BT2, A BTB SCAFFOLD PROTEIN, MEDIATES RESPONSES TO MULTIPLE BIOTIC AND ABIOTIC SIGNALS IN *ARABIDOPSIS*

A Dissertation

by

KRANTHI KIRAN MANDADI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Molecular and Environmental Plant Sciences

BT2, A BTB SCAFFOLD PROTEIN, MEDIATES RESPONSES TO MULTIPLE BIOTIC AND ABIOTIC SIGNALS IN *ARABIDOPSIS*

A Dissertation

by

KRANTHI KIRAN MANDADI

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Approved by:

Chair of Committee,	Thomas D. McKnight
Committee Members,	Dorothy E. Shippen
	Wayne K. Versaw
	Kathryn J. Ryan
Intercollegiate Faculty Chair,	Jean H. Gould

August 2010

Major Subject: Molecular and Environmental Plant Sciences

ABSTRACT

BT2, a BTB Scaffold Protein, Mediates Responses to Multiple Biotic and Abiotic Signals in *Arabidopsis.* (August 2010)

Kranthi Kiran Mandadi, B.S., ANGR Agricultural University, Hyderabad, India; M.S., Texas A&M University, Kingsville

Chair of Advisory Committee: Dr. Thomas D. McKnight

We previously described BT2, a BTB/POZ domain containing protein, as an activator of telomerase in *Arabidopsis thaliana*. In the current study, I present evidence of its interesting roles in mediating multiple hormone, stress and metabolic responses in plants. Steady-state expression of *BT2* mRNA was regulated diurnally and was under the control of circadian clock, with a maximum expression in the dark. *BT2* mRNA was responsive to nutrient status and to multiple biotic and abiotic stress signals. Using *bt2* loss-of-function and BT2 over-expressing lines, I show that BT2 suppresses sugar and ABA-mediated responses during germination. BT2 is also essential for transcriptional gene activation mediated by CaMV *35S* enhancers in *Arabidopsis.* Loss of BT2 in several well-characterized *35S* enhancer activation-tagged lines such as *yucca1d*, *pap1d*, *jaw1d* etc., resulted in suppression of the activation phenotypes. The suppression of the phenotypes was due to decreased transcription of the activation-tagged genes. I further demonstrate that BT2

genetically interacts with CULLIN3. I propose that BT2 and CULLIN3 are components of a ubiquitin ligase complex. Together with associated proteins BET9 and BET10, the BT2 complex is required for CaMV *35S* enhancer-mediated activation of gene expression and may regulate expression of target genes involved in multiple responses to fluctuating biotic and abiotic conditions.

I also found that BT2 protein levels are tightly regulated in plants. BT2 protein was primarily localized in the nucleus and was developmentally regulated. BT2 turn-over was regulated in part by the 26S-proteosome, and rare codons present in its open reading frame affected BT2 protein accumulation. In addition to BT2, its orthologs, BT1, BT3, BT4 and BT5, also responded to light, clock and nutrients, with some differences. Moreover, BT1, BT3 and BT4 were also required for *35S* enhancer-mediated activation of gene expression. I propose that BT family proteins assemble into multi-protein complexes to mediate multiple responses to changing environmental and nutritional conditions.

DEDICATION

To my parents, Usha Rani Dogga and Haranath Mandadi, my sister Vamsi for their encouragement, love and patience.

To my wife, Sonia C Irigoyen, for her love and encouragement.

To all my friends, Sreenath, Adriana, Arlene, Veronica, Candace, Sheetal, Madhuri, Madhu, Vivek, Raj, Jose, Chebrolu, Mahesh, with whom I shared some of my memorable experiences here at Texas A&M University.

ACKNOWLEDGEMENTS

This dissertation is a result of dedicated effort and encouragement of many individuals, several of whom deserve special mention.

Foremost, I thank my advisor, Dr. McKnight, who provided me the opportunity to work on this project and to become a part of his lab in 2005. I thank him for his constant direction, support and encouragement to find solutions for problems on several occasions throughout my Ph.D. studies.

I also express my thanks and regards to my committee members, Dr. Shippen, Dr. Versaw and Dr. Ryan, for their guidance and help in the project. With their valuable suggestions and helpful comments on my project, both during lab and committee meetings, they led and kept my research on track.

I would also like to thank several faculty of MEPS and the Biology Department, Drs. Jean Gould, Marla Binzel, Bhimu Patil, Carol Loopstra, Mike Kolomiets, Scott Finlayson, Dirk Hays, Greg Cobb, Libo Shan, Terry Thomas, Tim Hall, Debby Siegele, Michael Benedik, and Hongmin Qin, for their valuable suggestions on my project at research symposiums, and to encourage my participation in MEPS student association activities.

Last, but not the least, I would like to thank present and past lab members of Drs. McKnight, Shippen, Versaw, Thomas and Hall; Ren, Ketan, Beth, Anjali, Yulia, Song, Eugene, Matt, Laurent, John, Michelle, Kalpana, Madhuri, Cathy, Andrew, Ross, Biwei, Tiffany, Pallavi, Sonia, Yichun, Veria, Jungim, Andy, Phil, Danny, Carol, Ann and Sunee, who helped me on several occasions with my project and also to have contributed to my motivation to finish my Ph.D. studies. Special thanks to Ginger Stuessy for her help with maintaining plant growth chambers in the Biology Department.

TABLE OF CONTENTS

	Page
ABSTRACT	iii
DEDICATION	v
ACKNOWLEDGEMENTS	vi
TABLE OF CONTENTS	viii
LIST OF FIGURES	x
LIST OF TABLES	xii
CHAPTER	
I INTRODUCTION AND LITERATURE REVIEW	1
Background	1
II BT2, A BTB PROTEIN, MEDIATES MULTIPLE RESPONSES TO NUTRIENTS, STRESSES, AND HORMONES IN *ARABIDOPSIS*	15
Summary	15
Introduction	16
Results	21
Discussion	36
Materials and Methods	43

CHAPTER Page

III BT2 IS REQUIRED FOR CAULIFLOWER MOSAIC VIRUS *35S* ENHANCER-MEDIATED ACTIVATION OF GENE EXPRESSION 48

Summary 48
Introduction 49
Results 54
Discussion 74
Materials and Methods 81

IV BIOCHEMICAL CHARACTERIZATION OF BT2 AND RESPONSES OF *BT* FAMILY GENES 87

Summary 87
Introduction 88
Results 91
Discussion 108
Materials and Methods 111

V CONCLUSIONS AND FUTURE DIRECTIONS 117

REFERENCES 126

VITA 140

LIST OF FIGURES

FIGURE		Page
1	BTB-domain proteins function as CULLIN3-based ubiquitin ligases	3
2	BT2 mediates multiple responses	5
3	*BT2* expression is diurnally regulated and is controlled by a circadian clock	22
4	Light modulates *BT2* expression, independent of circadian regulation	24
5	*BT2* is repressed by sugars and induced by nitrates	27
6	BT2 suppresses sugar-mediated inhibition of germination	29
7	BT2 suppresses ABA-mediated inhibition of germination	31
8	Loss of BT2 suppresses the high-auxin phenotypes in *yucca1d*	33
9	*BT2* expression is modulated by multiple stress signals	35
10	BT2 may function as an integrator of light, nutrient, hormone and stress signals and maintains the required homeostatic responses	41
11	BT2 does not affect auxin-responsive gene expression	55
12	BT2 regulates *YUCCA1* transcript abundance in *yucca1d*, but not in WT	57
13	Loss of BT2 abolishes *35S* enhancer activation tagged Phenotypes	59
14	BT2 regulates expression of genes from the *35S* enhancer in the activation lines	61
15	BT2 does not affect *YUCCA1* mRNA turn-over in *yucca1d*	63

FIGURE		Page
16	BT2 affects transcription in the *35S* enhancer activation lines	64
17	Expression of *CAB2* and *BT2* in light and dark conditions	65
18	BT2 is not required for CaMV *35S* and *NOS* promoter activity	67
19	CaMBD of BT2 is essential for *35S* enhancer activity	69
20	CULLIN3 is required for BT2 function	71
21	Over-expression of BT2 TAZ domain is a dominant suppressor of *yucca1d* phenotypes	73
22	Working model for function of BT2 to regulate CaMV *35S* enhancer activity	80
23	BT2 protein is diurnally and developmentally regulated	92
24	BT2 is primarily localized in nucleus and is turned-over by 26S proteosome	94
25	Codon usage affects expression of recombinant BT2	96
26	Codon usage of BT2 in *Arabidopsis*	97
27	Codon usage affects expression of BT2 in *Arabidopsis*	99
28	Expression of *BT1, BT2* and *BT5* is diurnally regulated	101
29	Expression of *BT1, BT2* and *BT5* is modulated by nutrients	104
30	Structure of BT family genes	106
31	Loss of BT1, BT2, BT3 and BT4 abolishes *35S* enhancer activation tagged phenotypes	107
32	Affinity purification of BT2::STII and GFP::STII in *Arabidopsis*	123

LIST OF TABLES

TABLE Page

1 Primers used for analysis of BT2 function to mediate multiple responses.. 47

2 Primers used for analysis of BT2 function to regulate *35S* enhancer activity 83

3 Primers used for BT2 modification and analysis of BT gene family function... 116

4 In silico promoter analysis of BT2 and identification of putative *cis*-regulatory elements 119

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

Background

Plants have evolved sophisticated mechanisms to detect and respond to multiple environmental and physiological signals simultaneously. Many responses primarily involve changes in transcription patterns of numerous genes. **B**road-Complex, **T**ramtrack, and **B**ric-a-Brac/**Po**xvirus and **z**inc finger (BTB/POZ) is a conserved protein-protein interaction domain found in diverse groups of proteins involved in transcriptional regulation, cytoskeletal organization, ion-channels, and cell-cycle regulation (Collins et al., 2001; Roberto et al., 2006). BTB/POZ domains often combine with other domains such as Meprin and TRAF-homology (MATH) domain, **T**ranscriptional **A**daptor **Z**inc finger (TAZ) domain, ankyrin repeats, armadillo/β-catenin-like repeats etc (Roberto et al., 2006). *Arabidopsis* has ~80 BTB/POZ domain containing proteins. However, only a few of them such as *ETHYLENE OVERPRODUCER1 (ETO1), NON-PHOTOTROPIC HYPOCOTYL3 (NPH3), NAKED PINS IN YUC MUTANTS1 (NPY1), BLADE ON PETIOLE1 (BOP1),* and *NONEXPRESSER OF PR-GENES1 (NPR1)* etc., have identified functional roles in ethylene signaling, blue-light signal transduction, auxin signaling, leaf morphogenesis,

---

This dissertation follows the style and format of The Plant Cell.

and salicylic acid signaling, respectively (Zhang et al., 1999; Wang et al., 2004a; Ha et al., 2004; Cheng et al., 2007; Pedmale and Liscum, 2007). Despite their diversity in protein structure and function, BTB/POZ domain proteins do have a characteristic feature. The BTB/POZ domain mediates interaction with CULLIN3. Thus, BTB proteins act as substrate-specific adapters in CULLIN3-based ubiquitin ligases and target proteins for degradation by poly-ubiquitination (Figure 1). In addition to binding to CULLIN3, BTB proteins also bind to transcription factors such as ETHYLENE RESPONSE FACTOR/APETALA2 (ERF/AP2), bZIP family proteins via their other combined domains, thus implicating them in transcriptional regulation (Zhang et al., 1999; Weber and Hellmann, 2009). Moreover, other domains of BTB proteins also function as molecular-switches to sense environmental signals and regulate their activity. For example, the C-terminal trans-activation (TA) domain of NPR1 has two cysteine residues that act as a redox-sensor and regulates NPR1 activity to modulate expression of *PATHOGENESIS RELATED* (*PR1*) in response to salicylic acid (Rochon et al., 2006).

BT2 contains a BTB/POZ domain and belongs to a family of five proteins with similar domain architecture (Du and Poovaiah, 2004). In addition to the N-terminal BTB/POZ domain, BT2 has two other protein-protein interaction domains: a central TAZ domain and a C-terminal **Ca**lmodulin-**B**inding **D**omain (CaMBD). The N-terminal BTB/POZ domain interacts with CULLIN3 in vitro (Figueroa et al., 2005) and a bromodomain protein, BET10 in yeast 2-hybrid

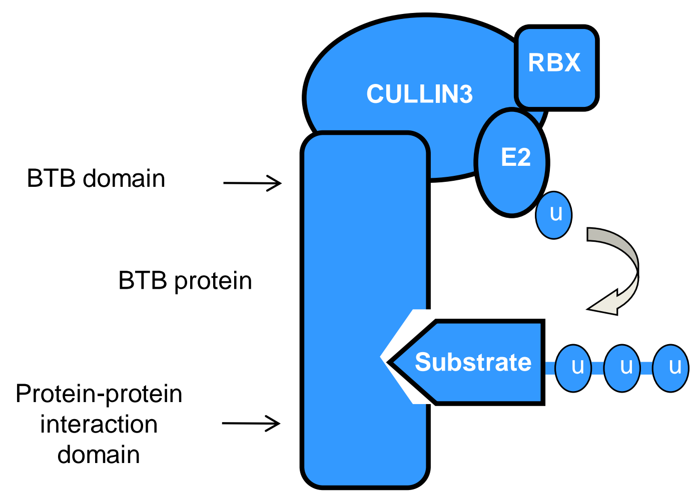

Figure 1. BTB-domain proteins function as CULLIN3-based ubiquitin ligases.

(Y2H) assay (Du and Poovaiah, 2004). I found that BT2 responds to multiple environmental and physiological signals including light, sugars, nitrates; hormones such as ABA, cytokinin; and abiotic and biotic stresses such as cold, H_2O_2 and methy-jasmonate (Mandadi et al., 2009, also in Chapter II). Moreover, BT2 is required for appropriate downstream responses to many of the same signals that regulate its expression (Figure 2) (Mandadi et al., 2009, also in Chapter II). BT2 functional analysis, interacting proteins and its domain architecture suggest a model for its action. It appears to function as a substrate adapter in a CULLIN-3 based ubiquitin ligase to target transcription factors for degradation. In response to multiple signals, and secondary messengers such as Ca^{++} that may be sensed by BT2s CaMBD domain, BT2-CULLIN3 complex may be recruited to gene promoters or enhancers. The recruitment could be mediated by bromodomain proteins BET10 or BET9, which recognize acetylated histones on chromatin. The TAZ domain of BT2 could provide specificity to recognize target proteins for poly-ubiquitination.

Light and circadian regulation of gene expression

Light controls almost every aspect of plant growth and development. It promotes seed germination, vegetative growth, transition to reproductive phase and flowering. Plants have therefore evolved sophisticated mechanisms to respond to light. Although the fine details and precise molecular mechanisms

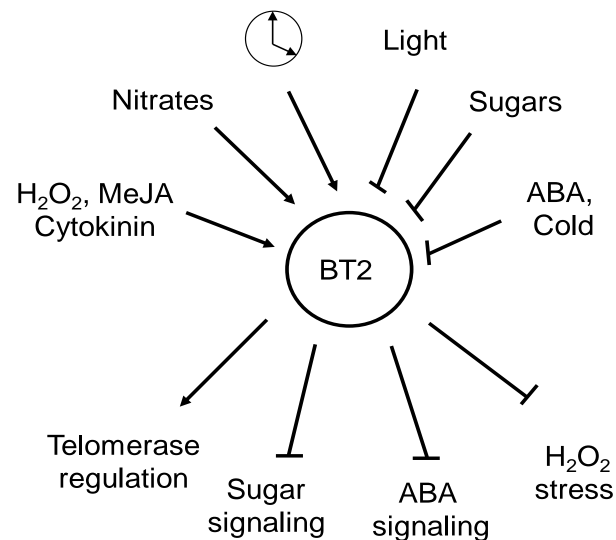

Figure 2. BT2 mediates multiple responses.

are not yet completely understood, significant progress was achieved in recent years in our understanding of light signal transduction. The process begins with sensing of light by a variety of photoreceptors. Plants can sense red/far-red, UV-A/blue, UV-B light signals using phytochrome, phototropin and cryptochrome, and UV-B photoreceptor molecules, respectively (Briggs and Christie, 2002;

Quail, 2002; Lin and Shalitin, 2003). The perception causes conformational changes or alteration in subcellular localization of the receptors and affects their activity (Huala et al., 1997; Quail, 2002; Shalitin et al., 2002; Lin and Shalitin, 2003). Furthermore, light triggers transcriptional and post-transcriptional changes in expression of numerous proteins, including transcription factors, to eventually control cell division, expansion, and differentiation (Chen et al., 2004). Several light responses such as germination and seedling development, entrainment of circadian clock, and photoperiodic flowering often require a complex and coordinated action of more than one photoreceptor (Chory et al., 1996; Neff et al., 2000).

Moreover, light signals are often inter-connected with the circadian clock to control expression of numerous genes that have indirect consequences on several metabolic and physiological processes (Fankhauser and Chory, 1997; Till and Russell, 1997; Harmer et al., 2000; McClung et al., 2002). Genetic studies have identified mutants that are simultaneously defective in circadian regulation and light signaling, and demonstrated cross-talk between the two processes (Anderson and Kay, 1996; Mandy and Andrew, 1999; Devlin and Kay, 2000). Many photosynthetic genes are light regulated and also under the control of the circadian clock (Millar and Kay, 1996). Clock control of expression of light regulated genes provides an advantage to plants in saving net energy. In the current study, I found that expression of *BT2* was regulated by both light and the circadian clock, with maximum expression in the dark (Mandadi et al., 2009,

Chapter II). In all eukaryotes, molecular mechanisms of circadian clock consist of interlocked transcriptional/translational feedback loops (Dunlap, 1999). In *Arabidopsis,* it consists of three interlocked feedback loops, involving two Myb transcription factors, *CIRCADIAN AND CLOCK ASSOCIATED1 (CCA1)*, and *LATE ELONGATED HYPOCOTYL (LHY*); *TIMING OF CAB1 (TOC1)*; *PSEUDO-RESPONSE REGULATOR5* (*PRR5*), *PRR7* and *PRR9*; and *LUX ARRHYTHMO* (*LUX*) (Schaffer et al., 1998; Green and Tobin, 1999; Makino et al., 2002; Salome and McClung, 2004; Mizuno and Nakamichi, 2005). In addition to transcriptional regulation, post-transcriptional regulations such as reversible protein phosphorylation, and proteosome degradation are essential mechanisms of clock regulation (Harms et al., 2004). Nevertheless, the circadian clock is a robust mechanism that controls ~35% of the *Arabidopsis* transcriptome (Michael and McClung, 2003), and *BT2* appears to be one of them.

Sugar sensing and signaling in plants

In light, plants fix carbon into sugars and store it in the form of starch to support metabolism, growth and development. During the night, stored carbon is depleted due to net consumption. Hence, sugar levels undergo marked diurnal changes and can act as an index for measuring the external environment. Indeed, global gene expression studies have identified sugars, in addition to circadian clock, as inputs to diurnal changes in gene expression (Blasing et al., 2005). Often, sugars seem to have an additive affect on the clock output pathways and suggest a synergism in the regulation of both processes. *BT2*

expression was in fact repressed by sugars, which is consistent with its activation in the absence of light, and supports the notion of interplay between light, clock and sugars to regulate gene expression.

In addition to acting as substrates for energy metabolism, and modulating gene expression, sugars possess hormone-like functions as signaling molecules (Rolland et al., 2002; León and Sheen, 2003; Ramon et al., 2008). However, identifying sugar sensors in cells has been complicated due to the dual nature of sugars acting as substrates and signaling molecules. Recently, a role for plant *HEXOSE KINASE (HXK)* was demonstrated in sensing glucose (Moore et al., 2003). Alternatively, the presence of sugars could be sensed via any of the downstream metabolites. Although sugars normally promote growth, at high concentrations they suppress germination and post-germination development (Jang and Sheen, 1994; Moore et al., 2003). During germination and seedling development, sugars inhibit nutrient mobilization, hypocotyl elongation, cotyledon greening, and shoot development. Using screens for either resistance or sensitivity of germination to high sugars, numerous sugar-insensitive and hypersensitive mutants have been identified (Zhou et al., 1998; Laby et al., 2000; Pego et al., 2000; Rolland et al., 2002). I found that germination of the *bt2-1* loss-of-function line was sensitive to high levels of sugars, and suggested that BT2 could modulate sugar signaling at germination. In addition to regulating germination, sugar signaling regulates photosynthetic activity, leaf senescence, and plant responses to biotic and abiotic stress stimuli, such as salinity, drought,

wounding, and infection by pathogens (Rolland et al., 2002). Diverse sugar signals can activate multiple HXK-dependent and HXK-independent pathways to control transcription, translation, stability and activity of downstream targets in the respective developmental pathways (Rolland et al., 2002; Ramon et al., 2008) and BT2 could be one of them.

Nitrate responses in plants

An interconnection between carbon (C) and nitrogen (N) metabolic pathways is anticipated to maintain the C:N homeostasis in plants (Coruzzi and Zhou, 2001). Recently, similar to sugar signals, nitrate signals were demonstrated to serve as inputs to control circadian regulation of gene expression (Gutierrez et al., 2008). Nitrate levels also modulate expression of numerous transcription factors, protein kinases/phosphatases, and enzymes involved in nitrate reduction and assimilation, amino-acid biosynthesis, glycolysis, and iron and sulfate metabolism (Scheible et al., 2004). Several genes involved in nitrate transport and assimilation including *NITRATE TRANPORTER1* (*NRT1), NRT2, AMMONIUM TRANPORTER1* (*AMT1), NITRATE REDUCTASE* (*NR*), *GLUTAMINE SYNTHETASE,* and *ASPARAGINE SYNTHETASE* are also co-regulated by sugars (Lejay et al., 2003). Moreover, nitrate availability affects expression of sugar-regulated genes such as *CHLOROPHYLL A/B BINDING PROTEIN1* (*CAB1*) and *RIBULOSE-1, 5-BISPHOSPHATE CARBOXYLASE SMALL SUBUNIT* (*RBCS*) (Moore et al., 2003). Interestingly, the inhibitory effects of sugars on germination,

photosynthetic gene expression and chlorophyll accumulation are also antagonized by nitrates, implicating interplay between carbon and nitrogen signals in controlling plant development (Moore et al., 2003). In contrast to the sugar effect on *BT2* expression, nitrates activated *BT2* expression. The molecular mechanisms underlying nitrate sensing are relatively less understood. Recently, using mutants defective in nitrate reductase (NR) activity, nitrate was demonstrated to serve as a metabolic signal to regulate gene expression (Gutierrez et al., 2008). Alternatively, assimilated forms of nitrogen such as glutamate and or glutamine could act as signaling molecules.

Hormone and stress signaling

Plants produce several hormones such as auxin, cytokinin, abscisic acid, gibberllin, brassinolide, ethylene, jasmonic acid and salicylic acid etc., to control growth and development (Davis, 2004). Indole-3-acetic acid (IAA), the major form of endogenous auxin, regulates numerous aspects of plant growth such as cell division, cell expansion, root and shoot development, apical dominance and flowering (Quint and Gray, 2006; Teale et al., 2006). Cytokinins are produced in root tips and developing seeds and control cell division, lateral bud emergence, leaf senescence, and other important developmental events (Maxwell and Kieber, 2004; Ferreira and Kieber, 2005; Sakakibara, 2006). Abscisic acid is produced in roots and mature leaves, and is transported to seeds. It controls storage protein synthesis, germination, shoot growth and stomatal closure (Finkelstein et al., 2002). Ethylene controls germination, shoot and root

differentiation, leaf and fruit abscission and flowering (Guo and Ecker, 2004). Hormones also mediate biotic and abiotic stress responses. Jasmonic acid, salicylic-acid, and ethylene play crucial role in plant defenses and promote resistance against pathogens (Fujita et al., 2006). ABA mediates responses to abiotic stresses such as drought, cold, and osmotic stress (Fujita et al., 2006). In contrast, ABA acts as a negative regulator of disease resistance by antagonizing responses of salicylic and jasmonic acid (Mauch-Mani and Mauch, 2005). In addition to plant hormones, abiotic and biotic stress responses, are mediated by nitric oxide, Ca^{++} and reactive oxygen species such as H_2O_2 (Klusener et al., 2002; Ludwig et al., 2004; Wendehenne et al., 2004). Expression of *BT2* was modulated by several hormones and stresses such as ABA, cytokinin, cold, methyl jasmonate, and H_2O_2. Furthermore, BT2 was required for appropriate downstream responses to ABA at germination and H_2O_2 during vegetative development.

In general, hormone signals are perceived by receptor proteins and are transmitted to the nucleus to modulate transcription of downstream signaling genes (Santner and Estelle, 2009). Interestingly, perturbation of one hormone signaling pathway affects expression of genes involved in other hormone responses suggesting an inter-connection between different hormone signaling pathways (Santner and Estelle, 2009). Indeed, genetic and molecular analysis revealed that hormone signaling is a highly inter-connected network. Moreover, hormone signaling has connections with light, nutrient and stress signaling in

plants. However, very little is known about how plants simultaneously integrate and transduce multiple and often overlapping signals. Transcriptional regulators that are simultaneously regulated by numerous signals could connect different pathways. Recently, bZIP family transcription factors, and snf1-related kinases (SnRK) were shown to synergistically activate transcription of numerous genes involved in nutrient, light and stress responses. Interestingly, BT2 was among the numerous targets of SnRK and bZIP transcription factors (Baena-Gonzalez et al., 2007; Hanson et al., 2008). My molecular and genetic analysis of BT2 confirms its role in connecting multiple signaling pathways.

Regulation of 35S *enhancer activity*

Transcription is a predominant mechanism to regulate gene expression. Core-promoter and promoter-proximal regions of genes are characterized by presence of several *cis*-regulatory elements. These *cis*-elements are binding sites for numerous transcription factors and regulators that promote transcription. DNA enhancers are elements further upstream from core-promoters. They can be found as far as 80 kb upstream of the transcription start site (Jack et al., 1991). Moreover, enhancers activate transcription of heterologous genes in a position-, orientation- and sequence-independent manner. Studies on classical enhancers such as *Drosophila* wing margin enhancer (Jack et al., 1991), human interferon-β gene enhancer (Goodbourn et al., 1985), human immunodeficiency virus-1 (HIV-1) enhancer (Jakobovits et al., 1988), and simian virus40 (SV40) enhancer (Weiher et al., 1983) led to a model

of enhancer action. In general, enhancers direct recruitment of transcription machinery including global transcription factors (GTFs), mediators, histone acetyl transferases (HATs), chromatin remodelers, etc. to the promoter and/or promote chromatin modifications to permit binding of the transcriptional machinery and lead to transcriptional activation (Blackwood and Kadonaga, 1998). Furthermore, enhancer action may involve formation of chromatin loops that bring the enhancer elements in proximity to the core promoter elements (Rubtsov et al., 2006). Such looping mechanism can explain the apparent ability of enhancers to act over large distances. The dynamics and constraints on the intervening DNA that promote chromatin looping, however, are not completely understood.

35S enhancers are derived from domain B of the constitutive *35S* Cauliflower Mosaic Virus (CaMV) promoter and can function independently to activate gene expression (Fang et al., 1989; Hayashi et al., 1992). In the last decade, multimers of *35S* enhancers have been used for activation tagging genes in plants, and led to discovery of function of numerous genes (Weigel et al., 2000; Johnson et al., 2007; Pogorelko et al., 2008). Classic examples of *35S* enhancer activation tagged lines in *Arabidopsis* include *yucca1d*, *pap1d*, *jaw1d* etc. These lines possess increased levels of *YUCCA1*, *PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1)* and a microRNA (miR-JAW) precursor, respectively (Borevitz et al., 2000; Zhao et al., 2001; Palatnik et al., 2003). Although *35S* enhancer activation tagging became a valuable and popular tool in

plant functional genomics, the basic mechanism of *35S* enhancer action and the identity of its cellular regulators remain unknown. I serendipitously discovered that BT2 was a regulator of *35S* enhancer mediated gene expression.

The aim of my dissertation research was primarily to characterize the function of BT2. In the second chapter, I describe BT2s function in regulating multiple responses to light, nutrients, hormones and biotic and abiotic stress signals, and thus acting as a key component in an inter-connected signaling network (Mandadi et al., 2009). In the third chapter, I describe BT2's role in regulating *35S* enhancer activity and propose a mechanism for its action. In the fourth chapter, I describe biochemical characterization of BT2 and identify mechanisms that regulate BT2 protein levels in plants. BT2 belongs to a family of five proteins. In the fourth chapter, I also describe analysis of four other members of BT family, *BT1, BT3, BT4* and *BT5,* and propose a model for their seemingly identical functions.

CHAPTER II

BT2, A BTB PROTEIN, MEDIATES MULTIPLE RESPONSES TO NUTRIENTS, STRESSES, AND HORMONES IN *ARABIDOPSIS**

Summary

The *Arabidopsis thaliana* gene *BT2* encodes a 41-kilodalton protein that possesses an N-terminal BTB domain, a central TAZ domain, and a C-terminal calmodulin-binding domain. We previously demonstrated that *BT2* could activate telomerase expression in mature *Arabidopsis* leaves. Here, I report its distinct role in mediating diverse hormone, stress and metabolic responses. I serendipitously discovered that steady-state expression of *BT2* mRNA was regulated diurnally and controlled by the circadian clock, with maximum expression in the dark. This pattern of expression suggested that BT2 mRNA could be linked to the availability of photosynthate in the plant.

---

* Used with permission from **Mandadi, K.K., Misra, A., Ren, S., and McKnight, T.D.** (2009). BT2, a BTB protein, mediates multiple responses to nutrients, stresses, and hormones in *Arabidopsis*. Plant Physiol. **150:** 1930-1939. www.plantphysiol.org, Copyright American Society of Plant Biologists.

Exogenous sugars decreased BT2 expression, whereas exogenous nitrogen increased expression. *bt2* loss-of-function mutants displayed a hypersensitive response to both sugar-mediated inhibition of germination and to abscisic-acid (ABA)-mediated inhibition of germination, thus supporting a role of ABA in sugar signaling in germination and development. Moreover, constitutive expression of *BT2* imparted resistance to both sugars and ABA at germination, suggesting that *BT2* suppresses sugar and ABA responses. I found that *BT2* positively regulated the high-auxin mutant, *yucca1d,* responses, as revealed by knocking down *BT2* in *yucca1d*. Accumulation of *BT2* mRNA was affected by a variety of hormones, nutrients and stresses, and *BT2* was required for response to many of these same factors. Together, these results suggest that BT2 is a central component of an interconnected signaling network that detects and responds to multiple inputs.

