* transgene, limiting CK production to conditions of water stress and onset of senescence.
3. Study the physiological and morphological responses of *IPT* transgenic and non-transgenic plants to exogenous CK supplied during water stress conditions.
4. Examine the hormonal and molecular changes in *IPT* transgenic and non-transgenic sugarcane under water stress conditions.

This thesis contains 6 chapters. Chapter 1 contains introduction and literature review. Chapter 2 presents the results of exogenous application of CK to sugarcane. Chapter 3 details the production, screening and selection of *IPT* transgenic sugarcane lines. Chapter 4 contains the results of experiments on the physiological responses of *IPT* transgenic and non-transgenic sugarcane supplied with CK externally. Chapter 5 presents changes in hormone levels in responses to water stressed conditions in *IPT* transgenic and non-transgenic sugarcane. Finally, Chapter 6 concludes the thesis with a general discussion.

CHAPTER 2: EFFECT OF EXOGENOUS CYTOKININ APPLICATION ON SUGARCANE PLANTS GROWN UNDER WATER STRESS

2.1 Introduction

Studies on phytohormones capable of enhancing water stress tolerance in crop species are important because phytohormones play a role in the perception and transduction of environmental and endogenous signals that modulate growth and development. Also, phytohormones are involved in root-to-shoot communication in response to abiotic and biotic stimuli (Wilkinson et al., 2012, Kohli et al., 2013). Consequently, changes to the hormonal balance play a key role in the sequence of events induced by stress. Cytokinins (CKs), naturally occurring plant hormones that promote cell division, are essential for normal plant growth and development (Howell et al., 2003, Mok and Mok, 2001), and control plant adaptation to abiotic and biotic stresses (Nishiyama et al., 2011, Ha et al., 2012). N6-benzyladenine (BA), a first-generation synthetic CK, is a good candidate for exogenous application because it is (i) considerably more stable than naturally occurring CK (Letham and Palni, 1983), (ii) readily taken up by the plant (Vogelmann et al., 1984, Cedzich et al., 2008), and (iii) not (or only slightly) degraded by CK oxidase (Sakakibara, 2010, Davies, 2010f).

Recent studies have demonstrated that exogenous treatment with BA (see Table 1-2 for more detail) is effective in delaying chlorophyll degradation in *in vitro* apple shoot cultures (Dobránszki and Mendler-Drienyovszki, 2014), eggplant (Wu et al., 2014), barley (Kravtsov et al., 2011), broccoli (Costa et al., 2005, Xu et al., 2012), and banana (Aghofack-Nguemezi and Manka'abiengwa, 2012). A number of studies also reported that exogenous BA application increases stomatal conductance (g_s) and improves photosynthesis in bean (Pospisilova et al., 2001), pineapple (Tanaka et al. 2006) Kentucky bluegrass (Hu et al., 2013), bean (Metwally et al., 1997), cucumber (Can, 2009) and sugar maple (Reeves et al., 2007). BA application is also reported to enhance plant growth under water stress conditions in pineapple (Al-Saif et al., 2011), *Arabidopsis* (Srivastava et al. 2007) Kentucky bluegrass (Hu et al. 2012), and ryegrass (Wang et al., 2012). These examples highlight that BA has beneficial effects on diverse plant taxa.

Conversely, high concentrations of CK can inhibit cell proliferation and induce programmed cell death in carrot (*Daucus carota* L.) and *Arabidopsis thaliana* (L.) cell cultures [13 and 27 µM, respectively] (Carimi et al., 2004). Treating plants with CK reduces apical dominance and promotes the release of axillary buds which results increased branching of shoots, and inhibition of root formation (Johnston and Jeffcoat, 1977). This effect is exploited in tissue culture of sugarcane for commercial-scale propagation (Khan et al., 2008, Ali et al., 2008). To the best of our knowledge, there is no reported literature on the effects of CK application in glasshouse or field-grown sugarcane.

The aims of the investigation here were to 1) determine the effects of CK application on sugarcane's physiological responses under stress conditions, and 2) determine the effect of root *versus* foliar application of BA in sugarcane. Three experiments were conducted to achieve these objectives. They are (i) detached leaf senescence assay, (ii) use of different concentrations of BA on growth of sugarcane plants and (iii) root *versus* foliar application of BA.

2.2 Methods and materials

2.2.1 Effects of 6-benzyladenine on senescence of detached leaves of sugarcane

2.2.1.1 Plant material and growth conditions

Sugarcane (*Saccharum officinarum L.* var. Q208^{A}) plantlets were produced using apical shoot meristem culture on Murashige and Skoog (MS) media supplemented with 2 µM of 6-benzyladenine (BA; *Phyto*Technology Laboratories, LLC™) under sterile conditions at 24±1 ˚C in 16 h light (250-300 µmol m^{-2} s^{-1}). Sugarcane shoot clumps produced were separated into single shoots and rooted on MS solid medium at the same growth conditions as above. Flag leaves of well-developed *in vitro* plantlets were collected under sterile condition and leaf segments (~2-3 cm long) were placed on a Whatman filter paper (Qualitative Circles 90 mm, GE Healthcare Australia Pty. Ltd.), moistened with 10 mL of the incubation solution (see Table 2-1) in sterile Petri dishes (90 mm × 25 mm). The sealed containers were maintained in a culture room (24±1˚C; 16 h light 250-300 µmol m^{-2} s^{-1}). All tissue isolation and setting up of leaf assays were performed in sterile conditions.

2.2.1.2 Osmotic stress and preparation of 6-benzyladenine treatment solutions

In the 'detached leaf experiment', an osmotic potential of -1.5 MPa was created by dissolving 250 g of polyethylene glycol (PEG 15,000 g mol-1) in 1 L of water (Steuter et al., 1981). Three different treatment solutions (0 µM BA, 5 µM BA and 10 µM BA) were tested for their ability to delay chlorophyll degradation in detached leaves of sugarcane. BA stock solutions were prepared by dissolving BA powder (m.w. 225.25, *Phyto*Technology Laboratories, LLC™) in 100 mM KOH and made up to the required volume with distilled water .

2.2.1.3 Experimental design and measurements

The experiment consisted of six incubation solutions containing 0, 5 and 10 µM BA with and without PEG added to induce osmotic stress (see Table 2-1). There were four biological replicates. Detached leaves were maintained in Petri dishes at 24±1 ˚C in 16 h light (250-300 µmol m^{-2} s^{-1}) for 14 days. Leaf greenness was observed visually and photographed at day 14 of incubation.

Table 2-1: Detached leaf experiment testing the effect of 6-benzyladenine treatments in non-stress and stress applications

Incubated solution (treatments, T)	T1	T2	T3	T4	T5	T6
BA concentrations (µM)	0	5	10	0	5	10
Stress applications	Without PEG (non-stress)			With PEG (stress)		

2.2.2 Effects of 6-benzyladenine on sugarcane growth and development

2.2.2.1 Plant material and growth conditions

To investigate the effects of BA concentration on whole plants, experiments were conducted in a naturally lit glasshouse (University of Queensland, Long Pocket, Australia) from December 2011 to April 2012. Sugarcane (*Saccharum officinarum L.* var. QC91-580) plantlets were produced in tissue culture (see section 2.2.1.1) and transplanted into potting trays (5 × 6 wells) in December 2011. Plantlets were acclimatised in potting trays in a growth room (24±1 ˚C, 16 h light at 250-300 µmol m^{-2} s^{-1}) for 4 days before being transported to the glasshouse. Twenty-eight-day-old plantlets were transplanted into 1.3 L pots (Garden city plastic, Australia) with sand and perlite (50:50v/v) for 28 days before applying the stress treatment. Nutrient solution (Thrive Soluble All Purpose, Yates

Australia) was applied once a week through irrigation. The transplanted seedlings were grown in a greenhouse at 30-35 ˚C for 2 weeks and uniform seedlings were chosen for the study.

2.2.2.2 Water stress condition

The water holding capacity (field capacity, FC) of the potting mix was estimated according to the method described by Earl (2003). To calculate 100% FC of potting mix, oven-dried potting mix was used for "dry weight" value (W_D). It was saturated with water and allowed to drain until a constant weight was reached ("wet weight" W_W). Field capacity was determined gravimetrically. Water stress levels were adjusted by adding the amount of water lost to return pots to the desired % FC ("target weight" (W_T) of water stressed pots. Plants in the water stress treatment were maintained at 40% FC while well-watered plants were watered daily to achieve 100% FC. Pots were watered daily according to the weight loss of each pot to create water stress.

2.2.2.3 6-Benzyladenine treatments

During the experiment, at the end of every 48-h cycle 25 mL of 10, 50, 100 or 200 µM BA solution was supplied (between 08.00 am – 10.00 am) to the roots of well-watered and water stressed plants *via* irrigation.

2.2.2.4 Measurement of physiological and morphological parameters

Chlorophyll content index (CCI), and arbitrary reading of greenness estimated by the CCM-200 plus chlorophyll meter (Opti-Sciences, Inc., USA), was measured in the middle portion of the youngest fully expanded leaves. The CCI values were measured once a week after water stress treatment commenced.

Stomatal conductance (g_s) was measured at the middle portion of the youngest fully expanded leaves using a handheld Leaf Porometer (Model SC-1, Decagon Devices, Inc.) once a week between 10.00 am and 14.00 pm.

Plant elongation was calculated from the differences between plant height on the first day of experiment and at the end of the experiment. Plant height was measured from surface soil level to the top visible dewlap. Tiller number and green leaf area data were collected at the end of

experiment. Green leaf area was measured using a Portable Leaf Area Meter (LI-3000C and LI-3050C, LI-COR Biosciences, Inc., USA). Dry mass was determined after oven-drying the tissue at 60 °C to a constant weight. Total plant aerial biomass was the sum of main stem, tiller stem, main leaves, and tiller leaves.

2.2.2.5 Experimental design and statistical analysis

The experiment was arranged in a split-plot design with five replications. Plants were grown under well-watered and water stress condition and they were treated with 0 to 200 µM BA (see Table 2-2). Analysis of variance (ANOVA) was used to determine if the means of the treatments were significantly different. When significant differences were noted, the Tukey HSD comparison method was used to test if differences were significant at $P<0.05$. Means of five plants are presented. Statistical analyses were conducted using Statistix 10 software (Analytical Software. Tallahassee, USA).

Table 2-2: Treatment conditions for dose-response effects of 6-benzyladenine concentrations.

Treatments (T)	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10
BA concentrations (µM)	0	10	50	100	200	0	10	50	100	200
Water conditions	Well-watered					Water stress				

2.2.3 Effects of 6-benzyladenine application methods (leaf and root) on sugarcane plants

2.2.3.1 Plant material and growth conditions

Sugarcane (*Saccharum officinarum L.* var. QC91-580) plantlets were produced and were subjected to water stress treatment as described above (section 2.2.2). This experiment was conducted at a naturally lit glasshouse (see above) from September to December 2012.

2.2.3.2 6-Benzyladenine application method

BA solutions were supplied through root *via* irrigation or spraying on leaf with 25 mL of 100 µM BA in both well-watered and water stress conditions every 24 h. Equal quantity of BA was supplied using both methods. For non-BA treatment, 25 mL of water was supplied or sprayed instead.

2.2.3.3 Measurement of physiological and morphological parameters

See section 2.2.2.4.

2.2.3.4 Experimental design and statistical analysis

The experiment was carried out in a randomised complete design with three replications. There were eight treatments (Table 2-3). Each treatment consisted of three pots with one plant in each pot. Analysis of variance (ANOVA) was used to determine if the means of the treatments were significantly different. 2-way ANOVA was used to examine application method and water regime interaction. When significant differences were noted, the Tukey HSD comparison method was used to determine homologous groups. Means of three plants are presented. Statistical analyses were conducted using Statistix 10 software (Analytical Software. Tallahassee, USA).

Table 2-3: Treatments to compare the effect of 6-benzyladenine application methods (leaf and root) in well-watered and water stress growth conditions.

Treatments (T)	T1	T2	T3	T4	T5	T6	T7	T8
Application methods	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
BA concentrations (µM)	0		100		0		100	
Water conditions	Well-watered				Water stress			

2.3 Results

2.3.1 Effect of 6-benzyladenine treatments on senescence of detached leaves of sugarcane

After 14 days of incubation, BA treatment showed distinct effects on the greenness of leaves in the control treatment without added PEG (Figure 2-1 T1-T3). With osmotic stress, all leaves senesced progressively, as evidenced by leaf yellowing (Figure 2-1 T4-T6). This result shows that application of BA strongly inhibits the process of leaf senescence only in the non-stress conditions.

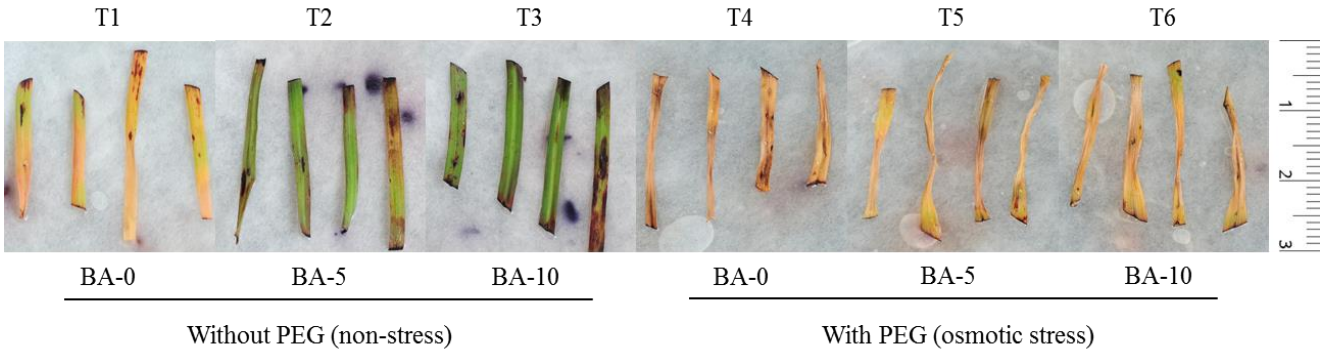

Figure 2-1: Effects of 6-benzyladenine treatments (0, 5 and 10 µM) on senescence in detached leaves of sugarcane incubated with and without osmotic stress. T1-T3 were kept in non-stressed conditions while T4-T6 were stressed by being treated with PEG to induce a low osmotic potential (-1.5 MPa). The bar on the right has a length 30 mm.

2.3.2 Effects of 6-benzyladenine on sugarcane growth and development

2.3.2.1 Chlorophyll content index (CCI)

The average CCI value was ~25 in well-watered plants and did not differ with BA concentrations (Figure 2-2). Significant interaction between dose and water regime on CCI was observed in week 4 (See appendix 2-1). After 4 weeks of induced water stress conditions, CCI value of all treatments significantly ($P<0.05$) decreased by 64% in non-BA plants, 60% in 10 µM BA plants, and ~40% in higher BA concentration treatments (Figure 2-2). Water stressed BA-treated plants (except 10 µM BA treated plants) had significantly higher CCI than non-BA plants in week 3 (40% higher in 50 µM BA-treated plants and ~55% higher in 100 and 200 µM BA-treated plants; Figure 2-2). In week 4, CCI was ~35-40% greater ($P<0.05$) in plants receiving 50 to 200 µM BA, compared to control and 10 µM BA-treated plants under water stress conditions (Figure 2.2).

2.3.2.2 Stomatal conductance (g_s)

Under well-watered conditions, stomatal conductance (g_s) was similar across treatments until week 3 of the experiment. From week 4, all BA treated plants, except those received 10 µM BA, had significantly ($P<0.05$) greater g_s (~30%) than non-BA treated plants. The effects of dose and water regime on stomatal conductance significantly interacted after week 3 of the experiment for the remaining sampling times of the experiment (See appendix 2-1). Under water stress conditions, g_s at week 4 relative to week 0 was significantly ($P<0.05$) reduced. For example, gs in non-BA control

plants was reduced by 73%, but only by ~60% in plants treated with higher BA concentrations (Figure 2-3). In week 4, only plants treated with 100 µM BA had significantly ($P<0.05$) higher g_s than non-BA treated plants (41% higher; Figure 2-3).

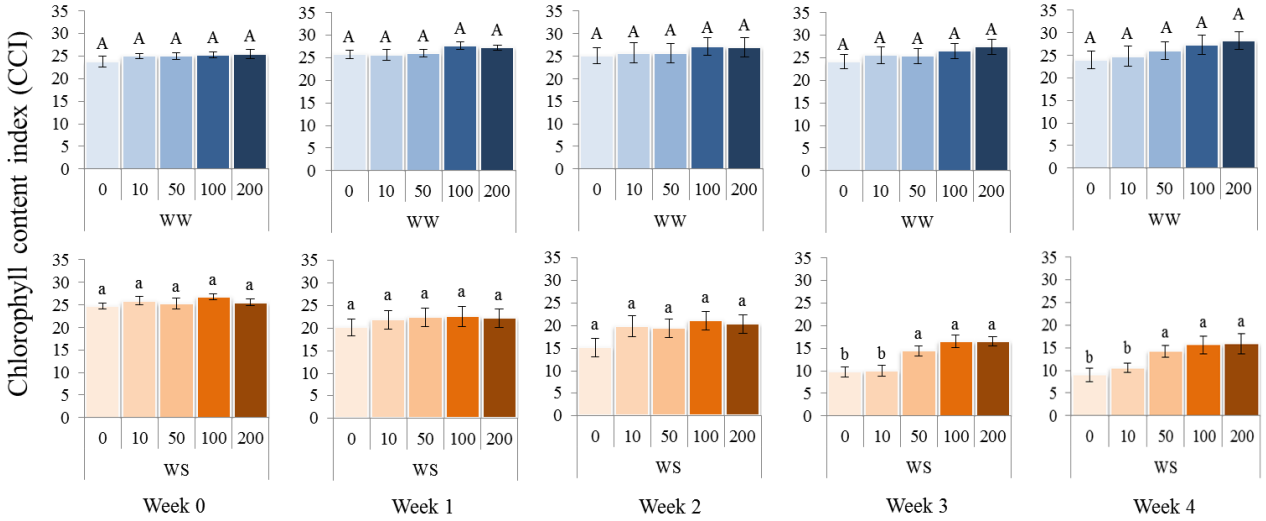

Figure 2-2: Effect of 6-benzyladenine on chlorophyll content index (CCI) of sugarcane grown under well-watered (WW, top row) and water stress (WS, bottom row) conditions. Youngest fully expanded leaves were measured once a week. X-axis values represent concentrations of 0, 10, 50, 100, and 200 µM BA applied to plants. Error bars indicate standard error (±SE). Different letters above bars indicate significantly ($P<0.05$) different mean values within the water regime treatment (Tukey HSD test, n = 5).

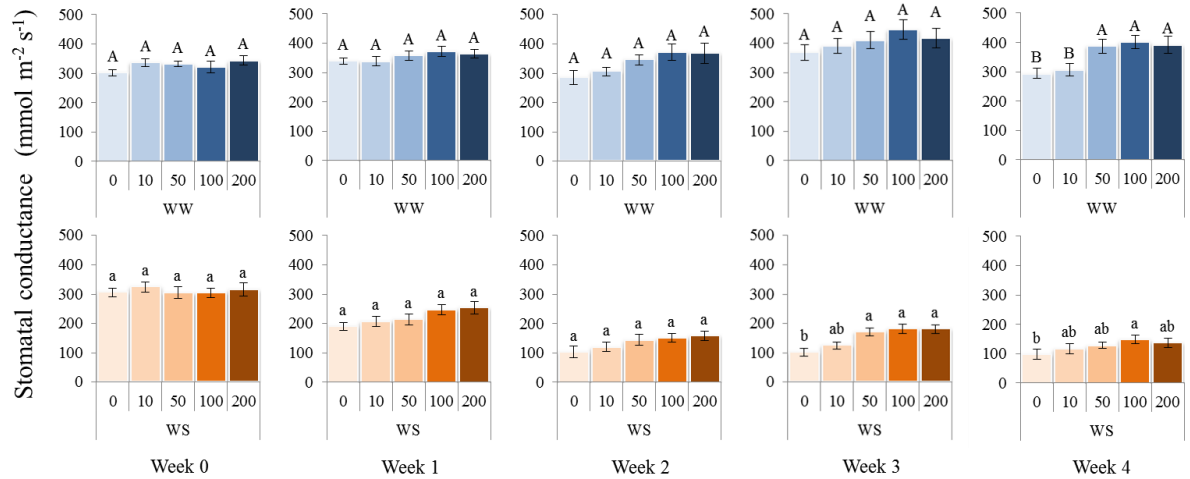

Figure 2-3: Effect of 6-benzyladenine on stomatal conductance of sugarcane grown under well-watered (WW, top row) and water stress (WS, bottom row) conditions. Youngest fully expanded leaves were measured once a week. X-axis values represent concentrations of 0, 10, 50, 100, and 200 µM BA applied to plants. Error bars indicate standard error (±SE). Different letters above bars indicate significantly ($P<0.05$) different mean values within the water regime treatment (Tukey HSD test, n = 5).

2.3.2.3 Green leaf area

Green leaf area was reduced by ~60% in water stressed plants compared to well-watered plants, except those receiving 100 µM BA where leaf area was reduced by only 55% (Figure 2-4). Leaf area of well-watered BA treated plants was similar to 0 BA control (Figure 2-4A). Under water stress, green leaf area was significantly (P<0.05) higher (~25%) in 100 µM BA treated plants compared to non-BA controls (Figure 2-4B). There was no significant BA and water treatment interaction for this parameter.

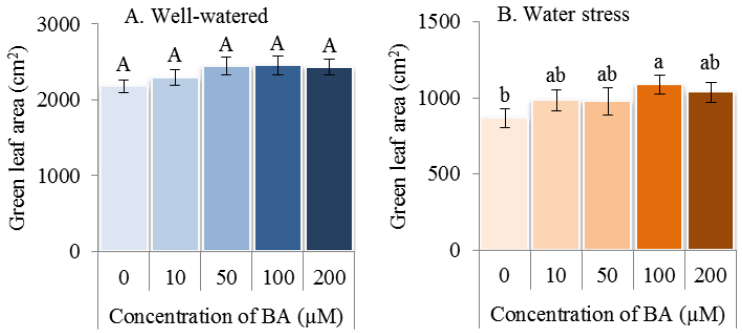

Figure 2-4: Effect of 6-benzyladenine on green leaf area of sugarcane after 4 weeks of growth under well-watered (A) and water stress (B) conditions. Error bars indicate standard error (±SE). Different letters above bars indicate significantly (p<0.05) different means within the water regime treatment (Turkey HSD test, n = 5).

2.3.2.4 Stalk elongation

Water stress reduced stalk elongation in control and BA treated plants (Figure 2-5). Under well-watered conditions, stalk elongation was significantly (P<0.05) increased in 100 µM BA plants by 35% compared to control plants (Figure 2-5A). A similar trend of increasing stem elongation was observed in water stress BA-treated plants, but this was not statistically significant (Figure 2-5B).

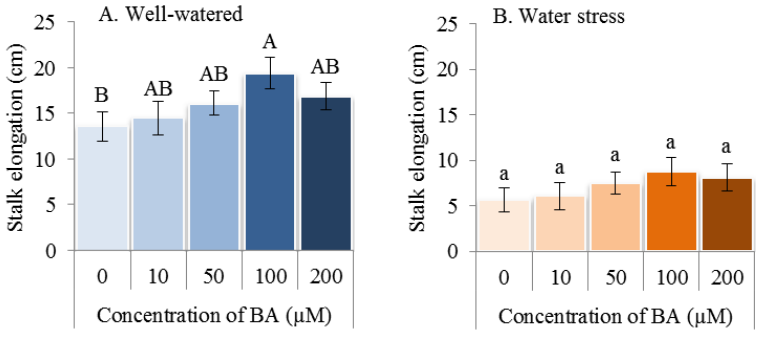

Figure 2-5: Effect of 6-benzyladenine on stalk elongation of sugarcane after 4 weeks of growth under well-watered (A) and water stress (B) conditions. Error bars indicate standard error (±SE). Different letters above bars indicate significantly (p<0.05) different means within the water regime treatment (Turkey HSD test, n = 5).

2.3.2.5 Tiller numbers

Water stress greatly reduced tillering but BA treatments increased tiller numbers (Figure 2-6). Under well-watered conditions, plants treated with 200 µM BA had significantly (P<0.05) increased tiller numbers, 1.6-fold greater than non-BA treated controls (Figure 2-6A). A similar result was observed with water stress where tiller numbers of 200 µM BA-treated plants were significantly (P<0.05, 3.5-fold) higher than non-BA treated plants (Figure 2-6B). There was no significant BA and water treatment interaction for tillering for range of BA dose tested here.

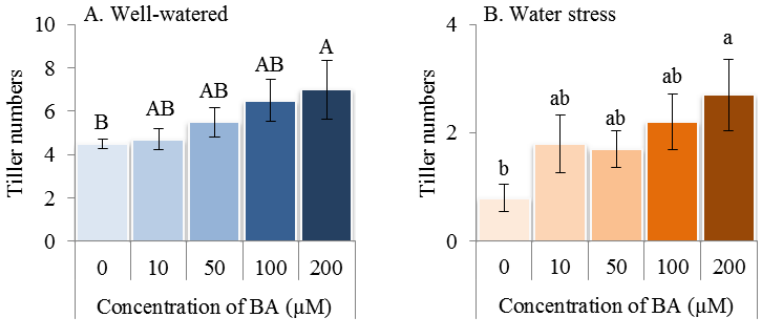

Figure 2-6: Effect of 6-benzyladenine on tiller numbers of sugarcane after 4 weeks of growth under well-watered (A) and water stress (B) conditions. Error bars indicate standard error (±SE). Different letters above bars indicate significantly (p<0.05) different means within the water regime treatment (Turkey HSD test, n = 5).

2.3.2.6 Above-ground biomass

Water stress decreased above-ground biomass in all treatments relative to well-watered plants (Figure 2-7). In well-watered conditions, plants receiving 100 and 200 µM BA had significantly (P<0.05) greater (~30%) biomass than non-BA treated controls (Figure 2-7A). In water stressed plants, above-ground biomass of 100 and 200 µM BA plants was significantly higher (P<0.05, 39 and 28%, respectively) than non-BA controls (Figure 2-7B). Like tillering there was no significant BA and water treatment interaction for above-ground biomass.

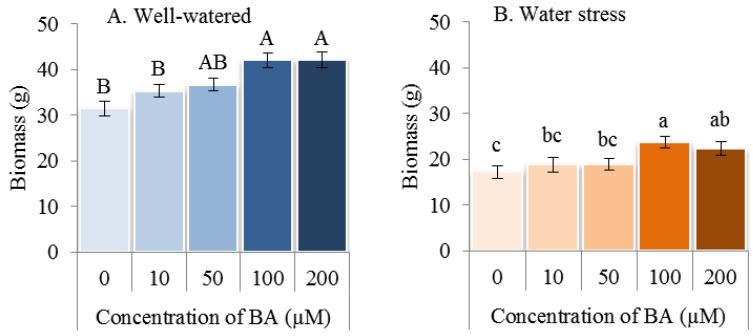

Figure 2-7: Effect of 6-benzyladenine on above-ground biomass of sugarcane after 4 weeks of growth under well-watered (A) and water stress (B) conditions. Error bars indicate standard error (±SE). Different letters above bars indicate significantly (p<0.05) different means within the water regime treatment (Turkey HSD test, n = 5).

2.3.2.7 Relative sensitivity of measured traits to cytokinin concentrations

In general, the response of measured traits was increased with increasing concentrations of cytokinin. Among the different traits studied, tiller number was the most sensitive to BA applications in all water regimes (Figure 2-8) followed by chlorophyll content index (CCI) in the well-watered treatment (Figure 2-8A) and stomatal conductance and shoot mass in water stress conditions (Figure 2-8B).

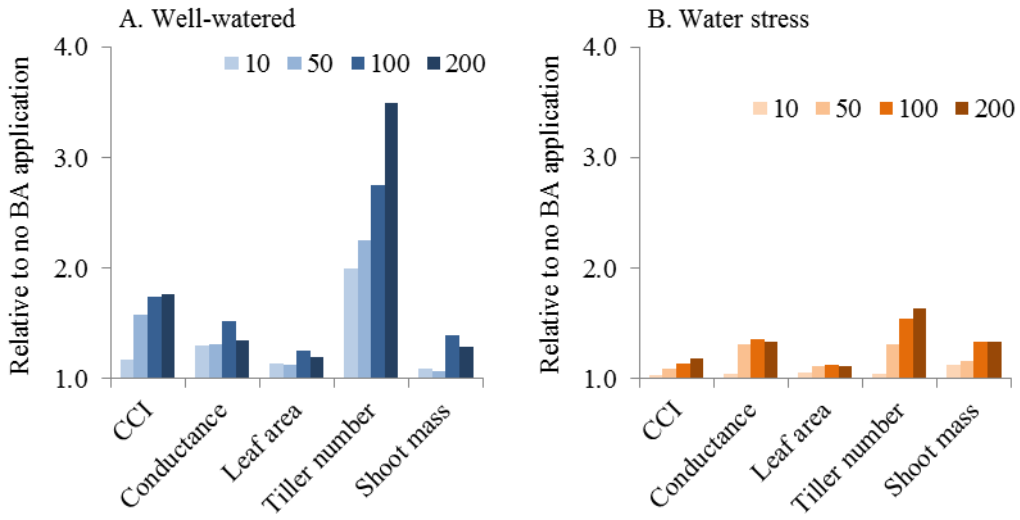

Figure 2-8: Summary figure to compare sensitivity (as fold change) of various parameters to different cytokinin concentrations relative to the "no BA application" treatment.