Introduction

Plants have evolved sophisticated mechanisms to perceive and transduce diverse environmental signals. Changes in light, the circadian clock, and nutrient status serve as major inputs to modulate the diurnal expression of networks of

genes that regulate growth and development (Blasing et al., 2005; Gutierrez et al., 2008; Usadel et al., 2008). The circadian clock further serves as an input to regulate or “gate” the expression of multiple genes involved in metabolism, growth, and development, thereby rendering a physiological advantage for plant growth and survival (reviewed by McClung, 2006). In addition to the intrinsic clock function, diurnal changes in nutrient status modulate expression of several genes (Blasing et al., 2005). Availability of sugars activates “feast” genes involved in growth and biosynthesis, while low sugar concentrations activate “famine” genes that mobilize carbon from primary reserves or other cellular components (Koch, 1996; Yu, 1999). Similarly, changes in nitrogen status modulate expression of numerous transcription factors, protein kinases/phosphatases, and enzymes involved in nitrate reduction and assimilation, amino-acid biosynthesis, glycolysis, and iron and sulfate metabolism (Scheible et al., 2004; Wang et al., 2004b)

Sugars and nitrates primarily affect plant growth by serving as building blocks for anabolic metabolism. They also function as signaling molecules that interact with light, hormones including ABA and ethylene, and stress signals to control vital processes of growth and development (Zhou et al., 1998; Stitt, 1999; Stitt and Krapp, 1999; León and Sheen, 2003; Wang et al., 2004b). Sugars normally promote growth; however, high sugar concentrations suppress germination and post-germination development. Interestingly, these inhibitory effects are antagonized by nitrates, suggesting interplay between carbon and nitrogen status in the control of germination (Moore et al., 2003; Bi et al., 2005). Using screens for either resistance

or sensitivity of germination to high sugar concentrations, sugar-insensitive or hypersensitive mutants have been identified (Zhou et al., 1998; Laby et al., 2000; Pego et al., 2000; Rolland et al., 2002). Surprisingly, many sugar-insensitive mutants, such as *sugar-insensitive4*/ *glucose-insensitive1* (*sis4*/*gin1*) and *sis5/gin6,* are allelic to ABA synthesis (*aba2*) and ABA-insensitive mutants (*abi4*), respectively (Arenas-Huertero et al., 2000; Cheng et al., 2002). Moreover, exogenous glucose specifically increases expression of ABA synthesis genes and affects endogenous ABA concentrations, revealing an intimate connection between ABA and sugar signaling (Cheng et al., 2002; Price et al., 2003). ABA itself mediates seed dormancy, leaf senescence, stomatal closure, and several other plant stress responses (Fedoroff, 2002; Gubler et al., 2005). ABA signaling also has antagonistic interconnections with other hormones, including auxin and ethylene. The ABA-hypersensitive mutant *hyponastic leaves1* (*hyl1*) is simultaneously resistant to auxin and cytokinin (Lu and Fedoroff, 2000). During lateral root initiation, auxin promotes initiation by down-regulating cell cycle inhibitors such as kip-related proteins (KRPs) (Richard et al., 2001; Himanen et al., 2002). In contrast, ABA inhibits lateral root initiation by activating KRPs (Verkest et al., 2005). Also, several genes involved in promoting lateral root initiation, including *AUXIN INDUCED IN ROOT CULTURES 12* (*AIR12*), and *INDOLE-3 ACETIC ACID 19* (*IAA19*), are repressed by ABA (Hoth et al., 2002).

Although there has been significant progress in understanding how plants perceive light, nutrient, hormone and stress signals, major questions persist

regarding how plants simultaneously integrate and transduce these different signals. Global gene expression studies in *Arabidopsis* have revealed that specific signals modulate extensive networks of genes. These networks typically include genes encoding putative transcription factors and protein kinases, along with genes involved in protein synthesis and ubiquitin-mediated protein degradation (Wang et al., 2004b; Blasing et al., 2005; Gutierrez et al., 2008; Usadel et al., 2008). Members of bZIP family of transcription factors characterized as G-box (CACGTG) binding factors (GBFs), such as bZIP2/GBF5 and bZIP11/ATB1, together with snf1-related kinases (SnRK), KIN10/11, orchestrate synergistic transcriptional networks in response to sugar, energy deprivation and diverse stresses (Baena-Gonzalez et al., 2007; Hanson et al., 2008). However, molecular mechanisms of the components downstream of bZIP/SnRK that affect the adaptive responses remain elusive.

We previously identified BT2 (At3g48360) as an activator of telomerase in mature leaves of *Arabidopsis* (Ren et al., 2007). BT2 is a ~41 kD protein with an N-terminal BTB/POZ (Broad-Complex, Tramtrack, and Bric-a-Brac/Poxvirus and Zinc finger) domain, a central TAZ (Transcriptional Adaptor Zinc finger) domain and a C-terminal calmodulin-binding domain (CaMBD). The *Arabidopsis* genome encodes four additional proteins with a similar domain structure (Du and Poovaiah, 2004).

Recently, members of this BT family, including BT2, were demonstrated to play crucial roles in gametophyte development in *Arabidopsis* and were further shown to compensate for loss of one another by reciprocal transcriptional regulation (Robert et al., 2009). Here, I report distinct and broader functions of *BT2* in

responding to changes in light signals, nutrient status, hormones and certain stresses. During the diurnal cycle, *BT2* expression peaked in the dark, and its expression was regulated by the circadian clock. Nutrient status also modulated *BT2* expression; sugars repressed *BT2* expression, while nitrates increased *BT2* expression. Using *BT2*-null mutants and constitutively expressing *BT2* lines, I demonstrated that *BT2* modulated hormone responses. *BT2* negatively regulates ABA- and sugar-mediated inhibition of germination. Loss of *BT2* in the auxin accumulating mutant *yucca1d* suppresses many of the phenotypes associated with high auxin concentrations. This result confirms our previous conclusion from *BT2*-overexpressing lines that *BT2* potentiates *yucca1d* responses in post-germination and vegetative development (Ren et al., 2007). Furthermore, *BT2* expression was modulated by multiple abiotic and biotic stresses including ABA, cold, methyl jasmonate, and H_2O_2. Loss of BT2 function resulted in sensitivity to H_2O_2. Because *BT2* expression is affected by multiple physiological and environmental conditions, and because it is also required for responses to many of these same conditions, the BT2 protein appears to be a key element in an interconnected network that detects and integrates responses to diverse signals.

Results

Circadian and light regulation of BT2

In our previous characterization of BT2's role in activating telomerase in mature leaves (Ren et al., 2007), we noticed that the level of *BT2* message fluctuated among RNA samples harvested at different times. To uncover the cause of this fluctuation, I analyzed *BT2* mRNA levels throughout the diurnal cycle. *BT2* was highly expressed in the dark phase (19, 24, 43 and 48 h) and was almost undetectable in the light phase (7 and 31 h) (Figure 3A). However, its abundance increased slightly towards the end of the light phase (14 and 38 h), in apparent anticipation of the dark phase. Rhythmic expression of *BT2* suggested that it may be under control of a circadian clock. To test this hypothesis, 3-wk-old light-dark entrained plants were either transferred to continuous light or kept in a normal diurnal cycle, and RNA samples were subjected to quantitative (q) RT-PCR analysis. The rhythmic pattern of *BT2* expression seen in control plants under a normal diurnal cycle was maintained in plants transferred to continuous light (Figure 3B).

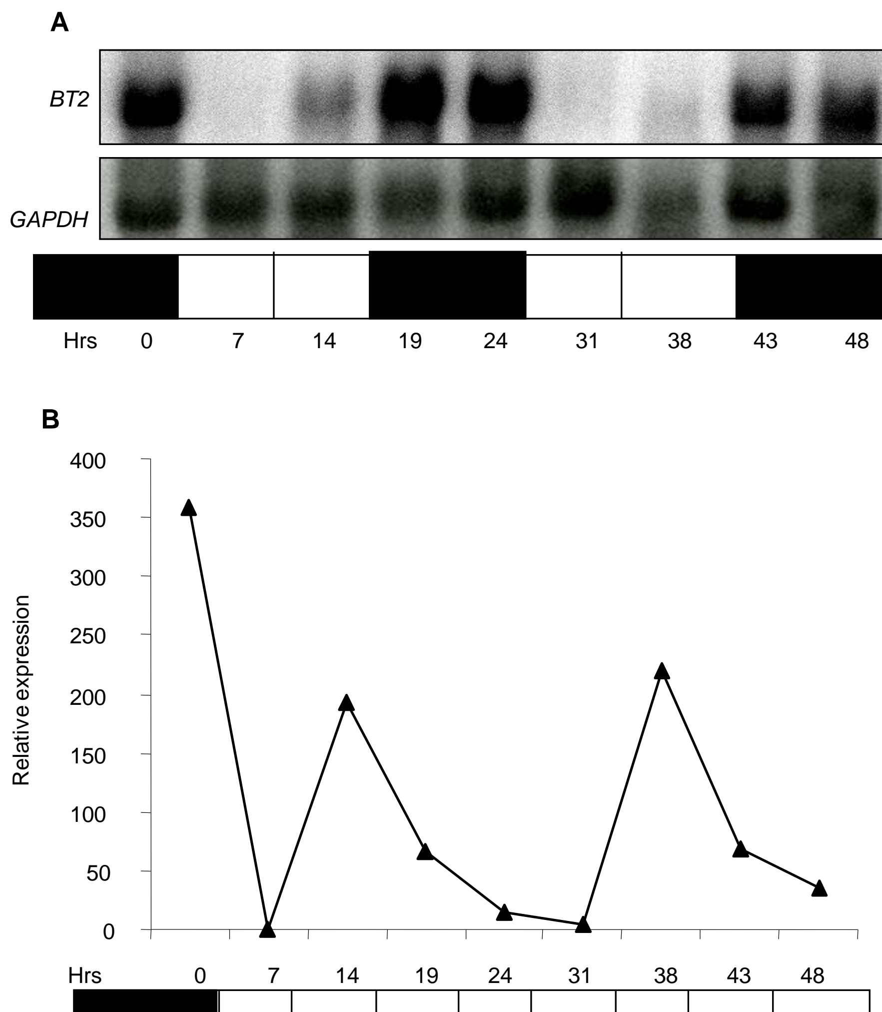

Figure 3. *BT2* expression is diurnally regulated and is controlled by a circadian clock. After entrainment of wild-type plants to 14:10 h light-dark (LD) cycles for 3 weeks, plants were either held in LD (A) or transferred to continuous light (B). Total RNA was extracted from rosette leaves harvested at the indicated times and was subjected to RNA gel blot analysis (A) or qRT-PCR (B). mRNA of *GLYCERALDEHYDE PHOSPHATE DEHYDROGENASE* (*GAPDH)* was used as a loading control for RNA gel blot. *EIF-4A2* was used to normalize the qRT-PCR data. Expression values plotted for *BT2* in (B) are the average of two biological replicates and are relative to the minimum value, which occurred at the 7 h time point. Light and dark bars at the bottom of each figure represent respective light conditions.

I then performed two experiments to determine whether light alone could modulate *BT2* expression. First, I subjected 3-wk-old light-dark entrained plants to an extended dark treatment. RNA samples were harvested at -1, 0, +1 and +2 h into the extended dark phase, along with control samples that were harvested from plants in a normal light-dark cycle, and subjected to qRT-PCR analysis. *BT2* expression was at its highest when the plants were in the dark at -1 and 0 h. As expected, *BT2* expression was down-regulated at +1 h and +2 h in control samples that were transferred to light. However, in plants that stayed in extended darkness, *BT2* expression remained higher (Figure 4A).

In the second experiment, 3-wk-old light-dark entrained plants were exposed to either 1h of dark during the light phase or 1 h of light during the dark phase, and BT2 expression was analyzed by RNA gel blots. *BT2* mRNA was increased by the brief exposure to dark during the light phase but decreased by the brief exposure to light during the dark phase (Figure 4B, 4C). Together, these results indicate that

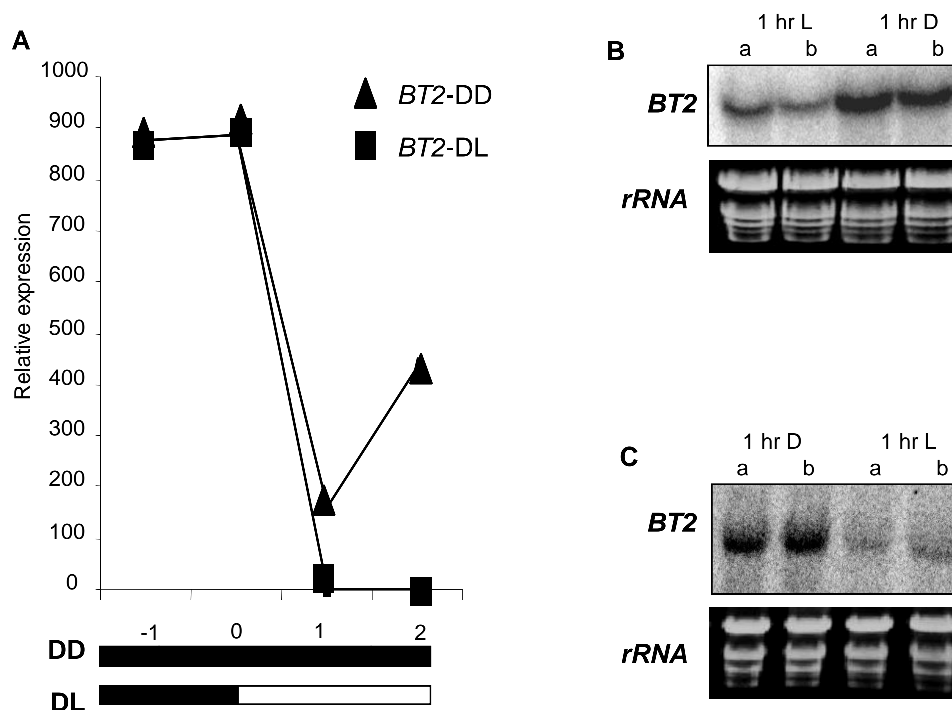

Figure 4. Light modulates *BT2* expression, independent of circadian regulation. (A) After entrainment of wild-type plants to 14:10 h light-dark cycles for 3 weeks, plants were either held in extended darkness (DD) or transferred to light (DL). Total RNA was extracted from rosette leaves harvested at the indicated times and subjected to qRT-PCR analysis. *18S rRNA* was used to normalize the qRT-PCR data and the expression values plotted for *BT2* are average of two biological replicates. Values are relative to the minimum value, which occurred at the +1 h time point. Light and dark bars at the bottom represent respective light conditions. (B) 3-wk-old wild-type plants were either exposed to 1 h of dark during the middle of light phase, or (C) 1 h of light during the middle of dark phase. Rosette leaves were harvested and analyzed by RNA gel blots. The first two lanes in both the blots represent controls that remained in their respective light and dark conditions. Replicate samples are indicated by “a” and “b”. Ethidium-stained rRNA was used as a loading control.

expression of *BT2* is modulated by light and also is under the control of a circadian clock.

BT2 *responds to changes in nutrient status of the plant*

To understand the functional significance of the diurnal regulation of *BT2*, I investigated whether its expression was correlated with metabolic changes. One of the major metabolic changes associated with a diurnal cycle is the concentration of sugars, which peak during the light and diminish in the dark. To test whether *BT2* expression responded to sugars, 3-wk-old, light-dark entrained plants that were in the middle of a dark phase were treated with either sucrose or mannitol (as an osmotic control) for 3 h in the dark. All samples were then subjected to RNA gel blot analysis. *BT2* transcript was repressed by sucrose even in the absence of the light (Figure 5A), suggesting that the low-sugar status of plants is a strong signal for *BT2* induction. Glucose also repressed *BT2* expression (Figure 5B). Mannitol had no effect on BT2 mRNA concentrations (Figure 5D).

Similar to sugars, changes in nitrogen status can affect resource allocation, growth and development in plants. Nitrogen status also modulates the circadian clock by serving as an input (Scheible et al., 2004; Gutierrez et al., 2008). Moreover, the C:N ratio in plants is tightly regulated, with interconnected sensing and signaling mechanisms (Coruzzi and Zhou, 2001). For example, addition of nitrates reverses sugar-mediated repression of gene expression (Moore et al., 2003). Because *BT2* expression was modulated by the circadian clock and responded to carbon signals, I wanted to determine whether nitrogen also modulated *BT2* expression. Three-wk-old, light-dark entrained plants were treated with either KNO_3 or KCl for 3 h in the middle of a light phase. *BT2* repression during the light phase was reversed by addition of nitrates (Figure 5C). Interestingly, nitrate induction of *BT2* was also observed when plants were treated during their dark phase (Figure 5C). KCl had no effect on *BT2* mRNA concentrations (Figure 5D).

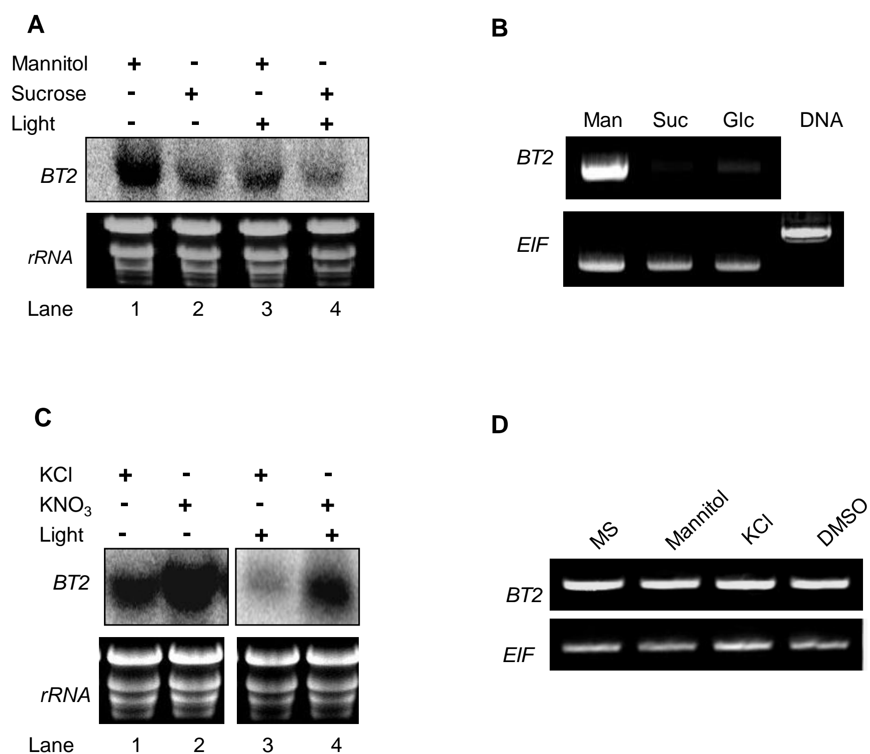

Figure 5. *BT2* is repressed by sugars and induced by nitrates. (A) 3-wk-old wild-type plants were treated with mannitol or sucrose in dark or in light for 3 h. (B) One week old seedlings, grown in continuous light, were treated with mannitol, sucrose or glucose for 3 h. Total RNA was isolated and subjected to RT-PCR analysis. *EIF4-A2* expression was used as a loading control. (C) 3-wk-old wild-type plants were treated with KCl (control) or KNO_3 in dark (lane 1, 2) or in light (lane 3, 4) for 3 h. (D) Conditions used as controls for this figure and for figure 7A had no effect on *BT2* mRNA concentrations.

BT2 *suppresses sugar signaling*

Sugars, in addition to their metabolic roles, act as signaling molecules and control key aspects of plant growth and development. High sugar levels early in plant development can inhibit germination and cotyledon emergence (Smeekens, 2000; Gazzarrini and McCourt, 2001; Moore et al., 2003). Because *BT2* expression was modulated diurnally and by the sugar status of the plant, I predicted a role for *BT2* in some aspects of sugar signaling. To test this hypothesis, seeds from *BT2*-null (*bt2-1*), constitutively expressing *BT2* (*BT2OE*) and WT lines were germinated on various concentrations of glucose (4%, 5%, and 6%) or mannitol (5%), and the percentage of seedlings with normal cotyledon emergence was determined. All lines had nearly 100% germination on 5% mannitol, however concentrations of 4% and 5% glucose were sufficient to inhibit WT germination. At similar concentrations, *bt2-1* seeds were hypersensitive, and *BT2OE* seeds were resistant to glucose inhibition of germination (Figure 6). Higher concentrations of glucose (6%) inhibited germination of all lines equally. These results suggest that *BT2* suppresses sugar signaling during germination and early vegetative development.

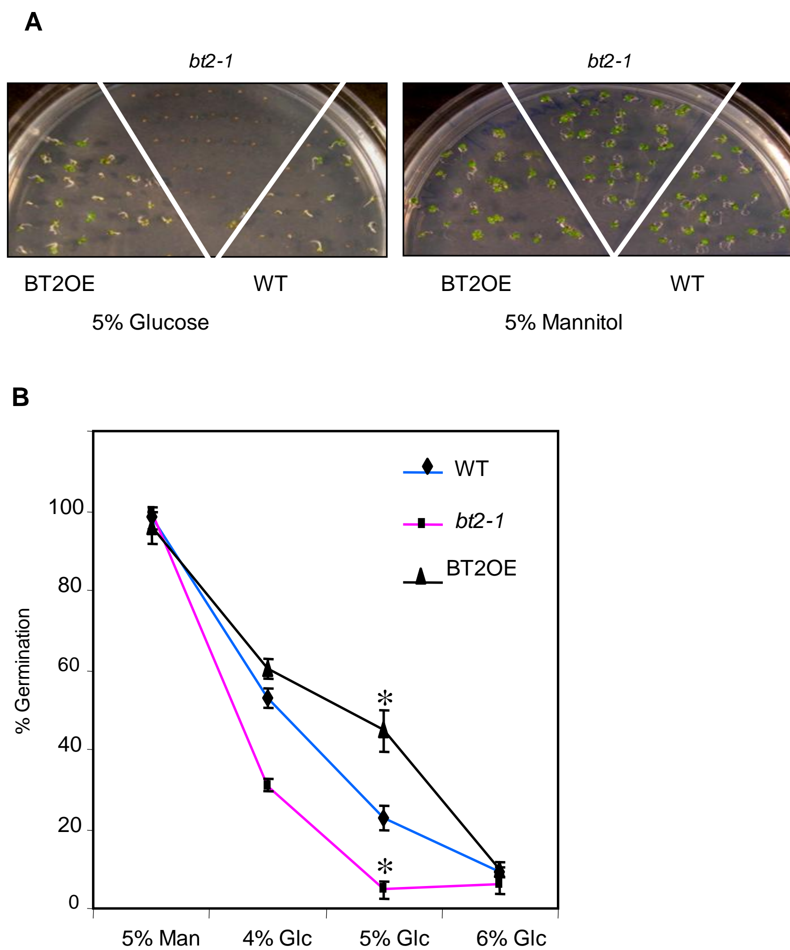

Figure 6. BT2 suppresses sugar-mediated inhibition of germination. (A) Visible phenotypes of 6-day-old wild-type, *bt2-1,* and BT2OE lines grown on either 5% glucose or 5% mannitol. (B) Quantification of germinated seedlings with normal cotyledons of wild-type, *bt2-1*, BT2OE lines grown on various concentrations of glucose (4%, 5% and 6%) or mannitol (5%). Approximately 30 seedlings per line per plate were assayed, and three plates per treatment were used. Error bars indicate standard deviation (SD). Asterisks indicate significant differences compared to wild-type ($p < 0.05$).

BT2 *modulates hormone responses in plants by suppressing ABA signaling while enhancing* yucca1d *responses*

Because ABA inhibits germination in a manner similar to sugars (Arenas-Huertero et al., 2000; León and Sheen, 2003), I performed two experiments to determine whether BT2 also affected ABA signaling and responses at germination. First, I germinated seeds from *bt2-1*, *BT2OE* and WT lines on various concentrations of ABA (0, 1, 2.5, 5 μM) and determined the percentage of seedlings with normal cotyledon emergence. In parallel to the results obtained for sugars, ABA concentrations as low as 2.5 μM reduced germination of WT. However, at similar concentrations the *bt2-1* line was sensitive, while the *BT2OE* line was resistant to ABA inhibition (Figure 7A), leading us to conclude that BT2 suppresses certain ABA signals or responses at germination. Next, I asked whether selected ABA signaling genes were differentially expressed in *bt2-1*, *BT2OE* and WT lines, when grown in the presence of high sugars (5% glucose). The results from RT-PCR experiments performed on *ABA-insensitive 3* (ABI3), *ABI4* and *ABI5* indicate that the relative abundance of the respective transcripts in the different lines remained unaffected (Figure 7B).

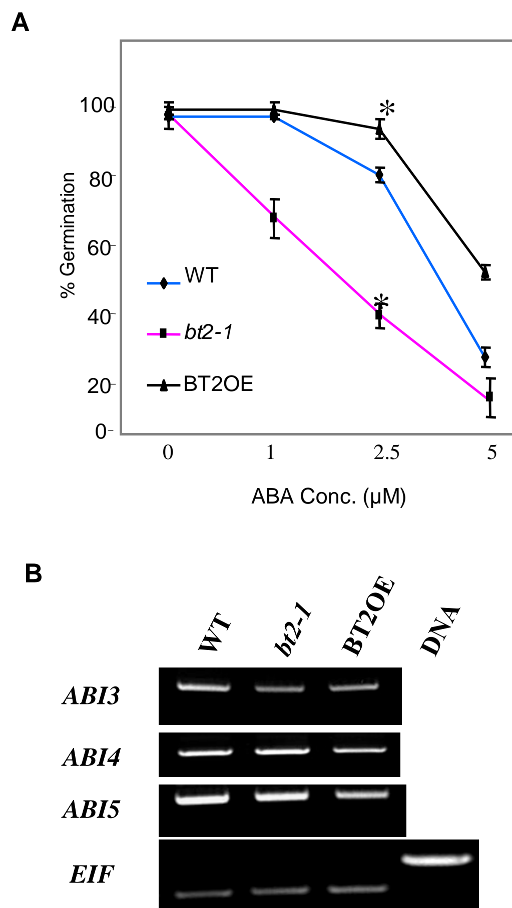

Figure 7. BT2 suppresses ABA-mediated inhibition of germination. (A) Quantification of the % germinated seedlings with normal cotyledons of wild-type, *bt2-1*, *BT2OE* lines grown on various concentrations of ABA (0, 1, 2.5, 5 µM). Approximately 30 seedlings per line per plate were assayed and three plates per treatment were used. Error bars indicate SD. Asterisks indicate significant differences compared to wild-type ($p < 0.05$). (B) 10-day-old wild-type, *bt2-1,* and *BT2OE* lines were grown on 5% glucose. Total RNA was isolated and subjected to RT-PCR analysis to determine expression of *ABI3, ABI4* and *ABI5*. *EIF4-A2* expression was used as a loading control.

We previously reported that *BT2* potentiates some responses to auxin. *bt2-1* seedlings are resistant to exogenous auxin, while constitutive expression of *BT2* in the high-auxin mutant *yucca1d* exacerbate its phenotype (Ren et al., 2007). Here, I found that loss of BT2 in *yucca1d* specifically suppressed its characteristic high-auxin phenotype of epinastic cotyledons, epinastic leaves, shorter primary roots, excess root hair, and delayed development (Figure 8). However, the elongated hypocotyl and petioles were less affected.

BT2 *appears to integrate multiple stress signals*

Because sugar and hormone signaling affect several responses to abiotic and biotic factors, and because BT2 has a role in both hormone and sugar signaling, I asked whether it also was required for stress responses. I started by analyzing changes in *BT2* expression in response to different stress signals by treating 3-wk-old plants with ABA, methyl-jasmonate (Me-JA), cold, wounding and H_2O_2.

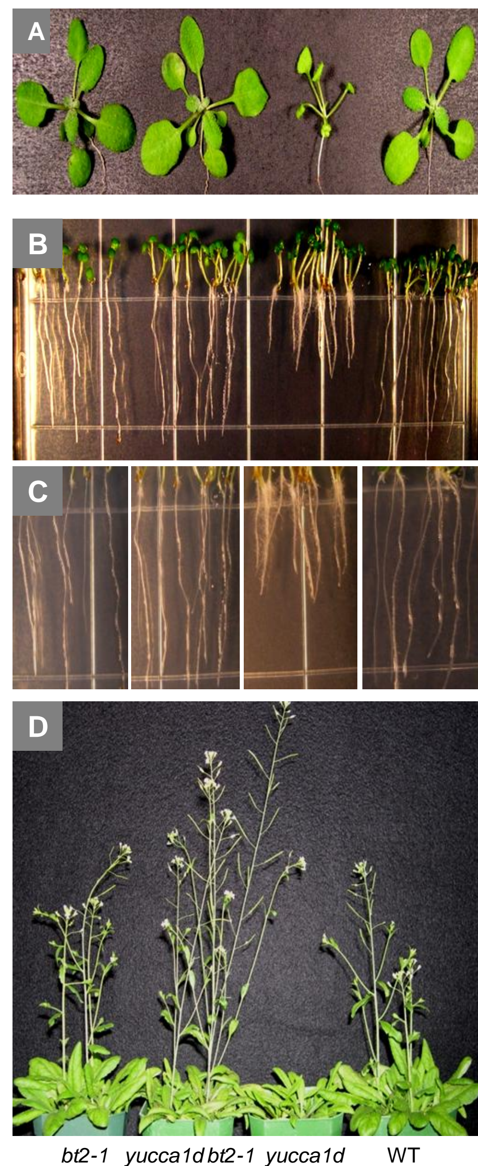

Figure 8. Loss of BT2 suppresses the high-auxin phenotypes in *yucca1d.* Suppression of epinastic cotyledon and leaf morphology (A), shorter primary root (B), excess root hairs (C), and delayed flowering (D) of *yucca1d* in the double mutant *yucca1d bt2-1.*

Treatment with ABA and cold lowered *BT2* mRNA levels (Figure 9A). Me-JA, which is antagonistic to ABA and mediates plant-pathogen defense signaling (Anderson et al., 2004), induced *BT2* expression along with cytokinin. However, wounding, auxin and brassinolide treatment did not effect the expression of *BT2* (Figure 9A). *BT2* expression was induced by H_2O_2, a major reactive oxygen species (ROS) in plants, but not by treatment with methyl viologen, an electron transport inhibitor that also leads to oxidative stress (Figure 9B). To ask whether loss of BT2 had any effect on plants challenged with free radical stress generated by H_2O_2, I grew *bt2-1*, *BT2OE*, and WT plants on media with and without 2 mM H_2O_2. Initially, H_2O_2 suppressed growth of all the lines equally (i.e., smaller leaves, shorter petioles and an overall inhibition of vegetative growth). However, after a period of ~ 3 weeks in the presence of H_2O_2, *bt2-1* plants were significantly smaller, whereas *BT2OE* was indistinguishable from WT (Figure 9C).

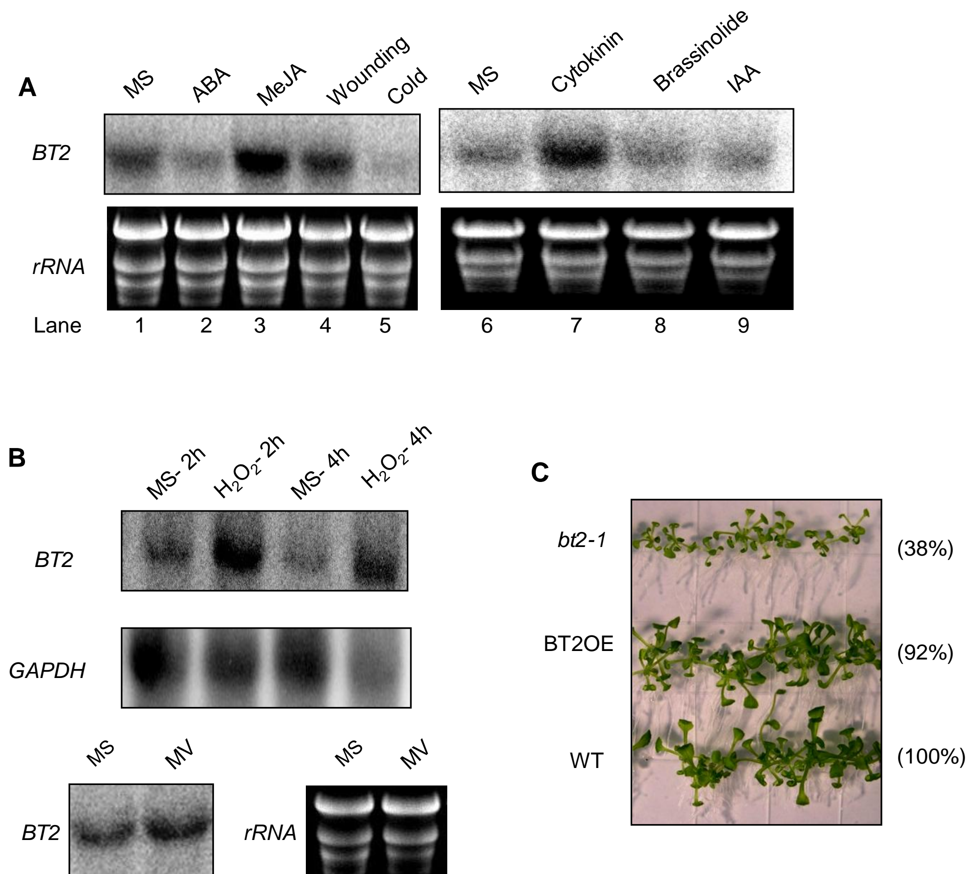

Figure 9. *BT2* expression is modulated by multiple stress signals. (A) 3-wk-old wild-type plants were treated with either ABA (lane 2), or Me-JA (lane 3) for 3 h; subjected to either wounding stress (lane 4) or cold stress (lane 5) for 3 h, treated with hormones cytokinin (lane 7), brassinolide (lane 8) and IAA (lane 9) for 3 h and (B) challenged with oxidative stress caused by either H_2O_2 or methyl-viologen for the indicated times. Total RNA was extracted from rosette leaves and subjected to RNA blot analysis. Ethidium-stained rRNA or *GAPDH* were used as loading controls. (C) Visible phenotype of 3-wk-old wild-type, *bt2-1* and *BT2OE* lines grown on MS media containing H_2O_2 (2 mM). Total fresh weight (mg) of 30 seedlings from two different plates and ratio of weights relative to WT (in parentheses) is shown on the right.

Discussion

We previously identified BT2 as an activator of telomerase activity in mature *Arabidopsis* leaves (Ren et al., 2007). Here, I report its roles in sugar signaling, its connections to hormone signaling, and its apparent function in integrating diverse biotic and abiotic stress signals. I showed that *BT2* expression was diurnally regulated. However, this is not surprising since 30% - 50% of *Arabidopsis* genes are diurnally regulated, and the circadian clock and sugar status are the major inputs driving the diurnal regulation (Blasing et al., 2005). *BT2* mRNA was more abundant (> 100-fold) in the dark, relative to the light (Figure 3). *BT2* expression was controlled by the circadian clock, because the diurnal anticipation of *BT2* expression at 14 h and 38 h (Figure 3) in entrained plants persisted even in a continuous light cycle. In light-dark conditions, *BT2* mRNA begins to accumulate at 14 h and 38 h, in a possible anticipation of the dark phase (Figure 3A). An initially puzzling feature of *BT2* expression in continuous light was, instead of peaking at the end of dark period (24 h and 48 h), the peaks of *BT2* mRNA occurred at end of the light period at 14 h and 38 h (Figure 3B). *BT2* transcript failed to accumulate after the anticipatory period in continuous light, possibly because of the continuous presence of the abnormal light signal or another metabolic signal. I also found that *BT2* was repressed and induced by brief exposure to light and dark alone, respectively (Figure 4). These results suggest that light can also modulate *BT2* expression independent of the circadian control. However, under prolonged exposure to an inappropriate signal, as in my continuous light experiment, the circadian regulation

can over-ride the abnormal signal, in an apparent attempt to restore an appropriate level of *BT2* transcript.

Although the circadian clock drives the diurnal regulation of many genes, diurnal changes in sugar concentration also play a major role (Blasing et al., 2005), and *BT2* expression was repressed by sugars (Figure 5A). This result was also consistent with my finding that relative expression of *BT2* was lowest during the light phase, possibly because of repression caused by higher levels of sugars produced by photosynthesis. In the dark, however, expression of *BT2* was induced, possibly in response to sugar depletion. I also found that *BT2* was induced by nitrates both during light and dark phases (Figure 5C). Addition of nitrates antagonizes sugar repression of gene expression (Moore et al., 2003). This antagonism could be due, in part, to competition for a limited amount of carbon in the cell that can be either diverted to produce organic acids and amino-acids by nitrogen metabolism or to produce sugars and starches by carbon metabolism (Stitt and Krapp, 1999). My results are consistent with gene expression databases, which indicate *BT2* is repressed by sugars and induced by nitrates (Scheible et al., 2004; Wang et al., 2004b; Blasing et al., 2005; Usadel et al., 2008).

To determine whether BT2 was involved in responses to sugar signaling, I analyzed germination in the presence of inhibitory glucose concentrations and found that constitutive over-expression of BT2 imparted resistance to inhibition of germination and early vegetative development by glucose. In contrast, loss of BT2 resulted in significantly increased sensitivity to inhibitory glucose levels (Figure 6).

High concentrations of sugars impart their inhibitory effect on germination by modulating ABA signaling (León and Sheen, 2003). Also, several mutations identified as glucose-insensitive or sugar-insensitive such as *gin1*, *gin5*, *sis4, sis7* and *sis10* are allelic to ABA-insensitive/deficient mutations (Arenas-Huertero et al., 2000; Cheng et al., 2002). After identifying a role for BT2 in modulating sugar signaling/responses at germination, I wanted to determine whether ABA responses also were modulated by BT2. I found that, parallel to the sugar responses, *bt2-1* was sensitive to ABA inhibition of germination, while *BT2OE* lines were resistant (Figure 7A). Again, similar to sugars, ABA repressed the expression of *BT2* (Figure 9A). This pattern of reciprocal negative feedback (where BT2 suppresses ABA signaling and ABA suppresses BT2 expression) suggests that BT2 may normally function to prevent inappropriate signaling at low concentrations of ABA, but this function can be abrogated at higher concentrations of ABA.

BT2 does not appear to affect expression at the mRNA level for ABA signaling genes. This conclusion is supported by our previous micro-array studies performed on the *tac1-1d* mutant line, which has increased *BT2* expression (Ren et al., 2007). When compared to WT, *tac1-1d* lines did not display any significant changes in transcript levels for genes in ABA signaling pathways. This lack of influence on ABA signaling genes could be due to either insufficient expression of BT2 at the protein level in 35S::BT2 and *tac1-1d* lines or the well-documented redundancy among BT family members (Robert et al., 2009) in the *bt2-1* null mutant. Alternatively, BT2 itself may be a downstream target of the ABA signaling

genes. Regardless of the mechanism, my current results strongly support a role for BT2 in modulating sugar and ABA responses at germination.

Cold signaling is intricately associated with ABA, and cold stress and treatment with ABA, repressed *BT2* expression (Figure 9A). In fact, treatment with cold leads to an increase in the levels of ABA (Lee et al., 2001). Hence, it is possible that the cold repression of *BT2* was an indirect effect of increased ABA levels and/or signaling. Jasmonate is antagonistic to ABA in modulating defense gene expression (Anderson et al., 2004) and salt stress-inducible gene expression in rice (Moons et al., 1997). In contrast to ABA and cold, Me-JA and cytokinin induced *BT2* expression, thus suggesting a possible role of BT2 in JA signaling/pathogen defense and cytokinin signaling. However, further experiments are necessary to directly implicate BT2 in defense or cytokinin signaling.

ABA, often dubbed 'the universal stress hormone', is associated with response to reactive oxygen species (ROS) and cross-talks with multiple hormones, biotic and abiotic signals (Roitsch, 1999; Fedoroff, 2002; Couee et al., 2006). *BT2* is induced by H_2O_2, a major ROS in plants, and loss of BT2 renders the plants sensitive to external H_2O_2 (Figure 9). This sensitivity, however, was not observed when the *bt2-1* lines were subjected to a different ROS stress, super-oxide anion generated by methyl-viologen (MV) (data not shown). Moreover, *BT2* expression was not induced by ROS stress caused by MV (Figure 9B), suggesting that the response of *BT2* to H_2O_2 is specific, and not due to general ROS-related stress.

We previously reported that BT2 enhances certain auxin responses (Ren et al., 2007). Here, I present additional evidence for its role in potentiating *yucca1d* responses. Loss of BT2 in the high auxin mutant *yucca1d* reversed several of its high-auxin phenotypes, including its characteristic epinastic cotyledons, epinastic leaves, shorter primary root, excess root hair, and delayed flowering (Figure 8A, B, C, D). From my current results and previous studies (Ren et al., 2007), BT2 seems to potentiate *yucca1d* responses.

Although BT2's initially described function was in regulating telomerase activity in mature leaves (Ren et al., 2007), and it was recently shown to function in gametophyte development along with other BT family genes (Robert et al., 2009), it now appears to play a much broader role. The gene itself responds to multiple biotic and abiotic signals, including light, circadian clock, phytohormones, and nutrients, and BT2 is required for appropriate response to many of these same signals (Figure 10). I propose that BT2 occupies an integral position in a complex signaling network that perceives, integrates, and responds to multiple, and sometimes competing, signals. Preliminary results from our lab indicate that similar to *BT2*

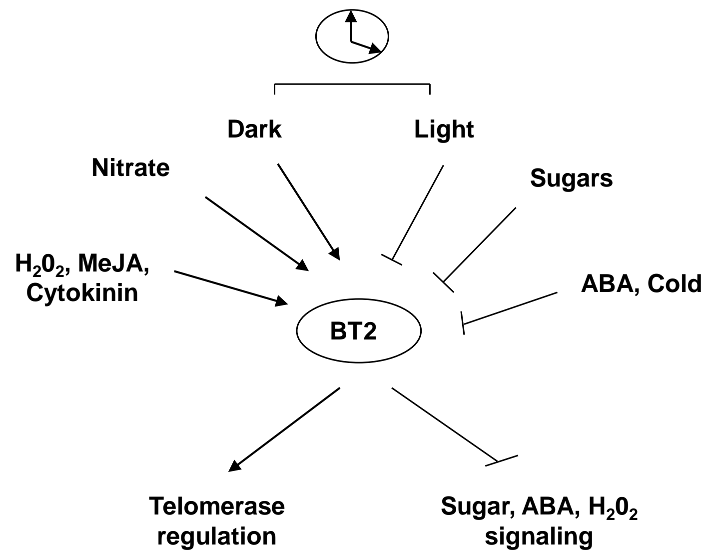

Figure 10. BT2 may function as an integrator of light, nutrient, hormone and stress signals and maintains the required homeostatic responses

responses, expression of *BT1* and *BT5* is also responsive to sugars and nitrates (Mandadi et al., unpublished; also in Chapter IV), consistent with previous reports of functional redundancy in the BT gene family (Robert et al., 2009).

It is not yet clear how BT2 affects multiple signaling pathways. Earlier studies from other labs, using recombinant proteins, in vitro pull-down assays, or yeast two-hybrid (Y2H) screens, identified BT2, along with other BT family members, as interacting with either CULLIN3 (Figueroa et al., 2005) or with the BET9 and BET10 bromodomain proteins (Du and Poovaiah, 2004). Although BT2's in vivo partners are yet to be identified, I hypothesize that it assembles in multi-protein complexes. If the complex requires CULLIN3 or a similar protein, it may function as an ubiquitin ligase and target specific proteins for degradation. Alternatively, if the BT2 complex requires the BET9 or BET10 bromodomain proteins, the complex may work by recognizing the chromatin state of target gene promoters. Identification of proteins that interact with BT2 in vivo will be required to resolve the possible modes of action.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* (Col-0) or mutant plants were grown in soil in 14 h light/10 h dark at 21 °C and a light intensity of ~120-130 μmol/m^{2}s with 70% relative humidity. For germination assays, seeds were surface sterilized with 50% (v/v) bleach and 0.1% (v/v) Triton X-100 for seven minutes, cold treated at 4 °C for 3-4 days, and then grown on Murashige and Skoog (MS) medium (Sigma) with 0.8% (w/v) phytagar under continuous low light (~ 30 μmol/m^{2}s). All media contained 1% sucrose, unless stated otherwise. *BT2* overexpression lines and the *bt2-1* null line were previously described (Ren et al., 2007). To examine the effect of loss of BT2 on the high-auxin phenotype of *yucca1d* (Zhao et al., 2001), I generated and examined the F2 progeny of *yucca1d bt2-1*cross.

Treatments and expression analysis

For circadian experiments and light-dark treatments, whole rosettes of 3-wk-old wild-type plants (prior to flowering) were harvested at the indicated times of the diurnal cycle. Two biological replicates each containing two rosettes were harvested and subjected to RNA gel blot and quantitative real-time PCR (qRT-PCR) analysis. Total RNA was isolated using TRI reagent following manufacturer's protocol (Ambion). For RNA gel blots, 20 μg of total RNA from each sample was separated on a 1.2% formaldehyde denaturing gel and transferred to Hybond N$^+$ membrane (Amersham). Blots were then probed with ^{32}P-labeled PCR products obtained from amplification of *BT2* cDNA using the primers listed (Table 1). Subsequently, the blot

was stripped and re-probed for *GAPDH* mRNA as a loading control. For qRT-PCR, 1 µg RNA was used to make cDNA using SuperScript first-strand cDNA synthesis kit (InVitrogen). Amplification by PCR was performed as described previously (Guo et al., 2008) using Power SYBR Green Master Mix (Applied Biosystems) and the ABI Prism 7500 sequence detection system (Applied Biosystems). The primers used for qRT-PCR are listed (Table 1). *EIF-4A2* (At1g54270) and *18S rRNA* (At2g01010) were used to normalize the expression and fold changes of *BT2* expression were calculated following the $^{\Delta\Delta}C_T$ method (Livak and Schmittgen, 2001; Guo et al., 2008).

To determine the effects of sugars and nitrates, leaves from 3-wk-old wild-type plants were excised at the indicated times. To maintain transpiration flow petioles were immediately re-cut in liquid media supplemented with 100 mM of sucrose or mannitol and 50 mM of KNO_3 or KCl, respectively (Chiou and Bush, 1998). Treatments were conducted for 3 h in the appropriate light conditions, and the samples were subsequently subjected to RNA gel blot analysis as described above to detect *BT2* expression. Ethidium-bromide stained rRNA was used as a loading control. For glucose treatments, seedlings were grown in MS liquid media for 5 days in continuous light and later transferred into media without any sugars for 2 days. After the seventh day, the medium was supplemented with 50 mM glucose, 50 mM sucrose or 50 mM mannitol, and the seedlings were treated for 3 h (Scheible et al., 2004; Blasing et al., 2005). Subsequent analysis of *BT2* expression was performed by RT-PCR using 5 µg of total RNA to prepare cDNA. To determine

expression of *ABI3, ABI4* and *ABI5*, 10-day old WT, *bt2-1* and *BT2OE* lines were grown on 5% glucose and analyzed by RT-PCR (25 cycles). The primers used for RT-PCRs are listed (Table 1).

To determine the effects of various stresses on *BT2* expression, 3-wk-old wild-type plants were subjected to various stress stimuli during the light phase. Cold treatment was performed by floating leaves in MS liquid medium on ice for 3 h; for wounding, leaves were punctured with forceps at several places and transferred to MS liquid medium for 3 h; for stress hormones, leaves were treated for 3 h in MS liquid media consisting of ABA (100 μM, mixed isomers), 6-benzyl adenine (100 μM), IAA (10 μM), brassinolide (100 μM) and Me-JA (100 μM), or DMSO (0.1%); for oxidative stress, leaves were treated for the indicated times in MS liquid media consisting of H_2O_2 (10 mM) and methyl-viologen (100 μM). *BT2* expression was analyzed by RNA gel blot analysis using 20 μg of total RNA as described above. Ethidium-bromide stained rRNA was used as a loading control. All the treatments were repeated at least twice at different periods, and the results described are representative of the consistent data obtained in the replicated experiments. DMSO did not affect *BT2* expression.

Glucose, ABA and H_2O_2 sensitivity assays

For glucose inhibition assays, wild-type, *bt2-1,* and *BT2OE* lines were germinated on solid MS medium with various concentrations of glucose (4%, 5% and 6% w/v) or mannitol (5% w/v), as described previously (Bi et al., 2005). After 5 or 6 days, seedlings with normal cotyledons were counted. For ABA inhibition

assay, wild-type, *bt2-1* and *BT2OE* lines were germinated on MS solid media with various concentrations of ABA (0, 1, 2.5, 5 μM), as described previously (Xiong et al., 2002). After 5 or 6 days, seedlings with normal cotyledons were counted. Three replicate plates for each treatment were used to calculate the % germination rates and significant differences were determined by student t-test. For H_2O_2 sensitivity assay, wild-type, *bt2-1* and *BT2OE* lines were germinated on MS solid media with or without 2 mM of H_2O_2 (Miao et al., 2006) and were kept vertically in continuous low light (~30 μmol/m^{2}s) for 3 weeks. Mean fresh weight of the seedlings was determined from averages of two replicate plates.

Accession numbers

AGI locus numbers for genes used in this chapter are: *BT2*, At3g48360; *ABI3*, At3g24650; *ABI4*, At2g40220; *ABI5*, At2g36270; *EIF-4A2*, At1g54270; *18S* rRNA, At2g01010; *GAPDH*; At3g04120.

Table 1. Primers used for analysis of BT2 function to mediate multiple responses

RNA blot analysis:	
BT2	F-ATGGAAGCTGTTCTTGTCGCAATGTCCG
	R-TAAACCCCTTGTGCTTGTTCACATTTG
GAPDH	F-GACCTTACTGTCAGACTCGAG
	R-CGGTGTATCCAAGGATTCCCT
qRT-PCR analysis:	
BT2	F-CACAACGGAAGACGACGGAT
	R-CAGTACCGGTGAAGCTGAA
EIF-4A2	F-CAAGGTGTCAAGGTTCATGC
	R-CAACGACAACATGAACACCA
18S rRNA	F-GTCATCAGCTCGCGTTGACTAC
	R-GAGCGACGGGCGGTG
RT-PCR analysis:	
BT2	F-ACATGGTCACCCAGCTGAAG
	R-CAGACACAACCCTTGTCACC
AB13	F-CACAGCCAGAGTTCCTTCCTTT
	R-AGGTTACCCACGTCGCTTTGCT
AB14	F-TCGCAAGTGGCTTGGTACTTTCG
	R-CGGATCCAGACCCATAGAACA
ABI5	F-GGAGGTGGCGTTGGGTTT
	F-GGACAACTCGGGTTCCTCATCA
EIF-4A2	F-GCAAGAGAATCTTCTTAGGGGTATCTATGC
	F-GGTGGGAGAAGCTGGAATATGTCATAG

CHAPTER III

BT2 IS REQUIRED FOR CAULIFLOWER MOSAIC VIRUS *35S* ENHANCER-MEDIATED ACTIVATION OF GENE EXPRESSION

Summary

The *Arabidopsis* BT2 protein contains a BTB domain at its N-terminus, a central TAZ zinc-finger protein-protein interaction domain, and a C-terminal calmodulin binding domain. In the previous chapter I demonstrated that BT2 mediates multiple responses to nutrients, hormones, abiotic and biotic stresses in *Arabidopsis*. Here, I report a significant role of BT2 in regulating transcriptional activation of genes by CaMV *35S (35S)* enhancers. Loss of BT2 (*bt2-1*) in several well-characterized *35S* enhancer activation-tagged lines such as, *yucca1d*, *pap1d*, *jaw1d,* resulted in suppression of the activation phenotypes. Suppression of the phenotypes was due to decreased transcript abundance of the tagged genes *YUCCA1*, *PAP1*, and microRNA *miR-JAW* precursor, respectively.

Nuclear run-on assays and mRNA decay studies in activation-tagged lines indicated that BT2 controls transcript abundance by regulating transcription from the *35S* enhancer, and not by altering mRNA turn-over. BT2 is specifically required only for multi-merized *35S* enhancer function, and does not affect activity of full constitutive promoters such as the entire CaMV *35S* or *NOS* promoters. Moreover, the Ca^{++}/calmodulin binding domain of BT2 is essential for regulating *35S* enhancer activity. I further demonstrate that CULLIN3 genetically interacts with BT2. Loss of CULLIN3 in *yucca1d* lines suppressed activation phenotypes and decreased *YUCCA1* expression. I propose that BT2, and CULLIN3 are components of a ubiquitin ligase complex. Along with two bromodomain proteins BET9 and BET10, the complex is required for *35S* enhancer-mediated activation of gene expression.

Introduction

Regulation of transcription is a coordinated process and requires synergistic action of numerous cellular factors. Enhancers are DNA elements that activate transcription (Khoury and Gruss, 1983; Serfling et al., 1985). They are distinct from the core promoter and promoter-proximal elements in several

aspects. First, enhancers function even when far (~80 kb) away from the transcription start site (Jack et al., 1991). Second, enhancers function in a sequence-, position-, and orientation-independent manner and activate transcription even when present upstream or downstream of a gene (Weigel et al., 2000). Third, enhancers and core promoters may possess different *cis*-elements that bind different general transcription factors (GTFs) and regulators (Blackwood and Kadonaga, 1998). Classical examples of enhancers include the *Drosophila* wing margin enhancer (Jack et al., 1991), human interferon-β gene enhancer (Goodbourn et al., 1985), human immunodeficiency virus-1 (HIV-1) enhancer (Jakobovits et al., 1988), and simian virus40 (SV40) enhancer (Weiher et al., 1983).

35S enhancers are derived from the genome of a plant double-stranded (ds) DNA virus, Cauliflower Mosaic Virus (CaMV), which belongs to the Caulimovirus genus (supergroup: pararetrovirus) (Ow et al., 1987; Muriel et al., 2002). In plant cells, CaMV replicates by an RNA intermediate and produces two major viral transcripts, the *35S* and *19S* RNAs. The CaMV *35S* promoter is a very strong promoter, which renders constitutive expression to heterologous genes without any requirement of CaMV proteins (McKnight and Meagher, 1981; Odell et al., 1985). Hence, over the past two decades the *35S* promoter has been used extensively to over-express genes in plants. Moreover, the architecture of *35S* promoter is well studied. It consists of two domains, A and B (Ow et al., 1987; Benfey et al., 1989). Domain A comprises nucleotides -90 to +8

relative to the transcription start site (hereafter termed *35S* core promoter). *35S* core promoter contains a tandem repeat of TGACG nucleotides separated by 7 bp that is called the *activating sequence 1* (*as-1*) element (Lam et al., 1990). A similar sequence is also present in the *Agrobacterium tumefaciens NOPALINE SYNTHASE (NOS)* promoter, which is another constitutive promoter widely used in plants (Lam et al., 1990). Members of the TGA family of bZIP transcription factors, implicated in pathogenesis related (*PR*) gene expression, bind to the *as-1* element of *35S* promoter and contribute to its activity (Katagiri et al., 1989; Lam and Lam, 1995; Zhang et al., 1999).

Domain B consists of nucleotides -343 to -90 relative to the transcription start site, and constitutes the *35S* enhancer (Hayashi et al., 1992; Weigel et al., 2000). The enhancer consists of a binding element called *activation sequence-2* (*as-2*), which contains two GT motifs. The *as-2* element also has homology to core A enhancer element of SV40, Box II element of pea *rbcS* and GATA motif of light-responsive promoters (Lam and Chua, 1989). An activation sequence factor 2 (ASF2) from tobacco nuclear extracts interacts with *as-2* sequence (Lam and Chua, 1989). The identity of ASF2 is unclear (Teakle et al., 2002; Reyes et al., 2004), however, it is presumed to be a member of the GATA-binding family of transcription factors.

Domain A and domain B confer different developmental and tissue-specific expression patterns on reporter genes because of differences in their *cis*- and *trans*-regulatory elements (Benfey et al., 1989). Since *35S* enhancers can function independently of the *35S* core promoter, they have been widely used to activate genes in plants (Fang et al., 1989; Hayashi et al., 1992; Weigel et al., 2000; Johnson et al., 2007). Activation tagging of genes, often with multiple, tandem copies of enhancers, was a breakthrough technology in functional genomics. It led to the discovery of numerous dominant gain-of-function mutations that would have been otherwise difficult to obtain by traditional loss-of-function screens (Weigel et al., 2000). Currently, several variants of the *35S* enhancer-based activation tagging system exist and are used to generate activation-tagged populations (Dong and VonArnim, 2003; Pogorelko et al., 2008; Qu et al., 2008). The presumed mechanism of *35S* enhancer is that, when randomly inserted either upstream or downstream of a gene, the enhancer directs recruitment of transcription machinery including GTFs, mediators, histone acetyl transferases (HATs), chromatin remodelers, etc. to the promoter and/or promote chromatin modifications to permit binding and activation of transcriptional machinery. This hypothesis is based on studies with model enhancers such as those from wing margin, SV40, and HIV-1 (Bondarenko et al., 2003). However, the identities of cellular factors that regulate *35S* enhancer activity in plants are currently unknown.

While investigating the function of the *Arabidopsis* BTB-domain protein BT2, I discovered that it is an essential regulator of *35S* enhancer activity. Loss of BT2 in multiple activation tagged lines suppressed the activation phenotypes. This suppression is due to a decrease in the tagged-gene expression. Nuclear run-on assays showed that BT2 affected transcription of the activated genes in the activation lines. The C-terminal Ca^{++}/calmodulin binding domain (CaMBD) of BT2 was essential for *35S* enhancer activity. Furthermore, BT2 requires the function of two bromodomain proteins, BET9 and BET10, and CULLIN3 to regulate transcription from 35S enhancers. I propose that BT2 and CULLIN3 are key components of a ubiquitin ligase that is directed to transcriptionally competent regulatory regions by the BET9 and BET10 bromodomain proteins. The BT2 complex may affect transcription by targeting transcription factors (TFs) for degradation by polyubiquitination, or by stabilizing TFs through monoubiquitination.

Results

BT2 *is essential for the high-auxin phenotypes in* yucca1d*, but does not affect auxin signaling in* yucca1d

In my previous characterization of BT2 function, I demonstrated that loss of BT2 (*bt2-1*) in a high-auxin accumulating mutant, *yucca1d,* suppressed its characteristic high-auxin phenotypes such as epinastic cotyledons and leaves, shorter primary roots, excessive root-hairs and delayed flowering (Mandadi et al., 2009; also in Chapter II). The reversal of *yucca1d* phenotypes due to loss of BT2 suggested that BT2 could affect auxin signaling, auxin levels and/or auxin gradients in *yucca1d.*

To investigate the role of BT2 in *yucca1d* responses, I analyzed whether auxin signaling was affected in *yucca1d bt2-1*. Using quantitative (q) RT-PCR, I analyzed the expression of several auxin signaling genes (Aux/IAA gene family) including *IAA1, IAA2, IAA5, IAA6, IAA19* and *GH3* (Abel et al., 1994). I found that expression of Aux/IAA genes was suppressed in *yucca1d bt2-1,* when compared to *yucca1d* (Figure 11). However, I did not detect any change in expression of those Aux/IAA genes in the *bt2-1* line, or in an over-expression line of BT2 (*BT2OE),* when compared to wild-type plants. Next, I wanted to determine whether BT2 was required for auxin-induced gene expression. When treated with exogenous auxin (IAA), I found that Aux/IAA genes responded similarly in *yucca1d* and *yucca1d bt2-1,* suggesting that BT2 was not essential for auxin-induced gene expression in *yucca1d* (Figure 11). Moreover, the

response of Aux/IAA genes to IAA in *bt2-1* and *BT2OE* remained similar to wild-type (Figure 9).