2.3.3 Effects of 6-benzyladenine application methods (leaf and root) on sugarcane plants

The previous experiment showed that 100 µM BA delivered to roots had the strongest positive effects on plant performance and growth. Here we tested the effects of BA (100 µM BA) delivery *via* foliar or root application in well-watered and water stress conditions over 30 days.

2.3.3.1 Chlorophyll content index (CCI)

Chlorophyll content index (CCI) was considerably decreased in all plants under water stress (Figure 2-9). BA application had no effect on well-watered plants, but a significant (P<0.05) CCI increase of ~45% was observed with both BA application methods in the water stress treatment (Figure 2-9).

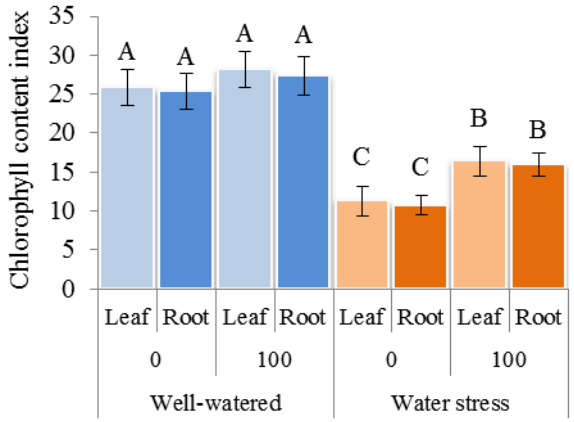

Figure 2-9: Effects of 6-benzyladenine application *via* leaf or roots on chlorophyll content index (CCI) of sugarcane after 4 weeks of growth under well-watered and water stress conditions. Error bars indicate standard error (±SE). X-axis values represent 0 and 100 µM BA concentrations. Different letters above bars indicate significantly (P<0.05) different means within the water regime treatment (Tukey HSD test, n = 3).

2.3.3.2 Stomatal conductance (g_s)

After 4 weeks of treatment, significantly decreased stomatal conductance (g_s) was observed in all water stressed plants (Figure 2-10). BA treated plants had significantly (P<0.05) greater g_s than non-treated ones in both water conditions (Figure 2-10). Stomatal conductance was ~25% greater in well-watered plants and nearly 40% in stressed plants treated with BA. No significant difference was observed between BA application methods in either treatment.

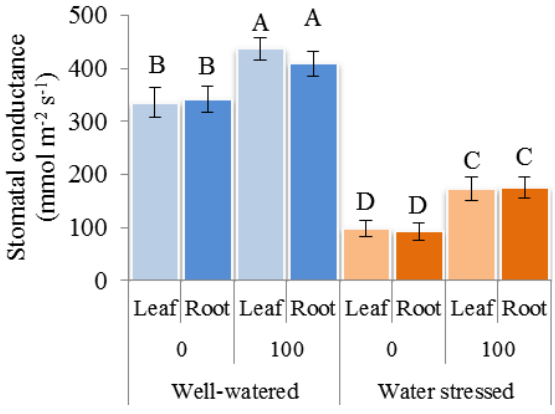

Figure 2-10: Effect of 6-benzyladenine application *via* leaf or root on stomatal conductance of sugarcane after 4 weeks of growth under well-watered and water stress conditions. Error bars indicate standard error (±SE). X-axis values represent 0 and 100 µM BA concentrations. Different letters above bars indicate significantly (P<0.05) different means within the water regime treatment (Tukey HSD test, n = 3).

2.3.3.3 Green leaf area

Loss of green leaf area due to water stress was drastic (Figure 2-11) with nearly 50% lower leaf area in water stressed plants. BA-treatment significantly (P<0.05) improved total green leaf area in both water treatments with plants under stress showed a proportionally stronger response (Figure 2-11). The increase in green leaf area with BA application was similar with leaf or root application in both water conditions.

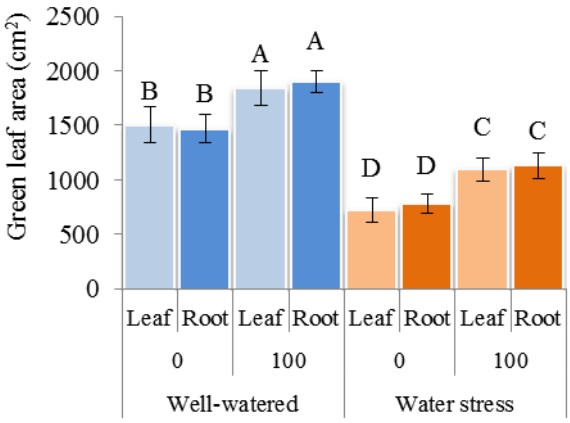

Figure 2-11: Effects of 6-benzyladenine application *via* leaf or root on green leaf area of sugarcane after 4 weeks of growth under well-watered and water stress conditions. Error bars indicate standard error (±SE). X-axis values represent 0 and 100 µM BA concentrations. Different letters above bars indicate significantly (P<0.05) different means within the water regime treatment (Tukey HSD test, n = 3).

2.3.3.4 Stalk elongation

Similar to leaf area loss, stalk elongation was severely affected in plants subjected to water stress. Stem elongation was reduced (P<0.05) by ~50% in plants under water stress (Figure 2-12). Root-BA treatment significantly (P<0.05) increased stalk elongation under well watered and water stress conditions (Figure 2-12).

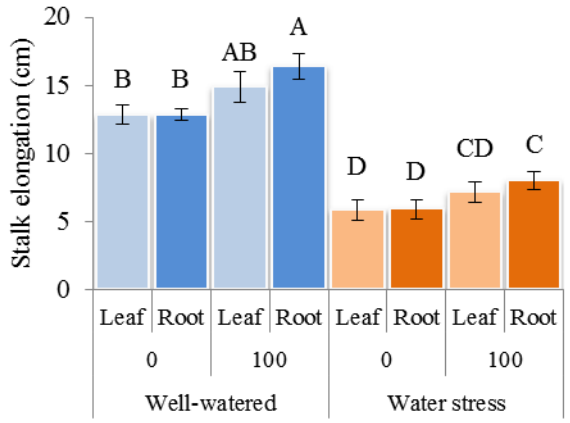

Figure 2-12: Effects of 6-benzyladenine application *via* leaf or root on stalk elongation of sugarcane after 4 weeks of growth under well-watered and water stress conditions. Error bars indicate standard error (±SE). X-axis values represent 0 and 100 µM BA concentrations. Different letters above bars indicate significantly (P<0.05) different means within the water regime treatment (Tukey HSD test, n = 3).

2.3.3.5 Above-ground biomass

Water stress significantly (P<0.05) reduced above-ground biomass in all tested plants (Figure 2-13). Under well-watered conditions, above-ground biomass was increased (P<0.05) by 20 and 30% in BA-foliar and BA-root application plants, respectively. Above-ground biomass was increased (P<0.05) by ~45% in water stressed plants treated with BA by foliar or root application, respectively. Foliar and root application of BA yielded similar results (Figure 2-13).

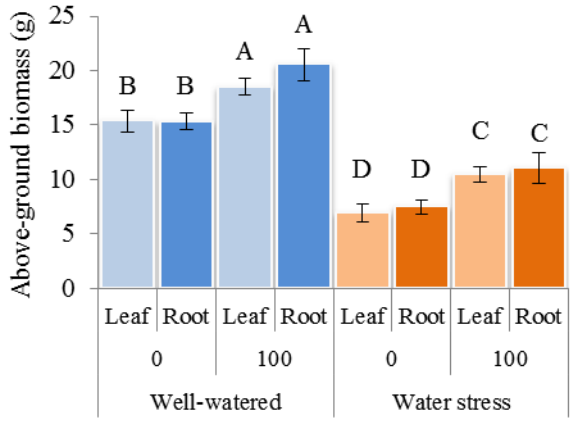

Figure 2-13: Effects of 6-benzyladenine application *via* leaf or root on above-ground biomass of sugarcane after 4 weeks of growth under well-watered and water stress conditions. Error bars indicate standard error (±SE). X-axis values represent 0 and 100 µM BA concentrations. Different letters above bars indicate significantly (P<0.05) different means within the water regime treatment (Tukey HSD test, n = 3).

2.3.3.6 Root biomass

Root biomass of plants in the water stress treatment was decreased by ~61% but this decline in root biomass was significantly reduced in the presence of BA (Figure 2-14). Under well-watered condition, BA supplied plants had significantly higher root biomass (P<0.05) compared to non-BA plants, with similar results with both methods of BA application (Figure 2-14).

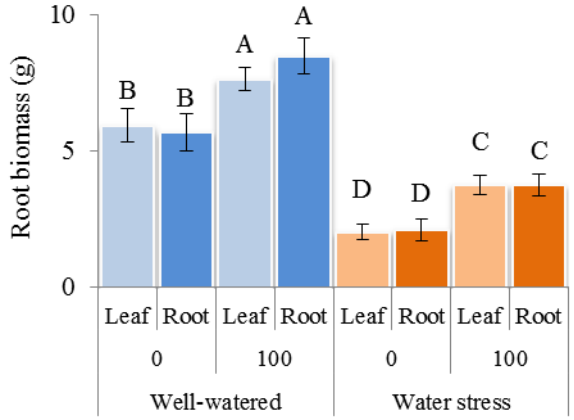

Figure 2-14: Effects of 6-benzyladenine application *via* leaf and root on root biomass of sugarcane after 4 weeks of growth under well-watered and water stress conditions. Error bars indicate standard error (±SE). X-axis values represent 0 and 100 µM BA concentrations. Different letters above bars indicate significantly (P<0.05) different means within the water regime treatment (Tukey HSD test, n = 3).

2.3.3.7 Relative sensitivity of measured traits to cytokinin application methods

There was no interaction between method of BA application and water stress for all studies parameters in the test plants. The traits most sensitive to CK application were stomatal conductance and root mass under water stress conditions in which root application had a higher effect on conductance than leaf application but this was reversed for root biomass (Figure 2-15B). In the well-watered treatment root mass was the trait most sensitive to CK, especially for the root application method (Figure 2-15A). The root application method produced greater changes in all measured traits except stomatal conductance in well-watered treatment (Figure 2-15A).

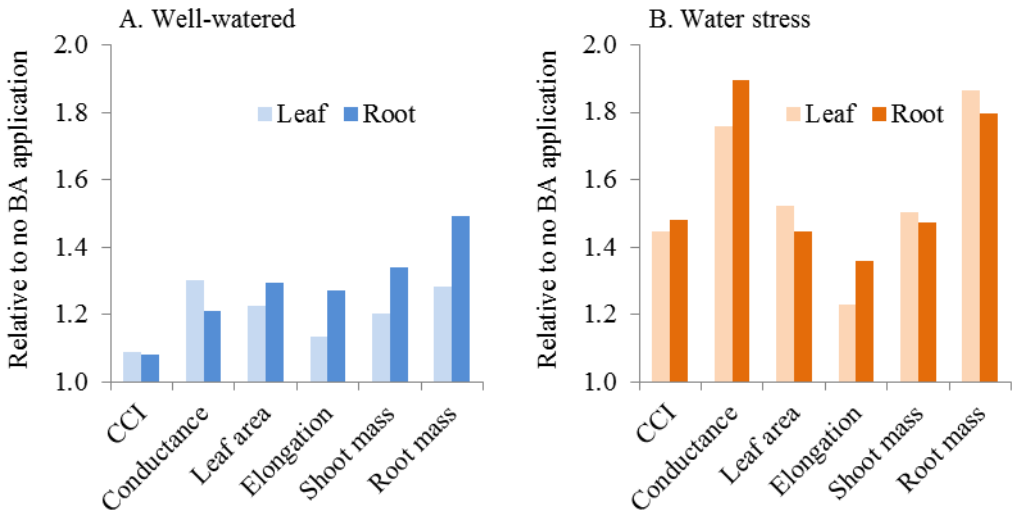

Figure 2-15: Summary figure to compare relative sensitivity of the different variables as fold change of the "no BA application" treatment to different cytokinin application methods.

2.4 Discussion

In this chapter we evaluated the effect of BA on senescence in detached sugarcane leaves and how BA affect water stress response in young sugarcane plants. We also investigated whether the method of hormone application (leaf *versus* root) affects stress alleviation and growth responses.

2.4.1 6-Benzyladenine treatment delays senescence in detached sugarcane leaves

Exogenous application of BA has been reported to delay senescence (Gan and Amasino, 1996). Here we show that BA-treated leaves retained considerable level of greenness even after 14 days of

incubation. This result is consistent with the effects of exogenous BA application to leaves in other species including bean (Rulcova and Pospisilova, 2001), barley (Kravtsov et al., 2011) and apple (Dobránszki and Mendler-Drienyovszki, 2014). The results of the leaf assay confirm that CK is effective in reducing senescence in non-stressed sugarcane leaves, but not so in the tissue exposed to water stress. The lack of CK effect in water stressed leaves could have had several reasons: (i) leaf tissue being developed in *in vitro* condition is quite fragile and the level of osmotic stress used in our experiment may have been too high and compromised the structural integrity of the tissue, (ii) because of the sudden exposure to osmotic stress, cytokinin influx to leaf tissue may be minimal. The 'detached leaf experiment' was a pre-cursor study to the 'whole plant experiments' that form the focus of this thesis and we did not advance this research with modified conditions, for example reduced osmotic stress and varying the application of BA.

2.4.2 6-Benzyladenine application improves growth in sugarcane affected by water stress

The second and main component of study reported in this chapter was to examine the physiological and morphological responses of sugarcane plants grown in glasshouse under well-watered and water stress conditions and how responses are modulated by BA concentration and application method. Water stress caused a significant reduction in the growth and development of all tested plants and the negative effect of water stress was more pronounced in non-BA treated control plants. With experiments aimed at identifying the most effective concentration of BA in the tested range (10 to 200 µM BA), we identified 100 µM BA as the optimal concentration for reducing water stress effects in glasshouse-grown plants. This result was consistent with a study in ryegrass where 35 µM BA enhanced above-ground productivity (Wang et al., 2012). Similarly, Metwally et al. (1997) and Sergiev et al. (2007) reported increased chlorophyll content after applying 100 µM BA to bean plants (*Phaseolus vulgaris* L.) and *Arabidopsis* (respectively) grown under water stress.

Similar to changes in above-ground growth parameters, chlorophyll content and stomatal conductance was significantly greater in BA-treated plants, irrespective of water status. Our result is similar to that reported in cucumber (Can, 2009) and sugar maple (Reeves et al., 2007) where application of 100 µM BA increased stomatal conductance. This was also true in Kentucky

bluegrass under water stress conditions with a low BA concentration (10 μM BA) (Hu et al., 2013) and in eggplant grown under salt stress (Wu et al., 2014).

The method of hormone application is critically important in maximising the effect of agrochemicals, especially plant growth regulators. In this study BA was applied via roots or leaves and observed similar results with either method similar to research with Kentucky bluegrass (Hu et al., 2013). These results demonstrate that physiologically activity of BA was not affected by the methods of application tested here, similar to previous report and BA can be administered to both organs and stability in *Pinus sylvestris* (Vogelmann et al., 1984) and *Arabidopsis* (Cedzich et al., 2008). From a practical perspective, root application *via* irrigation may be easier to implement as is not affected by the shoot size. From a practical crop production perspective, root application via irrigation may be easier to implement as is not affected by the shoot size.

Our results showed an overall improvement in sugarcane growth with BA application. We also did not observed CK inhibition on root development, which is contrary to earlier reports in other species. For example, Johnston and Jeffcoat (1977) showed that application of 100 μM BA markedly increased tillering but arrested root growth in cereals (oat, wheat, barley), although reasons for the impact of BA on root growth is unclear. However, in sugarcane grown under water stress, it is possible that water deficit triggered production of ABA and ethylene. Since it is established that water stress reduces CK production in plants (Ha et al., 2012) which amplifies ABA and ethylene effect, external application BA may have counteracted the effects of increased ABA and ethylene production (Davies, 2010f). We examined this possibility at molecular level and reported it in chapter 5. In brief, external application of BA had a significant growth stimulation effect that was more pronounced in non-stress plants, possibly due to the larger leaf area in response to BA application and the consequent increase in C-fixation and growth.

2.4.3 Conclusions

The key conclusions of the research presented here testing the effect of CK (in the form of BA) on sugarcane growth and development are:

1. CK is effective in controlling leaf senescence and retention of leaf area in sugarcane;

2. CK improves overall growth and development in well-watered and water stress conditions;

3. Root growth is not significantly inhibited by CK in sugarcane in the conditions tested here;

4. CK application methods *via* irrigation of roots or foliar spray did not elicit noticeable differences in growth or physiology in either water condition.

5. From the practical crop production perspective, CK application *via* root method is more convenient and possibly cheaper than foliar application method.

CHAPTER 3: CONDITIONAL UP-REGULATION OF CYTOKININ BIOSYNTHESIS IN SUGARCANE AND IDENTIFICATION OF TRANSGENIC LINES TOLERANT TO WATER STRESS.

3.1 Introduction

Transgenic plants have become an essential tool for investigations into various aspects of plant biology such as physiology, genetics, developmental biology, and molecular biology. Further, advocates of genetic engineering as a plant breeding tool claim its precision provides a major advantage over other breeding techniques. The presumption is that genetic engineering results in (1) specific and known genotypic changes to the engineered plant (the simple insertion of a defined DNA sequence - the transgene) and (2) known and specific phenotypic changes [the intended trait(s) encoded by the transgene].

Molecular improvement strategies have the potential to enhance agronomic performance, or produce novel compounds, by manipulating endogenous genes or introducing foreign genes into sugarcane following the establishment of an efficient sugarcane transformation system (Bower and Birch, 1992). Although researchers globally have introduced many transgenes conferring agronomic traits into crop plants, maize and sugarcane remain the only commercialised transgenic drought tolerant crops so far. While the *csp-B* gene coding an RNA chaperone makes Monsanto's DroughtGard maize drought adapted, betain dehydrogenase over-expression and resultant accumulation of glycinebetain confers drought tolerance in sugarcane (Marshall, 2014). Drought tolerance is one of the priority research areas in major agribusiness companies. As with the broad-acre crops, substantial research effort is currently underway in developing drought tolerant sugarcane *via* transgenic and conventional strategies (Basnayake et al., 2012, Lakshmanan and Robinson, 2014).

Many projects aimed at transgenically engineering drought tolerance have focused on components of cytokinin (CK) production or signaling pathway (Wilkinson et al., 2012). Recently, Rubia et al.

(2014) reported that low CK levels were associated with growth inhibition and onset of senescence. Regulation of CK biosynthesis and action could be an important avenue to develop drought tolerant crops. Since the decline in CK content in stressed plants is likely to enhance sensitivity to ABA-induced stomatal closure, it may be beneficial to prevent CK decline.

In this regard, a number of studies have dealt with over-expression of the *IPT* gene with different promoters to increase CK biosynthesis and alter signaling for drought tolerance. However, the expression of *IPT*, the regulatory gene in the CK biosynthesis pathway, with its native promoter results in extreme over-production of CK in plant cells and this results in almost complete inhibition of root growth and abnormal shoot development (Hewelt et al., 1994, Wang et al., 1997). Thus, constitutive up-regulation of CK production is undesirable from a crop yield perspective. In Australia, commercial sugarcane crops experience two reasonably long periods of water stress, about 3 months before and after the (generally) rainy summer, during the 12-month crop cycle (Basnayake et al., 2012). Hence, for crop improvement a logical approach would be to increase CK production during stress periods by bringing *IPT* transgene expression under the control of a stress-inducible promoter. This approach has been successful in tobacco and rice plants with enhanced drought tolerance (Rivero et al., 2007, Rivero et al., 2009, Peleg et al., 2011). For example, by fusing the *IPT* coding sequence to the promoter sequence of a senescence-associated gene (*SAG12*), the *IPT* transgene is primarily transcribed during the senescence period (Zhang et al., 2010). In sugarcane, expression of *IPT* driven by a cold-inducible promoter did not affect plant growth but resulted in greater tolerance to cold stress (Belintani et al., 2012). Response of plants to water deficit and the underlying mechanisms of tolerance to water deficit vary between plant species. Although *SAG12* promoter-driven *IPT* up-regulation was effective in reducing drought-induced growth effects in many crops, it may not be universally applicable for all crops due to considerable promoter-dependent variation in CK production and drought tolerance (Merewitz et al., 2011a). There is no study on *IPT* transgenic sugarcane in relation to drought tolerance, and little is known about the hormonal or molecular basis of drought tolerance in sugarcane (Lakshmanan and Robinson, 2014). It is essential to obtain phenotypically normal transgenic plants containing a conditionally expressing *IPT* gene (only responding to stress conditions) to study the CK roles in sugarcane. To generate plants with normal phenology and upregulated CK levels, we therefore transformed sugarcane with the *IPT* coding sequence fused to one of three promoters (discussed in the next section). The promoters selected here are from the *Senescence associated gene 12*

(*SAG12*), *ABA-responsive gene* (*RAB17*) and *Ubiquitin gene* (*Ubi*). The first two promotors were selected because their stress-responsiveness in many plants is well established, while the *Ubi* promotor was chosen as a constitutively expressing control known to function in transgenic sugarcane under field condition (Joyce et al., 2014).

3.1.2 Function of selected promoters

The promoter is a critical regulatory element of gene expression. Differential gene expression in response to external and internal cues determines growth, development and adaptation to its environment. Differential gene expression, such stress-responsiveness, is often achieved by variation in structural attributes of promoters. Because sugarcane growth is most sensitive to water deficit, followed by loss of green leaves as the second most pronounced response to water deficit, senescence related and an ABA-responsive promoter was selected to limit CK up-regulation to stress periods.

3.1.2.1 *Senescence associated gene 12* (*SAG12*) promoter

Senescence associated gene 12 (*SAG12*), the gene encoding a cysteine protease in *Arabidopsis*, is expressed only in leaf senescing tissue (Noh and Amasino, 1999). *SAG12* expresses during developmentally controlled senescence, in an organ-specific manner, and shows enhanced expression under conditions of stress, such as drought (Gombert et al., 2006). The specific induction of *SAG12* during leaf senescence may indicate that it has a more specialised role in protein breakdown during senescence (Grbic, 2003). The expression profile of the *SAG12* gene has been determined using quantitative real-time PCR with mRNA extracted from the leaves of plants harvested at different stages of maturity and exposed to different levels of stress (Zhang et al., 2010, Gombert et al., 2006). The *SAG12* promoter has been used to drive *IPT* gene transcription in numerous plants species with positive effects on growth under water stress (Zhang et al., 2010, Sykorova et al., 2008).

3.1.2.2 *ABA-responsive gene* (*RAB17*) promoter

The *ABA-responsive gene* (*RAB17*) is induced during late embryogenesis when ABA levels are high (Goday et al., 1994). It is also induced during maize embryo maturation and in vegetative tissues

under water stress conditions (Busk et al., 1997, Figueras et al., 2004, Pla et al., 1989). Previous studies showed that the *RAB17* protein plays a role in nuclear protein transport to assist ABA signaling under stress conditions (Goday et al., 1994). The structure of the gene is well characterised, with two exons, one intron in the transcription region and nine transcription factor binding sites in the promoter region of the *RAB17* gene (Busk et al., 1997). These *cis*-elements mediate responses to ABA and dehydration to regulate gene expression during seed and vegetative development (Kizis and Pages, 2002). Increased production of ABA is a very common response of plants experiencing water stress (Vankova, 2012).

3.1.2.3 *Ubiquitin gene* (*Ubi*) promoter

The *Ubiquitin gene* (*Ubi*) encodes a protein found in eukaryotic cells and its sequence is highly conserved among organisms. The structure of the gene is well described, with a 899 bp promoter sequence, 83 bp 5' untranslated exon, and 1010 bp first intron sequence (Christensen and Quail, 1996). The *Ubi* promoter is considered to be constitutive as the *Ubi* gene is transcribed in all tissues regardless of the surrounding environment and development stage of the organism, and will turn on transcription of the downstream gene at all times throughout the organism's lifetime (Christensen and Quail, 1996). Thus, the *Ubi* promoter may provide information about the effect of constitutive over-expression of *IPT* gene in sugarcane. Several reports demonstrate that oversupply of CK reduces apical dominance and release axillary buds which results in highly branched shoots, and inhibition or retardation of root formation (Miyawaki et al., 2004, Pospisilova et al., 2000). Oversupply of CK, *via* constitutive over-expression of the *IPT* gene, will indicate if a threshold CK concentration is reached or exceeded. Further, as the activity of the *SAG12* and *RAB17* promoters have not been well described in sugarcane, it was important to include the *Ubi* promoter (that is known to produce strong constitutive gene expression in sugarcane) as a positive control for this transgenic study.

This chapter explores the challenges in manipulation and application of promoters to regulate *IPT* gene over-expression in sugarcane. This approach may lead to avenues for molecular breeding for water stress tolerance in sugarcane. Three DNA constructs (P_{SAG12}*::IPT*, P_{RAB17}*::IPT*, and P_{Ubi}*::IPT*) were cloned and introduced into wild-type sugarcane (Australian commercial variety $Q208^{A}$). We aimed to determine (i) whether stress-responsive up-regulation of CK production by expressing

either P_{SAG12}*::IPT* or P_{RAB17}*::IPT* at the time of water deficit can improve drought tolerance in sugarcane and if so how this is being achieved, and (ii) the effect of continual CK up-regulation in P_{Ubi}*::IPT* transgenic plants.

Another aim of this study was to select drought tolerant P_{SAG12}*::IPT* or P_{RAB17}*::IPT* clones from a large population of independent transgenic events to further study plant responses to water deficit and link phenotypic traits with genotype. The main characteristic used for clone selection was the retention of green leaf area under conditions of water stress that has been used as an indicator of drought tolerance in breeding programs in wheat (Vassileva et al., 2011), sorghum (Xu et al., 2000) and maize (Monneveux et al., 2008).

3.2 Materials and methods

3.2.1 Plant transformation

3.2.1.1 Plant material and growth conditions

The tops of sugarcane (*Saccharum officinarum* L. var. $Q208^{A}$) were provided by Sugar Research Australia (SRA), formerly BSES limited. Immature sugarcane leaf whorls were cut into approximately 2 mm thick transverse sections and cultured on MS medium (Murashige and Skoog, 1962) containing 1 mg of 2,4 dichlorophenoxyacetic acid per L (MS1 Media) and kept in the dark at 24±1 °C for embryogenic callus production. Cultured leaf tissues were transferred to fresh medium every 2 weeks. After 6 weeks of culture, calli produced were removed from the leaf whorl and placed directly onto MS1 medium and maintained at 24±1 °C in the dark for 2 weeks or until such time when sufficient callus was produced for transformation as descried earlier (Bower and Birch, 1992).

3.2.1.2 Plasmid construction

P_{SAG12}::IPT

Vectors for *SAG12* regulated expression of *IPT* were based on the 58 kb P_{SG516} binary vector, supplied by Sugar Research Australia (Figure 3-1). The P_{SG516} vector contained the 2180 bp *SAG12* promoter upstream of the 720 bp *IPT* coding sequence followed by the 274 bp *Agrobacterium tumefaciens NOS* (*nopaline synthase*) terminator as described in Gan and Amasino (1995). P_{SAG12}::*IPT* sequences are shown in Appendix 1.

Figure 3-1: Schematic diagram of constructs showing the locations of the *SAG12* promoter, *IPT* CDS, and terminator.

P_{RAB17}::IPT

In order to construct the P_{RAB17}::*IPT* plasmid (Figure 3-2), the *SAG12* promoter was excised from P_{SAG12}::*IPT* by digestion with *Nco*I and *Not*I to yield an *IPT* backbone fragment (3,680bp). The *RAB17* promoter including the 5'UTR (603 bp) was amplified by PCR with 5'-GCGGCCGCTATAGTATTTTAAAATTGC-3' (forward primer incorporating the *Not*I site) and 5'-CCATGGGGTGCTTGCACGGCTTGGTGG-3' (reverse primer incorporating the *Nco*I site) from a plasmid template supplied by Sugar Research Australia. The *RAB17* promoter and 5'UTR was digested with *Nco*I and *Not*I and then ligated with the *IPT* backbone fragment using T4 DNA Ligase (Promega, Australia) to create P_{RAB17}::*IPT*, and verified by PCR and sequencing. P_{RAB17}::*IPT* sequences are shown in Appendix 2.

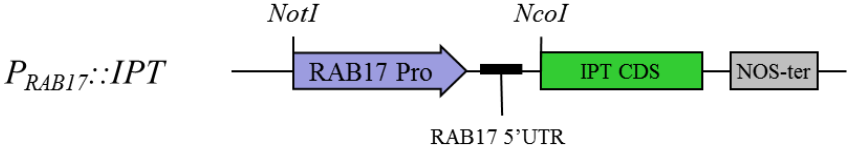

Figure 3-2: Schematic diagram of constructs showing the locations of the *RAB17* promoter, *RAB17* 5' UTR, *IPT* CDS, and terminator.