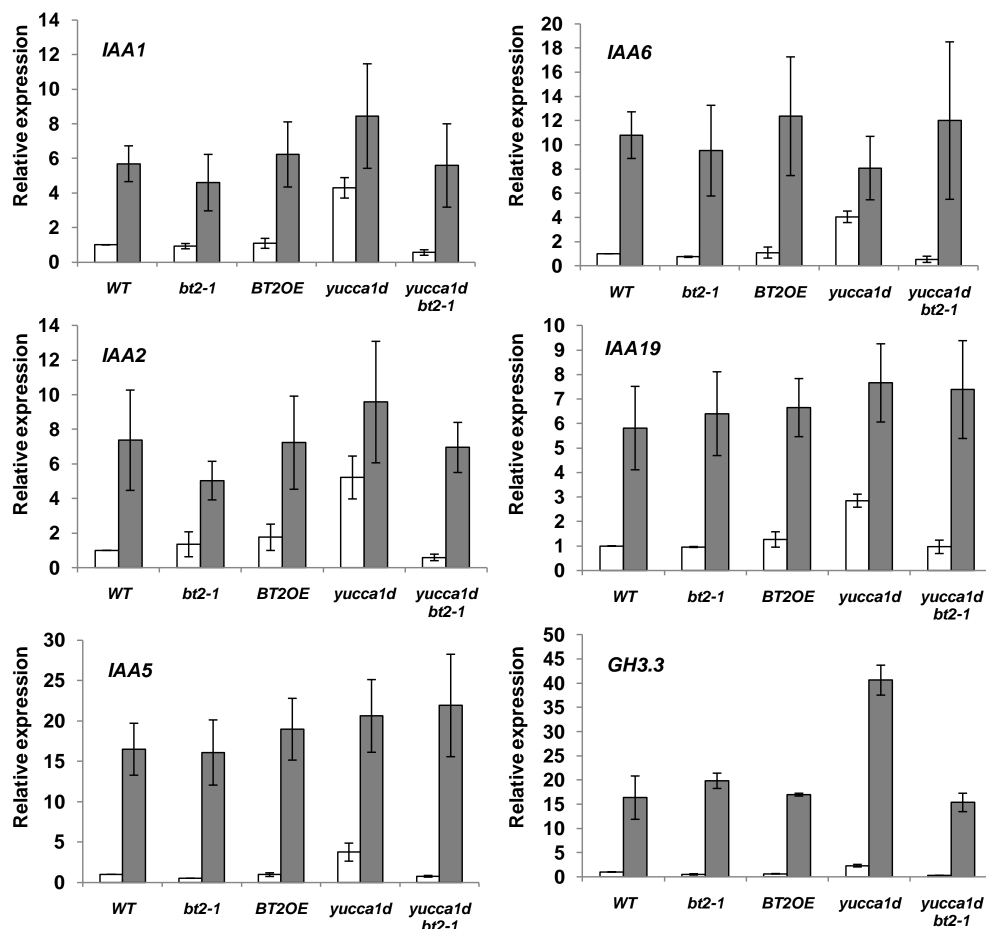

Figure 11. BT2 does not affect auxin-responsive gene expression. Wild-type*, bt2-1, BT2OE, yucca1d, yucca1d bt2-1* seedlings were either mock treated with DMSO (white bars) or treated with 10 µM IAA (grey bars), for 3 hours in MS liquid medium**.** Total RNA was extracted and was subjected to qRT-PCR. *EIF-4A2* was used to normalize the qRT-PCR data. Expression values plotted are relative to mock treated wild-type.

BT2 *regulates* YUCCA1 *transcript levels in* yucca1d*, but is not required for endogenous* YUCCA1 *expression*

Since loss of BT2 did not affect Aux/IAA gene responses to exogenous auxin in *yucca1d*, I asked whether *YUCCA1* expression itself was altered in *yucca1d bt2-1*. I performed qRT-PCR analysis and found that *YUCCA1* expression was indeed suppressed in *yucca1d bt2-1* (Figure 12). I also performed a similar experiment with *a* second *bt2* loss-of-function allele, *bt2-2.* Similar to *yucca1d bt2-1, yucca1d bt2-2* showed suppression of *yucca1d* phenotypes and possessed lower levels of *YUCCA1* transcript. Moreover, similar results were obtained when the parent lines were used for reciprocal crosses (data not shown).

Since BT2 was required for *YUCCA1* expression in *yucca1d*, I next determined whether BT2 was also required for expression of endogenous *YUCCA1* in wild-type plants. Using qRT-PCR analysis I determined the levels of endogenous *YUCCA1* mRNA and found that it remained unaltered in *bt2-1* and *BT2OE* lines (Figure 12), when compared to wild-type plants. This result suggests that BT2 did not regulate endogenous expression of *YUCCA1*, but it did regulate *YUCCA1* transcript abundance in the *yucca1d* activation-tagged line.

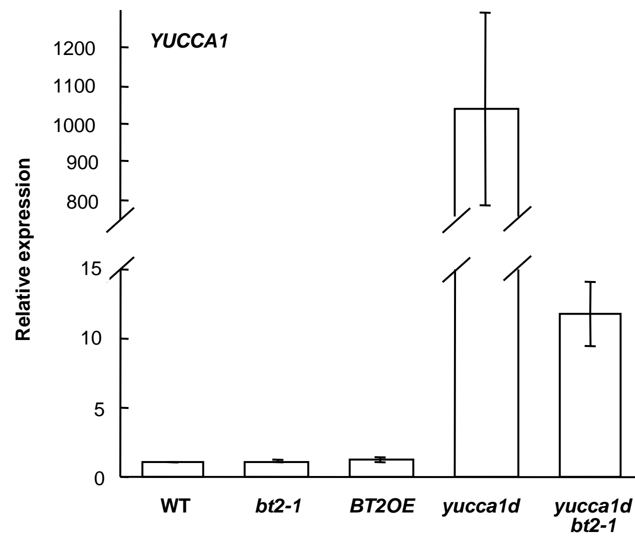

Figure 12. BT2 regulates *YUCCA1* transcript abundance in *yucca1d,* but not in WT. Expression of *YUCCA1* in wild-type, *bt2-1, BT2OE, yucca1d,* and *yucca1d bt2-1* lines. Total RNA from the respective genotypes was extracted and subjected to qRT-PCR. *EIF-4A2* was used to normalize the qRT-PCR data. Expression values are plotted relative to wild-type plants.

BT2 *is essential for activation of genes by* 35S *enhancers*

Since BT2 did not appear to affect *YUCCA1* expression in wildtype but did affect *YUCCA1* expression in a *35S* enhancer line, I hypothesized that BT2 could be a regulatory factor required for *35S* enhancer function. To test this hypothesis, I obtained unrelated but well-characterized *35S* enhancer activation-tagged lines *pap1d*, *jaw1d* and *e134d* (Borevitz et al., 2000; Palatnik et al., 2003; Pogorelko et al., 2008). Each activation line has a characteristic phenotype associated with activation of a single gene by the *35S* enhancers. *pap1d* plants have bright purple pigmentation in their organs due to accumulation of anthocyanins, caused by over-expression of a MYB family transcription factor, *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*) (Borevitz et al., 2000) from the *35S* enhancers located in the 3' untranslated region (UTR). *jaw1d* and *jaw3d* plants have uneven leaf shape and curvature caused by over-expression of a microRNA (miR-JAW) precursor from the *35S* enhancers located in the 3' and 5' UTR, respectively. The *jaw* mutants also have decreased expression of TCP-family of transcription factor genes that are targets of the miR-JAW microRNA (Palatnik et al., 2003). *e134d* plants have serrated and irregular leaf shape caused by over-expression of a proline-rich protein (At5g13760) of unknown function (Pogorelko et al., 2008) from the 35S enhancers located in the

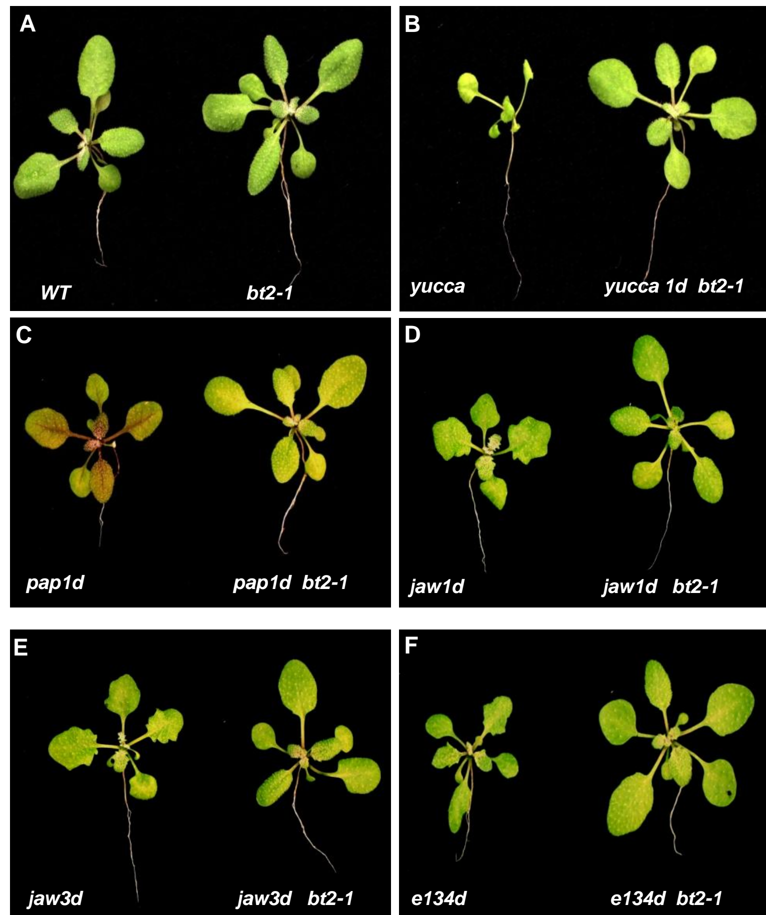

Figure 13. Loss of BT2 abolishes *35S* enhancer activation tagged phenotypes. **(A)** Wild-type and *bt2-1* lines. Suppression of epinastic leaves of *yucca1d* in *yucca1d bt2-1* **(B)**; purple leaves of *pap1d* in *pap1d bt2-1* **(C)**; un-even leaf shape of *jaw1d* in *jaw1d bt2-1* **(D)**, un-even leaf shape of *jaw3d* in *jaw3d bt2-1* **(E)**, and irregular leaf shape of *e134d* in *e134d bt2-1* **(F)**.

5' UTR. All the activation lines were crossed to *bt2-1* plants to generate *pap1d bt2-1*, *jaw1d bt2-1*, *jaw3d bt2-1*, *e134d bt2-1*. Surprisingly, loss of BT2 completely reversed the *35S* enhancer activation phenotypes in all the activation lines tested (Figure 13A-13F).

To determine whether the BT2 mechanism was similar in different activation tagged lines, I analyzed expression of the activated genes. As predicted, and similar to *yucca1d bt2-1*, expression of *PAP1*, *miR-JAW* precursor and At5g13760 in *pap1d bt2-1*, *jaw1d bt2-1* and *e134d bt2-1* was suppressed when compared to *pap1d, jaw1d* and *e134d* plants, respectively (Figure 14A-14D). Furthermore, *jaw1d bt2-1* plants have restored expression levels of *TCP3,* the micro RNA target (Figure 14D). These results strongly suggest that BT2 is an essential regulator of *35S* enhancer activity. For further experimentation, I chose the *yucca1d* activation line as my working model because of high levels of *YUCCA1* expression in *yucca1d* and its characteristic phenotype, which is amenable for visual analysis.

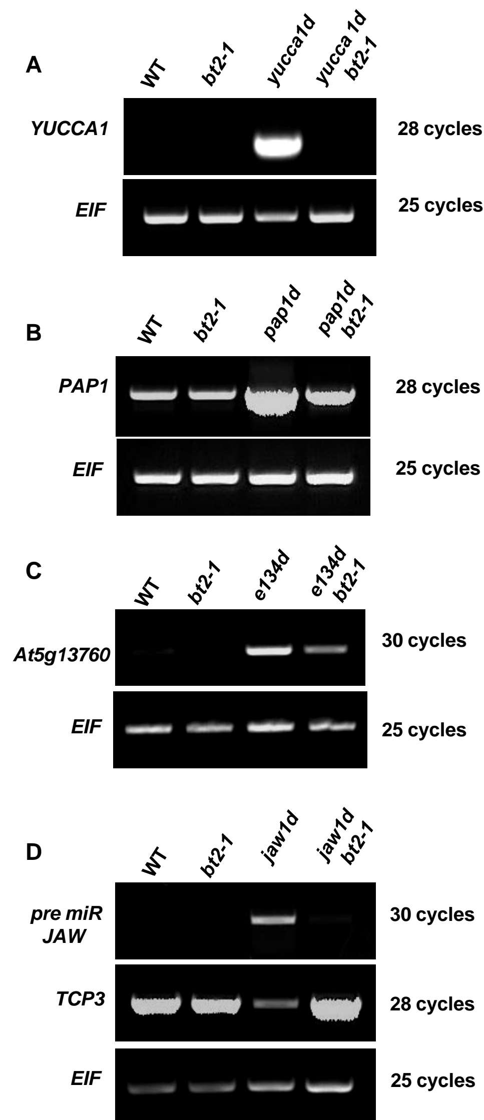

Figure 14. BT2 regulates expression of genes from the *35S* enhancer in the activation lines. Expression of *YUCCA1* **(A)**, *PAP1* **(B),** *At5g13760* **(C),** precursor of miR-JAW and its target microRNA *TCP3* **(D)** was determined in WT, *bt2-1, yucca1d, yucca1d bt2-1, pap1d, pap1d bt2-1, e134d, e134d bt2-1, jaw1d, jaw1d bt2-1* lines, respectively. Total RNA from respective seedlings was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control.

BT2 *does not affect mRNA turn-over, but regulates transcription in the activation lines*

BT2 could affect transcript levels in the activation lines by affecting transcription or by altering mRNA stability post-transcriptionally. To determine whether BT2 affected mRNA stability, I performed mRNA stability assays using the transcription inhibitor cordycepin (Gutierrez et al., 2002) and *yucca1d* lines. *YUCCA1* mRNA was unstable in wild-type and was rapidly turned-over to half its abundance within ~30 minutes after transcription inhibition, when compared to a stable *EIF* mRNA (Figure 15). Although there was an apparent increase in turnover rate in *yucca1d,* which could be due to very high levels of *YUCCA1* in *yucca1d* when compared to wild-type*, YUCCA1* mRNA turnover rate remained unaffected in *bt2-1* and *yucca1d bt2-1*, indicating that BT2 does not decrease *YUCCA1* mRNA turnover.

Next, to determine whether BT2 affected transcription, I performed nuclear run-on assays. As expected, the rate of *YUCCA1* transcription was higher in *yucca1d* when compared to wild-type, because of *35S* enhancers (Figure 16A). The activation of *YUCCA1* transcription, however, was suppressed in *yucca1d bt2-1,* suggesting that BT2 is required to activate *YUCCA1* transcription in *yucca1d* (Figure 16A). I also performed nuclear-run on analysis on *pap1d* and *pap1d bt2-1,* and the results support BT2 function in regulating transcription activated by *35S* enhancer (Figure 16B). Due to low level of expression of *miR-JAW* precursor and At5g13760, I was unable to detect any

signal from nuclear run-on analysis on *jaw1d* and *e134d* activation lines (data not shown). Transcription of *CAB2* and *BT2* in light and dark was estimated to serve as a positive control for changes in transcription (Figure 17).

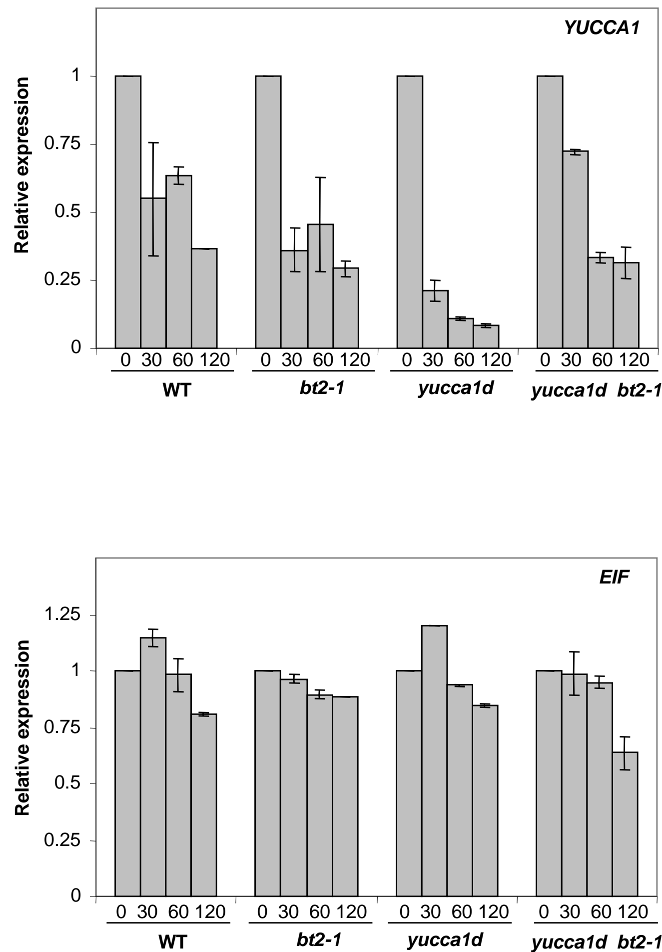

Figure 15. BT2 does not affect *YUCCA1* mRNA turn-over in *yucca1d*. Wild-type*, bt2-1, yucca1d, yucca1d bt2-1* seedlings were treated with transcription inhibitor, cordecypin, for the indicated times. Total RNA was extracted and subjected to qRT-PCR. Upper and lower panels represent expression of *YUCCA1* and *EIF-4A2,* respectively. Expression values plotted are relative to “0 minute” which was set at 1.

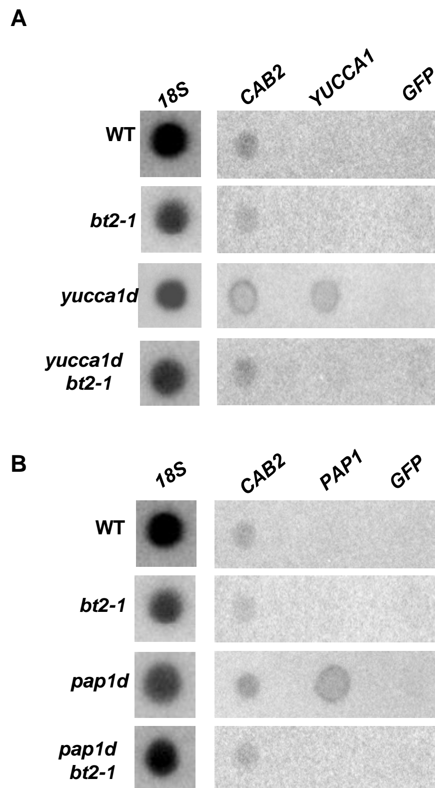

Figure 16. BT2 affects transcription in the *35S* enhancer activation lines. Active nuclei from wild-type, *bt2-1, yucca1d, yucca1d bt2-1* **(A)** and *pap1d, pap1d bt2-1* **(B)** lines were isolated and subjected to *in vitro* run-on transcription reactions with ^{32}P-UTP. Nascent labeled RNA was extracted and hybridized to nylon membranes bound with denatured *18S, CAB2, YUCCA1, PAP1* and *GFP* cDNA sequences. Intensity of radioactive signals represent rate of transcription of the respective genes. *18S rRNA* and *CAB2* signals were used as positive controls for labeling and hybridization. *GFP* signal was used as a control for non-specific binding.

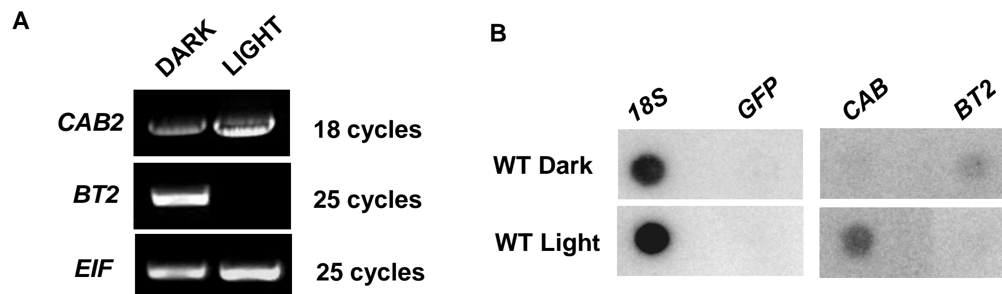

Figure 17. Expression of *CAB2* and *BT2* in light and dark conditions. RT-PCR analysis of *CAB2* and *BT2* expression **(A)**; and transcription of *CAB2* and *BT2* **(B)** in light and dark conditions. Active nuclei from wild-type plants, harvested in light and dark phase, were isolated and subjected to *in vitro* run-on transcription reactions with ^{32}P-UTP. Nascent labeled RNA was extracted and hybridized to nylon membranes bound with denatured *18S, CAB2, BT2* and *GFP* cDNA sequences. Intensity of radioactive signals represent rate of transcription of the respective genes. *18S* signal was used as hybridization control. *GFP* signal was used as control for non-specific binding.

BT2 *is not required for constitutive CaMV* 35S *and* NOS *promoter function*

35S enhancers are derived from CaMV *35S* promoter (Hayashi et al., 1992). Moreover, c*is*-elements present in *35S* promoter are also present in the *NOS* promoter. Since BT2 is essential for *35S* enhancer activity, I next determined whether it was also required for expression of genes controlled by full-length *35S*, and *NOS* promoters. I cloned *GREEN FLUORESCENT PROTEIN* (*GFP*) under the control of *35S* promoter and generated *35S:GFP bt2-1* lines. Using RT-PCR I analyzed expression of *GFP,* and found that loss of BT2 did not affect *GFP* expression controlled by *35S* promoter (Figure 18A). I also over-expressed *YUCCA1* under the control of *35S* promoter (*35S:YUC1*) and determined the effect of loss of BT2. Loss of BT2 did not alter the high auxin-phenotypes of *35S:YUC1* in the *bt2-1* background (Figure 18B) and did not suppress *YUCCA1* transcript levels (Figure 18C). Together, these results suggest that BT2 is not required for constitutive expression of genes controlled by the intact *35S* promoter.

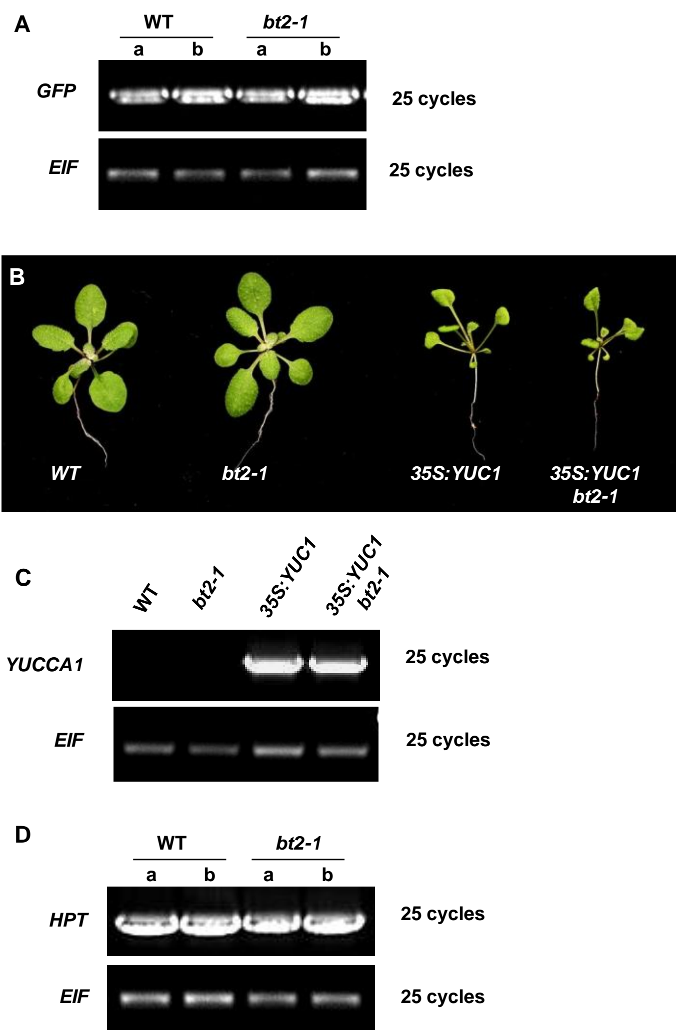

Figure 18. BT2 is not required for CaMV *35S* and *NOS* promoter activity. **(A)** Expression of *GFP* driven by *35S* promoter in wild-type and *bt2-1* lines. Total RNA from the respective genotypes was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control. Replicate samples are indicated by “a” and “b”. **(B)** Phenotypes and **(C)** expression of *YUCCA1* in *35S:YUC1* and *35S:YUC1 bt2-1* lines. **(D)** Expression of *HPT* controlled by *NOS* promoter in wild-type and *bt2-1* lines. Total RNA from respective genotypes was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control. Replicate samples are indicated by “a” and “b”.

Next, to determine whether BT2 affected *NOS* promoter activity, I obtained transgenic lines expressing *HYGROMYCIN PHOSPHOTRANSFERASE II* (*HPT*) under the control of *NOS* promoter and determined the effect of loss of BT2. Loss of BT2 did not affect hygromycin resistance of HPT expressing lines, and did not alter expression of the *HPT* (Figure 18D) in *bt2-1* background. This result suggested that BT2 is not required for constitutive expression of genes controlled by the full-length *NOS* promoter.

Calmodulin binding domain of BT2 is critical for 35S enhancer activity

Recently, Uno et al., (2009) found BT2 to interact with two identical protein kinases, CALCIUM DEPENDENT PROTEIN KINASE (CPK) 3 and CPK11 in yeast 2-hybrid assays. Moreover, BT2 has a CaMBD domain at its C-terminus and interacts with calmodulin in a calcium dependent manner (Du and Poovaiah, 2004). To understand the role of CaMBD in BT2 function, I generated a mutant form of BT2 ($BT2_{mut}$) that has altered sites (K333E, W334K, and K335E) in the conserved calmodulin binding domain (Du and Poovaiah, 2004). Over-expression of $BT2_{mut}$ in *yucca1d* suppressed its high auxin-related phenotypes (Figure 19A) and decreased levels of *YUCCA1* expression (Figure 19B).

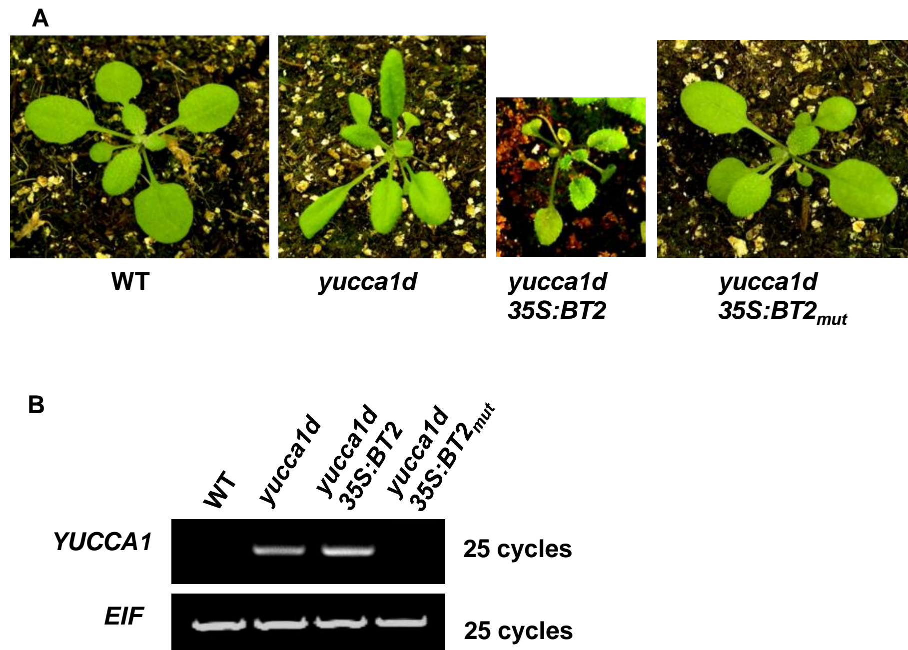

Figure 19. CaMBD of BT2 is essential for *35S* enhancer activity. Suppression of *yucca1d* phenotypes **(A)** and expression of *YUCCA1* **(B),** in *yucca1d 35S:BT2*$_{mut}$. Total RNA from respective genotypes was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control.

BT2 *interacting protein* CULLIN3 *is required for* 35S *enhancer activity*

Previous studies using yeast two-hybrid (Y2H) and in vitro pull down assays have demonstrated that BT2 interacts with BET10 bromodomain protein (Du and Poovaiah, 2004), and CULLIN3 (Figueroa et al., 2005) through its N-terminal BTB domain. Recently, we demonstrated that BET9 and BET10 genetically interact with BT2 in mediating nutrient and hormone responses (Misra et al., 2010). Moreover, similar to *yucca1d bt2-1*, loss of BET9 or BET10 in *yucca1d* suppresses its high auxin phenotypes (Misra et al., 2010). In the current study, after discovering that BT2 regulated *35S* enhancer function, I wanted to determine whether CULLIN3 was also required for *35S* enhancer activity. *Arabidopsis* has two redundant genes for CULLIN3, *CUL3A* and *CUL3B*. *CUL3A* is the predominantly expressed form, and the loss-of-function allele *cul3a-1* has decreased levels of CULLIN3 protein (Figueroa et al., 2005). I crossed *cul3a-1* to *yucca1d* plants. Similar to *yucca1d bt2-1,* loss of CULLIN3 in *yucca1d* suppressed the high auxin phenotypes and reduced expression of *YUCCA1* (Figure 20A). Taken together, my experiments indicate that all three interacting proteins, BET9, BET10 and CULLIN3, are required to regulate *35S* enhancer activity.

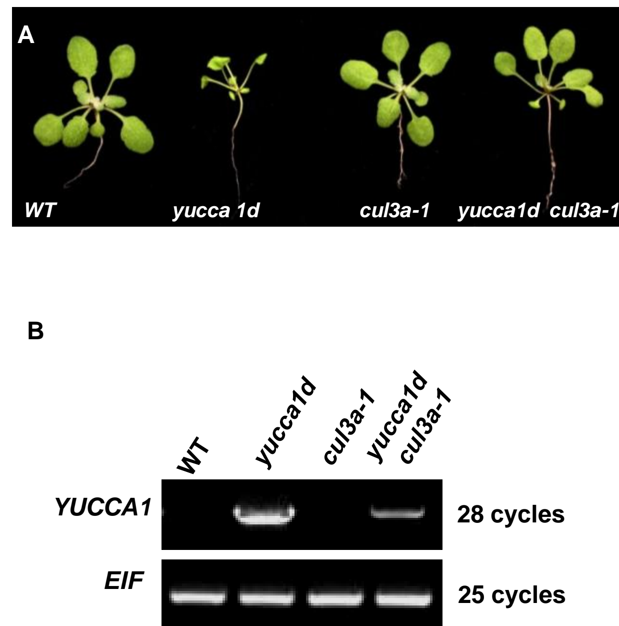

Figure 20. CULLIN3 is required for BT2 function. Suppression of *yucca1d* phenotypes **(A)** and expression of *YUCCA1* **(B)** due to loss of CULLIN3 in *yucca1d cul3a-1*. Total RNA from respective genotypes was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control.