P_{Ubi}::IPT

To construct the *P_{Ubi}::IPT* plasmid (Figure 3-3), the *SAG12* promoter was excised from *P_{SAG12}::IPT* by digestion with *Nco*I and *Not*I to yield an *IPT* backbone fragment (3,680bp). The maize *Ubiquitin* promoter, 5'UTR, and intron (1991 bp) was amplified by PCR with 5'-ATGCGGCCGCCTG CAGTGCAGCGTGACCCGGT-3' (forward primer incorporating the *Not*I site) and 5'-ATCCATGGTGCAGAAGTAACACCAAACAA-3' (reverse primer incorporating the *Nco*I site) from a plasmid template supplied by Sugar Research Australia. The PCR product was digested with *Nco*I and *Not*I to yield two products due to an internal *Nco*I site. The two products were then sequentially ligated with the *IPT* backbone fragment to create *P_{Ubi}::IPT*, and confirmed by PCR screening and sequencing. *P_{Ubi}::IPT* sequences are shown in Appendix 3.

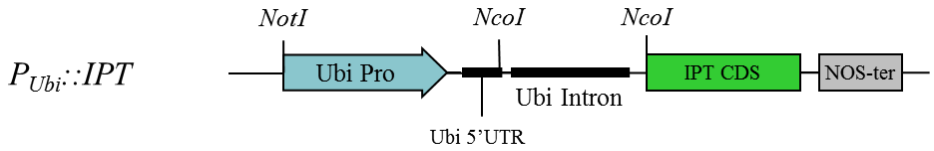

Figure 3-3: Schematic diagram of constructs showing the locations of the *Ubi* promoter, *Ubi* 5'UTR, *Ubi* Intron, *IPT* CDS, and terminator.

All plasmid constructs were verified by direct sequencing following the recommended amounts of template and primer provided by Australian Genome Research Facility (AGRF), University of Queensland, St Lucia, Australia.

3.2.1.3 Plant transformation

P_{SAG12}::IPT, *P_{RAB17}::IPT* and *P_{Ubi}::IPT* constructs were introduced into sugarcane (*Saccharum* hybrid. var. Q208^{A}) callus *via* microprojectile bombardment (Bower and Birch, 1992). They were co-bombarded with *P_{Ubi}::NPTII* (supplied by Sugar Research Australia) which allows for antibiotic selection of transformed cells using Geneticin (A.G. Scientific, Inc. USA). Transformed calli were maintained on MS1 medium for 4-7 days and then transferred to MS1G50 medium containing 50 µg mL^{-1} of Geneticin as a selective antibiotic in the dark for 4 weeks. Transformed calli were transferred to MSG50 medium and cultured in the light. A plantlet from each callus clump was sub-

cultured onto MSG50 medium and labeled as an independent line. They were maintained on MSG50 medium and sub-cultured every 2-3 weeks.

3.2.2 Molecular screening for *IPT* transgenic plants

A total of 155 putative transgenic lines; 60lines transformed with P_{SAG12}*::IPT*, 45 lines transformed with P_{RAB17}*::IPT*, and 50 lines transformed with P_{Ubi}*::IPT* construct were generated through micro-projectile bombardment transformation. The integration of transgene was confirmed by genomic PCR.

3.2.2.1 Genomic DNA preparations

Leaf tissue from putative transgenic plants transformed with P_{SAG12}*::IPT*, P_{RAB17}*::IPT*, and P_{Ubi}*::IPT* plasmids were collected and total genomic DNA was isolated using the Template Preparation Solution (TPS) extraction protocol. Leaf tissues were sampled into a 2 mL screw cap containing a glass bead, and 200 µL of TPS buffer added to each tube and ground in a FastPrep®-24 (MP Biomedicals) for 20 sec at 4 m/s. Sample were incubated at 95 ˚C for 10 min and chilled on ice for 1 min. Tubes were spun at 13,000 rpm at room temperature for 5 min and 20 µL of the supernatant was diluted with 180 µL of distilled H_2O. Diluted sample was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). All DNA samples were standardised by diluting to 100 ng μL^{-1}. Diluted sample was used as the template in PCR reaction. TPS buffer preparation and components are descripted in Table 3-1.

Table 3-1: TPS buffer preparation and components

Components	In 100 mL (dH_2O)
100 mM Tris	1.21 g
1M KCl	7.46 g
10mM Na_2EDTA	0.37 g
pH 9.5	

Solution was autoclaved at 121 ˚C, 100 kPa for 20 min.

3.2.2.2 PCR amplification and thermal cycling conditions

PCR reactions were performed using 1 µL of 100 ng μL^{-1} genomic DNA extract in a 25 µL total reaction volume containing 12.5 µL of GoTaq® Green Master Mix (Promega, Australia), and 0.5 µL of each primer (10 µM). 0.5 µL of 100 ng μL^{-1} of plasmid was used as a positive control template.

The thermal profile was 95 °C for 2 min initial denaturation, 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 10 min. Four primer sets were generated and used to screen plants carrying the *IPT* transgene in this experiment (Table 3-2) and primer positions are displayed in Figure 3-4.

Table 3-2: List of primers and their sequence used in PCR screening of putative transgenic lines

Target clone	Primer	Sequence	Product
P_{SAG12}::*IPT*	SAG12-F IPT-R	5'-CATGAAAGGTACCTACGTACTAC-3' 5'-GCAAGTTGGACCGAAAATTAGATGC-3'	627 bp
P_{RAB17}::*IPT*	RAB17-F IPT-R	5'-GCATTAACAAACATGTCCTAATTGG-3' 5'-GCAAGTTGGACCGAAAATTAGATGC-3'	620 bp
P_{Ubi}::*IPT*	Ubi-F2 IPT-R	5'-GCAGACGGCACGGCATCTC-3' 5'-GCAAGTTGGACCGAAAATTAGATGC-3'	1471 bp
P_{Ubi}::*IPT*	Ubi-F2 Ubi-R	5'-GCAGACGGCACGGCATCTC-3' 5'-AATAGCGTATGAAGGCAGGGC-3'	1385 bp

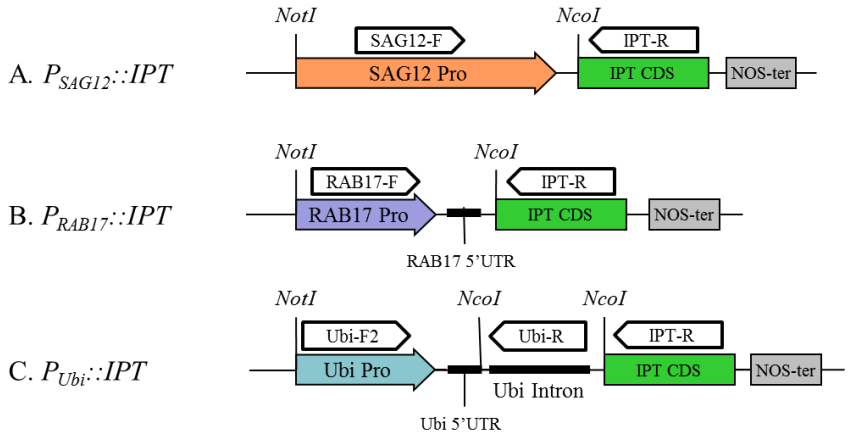

Figure 3-4: Schematic diagram of constructs showing the locations of the primer sets used for PCR. A: The locations of SAG12-F and IPT-R primers on the P_{SAG12}::*IPT* plasmid; B: The locations of RAB17-F and IPT-R primers on P_{RAB17}::*IPT*; C: The locations of Ubi-F2, Ubi-R, and IPT-R primers on the P_{Ubi}::*IPT* plasmid.

3.2.3 Phenotypic selection for transgenic plants

3.2.3.1 Plant material and growth conditions

The phenotypic selection of transgenic plants was carried out in April to July 2013 at Sugar Research Australia PC2 facilities (Indooroopilly, Australia). A total of 30 transgenic lines consisting of ten independent lines from each construct (3 constructs) showing normal growth (~5-7 cm stem height) and had sufficient shoot and root material, were evaluated in this experiment. The developing plants were transferred from tissue culture to potting trays with peat and sand (60:40 v/v) in a PC2 glasshouse under natural light at 30-35 °C. After 30 days of acclimation, healthy plantlets of similar size were transferred to 0.7 L pots (Garden city Plastics, Australia) in peat and sand (60:40). To each pot, 100 mL of Thrive Soluble All Purpose Plant Food (8 g / 4.5 L water; Yates, Australia) was applied every fortnight. Water was automatically supplied through drip irrigation twice a day. The transplanted seedlings were grown in a greenhouse at 30-35 °C for 4 weeks; uniformly grown seedlings were chosen for the study.

3.2.3.2 Water stress treatment

In this experiment, the well-watered plants continued to receive full irrigation (i.e. were kept at 100% field capacity, FC). For the stress treatment, water stress was imposed by gradually withdrawing irrigation until the soil reached 30% FC, determined gravimetrically (Earl, 2003). Pots of both treatments were weighed and watered daily to maintain 100% FC in the well-watered treatment and 30% FC in the stress treatment.

3.2.3.3 Experimental design

The experiment was carried out using a split-plot design with three replications. In each replication two sub-plots, one for 100% FC and the other for 30% FC, were maintained. Each treatment had thirty-three independent lines; ten independent lines from each *IPT* construct and three independent non-transgenic lines (produced from independent clumps of calli and had gone through the process of transformation except the integration of transgene and antibiotic selection process) from wild-type sugarcane ($Q208^{A}$) as a control.

3.2.3.4 Clone evaluation parameters

In this experiment, four variables; chlorophyll content index (CCI), number of green leaves, stem elongation, and above ground biomass were quantified. CCI values were measured in the middle portion of the youngest fully expanded leaves using a hand-held CCM-200 plus chlorophyll meter (Opti-Sciences, Inc., USA) with the average obtained from three readings. The number of leaves with a clearly visible dewlap with at least 50% green area and attached to the main stem were counted (Robertson et al., 1999). Plant elongation was calculated from the difference between plant height on the first day and at the end of the experiment. Plant height was measured from soil level to the point of the top visible dewlap. Biomass data was collected at the end of experiment. Above-ground parts were collected and dried at 60 °C for 4 days.

3.2.3.5 Quantitative reverse transcription PCR analysis

Total RNA was extracted from the youngest fully expanded leaf of transgenic and wild-type plants, using RNeasy Mini Kits (Qiagen). RNA was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). A total of 1 µg RNA was treated with 2 units of RQ1 RNase-Free DNase (Promega, USA). First strand cDNA was synthesised in a 20 µL reaction volume containing Improm-II 5× Reaction Buffer (Promega), 0.5 µg random primers (Invitrogen), dNTPs equating to a final concentration of 0.5 mM (Promega), and 1 µL Improm-II Reverse Transcriptase (Promega). Samples were incubated at 25 °C for 5 min, 42 °C for 60 min and then 70 °C for 15 min. After cDNA synthesis, PCR was used to check for gDNA contamination using sugarcane *phosphofructokinase 5* (ScPFK5) PFK5 forward (5'–AGCCACATCAGATCAACAAG–3') and PFK5 reverse (5'–TGAAGTTATACCCTGCCA TT–3') primers that are positioned either side of an intron. Contamination of cDNA with gDNA results in a larger PCR product (450 bp). Before qRT-PCR, all cDNA samples were standardised by diluting to 5 ng μL^{-1}.

Quantitative reverse transcription PCR primers were designed using an online primer design program (http://www.genscript.com/). Primer sequences were as follows: IPT-RT-F3 forward (5'-GACGACCAACAGTGGAAGAA-3') and IPT-RT-R3 reverse (5'-TCAGCCTATGATGAGCTTG C-3'). Primers were initially tested for specificity before use in qRT-PCR analysis. Primers were used in standard PCR with sugarcane wild-type and transgenic cDNA template, and products were visualised using electrophoresis with a 1% agarose gel and 0.5 X TBE (Tris, Borate, EDTA) buffer

at 100 V for 60 min. The thermal profile used was 94°C for 5 min initial denaturation, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 3 min. The amplified PCR products were sequenced to confirm the sequence identity at AGRF. Optimisation of qRT-PCR primer concentrations and melt curve analyses were also done before quantification.

A ViiA™ 7 Real-Time PCR System (Life technologies, Australia) was used to measure samples. Reagents were mixed by hand and each reaction contained 7.5 µL of SensiMix SYBR Low-ROX (Bioline, Australia), 0.3 µL (300 nM) of each gene specific forward and reverse primer and 0.9 µL H_2O. An epMotion 5073 liquid handler (Eppendorf) was used to aliquot the reagent mix and 5 µL of 5 ng μL^{-1} cDNA into MicroAmp® Fast Optical 96-Well Reaction Plates (Life Technologies, Australia). The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a dissociation step of 95 °C for 2 min, 60 °C for 15 s. No template controls (NTC) were used to check for contamination and primer dimers.

All qRT-PCR data generated was analysed using the DataAssist™ Software (Life Technologies, Australia). For each cDNA sample, an average gene expression level was calculated from three duplicate PCR reactions (technical replicates). This average amplification for each gene was normalised against the average amplification of a reference gene (*ADF*, *actin depolymerizing factor*) expression level, the purpose of which was to account for template variations between samples. Then each expression level was compared relatively to a reference sample according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

3.2.3.6 Statistical analysis

Data were subjected to analysis of variance (ANOVA). When significant differences were noted, the Tukey HSD comparison method was used to determine the level of differences. Means of one representative experiment are presented; n = 3. Statistical analyses were conducted using Statistix 10 software (Analytical Software. Tallahassee, USA).

3.3 Results

3.3.1 PCR screening for *IPT* transgenic plants

Genomic PCR was used to screen putative transgenic plants and the number of PCR positive lines (Table 3-3). Approximately 30% of the putative lines were non-transgenic or did not contain the intact transgene cassette. An example of the PCR analysis of *IPT* transgene is shown in Figure 3-5.

Table 3-3: Number of putative lines and number of positive lines screening by PCR

Construct	Putative lines	Positive lines
P_{SAG12}::*IPT*	60	46
P_{RAB17}::*IPT*	45	33
P_{Ubi}::*IPT*	50	34

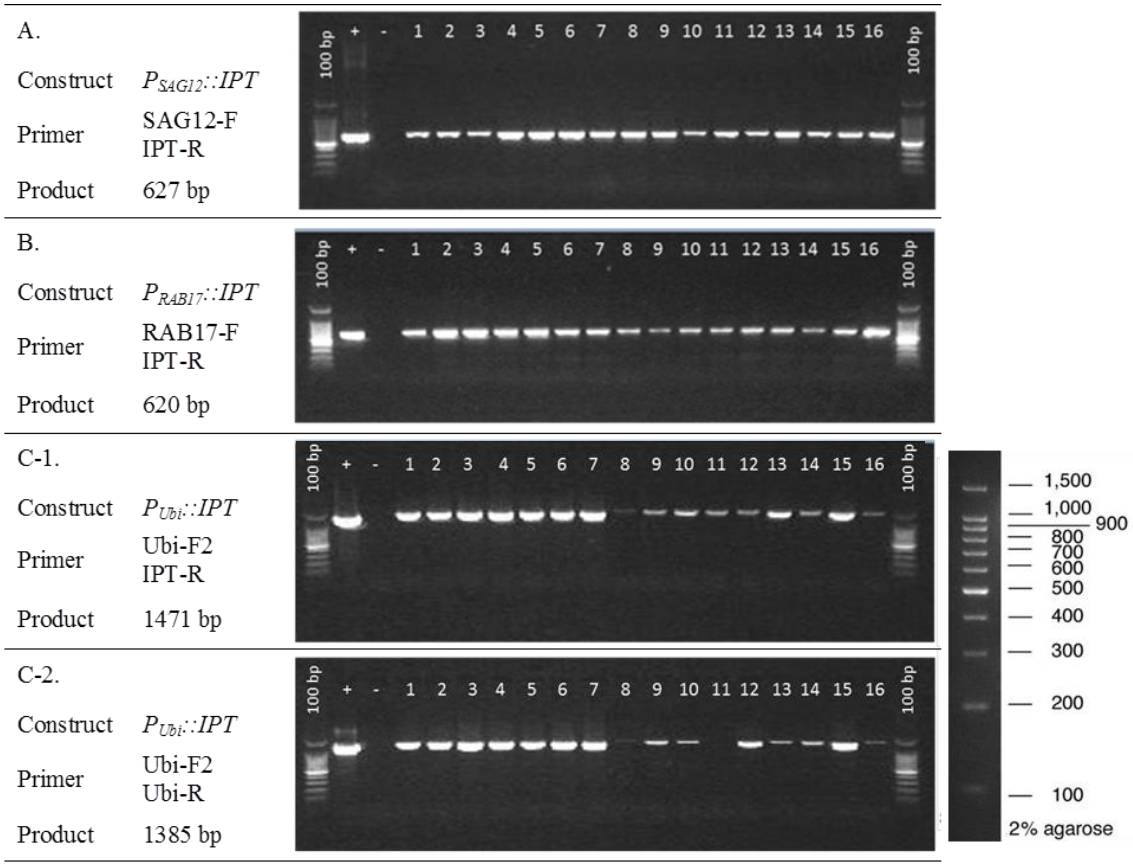

Figure 3-5: PCR analysis of putative *IPT* transgenic sugarcane plants. A, P_{SAG12}::*IPT*; B, P_{RAB17}::*IPT*; C, P_{Ubi}::*IPT* (C-1, Ubi-F2 and IPT-R; C-2, Ubi-F2 and Ubi-R). The numbers represent independent transgenic lines. A 100 bp DNA ladder (Promega, Australia) was used as a DNA molecular weight marker; (+) represents a positive control; (-) represents a negative control.

3.3.2 Phenotypic selection results

Confirmed transgenic lines were assessed for leaf senescence response under water stress condition. With the gradual reduction in irrigation, water stress intensity progressed and the number of green leaves and CCI in the leaves of most of the transgenic plants and wild-type plants declined throughout the experiment. Among 30 transgenic lines, RAB19, RAB20, RAB25, RAB32 and SAG32 maintained green leaves after 60 days of severe water stress treatment (30% FC) (Figure 3-6).

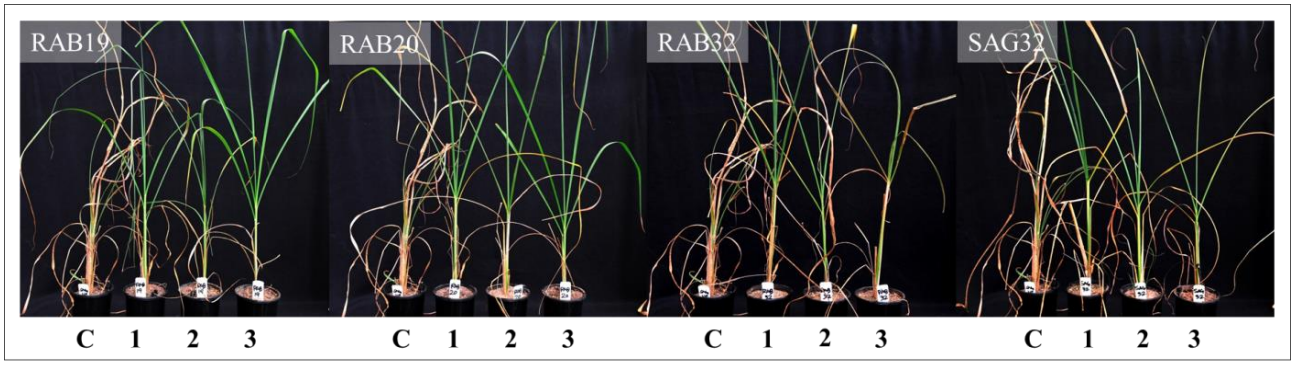

Figure 3-6: Phenotypes of wild-type and transgenic plants after 60 days of water stress treatment in glasshouse. C represents a wild-type control plant and numbers 1, 2, and 3 are replications of each transgenic line.

3.3.2.1 Chlorophyll content index (CCI)

After 60 days of water stress treatment, RAB19, RAB20, RAB25, RAB32 and SAG32 plants had significantly ($P<0.05$) higher CCI value, at least 2-fold higher than the wild-type (Figure 3-7). There was no significant difference in CCI value between different transgenic types within well-watered plants. Moreover, interaction between line and water regime was observed in CCI after 60 days of treatment (See appendix 2-3 and 2-4) where, CCI value was significantly ($P<0.05$) decreased by 70-80% in general and ~40% in RAB19, RAB20, RAB25, RAB32 and SAG32 plants (Figure 3-7).

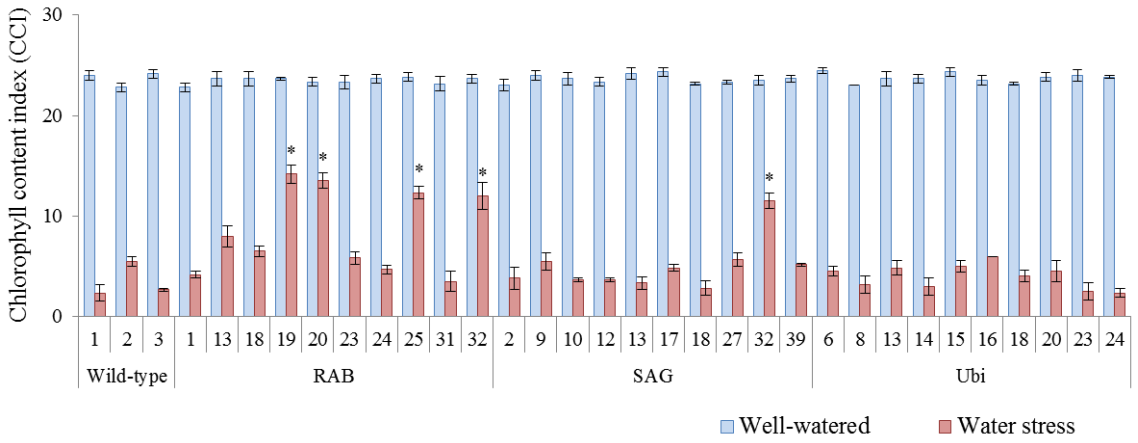

Figure 3-7: Chlorophyll content index (CCI) values of sugarcane plants after 60 days under well-watered (100% field capacity) and water stress (30% field capacity) conditions. SAG = P_{SAG12}*::IPT*; RAB = P_{RAB17}*::IPT*; Ubi = P_{Ubi}*::IPT*. Values are mean of each line. The error bars show standard error (SE±). * indicates a significant difference at P<0.05 (Tukey HSD test) compared to wild-type and other transgenic lines within water regime.

3.3.2.2 Number of green leaves

Similar to CCI, RAB19, RAB20, RAB25, RAB32 and SAG32 plants had significantly (P<0.05) higher number of green leaves compared to other transgenic lines and wild-type plants exposed to water deficit (Figure 3-8). In the full FC treatment, number of leaves did not differ among test plants. The effect of genotype on green leaf number exhibited interaction with water regime after 60 days of experiment (See appendix 2-3 and 2-4). The number of green leaves of all tested lines was significantly (P<0.05) decreased by 70-80% except in RAB19, RAB20, RAB25, RAB32 and SAG32 plants where the number of green leaves was decreased by ~40-50% (Figured 3-8).

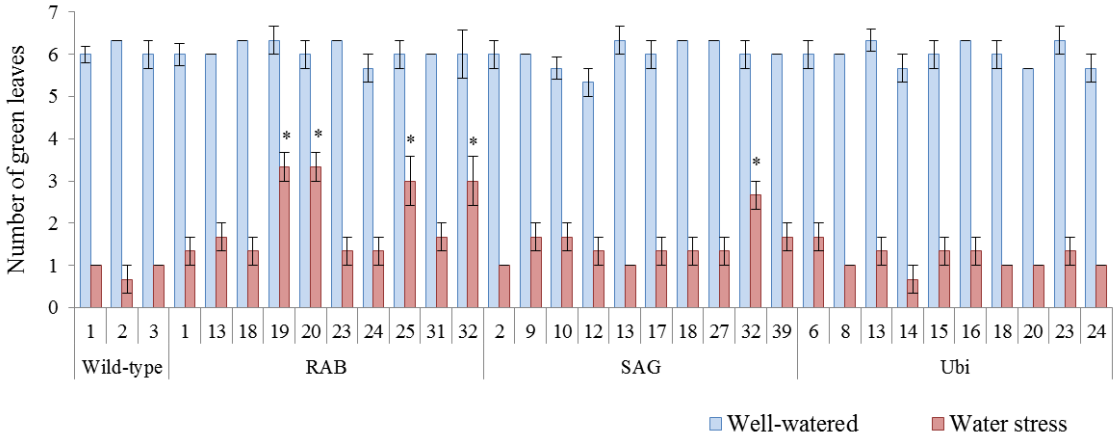

Figure 3-8: Number of green leaves of sugarcane plants after 60 days under well-watered (100% field capacity) and water stress (30% field capacity) conditions. SAG = P_{SAG12}*::IPT*; RAB = P_{RAB17}*::IPT*; Ubi = P_{Ubi}*::IPT*. Values are mean of each line; n = 3. The error bars show standard error (SE±). * indicates a significant difference at P<0.05 (Tukey HSD test) compared to wild-type and other transgenic lines within water regime.

3.3.2.3 Stalk elongation

Water stress treatment dramatically reduced stalk elongation among all plants evaluated in the experiment. On average stem elongation of plants in the water stress treatment were almost 10-times shorter than those grown at 100% field capacity. There was no significant difference in stalk elongation between different lines in both water conditions (Figure 3-9).

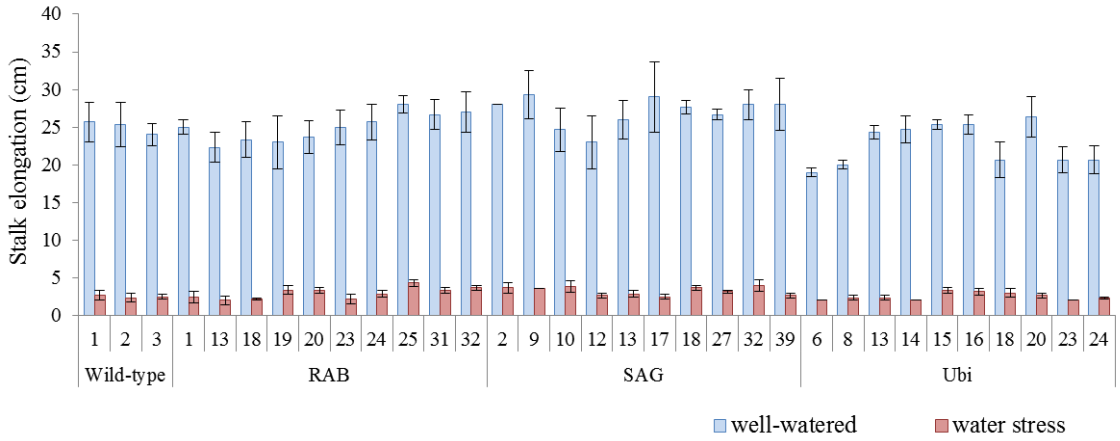

Figure 3-9: Stalk elongation of sugarcane plants after 60 days under well-watered (100% field capacity) and water stress (30% field capacity) conditions. SAG = P_{SAG12}*::IPT*; RAB = P_{RAB17}*::IPT*; Ubi = P_{Ubi}*::IPT*. Values are mean of each line; n = 3. The error bars show standard error (SE±).

3.3.2.4 Above-ground biomass

Above-ground biomass of water stressed plants was reduced by ~75% compared to the non-stressed controls after 60 days of treatment (Figure 3-10). Variation in biomass production was observed among lines within each water treatment but the difference was not significant.

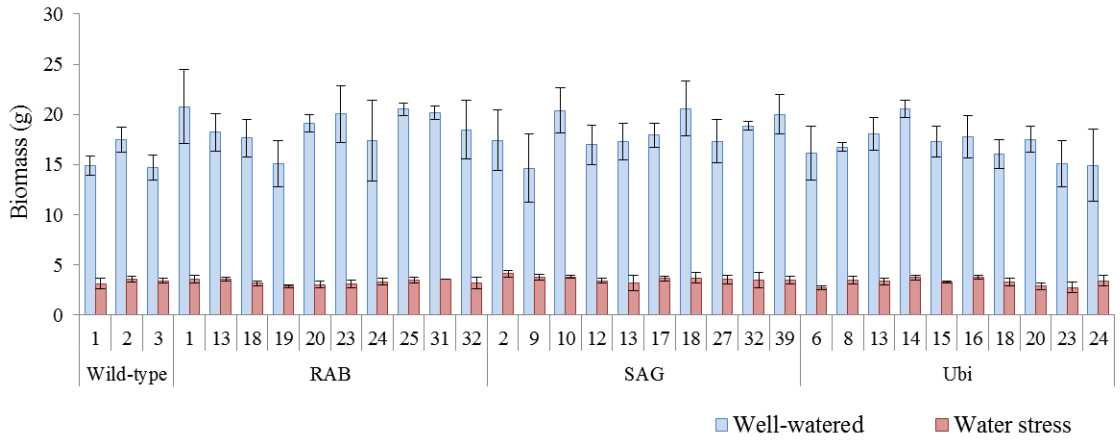

Figure 3-10: Above-ground biomass of sugarcane plants after 60 days under well-watered (100% field capacity) and water stress (30% field capacity) conditions. SAG = P_{SAG12}*::IPT*; RAB = P_{RAB17}*::IPT*; Ubi = P_{Ubi}*::IPT*. Values are mean of each line; n = 3. The error bars show standard error (SE±).

3.3.3 Quantitative reverse transcription PCR result

Results of *IPT* transgene expression studies confirmed the conditional (stress-dependent) activation of *SAG12* and *RAB17* promoters as expected (Figure 3-11). There was no *IPT* transgene transcript found in wild-type plants. The relative *IPT* expression levels were variable across all lines and active under water stress compared to well-watered conditions. In contrast to plants expressing *IPT* driven by stress-inducible promoters *SAG12* and *RAB17*, in *Ubi* promoter lines, the *IPT* expression was high in both water conditions (Figure 3-11). Ubi6, Ubi8, Ubi16, Ubi23 and Ubi24 plants had significantly ($P<0.05$) higher *IPT* expression than other transformants in both water supply conditions (Figure 3-11). However, there were significant genotype lines and water treatment effects but there was no interaction between genotype and water treatments (Appendix 2-3 and 2-4).

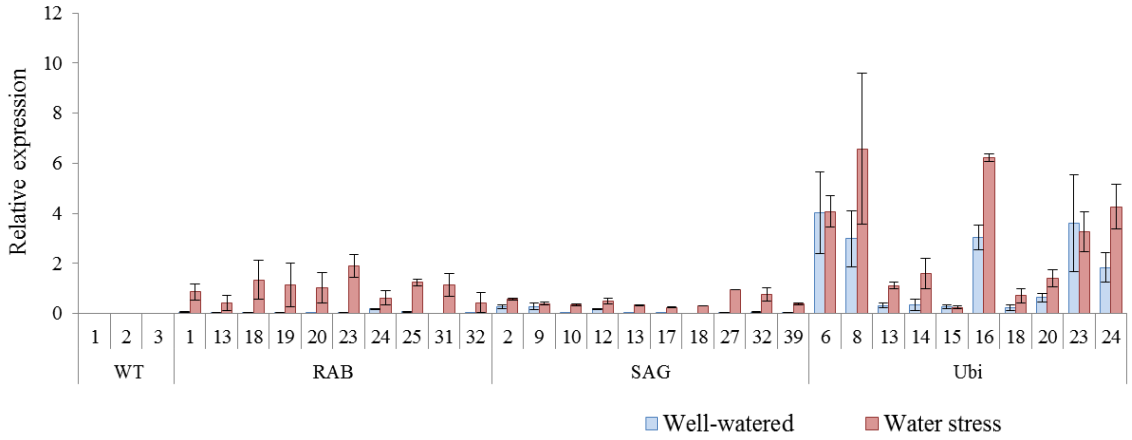

Figure 3-11: Relative expression of *IPT* genes in leaf tissue of wild-type and transgenic plants under well-watered (100% field capacity) and water stress (30% field capacity) conditions after 60 days of treatment. SAG = P_{SAG12}*::IPT*; RAB = P_{RAB17}*::IPT*; Ubi = P_{Ubi}*::IPT*. Values were calculated and normalised against the average amplification of a reference gene (*ADF*, *Actin depolymerising factor*) as an internal control. Expression values were compared relative to a reference sample according to the $2^{-\Delta\Delta Cq}$ method. The values presented are the average of three replications. Bars indicate the standard error (±SE) of the mean.

3.4 Discussion

The primary objective of the experiment reported here was to identify lines that show tolerance to water stress for more detailed mechanistic studies. Accordingly, screening parameters were related to growth and senescence. We hypothesised that *SAG12* and *RAB17*-driven *IPT* transgenic lines will have a superior performance under water deficit as they were expected to up-regulate CK production only during stress induction. We expected that a refined conditional expression of *IPT*

will negate the action of ABA and ethylene and lead to adaptation to water stress. We also assumed that *Ubi*-driven *IPT* lines may be stress tolerant but be morphologically abnormal with high tillering phenotypes due to accumulated CK with constitutive expression of *IPT*. However, we obtained some unexpected results.

Four P_{RAB17}*::IPT* lines (RAB19, RAB20, RAB25 and RAB32) and one P_{SAG12}*::IPT* line (SAG32) showed distinct stress tolerance under prolonged stress condition. Yet neither of these lines nor other transgenic lines tested showed a significant growth advantage compared to wild-type plants under water stress or under full irrigation. This is not consistent with the results obtained with the external application of synthetic CK to non-transgenic sugarcane under well-watered and water stress condition, where CK-treated plants showed significant growth improvement irrespective of water availability. The observed lack of growth improvement could be due to the following reasons:

1. The stress condition imposed in the screening experiment may be too severe as all plants in the stress treatment showed very limited growth. This possibility was expected, but with RAB19, RAB20, RAB25, RAB32 and SAG32 showed stress tolerance, we maintained low water regime (30% FC) until the end of the 60-day screening to evaluate the survival of those lines, which they did. The uniformity of growth inhibition in all transgenic lines indicates the severity of water stress imposed in the experiment. This is supported by the observation that sugarcane growth is highly sensitive to water deficit (Nable et al., 1999) and a moderate level stress would be more appropriate for screening purpose.

2. The conditional expression of *IPT* may not be translating to the level of CK required to reduce growth inhibition in the stressed plants. The evidence for this can be derived from the gene expression and the phenotype of *Ubi-IPT* lines. *Ubi-IPT* lines showed much higher transgene *IPT* expression than *RAB17* and *SAG12* lines in stressed and non-stressed plants. But these lines did not outperform *RAB17* or *SAG12* lines under water deficit. Surprisingly, *Ubi-IPT* lines did not show morphological anomalies under full irrigation. This indicates that either *Ubi-IPT* lines may not be producing sufficient CK, possibly due to post-translational gene silencing (PTGS) which is not uncommon in sugarcane (Iyer et al., 2000).

3. Cytokinins vary in their biological activity with synthetic CK like 6-benzyladenine (BA) being very stable and highly bioactive compared to native CK like zeatin and 2-IP (2, iso-

pentenyladenine) (Davies, 2004). Five transgenic lines showed clear signs of stress tolerance such as green leaf area retention and high chlorophyll content which indicates that the stress-inducible promoter may be activating CK at the right time of stress induction and reduce senescence. However, native CK may be not as bioactive as the synthetic ones or may be more prone to degradation by cytokinin oxidase and hence the growth differential observed between 6-benzyladenine-treated and transgenic lines under water stress.

Although the transgenic lines did not have a growth benefit, the results show that under progressive drought stress conditions, RAB19, RAB20, RAB25, RAB32 and SAG32 retained significantly higher CCI and more green leaves than wild-type and other transgenic plants under water deficit. Similar results were also reported in *IPT* transgenic cotton (Kuppu et al., 2013), peanut (Qin et al., 2011) and tobacco (Rivero et al., 2010) with reduced water supply.

Analysis of *IPT* transgene expression revealed a greater expression in water stressed plants than well-watered plants which was expected from the stress-inducible promoters used here. This result is in line with the reports of *IPT* up-regulation in P_{SAG12}*::IPT* transgenic tobacco (Cowan et al., 2005), wheat (Sykorova et al., 2008) creeping bentgrass (Xu et al., 2009) and cassava (Zhang et al., 2010) in response to drought stress treatment. In addition, we observed that *IPT* expression was also up-regulated under well-watered treatment in most of the P_{Ubi}*::IPT* plants which is expected as *Ubi* is a constitutive promoter (Christensen and Quail, 1996), however there was no obvious difference in the phenotype of these lines. Although a range of plants were produced with varying *IPT* transcription levels, none of the phenotypic measurements correlated with transgene expression levels in our experiment. This result is in agreement with (Hewelt et al., 1994) who showed that tobacco had the same phenotype although *IPT* was up-regulated. Such lack of correlation between transgene expression and phenotype has also been reported in field-grown sugarcane (Joyce et al., 2014).

Positional effects of the transgene on region genome could be an explanation for the variation in transgene transcription observed here. Transgenes integrated into an inactive region of the genome may not be transcribed (Cremer et al., 2006). This could be due to repressive influences exerted by flanking plant DNA on transgenes or positioning of transgenes in an unfavorable chromosomal

location (Matzke and Matzke, 1998). In addition, transgene silencing is another explanation for the lack of transgene activity that is observed in sugarcane (Iyer et al., 2000).

For further mechanistic studies four *IPT* transgenic lines (RAB19, RAB20, RAB25, and SAG32) that showed a delayed senescence phenotype were selected and the results presented in the following chapters.

CHAPTER 4: GROWTH AND PHYSIOLOGICAL RESPONSES OF TRANSGENIC SUGARCANE WITH ALTERED CYTOKININ BIOSYNTHESIS TO WATER STRESS

4.1 Introduction

Sugarcane is one of the fastest-growing crops requiring large quantities of water and nutrients to sustain its productivity (Inman-Bamber, 2014, Lakshmanan and Robinson, 2014, Robinson et al., 2013). Despite intensive breeding and advanced agronomy, even the currently best commercial crop yield is reported to be ~50-60% of the potential yield (Inman-Bamber, 2014). The productivity of sugarcane is largely defined by water availability and radiation use efficiency (Basnayake et al., 2012, Muchow et al., 1997). Inhibition of photosynthesis is one of the primary physiological functions affected by drought stress (Lakshmanan and Robinson, 2014). By regulating stomatal activity, plants balance water loss and CO_2 uptake when environmental conditions are unfavorable (Arve et al., 2011).

In addition to stomatal limitation, inhibition of photosynthesis occurs through reductions in leaf area and chlorophyll content (Sage et al., 2013). In sugarcane, water restriction affects crop canopy development negatively by inhibiting leaf expansion and accelerating leaf senescence (Inman-Bamber, 2004) which adversely affects sugarcane yield and productivity by reducing interception of radiation and photosynthesis, and consequently biomass production (Lawlor and Tezara, 2009, Gan, 2010, Ribeiro et al., 2013).

Since photosynthesis is a major driver of biomass production, enhancing productivity during water deficit by optimising stomatal response, preserving chlorophyll content and photosynthetic potential is likely to be valuable. One approach to reduce or delay the stress-induced responses, especially stomatal closure and senescence, is by maintaining hormone balance, especially cytokinin (CK) which decreases in stressed plants, through external supply or transgenic approaches (Ha et al., 2012, Spichal, 2012).

Application of exogenous CK to plants increases transpiration under water stress and non-stressed conditions (Davies and Zhang, 1991, Pospíšilová and Baťková, 2004, Pospisilova et al., 2005). Similarly, water stressed plants treated with CK had increased chlorophyll content and photosynthesis (Rulcova and Pospisilova, 2001, Hu et al., 2012). There are numerous reports of delayed senescence and chlorophyll degradation and preservation of structural and functional integrity of leaf cells by CK (Botha et al., 2014) under non-stress conditions.

In addition to exogenous manipulation, following the discovery of an *isopentenyltransferase gene* (*IPT*) from *Agrobacterium tumefaciens* and its role in cytokinin biosynthesis (Barry et al., 1984), efforts were made to express this gene to up-regulate the production of CK to improve growth under water stress conditions (Kuppu et al., 2013, Qin et al., 2011, Merewitz et al., 2011d, Rivero et al., 2010). The above findings point to potential applications of CK for improving or stabilising agricultural yields where crop growth is limited by water (Ha et al., 2012).

The *IPT* transgenic sugarcane plants showing drought tolerance and non-transgenic lines (for external CK treatment) were used to study their physiological responses to water deficit. It is hypothesised that CK may enhance water stress tolerance by reducing the effects of water deficit on photosynthesis and delaying stress-induced senescence in sugarcane. The results of the experiment conducted to test this hypothesis are presented below.

4.2 Methods and materials

4.2.1 Plant material and growth conditions

Please refer to Chapter 3 for plant material and growth conditions of non-transgenic and *IPT* transgenic lines (RAB19, RAB20, RAB25 and SAG32) used in the work reported here. These lines were derived from Australian commercial sugarcane variety $Q208^{A}$. A pot experiment was conducted from October to December 2013 in a PC2 glasshouse located at Sugar Research Australia, Indooroopilly, Australia. Average temperature in glasshouse was 35°C/20°C day/night, and the relative humidity ranged between 30 and 60%. At midday, the light intensity was ~800-1000 µmol s^{-1} m^{-2}. Nutrients (Thrive Soluble All Purpose, Yates, New Zealand) were applied through irrigation every fortnight. To minimise soil evaporation, the soil surface was covered with

2 cm polyethylene beads (Qenos Pty Ltd, Australia). At the beginning of the experiments, pots were water saturated and allowed to drain freely until there was no change in weight. The difference between wet and dry weight of potting mix was used to calculate the field capacity (FC). To quantify evaporation through the polyethylene beads, six pots covered with polyethylene beads but without plants were included in the experiment. Evaporation from the pots was less than 5 g/day and was therefore considered insignificant as each pot received on average 900 mL of water.

4.2.2 Water stress treatment

Each pot consisted of approximately 2500 (±50) g of peat and sand (60:40 v/v) and had one plant. Unlike in the screening study, in this experiment, plants water stress (WS) was kept at moderate level, at 50% FC while well-watered (WW) plants were watered daily to maintain 100% FC. Pots were watered daily according to the weight loss of each pot to achieve the target level of water stress (50% FC). Experiment was conducted for a period of 50 days from the day irrigation was withdrawn for the gradual progression of water deficit to reach 50% FC. Under the glasshouse conditions of this experiment it took 5 days for the pots in the water stress treatment to reach 50% FC.

4.2.3 Exogenous cytokinin application

Each pot in the external CK supply (WT+CK) treatment received 25 mL of 100 µM BA applied through irrigation for both WW and WS condition every 48 h.

4.2.4 Measurement of physiological and morphological responses

Chlorophyll content index (CCI) measurement was performed in the middle portion of the youngest fully expanded leaves using a CCM-200 plus chlorophyll meter (Opti-Sciences, Inc., USA). The CCI values were measured once a week. In each treatment measurements (three readings/plant) were taken from three plants/replicate.

Stomatal conductance (g_s) was collected once a week from the middle portion of the youngest fully expanded leaves using a Leaf Porometer (Model SC-1, Decagon Devices, Inc.) between 10.00 am

and 2.00 pm. In each treatment measurements (one reading/plant) were taken from three replicate plants.

Photosynthetic rate (P_N) was measured using a LiCOR 6400 Photosynthesis system (LiCOR Inc., Lincoln, Nebraska, USA) from the middle portion of the youngest fully expanded leaves between 9.00 am and 3.00 pm. The measurements were taken when a steady-state (around 3–5 min) was obtained, at 2000 µmol photon m^{-2} s^{-1}, cuvette temperature set to 30 ˚C, 400 µmol mol^{-1} CO_2. In each treatment measurements (five readings per plant) were taken from three plants/replicate.

4.2.5 Measurement of plant growth parameters

Plant height was recorded from the base at soil level to the top visible dewlap (of youngest fully expanded leaf) of the main stalk. Tiller number was recorded at the end of the experiment.

Green leaf area of the entire plant (main stem + tillers) was determined with a Portable Leaf Area Meter (LI-3000C and LI-3050C, LI-COR Biosciences, Inc., USA).

Fresh and dry weight was determined at the end of the experiment. Aerial tissue was separated into main stem, tiller stem, main leaves and tiller leaves. Root tissue was collected by washing off the potting mix. Care was taken to recover taproot and most of the fine roots. Tissues were oven dried at 60 °C to a constant weight for biomass determination. Total plant biomass was the sum of main stem, tiller stem, main leaves, tiller leaves, dead leaves and root. Dead leaves were collected during the course of the experiment for final biomass determination. Biomass-to-irrigation ratio was determined by dividing the total volume of water used (upper surface of potting medium was fully covered with plastic beads to prevent evaporation) over the entire period of plant growth by biomass produced.

4.2.6 Experimental design

The experiment was carried out in a glasshouse with a 2-factor randomised complete block design with six replicates. In this experiment, a wild-type plant (WT; tissue culture derived), a WT with external CK supplied (WT+CK), three independent P_{RAB17}*::IPT* transgenic lines (RAB19, RAB20 and RAB25) and a P_{SAG12}*::IPT* transgenic line (SAG32) were studied under well-watered and water stress conditions. Two factor analysis of variance (ANOVA) was used to determine whether

genotype, water treatment and genotype by water treatments interactions were significantly different. When significant differences were noted, the Tukey HSD comparison method was used to determine where differences existed at $P<0.05$ levels. Means of one representative experiment are presented; n = 6. Statistical analyses were conducted using Statistix 10 software (Analytical Software. Tallahassee, USA).

4.3 Results

4.3.1 Growth responses to water stress

4.3.1.1 Total leaf area

Water stress caused considerable green leaf area reductions in all affected plants after 50 days of experiment, but the impact was significantly more severe in wild-type plants compared to transgenic and CK-treated plants (62% reduction in wild-type *versus* 52% reduction in WT+CK, 45% reduction in RAB19 and ~40% reduction in RAB20, RAB25 and SAG32).There was no significant difference in leaf area among tested lines grown with full irrigation (Figure 4-1A). On day 50, green leaf area of all transgenic and CK-treated plants grown under water stress was significantly ($P<0.05$) higher than wild-type plants (Figure 4-1B).

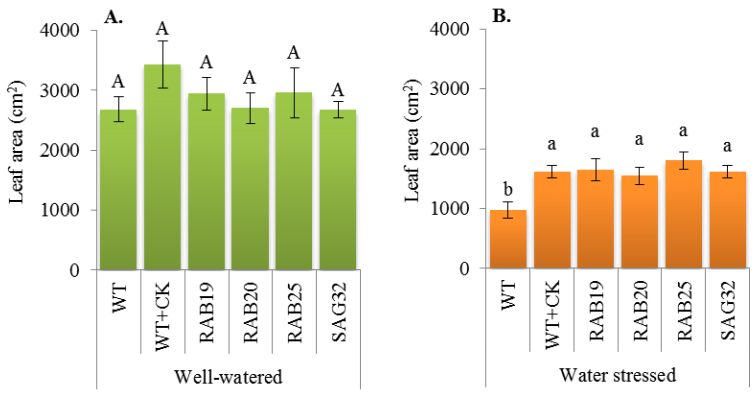

Figure 4-1: Leaf area of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Leaf area was quantified at the end of the 50-day experiment. Values are averages of six replications. Bars indicate standard error (±SE). Different letters above bars indicate significant differences among lines in the same water condition (Tukey HSD test at $P<0.05$).

4.3.1.2 Main stalk height

There was considerable variation in stalk height among transgenic lines in both water treatments. RAB25 plants were tallest (P<0.05) while clones of line RAB19 were shortest (P<0.05) in both water treatments (Figure 4-2A and 4-2B). There was a significant interaction between line and water treatment for main stalk height after 50 days of experiment (See appendix 2-5). Compared to well-watered wild-type plants, the water stressed counterpart and SAG32 plants were shorter by 35%, WT+CK plants by 39%, RAB19 and RAB20 plants by 37%, and RAB25 plants by 33% after 50 days of water stress (Figure 4-2B). A somewhat similar trend was observed by day 50 of the experiment with stalk height of plants under water stress treatment where RAB25 plants were significantly (P<0.05) taller (23 %) than wild-type plants and RAB19 plants were significantly (P<0.05) shorter (28 %) than wild-type plants (Figure 4-2B).

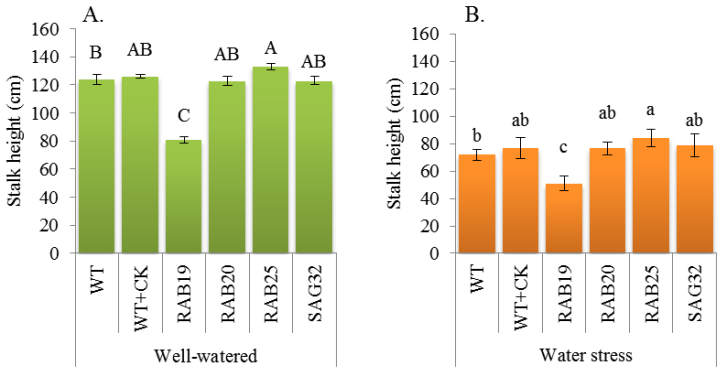

Figure 4-2: Main stalk height of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data was collected at the end of the 50-day experiment. Values are the average of six replicates. Bars indicate the standard error (±SE). The different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.3.1.3 Tiller numbers

While water stress reduced tiller numbers of wild-type plants by 50%, WT+CK and RAB19 plants displayed similar tiller numbers in both water conditions. In response to water stress, tiller numbers of RAB20, RAB25 and SAG32 were reduced by 22, 18 and 27%, respectively (Figure 4-3). Under well-watered conditions, RAB19 had at least 2-fold (P<0.05) more tillers than wild-type plants (Figure 4-3A). Other tested lines had similar tiller numbers under well-water conditions (Figure 4-7A). Under water stress conditions, RAB19, WT+CK and RAB25 had significantly (P<0.05) more tillers than wild-type plants (Figure 4-3B). Tiller numbers of RAB19 was 3.5-fold, RAB25 and WT+CK was 2-fold higher than those of wild-type plants (Figure 4-3B).

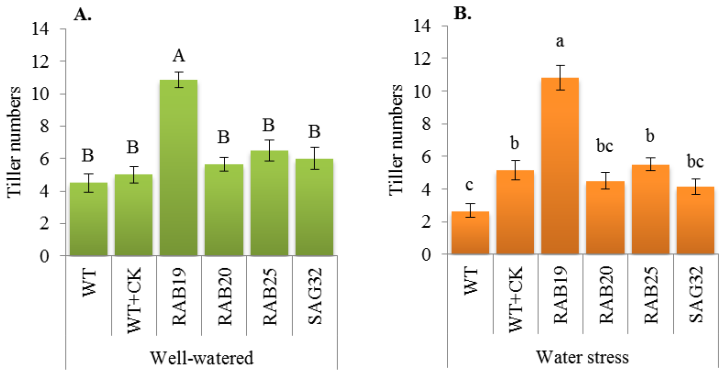

Figure 4-3: Tiller numbers in wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data was collected at the end of the 50-day experiment. Values are the average of six replications. Bars indicate the standard error (±SE). The different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.3.2 Biomass and biomass allocation

4.3.2.1 Total biomass

The interaction between line and water regime significantly (p=0.0032) effected total biomass after 50 days of experiment (See appendix 2-5). After 50 days under water stress conditions, total biomass was reduced in all six tested lines compared with those in the well-watered condition (~55% in wild-type, WT+CK plants and RAB19, ~50% in RAB20 and RAB25 and 40% in SAG32; Figure 4-4). Under well-watered conditions, RAB25 and WT+CK plants had at least 35% higher biomass than wild-type plants (P<0.05), while the biomass of other CK up-regulated plants was similar (Figure 4-4A). However, all tested lines, except RAB19, had significantly (P<0.05) higher biomass than wild-type plants (50% in RAB25, 40% in SAG32, 31% in WT+CK and 20% in RAB20) in the water stress treatment (Figure 4-4B).

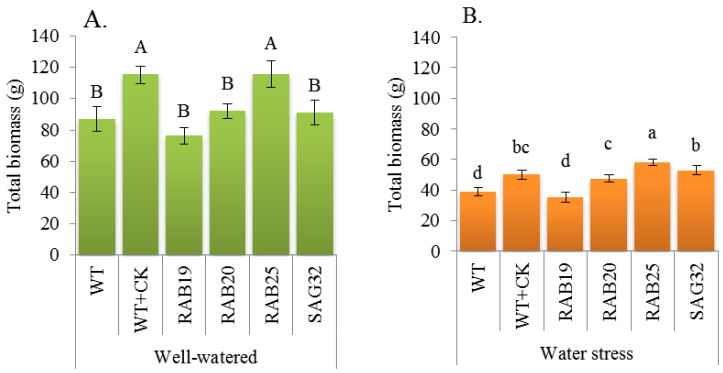

Figure 4-4: Total biomass of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data was collected at the end of the 50-day experiment. Values are the average of six replications. Bars indicate the standard error (±SE). The different letters above bars indicate significant differences among the lines in the same water conditions (Tukey HSD test at P<0.05).

4.3.2.2 Main stalk biomass

Main stalk biomass was reduced by water stress in all tested lines with 55% in wild-type, and between 40-50% in other lines after 50 days of experiment (Figure 4-5B). RAB19 had the lowest ($P<0.05$) main stalk biomass among all tested lines in both water treatments (Figure 4-5A and 4-5B). Similar to total biomass in water stress conditions, all CK up-regulated plants, except RAB19, had significantly ($P<0.05$) higher main stalk biomass than wild-type plants (Figure 4-5B). At the end of the experiment, the highest main stalk biomass was observed in RAB25 plants (65% higher than wild-type plants) followed by RAB20 and SAG32 (~30% higher than wild-type plants) and WT+CK (20% higher than wild-type plants) in the water stress treatment (Figure 4-5B). Significant ($P=0.0218$) interaction between line and water conditions was observed for main stalk biomass (See appendix 2-5).

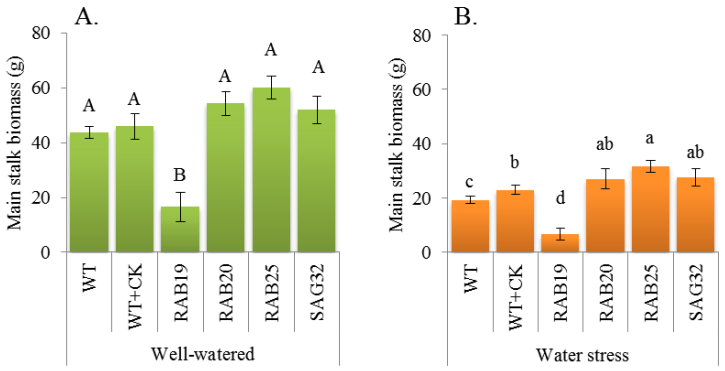

Figure 4-5: Main stalk biomass of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stressed conditions. Data was collected at the end of the 50-day experiment. Values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at $P<0.05$).

4.3.2.3 Tiller biomass

Wild-type plants treated with CK and RAB19 had the highest tiller biomass in fully irrigated plants (Figure 4-6A). Tiller biomass was reduced by water stress in all tested lines with wild-type showing the largest reduction (Figure 4-6B). Among the lines grown under water deficit WT+CK and RAB19 had significantly ($P<0.05$) higher tiller mass than wild-type plants (2.5 fold and 3.5 fold higher, respectively; Figure 4-6B). There was a significant ($P=0.0146$) interaction between line and water regime on tiller biomass after 50 days of experiment (See appendix 2-5).

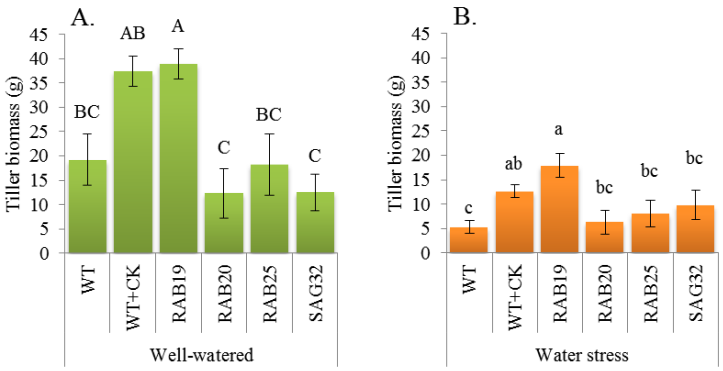

Figure 4-6: Tiller biomass of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data was collected at the end of the 50-day experiment. Values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.3.2.4 Dead leaf biomass

Water stress significantly increased (P<0.05) dead leaf biomass 2-fold in wild-type plants. CK-treated plants and transgenic lines had relatively smaller increases in senesced leaves under water stress compared to wild-type plants (Figure 4-7B). In general, there was no significant difference in dead leaf biomass under well-water conditions (Figure 4-7A). The interaction between line and water treatment was significant (P=0.0462) for dead leaf biomass (See appendix 2-5).