The TAZ *domain of* BT2 *interacts with* 35S *enhancer regulatory proteins*

BT2 protein has a central zinc-finger protein-protein interaction domain (Du and Poovaiah, 2004). TAZ domains are also commonly found in transcriptional co-activator proteins such as CREB binding protein (CBP) and p300 family proteins, and it is a bonafide protein-protein interaction domain (De Guzman et al., 2000). To determine the role of the BT2 TAZ domain, I generated transgenic lines over-expressing only the TAZ domain of BT2 ($35S{:}TAZ_{BT2}$). I crossed the $35S{:}TAZ_{BT2}$ lines to *yucca1d* plants and analyzed the responses. Interestingly, over-expression of TAZ domain alone completely suppressed *yucca1d* phenotypes (Figure 21A) and decreased levels of *YUCCA1* expression (Figure 21B). This dominant-suppressor effect of $35S{:}TAZ_{BT2}$ suggests that the TAZ domain interacts with cellular factors that are essential for *35S* enhancer function.

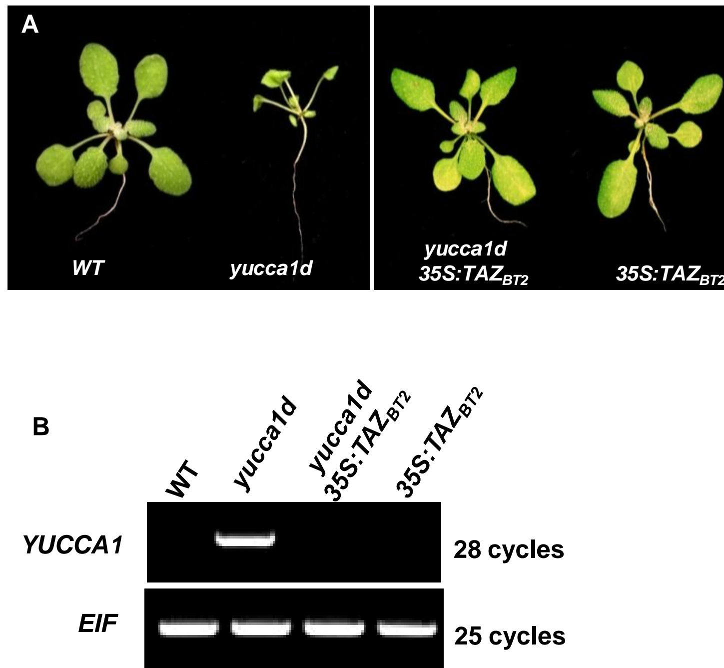

Figure 21. Over-expression of BT2 TAZ domain is a dominant-suppressor of *yucca1d* phenotypes. Suppression of *yucca1d* phenotypes **(A)** and expression of *YUCCA1* **(B)** in *yucca1d 35S:TAZ*$_{BT2}$. Total RNA from respective genotypes was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control.

Discussion

I previously reported that BT2 mediated multiple responses to nutrients, stresses and hormones in *Arabidopsis* (Mandadi et al., 2009). I also hypothesized that BT2 affected auxin responses in *yucca1d* because of the suppression of auxin related phenotypes in *yucca1d bt2-1.* In the current study, I found that BT2 is not required for auxin responses in *yucca1d*. Instead, I show that BT2 is an essential regulator of transcriptional activation via *35S* enhancers.

Although BT2 seemed to be required for auxin responses in *yucca1d*, expression of auxin signaling genes (Aux/IAA) in response to exogenous IAA was not affected by loss of BT2 in *yucca1d bt2-1* (Figure 11). Moreover, Aux/IAA gene expression was not altered in *bt2-1* and *BT2OE* lines when compared to wildtype, either in the presence or absence of exogenous IAA*.* Hence, I conclude that BT2 may not affect auxin signaling in response to IAA (Figure 11), but, is required for auxin responses in *yucca1d*. Surprisingly, I found that BT2 was required to maintain *YUCCA1* mRNA levels in *yucca1d* because the levels of *YUCCA1* mRNA decreased in *yucca1d bt2-1* compared to *yucca1d* (Figure 12). This result also explained the cause of suppression of the numerous auxin-related phenotypes in *yucca1d bt2-1.* Moreover, it clarified the seemingly contradictory result that BT2 was required for the high-auxin phenotypes in *yucca1d,* but did not affect auxin signaling in *yucca1d.* However, loss of BT2 and over-expression of BT2 had no apparent effect on endogenous *YUCCA1* expression (Figure 12), and suggested that BT2 is not required for endogenous

YUCCA1 expression. Because *YUCCA1* is primarily localized to shoot apical meristem and flower primordia (Cheng et al., 2006), it is formally possible that BT2 affects spatio-temporal expression patterns of *YUCCA1*.

Since BT2 did not affect auxin signaling and endogenous *YUCCA1* expression but is required for *YUCCA1* expression in the *35S* enhancer activation line *yucca1d,* I hypothesized that BT2 could be a general regulator of *35S* enhancers. Indeed, loss of BT2 suppressed the phenotypes associated with multiple activation lines (Figure 13A-13F). The reversal of phenotypes was due to loss of expression of the activated genes (Figure 14A-14D). Moreover, BT2 regulated expression of genes in the different activation lines independent of the position or location of the enhancers from the transcription start site, characteristic of a true enhancer and/or its regulator (Blackwood and Kadonaga, 1998). Using nuclear run-on assays and mRNA turn-over experiments, I found that BT2 did not affect stability of mRNA (Figure 15), but regulated transcription (Figure 16) in the activation lines. Based on the above phenotypic and molecular analysis I conclude that BT2 is a regulator of *35S* enhancer mediated transcription.

Although *35S* enhancers are derived from the *35S* promoter BT2 did not affect full-length *35S* promoter activity (Figure 18A-18C) or *NOS* promoter activity (Figure 18D). There are two plausible explanations for this result. Firstly, the full promoter possesses only one copy of enhancer sequence, and together with the core promoter elements renders strong activity to the promoters.

However, multimers of *35S* enhancers seem to be required for high *35S* enhancer activity (Lam and Chua, 1989). In fact, the activation lines used in this study possess four copies of *35S* enhancers. Hence, it is possible that the mechanism of *35S* enhancer function could be inherently different from *35S* promoter. Secondly, the CaMV *35S* promoter functions as a combinatorial module (Benfey and Chua, 1990). The *35S* core promoter region (domain A) can itself recruit numerous transcription factors, and BT2 may not be required for this activity. Moreover, *35S* enhancer region of the full promoter conferred different development and tissue-specific expression pattern to reporter genes (Benfey et al., 1989) and suggests differences in regulation from core promoter.

Interestingly, mutations to *as-2* element in *35S* enhancer, the only known binding site for an unidentified tobacco nuclear factor ASF2 (Lam and Chua, 1989), did not significantly affect *35S* enhancer activity in leaves (Fang et al., 1989), which suggested that other elements regulate its activity. High A/T nucleotide content is a general feature of nuclear matrix interacting regions (MARs), and MARs activate transcription similar to enhancers (Käs et al., 1989; Sandhu et al., 1998). A pea plastocyanin gene enhancer/MAR (*PetE*) sequence, rich in A/T nucleotides, interacts with nuclear matrices and mediated acetylation of histones at the linked promoters (Sandhu et al., 1998; Chua et al., 2003). *35S* enhancer sequences do not resemble *PetE*-enhancer or any related sequences, and are not rich in A/T nucleotides (data not shown). Hence, they may not

function as MARs. However, *35S* enhancers could be substrates for chromatin modification and recruit transcriptional machinery.

In general, enhancers activate transcription by recruiting or enhancing interaction among transcription factors/regulators (Blackwood and Kadonaga, 1998). Based on its protein structure, BT2 does not seem to directly function as DNA-binding transcription factor or an activator. BT2 protein has three distinct domains: 1) an N-terminal BTB/POZ domain, 2) a central TAZ domain, and 3) a C-terminal calmodulin binding domain.

BT2 interacts with calcium-dependent protein kinases (Uno et al., 2009) and calmodulin in a calcium dependent manner (Du and Poovaiah, 2004). I found that CaMBD of BT2 was required to mediate *35S* enhancer activity because over-expression of mutated BT2 with altered calmodulin binding sites acted as a dominant-suppressor of the activation phenotypes in *yucca1d* (Figure 19A) and had decreased *YUCCA1* expression (Figure 19B). These results implicate Ca^{++} in regulating *35S* enhancer mediated transcription via BT2 CaMBD.

BT2 interacts with the bromodomain protein BET10 (Du and Poovaiah, 2004), and CULLIN3 (Figueroa et al., 2005) through its N-terminal BTB domain. Recently, we demonstrated that BET9 and BET10 proteins genetically interact with BT2 to regulate *YUCCA1* expression in *yucca1d* lines (Misra et al., 2010). In the current study, I demonstrated that similar to loss of BT2, BET9, BET10, loss of CULLIN3 also results in suppression of *yucca1d* phenotypes (Figure

20A). The suppression of *yucca1d* phenotypes was due to a decrease in *YUCCA1* expression (Figure 20B).

The TAZ domain of BT2 is similar to the TAZ domain of transcriptional scaffold and activator proteins such as CREB binding protein (CBP) and p300 (De Guzman et al., 2000). The latter proteins are involved in transcriptional regulation of genes in a variety of biological functions by interacting with numerous cellular factors via TAZ domains (Goodman and Smolik, 2000). Over-expression of TAZ domain of BT2 in *yucca1d* suppressed the activation phenotypes (Figure 21A) and decreased *YUCCA1* expression (Figure 21B). This dominant-suppressor effect could be due to sequestering of cellular factors that interact with endogenous BT2 and are required for *35S* enhancer activity.

Based on the current results and previously identified biochemical interactions (Du and Poovaiah, 2004; Figueroa et al., 2005), I propose that BT2 assembles into a functional complex that contains BET9, BET10 and CULLIN3 and is required for *35S* enhancer activity (Figure 22). Moreover, BT2 and CULLIN3 may function as a CULLIN3-based ubiquitin ligase and target certain

proteins for destruction by poly-ubiquitination or stabilizing them by mono-ubiquitination to affect transcription.

Alternatively, the complex may serve as a scaffold and promote interactions among transcription factors. The TAZ domain of BT2 could provide specificity to recognize specific targets. Calcium signals could affect BT2 activity via its calmodulin binding domain. The exact mechanism for how BT2 complex regulates *35S* enhancer activity is currently unknown. I hypothesize that *35S* enhancers are marked by certain chromatin modifications and are recognized by the BET9 and BET10 chromatin remodelers, which simultaneously interact with BT2. It is likely that the BT2 complex is also recruited to promoters or enhancers of endogenous target genes involved in the multiple pathways affected by BT2. Identification of signals on *35S* enhancers and protein targets of BT2 complex will enhance our understanding of the mechanism of enhancer regulation in general.

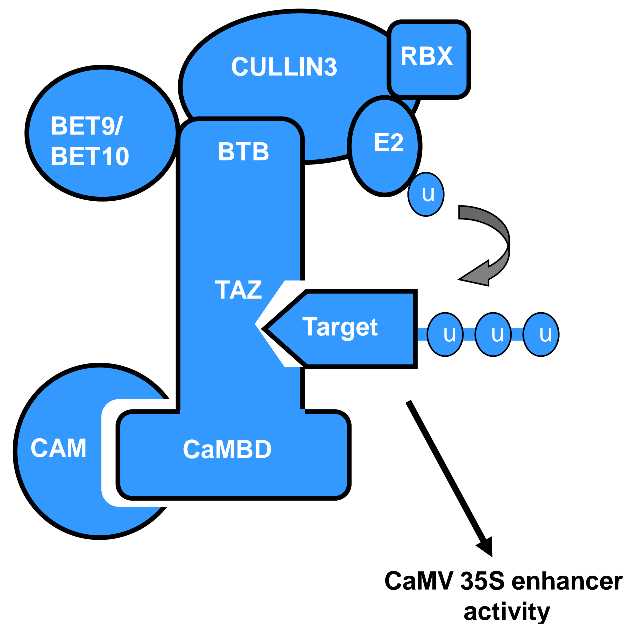

Figure 22. Working model for function of BT2 to regulate CaMV *35S* enhancer activity. BT2 assembles into a functional complex that constitutes of proteins BET9, BET10 and/or CULLIN3 and might function as a CULLIN3-based ubiquitin ligase required for *35S* enhancer activity in plants. Figures are not scaled to size of the respective proteins.

Materials and methods

Plant growth conditions and materials

Plants were grown in soil in 14 h light/10 h dark at 21 °C and a light intensity of ~120-130 μmol/m^2s with 70% relative humidity, unless otherwise stated. For growth in nutrient media, seeds were surface sterilized with 50% (v/v) bleach and 0.1% (v/v) Triton X-100 for seven minutes, cold treated at 4°C for 3-4 days, and grown on Murashige and Skoog (MS) medium (Sigma) with 0.8% (w/v) phytagar under continuous low light (~ 30 μmol/m^2s). All media contained 1% sucrose.

Transgenic lines, plasmids and constructs

BT2 overexpression lines, *35S:YUC1*, *yucca1d*, *bt2-1*, *bet9-1*, and *bet10-1* lines were previously described (Zhao et al., 2001; Ren et al., 2007, Misra et al., 2010). *cul3a-1*, *pap1d, jaw1d, jaw3d*, and *e134d* lines were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at the Ohio State University. $35S_{pro}$*:GFP* plasmid was constructed in the binary vector pCBK05, which confers phosphinothricin (BASTA) herbicide resistance as selectable marker and was used to generate *35S:GFP* transgenic lines. The NOS_{pro}*:HPT* selectable marker in the binary vector pER8 (Jianru et al., 2000; Ng et al., 2006) was used to construct *NOS:HPT* transgenic lines. The sequences corresponding to TAZ domain of BT2 (Du and Poovaiah, 2004) were cloned under the control of CaMV *35S* promoter in the binary vector pCBK05. $BT2_{mut}$ construct containing mutations K333E, W334K, K335E in the BT2 CaMBD domain (Du and Poovaiah, 2004), was generated by

QuickChange II mutagenesis system following manufacturer's instructions (Stratagene). All clones were verified by sequencing.

Treatments, RNA isolation, and expression analysis

For treatments with exogenous IAA, 7 to 8-day-old seedlings grown on MS agar medium were transferred to MS liquid medium containing either IAA (10 μM) or DMSO (0.1%) and were treated for 3 h. Expression of auxin responsive genes was determined by quantitative real-time PCR (qRT-PCR) analysis. Total RNA was isolated using TRI reagent following manufacturer's protocol (Ambion). 5 μg RNA was used to make cDNA using SuperScript first-strand cDNA synthesis kit (Invitrogen). Amplification was performed using Power SYBR Green Master Mix (Applied Biosystems) and the ABI Prism 7500 sequence detection system (Applied Biosystems). *EIF-4A2* (At1g54270) was used to normalize the expression and fold changes were calculated following the $^{\Delta\Delta}C_T$ method (Livak and Schmittgen, 2001; Guo et al., 2008). The primers used for qRT-PCR and RT-PCR are listed (Table 2). All treatments were repeated at least twice at different periods using three biological replicates, and the results described are representative of consistent data obtained from replicated experiments.

Nuclear isolation and nuclear run-on analysis

Nuclei were isolated according to the protocol described previously (Folta and Kaufman, 2006) with minor modifications. 3 to 4 g of one-week-old *Arabidopsis* seedlings were harvested and briefly rinsed in 3 tissue volumes of ice-cold anhydrous ethyl-ether (Sigma). Tissues were washed 3 times with 3 tissue volumes

Table 2. Primers used for analysis of BT2 function to regulate *35S* enhancer activity

Gene	Primer
qRT-PCR analysis:	
IAA1	F-TGG ACG GAG CTC CAT ATC TC
	R-ACC GAC CAA CAT CCA ATC TC
IAA2	F-ATC ACC AAC CAA CAT CCA GTC
	R-TGG ACG GAG CTC CAT ATC TC
IAA5	F-CCG GAG AAA GAA CAG TCT CG
	R-TCC AAG GAA CAT TTC CCA AG
IAA6	F-ACT GCC GGT TGT GAA GAG TC
	R-AAC TGT TGC TCG AAC CAA GG
IAA19	F-GAC TCG GGC TTG AGA TAA CG
	R-CGT GGT CGA AGC TTC CTT AC
GH3.3	F-TCCACTAAGGACGTGAAGGCTCTAAG
	R-TGCTGGTAATCCACCGGGAGTCTTCG
YUCCA1	F-ATCGTTCACGCGAGTGAGTA
	R-AGTATCTCCCTTGGCAACAC
EIF-4A2	F-CAAGGTGTCAAGGTTCATGC
	R-CAACGACAACATGAACACCA
RT-PCR analysis:	
YUCCA1	F-ATGGAGTCTC ATCCTCACAAC
	R-CAGCGATCTTAACGGCGTCA
PAP1	F-ATGGAGGGTTCGTCCAAAG
	R-TCAACGTCAAAAGCCAAGGT
JAW1	F-TCCTCGCATCTACCATCCCT
	R-TGTTGAAACCGCCAATTCCCAGAG
TCP3	F-CACATTGTTCGGTCAACAGG
	R-TGCCTGGAATAGCAGATTGG
At5g13760	F-TCACACCTACCCCTCAACAA
	R-ACGCCTCTGAAGACAAGGAA
BT2	F-ACATGGTCACCCAGCTGAAG
	R-CAGACACAACCCTTGTCACC
CAB2	F-ATGGCCGCCTCAACAATGGCT
	R-CACTTTCCGGGAACAAAGTTGG
18S rRNA	F-ACCTGGTTGATCCTGCCAG
	R-GATCGTCTTCGAGCCCCCAAC
EIF-4A2	F-GCAAGAGAATCTTCTTAGGGGTATCTATGC
	R-GGTGGGAGAAGCTGGAATATGTCATAG
HPT	F-TGAACTCACCGCGACGTCTGT
	R-TCGGTTTCCACTATCGGCGA

of extraction buffer (2.0 M hexylene glycol, 20 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol). Tissues were re-suspended in 3 tissue volumes of extraction buffer and homogenized. The homogenate was filtered through 3 layers of miracloth, and Triton X-100 (1%) solution was added to the final volume and mixed gently. The extract was overlayed on a gradient of 80% and 30% percoll solutions (Sigma) prepared in gradient buffer (0.5 M hexylene glycol, 5 mM PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, 5 mM β-mercaptoethanol and 1% Triton X-100) and centrifuged at 2000 g for 30 minutes at 4 °C. After centrifugation, the nuclei accumulated at the interface were collected and suspended in 10 ml of gradient buffer. The nuclei were overlayed again on a 30% percoll solution in gradient buffer and centrifuged at 2000 g for 15 minutes at 4 °C. The final nuclei pellet was suspended in nuclei storage buffer (50 mM Tris-HCl (pH 7.8), 10 mM β-mercaptoethanol, 20% glycerol, 5 mM $MgCl_2$ and 0.44 M sucrose) and stored at -80 °C in aliquots of 50 µL.

Batches of nuclei obtained similarly at the same time from different genotypes were used directly for the nuclear run-on reactions. 20 U RNAsin (Promega) was added to 50 µL of nuclei and incubated at 30 °C for 10 minutes. 100 mM of ATP, GTP, CTP (Epicenter) and 100 µCi of ^{32}P-UTP (Amersham) were

added to the nuclei along with 10X transcription assay buffer (250 mM Tris–HCl (pH 7.8), 375 mM NH_4Cl, 50 mM $MgCl_2$ and 50 % (v/v) glycerol) to a final reaction volume of 100 µL. The reaction was incubated at 30 °C for 30 minutes. 10U of DNase I was added and incubated at 30 °C for another 10 minutes. The reaction was completely terminated by adding 2 reaction volumes of termination buffer (7.5 M Urea, 0.5% SDS, 20 mM EDTA (pH 7.5) and100 mM LiCl). Total radio-labeled RNA was isolated using TRI reagent (Ambion). Target gene coding sequences (CDS) were amplified and 1 µg of each CDS was dot-blotted to Hybond N+ membrane (Amersham). Blots were then probed with ^{32}P-labeled in vitro synthesized nascent transcripts. Following hybridization and washing, the radioactive signals were quantified using a phosorimager. Expression of *18S* and *CAB2* RNAs was used as internal hybridization and loading controls. *GFP* was used as a negative control for non-specific binding of RNA to DNA. Changes in *CAB2* and *BT2* transcription during light and dark phase was used as a positive control for quantifying differences in transcription using this protocol (Figure 17).

Cordecypin treatments and mRNA turnover

mRNA turnover was analyzed as described previously (Gutierrez et al., 2002) with minor modifications. One-week-old *Arabidopsis* seedlings grown on MS agar plates were transferred to MS liquid media for a pre-incubation period of 30 minutes. To inhibit transcription, 0.6 mM of cordecypin (Sigma) was added to the MS solution. Tissue samples were harvested at 0, 30, 60, and 120 minutes after cordecypin addition and frozen in liquid nitrogen. Total RNA was isolated using TRI reagent (Ambion). Expression of *YUCCA1* and *EIF-4A2* was analyzed by qRT-PCR. *EIF-4A2* expression was used to normalize the data.

Accession numbers

AGI locus numbers for genes used in this chapter are: *BT2*, At3g48360; *BET9,* At5g14270; *BET10,* At3g01770; *CULLIN3A,* At1g26830; *YUCCA1,* At4g32540; *PAP1,* At1g56650; *JAW;* At5g13760; *EIF-4A2*, At1g54270; *18S*, At2g01010; *CAB2,* At1g29920. SALK seed stock numbers for activation lines are*:* *pap1d,* CS3884; *jaw1d,* CS6948; *jaw3d*, CS6950; *e134d,* CS16283.

CHAPTER IV

BIOCHEMICAL CHARACTERIZATION OF BT2 AND RESPONSES OF *BT* FAMILY GENES

Summary

In the previous chapters I demonstrated that BT2 mediates multiple responses to nutrients, stresses and hormones and is required for CaMV *35S* enhancer-mediated activation of genes in *Arabidopsis*. In the current chapter, I further characterize BT2 protein and examine other BT family genes. BT2 protein was expressed in very low levels in plants. In a diurnal cycle, BT2 protein accumulated in dark, and was relatively more abundant in early vegetative stages of development. BT2 protein was primarily localized in the nucleus and was turned-over, in part, by the 26S proteosome. Moreover, BT2 coding sequence has numerous rare codons that affected its accumulation. Expression of *BT1* and *BT5* was diurnally regulated. Similar to *BT2*, expression of *BT1* was circadian. Also similar to *BT2*, expression of *BT1* and *BT5* was repressed and induced by sugars and nitrates, respectively. Finally, I found that BT1, BT3, and BT4 were also required for *35S* enhancer activity. I propose that BT family proteins assemble into multi-protein complexes to mediate responses to changing environmental and nutritional conditions.

Introduction

Light and the circadian clock modulate expression of numerous genes involved in growth and development (Blasing et al., 2005; Gutierrez et al., 2008; Usadel et al., 2008). Many genes are also regulated by multiple and overlapping signals of light, clock and nutrients (Blasing et al., 2005). Sugars induce genes involved in growth and biosynthesis and low sugar concentrations activate genes that mobilize carbon from primary reserves (Koch, 1996; Yu, 1999). Nitrogen status also affects expression of numerous transcription factors, protein kinases, phosphatases, and enzymes involved in nitrate metabolism (Scheible et al., 2004; Wang et al., 2004b). Global gene expression studies revealed extensive gene networks and several transcription factor and protein kinases, genes involved in protein synthesis and ubiquitin-mediated protein degradation modulated by nutrient and light signals. For example, bZIP transcription factors such as bZIP2/GBF5, bZIP11/ATB1, and snf1 related kinases (SnRK), KIN10/11 orchestrate synergistic transcriptional networks in response to sugar, energy deprivation and diverse stresses (Blasing et al., 2005, Usadel et al., 2008, Gutierrez et al., 2008).

Expression of the *Arabidopsis* BTB-domain containing protein *BT2* is regulated by multiple signals (Mandadi et al., 2009). *BT2* expression is diurnally regulated with a maximum expression in the dark, and it is regulated by the circadian clock. *BT2* expression is also repressed by sugars and induced by nitrates. Moreover, expression of *BT2* is affected by multiple hormone and biotic

and abiotic signals. Recently, I discovered that BT2 is required for *35S* enhancer activity (Mandadi et al., 2010; also in Chapter III).

BT2 is a ~40 kD protein with an N-terminal BTB/POZ (**B**road-Complex, **T**ramtrack, and **B**ric-a-Brac/**Po**xvirus and **z**inc finger) domain, a central TAZ (**T**ranscriptional **A**daptor **Z**inc finger) domain and a C-terminal calmodulin-binding domain (CaMBD). It belongs to a family of five highly similar proteins that include BT1, BT3, BT4, and BT5 (Du and Poovaiah, 2004). All five proteins possess the three conserved domains i.e., BTB domain, TAZ domain, and CaMBD domain. Multiple members of the BT family interact with the bromodomain protein BET10 (Du and Poovaiah, 2004), and CULLIN3 (Figueroa et al., 2004). BET10 and its ortholog BET9 genetically interact with BT2 to mediate responses to sugars and hormones (Misra et al., 2010). Moreover, BT family genes redundantly regulate gametophyte development in plants (Robert et al., 2009). In the latter study, BT family genes were also shown to compensate for each other, because loss-of-function of a member affected expression of other BT genes.

In the current study I biochemically characterized BT2 and analyzed the responses of other BT family genes. Using anti-BT2 antibodies I show that BT2 protein was low in abundance in plants. In a diurnal cycle BT2 protein accumulated in dark, consistent with its mRNA levels. BT2 was primarily localized in the nucleus and was developmentally regulated with maximum expression during early vegetative phases. BT2 was turned-over by the 26S proteosome, and I demonstrated that a codon bias in BT2 coding sequence affected BT2 protein accumulation. Furthermore, in a diurnal cycle, expression of *BT1* and *BT5* overlapped *BT2* expression. Like BT2, expression of *BT1* and *BT5* was modulated in response to sugars and nitrates. Finally, using loss-of-function alleles, *bt1-1, bt3-1,* and *bt4-1,* I demonstrated that BT1, BT3, and BT4 also are required for *35S* enhancer activity. I propose that BT proteins assemble into multi-protein complexes and mediate similar responses.

Results

BT2 *protein is expressed at low level and is developmentally regulated*

I produced antibodies against full length recombinant BT2. The anti-BT2 anti-antibodies were able to detect upto 4 ng of recombinant BT2 protein (data not shown). Next, I performed immuno-detection of BT2 in plants. Endogenous levels of BT2 protein in mature 3-week-old wild-type plants were very low (Figure 23B). However, at the end of the dark phase I observed slight accumulation, a result consistent with the diurnal expression pattern of *BT2* mRNA (Figure 23A). In a *BT2* overexpression line (*BT2OE*), BT2 was constitutively present. However, relative to the level of *BT2 mRNA* in both wild-type and *BT2OE* lines, BT2 protein was significantly lower. To determine if BT2 protein was developmentally regulated, I analyzed the level of BT2 protein in 1, 2 and 3 week-old plants. In wildtype, BT2 protein was relatively higher in younger stages (1st and 2nd week), and its levels decreased when plants mature (3rd week) (Figure 23C).

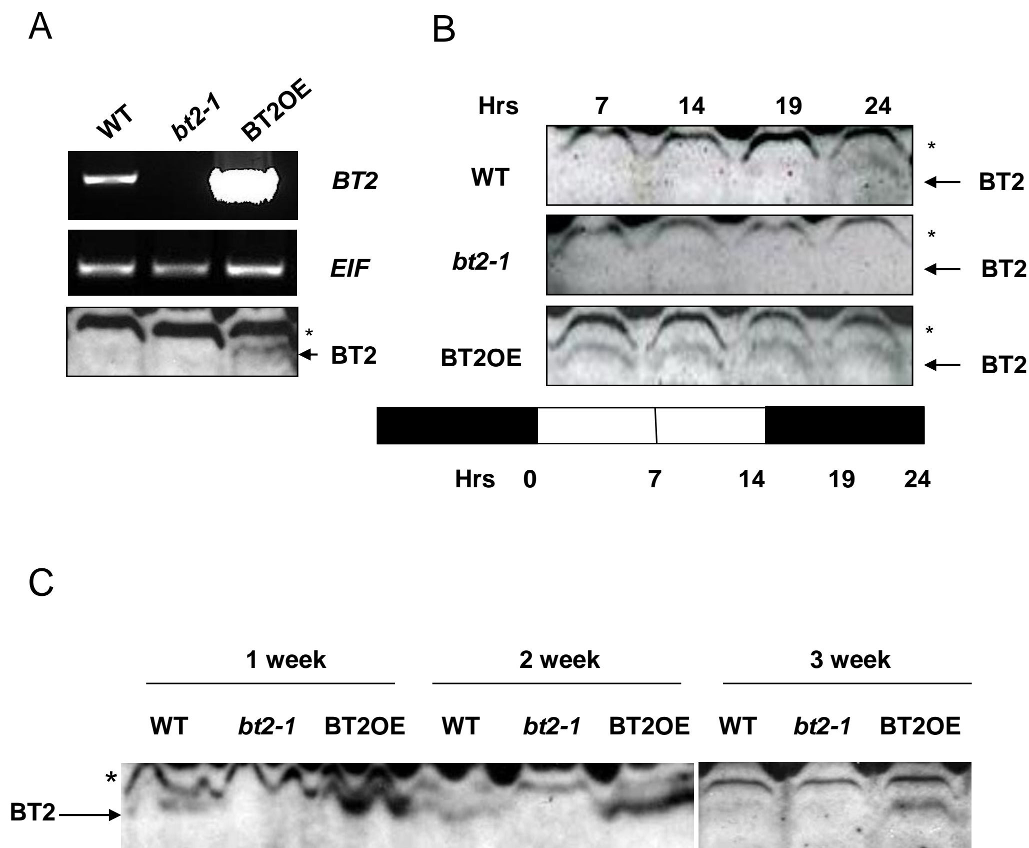

Figure 23. BT2 protein is diurnally and developmentally regulated. **(A)** Rosette leaves from 3-week-old wild-type, *bt2-1*, BT2OE lines were either harvested in light or, **(B)** harvested at the indicated times of a diurnal cycle and were subjected to immunoblot analysis using anti-BT2 antibodies. For gene expression analysis, total RNA was isolated and subjected to RT-PCR analysis. *EIF2A* expression was used as a loading control. **(C)** Rosette leaves from 1, 2 and 3-week-old-wild-type, *bt2-1* and BT2OE lines were harvested in light and subjected to immunoblot analysis using anti-BT2 antibodies. Arrows indicate BT2 protein. An asterisk represents a cross-reacting protein and also serves as loading control.