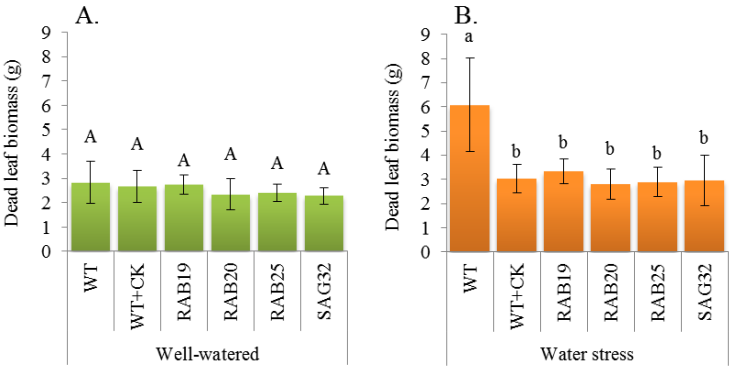

Figure 4-7: Dead leaf biomass of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data was collected at the end of the 50-day experiment. Values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.3.2.5 Root biomass

CK increased root mass production in water stressed plants and in RAB25 and WT+CK receiving full irrigation. RAB25 and WT+CK had significantly (P<0.05) higher (60 and 35%, respectively) root biomass relative to wild-type plants in well-watered treatment (Figure 4-8A). Water stress caused an extensive inhibition in root production of all tested lines relative to well-watered plants (Figure 4-8B). Under water stress conditions, WT+CK and transgenic lines (except RAB19) had significantly (P<0.05) higher root biomass than wild-type plants (70% higher in RAB25 and ~50%

higher in WT+CK, RAB20 and SAG32; Figure 4-8B). After 50 days of treatment, a significant interaction effect (P=0.038) on root biomass was observed between line and water treatment (See appendix 2-5).

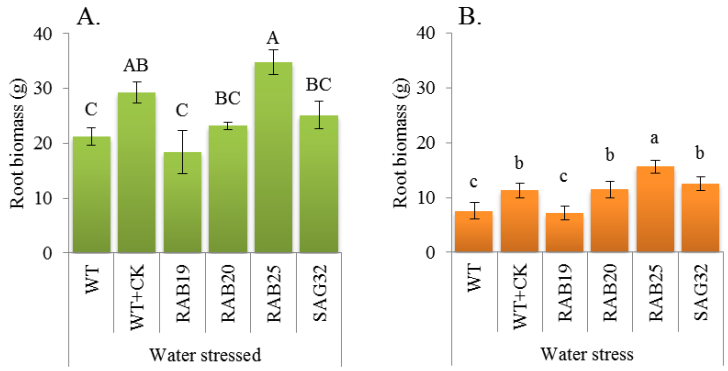

Figure 4-8: Root biomass of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data was collected at the end of the 50-day experiment. The values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.3.2.6 Root-to-shoot weight ratio

After 50 days of treatment, root-to-shoot ratio of RAB25 plants grown under water stress was significantly (P<0.05) higher than wild-type plants (Figure 4-9B). However, there was no significant difference in root-to-shoot ratio among tested lines grown with full irrigation (Figure 4-9A).

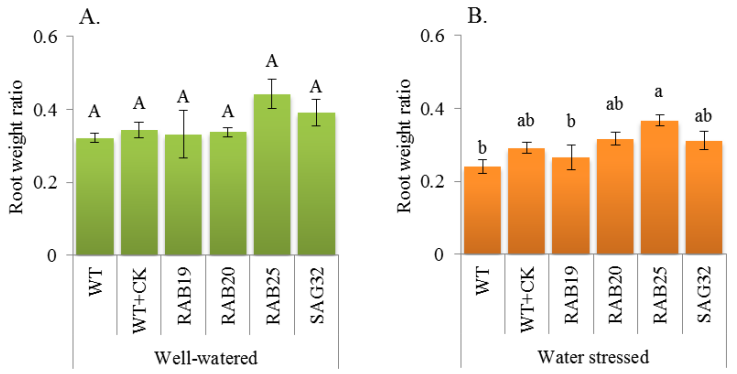

Figure 4-9: Root-to-shoot ratio of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Ratio was calculated from total root biomass to total shoot biomass after 50 days of experiment. The values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.3.2.7 Biomass-to-irrigation ratio

Biomass-to-irrigation ratio is defined here as plant's capacity to convert water into plant biomass, an estimate of water use efficiency (WUE). Enhanced cytokinin status significantly increased biomass-to-irrigation ratio in WT+CK and RAB25 in well-watered treatment and all CK-treated plants except RAB19 under water stress conditions relative to wild-type plants (Figure 4-10A and

4-10B). There was a significant (P=0.0391) interaction between line and water regime on biomass-to-irrigation ratio after 50 days of treatment (See appendix 2-5).

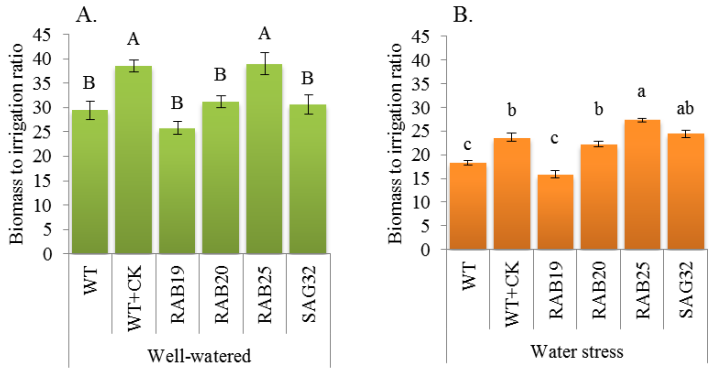

Figure 4-10: Biomass-to-irrigation ratio of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Ratio was calculated from total biomass to total amount of water used after 50 days of experiment. The values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

WT plant, a WT+CK plant, RAB19, RAB20, RAB25 and SAG32 transgenic lines showed no obvious phenotypic differences between lines in the well-watered treatment except that RAB19 plants were shorter with more tillers than others (Figure 4-11). Under water-stress conditions, wild-type plants showed reductions in overall growth compared to WT+CK and transgenic plants apart from RAB19 (Figure 4-11).

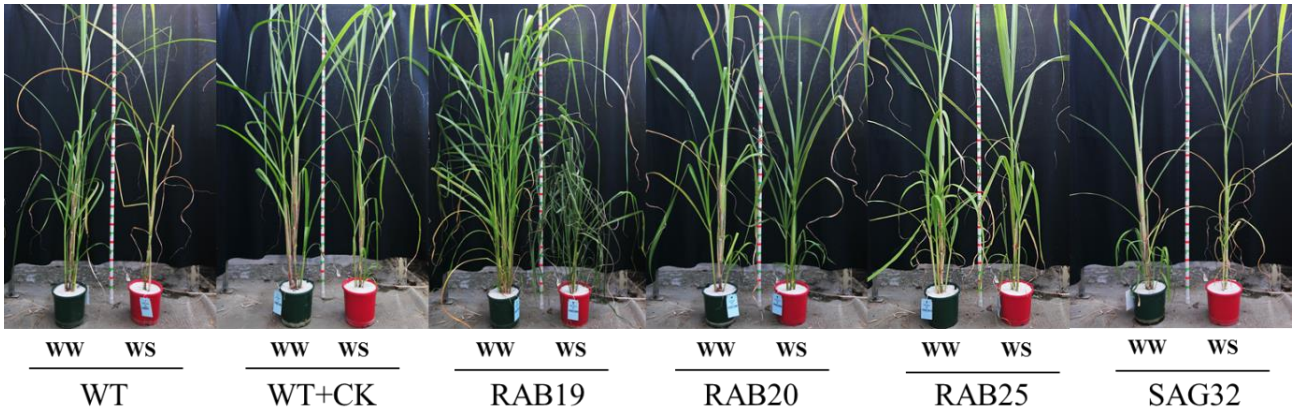

Figure 4-11: Phenotypes of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) grown under well-watered (WW) and water stress (WS) conditions after 50 days of treatment in the glasshouse.

4.3.3 Physiological responses to water stress

4.3.3.1 Stomatal conductance (g_s)

Stomatal conductance ranged between 300 to 400 mmol m^{-2} s^{-1} in all lines under well-watered condition with no significant differences throughout the experiment (Figure 4-12A). Stomatal conductance decreased progressively in all lines when plants were water restricted (Figure 4-12B). WT+CK, RAB25 and SAG32 had significantly ($P<0.05$) higher g_s relative to wild-type plants after 36 days of water stress treatment (Figure 4-12B). The g_s of RAB25 plants was ~2.3 -fold and WT+CK and SAG32 plants was ~1.8 fold greater than that of wild-type plants (Figure 4-10B). The g_s of wild-type plants was almost zero in the last two weeks of the experiment while RAB25 and SAG32 plants was ~150 and ~100 mmol m^{-2} s^{-1} on day 43 and day 50 (Figure 4-12B).

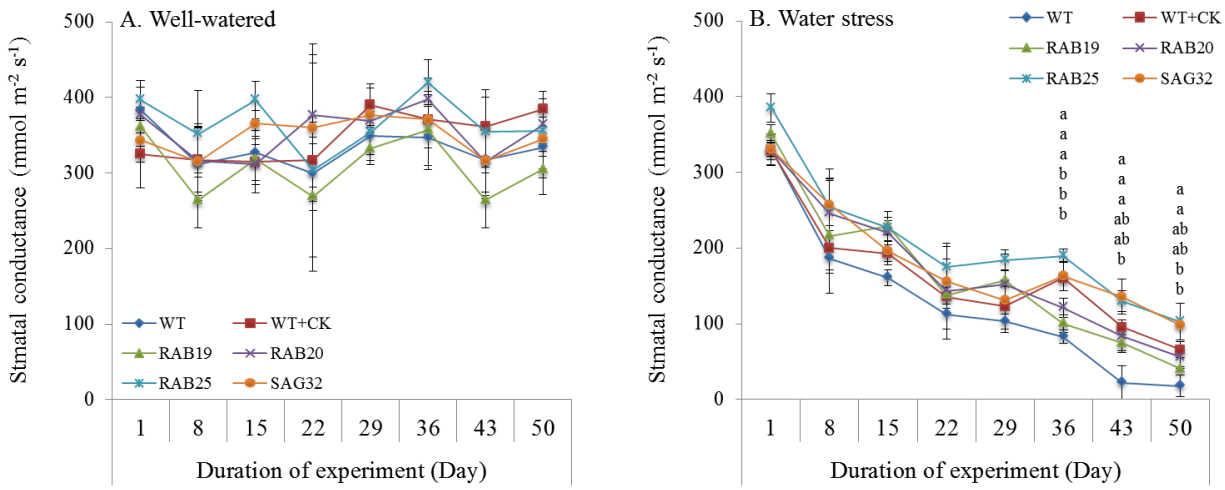

Figure 4-12: Stomatal conductance of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data were collected once a week using the youngest first fully expanded leaf under well-watered (A) and water stress (B) conditions. The values are averages of six replications. Bars indicate the standard error (±SE). Different letters at each panel indicate significant differences between lines, in order of top to base on each panel, according to Tukey HSD test at $P<0.05$.

4.3.3.2 Chlorophyll content index (CCI)

Under well-watered conditions CCI was similar (~26) in all lines throughout the experiment (Figure 4-13A). In water stress conditions, CCI of wild-type plants showed a much higher level of reduction

than that of CK up-regulated and CK supplied plants by day 36 (Figure 4-13B). CCI differed significantly (P<0.05) between lines under water stress after day 43 such that CCI was nearly 40% higher in RAB25 and SAG32 plants than wild-type by the end of the experiment (Figure 4-13B). Significant (P=0.0131) interaction between line and water condition was observed on CCI after 50 days of experiment (See appendix 2-5).

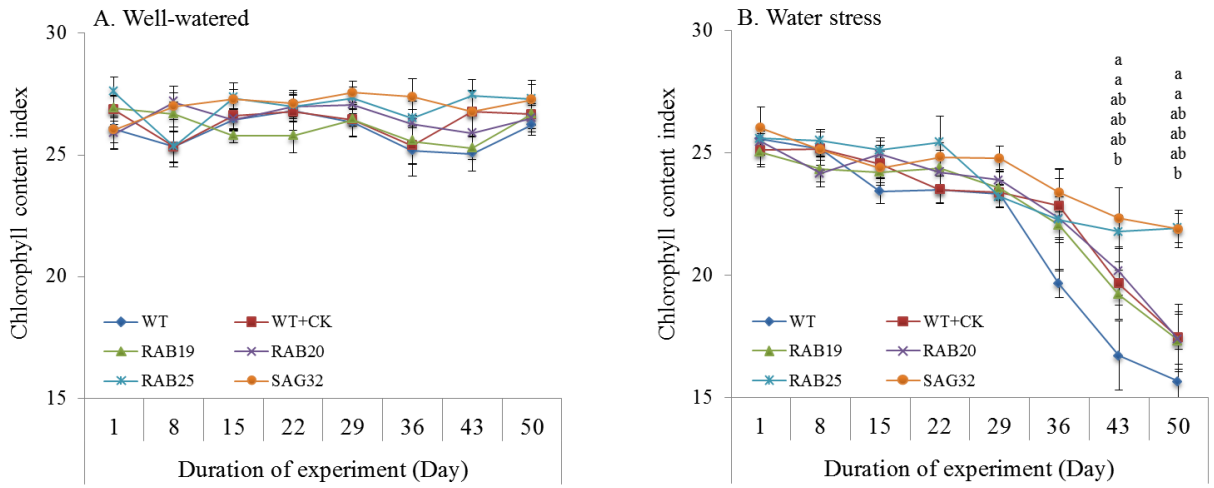

Figure 4-13: Chlorophyll content index (CCI) of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data were collected once a week using the youngest first fully expanded leaf under well-watered (A) and water stress (B) conditions. The values are averages of six replications. Bars indicate the standard error (±SE). Different letters at each panel indicate significant differences between lines, in order of top to base on each panel, according to Tukey HSD test at P<0.05.

4.3.3.3 Photosynthetic rates (P_N)

Photosynthetic rate (P_N) did not differ between the six tested lines during the experiment under well-watered conditions (Figure 4-14A). Photosynthetic rate decreased as a result of the imposition of water stress in all lines, and significant differences were observed on days 43 and 50 of the experiment (Figure 4-14B). On day 43, P_N of RAB25 and SAG32 was ~2-fold and 1.7 fold, respectively **(**P<0.05), higher than of wild-type plants (Figure 4-14B). This difference increased with time and RAB25 and SAG32 plants still had a considerable level of photosynthetic activity on day 50 under water stress conditions (Figure 4-14B).

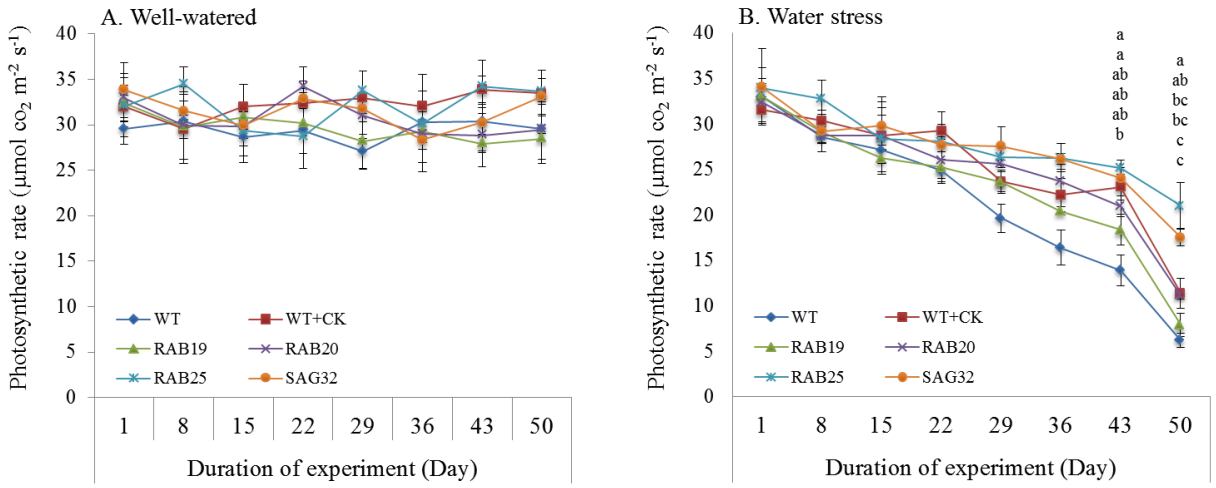

Figure 4-14: Photosynthetic rate of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Data were collected once a week using the youngest first fully expanded leaf under well-watered (A) and water stress (B) conditions. The values are averages of three replications. Bars indicate the standard error (±SE). Different letters at each panel indicate significant differences between lines, in order of top to base on each panel, according to Tukey HSD test at P<0.05.

4.3.3.4 Photosynthesis-to-transpiration ratio

In general, there was no significant difference in photosynthesis to transpiration ratio under well-watered conditions (Figure 4-15A). However, this ratio this ratio was significantly increased (P<0.05), more than 2-fold, in RAB25 plants under water stress, while other CK-treated water-stressed plants had a relatively smaller increase compared to wild-type plants (Figure 4-15B).

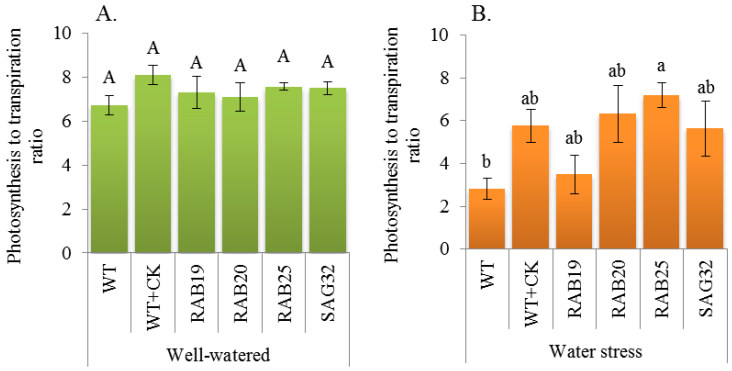

Figure 4-15: Photosynthesis-to-transpiration ratio of wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32) compared to WT plants under well-watered and water stress conditions. Ratio was calculated from photosynthesis rate to transpiration rate at 50-day experiment. The values are the average of six replications. Bars indicate the standard error (±SE). Different letters above bars indicate significant differences among the lines in the same water condition (Tukey HSD test at P<0.05).

4.4 Discussion

Reducing growth to conserve water is an adaptive response to water deficit of most plants (Chaves et al., 2002). Often this is achieved by, or accompanied by, reduced carbon fixation and resultant conservation of water. This aspect has not been extensively researched in sugarcane and the mechanistic basis of water stress responses is not well understood (Lakshmanan and Robinson, 2014). The result presented here clearly shows that CK plays a regulatory role in stomatal function, photosynthesis and senescence in sugarcane, and response to water deficit that showed improved biomass production under water deficit. The results presented here also show that stomatal conductance was the most sensitive to CK up-regulation treatments under water stress conditions (Figure 4-16).

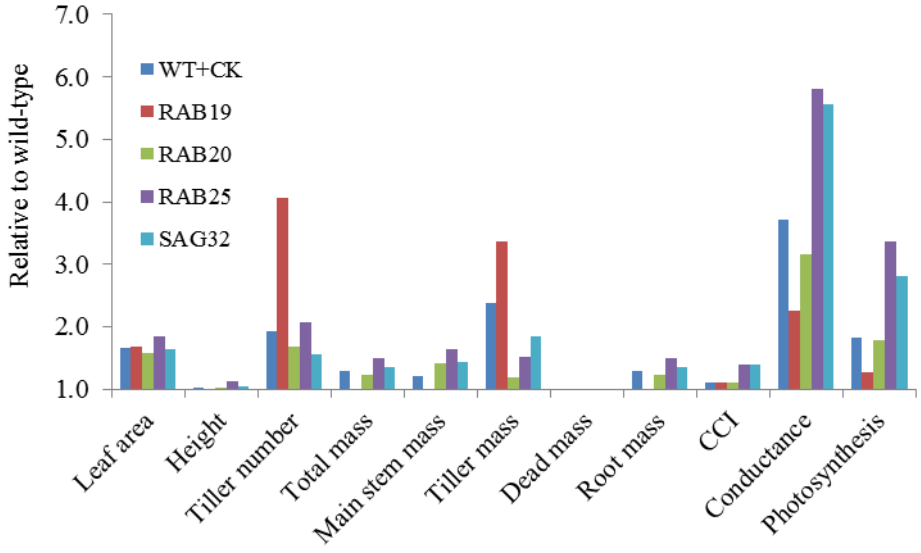

Figure 4-16: The relative sensitivity of different growth parameters of different experimental plants [wild-type (WT) plants supplied with cytokinin (WT+CK) and cytokinin up-regulated transgenic sugarcane lines (RAB19, RAB20, RAB25 and SAG32)] to water stress. The data are collected after 50 days of stress treatment.

Stomata are a highly effective at balancing water loss and carbon gain in plants. Limiting g_s is a common response of sugarcane to water deficit, and there is large genetic variability for this trait (Basnayake et al., 2012). Stomata respond to most environmental and many internal cues to optimise water and carbon relations and growth under stress and non-stress conditions (Dodd, 2003). By virtue of being the nexus of various regulatory inputs related to water relations, stomates are also the point of cross-talk between various local and long-distance signaling networks, many of which are under hormone control (Kohli et al., 2013). RAB25 and SAG32 transgenic plants and

those that received external supply of CK displayed statistically significantly higher g_s than wild-type plants after 36 days of water stress treatment. Similar results were observed in *IPT* up-regulated transgenic peanut under water stress conditions (Qin et al., 2011) and tomato under salt stress conditions (Ghanem et al., 2011) and tobacco (Wang et al., 1997) in non-stressed condition. These results suggests that conditional over-expression of the *IPT* transgene reduces the effects of water stress on stomatal function. The possible mechanism(s) supporting stomatal opening in sugarcane is speculative but some logical explanation may describe the observed phenotype.

When the plant experiences water deficit at the root level, it signals stomates to limit water loss (Lakshmanan and Robinson, 2014). Abscisic acid is implicated in this long-distance signaling as it is one of the first rapid biochemical changes occurs in plants with a gradual reduction in growth promoting hormones, particularly CK and gibberellins (Botha et al., 2014). Because CK is antagonistic to ABA, its down-regulation amplifies ABA action and leads to rapid stomatal closure (Wilkinson et al., 2012). This appears to be the case in sugarcane as g_s was greatly reduced by water stress and this effect was considerable reversed by external supply of CK (Figure 2-9) or by increased endogenous production by transgenesis. What is interesting is that stress-induced up-regulation of CK or external supply of CK *via* irrigation caused increased root production in our plants, which is opposite of what is generally seen in CK over-producing plants (Hewelt et al., 1994, Wang et al., 1997). CK is an inhibitor of root production while ABA may promote rooting in stress conditions in a species dependent manner (Kohli et al., 2013, Ha et al., 2012). Increased rooting in response to CK application may be a reflection of ABA-induced rooting and CK's effect in moderating ABA actions on stomatal function. Plants with greater root mass sustain more water supplies thus facilitating higher transpiration rates (Inman-Bamber et al., 2012). Similar to our study, improved root growth and drought tolerance was reported in P_{SAG12}*::IPT* creeping bentgrass (Merewitz et al., 2010, Xu et al., 2009). Our results also agree with Endres et al. (2010) who suggested that a higher root density allows sugarcane to maintain stomatal opening over a broad range of leaf water potential.

While the present study revealed the responses of g_s to water stress, non-stomatal limitation is also considered an important cause of reduced in photosynthesis and growth in plants exposed to water stress (Flexas et al., 2006, Ghannoum, 2009, Endres et al., 2010, Singh and Raja Reddy, 2011, Pinheiro and Chaves, 2011). In sugarcane, light and dark reactions are less sensitive to stress than

stomates as photosynthesis continues for a considerable time when stomates are closed (Sage et al., 2013). However, severe or prolonged water deficit affects the integrity of photosynthetic machinery and contributes to the inhibition of photosynthesis. An inevitable consequence is chlorophyll breakdown and leaf senescence. In the presence of CK the impact of stress on all these processes was considerably reduced. Cytokinins protect nucleic acids, proteins and membranes and preserve structural and functional integrity of cellular organelles including chloroplast (Davies, 2004, Taiz and Zeiger, 2010). The data presented here demonstrate that CK up-regulated plants were able to maintain higher chlorophyll content and had less dead leaf biomass than wild-type plants over 50 days of drought exposure. CK is the key regulator of leaf senescence which is delayed by CK application or transgenic up-regulation with *IPT* genes under water stress conditions (Rivero et al., 2010, Merewitz et al., 2011d, Qin et al., 2011, Kuppu et al., 2013). Leaf area is an important component for photosynthesis (Inman-Bamber, 2014) and results obtained here demonstrate that the leaf area of CK up-regulated plants was significantly higher than wild-type plants under water stress. These findings are consistent with reports of drought-tolerant genotypes in a wheat stay-green mutant (Tian et al., 2012, Saint Pierre et al., 2012).

In addition, this study shows that water stress reduced growth and development as evidenced by the decreased biomass production of all plants in the stress treatment. Reduction in stalk elongation, stalk biomass and tillering are common responses in sugarcane subjected to drought stress (Lakshmanan and Robinson, 2014, Ribeiro et al., 2013). In this study, water stress significantly decreased stalk elongation, stalk biomass and tiller production in wild-type plants but to a lesser extent in plants with higher CK status. The reason for the CK-dependent growth stimulation in sugarcane is not known but as discussed in the previous sections, additional CK may be negating the growth inhibitory effects of stress hormones, ABA and ethylene (Ha et al., 2012, Zalabák et al., 2013). Water stress also inhibits GA production or action (Colebrook et al., 2014) which may be the direct cause of water-stress induced inhibition in stalk elongation. Increased CK levels may indirectly improve endogenous GA levels and improving stalk growth by its action on ethylene and ABA.

4.4.1 Conclusions

Based on the data presented in this chapter, the following conclusions can be made:

1. The stress level used in the screening experiments (30% FC) was too high and hence 50% FC was used to study the mechanistic basis of CK regulation of water stress response in sugarcane. It is possible that the stress levels experienced by plants here are similar to the moderate level of recurring water stress occurring in commercial production conditions as observed in a related sugarcane study under field condition (Basnayake et al., 2012, Basnayake et al., 2015). To confirm this statement, measurements of leaf water relations need to be undertaken.

2. Increasing the production of CK at the time of stress by driving transgenic *IPT* with stress-responsive promoters appears to be an effective strategy to improve water stress tolerance in sugarcane.

3. Cytokinin has a regulatory role in sugarcane stomatal functioning and photosyntheis. Physiological and phenological responses of plants under water stress receiving an external supply of CK or up-regulated endogenous CK levels included higher stomatal conductance, increased photosynthesis and reduced leaf senescence.

4. Increasing endogenous CK content by transgenesis or external supply at the time of stress improves overall growth, development and biomass production.

CHAPTER 5: WATER STRESS-INDUCED CHANGES IN HORMONE CONTENT AND HORMONE-RELATED GENE EXPRESSION IN TRANSGENIC SUGARCANE WITH ALTERED CYTOKININ PRODUCTION

5.1 Introduction

Plants respond to water deficit and adapt to drought stress through various physiological, biochemical and molecular mechanisms (Mantri et al., 2012, Skirycz and Inze, 2010, Wilkinson et al., 2012). A component central to these adaptive and growth regulatory mechanisms is plant hormones, with a major role in stress signal perception, signal transduction and regulating various cellular and growth process in response to water deficit (Dodd, 2003, Dodd and Davies, 2010, Wilkinson et al., 2012).

Plant hormones are small molecules that regulate growth and development in responses to changing environmental conditions (Davies, 2010a). Hormones modify gene expression, determine cells, tissue and organ differentiation, and facilitate the necessary growth and developmental plasticity to complete the life cycle of an organism, including plants (Santner and Estelle, 2010). It is now well established that the level of endogenous hormones changes in response to both biotic and abiotic stresses (Taiz and Zeiger, 2010, Davies, 2010f, Dodd and Davies, 2010). Ha et al. (2012) reported low CK levels, shoot growth reduction and onset of senescence in response to water stress.

Cytokinins (CK) are a class of growth substances or phytohormones that promote cell division, or cytokinesis, in roots and shoots. Cytokinins are involved primarily in cell growth and differentiation, but also affect apical dominance, axillary bud growth, and leaf senescence (Mok and Mok, 2001). Xylem exudate and/or leaves of drought-affected plants usually exhibit reduced CK content and activity. The explanation for reduce CK content under water stress is either a reduction in CK biosynthesis or enhanced CK degradation (Hwang et al., 2012). Under drought stress, biologically important CKs isopentenyladenine (iP), isopentenyladenosine (iPR), and zeatin-types

CK decreased in leaves of tobacco (Cowan et al., 2005), wheat (Sykorova et al., 2008), creeping bentgrass (Xu et al., 2009) and cassava (Zhang et al., 2010).

In response to abiotic stress, especially water stress, abscisic acid (ABA) signaling and ABA-responsive genes are up-regulated (Vankova, 2012). ABA synthesis is one of the fastest responses of plants to water deficit, triggering ABA-inducible gene expression (Yamaguchi-Shinozaki and Shinozaki, 2006) and causing stomatal closure, thereby reducing water loss *via* transpiration (Wilkinson and Davies, 2010) and eventually restricting cellular growth. The first committed step in the ABA synthesis is catalysed by an epoxy-carotenoid dioxygenase (Schwartz et al., 2003, Tan et al., 1997). For e.g. in maize, the *Viviparous14* (*Vp14)* gene is involved in the synthesis and perception of ABA, and expression induced in leaves by drought stress (Tan et al., 1997).

Ethylene is up-regulated under stressful conditions such as heat and drought, and in stress-induced leaf senescence and abscission (Abeles et al., 1992, Morgan and Drew, 1997). Biosynthesis of ethylene occurs *via* a well-characterised biochemical pathway with two enzymes, ACC synthase (ACS) and ACC oxidase (ACO), playing regulatory role (Argueso et al., 2007). Under stress conditions, the generation of ethylene increased in shoots by up-regulating the synthesis and xylem transport from roots to shoots of the ethylene precursor (Sobeih et al., 2004, Belimov et al., 2009). Water stress induces ACS, raising the concentration of the ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC) (Morgan and Drew, 1997) which is followed by its conversion to ethylene by ACO (Morgan and Drew, 1997). ACC and ACS have been examined when studying the signaling and regulatory network of ethylene metabolism and action in many plant species (Argueso et al., 2007) including sugarcane (Wang et al., 2006) under a variety of environmental conditions.

Evidence is mounting for a central role for the hormone gibberellin (GA) in the regulation of plant growth under various environmental constraints (Colebrook et al., 2014, Yamaguchi, 2008). Reduction of GA levels and signaling contributes to plant growth restriction upon exposure to several stresses, including water stress (Colebrook et al., 2014). Suppression of GA signaling is a general response to abiotic stresses, with transcriptional up-regulation of *GA 2-oxidase* (*GA2ox*) and down-regulation of *GA 20-oxidase* (*GA20ox*) (Colebrook et al., 2014). Another key regulator of the GA signaling pathway is DELLA proteins (Achard and Genschik, 2009). Under stress conditions, DELLA proteins act as growth repressors, and GA induces the degradation of these proteins

(Achard and Genschik, 2009, Claeys et al., 2012). Transcription of genes encoding GA2ox and DELLA proteins demonstrate GA catabolism and repressive signaling represents a major route for control of growth and physiological adaptation in response to various environmental signals and stress conditions including drought (Zawaski and Busov, 2014). In addition, the reduction in *GA20ox* transcription results in low amounts of active GA in leaves of rice (Ashikari et al., 2002).

Auxin, one of the major plant hormones has been implicated primarily in developmental processes (Peer et al., 2011, Simon and Petrášek, 2011) and its involvement in stress/defense responses is now well documented (Ghanashyam and Jain, 2009). Recent evidence suggests a crosstalk between auxin and CK in non-stress conditions (Moubayidin et al., 2009), with CK and auxin interactions being fundamental for normal shoot and root development (Dello Ioio et al., 2008). However, the crosstalk between CK and auxin under stress conditions remains largely unclear.

Results obtained in this study have shown that the stress-induced stomatal closure and leaf senescence was considerably reduced and biomass increased in transgenic sugarcane plants expressing an *isopentenyltransferase* (*IPT*) gene under the control of P_{RAB17}, an ABA-responsive promoter. Here we hypothesise that in *IPT* transgenic plants the synthesis of other plant hormones is altered and that such changes may in part be responsible for the growth improvement observed in transgenic plants exposed to water deficit. A number of genes involved in hormone biosynthesis, especially ones that regulate responses to stress conditions, have been targeted here for gene expression analysis. However, homology searches for the native genes in sugarcane were not successful for all gene targets including native endogenous *IPT*. Only ACC *oxidase*, *ACC synthase*, *DELLA*, *GA 2-oxidase*, *GA 20-oxidase* and *Vp14* were successfully identified from published sugarcane sequence libraries. For hormone analysis, the phytohormone selection was focused on the physiological effects on plant's response to stress conditions including CK, ABA, GA and Auxin. However, ethylene and other known hormones were not analysed due to the limited resource for analyses. We therefore quantified biologically important CKs isopentenyladenine (iP), isopentenyladenosine (iPR), and *trans*-zeatin (tZ), ABA, gibberellin and indole-3-acetic acid (IAA) along with the expression of genes associated with their metabolism in leaf and root tissues of wild-type and transgenic sugarcane (RAB25) exposed to water stress.

5.2 Methods and materials

5.2.1 Plant material and growth conditions

Sugarcane (*Saccharum officinarum* L. var. Q208^{A}) plants of a selected transgenic line (RAB25) and wild-type were used in this study. The experiment was conducted from April to July 2014 in a PC2 glasshouse located at Sugar Research Australia, Indooroopilly, Australia. Plantlets used in this experiment were produced in tissue culture and transplanted into potting trays (5 × 6 wells) with peat and sand (25:75v/v) in April 2014. Thirty-day-old plantlets were transferred to 2.4 L pots (Garden city plastic, Australia) with peat and sand (25:75v/v) with full irrigation in the glasshouse. Nutrient solution (Thrive Soluble All Purpose, Yates Australia) was applied once a week through irrigation. The transplanted seedlings were grown in a greenhouse at 25-35 ˚C for 30 days and uniform seedlings were chosen for the study. To minimise soil evaporation, the soil surface was covered with polyethylene beads (Qenos Pty Ltd, Australia). Seedlings with uniform growth were selected as experimental materials, and water stress treatment started.

5.2.1.1 Water stress treatment and sampling time points

In this experiment, a well-watered treatment (control) was maintained at 100% field capacity (FC) across all sampling time points. The water stress treatment involves a gradual increase in water stress by withdrawing irrigation water. Target FC of 75, 50 and 25% were achieved by monitoring weight loss of pots in the stress treatment (Earl, 2003). At day 0 of the experiment, all tissue samples were collected with pots kept at 100% FC. At day 3, 6 and 10 of the experiment, plants in the water stress treatment were at 75, 50 and 25% FC, respectively.

5.2.1.2 Experimental design

The experiment was carried out with a randomised complete block design with four replications (four separate plants for each treatment/time point) for the gene expression study in each sampling time point. Due to high cost for hormone analysis, only two replicates (two separate plants for each treatment/time point) were used in each sampling time point. Sampling time points were designed to investigate molecular and hormonal changes at different water stress levels and samples were collected between 11.00 am – 1.00 pm. Data of each sampling time point were analysed separately.

One-way analysis of variance (ANOVA) was used to determine if means of four treatments (WW-Wild-type, WW-RAB25, WS-Wild-type and WS-RAB25) were significantly different. When significant differences were noted, the Tukey HSD comparison was used to establish the level of significance. Means of four plants are presented in the gene expression study and two plants in the hormone analysis study. Statistical analyses were conducted using Statistix 10 software (Analytical Software. Tallahassee, USA).

5.2.2 Gene expression analysis

5.2.2.1 Samples preparation for quantitative reverse transcription PCR

Total RNA was extracted from the youngest fully expanded leaf of transgenic and wild-type plants, using RNeasy Mini Kits (Qiagen). RNA concentration was quantified using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). A total of 1 µg RNA was treated with 2 units of RQ1 RNase-Free DNase (Promega, USA). First strand cDNA was synthesised in a 20 µL reaction volume containing Improm-II 5× Reaction Buffer (Promega), 0.5 µg random primers (Invitrogen), dNTPs equating to a final concentration of 0.5 mM (Promega), and 1 µL Improm-II Reverse Transcriptase (Promega). Samples were incubated at 25 °C for 5 min, 42 °C for 60 min and then 70 °C for 15 min. After cDNA synthesis, PCR was used to check for gDNA contamination using *sugarcane phosphofructokinase 5* (*ScPFK5*) specific primers PFK5 forward (5'-AGCCACATCA GATCAACAAG-3') and PFK5 reverse (5'-TGAAGTTATACCCTGCCATT-3') that are positioned either side of an intron. These primers gave different sized PCR products, depending on whether the template was cDNA or gDNA. Contamination with gDNA results in a larger PCR product (450 bp). Before qRT-PCR, all cDNA samples were standardised by diluting to 5 ng μL^{-1}.

5.2.2.2 Primers preparation for quantitative reverse transcription PCR

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) primers were designed using an online primer design program (http://www.genscript.com/). Due to limited available expressed sequence tag (EST) information in sugarcane, the target sequences for genes of interest were based on other well-characterised plant genomes (e.g. maize, barley). Consensus sequences of genes of interest were aligned with sorghum genes to identify homologous sequences. The sorghum homologues were used as the primary target sequence to design degenerate primers for use in

sugarcane qRT-PCR because the sugarcane and sorghum genomes are mostly collinear in the genic regions, and the sorghum genome can be used as a template for assembling much of the genic DNA of the autopolyploid sugarcane genome (Wang et al., 2010). ESTs with high similarity to the target gene in the sorghum genome sequence database were analysed, collected and assembled.

Three primer sets for each target gene were initially tested for specificity before use in qRT-PCR analysis. Primers were used in standard PCR with cDNA template from wild-type and transgenic sugarcane, and products were visualised using electrophoresis with a 1% agarose gel and 0.5 X TBE (Tris, Borate, EDTA) buffer at 100 V for 60 min. The thermal profile used was 94 °C for 5 min initial denaturation, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 3 min. The amplified PCR products were sequenced at AGRF to confirm the sequence identity. Optimisation of qRT-PCR primer concentrations and melt curve analyses were also performed before quantification. The primers are listed in Table 5-1.

Table 5-1: The list of primers and sequences used in quantitative reverse transcription polymerase chain reaction analysis.

Genes of interest	Primer	Sequence 5' to 3' orientation
ACC oxidase	SoACO F2	GCAGGTACAAGAGCGTGATG
	SoACO R2	GTAGAAGGACGCGATGGAC
ACC synthase	SoACS2 F2	GCTTCGCTAACATGAGCTTG
	SoACS2 R2	GCTGCGTCGATACTGTTGTT
DELLA	SoDella F1	CACCATGTTCGATTCTCTCG
	SoDella R1	CAGGTACACCTCGGACATGA
GA 2-oxidase	SoGA2ox F2	GAGGCCGTCAGGTTCTTC
	SoGA2ox R2	GCGAGGAGGAGGTACTCG
GA 20-oxidase	SoGA20ox F2	GCTTCTTCCAGGTGGTCAAC
	SoGA20ox R2	CGTGAAGAAGGCGTCCAT
Vp14	SoVP14 F2	GTGCTGGACATGGAGAAGAC
	SoVP14 R2	AGGTGGAAGCAGAAGCAGTC

Note: So = *Saccharum officinarum*

5.2.2.3 Quantitative reverse transcription PCR analysis

A ViiA™ 7 Real-Time PCR System (Life technologies, Australia) was used to measure samples. Reagents were mixed by hand and each reaction contained 5.25 µL of SensiMix SYBR Low-ROX (Bioline, Australia), 0.21 µL (200 nM) of each gene specific forward and reverse primer and 1.33 µL H_2O. An epMotion 5073 liquid handler (Eppendorf) was used to aliquot the master mix and 3 µL of 5 ng μL^{-1} cDNA into MicroAmp® Fast Optical 384-well reaction plates (Life technologies,

Australia). The thermal profile was 50 °C for 2 min, 95 °C for 10 min, 45 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by a dissociation step of 95 °C for 2 min, 60 °C for 15 s. No template controls (NTC) were used to check for contamination and primer dimers.

All qRT-PCR data generated was analysed using the DataAssist™ Software (Life technologies, Australia). For each cDNA sample, an average gene amplification level was calculated from three duplicate PCR reactions (technical replicates). This average expression for each gene was normalised against the average expression level of a reference gene (*ADF, Actin depolymerising factor*), the purpose of which was to account for template variations between samples. Then each expression level was compared relatively to a reference sample according to the $2^{-\Delta\Delta Cq}$ method (Livak and Schmittgen, 2001).

5.2.3 Hormone analysis

5.2.3.1 Sample preparation for hormone analysis

All samples were prepared at Sugar Research Australia, Indooroopilly, Australia. Freeze-dried sugarcane leaves were weighed (approximately 100 mg each sample) and placed in a screw-capped micro-centrifuge grinding tube along with a metal bead. The samples were homogenised at 4.5 m/s for 5 min and dispatched to the National Research Council of Canada, Saskatoon, for hormone analysis.

5.2.3.2 Extraction and purification for hormone analysis

An aliquot (100 µL) containing all the internal standards, each at a concentration of 0.2 ng μL^{-1}, was added to homogenised sample (approx. 50 mg). 3 mL of isopropanol:water:glacial acetic acid (80:19:1, v/v/v) were further added, and then samples were shaken in the dark for 14-16 h at 4 ºC. Samples were then centrifuged and the supernatant was isolated and dried on a Büchi Syncore Polyvap (Büchi, Switzerland). Further, samples were reconstituted in 100 µL acidified methanol, adjusted to 1 mL with acidified water, and then partitioned against 2 mL of hexane. After 30 min, the aqueous layer was isolated and dried as above. Dry samples were reconstituted in 800 µL acidified methanol and adjusted to 1 mL with acidified water. The reconstituted samples were passed through equilibrated Sep-Pak C18 cartridges (Waters, Mississauga, ON, Canada), and then

the final eluate was split in two (2) equal portions. One portion (#1) was dried completely (and stored) while the other portion was dried down to the aqueous phase on a LABCONCO centrivap concentrator (Labconco Corporation, Kansas City, MO, USA). The second portion was partitioned against ethyl acetate (2 mL) and further purified using an Oasis WAX cartridge (Waters, Mississauga, ON, Canada). The GA enriched fraction (#2) was eluted with 2 mL of acetonitrile:water (80:20, v/v) and then dried on a centrivap as described above. An internal standard blank was prepared with 100 µL of the deuterated internal standards mixture. A quality control standard (QC) was prepared by adding 100 µL of a mixture containing all the analytes of interest (Table 5-2), each at a concentration of 0.2 ng μL^{-1}, to 100 µL of the internal standard mix. Finally, fractions #1 and #2, blanks, and QCs were reconstituted in a solution of 40% methanol (v/v), containing 0.5% acetic acid and 0.1 ng μL^{-1} of each of the recovery standards.

5.2.3.3 Hormone quantification

Quantification of ABA, cytokinin, auxin, and gibberellin in sugarcane leaf was conducted at the National Research Council of Canada, Saskatoon, by high-performance liquid chromatography-electrospray tandem mass spectrometry (HPLC-ESI–MS/MS). The procedure for quantification in plant tissue was performed as described in Lulsdorf et al. (2013). Samples were injected onto an ACQUITY UPLC® HSS C18 SB column (2.1 × 100 mm, 1.8 µm) with an in-line filter and separated by a gradient elution of water containing 0.02% formic acid against an increasing percentage of a mixture of acetonitrile and methanol (50:50, v/v).

Briefly, the analysis uses the Multiple Reaction Monitoring (MRM) function of the MassLynx v4.1 (Waters Inc) control software. The resulting chromatographic traces are quantified off-line by the QuanLynx v4.1 software (Waters Inc) wherein each trace is integrated and the resulting ratio of signals (non-deuterated/internal standard) is compared with a previously constructed calibration curve to yield the amount of analyte present (ng per sample). Calibration curves were generated from the MRM signals obtained from standard solutions based on the ratio of the chromatographic peak area for each analyte to that of the corresponding internal standard, as described by Ross et al. (2004). The QC samples, internal standard blanks and solvent blanks were also prepared and analyzed alongside each batch of tissue samples.

Table 5-2: The analytes of interest in hormone analysis.

Hormone	Analytes of interest	
Abscisic acid and metabolites	ABA	*cis*-Abscisic acid
	ABAGE	Abscisic acid glucose ester
	DPA	Dihydrophaseic acid
	PA	Phaseic acid
	7'OH-ABA	7'-Hydroxy-abscisic acid
	neo-PA	*neo*-Phaseic acid
	t-ABA	*trans*-Abscisic acid
Auxins	IAA	Indole-3-acetic acid
	IAA-Asp	N-(Indole-3-yl-acetyl)-aspartic acid
	IAA-Glu	N-(Indole-3-yl-acetyl)-glutamic acid
	IAA-Ala	N-(Indole-3-yl-acetyl)-alanine
	IAA-Leu	N-(Indole-3-yl-acetyl)-leucine
	IBA	Indole-3-butyric acid
Cytokinins	*t*-ZOG	(*trans*) Zeatin-O-glucoside
	c-ZOG	(*cis*) Zeatin-O-glucoside
	t-Z	(*trans*) Zeatin
	c-Z	(*cis*) Zeatin
	dhZ	Dihydrozeatin
	t-ZR	(*trans*) Zeatin riboside
	c-ZR	(*cis*) Zeatin riboside
	dhZR	Dihydrozeatin riboside
	iP	Isopentenyladenine
	iPR	Isopentenyladenosine
Gibberellins	GA1	Gibberellin 1
	GA3	Gibberellin 3
	GA4	Gibberellin 4
	GA7	Gibberellin 7
	GA8	Gibberellin 8
	GA9	Gibberellin 9
	GA19	Gibberellin 19
	GA20	Gibberellin 20
	GA24	Gibberellin 24
	GA29	Gibberellin 29
	GA34	Gibberellin 34
	GA44	Gibberellin 44
	GA51	Gibberellin 51
	GA53	Gibberellin 53

5.3 Results

5.3.1 Cytokinin

5.3.1.1 Transcription of the *isopentenyltransferase* (*IPT*) transgene

The relative expression of the *IPT* transgene in leaves and root was investigated by qRT-PCR. Gene expression results confirmed the expression of *IPT* transcript in the leaves and roots of transgenic plants, and the transcript was not detected in wild-type plants (data not shown). Gene expression data was collected for four different days during the experiment. Each day represents a different level of field capacity (FC); day 0 = 100%, day 3 = 75%, day 6 = 50% and day 10 = 25% FC in the water stress (WS) treatment and 100% FC for well-watered treatment (WW) for all time points. Transgenic *IPT* transcription of plants in water stress treatment was significantly ($P<0.05$) induced on day 6 and 10 in both tissues (Figure 5-1A and Figure 5-1B). In leaf and root tissues, *IPT* transcript levels were at least 2-fold greater in water stressed than well-watered plants by day 10 (Figure 5-1A and Figure 5-1B).

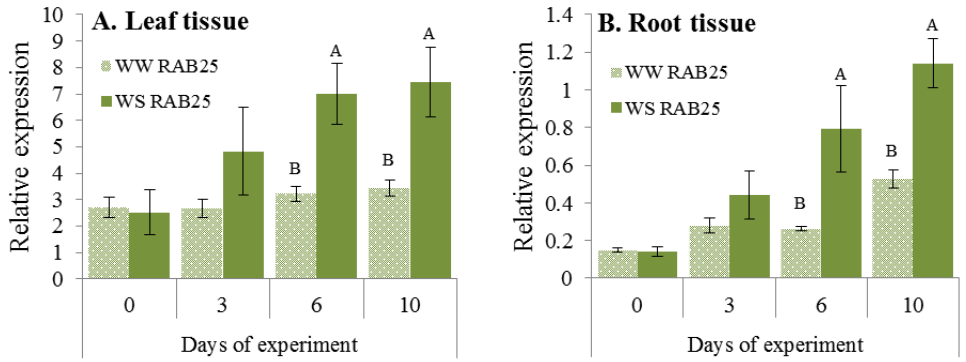

Figure 5-1: Relative expression of *isopentenyltransferase* (*IPT*) transgene in leaf and root tissues of transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the treatments for the same day (Tukey HSD test ($P<0.05$); n = 4).

5.3.1.2 Endogenous level of bioactive cytokinins

Changes in the levels of bioactive CKs were observed in the youngest fully expanded leaves of *IPT* transgenic plants (RAB25) and wild-type plants at four different field capacities (FC). Isopentenyladenosine (iPR), isopentenyladenine (iP) and *trans*-zeatin (tZ) occurred in leaf tissue of water stressed and well-watered transgenic and non-transgenic sugarcane (Figure 5-2, 5-3 and 5-4).

Isopentenyladenosine (iPR)

Higher concentrations of isopentenyladenosine (iPR) content was observed in transgenic than in wild-type plants at all field capacities (Figure 5-2). iPR content showed an up-ward trend with increasing water stress in transgenic plants but iPR was undetectable in wild-type plants from day 3.

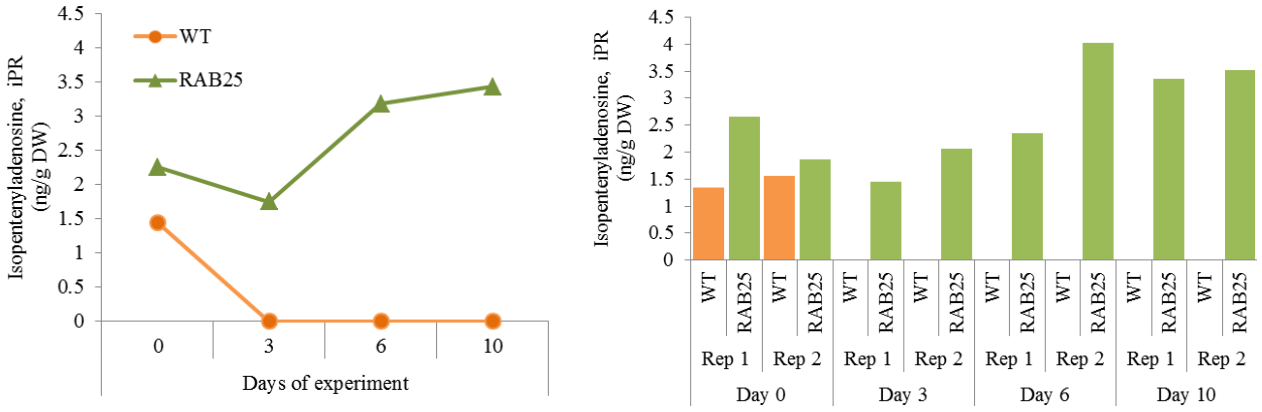

Figure 5-2: Endogenous level of Isopentenyladenosine (iPR) in leaf tissue of wild-type (WT) and transgenic (RAB25) plants. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC. The values on line chart refer to the average of two replicate plants. Bar chart represents value of individual plant in each replicate (Rep).

Isopentenyladenine (iP)

Similar to iPR, isopentenyladenine (iP) content of transgenic plants was higher than wild-type plants at all water stress levels and increased with increasing stress level in transgenic lines (Figure 5-3). At day 6 and day 10, iP content was undetectable in wild-type plants.

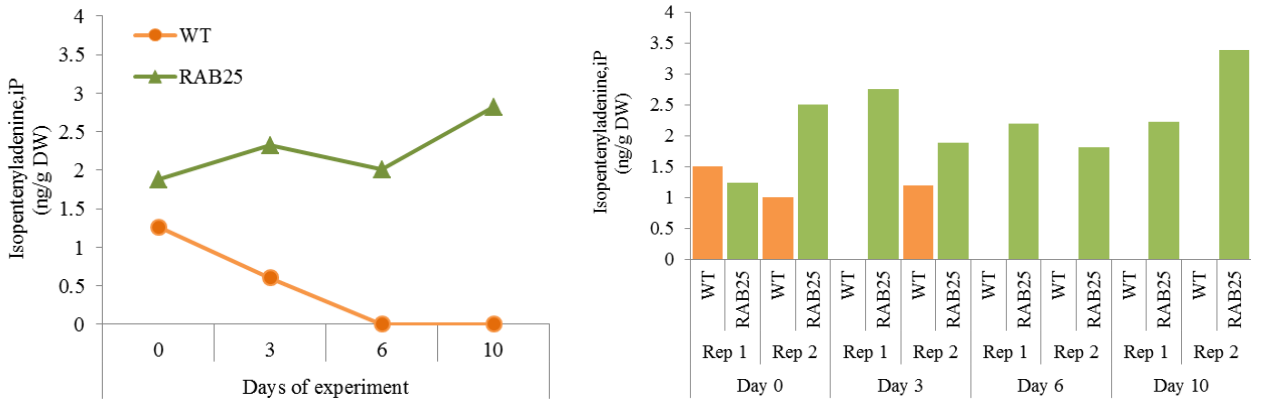

Figure 5-3: Endogenous level of Isopentenyladenine (iP) in leaf tissue of wild-type (WT) and transgenic (RAB25) plants. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC. The values on line chart refer to the average of two replicate plants. Bar chart represents value of individual plant in each replicate (Rep).

trans-Zeatin (tZ)

A decreasing trend of tZ content was observed in transgenic and wild-type plants (Figure 5-4). At 50 (day 6) and 25% FC (day 10), tZ content in transgenic plants was measured at ~1.4 ng/g DW, whereas tZ content of wild-type plants was below the detection limit.

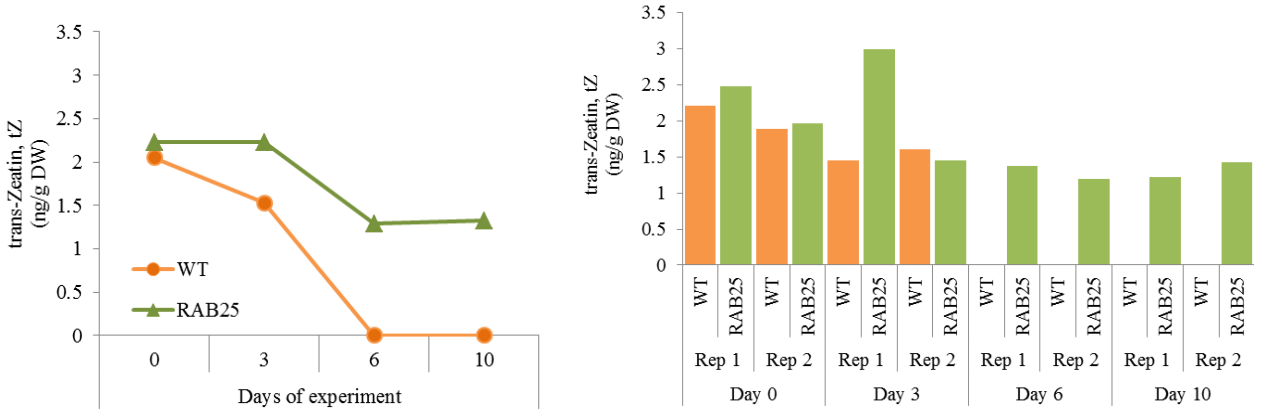

Figure 5-4: Endogenous level of *trans*-Zeatin (tZ) in leaf tissue of wild-type (WT) and transgenic (RAB25) plants. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC. The values on line chart refer to the average of two replicate plants. Bar chart represents value of individual plant in each replicate (Rep).

5.3.2 Abscisic acid (ABA)

5.3.2.1 Transcription of *Viviparous14* (*Vp14*)

In leaf tissue, *Vp14* expression in water stressed wild-type and transgenic plants was significantly ($P<0.05$) higher than in well-watered plants on day 6 (2.5-fold higher in wild-type plants and 2.3-fold higher in transgenic plants) and day 10 (4.5-fold higher in wild-type and 6-fold in transgenic plants; Figure 5-5A). In root tissue, *Vp14* expression was significantly ($P<0.05$) increased in water stressed plants on day 10 (4.3-fold higher in wild-type plants and 4.6-fold higher in transgenic plants; Figure 5-5B). However, *Vp14* expression within water treatment was similar in wild-type and transgenic plants at all sampling time points.

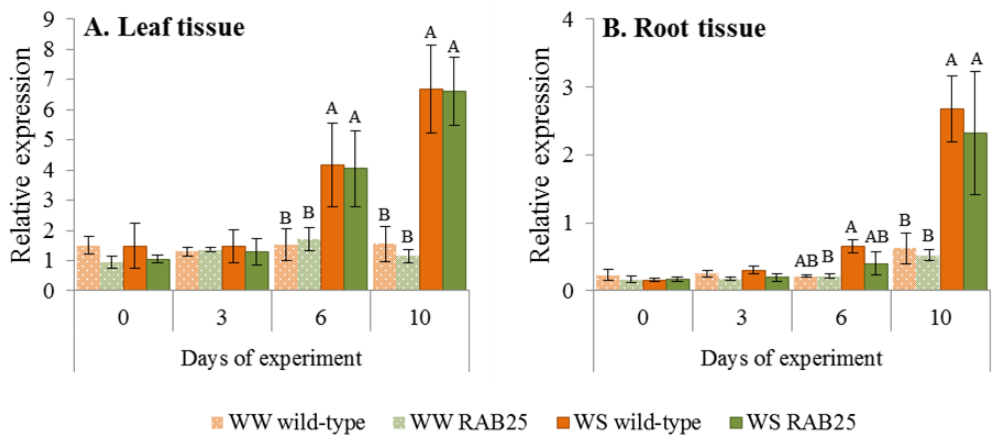

Figure 5-5: Relative expression of *Viviparous14* (*Vp14*) in leaf and root tissues of wild-type and transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the four treatments for the same day (Tukey HSD test ($P<0.05$); n = 4).

5.3.2.2 Endogenous level of abscisic acid (ABA)

ABA content was correlated with the degree of applied water stress (Figure 5-6). At 25% FC (day 10), while ABA content increased substantially in both plant types, wild-type plants had higher ABA content, about 30% more, than transgenic plants. ABA content of wild-type was ~2-fold higher at 50% FC (day 6).