BT2 *protein is primarily localized in the nucleus and is turned-over by 26S proteosome*

I fractionated cellular proteins to determine whether BT2 was a nuclear or cytosolic protein. BT2 was primarily localized in nucleus (Figure 24A). Post-transcriptional and post-translational processes, including 26S proteosome-mediated turnover, regulate abundance of many proteins (Dreher and Callis, 2007). Since BT2 protein levels were low, I tested the hypothesis that BT2 protein was degraded by 26S proteosome. I treated an overexpression line of BT2 that is under the control of β-estradiol inducible promoter (BT2modOE) with MG132, a specific inhibitor of 26S proteosome function (Shen et al., 2005). Results from immuno-detection performed after treatment indicated that BT2 protein was at least partly turned over by 26S proteosome, because BT2 protein modestly accumulated with MG132 (Figure 24B). Since the level of BT2 protein recovered with MG132 was still not very high, other mechanisms controlling its accumulation must exist.

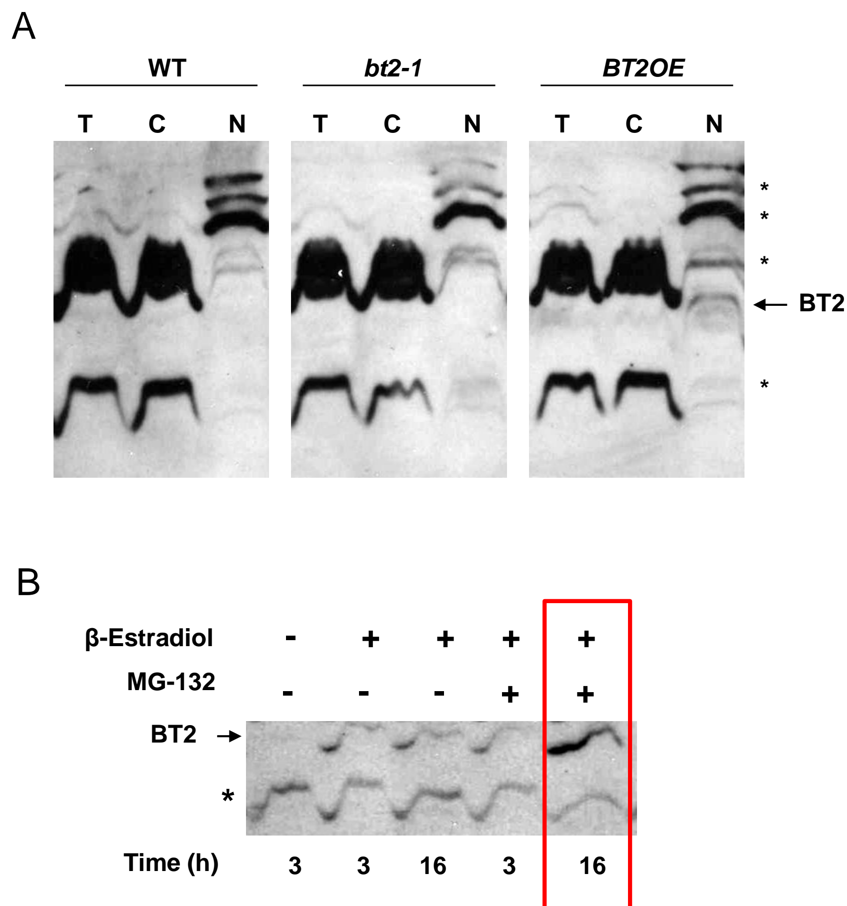

Figure 24. BT2 is primarily localized in nucleus and is turned-over by 26S proteosome. **(A)** 3-week-old wild-type, *bt2-1*, BT2OE lines were harvested and total (T), cytoplasmic (C) and nuclear (N) proteins were extracted and were subjected to immunoblot analysis. **(B)** 1-week-old BT2modOE seedlings were treated with β-estradiol (50 μM) to induce *BT2* expression in the presence or absence of MG132 (50 μM) for 3 h or 16 h. DMSO (0.1%) was used for mock-control. All the samples were subjected to immunoblot analysis using anti-BT2 antibodies. Arrows indicate BT2 protein. An asterisk represents cross-reacting proteins and also serves as loading control. Red outlined box indicates accumulation of BT2 protein upon MG132 treatment.

Rare codons affect BT2 *protein accumulation*

During my attempts to express recombinant BT2 protein in *E. coli* cells, I noticed that BT2 failed to accumulate. However, when supplemented with additional tRNAs for rare codons (AUA, AGG, AGA, CUA, CCC and GGA), BT2 protein accumulated to very high levels (Figure 25). This observation led us to analyze whether codons in the BT2 open reading frame (ORF) affected its protein accumulation in *Arabidopsis*. Using a publicly available program (Fuhrmann et al., 2004), I found that the BT2 ORF had relatively high number of codons that are used much less frequently than their synonymous codons in *Arabidopsis* (Figure 26A). Using a nested PCR-based approach, I re-engineered BT2 (BT2mod) with 39 favorable synonymous codons (Figure 26B). Analysis of transgenic lines expressing BT2mod revealed that rare codons did affect accumulation of BT2 protein, because the amount of BT2 protein relative to its mRNA in the BT2mod expression lines was higher compared to the unmodified BT2 expression line (Figure 27). I could not increase levels of *BT2* mRNA beyond the achieved levels, and BT2 protein accumulated only to a certain level in the over-expression lines (Figure 27). This result suggested that BT2 expression was very tightly controlled at the protein level.

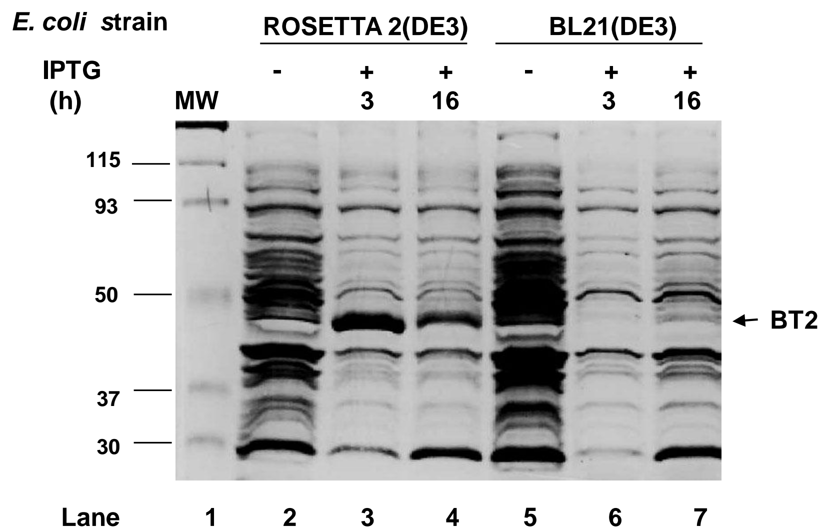

Figure 25. Codon usage affects expression of recombinant BT2. *E. coli* Rosetta2 (DE3) strain (lane 2, 3, and 4) and BL21 (DE3) strain (lane 5, 6, and 7) transformed with BT2 were grown in the presence of IPTG at 37 °C. Total proteins were extracted and separated by 10 % SDS-PAGE and visualized by Coommassie-blue stain. Arrows indicate BT2 protein, which was over-expressed only in Rosetta cells, an *E. coli* strain supplemented with tRNAs for 7 rare codons (AGA, AGG, AUA, CUA, GGA, CCC, and CGG). Lane 1 indicates a protein molecular weight (MW) ladder in kD.

A

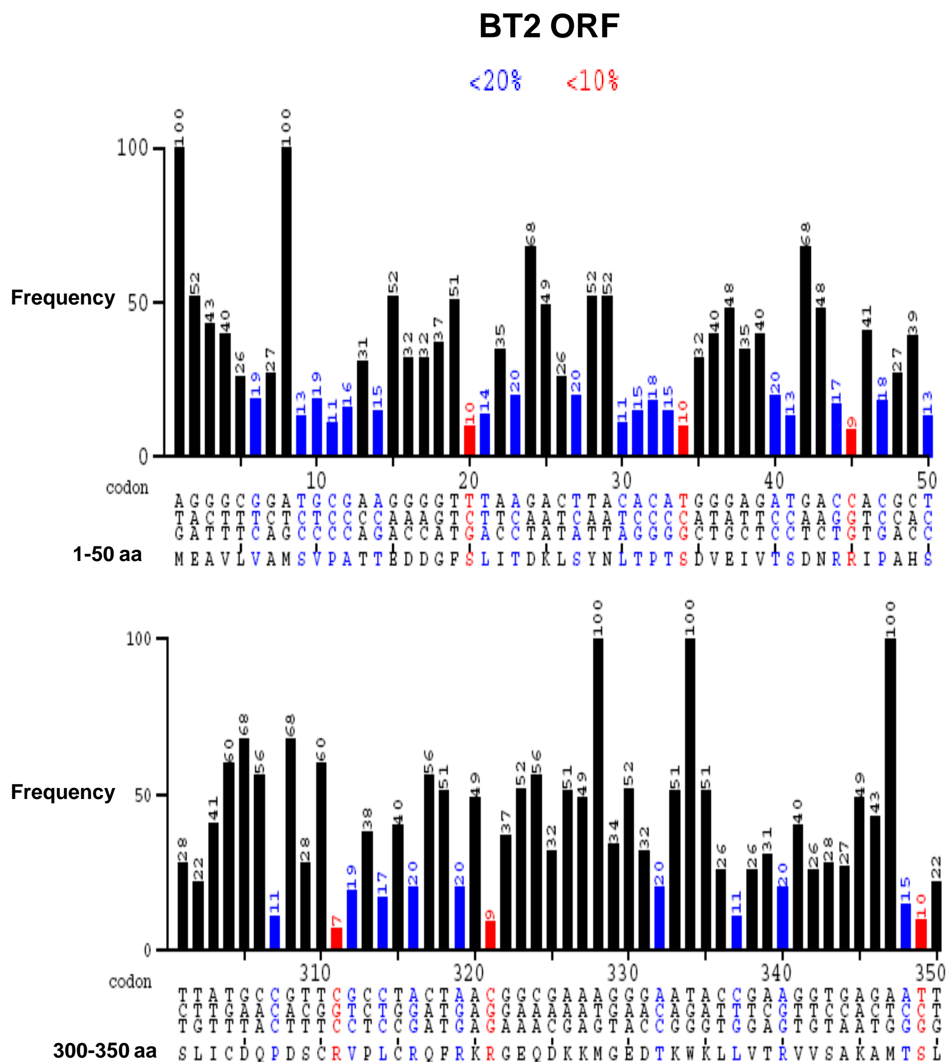

Figure 26. Codon usage of BT2 in *Arabidopsis*. **(A)** Representation of codon usage of N-terminus (1-50 aa) and C-terminus (300-350 aa) regions of BT2. Bars indicate relative frequency or abundance of the tRNAs for the respective codons in *Arabidopsis*. Blue and red colors represent critically rare codons, whose tRNAs are less that 20% and 10%, respectively, in abundance. **(B)** Representation of codon usage of N-terminus (1-50 aa) and C-terminus (300-350 aa) regions of modified BT2 (BT2mod).

B

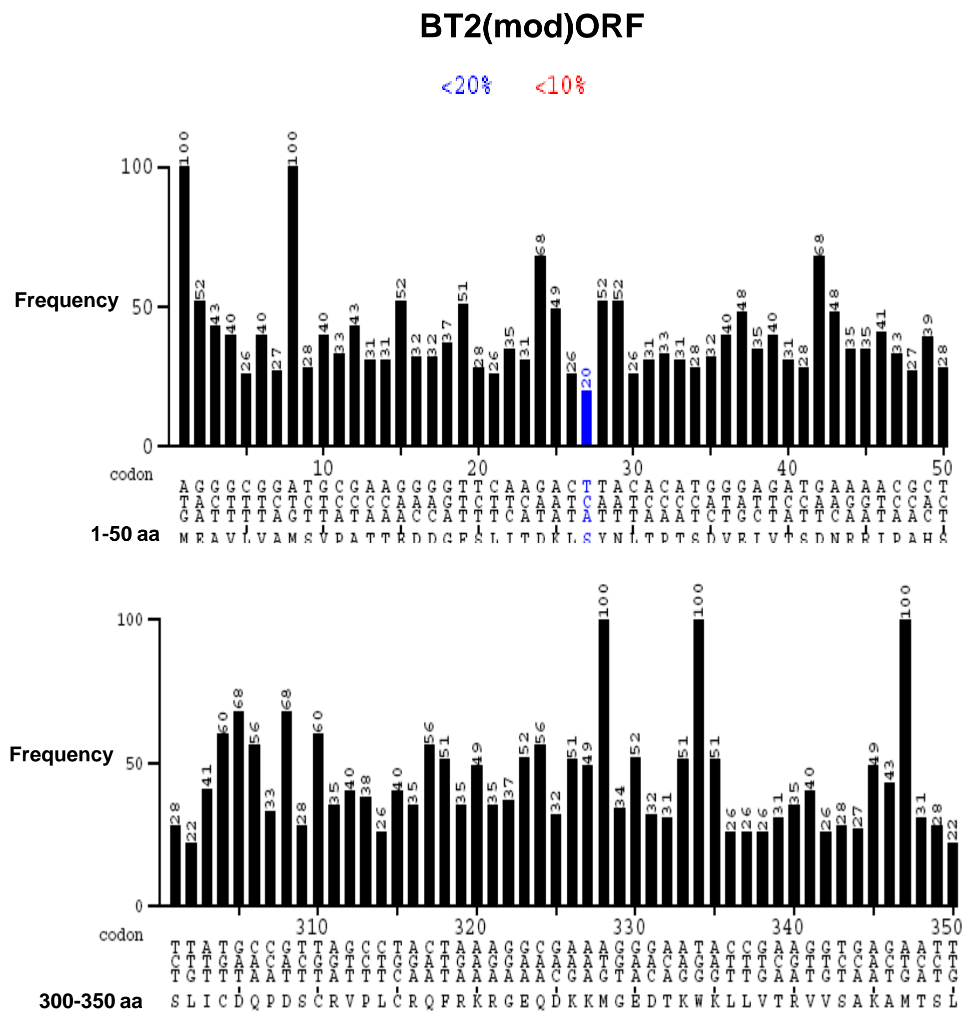

Figure 26. Continued.

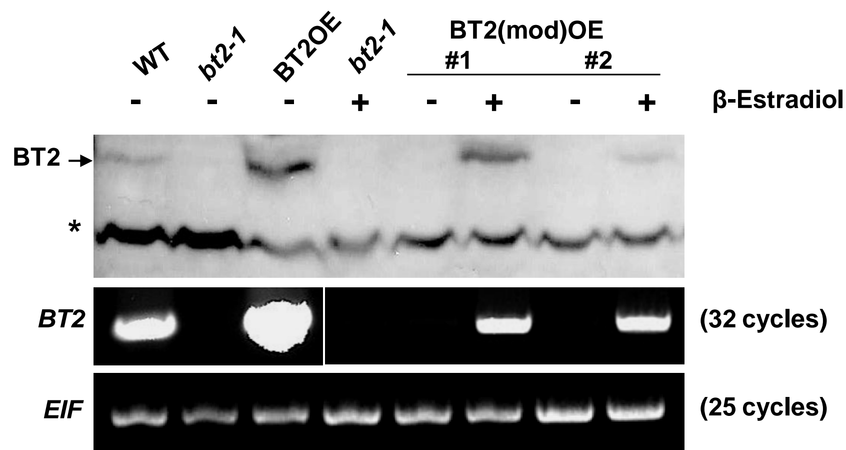

Figure 27. Codon usage affects expression of BT2 in *Arabidopsis.* One-week-old independently transformed BT2modOE lines (#1 and #2) were treated with β-estradiol (50 μM) or DMSO (0.1%) for 6 h. Treated samples, along with wild-type, *bt2-1* and BT2OE seedlings were subjected to immunoblot analysis using anti-BT2 antibodies. Arrows indicate BT2 protein. An asterisk represents cross-reacting protein and also serves as loading control. For gene expression analysis, total RNA was isolated and subjected to RT-PCR analysis. *EIF2A* expression was used as a loading control. Number in parenthesis represents PCR cycles.

Expression of BT1 *is diurnally regulated and is controlled by circadian clock*

BT2 belongs to a family of five proteins and is 75% identical to BT1, its closest homolog. To determine whether other *BT* family members are regulated similar to *BT2*, I first analyzed their expression in a diurnal cycle using qRT-PCR. As previously reported *BT2* expression is diurnally regulated with maximum expression in the dark and is circadian regulated (Figure 28B). Interestingly, expression of *BT1* also was diurnally regulated and controlled by circadian clock. *BT1* expression was lowest during the light phase (7, 14, 31, and 38 h) and gradually accumulated in dark (24 and 48 h). In continuous light, *BT1* expression was lowest during the subjective light phase (7, 14, 31 and 38 h), however, its levels increased during the subjective dark phase (19, 24, and 43 h), suggesting that it is regulated by the circadian clock (Figure 28A). Similar to *BT2,* in a diurnal cycle *BT5* expression was lowest during the light phase (7, 14, 31 and 38 h), and gradually accumulated in the dark (24 and 48 h) (Figure 28E). However, in continuous light *BT5* mRNA, although modestly increased at 24 h in the first cycle, did not consistently accumulate, suggesting that it is not circadian regulated. *BT3* and *BT4* expression did not change in a diurnal pattern (Figure 28C, 28D).

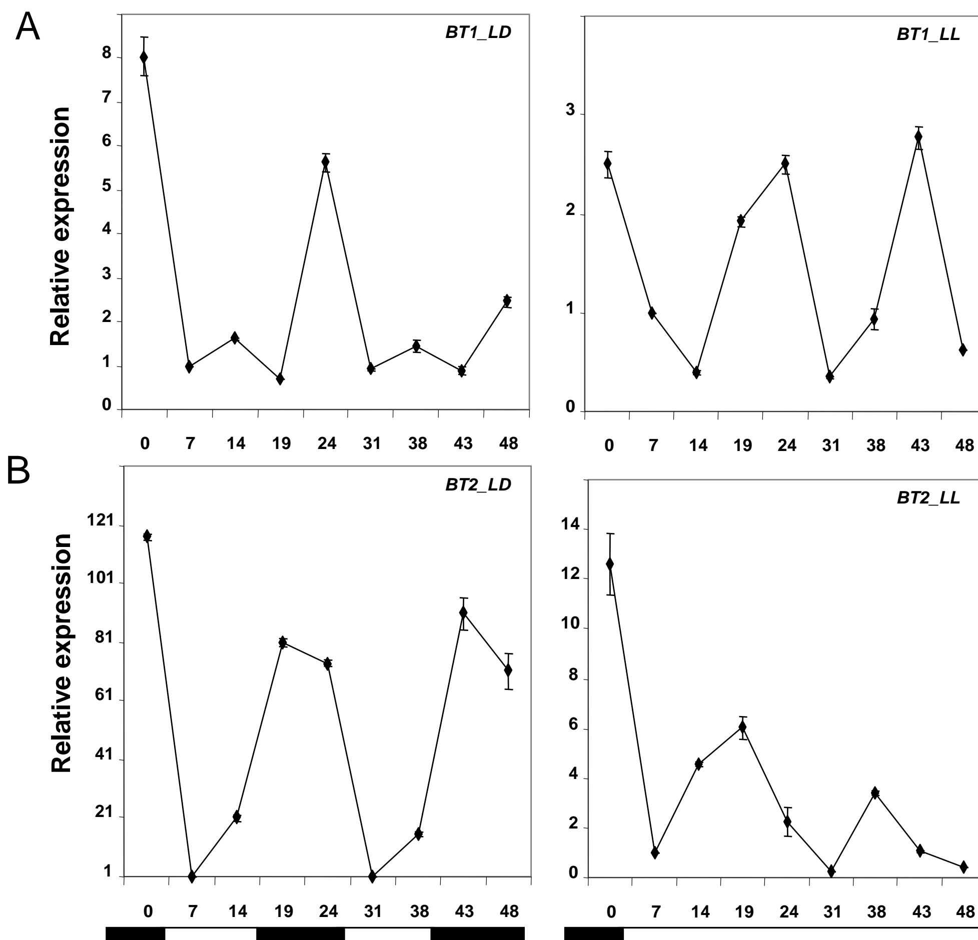

Figure 28. Expression of *BT1, BT2* and *BT5* is diurnally regulated. Wild-type plants entrained to 14 h light and 10 h dark (LD) cycles for 3 weeks were either held in LD or transferred to continuous light (LL)**.** Total RNA was extracted from rosette leaves harvested at the indicated times on X-axis, and was subjected to qRT-PCR analysis. *EIF-4A2* was used to normalize the qRT-PCR data. Expression values for *BT1* **(A)**, *BT2* **(B),** *BT3* **(C)**, *BT4* **(D)** and *BT5* **(E)** are relative to 7 h and were set to 1. Light and dark bars represent respective light conditions.

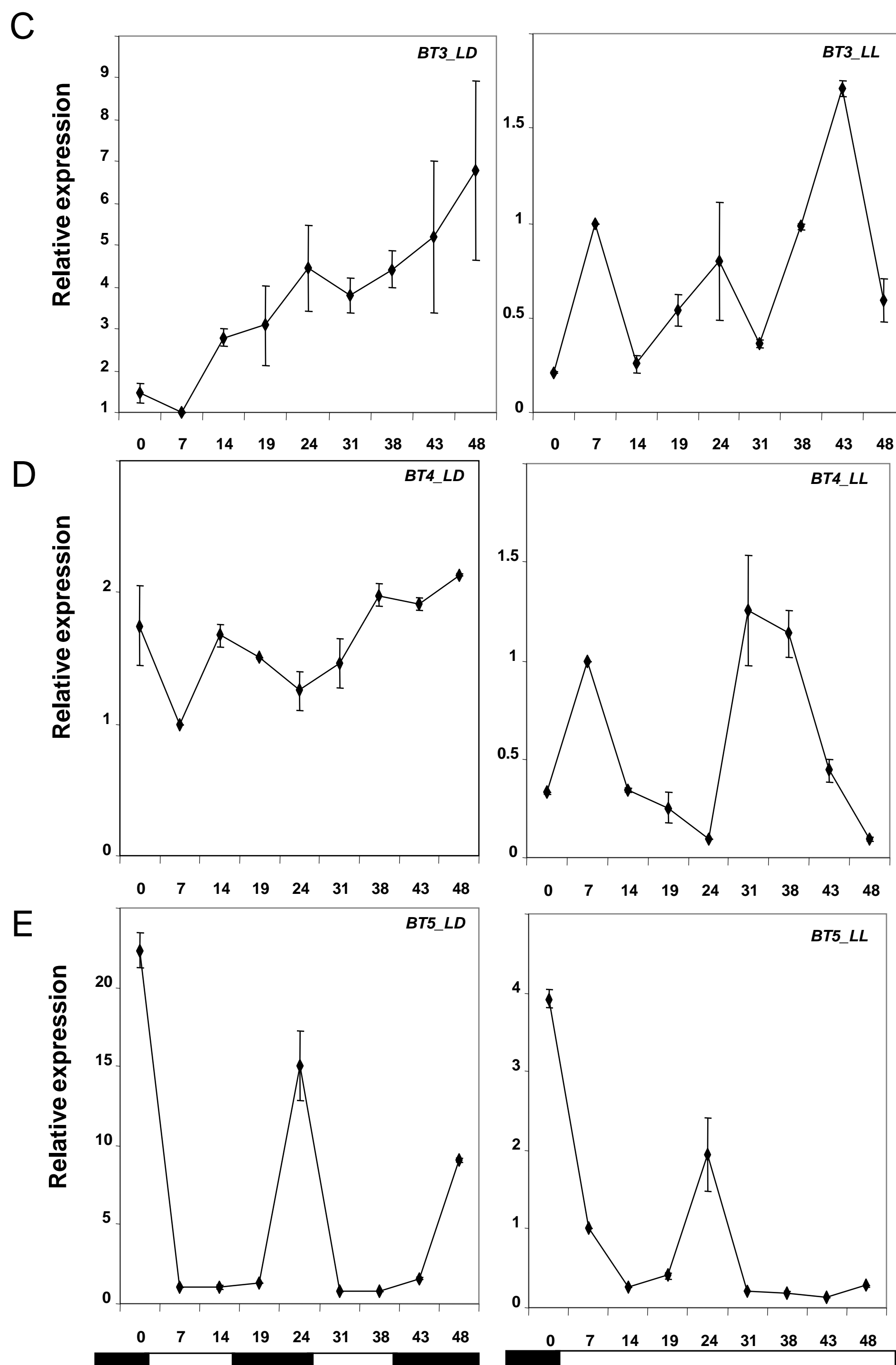

Figure 28. Continued .

BT1 *and* BT5 *expression is modulated by sugars and nitrates*

Since *BT1* and *BT5* also are diurnally regulated, I next determined if they also responded to sugars and nitrates. As expected, expression of *BT2* was derepressed by starvation (-C, -N), repressed by addition of sugars (+C), and induced by addition of nitrates (+N) (Figure 29B). Interestingly, expression of *BT1*, and *BT5* were induced by starvation, repressed by sugars and induced by nitrates (Figure 29A, 29E). However, when sucrose and nitrate were supplemented together (+C, +N), the sucrose repression was dominant.

Loss of BT1*,* BT3*, and* BT4 *suppresses* 35S *enhancer activation phenotypes*

I obtained loss-of-function alleles for *BT1* (*bt1-1*), *BT3* (*bt3-1*), and *BT4* (*bt4-1*) (Figure 30) and determined their effect on *35S* enhancer activity in the *yucca1d* line. Loss of *BT1, BT3,* and *BT4* suppressed *yucca1d* phenotypes and possessed decreased *YUCCA1* mRNA levels (Figure 31A, 31B) similar to the effect of loss of *BT2* (Mandadi et al., 2010). However, there were certain differences in the degree of suppression. For example, loss of *BT2* or *BT4* suppressed both epinastic cotyledon and leaf phenotypes of *yucca1d,* while loss of *BT1* and *BT3* suppressed only epinastic leaves. Although I still detect *BT3* transcript in the *bt3-1* line, I conclude that it is a true loss-of-function allele with dysfunctional *BT3* mRNA, due to the apparent suppression of *yucca1d* phenotypes and *YUCCA1* expression in *yucca1d bt3-1* line.

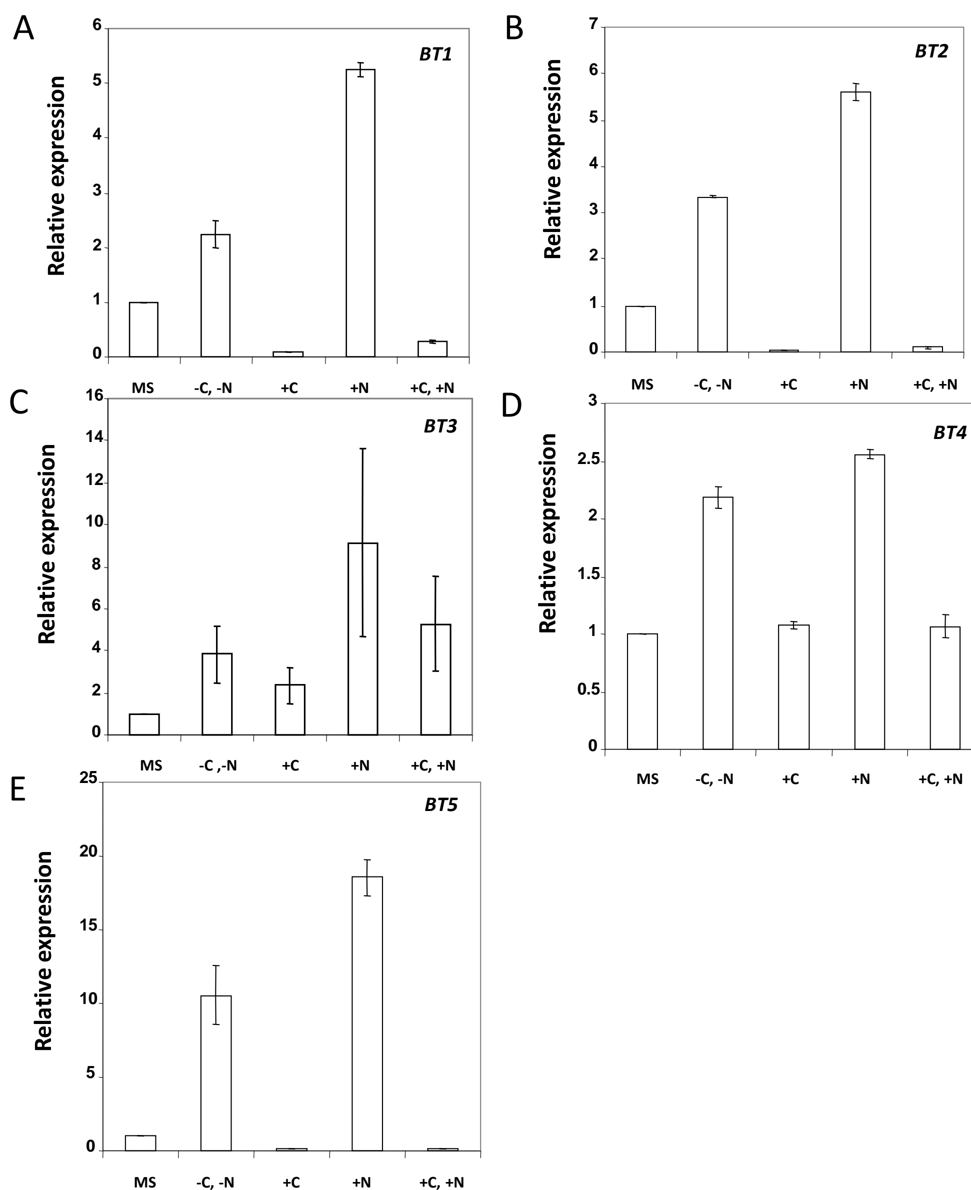

Figure 29. Expression of *BT1, BT2* and *BT5* is modulated by nutrients. Five-day-old seedlings grown in MS medium were deprived for nutrients (-C,-N) for two days. Subsequently the medium was supplemented with either 50 mM sucrose (+C); 50 mM KNO_3 (+N); 50mM of sucrose + 50 mM KNO_3 (+C,+N) or mock-treated with 50 mM mannitol and 50 mM KCl (MS). All the seedlings were treated for 3 h and total RNA was isolated and subjected to qRT-PCR analysis. *EIF-4A2* was used to normalize the qRT-PCR data. Expression values for *BT1* **(A)**, *BT2* **(B),** *BT3* **(C)**, *BT4* **(D)** and *BT5* **(E)** are relative to control seedlings (MS) and were set to 1.