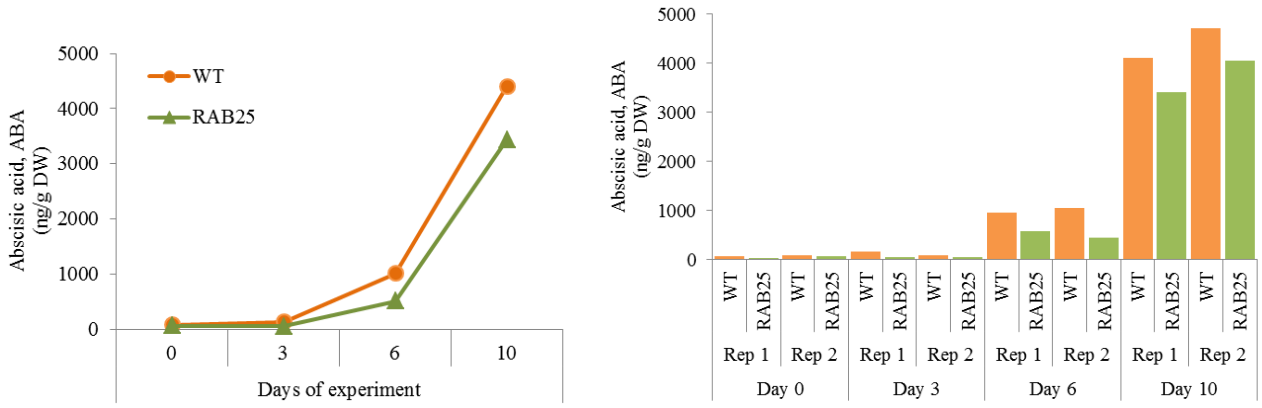

Figure 5-6: Endogenous levels of abscisic acid (ABA) in leaf tissue of wild-type (WT) and transgenic (RAB25) plants. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC. The values on line chart refer to the average of two replicate plants. Bar chart represents value of individual plant in each replicate (Rep).

5.3.3 Ethylene

5.3.3.1 Transcription of *ACC synthase* (*ACS*)

The transcription of *ACC synthase* (*ACS*) in leaves of transgenic and wild-type plants during water stress and well-watered condition is shown in Figure 5-7. On day 10, the expression of *ACS* in water stressed wild-type plants was 1.8 and 2.5-fold ($P<0.05$) higher than transgenic and well-watered plants, respectively. The expression of *ACS* in water stressed and well-watered transgenic plants was similar (Figure 5-7A). In root tissue on the other hand, the expression of *ACS* was significantly ($P<0.05$) decreased in all water stressed plants from day 3 (Figure 5-7B), with similar expression of transgenic and wild-type plants in both water treatments.

5.3.3.2 Transcription of *ACC oxidase* (*ACO*)

The transcription of *ACC oxidase* (*ACO*) in transgenic and wild-type plants in water stress and well-water treatment is shown in Figure 5-8. In leaf tissue, *ACO* expression showed a pattern similar to that of *ACS*. In water stressed wild-type plants, *ACO* expression was 1.6-fold higher than in transgenic plants on day 10 ($P<0.05$; Figure 5-8A). *ACO* transcription was not significantly

different in root tissue of both plant types but an increasing trend was observed in water stressed plants (Figure 5-8B).

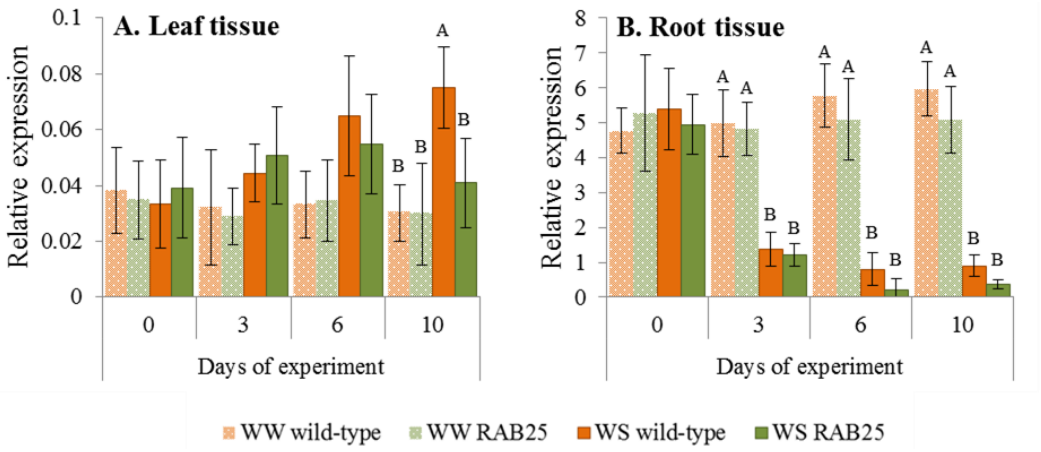

Figure 5-7: Relative expression of *ACC synthase* (*ACS*) in leaf and root tissues of wild-type and transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the four treatments for the same day (Tukey HSD test ($P<0.05$); n = 4).

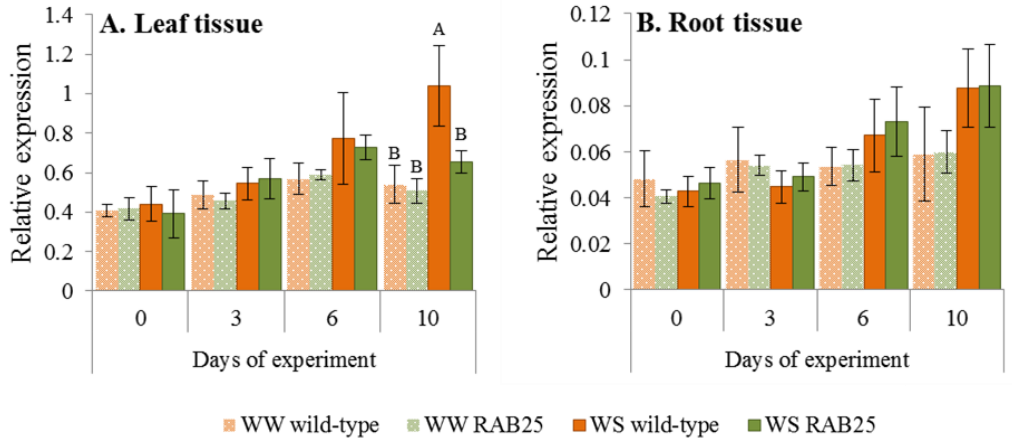

Figure 5-8: Relative expression of *ACC oxidase* (*ACO*) in leaf and root tissues of wild-type and transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the four treatments for the same day (Tukey HSD test ($P<0.05$); n = 4).

5.3.4 Gibberellin (GA)

5.3.4.1 Transcription of *GA 20-oxidase* (*GA20ox*)

Down-regulation of *GA 20-oxidase* (*GA20ox*) was observed in leaves of water stressed plants while increased expression was recorded in root tissue (Figure 5-9). On day 6 (50% FC), water stressed wild-type plants had significantly (P<0.05) lower *GA20ox*, about 5-fold less, than well-watered plants and this was further reduced by day 10 (P<0.05; Figure 5-9A). In comparison, leaf tissue of transgenic plants maintained significantly (P<0.05) higher, nearly 3-fold more, *GA20ox* expression than wild-type plants by day 10 (25% FC) (Figure 5-9A). In root tissue, *GA20ox* expression was similar in transgenic and wild-type plants in both water treatments throughout the experiment (Figure 5-9B).

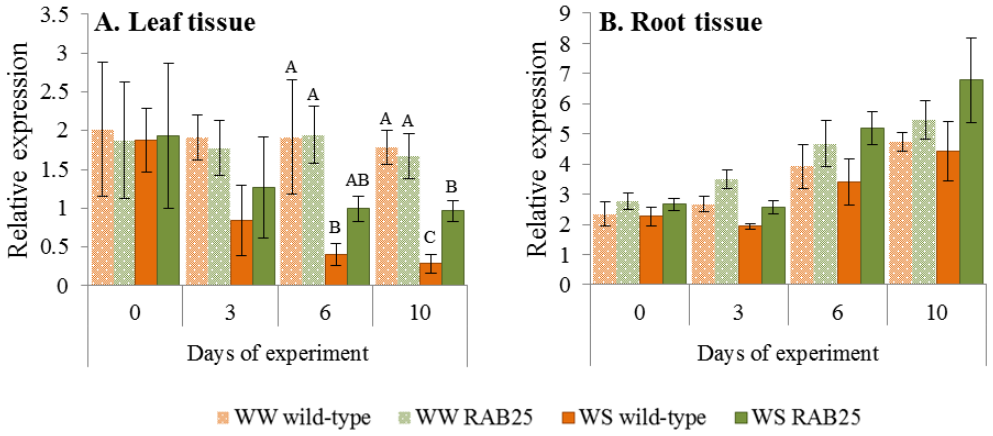

Figure 5-9: Relative expression of *GA 20-oxidase* (*GA20ox*) in leaf and root tissues of wild-type and transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the four treatments for the same day (Tukey HSD test (P<0.05); n = 4).

5.3.4.2 Transcription level of *GA 2-oxidase* (*GA2ox*)

GA 2-oxidase (*GA2ox*) expression in leaf tissue of water stressed wild-type plants was 6 and 14-fold (P<0.05) higher than all other plants including water stressed transgenic plants between day 6 and 10 (Figure 5-10A). *GA2ox* expression did not change significantly in leaf tissues of transgenic plants experiencing increasing water deficit (Figure 5-10A). A somewhat similar trend, though of

less magnitude, was evident in root tissue, except that *GA2ox* expression was significantly elevated in transgenic plants by day 6 and day 10 (Figure 5-10B).

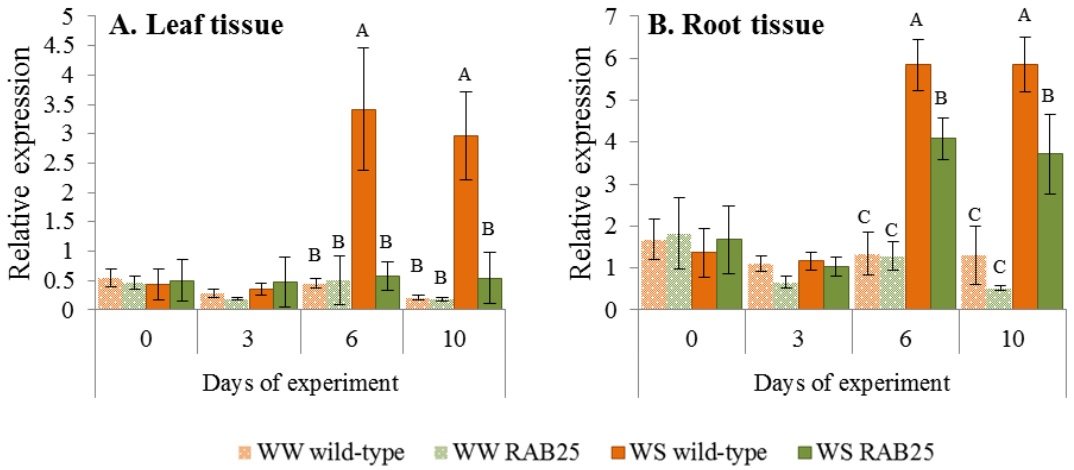

Figure 5-10: Relative expression of *GA 2-oxidase* (*GA2ox*) in leaf and root tissues of wild-type and transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the four treatments for the same day (Tukey HSD test ($P<0.05$); n = 4).

5.3.4.3 Transcription level of *DELLA*

DELLA gene expression was recorded in leaf and root tissue (Figure 5-11). In leaf tissue, on day 10, *DELLA* transcription was significantly ($P<0.05$) higher in water stressed wild-type and transgenic plants (1.6- and 2-fold higher, respectively) compared to well-watered plants (Figure 5-11A). However, there was no difference between lines in the same water treatment.

In root tissue, on the other hand, *DELLA* transcription in all water stressed plants was significantly ($P<0.05$) higher from day 3 onwards (Figure 5-11B). On day 3, water stressed transgenic and wild-type plants had significantly ($P<0.05$) higher *DELLA* transcription than well-watered transgenic and wild-type plants (~1.6-fold higher in both plants; Figure 5-11B). At the end of the experiment when plants were exposed to the highest water stress level, *DELLA* expression in water stressed wild-type and transgenic plants was 3.9- and 3.5-fold higher than in well-watered plants, respectively (Figure 5-11B). However, there was no significant difference between transgenic and wild-type plants in the same treatment.

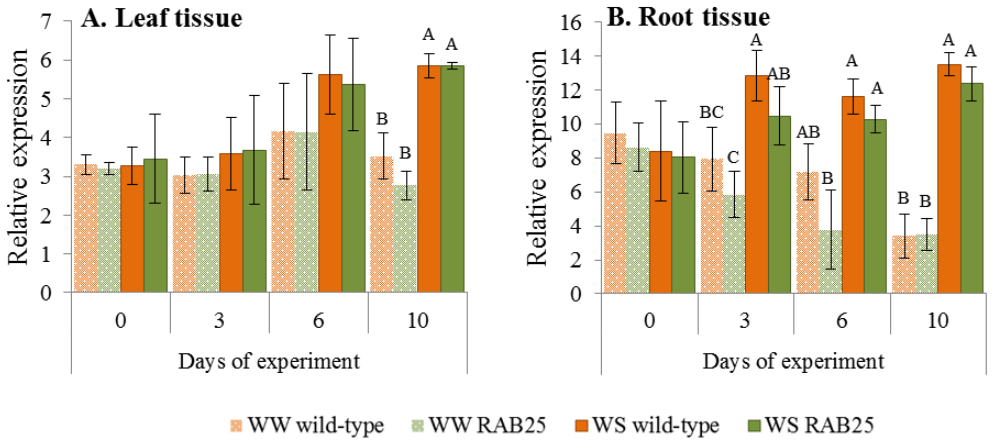

Figure 5-11: Relative expression of *DELLA* in leaf and root tissues of wild-type and transgenic (RAB25) plants grown under well-watered (WW) and water-stress (WS) conditions. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC and 100% FC maintained in the WW treatment. Values represent averages of four replicate plants. Bars indicate standard error (±SE). Different letters above bars indicate significant differences between the four treatments for the same day (Tukey HSD test ($P<0.05$); n = 4).

5.3.4.4 Endogenous level of bioactive gibberellins

Bioactive gibberellin 3 (GA_3) was higher in leaves of transgenic than wild-type plants and the level GA_3 did not decline with increasing water stress in the transgenic line (Figure 5-12). GA_3 content, however, decreased in wild-type plants as water stress increased and were below the detection levels in plants grown at day 10.

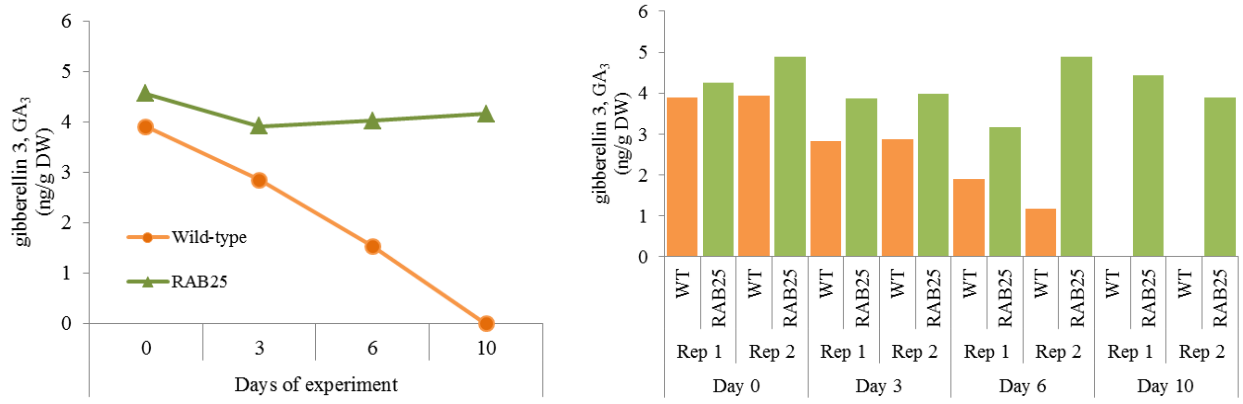

Figure 5-12: Endogenous level of gibberellin 3 (GA_3) in leaf tissue of wild-type (WT) and transgenic (RAB25) plants. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC. The values on line chart refer to the average of two replicate plants. Bar chart represents value of individual plant in each replicate (Rep).

5.3.5 Auxin

5.3.5.1 Endogenous level of indole-3-acetic acid (IAA)

Contrary to other hormones, IAA content in leaves of wild-type and transgenic plants were similar at day 0 and 3 (Figure 5-13). Water stress treatment caused IAA content to increase in all plants at day 6 and day 10 with transgenic plants showing 2.5- and 1.5-fold higher content than wild-type plants, respectively.

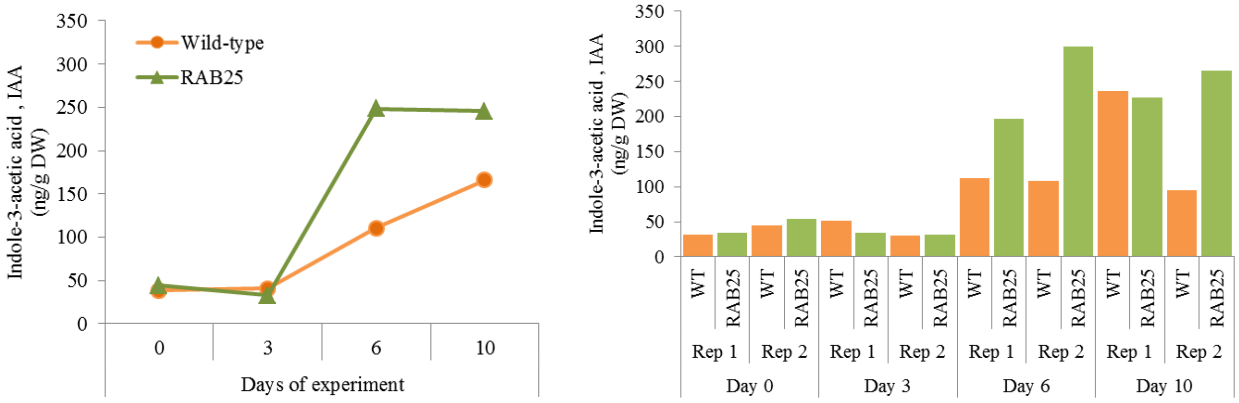

Figure 5-13: Endogenous level of indole-3-acetic acid (IAA) in leaf tissue of wild-type (WT) and transgenic (RAB25) plants. Data was collected at four time points with each day representing a different field capacity (FC) in WS treatment; 0 = 100%, 3 = 75%, 6 = 50% and 10 = 25% FC. The values on line chart refer to the average of two replicate plants. Bar chart represents value of individual plant in each replicate (Rep).

5.3.6 Correlation between the content of hormones

A correlation of hormone contents was conducted to determine if hormones varied in synchrony. The correlations procedure computes a correlation matrix for a total of 16 samples (n=16) using Statistix 10 software. All CK bioactive hormones (iP, iPR and tZ) were positively correlated ($P<0.05$), with GA_3, while ABA content was negatively correlated to GA_3 (Table 5-3). In contrast, ABA was positively correlated to IAA but negatively correlated to tZ (Table 5-3). The positive correlation was also shown in between CK bioactive hormone in which iP had significantly positive correlation to both tZ and iPR (Table 5-3).

Table 5-3: Table of correlations efficiency and P values of the relationship between hormones.

	ABA	tZ	iP	iPR	IAA	GA3
ABA	1.0000					
p-value	0.0000					
tZ	-0.5872	1.0000				
p-value	0.0168	0.0000				
iP	-0.1047	0.6482	1.0000			
p-value	0.6995	0.0066	0.0000			
iPR	-0.018	0.4359	0.7852	1.0000		
p-value	0.9474	0.0915	0.0003	0.0000		
IAA	0.5846	-0.4238	0.1986	0.4824	1.0000	
p-value	0.0174	0.1019	0.461	0.0585	0.0000	
GA3	-0.5393	0.7778	0.7622	0.7707	-0.0537	1.0000
p-value	0.0311	0.0004	0.0006	0.0005	0.8434	0.0000

Note: = positive correlation (where P<0.05)
= negative correlation (where P<0.05)

5.4 Discussion

Multiple hormones are involved in the regulation of a given biological process (Davies, 2010a). Results presented in the previous chapters have shown that the increased availability of CK, achieved transgenically by up-regulation of *IPT* or by external application, enhanced drought tolerance and biomass production in sugarcane plants subjected to moderate water stress. *IPT* transgenic plants produced more roots, had improved gaseous exchange and photosynthesis, and had larger functional green leaf area than non-transgenic wild-type plants under water stress conditions. This overall enhancement in physiology, growth and development occurred with the introduction of conditionally expressing *IPT* gene when required and may involve the interplay of a number hormones (Kohli et al., 2013). The hormone and gene expression data reported in this chapter support these notions and results are summarised in Table 5-4.

Cytokinin homeostasis and signaling are rapidly altered under various water-deficit conditions. The current studies show that bioactive CK content was reduced by water stress in sugarcane (Table 5-4). This is in line with reports in various plant species which demonstrated a reduction of biosynthesis, metabolism, perception and signal transduction of CK in response to environmental stress (Argueso et al., 2009, Nishiyama et al., 2011, Schachtman and Goodger, 2008, Havlova et al.,

2008). However, during the experiment, simultaneously with the development of stress levels, *P_{RAB17}::IPT* plants produced substantially more bioactive CKs than wild-type plants. This is a consequence of *IPT* transgene driven by the ABA-responsive *RAB17* promoter. This is an important result as it confirms that *RAB17* promoter is active in sugarcane, is induced by stress (*via* stress-induced ABA), and facilitated the production of biologically active CKs in quantities required to elicit physiological and growth responses. This is also consistent with the strategy of conditional expression of *IPT* driven by other promoters like *P_{SAG12}* in tobacco (Cowan et al., 2005), wheat (Sykorova et al., 2008), creeping bentgrass (Xu et al., 2009) and cassava (Zhang et al., 2010) under drought stress.

Table 5-4: Summary of hormone content and expression of related genes of *P_{RAB17}::IPT* transgenic (IPT) and wild-type (WT) sugarcane plants in four field capacities ranging from well-watered (100% FC) to severe water stress (25% FC).

Hormones	Gene expression	Hormone content	Field capacity								Figure
			100%		75%		50%		25%		
			WT	IPT	WT	IPT	WT	IPT	WT	IPT	
Cytokinin (CK)	*IPT*		N/A	=	N/A	↑↑	N/A	↑↑↑	N/A	↑↑↑	5-1
		iPR	=	=	↓	=	↓↓	↑	↓↓↓	↑	5-2
		iP	=	=	↓	=	↓↓	=	↓↓↓	↑	5-3
		tZ	=	=	↓	=	↓↓	↓	↓↓↓	↓	5-4
Abscisic acid (ABA)	*Vp14*		=	=	↑	↑	↑↑	↑↑	↑↑↑	↑↑↑	5-5
		ABA	=	=	↑	↑	↑↑	↑	↑↑↑	↑↑	5-6
Ethylene	*ACC ox*		=	=	↑	↑	↑↑	↑	↑↑↑	↓	5-7
	ACC syn		=	=	↑	↑	↑↑	↑	↑↑↑	↓	5-8
Gibberellin (GA)	*GA2ox*		=	=	=	=	↑↑↑	=	↑↑	=	5-9
	GA20ox		=	=	↓	↓	↓↓	↓	↓↓↓	↓	5-10
	DELLA		=	=	↑	↑	↑↑	↑↑	↑↑	↑↑	5-11
		GA3	=	=	↓	=	↓↓	=	↓↓↓	=	5-12
Auxin		IAA	=	=	=	=	↑	↑	↑↑	↑↑	5-13

Note: =, No difference; ↑, increased; ↓, decreased.

ABA levels observed in the plants here strongly correlated with the degree of stress. ABA is an important component of the plant stress defense system and this is reflected by increased production and accumulation of ABA with increasing level of stress. The gene expression data of *Vp14*, a gene encoding the rate-limiting step of ABA biosynthesis, during water stress treatment further supports

this observation. Ye et al. (2012) and Mahdid et al. (2011) reported up-regulation of *Vp14* during drought condition in rice and wheat, respectively. However, the expression of *Vp14* in water stressed sugarcane was not significantly different between P_{RAB17}*::IPT* and wild-type plants, which indicate that regulation of *Vp14* levels are not altered in P_{RAB17}*::IPT* transgenic plants.

Interestingly, while our result shows no difference in *Vp14* expression between transgenic and wild-type plants in stress treatment, a significantly lower ABA content was observed in P_{RAB17}*::IPT* plants. Increased bioactive CK content reduces ABA content during water stress (Rivero et al., 2010) and after heat-shock treatment (Teplova et al., 2000). Moreover, reduction of ABA content was also observed in Kentucky bluegrass treated with BA and exposed to drought stress (Hu et al., 2012). Based on these findings in other species and the results reported here, this suggests a possible direct intervention of CK on the status and physiological activity of ABA in sugarcane. Another noteworthy observation is the lack of response of *Vp14* in roots of transgenic and wild-type plants even under 50% FC (Day 6) which is surprising and difficult to explain, and warrants detailed investigation.

Ethylene is another important stress hormone likely but its impact in water stress tolerance in sugarcane is unclear. While water stress caused the increased expression of *ACC synthase* and *ACC oxidase* in leaves of wild-type plants, P_{RAB17}*::IPT* plants had significantly decreased transcription levels of these two genes compared to wild-type during severe drought stress. A similar report was presented by Goncalves et al. (2013), where over-expression of antisense *ACC oxidase* resulted in increased accumulation of bioactive CKs in melons. This result suggests that the ethylene and CK pathways are probably able to be regulated in either direction. For example, increased CK causes decreased ethylene in *IPT* transgenic plants, and in reverse, decreased ethylene causes increased CK in antisense expression of ethylene biosynthetic genes. Going by the data of the key biosynthesis regulatory genes, *Vp14* for ABA and *ACS* for ethylene, the above cross-regulation are proving to be true in roots but not in leaves. This result suggests that CK up-regulation may at least repress the biosynthesis of ethylene in sugarcane roots during water stress conditions and alleviates ethylene-induced stress effects.

A comparison between the P_{RAB17}*::IPT* and wild-type plants revealed the up-regulation of bioactive gibberellin (GA) in the P_{RAB17}*::IPT* plants under water stress conditions while down-regulated GA was observed in wild-type plants, suggesting an interaction between CK and GA synthesis. This

interaction was further supported by measuring the transcription of major genes involved in GA metabolism (*GA 20-oxidase* and *GA 2-oxidase*) and the regulation of GA action (*DELLA*) in P_{RAB17}*::IPT* plants. GA 20-oxidase, an enzyme that catalyses the formation of bioactive GAs, was up-regulated in the P_{RAB17}*::IPT* plants under water stress conditions, more strongly in the leaf tissue. On the other hand, the expression of GA 2-oxidase, an enzyme that catalyses the formation of inactive GAs, had reduced transcription in the P_{RAB17}*::IPT* plants. Taken together, a link between down-regulation of GA 2-oxidase and up-regulation of GA 20-oxidase was possibly the reason behind increased bioactive GAs in P_{RAB17}*::IPT* plant relative to wild-type plants. It is logical to assume that increased GA will facilitate drought tolerance, but the situation is not straightforward. Mounting evidence suggests that increased GA or GA action and the consequent growth and vigour can be counterproductive in a terminal drought environment as shown in the model species *Arabidopsis*, where the reduction in endogenous bioactive GAs was observed in plants exposure to water stress (Colebrook et al., 2014). Indeed reduction in growth by reducing GA content or GA sensitivity is implicated in drought tolerance by inhibiting GA biosynthesis (Rademacher, 2000). However the validity of this notion remains to be tested in a commercial production situation where stress level is often mild to moderate and boosting growth by up-regulating GA production or GA action may be beneficial. This thinking is consistent with the evidence on gibberellin signaling, especially the involvement of DELLA proteins in plant responses to abiotic stress discussed by Colebrook et al. (2014).

Zentella et al. (2007) suggested that *DELLA* transcription in *Arabidopsis* was related to a reduction in bioactive GA levels and growth inhibition. However, expression of *DELLA*, a gene involved in growth inhibition and regulated by bioactive GAs, was similar in leaves of P_{RAB17}*::IPT* and wild-type plants. While our result demonstrated that transcription of *DELLA* was up-regulated in both water stressed P_{RAB17}*::IPT* and wild-type plants, significantly higher levels of bioactive GAs were found in P_{RAB17}*::IPT* plants. This result suggests that P_{RAB17}*::IPT* plants probably had less sensitivity to the negative effect of *DELLA* expression as observed in higher bioactive GA in P_{RAB17}*::IPT* plants. It seems that CK up-regulation had a strong effect on GA synthesis pathways, either through repressing *GA 2-oxidase* and inducing *GA 20-oxidase* transcription, or reducing sensitivity to DELLA responses.

Auxins play a critical role in plant development. In the current study attempts to identify auxin-related genes in sugarcane were unsuccessful and consequently were not analysed. However, the hormone analysis results showed that levels of free auxin (indole-3-acetic acid, IAA) were elevated in all plants under water stress conditions. The increase in IAA content was higher in severely stressed leaves of P_{RAB17}*::IPT* than wild-type plants, a finding consistent with the increased bioactive CK and IAA in drought-affected tobacco (Havlova et al., 2008). The P_{RAB17}*::IPT* finding in sugarcane is in line with Bertell and Eliasson (1992) who detected increased in IAA content when CK (6-benzyladenine) was applied to pea plants. This suggests that an increase in bioactive CK content might act as a positive regulator for IAA in sugarcane under water stress conditions.

Stress-induced up-regulation of CK resulted in considerable alteration in ABA, GA, IAA and possibly ethylene levels in P_{RAB17}*::IPT* plants. The correlation analysis indicated that iPR, iP and tZ were positively correlated to GA_3. On the other hand, tZ and GA_3 was negatively correlated to ABA. From the data available here it is difficult establish the exact cause of these changes. All hormones directly influence the metabolism of other hormones to achieve the desired physiology and growth effect (Davies, 2010a, Davies, 2010f). But this cross-regulation is dependent on the developmental stage, growth conditions and species. It is unclear as to how much the up-regulation of CK directly influenced the metabolism of other hormones, but it is logical to assume that the interrelationship of hormones might have played a significant role in the observed water stress tolerance and growth benefits in P_{RAB17}*::IPT* plants under stress. From a stress response perspective, the deliberately increased CK in P_{RAB17}*::IPT* plants might have directly counteracted the effect of stress hormones, ABA and ethylene. Therefore it is likely that the reduced action of ABA and ethylene and the alleviation of stress effects might have afforded a more conducive condition for growth promoters like GA and auxins leading to improved growth under water deficit.

5.4.1 Conclusions

The analysis of drought and hormone-related genes and hormone content displayed a contrasting response to water deficit between the wild-type and transgenic plants. Up-regulation of CK content clearly alters biosynthesis and metabolisms of other hormones and consequently the response of plants to water stress. P_{RAB17}*::IPT* plants displayed an increase in bioactive CK as a result of up-regulation of the *IPT* transgene. P_{RAB17}*::IPT* plants enhanced bioactive GA levels *via* increased

transcription of *GA 20-oxidase* and decreased *GA 2-oxidase* expression, and probably also reduced GA sensitivity to DELLA proteins. In contrast, P_{RAB17}*::IPT* plants reduced ABA content and ethylene biosynthesis pathways *via* down-regulated *ACC oxidase* and *ACC synthase*. Consequently, transgenic plants displayed reduction of growth inhibition response mechanisms and improved growth promotion responses under stress conditions.

CHAPTER 6: CONCLUSIONS

6.1 Introduction

Sugarcane is a major industrial crop accounting for nearly 80% of sugar produced worldwide. Most sugarcane is grown as a 12-month crop in rain-fed conditions. The long crop cycle and lack of irrigation causes rain-fed sugarcane crops to experience some degree of water stress in most years. The economic loss due to water stress is estimated to be ~$260 million in Australia alone, making water deficit the most important production constraint for sugarcane. To address this issue, research on genetic, physiological and molecular components of water relations of sugarcane is underway.

As part of that integrated effort, the work presented in this thesis explored a transgenic approach to aid understanding of molecular and physiological causalities in line with the need to develop future sugarcane varieties with greater resilience to water deficit. As hormones are central to plant growth and development and are implicated in controlling plant responses to environmental constraints including water stress, this project focused on the effect of cytokinin (CK), the hormone directly involved in the regulation of leaf senescence (Gan and Amasino, 1995, Mok and Mok, 2001, Ha et al., 2012, Hwang et al., 2012) and photosynthesis (Chernyad'ev, 2009). Specifically, this thesis aimed to determine whether water stress-induced loss of photosynthetic area, photosynthesis and growth inhibition in sugarcane can be reversed by up-regulation of CK production.

6.2 Initial assessment of hypothesis: external supply of cytokinin improved sugarcane growth under water stress and non-stress conditions.

Before embarking on an expensive high risk program on developing transgenic plants and their characterisation, the effectiveness of CK was tested for reducing the effects of water stress with non-transgenic young plants in a glasshouse study. Plants of a commercial sugarcane variety were grown and supplied with a synthetic CK (6-benzyladenine, 0 to 200 µM BA) to roots *via* irrigation and kept at 100 or 40% FC for 4 weeks. Cytokinin-treated plants outperformed non-CK controls in

shoot and root growth (Figure 2-7, 2-12 and 2-13). This result was confirmed in a further experiment using 100 µM BA, the optimal concentration identified in the previous experiment. The hormone application experiments established that CK (i) is effective in reducing leaf senescence (Figure 2-8) and maintaining photosynthetic function (Figure 2-9 and 2-10) under low water supply, (ii) improves growth of plants receiving full or reduced water supply (Figure 2-11 and 2-12), (iii) does not affect root growth when applied in optimal concentration for plant function identified here, and (iv) as foliar or root application is equally effective.

6.3 Cytokinin production at the time of water stress greatly reduced the impact of water deficit and improved sugarcane growth

Encouraged by the results of the CK application experiments, transgenic sugarcane plants were created to over-produce CK either at the time of stress or constitutively. To generate lines that over-produce CK at the time of stress, *IPT*, the gene coding for the key regulatory enzyme in the CK biosynthesis pathway, was driven by an ABA-responsive promoter (*RAB17*) or senescence-associated promoter (*SAG12*). Lines carrying the *IPT* gene under the control of *Ubi* promoter served as constitutive CK producers. A total of 113 independent transgenic lines; 46 lines transformed with P_{SAG12}*::IPT*, 33 lines transformed with P_{RAB17}*::IPT*, and 34 lines transformed with P_{Ubi}*::IPT* construct were successfully generated through micro-projectile bombardment transformation. These lines were screened in a glasshouse under more severe stress condition (30% FC) than water deficit conditions applied in the CK application experiments.

Four *RAB17* lines (RAB19, RAB20, RAB25 and RAB32) and one *SAG12* line (SAG32) showed distinct stress tolerance under prolonged stress conditions, yet none of transgenic lines tested in the phenotypic screening experiment displayed a significant growth advantage over wild-type plant under water stress or full irrigation (Figure 3-9 and 3-10). We showed that the lack of growth improvement was due to the high level of water stress (30% FC) which was too severe for young plants in pots. Although the transgenic lines did not demonstrate growth benefits in the experiment reported here, under progressive drought stress, RAB19, RAB20, RAB25, RAB32 and SAG32 retained significantly higher chlorophyll content (Figure 3-7) and more green leaves (Figure 3-8) than wild-type and other transgenic plants under water deficit. These transgenic lines were selected as the cluster of clones for further mechanistic studies.

6.4 Cytokinin improved water relation, carbon assimilation and leaf area retention in water stressed sugarcane

Since 30% FC was too severe for young plants to discern the effects of elevated CK levels, we reduced water stress to 50% FC for subsequent experiments and compared four transgenic lines (RAB19, RAB20, RAB25, and SAG32) with wild-type sugarcane. Wild-type plants were either treated with CK (100 µM BA applied *via* irrigation) or kept without CK application to serve as controls. The results validated the previous finding that increased production of CK at the time of stress by driving transgenic *IPT* with stress-responsive promoters is an effective strategy to improve tolerance to water stress. The experiments demonstrated that CK has a distinct regulatory role in sugarcane stomatal function and photosynthesis. Wild-type plants that received CK externally or transgenic plants with elevated endogenous CK levels in response of stress consistently had higher stomatal conductance, reduced leaf senescence and maintained higher photosynthesis rates (Figure 4-10, 4-11 and 4-12, respectively). Together these physiological and phenological responses provide a mechanistic explanation for the observed CK-induced growth benefits observed.

6.5 Transgenic up-regulation of cytokinin production in sugarcane altered the endogenous level of other hormones and the expression of hormone-related genes in water stressed and fully irrigated condition

Targeted up-regulation of CK altered biosynthesis and metabolism of other hormones, namely gibberellins, ethylene, auxin and ABA in addition to the level of bioactive forms of CK. Changes in hormone levels were evident in stress and non-stress conditions. Generally CK up-regulation increased GA and auxin content (Figure 5-12 and 5-13) and reduced ABA (Figure 5-6) and ethylene (based on ACC synthase expression data; Figure 5-7 and 5-8). The observed changes in hormonal status were supported by expression data of hormone biosynthetic or catabolism genes. The overall hormone changes are consistent with leaf function and phenology parameters and with the growth advantage of CK up-regulated transgenic plants under stress and non-stress conditions.

From testing transgenic plants, it is evident that there is a strong correlation between CK up-regulation and increased biomass production under water stress conditions. Under such conditions

plants are able to maintain higher rates of photosynthesis and photosynthetic leaf area. The results discussed in this thesis present a compelling reason to suggest a key role for CK in sugarcane stress responses. Results so far indicate an interplay of CK, ABA, ethylene, GA and auxin biosynthesis in water stressed sugarcane. Improved growth in the CK up-regulated plants under water stress was not related to avoidance mechanisms but rather could be due to enhanced tolerance mechanisms. We therefore hypothesize that CK up-regulation may be decreasing stress sensitivity, rather than directly regulating water loss and cellular dehydration. However without leaf water status of wild-type and transgenic plants under the test conditions, it is difficult to ascertain this possibility.

6.6 Future prospects and challenges

Plant growth and development requires integration of complex environmental signals. Water stress tolerance is a complex multi-genic trait with considerable environmental control. Hence these findings, while providing some useful scientific and potentially practical leads, must be tested in field conditions. Field assessment of *IPT* lines under commercially-relevant water deficit conditions will advance knowledge of the practical relevance of the results reported. A similar analysis of the efficacy of exogenous BA application in commercial settings would be useful to compare costs and benefits of transgenic and crop management strategies.

It is now emerging that CK plays a key role in integrating the environmental and intrinsic cues of sugarcane to adapt and grow under water stress conditions. While substantial progress has been made in identifying physiological and molecular responses of plants to water stress, further investigation into the mechanisms by which CK improves photosynthesis in water stressed plants is needed. Does CK improve drought tolerance by protecting photosystems (light reaction) and/or improved dark reaction (carbon-fixation), or both? Alternatively, is the beneficial effect of CK an indirect consequence of reducing ethylene and ABA production and/or action? Also, it would be very valuable to understand the molecular regulation of hormonal interplay and growth responses observed in this study. Such advances will provide very powerful biotechnological tools for crop improvement for both stressed and non-stressed crop production conditions. Research on crosstalk between hormone signals should also help to explain the various stress responses and offer strategies for improving drought tolerance in plants. Future research should assess how water stress affects hormone biosynthesis, transport and turnover. From a sugarcane crop production

perspective, impact of CK on sugar metabolism and sugar accumulation would be very relevant and important and must be studied as well.

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APPENDIX

Appendix 1: DNA Sequences

P_{SAG12}::IPT sequences, 5’ to 3’.

Arabidopsis thaliana SAG12 promoter

```
1      GATATCTCTT TTTATATTCA AACAATAAGT TGAGATATGT TTGAGAAGAG GACAACTATT CTCGTGGAGC
71     ACCGAGTCTG TTTTATATTA GAAACCCGAT TGTTATTTTT AGACTGAGAC AAAAAAGTAA AATCGTTGAT
141    TGTTAAAATT TAAAATTAGT TTCATCACGT TTCGATAAAA AAATGATTAG TTATCATAGC TAATATAGCA
211    TGATTCTAAA TTTGTTTTTT GACACCCTTT TTTTCTCTCT TTGGTGTTTT CTTAACATTA GAAGAACCCA
281    TAACAATGTA CGTTCAAATT AATTAAAAAC AATATTTCCA AGTTTTATAT ACGAAACTTG TTTTTTTAAT
351    GAAAAACAGTT GAATAGTTGA TTATGAATTA GTTAGATCAA TACTCAATAT ATGATCAATG ATGTATATAT
421    ATGAACTCAG TTGTTATACA AGAAATGAAA ATGCTATTTA AATACCGATC ATGAAGTGTT AAAAAGTGTC
491    AGAATATGAC ATGAAGCGTT TTGTCCTACC GGGTATCGAG TTATAGGTTT GGATCTCTCA AGAATATTTT
561    GGGCCATATT AGTTATATTT GGGCTTAAGC GTTTTGCAAA GAGACGAGGA AGAAAGATTG GGTCAAGTTA
631    ACAAAAACAGA GACACTCGTA TTAGTTGGTA CTTTGGTAGC AAGTCGATTT ATTTGCCAGT AAAAACTTGG
701    TACACAACTG ACAACTCGTA TCGTTATTAG TTTGTACTTG GTACCTTTGG TTAAGAAAAA GTTGATATAG
771    TTAAATCAGT TGTGTTCATG AGGTGATTGT GATTTAATTT GTTGACTAGG GCGATTCCTT CACATCACAA
841    TAACAAAGTT TTATAGATTT TTTTTTATAA CATTTTTGCC ACGCTTCGTA AAGTTTGGTA TTTACACCGC
911    ATTTTTCCCT GTACAAGAAT TCATATATTA TTTATTTATA TACTCCAGTT GACAATTATA AGTTTATAAC
981    GTTTTTACAA TTATTTAAAT ACCATGTGAA GATCCAAGAA TATGTCTTAC TTCTTCTTTG TGTAAGAAAA
1051   CTAACTATAT CACTATAATA AAATAATTCT AATCATTATA TTTGTAAATA TGCAGTTATT TGTCAATTTT
1121   GAATTTAGTA TTTTAGACGG TTATCACTTC AGCCAAATAT GATTTGGATT TAAGTCCAAA ATGCAATTTC
1191   GTACGTATCC CTCTTGTCGT CTAATGATTA TTTCAATATT TCTTATATTA TCCCTAACTA CAGAGCTACA
1261   TTTATATTGT ATTCTAATGA CAGGGAAACT TTCATAGAGA TTCAGATAGA TGAAATTGGT GGGAAACATC
1331   ATTGAACAGG AAACTTTTAG CAAATCATAT CGATTTATCT ACAAAAGAAT ACTTAGCGTA ATGAAGTTCA
1401   CTTGTTGTGA ATGACTATGA TTTGATCAAA TTAGTTAATT TTGTCGAATC ATTTTTCTTT TTGATTTGAT
1471   TAAGCTTTTA ACTTGCACGA ATGGTTCTCT TGTGAATAAA CAGAATCTTT GAATTCAAAC TATTTGATTA
1541   GTGAAAAGAC AAAAGAAGAT TCCTTGTTTT TATGTGATTA GTGATTTTGA TGCATGAAAG GTACCTACGT
1611   ACTACAAGAA AAATAAACAT GTACGTAACT ACGTATCAGC ATGTAAAAGT ATTTTTTTCC AAATAATTTA
1681   TACTCATGAT AGATTTTTTT TTTTTGAAAT GTCAATTAAA AATGCTTTCT TAAATATTAA TTTTAATTAA
1751   TTAAATAAGG AAATATATTT ATGCAAAACA TCATCAACAC ATATCCAACT TCGAAAATCT CTATAGTACA
1821   CAAGTAGAGA AAATAAATTT TACTAGATAC AAACTTCCTA ATCATCAATT ATAAATGTTT ACAAAACTAA
1891   TTAAACCCAC CACTAAAATT AACTAAAAAT CCGAGCAAAG TGAGTGAACA AGACTTGATT TCAGGTTGAT
1961   GTAGGACTAA AATGGCTACG TATCAAACAT CAACGATCAT TTAGTTATGT ATGAATGAAT GTAGTCATTA
2031   CTTGTAAAAC AAAAATGCTT TGATTTGGAT CAATCACTTC ATGTGAACAT TAGCAATTAC ATCAACCTTA
2101   TTTTCACTAT AAAACCCCAT CTCAGTACCC TTCTGAAGTA ATCAAATTAA GAGCAAAAGT CATTTAACTT
2171   TCCTAAAACA
```

Appendix

Agrobacterium tumefaciens IPT coding sequence

```
1      ATGGATCTAC GTCTAATTTT CGGTCCAACT TGCACAGGAA AGACATCGAC TGCGATAGCT CTTGCCCAGC
71     AGACTGGCCT CCCAGTCCTC TCGCTCGATC GCGTCCAATG CTGTCCTCAA CTATCAACCG GAAGCGGGCG
141    ACCAACAGTG GAAGAACTGA AAGGAACGAC TCGTCTGTAC CTTGATGATC GCCCTTTGGT AAAGGGTATC
211    ATTACAGCCA AGCAAGCTCA TGAACGGCTC ATTGCGGAGG TGCACAATCA CGAGGCCAAA GGCGGGCTTA
281    TTCTTGAGGG AGGATCTATC TCGTTGCTCA GGTGCATGGC GCAAAGTCGT TATTGGAACG CGGATTTTCG
351    TTGGCATATT ATTCGCAACG AGTTAGCAGA CGAGGAGAGC TTCATGAGCG TGGCCAAGAC CAGAGTTAAG
421    CAGATGTTAC GCCCCTCTGC AGGTCTTTCT ATTATCCAAG AGTTGGTTCA ACTTTGGAGG GAGCCTCGGC
491    TGAGGCCCAT ACTGGAAGGG ATCGATGGAT ATCGATATGC CCTGCTATTT GCTACCCAGA ACCAGATCAC
561    GCCCGATATG CTATTGCAGC TCGACGCAGA TATGGAGAAT AAATTGATTC ACGGTATCGC TCAGGAGTTT
631    CTAATCCATG CGCGTCGACA GGAACAGAAA TTCCCTTTGG TGGGCGCGAC AGCTGTCGAA GCGTTTGAAG
701    GACCACCATT TCGAATGTGA
```

Cauliflower mosaic virus *Nopaline synthase* (NOS) terminator sequence

```
1      AGCTCGAATT TCCCCGATCG TTCAAACATT TGGCAATAAA GTTTCTTAAG ATTGAATCCT GTTGCCGGTC
71     TTGCGATGAT TATCATATAA TTTCTGTTGA ATTACGTTAA GCATGTAATA ATTAACATGT AATGCATGAC
141    GTTATTTATG AGATGGGTTT TTATGATTAG AGTCCCGCAA TTATACATTT AATACGCGAT AGAAAACAAA
211    ATATAGCGCG CAAACTAGGA TAAATTATCG CGCGCGGTGT CATCTATGTT ACTAGATCGG GAAT
```

P_{RAB17}*::IPT* sequences, 5’ to 3’.

Zea mays RAB17 promoter

```
1      CTATAGTATT TTAAAATTGC ATTAACAAAC ATGTCCTAAT TGGTACTCCT GAGATACTAT ACCCTCCTGT
71     TTTAAAATAG TTGGCATTAT CGAATTATCA TTTTACTTTT TAATGTTTTC TCTTCTTTTA ATATATTTTA
141    TGAATTTTAA TGTATTTTAA AATGTTATGC AGTTCGCTCT GGACTTTTCT GCTGCGCCTA CACTTGGGTG
211    TACTGGGCCT AAATTCAGCC TGACCGACCG CCTGCATTGA ATAATGGATG AGCACCGGTA AAATCCGCGT
281    ACCCAACTTT CGAGAAGAAC CGAGACGTGG CGGGCCGGGC CACCGACGCA CGGCACCAGC GACTGCACAC
351    GTCCCGCCGG CGTACGTGTA CGTGCTGTTC CCTCACTGGC CGCCCAATCC ACTCATGCAT GCCCACGTAC
421    ACCCCTGCCG TGGCGCGCCC AGATCCTAAT CCTTTCGCCG TTCTGCACTT CTGCTGCCTA TAAATGGCGG
491    CATCGACCGT CACCTGCT
```

Zea mays RAB17 5’UTR

```
1      TCACCACCGG CGAGCCACAT CGAGAACACG ATCGAGCACA CAAGCACGAA GACTCGTTTA GGAGAAACCA
71     CAAACCACCA AGCCGTGCAA GCACC
```

Appendix

P_{Ubi}*::IPT* sequences, 5’ to 3’.

Zea mays Ubi promotor

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1      CTGCAGTGCA GCGTGACCCG GTCGTGCCCC TCTCTAGAGA TAATGAGCAT TGCATGTCTA AGTTATAAAA
71     AATTACCACA TATTTTTTTT GTCACACTTG TTTGAAGTGC AGTTTATCTA TCTTTATACA TATATTTAAA
141    CTTTACTCTA CGAATAATAT AATCTATAGT ACTACAATAA TATCAGTGTT TTAGAGAATC ATATAAATGA
211    ACAGTTAGAC ATGGTCTAAA GGACAATTGA GTATTTTGAC AACAGGACTC TACAGTTTTA TCTTTTTAGT
281    GTGCATGTGT TCTCCTTTTT TTTTGCAAAT AGCTTCACCT ATATAATACT TCATCCATTT TATTAGTACA
351    TCCATTTAGG GTTTAGGGTT AATGGTTTTT ATAGACTAAT TTTTTTAGTA CATCTATTTT ATTCTATTTT
421    AGCCTCTAAA TTAAGAAAAC TAAAACTCTA TTTTAGTTTT TTTATTTAAT AATTTAGATA TAAAATAGAA
491    TAAAATAAAG TGACTAAAAA TTAAACAAAT ACCCTTTAAG AAATTAAAAA AACTAAGGAA ACATTTTTCT
561    TGTTTCGAGT AGATAATGCC AGCCTGTTAA ACGCCGTCGA CGAGTCTAAC GGACACCAAC CAGCGAACCA
631    GCAGCGTCGC GTCGGGCCAA GCGAAGCAGA CGGCACGGCA TCTCTGTCGC TGCCTCTGGA CCCCTCTCGA
701    GAGTTCCGCT CCACCGTTGG ACTTGCTCCG CTGTCGGCAT CCAGAAATTG CGTGGCGGAG CGGCAGACGT
771    GAGCCGGCAC GGCAGGCGGC CTCCTCCTCC TCTCACGGCA CCGGCAGCTA CGGGGGATTC CTTTCCCACC
841    GCTCCTTCGC TTTCCCTTCC TCGCCCGCCG TAATAAATAG ACACCCCCTC CACACCCTCT
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Zea mays Ubi 5’UTR

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1      TTCCCCAACC TCGTGTTGTT CGGAGCGCAC ACACACACAA CCAGATCTCC CCCAAATCCA CCCGTCGGCA
71     CCTCCGCTTC AAG
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Zea mays Ubi Intron

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1      GTACGCCGCT CGTCCTCCCC CCCCCCCCTC TCTACCTTCT CTAGATCGGC GTTCCGGTCC ATGGTTAGGG
71     CCCGGTAGTT CTACTTCTGT TCATGTTTGT GTTAGATCCG TGTTTGTGTT AGATCCGTGC TGCTAGCGTT
141    CGTACACGGA TGCGACCTGT ACGTCAGACA CGTTCTGATT GCTAACTTGC CAGTGTTTCT CTTTGGGGAA
211    TCCTGGGATG GCTCTAGCCG TTCCGCAGAC GGGATCGATT TCATGATTTT TTTTGTTTCG TTGCATAGGG
281    TTTGGTTTGC CCTTTTCCTT TATTTCAATA TATGCCGTGC ACTTGTTTGT CGGGTCATCT TTTCATGCTT
351    TTTTTTGTCT TGGTTGTGAT GATGTGGTCT GGTTGGGCGG TCGTTCTAGA TCGGAGTAGA ATTCTGTTTC
421    AAACTACCTG GTGGATTTAT TAATTTTGGA TCTGTATGTG TGTGCCATAC ATATTCATAG TTACGAATTG
491    AAGATGATGG ATGGAAATAT CGATCTAGGA TAGGTATACA TGTTGATGCG GGTTTTACTG ATGCATATAC
561    AGAGATGCTT TTTGTTCGCT TGGTTGTGAT GATGTGGTGT GGTTGGGCGG TCGTTCATTC GTTCTAGATC
631    GGAGTAGAAT ACTGTTTCAA ACTACCTGGT GTATTTATTA ATTTTGGAAC TGTATGTGTG TGTCATACAT
701    CTTCATAGTT ACGAGTTTAA GATGGATGGA AATATCGATC TAGGATAGGT ATACATGTTG ATGTGGGTTT
771    TACTGATGCA TATACATGAT GGCATATGCA GCATCTATTC ATATGCTCTA ACCTTGAGTA CCTATCTATT
841    ATAATAAACA AGTATGTTTT ATAATTATTT TGATCTTGAT ATACTTGGAT GATGGCATAT GCAGCAGCTA
911    TATGTGGATT TTTTTAGCCC TGCCTTCATA CGCTATTTAT TTGCTTGGTA CTGTTTCTTT TGTCGATGCT
981    CACCCTGTTG TTTGGTGTTA CTTCTGCA
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Appendix 2: 2-way analysis of variance (P value) table

Appendix 2-1: 2-way analysis of variance (P value) table for dose and water regime interaction after 4 weeks of experiment (Chapter 2: section 2.3.2)

Variable	Week of Exp.	Source (P value)		
		Dose	Water	Dose by Water
Chlorophyll content index (CCI)	0	0.6205	0.3087	0.9732
	1	0.5376	<0.001	0.9266
	2	0.047	<0.001	0.526
	3	<0.001	<0.001	0.3195
	4	<0.001	<0.001	0.0039
Stomatal conductance	0	0.7797	0.3072	0.9637
	1	0.0033	<0.001	0.8866
	2	0.038	<0.001	0.9144
	3	<0.001	<0.001	0.0008
	4	<0.001	<0.001	0.0024
Leaf area	4	0.0192	<0.001	0.8276
Stalk elongation	4	0.0019	<0.001	0.7049
Tiller numbers	4	<0.001	<0.001	0.5563
Above-ground biomass	4	<0.001	<0.001	0.1988

Note: difference was significant at P<0.05

Appendix 2-2: 2-way analysis of variance (P values) table for application method and water regime interaction after 4 weeks of experiment (Chapter 2: section 2.3.3)

Variable	Source (P value)		
	Application	Water	Application by Water
Chlorophyll content index (CCI)	<0.001	<0.001	0.1445
Stomatal conductance	<0.001	<0.001	0.5894
Leaf area	<0.001	<0.001	0.6935
Stalk elongation	<0.001	<0.001	0.3292
Tiller numbers	<0.001	<0.001	0.42
Above-ground biomass	<0.001	<0.001	0.3907

Note: difference was significant at P<0.05

Appendix

Appendix 2-3: 2-way analysis of variance (P values) table for transformant and water regime interaction after 60 days of experiment (Chapter 2: section 3.3.2)

Variable	Source (P value)		
	Transformant	Water	Transformant by Water
Chlorophyll content index (CCI)	<0.001	<0.001	<0.001
Green leaf numbers	<0.001	<0.001	<0.001
Stalk elongation	0.0801	<0.001	0.0622
Above-ground biomass	0.1102	<0.001	0.1609
Relative expression	<0.001	<0.001	0.9104

Note: differences were significant at P<0.05

Appendix 2-4: 2-way analysis of variance (P values) table for constructs and water regime interaction after 60 days of experiment (Chapter 3: section 3.3.2)

Variable	Source (P value)		
	Construct	Water	Construct by Water
Chlorophyll content index (CCI)	<0.001	<0.001	<0.001
Green leaf numbers	<0.001	<0.001	<0.001
Stalk elongation	0.0912	<0.001	0.0950
Above-ground biomass	0.8429	<0.001	0.9842
Relative expression	<0.001	0.0021	0.3675

Note: difference was significant at P<0.05

Appendix

Appendix 2-5: 2-way analysis of variance (P values) table for line and water regime interaction (Chapter 4: section 4.3)

Variable	Week of Exp.	Source (P value)		
		Line	Water	Line x Water
Leaf area	7	0.0016	<0.001	0.1034
Stalk height	7	<0.001	<0.001	0.0004
Tiller numbers	7	<0.001	<0.001	0.1542
Total biomass	7	<0.001	<0.001	0.0032
Main stalk biomass	7	<0.001	<0.001	0.0218
Tiller biomass	7	<0.001	<0.001	0.0146
Dead leaf biomass	7	0.0013	0.0005	0.0462
Root biomass	7	<0.001	<0.001	0.0038
Root to shoot ratio	7	0.0019	0.0005	0.9129
Biomass to irrigation ratio	7	<0.001	<0.001	0.0391
Stomatal conductance	0	0.4054	0.0614	0.4237
	1	0.5982	0.0004	0.8956
	2	0.0592	<0.001	0.1879
	3	0.6519	<0.001	0.7857
	4	0.6588	<0.001	0.3238
	5	0.0215	<0.001	0.7035
	6	0.0698	<0.001	0.3859
	7	0.1185	<0.001	0.5492
Chlorophyll content index (CCI)	0	0.208	0.1935	0.6821
	1	0.1857	0.0525	0.3378
	2	0.1264	<0.001	0.5869
	3	0.3758	<0.001	0.5386
	4	0.0281	<0.001	0.6657
	5	0.1346	<0.001	0.8232
	6	0.0016	<0.001	0.4187
	7	0.0002	<0.001	0.0131
Photosynthesis rate	0	0.9548	0.6233	0.9803
	1	0.4198	0.3977	0.9863
	2	0.9614	0.2523	0.9701
	3	0.2237	0.0008	0.4643
	4	0.1561	0.0002	0.8873
	5	0.5789	0.0002	0.3683
	6	0.0934	<0.001	0.5954
	7	0.0006	<0.001	0.1347
Photosynthesis to transpiration ratio	4	0.0177	0.0002	0.0347

Note: difference was significant at $P<0.05$