I could not recover a BT5 loss-of-function line from the available seed stocks in the stock centers. However, I obtained an insertion line (GABI-Kat 771C08) with a T-DNA insertion in the 5' untranslated region (UTR) of *BT5*. By RT-PCR, I found that this line was not a loss-of-function line, but possessed increased levels of *BT5* transcript (Figure 30B). This could be due to over-expression of *BT5* by the *35S* promoter present in the T-DNA itself. It should be noted that this line was previously defined as a loss-of-function line by Robert et al., (2009) and was named *bt5-1.* Since this line possessed increased levels of *BT5*, I renamed it as *bt5-1d*. Interestingly, *yucca1d bt5-1d* exhibited poor-seed set, exacerbated *yucca1d* phenotype, especially at maturity and possessed increased levels of *YUCCA1* mRNA (Figure 31B). Therefore overexpression of *BT5* phenocopies effects of *BT2* overexpression (Ren et al., 2007), suggesting that *BT5* also regulates *35S* enhancers.

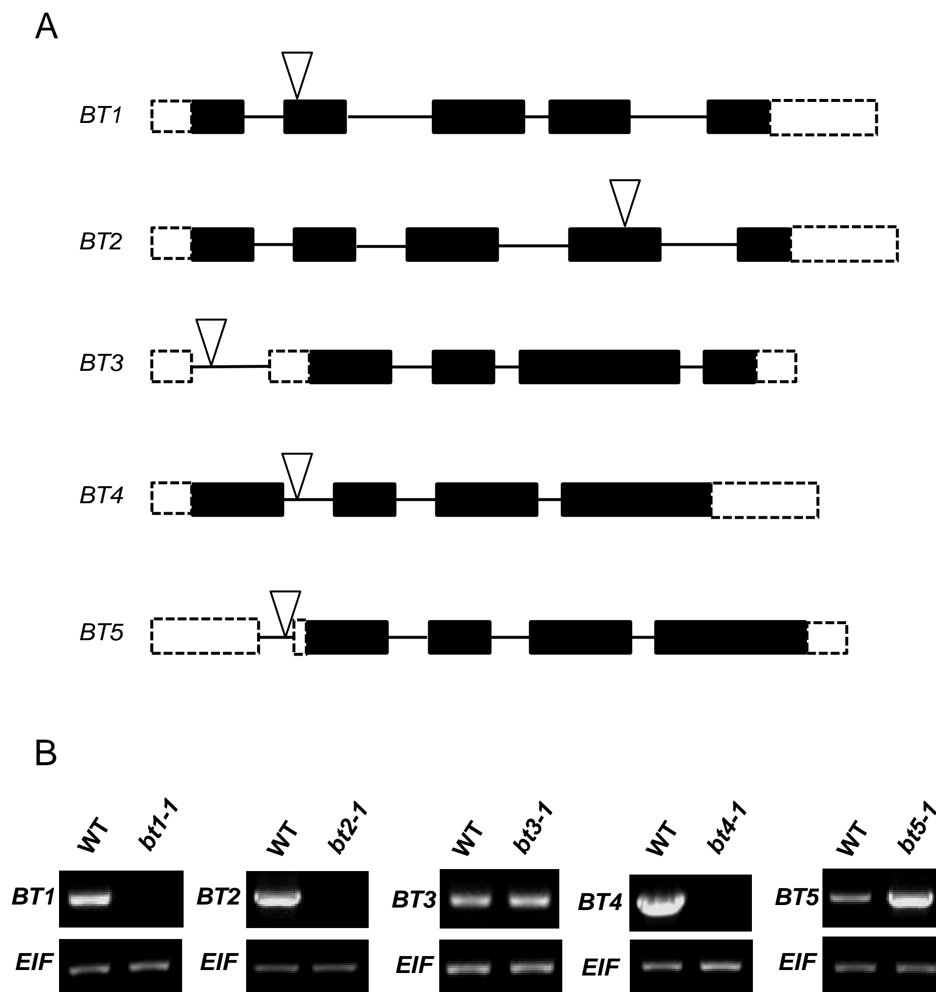

Figure 30. Structure of BT family genes. **(A)** Representation of *BT1, BT2, BT3, BT4* and *BT5* gene structure. Black bars represent exons. Black lines represent introns. Dashed bars represents untranslated regions. An open arrow head represents position of T-DNA insertion in *bt1-1, bt2-1, bt3-1, bt4-1* and *bt5-1d* alleles, respectively. **(B)** Expression *of BT1, BT2, BT3, BT4* and *BT5* in the respective alleles. Total RNA from seedlings was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control.

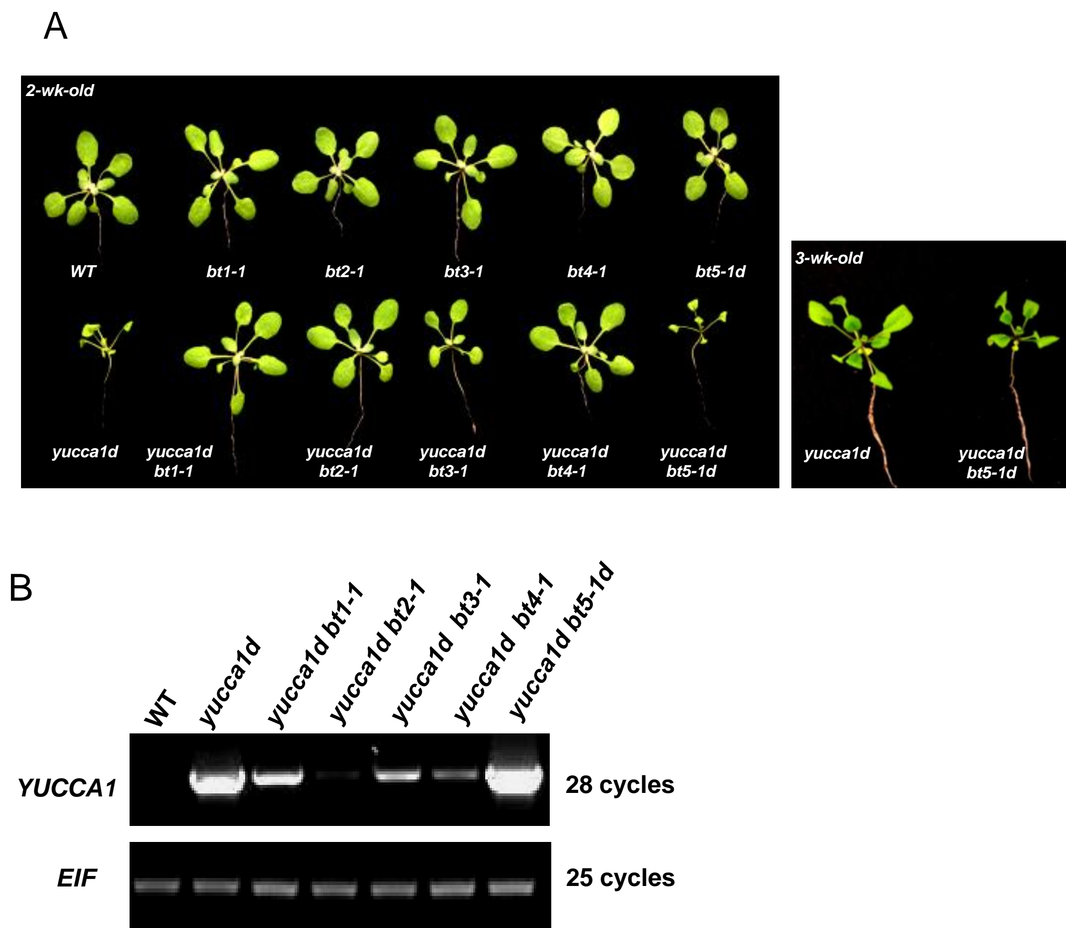

Figure 31. Loss of BT1, BT2, BT3 and BT4 abolishes *35S* enhancer activation tagged phenotypes. Suppression of epinastic leaves of *yucca1d* **(A),** and *YUCCA1* expression **(B)** in *yucca1d bt1-1, yucca1d bt2-1, yucca1d bt3-1*, *yucca1d bt4-1.* Total RNA from respective seedlings was extracted and subjected to RT-PCR. *EIF4-A2* expression was used as a loading control.

Discussion

I previously showed that BT2 mediates multiple responses to light, nutrients and a/biotic stresses (Mandadi et al., 2009; also in Chapter II), and is required for *35S* enhancer activity (Mandadi et al., 2010; also in Chapter III). In the current study, I found that BT2 was a low-abundance protein that accumulated in the dark (Figure 23), which is consistent with peak levels of BT2 mRNA in the dark. Moreover, expression of BT2 protein was developmentally regulated with the highest levels in early vegetative phases (1 and 2 week-old) (Figure 23C).

BT2 was predominantly localized in nucleus (Figure 24A) and was turned-over in part by 26S proteosome (Figure 24B). An analysis of the BT2 open reading frame (ORF) revealed that there are a relatively high number of rare codons (Figure 26A), whose tRNAs are less than 20% of all synonymous tRNAs in *Arabidopsis*. It is possible that during translation of *BT2* mRNA ribosome stalling at the rare codons affects processivity, and results in lower levels of BT2. In fact, by substituting the rare codons with favorable codons (Figure 26B), I demonstrated that BT2 codon usage had an impact on its protein levels (Figure 27). However, I simultaneously discovered that in those over-expression lines, the abundance of its mRNA is lower and the final abundance of BT2 protein seemed to be limited to a very low level (Figure 27). I do not yet know the mechanism for such fine-tuned regulation. Nevertheless, my results indicate that rare codons in the BT2 ORF contribute to the low level of BT2 protein.

BT2 expression is diurnally regulated, circadianly regulated, and is modulated by nutrients (Mandadi et al., 2009). Similar to *BT2* expression, expression of *BT1* and *BT5* was diurnally regulated with maximum expression in the dark (Figure 28A, 28E). Moreover, expression of *BT1* was circadian regulated (Figure 28A). *BT2* expression is affected by sugars and nitrates (Mandadi et al., 2009). Similar to *BT2* expression, *BT1* and *BT5* expression was also repressed by sugars and induced by nitrates (Figure 29A, 29E). My results are also consistent with global gene expression studies that have identified expression of *BT5*, along with *BT2*, to be modulated by sugars and nitrates (Blasing et al., 2005; Usadel et al., 2008). The over-lapping expression pattern of BT genes could be due to similar regulatory *cis*-elements in their promoters, and/or similar transcription factors regulating their expression.

Loss of BT2 in the high-auxin mutant *yucca1d* suppresses several of its auxin-related phenotypes, such as epinastic cotyledons, epinastic leaves, shorter primary root, excess root hair, and delayed flowering (Mandadi et al., 2009). BT2 is also required for *35S* enhancer activity in the *yucca1d* line (Mandadi et al., 2010; also in Chapter III). In the current study I found that BT1, BT3, and BT4 are also required for *35S* enhancer activity. Loss of any of these in the *yucca1d* background suppressed the activation phenotypes, and decreased expression of *YUCCA1* (Figure 31). My results provide genetic support to a hypothesis that BT family proteins could function similarly or together, and underscores the importance of their over-lapping expression

pattern. Although I did not obtain a null mutant of *BT5*, overexpression of this gene phenocopied overexpression of *BT2* in *yucca1d*, indicating that all five members of the BT family affect expression from 35S enhancers.

It is not yet clear why BT proteins mediate seemingly identical responses. Previous studies identified BT1, BT2, and BT5 as interacting partners with CULLIN3 (Figueroa et al., 2005) and BT1, BT2, and BT4 interaction with BET10 (Du and Poovaiah, 2004). However, there was no reported functional significance for these interactions. Although in vivo complexes are yet to be identified, based on my current results I hypothesize that BT family proteins assemble into multi-protein complexes. If the complex requires CULLIN3, it could function as an ubiquitin-ligase and target specific proteins for degradation and, if the complex requires BET9 or BET10 bromodomain proteins, the complex could recognize the chromatin state of target promoters. Future experiments to identify BT protein complexes and their targets will test the hypothesis.

Materials and methods

Plant materials and growth conditions

Wild-type *Arabidopsis thaliana* (Col-0) or mutant plants were grown in soil in 14 h light/10 h dark at 21 °C and a light intensity of ~120-130 μmol/m^{2}s with 70% relative humidity. The *bt2-1* null line, the *BT2* overexpression line, and *yucca1d bt2-1* lines were previously described (Ren et al., 2007; Mandadi et al., 2009). The *bt1-1* (WISCDSLOX354HO5), *bt3-1* (SALK_068395), and *bt4-1* (SALK_045370) alleles were obtained from the *Arabidopsis* Biological Resource Center (ABRC) at the Ohio State University. The *bt5-1d* (GABI-Kat 771C08) allele was obtained from European *Arabidopsis* Stock Center (NASC). All alleles were analyzed for T-DNA insertion by DNA genotyping, and transcript levels were analyzed by RT-PCR (Figure 30B). To examine the effect of altered BT gene expression on *yucca1d* (Zhao et al., 2001), we generated and examined the F2 progeny of *yucca1d bt1-1, yucca1d bt3-1*, *yucca1d bt4-1, yucca1d bt5-1d* crosses.

Treatments and expression analysis

For circadian experiments, whole rosettes of 3-wk-old wild-type plants before flowering were harvested at the indicated times of the diurnal cycle. Two biological replicates each containing two rosettes were harvested and subjected to quantitative RT-PCR (qRT-PCR). Total RNA was isolated using TRI reagent following manufacturer’s protocol (Ambion). For qRT-PCR, 5 μg RNA was used to make cDNA using SuperScript first-strand cDNA synthesis kit (Invitrogen).

Amplification by PCR was performed using Power SYBR Green Master Mix (Applied Biosystems) and the ABI Prism 7500 sequence detection system (Applied Biosystems). The primers used for quantitative qRT-PCR are listed (Table 3). *EIF-4A2* (At1g54270) was used to normalize the expression, and fold changes of expression was calculated following the $^{\Delta\Delta}$CT method (Livak and Schmittgen, 2001; Guo et al., 2008).

For treatment with sugars and nitrates, seedlings were grown in MS liquid medium for 5 days in continuous light, and transferred to medium without sugars or nitrates (-C,-N) for 2 days. On the seventh day, medium was supplemented with 50 mM sucrose (+C); 50 mM of KNO_3 (+N); 50mM of sucrose + 50 mM KNO_3 (+C, +N) or 50 mM mannitol and 50 mM KCl as mock-control (MS). All the seedlings were treated for 3 h (Scheible et al., 2004; Blasing et al., 2005). Subsequent analysis was performed by qRT-PCR using 5 µg of total RNA. The primers used for qRT-PCR are listed (Table 3).

Antibody generation and immunoblot analysis

Full-length BT2 coding sequence was cloned into pET28a (Novagen) vector, and was induced with 1 mM IPTG for 3 h at 37 °C in *E.coli* strain Rosetta2(DE3)*lysS*. BT2 protein was highly expressed in Rosetta cells which are supplied with tRNAs for codons AUA, AGG, AGA, CUA, CCC and GGA on a separate plasmid. The expressed protein was insoluble. Hence, the insoluble fraction was washed thoroughly with 5 M urea, solubilized in 2X Laemmli sample buffer, and then electrophoresed by SDS-PAGE. BT2 protein was excised from

the gel and injected into rabbits to produce anti-BT2 antibodies by Covance immunology services (Covance, Princeton, New Jersey).

Detection of BT2 protein in wild-type, *bt2-1* and BT2OE lines was performed by immunoblot analysis. Samples were harvested at the indicated times and homogenized directly in 2X Laemmli sample buffer as described previously (Martinez-Garcia et al., 1999). The extracts were boiled for 10 min and centrifuged at 13,000 g for 10 min. Supernatant was electrophoresed on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes (Amersham). Blots were incubated first with primary anti-BT2 antibodies (1:1000 dilutions), and later with peroxidase-conjugated light chain-specific mouse anti-rabbit secondary antibodies (Jackson Immunoresearch) at a 1:10,000 dilution. Visualization of proteins was performed by chemiluminescence using ECL plus detection reagents and Hyperfilm-ECL films (Amersham).

For analysis of BT2 turnover by 26S proteosome, 1-wk-old BT2modOE seedlings were incubated in MS liquid medium with 50 μM MG132 (Calbiochem) or DMSO (mock treatment) for 3 h and 16 h. Total proteins were extracted and analyzed by immunoblotting using anti-BT2 antibody as described above.

For analysis of BT2 localization, nuclear and cytoplasmic proteins were separated. 2-3 g of plant material was ground in liquid nitrogen and resuspended in 3 volumes of nuclei isolation buffer (50 mM Tris pH8.0, 10 mM KCl, 250 mM sucrose, 1.5 mM $MgCl_2$, 5 mM EDTA, 0.3% Triton X-100, 1 mM PMSF, 5 mM β-mercaptoethanol, 1 mM spermine, 1 mM spermidine, Protease inhibitor cocktail). After filtering through 2 layers of miracloth, an aliquot of the extract was saved to represent total protein fraction. The remaining extract was centrifuged at 3000 g for 30 min at 4 °C. After centrifugation, an aliquot of the supernatant was saved to represent cytoplasmic protein fraction. The pellet was suspended in 1 ml of Triton X-100 buffer (10 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.25 M sucrose, 1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 mM spermine, 1 mM spermidine, Protease inhibitor cocktail), and gently mixed. The suspension was centrifuged in steps of 2000 g, 1 min; 4000 g, 1 min; 8000 g, 2 min, respectively. The nuclear pellet was finally suspended in 2X Laemmli sample buffer. Approximately 75 μg of total, cytoplasmic and nuclear proteins were subjected to immunoblot analysis as described above.

BT2 *codon analysis and engineering of* BT2modOE *lines*

In silico codon analysis was performed using graphic codon usage analyzer software publicly available at http://gcua.schoedl.de/cite.html (Fuhrmann et al., 2004). To modify BT2 codons, overlapping sets of primers with the necessary changes were designed (Table 3). PCR was performed in steps and the final PCR product was cloned into an estradiol inducible binary vector pER8 (Jianru et al., 2000; Ng et al., 2006). Mutations were verified by DNA sequencing. *Arabidopsis* transgenic lines overexpressing BT2mod (BT2modOE) were generated by *Agrobacterium*-mediated transformation and subsequent selection on Hygromycin. For analysis of BT2mod protein in the transgenic lines, 1-wk-old BT2modOE seedlings were incubated in MS liquid medium with 50 µM β-estradiol (Sigma) or DMSO (mock) for 6 h. Total proteins were extracted and analyzed by immunoblotting using anti-BT2 antibodies as described above.

Accession numbers

AGI locus numbers for genes used in this study are: *BT1*, At5g63160; *BT2*, At3g48360; *BT3*, At1g05690; *BT4*, At5g67480; *BT5*, At4g37610; *BET9,* At5g14270; *BET10,* At3g01770; *CULLIN3A,* At1g26830; *YUCCA1,* At4g32540; *EIF-4A2*, At1g54270.

Table 3. Primers used for BT2 modification and analysis of BT gene family function

qRT-PCR analysis:	
BT1	F-GTCTTCGTCAGATTCCTCTA
	R-CACCGTTGCTTTAACTGAG
BT2	F-CACAACGGAAGACGACGGAT
	R-CAGTACCGGTGAAGCTGAA
BT3	F-GAAGCACTTGAAACTTG
	R-CAGAAGTGTCGCTATAACTG
BT4	F-TGTGGGATCGTCTTTTCAAT
	R-TATTGTATGCCACTTGCCATGT
BT5	F-CTCATGGAGCTGATGTTTTG
	R-TGTTGCTTCATCATTCCTCTG
EIF-4A2	F-CAAGGTGTCAAGGTTCATGC
	R-CAACGACAACATGAACACCA
RT-PCR analysis:	
BT1	F-CCTCCAGCCTCCAACAAC
	R-ACACTTGAGACAGAGGTCAG
BT2	F-ACATGGTCACCCAGCTGAAG
	R-CAGACACAACCCTTGTCACC
BT3	F-CGATGTCTAGTAGTACCAAGAAC
	R-GACCGAGTAGCAATGTGACAGA
BT4	F-ATGCAGGGAAGAGAAGATAAGC
	R-CAAGGAGTTGCCACATTCTCT
BT5	F-GGCTTCAGATGTAATCAGAGGA
	R-GGCCAAAGATTGGATCTTAGG
EIF-4A2	F-GCAAGAGAATCTTCTTAGGGGTATCTATGC
	R-GGTGGGAGAAGCTGGAATATGTCATAG
BT2 codon modification	
BT2FCSTII	ATAGGATCCATGGAAGCTGTTCTTGTTGCAATGTCTGTTCCAGCT
BT2F6-22	GTTGCAATGTCTGTTCCAGCTACAACAGAAGACGACGGATTTTCTCTTATC
BT2F17-33	GACGGATTTTCTCTTATCACAGATAAACTTTCATATAATCTTACACCAACA
BT2F28-44	TATAATCTTACACCAACATCTGACGTTGAGATCGTTACATCTGATAACAGA
BT2F39-55	GTTACATCTGATAACAGAAGAATTCCAGCACACTCTGGAGTTCTTGCTTCA
BT2F50-66	TCTGGAGTTCTTGCTTCAGCTTCACCAGTTCTTATGAACATCATGAAGAAA
BT2RCSTII	GGAGCTCTATTTTTCAAATTGAGGATGAGACCAAACTCCTTGTGCTTGTTC
BT2R362-346	TCCTTGTGCTTGTTCACATTTGTTCTTCTTTGACTGACACAAAGATGTCATAGC
BT2R350-334	CAAAGATGTCATAGCTTTTGCAGACACAACTCTTGTCACAAGAAGCTTCCA
BT2R339-323	TGTCACAAGAAGCTTCCACTTTGTGTCTTCACCCATTTTCTTGTCTTGTTC
BT2R328-312	CATTTTCTTGTCTTGTTCTCCTCTTTTTCTAAATTGTCTGCAAAGAGGAAC
BT2R317-301	TTGTCTGCAAAGAGGAACTCTACAAGAATCTGGTTGATCACAAATCAAAGA

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

We initially identified BT2 as an activator of telomerase in plants. BT2 functions downstream of the zinc-finger transcription factor *TELOMERASE ACTIVATOR 1 (TAC1),* and overexpression of *BT2* activates expression of *TERT* (Ren et al., 2007). In the current study I discovered that BT2 plays a much broader role in the physiology of the plant. The gene itself responds to multiple signals and is required for appropriate response to many of these same signals. *BT2* expression was affected by light, the circadian clock, and it responded to nutrients such as sugars and nitrates (Mandadi et al., 2009; also in Chapter II). Moreover, expression of *BT2* was modulated by hormones and signals such as ABA, cold, cytokinin, methyl-jasmonate and hydrogen-peroxide (Mandadi et al., 2009; also in Chapter II). It is possible that *BT2* expression is controlled by a master regulator or several transcription factors that integrate multiple signals.

The bZIP family of transcription factors such as bZIP2 and bZIP11, and snf1-related kinases (SnRK) KIN10 and KIN11, modulate transcription of numerous genes in response to sugar, energy deprivation and diverse stresses (Baena-Gonzalez et al., 2007; Hanson et al., 2008). Interestingly, *BT2* was among the numerous putative targets of bZIP11 and KIN10. However, further experiments will be needed to determine whether BT2 is a direct target of bZIP11 and/or KIN10, using ChIP assays or yeast one-hybrid analysis with the BT2 promoter and bZIP11/KIN10 proteins. An in silico analysis of the *BT2* promoter revealed several *cis*-regulatory elements that may regulate *BT2* expression (Table 4). These include the G-box/ABRE element (CACGTG, involved in light, ABA, and Me-JA signaling); starvation element (TATCCA, involved in nutrient responses); DOF transcription factor binding sites (A/TAAAG, involved in carbon/nitrogen signaling, hormone and stress responses). Further experiments are required to validate these predictions using promoter deletion or similar strategies. Nevertheless, identification of *cis*-elements in the *BT2* promoter and transcription factors that control *BT2* expression in response to multiple signals will contribute to our knowledge of synergistic transcriptional networks.

Table 4. In silico promoter analysis of BT2 and identification of putative *cis*-regulatory elements. In silico promoter analysis was performed using PLACE database search (Higo et al., 1999)

***cis*-element**	**Reported Sequence (5'→ 3')**	**Binding factor; function**	**Position in *BT2* promoter from *ATG*; matching sequence**
DOF TF binding site	(A/T)AAAG	Plant specific DOF TF's; C:N metabolism, hormone and stress signaling	-123, -226, -235, -300, -308, -558, -600, -920, -920, -1138 -1169, -1412, -1501, -1603, -1818; (**A/T)AAAG**
G-Box/ ABRE	CACGTG	bZIP family/ GBFs; Light, ABA, Methyl-jasmonate signaling	-2024; **CACGTG**
Starvation element	TATCCA	Induced during starvation	-274, -1530; **TATCCA**
GT box	AAACC(A/G)(A/G), (T/C)GGTTT	*AtMYB2;* hypoxic/anoxic signaling	-751, -2108; **AAACCAA, TGGTTT**

High levels of sugars inhibit germination by modulating ABA signaling, a key hormone that controls dormancy (León and Sheen, 2003). BT2 was required for appropriate responses to sugars and ABA during germination. Loss of BT2 resulted in sensitivity to germination in high sugars and ABA, while over-expression of BT2 imparted resistance to germination in high sugars and ABA (Mandadi et al., 2009; also in Chapter II). However, I did not detect changes in expression of ABA signaling genes such as *ABA-insensitive 3* (*ABI3*), *ABI4* and *ABI5* in *bt2-1* or *BT2OE* in the presence of sugars (Mandadi et al., 2009; also in Chapter II). It is possible that BT2 could be affecting different ABA and sugar signaling genes and/or BT2 itself could be the target. Future experiments to identify downstream targets of BT2 in sugar and ABA signaling will be important. A micro-array gene expression analysis of *bt2-1* or *BT2OE* treated with sugars and/or ABA can be performed to identify BT2 target genes. Alternatively, suppressor-screening using *bt2-1* lines could be employed.

Enhancers are DNA elements that activate transcription (Khoury and Gruss, 1983; Serfling et al., 1985). I found that BT2 is an essential regulator of CaMV *35S* enhancer-mediated activation of gene expression in *Arabidopsis*. Lack of BT2 in several activation-tagged lines suppressed activation phenotypes and decreased gene expression (Mandadi et al., 2010; also in Chapter III). Furthermore, I found that BT2 affected transcription in the activation lines (Mandadi et al., 2010; also in Chapter III). Currently, I do not know whether BT2 is recruited directly to *35S* enhancers. I hypothesize that *35S* enhancers are

marked by certain chromatin modifications and are recognized by BT2 or proteins associated with it. Further identification of the chromatin modifications on *35S* enhancers using ChIP assays will be important. I also do not know the exact mechanism of BT2 in activating transcription. Nevertheless, this discovery has provided us with a working model. Activation tagging of genes with multiple copies of *35S* enhancers is a major tool in functional genomics. Although activation tagging is popular in *Arabidopsis*, it is also increasingly being used in rice and other plant species. It will be interesting to determine whether BT2 orthologs in rice and other species are required for *35S* enhancer function.

I also demonstrated that BT2 genetically interacts with CULLIN3 (Mandadi et al., 2010; also in Chapter III) and two bromo-domain containing proteins, BET9 and BET10 (Misra et al., 2010). I propose that BT2 assembles in a multi-protein complex. The complex could consist of BT2, CULLIN3, and BET9 or BET10. Since bromodomains bind to acetylated histone proteins, BET9 or BET10 could anchor BT2-complex to gene promoters or enhancers by recognizing the acetylated histones. Because of the requirement of CULLIN3, BT2-complex could function as a CULLIN3-based ubiquitin ligase. My hypothesis is that BT2 targets certain transcription factors for destruction by poly-ubiquitination and/or activates factors by mono-ubiquitination. The TAZ domain of BT2 could provide specificity by recruiting the target proteins. Alternatively, the entire complex may serve as a scaffold to promote interactions among factors. BT2's CaMBD domain was critical for its function (Mandadi et al.,

2010; also in Chapter III). Ca^{++} acts as a secondary messenger and is involved in nearly every aspect of plant growth and development. Regulation of BT2 by Ca^{++} signals could connect the seemingly diverse roles of BT2. Further experiments will be necessary to test this hypothesis.

Using anti-BT2 antibodies I found that the abundance of BT2 protein is very low in plants (Chapter IV). Expression of BT2 protein was developmentally regulated and was regulated by 26S proteosome (Chapter IV). Furthermore, I found that the BT2 coding sequence has numerous rare codons that affected protein abundance (Chapter IV). Although I re-engineered BT2 with favorable codons and demonstrated that rare codons did affect BT2 protein accumulation, in those transgenic lines the abundance of *BT2* mRNA is reduced (Chapter IV). Nevertheless, I generated an over-expression line of BT2 with favorable codons and with a C-terminal StrepII affinity tag. Preliminary results from affinity purification of BT2 from the estradiol inducible *BT2mod* over-expression line, in the presence of MG132, demonstrated that this technique can be utilized to purify BT2 (Figure 32). Further experiments to pull down BT2-complex followed by mass spectrometry analysis are required to identify BT2 interacting proteins and targets. Alternatively, a proteomic analysis of *bt2-1* or *BT2OE* lines in response to sugars, ABA or *35S* enhancer can be performed to identify BT2 targets.

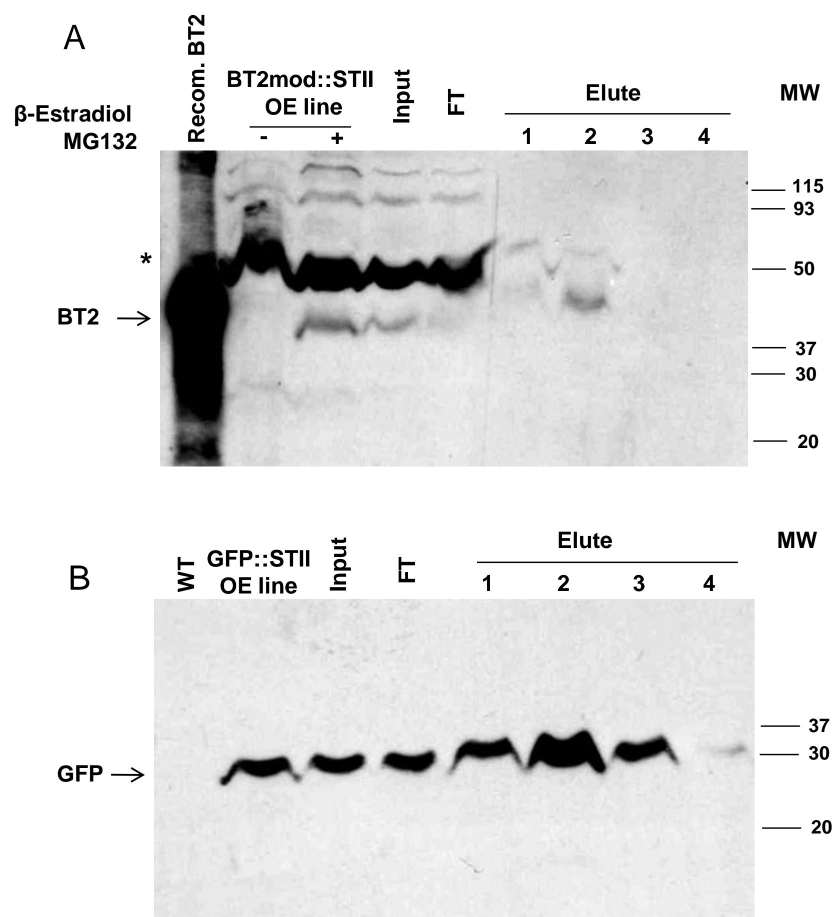

Figure 32. Affinity purification of BT2::STII **(A)** and GFP::STII **(B)** in *Arabidopsis.* One-week-old BT2modOE line was treated with β-estradiol (50 μM) and MG132 (50 μM) for 6 h. Treated samples, along with a positive control GFP::STIIOE seedlings were subjected to affinity purification using StrepTactin columns. After washing unbound proteins (FT) , desthiobiotin was used to elute bound proteins in four fractions. All the fractions were subjected to immunoblot analysis using anti-BT2 and anti-GFP antibodies. Arrows indicate BT2 and GFP proteins. An asterisk represents cross-reacting protein and also serves as loading control. Protein molecular weight (MW) of standards in kD are indicated by lines.

BT2 belongs to a family of five proteins, BT1 through BT5, and all the proteins possess the conserved BTB domain, TAZ domain and CaMBD domain (Du and Poovaiah, 2004). In addition to BT2, BT1 and BT4 interact with BET10 (Du and Poovaiah, 2004), while BT1 and BT5 interact with CULLIN3 (Figueroa et al., 2004). Robert et al., (2009) recently demonstrated that BT family genes redundantly regulate gametophyte development and regulate expression of each other. I have shown that BT family genes all respond similarly to sugars and nitrates, but respond differently to light and the circadian clock (Chapter IV). BT2 responds to multiple stimuli, and it is required for response to many of these same stimuli. Further analysis of loss-of-function and over-expression lines of BT genes will be necessary to determine if other BT family members are required for downstream responses to stimuli that affect their expression. Moreover, since BT genes respond similarly to multiple signals, a comparative analysis of their promoters to identify conserved *cis*-elements might reveal important regulatory features.

Several BT proteins are required for *35S* enhancer activity because I observed that loss of different *BT* genes in *yucca1d* lines suppressed the overall activation phenotypes (Chapter IV), although there were notable differences. For example, loss of BT2 or BT4 suppressed the activation phenotype in cotyledons, while loss of BT1 and BT3 did not. BT proteins could perform similar function biochemically; however, they could yield different effects because of their localization. A detailed study of temporal and spatial expression of BT genes will be used to test this hypothesis. Such regulation could provide specificity to their seemingly redundant functions.

It is not clear how BT proteins mediate similar responses, yet, they cannot compensate for each other in activating *35S* enhancers. My hypothesis is that BT proteins assemble into multi-protein complexes with CULLIN3. The complex could function as an ubiquitin ligase and target certain proteins for destruction by poly-ubiquitination, or activate certain proteins by mono-ubiquitination. It is also possible that BT proteins are themselves targets of each other. Further biochemical experiments are needed to test this hypothesis and identification of specific targets of BT family will further our knowledge about the mechanism of BTB domain containing proteins in general.

REFERENCES

Abel, S., Oeller, P.W., and Theologis, A. (1994). Early auxin-induced genes encode short-lived nuclear proteins. Proc. Natl. Acad. Sci. USA **91:** 326-330.

Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J, Ebert, P.R., and Kazan, K. (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell **16:** 3460-3479.

Anderson, S.L., and Kay, S.A. (1996). Illuminating the mechanism of the circadian clock in plants. Trends Plant Sci. **1:** 51-57.

Arenas-Huertero F, Arroyo A, Zhou L, Sheen J, Leon P (2000). Analysis of *Arabidopsis* glucose insensitive mutants, *gin5* and *gin6*, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. Genes Dev. **14:** 2085-2096.

Baena-Gonzalez, E., Rolland, F., Thevelein, J.M., and Sheen, J. (2007). A central integrator of transcription networks in plant stress and energy signaling. Nature **448:** 938-942.

Benfey, P.N., and Chua, N.-H. (1990). The cauliflower mosaic virus *35S* promoter: combinatorial regulation of transcription in plants. Science **250:** 959-966.

Benfey, P.N., Ren, L., and Chua, N.-H. (1989). The CaMV *35S* enhancer contains at least two domains which can confer different developmental and tissue-specific expression patterns. EMBO J. **8:** 2195-2202.

Bi, Y-M., Zhang, Y., Signorelli, T., Zhao, R., Zhu, T., and Rothstein, S (2005). Genetic analysis of *Arabidopsis* GATA transcription factor gene family reveals a nitrate-inducible member important for chlorophyll synthesis and glucose sensitivity. Plant J. **44:** 680-692.

Blackwood, E.M., and Kadonaga, J.T. (1998). Going the distance: a current view of enhancer action. Science **281:** 60-63.

Blasing, O.E., Gibon, Y., Gunther, M., Hohne, M., Morcuende, R., Osuna, D., Thimm, O., Usadel, B., Scheible, W.R., and Stitt, M. (2005). Sugars and circadian regulation make major contributions to the global regulation of diurnal gene expression in *Arabidopsis*. Plant Cell **17:** 3257-3281.

Bondarenko, V.A., Liu, Y.V., Jiang, Y.I., and Studitsky, V.M. (2003). Communication over a large distance: enhancers and insulators. Biochem. Cell. Biol. **81:** 241-251.

Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., and Lamb, C. (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12:** 2383-2393.

Briggs, W.R., and Christie, J.M. (2002). Phototropins 1 and 2: versatile plant blue-light receptors. Trends Plant Sci. **7:** 204-210.

Chen, M., Chory, J., and Fankhauser, C. (2004). Light signal transduction in higher plants. Annu. Rev. Genet. **38:** 87-117.

Cheng, W-H., Endo, A., Zhou, L., Penney, J., Chen, H-C., Arroyo, A., Leon, P., Nambara, E., Asami, T., Seo, M., Koshiba, T., and Sheen, J (2002). A unique short-chain dehydrogenase/reductase in *Arabidopsis* abscisic acid biosynthesis and glucose signaling. Plant Cell **14:** 2723-2743.

Cheng, Y., Dai, X., and Zhao, Y. (2006). Auxin biosynthesis by the *YUCCA* flavin monooxygenases controls the formation of floral organs and vascular tissues in *Arabidopsis*. Genes Dev. **20:** 1790-1799.

Cheng, Y., Qin, G., Dai, X., and Zhao, Y. (2007). NPY1, a BTB-NPH3-like protein, plays a critical role in auxin-regulated organogenesis in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **104:** 18825-18829.

Chiou, T-J., and Bush, D.R (1998). Sucrose is a signal molecule in assimilate partitioning. Proc. Natl. Acad. Sci. USA **95:** 4784-4788.

Chory, J., Chatterjee, M., Cook, R.K., Elich, T., Fankhauser, C., Li, J., Nagpal, P., Neff, M., Pepper, A., Poole, D., Reed, J., and Vitart, V. (1996). From seed germination to flowering, light controls plant development via the pigment phytochrome. Proc. Natl. Acad. Sci. USA **93:** 12066-12071.

Chua, Y.L., Watson, L.A., and Gray, J.C. (2003). The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. Plant Cell **15:** 1468-1479.

Collins, T., Stone, J.R., and Williams, A.J. (2001). All in the family: the BTB/POZ, KRAB, and SCAN domains. Mol. Cell. Biol. **21:** 3609-3615.

Coruzzi, G.M., and Zhou, L. (2001). Carbon and nitrogen sensing and signaling in plants: emerging `matrix effects'. Curr. Opin. Plant Biol. **4:** 247-253.

Couee I, Sulmon C, Gouesbet G, El Amrani A (2006). Involvement of soluble sugars in reactive oxygen species balance and responses to oxidative stress in plants. J. Exp. Bot. **57:** 449-459.

Davis, P.J. (2004). The plant hormones: their nature, occurrence, and functions. In Plant Hormones-Biosynthesis, Signal Transduction, Action, P.J. Davies, ed (New York: Springer), pp. 1-15.

De Guzman, R.N., Liu, H.Y., Martinez-Yamout, M., Dyson, H.J., and Wright, P.E. (2000). Solution structure of the TAZ2 (CH3) domain of the transcriptional adaptor protein CBP. J. Mol. Biol **303:** 243-253.

Devlin, P.F., and Kay, S.A. (2000). Cryptochromes are required for phytochrome signaling to the circadian clock but not for rhythmicity. Plant Cell **12:** 2499-2510.

Dong, Y., and VonArnim, A.G. (2003). Novel plant activation-tagging vectors designed to minimize *35S* enhancer-mediated gene silencing. Plant Mol. Biol. Rep. **21:** 349-358.

Du, L., and Poovaiah, B.W. (2004). A novel family of Ca^{2+}/calmodulin-binding proteins involved in transcriptional regulation: interaction with fsh/Ring3 class transcription activators. Plant Mol. Biol. **54:** 549-569.

Dunlap, J.C. (1999). Molecular bases for circadian clocks. Cell **96:** 271-290.

Fang, R.X., Nagy, F., Sivasubramaniam, S., and Chua, N.H. (1989). Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus *35S* promoter in transgenic plants. Plant Cell **1:** 141-150.

Fankhauser, C., and Chory, J. (1997). Light control of plant development. Annu. Rev. Cell Dev Biol **13:** 203-229.

Fedoroff, N.V. (2002). Cross-talk in abscisic acid signaling. Sci. STKE. **140:** re10.

Ferreira, F.J., and Kieber, J.J. (2005). Cytokinin signaling. Curr. Opin. Plant Biol. **8:** 518-525.

Figueroa, P., Gusmaroli, G., Serino, G., Habashi, J., Ma, L., Shen, Y., Feng, S., Bostick, M., Callis, J., Hellmann, H., and Deng, X.W. (2005). *Arabidopsis* has two redundant Cullin3 proteins that are essential for embryo development and that interact with RBX1 and BTB proteins to form multisubunit E3 ubiquitin ligase complexes in vivo. Plant Cell **17:** 1180-1195.

Finkelstein, R.R., Gampala, S.S.L., and Rock, C.D. (2002). Abscisic acid signaling in seeds and seedlings. Plant Cell **14:** S15-45.

Folta, K.M., and Kaufman, L.S. (2006). Isolation of *Arabidopsis* nuclei and measurement of gene transcription rates using nuclear run-on assays. Nat. Prot. **1:** 3094-3100.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K., and Shinozaki, K. (2006). Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks. Curr. Opin. Plant Biol. **9:** 436-442.

Gazzarrini, S., and McCourt, P (2001). Genetic interactions between ABA, ethylene and sugar signaling pathways. Curr. Opin. Plant Biol. **4:** 387–391.

Goodbourn, S., Zinn, K., and Maniatis, T. (1985). Human β-interferon gene expression is regulated by an inducible enhancer element. Cell **41:** 509-520.

Goodman, R.H., and Smolik, S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. **14:** 1553-1577.

Green, R.M., and Tobin, E.M. (1999). Loss of the circadian clock-associated protein 1 in *Arabidopsis* results in altered clock-regulated gene expression. Proc. Natl. Acad. Sci. USA **96:** 4176-4179.

Gubler, F., Millar, A.A., and Jacobsen, J.V. (2005). Dormancy release, ABA and pre-harvest sprouting. Curr. Opin. Plant Biol. **8:** 183–187.

Guo, B., Jin, Y., Wussler, C., Blancaflor, E.B., Motes, C.M., and Versaw, W.K (2008). Functional analysis of the *Arabidopsis* PHT4 family of intracellular phosphate transporters. New Phytol. **177:** 889-898.

Gutierrez, R.A., Ewing, R.M., Cherry, J.M., and Green, P.J. (2002). Identification of unstable transcripts in *Arabidopsis* by cDNA microarray analysis: rapid decay is associated with a group of touch- and specific clock-controlled genes. Proc. Natl. Acad. Sci. USA **99:** 11513-11518.

Gutierrez, R.A., Stokes, T.L., Thum, K., Xu, X., Obertello, M., Katari, M.S., Tanurdzic, M., Dean, A., Nero, D.C., McClung, C.R., and Coruzzi, G.M. (2008). Systems approach identifies an organic nitrogen-responsive gene network that is regulated by the master clock control gene CCA1. Proc. Natl. Acad. Sci. USA **105:** 4939-4944.

Ha, C.M., Jun, J.H., Nam, H.G., and Fletcher, J.C. (2004). BLADE-ON-PETIOLE1 encodes a BTB/POZ domain protein required for leaf morphogenesis in *Arabidopsis thaliana*. Plant Cell Physiol. **45:** 1361-1370.

Hanson, J., Hanssen, M., Wiese, A., Hendriks, M.M., Smeekens, S. (2008). The sucrose regulated transcription factor bZIP11 affects amino acid metabolism by regulating the expression of *ASPARAGINE SYNTHETASE1* and *PROLINE DEHYDROGENASE*2. Plant J **53:** 935-949.

Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.-S., Han, B., Zhu, T., Wang, X., Kreps, J.A., and Kay, S.A. (2000). Orchestrated transcription of key pathways in *Arabidopsis* by the circadian clock. Science **290:** 2110-2113.

Harms, E., Kivimae, S., Young, M.W., and Saez, L. (2004). Posttranscriptional and posttranslational regulation of clock genes. J. Biol. Rhyth. **19:** 361-373.

Hayashi, H., Czaja, I., Lubenow, H., Schell, J., and Walden, R. (1992). Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. Science **258:** 1350-1353.

Himanen, K., Boucheron, E., Vanneste, S., de Almeida Engler, J., Inzé, D., and Beeckman, T. (2002). Auxin-mediated cell cycle activation during early lateral root initiation. Plant Cell **14:** 2339–2351.

Hoth, S., Morgante, M., Sanchez, J-P., Hanafey, M.K., Tingey, S.V., and Chua, N-H. (2002). Genome-wide gene expression profiling in *Arabidopsis* thaliana reveals new targets of abscisic acid and largely impaired gene regulation in the *abi1-1* mutant. J. Cell Sci. **115:** 4891–4900.

Huala, E., Oeller, P.W., Liscum, E., Han, I.-S., Larsen, E., and Briggs, W.R. (1997). *Arabidopsis* NPH1: a protein kinase with a putative redox-sensing domain. Science **278:** 2120-2123.

Jack, J., Dorsett, D., Delotto, Y., and Liu, S. (1991). Expression of the cut locus in the *Drosophila* wing margin is required for cell type specification and is regulated by a distant enhancer. Development **113:** 735-747.

Jakobovits, A., Smith, D.H., Jakobovits, E.B., and Capon, D.J. (1988). A discrete element 3' of human immunodeficiency virus 1 (HIV-1) and HIV-2 mRNA initiation sites mediates transcriptional activation by an HIV trans activator. Mol. Cell. Biol. **8:** 2555-2561.

Jang, J.C., and Sheen, J. (1994). Sugar sensing in higher plants. Plant Cell **6:** 1665-1679.

Jianru, Z., Qi-Wen, N., and Nam-Hai, C. (2000). An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J. **24:** 265-273.

Johnson, A.A.T., Yu, S.-M., and Tester, M. (2007). Activation tagging systems in rice. In Rice Functional Genomics, N.M. Upadhyaya, ed (New York: Springer), pp. 333-353

Käs, E., Izaurralde, E., and Laemmli, U.K. (1989). Specific inhibition of DNA Binding to nuclear scaffolds and histone H1 by distamycin: the role of oligo(dA)·oligo(dT) tracts. J. Mol. Biol. **210:** 587-599.

Katagiri, F., Lam, E., and Chua, N.-H. (1989). Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340:** 727-730.

Khoury, G., and Gruss, P. (1983). Enhancer elements. Cell **33:** 313-314.

Klusener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V., and Schroeder, J.I. (2002). Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in *Arabidopsis* guard cells. Plant Physiol. **130:** 2152-2163.

Koch, K.E. (1996). Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant. Physiol. Plant Mol. Biol. **47:** 509-540.

Laby, R.J., Kincaid, M.S., Kim, D., and Gibson, S.I (2000). The *Arabidopsis* sugar-insensitive mutants *sis4* and *sis5* are defective in abscisic acid synthesis and response. Plant J. **23:** 587-596.

Lam, E., and Chua, N.H. (1989). ASF-2: A factor that binds to the cauliflower mosaic virus *35S* promoter and a conserved GATA motif in *CAB* promoters. Plant Cell **1:** 1147-1156.

Lam, E., and Lam, Y.K.-P. (1995). Binding site requirements and differential representation of TGA factors in nuclear ASF-1 activity. Nucl. Acids Res. **23:** 3778-3785.

Lam, E., Katagiri, F., and Chua, N.H. (1990). Plant nuclear factor ASF-1 binds to an essential region of the nopaline synthase promoter. J. Biol. Chem. **265:** 9909-9913.

Lee, H., Xiong, L., Gong, Z., Ishitani, M., Stevenson, B., and Zhu. J-K. (2001). The *Arabidopsis HOS1* gene negatively regulates cold signal transduction and encodes a RING finger protein that displays cold-regulated nucleocytoplasmic partitioning. Genes Dev. **15:** 912-924.

Lejay, L., Gansel, X., Cerezo, M., Tillard, P., Muller, C., Krapp, A., von Wiren, N., Daniel-Vedele, F., and Gojon, A. (2003). Regulation of root ion transporters by photosynthesis: Functional importance and relation with Hexokinase. Plant Cell **15:** 2218-2232.

León, P., and Sheen, J. (2003). Sugar and hormone connections. Trends Plant Sci. **8:** 110-116.

Lin, C., and Shalitin, D. (2003). Cryptochrome structure and signal transduction. Annu. Rev. Plant Biol. **54:** 469-496.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods **25:** 402-408.

Lu, C., and Fedoroff, N. (2000). A mutation in the *Arabidopsis HYL1* gene encoding a dsRNA binding protein affects responses to abscisic acid, auxin, and cytokinin. Plant Cell **12:** 2351-2366.

Ludwig, A.A., Romeis, T., and Jones, J.D.G. (2004). CDPK-mediated signalling pathways: specificity and cross-talk. J. Exp. Bot. **55:** 181-188.

Makino, S., Matsushika, A., Kojima, M., Yamashino, T., and Mizuno, T. (2002). The APRR1/TOC1 quintet Implicated in circadian rhythms of *Arabidopsis thaliana*: I. Characterization with APRR1-overexpressing plants. Plant Cell Physiol. **43:** 58-69.

Mandadi, K.K., Misra, A., Ren, S., and McKnight, T.D. (2009). BT2, a BTB Protein, mediates multiple responses to nutrients, stresses, and hormones in *Arabidopsis*. Plant Physiol. **150:** 1930-1939.

Mandadi, K.K., Misra, A., and McKnight, T.D. (2010). BT2 is required for Cauliflower Mosaic Virus *35S* enhancer-mediated activation of gene expression in *Arabidopsis*. In preparation.

Mauch-Mani, B., and Mauch, F. (2005). The role of abscisic acid in plant-pathogen interactions. Curr. Opin. Plant Biol. **8:** 409-414.

Maxwell, B.B., and Kieber, J.J. (2004). Cytokinin signal transduction. In Plant Hormones-Biosynthesis, Signal Transduction, Action, P.J. Davies, ed (New York: Springer), pp. 329-357.

McClung, C.R. (2006). Plant circadian rhythms. Plant Cell **18:** 792-803.

McClung, C.R., Salome, P.A., and Michael, T.P. (2002). The *Arabidopsis* circadian system. In The *Arabidopsis* Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), pp. 1-23.

McKnight, T.D., and Meagher, R.B. (1981). Isolation and mapping of small cauliflower mosaic virus DNA fragments active as promoters in *Escherichia coli*. J. Virol. **37:** 673-682.

Miao, Y., Lv, D., Wang, P., Wang, X-C., Chen, J., Miao, C., and Song, C-P. (2006). An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. Plant Cell **18:** 2749–2766.

Michael, T.P., and McClung, C.R. (2003). Enhancer trapping reveals widespread circadian clock transcriptional control in *Arabidopsis*. Plant Physiol. **132:** 629-639.

Millar, A.J., and Kay, S.A. (1996). Integration of circadian and phototransduction pathways in the network controlling *CAB* gene transcription in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **93:** 15491-15496.

Mizuno, T., and Nakamichi, N. (2005). Pseudo-Response Regulators (PRRs) or True Oscillator Components (TOCs). Plant Cell Physiol. **46:** 677-685.

Moons, A., Prinsen, E., Bauw, G., and Van Montagu, M. (1997). Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. Plant Cell **9:** 2243-2259.

Moore, B., Zhou, L., Rolland, F., Hall, Q., Cheng, W.H., Liu, Y.-X., Hwang, I., Jones, T., and Sheen, J. (2003). Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. Science **300:** 332-336.

Muriel, H., Marina, B., Angèle, G., Pierre, Y., and Mario, K. (2002). Cauliflower mosaic virus: still in the news. Mol. Plant Path. **3:** 419-429.

Neff, M.M., Fankhauser, C., and Chory, J. (2000). Light: an indicator of time and place. Genes Dev. **14:** 257-271.

Ng, D.W.K., Chandrasekharan, M.B., and Hall, T.C. (2006). Ordered histone modifications are associated with transcriptional poising and activation of the phaseolin promoter. Plant Cell **18:** 119-132.

Odell, J.T., Nagy, F., and Chua, N.-H. (1985). Identification of DNA sequences required for activity of the cauliflower mosaic virus *35S* promoter. Nature. **313:** 810-812.

Ow, D.W., Jacobs, J.D., and Howell, S.H. (1987). Functional regions of the cauliflower mosaic virus *35S* RNA promoter determined by use of the firefly luciferase gene as a reporter of promoter activity. Proc. Natl. Acad. Sci. USA **84:** 4870-4874.

Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., and Weigel, D. (2003). Control of leaf morphogenesis by microRNAs. Nature **425:** 257-263.

Pedmale, U.V., and Liscum, E. (2007). Regulation of phototropic signaling in *Arabidopsis* via phosphorylation state changes in the phototropin 1-interacting protein NPH3. J. Biol. Chem. **282:** 19992-20001.

Pego, J.V., Kortstee, A.J., Huijser, C., and Smeekens, S. (2000). Photosynthesis, sugars and the regulation of gene expression. J. Exp. Bot. **51:** 407–416.

Pogorelko, G.V., Fursova, O.V., Ogarkova, O.A., and Tarasov, V.A. (2008). A new technique for activation tagging in *Arabidopsis*. Gene **414:** 67-75.

Price, J., Li, T-C., Kang, S.G., Na, J.K., and Jang, J-C (2003). Mechanisms of glucose signaling during germination of *Arabidopsis*. Plant Physiol. **132:** 1424-1438.

Qu, S., Desai, A., Wing, R., and Sundaresan, V. (2008). A versatile transposon-based activation tag vector system for functional genomics in cereals and other monocot plants. Plant Physiol. **146:** 189-199.

Quail, P.H. (2002). Phytochrome photosensory signalling networks. Nat. Rev. Mol. Cell Biol. **3:** 85-93.

Quint, M., and Gray, W.M. (2006). Auxin signaling. Curr. Opin. Plant Biol. **9:** 448-453.

Ramon, M., Rolland, F., and Sheen, J. (2008). Sugar sensing and signaling. In The *Arabidopsis* Book, C.R. Somerville and E.M. Meyerowitz, eds (Rockville, MD: American Society of Plant Biologists), pp. 1-22.

Ren, S., Mandadi, K.K., Boedeker, A.L., Rathore, K.S., and McKnight, T.D. (2007). Regulation of telomerase in *Arabidopsis* by BT2, an apparent target of TELOMERASE ACTIVATOR1. Plant Cell **19:** 23-31.

Reyes, J.C., Muro-Pastor, M.I., and Florencio, F.J. (2004). The GATA family of transcription factors in *Arabidopsis* and rice. Plant Physiol. **134:** 1718-1732.

Richard, C., Granier, C., Inzé, D., and De Veylder, L. (2001). Analysis of cell division parameters and cell cycle gene expression during the cultivation of *Arabidopsis thaliana* cell suspensions. J. Exp. Bot. **52:** 1625–1633.

Robert, H.S., Quint, A., Brand, D., Vivian-Smith, A., and Offringa, R. (2009). BTB and TAZ domain scaffold proteins perform a crucial function in *Arabidopsis* development. Plant J. **58:** 109-121.

Roberto, P.-T., Daisuke, Y., and Pierre-Antoine, D. (2006). Born to bind: the BTB protein-protein interaction domain. Bio Essays **28:** 1194-1202.

Rochon, A., Boyle, P., Wignes, T., Fobert, P.R., and Despres, C. (2006). The coactivator function of *Arabidopsis* NPR1 requires the core of its BTB/POZ domain and the oxidation of C-terminal cysteines. Plant Cell **18:** 3670-3685.

Roitsch, T. (1999). Source–sink regulation by sugar and stress. Curr. Opin. Plant Biol. **2:** 198–206.

Rolland, F., Moore, B., and Sheen, J. (2002). Sugar sensing and signaling in plants. Plant Cell **14:** S185-205.

Rubtsov, M.A., Polikanov, Y.S., Bondarenko, V.A., Wang, Y.-H., and Studitsky, V.M. (2006). Chromatin structure can strongly facilitate enhancer action over a distance. Proc. Natl. Acad. Sci. USA **103:** 17690-17695.

Sakakibara, H. (2006). Cytokinins: Activity, biosynthesis, and translocation. Annu. Rev. Plant Biol **57:** 431-449.

Salome, P.A., and McClung, C.R. (2004). The *Arabidopsis thaliana* clock. J. Biol. Rhyth. **19:** 425-435.

Sandhu, J.S., Webster, C.I., and Gray, J.C. (1998). A/T-rich sequences act as quantitative enhancers of gene expression in transgenic tobacco and potato plants. Plant Mol.Biol. **37:** 885-896.

Santner, A., and Estelle, M. (2009). Recent advances and emerging trends in plant hormone signalling. Nature **459:** 1071-1078.

Schaffer, R., Ramsay, N., Samach, A., Corden, S., Putterill, J., Carré, I.A., and Coupland, G. (1998). The *late elongated hypocotyl* mutation of *Arabidopsis* disrupts circadian rhythms and the photoperiodic control of flowering. Cell **93:** 1219-1229.

Scheible, W.R., Morcuende, R., Czechowski, T., Fritz, C., Osuna, D., Palacios-Rojas, N., Schindelasch, D., Thimm, O., Udvardi, M.K., and Stitt, M. (2004). Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infrastructure of *Arabidopsis* in response to nitrogen. Plant Physiol. **136:** 2483–2499.

Serfling, E., Jasin, M., and Schaffner, W. (1985). Enhancers and eukaryotic gene transcription. Trends Gen. **1:** 224-230.

Shalitin, D., Yang, H., Mockler, T.C., Maymon, M., Guo, H., Whitelam, G.C., and Lin, C. (2002). Regulation of *Arabidopsis* cryptochrome 2 by blue-light-dependent phosphorylation. Nature **417:** 763-767.

Smeekens, S. (2000). Sugar-induced signal transduction in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **51:** 49-81.

Stitt, M. (1999) Nitrate regulation of metabolism and growth. Curr. Opin. Plant Biol. **2:** 178–186.

Stitt, M., and Krapp, A. (1999). The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ. **22:** 583-621.

Teakle, G.R., Manfield, I.W., Graham, J.F., and Gilmartin, P.M. (2002). *Arabidopsis thaliana* GATA factors: organisation, expression and DNA-binding characteristics. Plant Mol.Biol. **50:** 43-57.

Teale, W.D., Paponov, I.A., and Palme, K. (2006). Auxin in action: signalling, transport and the control of plant growth and development. Nat. Rev. Mol. Cell Biol. **7:** 847-859.

Till, R., and Russell, G.F. (1997). Twilight times: light and the circadian system. Photochem. Photobiol. **66:** 549-561.

Uno, Y.C., Milla, M.A.R., Maher, E., and Cushman, J.C. (2009). Identification of proteins that interact with catalytically active calcium-dependent protein kinases from *Arabidopsis*. Mol. Genet. Genomics **281:** 375-390.

Usadel, B., Blasing, O.E., Gibon, Y., Retzlaff, K., Hohne, M., Gunther, M., and Stitt, M (2008). Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in *Arabidopsis* rosettes. Plant Physiol. **146:** 1834-1861.

Verkest, A., Weinl, C., Inzé, D., Veylder, L.D., and Schnittger, A (2005). Switching the cell cycle. Kip-related proteins in plant cell cycle control. Plant Physiol. **139:** 1099-1106.

Wang, K.L.C., Yoshida, H., Lurin, C., and Ecker, J.R. (2004a). Regulation of ethylene gas biosynthesis by the *Arabidopsis* ETO1 protein. Nature **428:** 945-950.

Wang, R., Tischner, R., Gutiérrez, R.A, Hoffman, M., Xing, X., Chen, M., Coruzzi, G., and Crawford, N.M (2004b). Genomic analysis of the nitrate response using a nitrate reductase-null mutant of *Arabidopsis*. Plant Physiol. **136:** 2512–2522.

Weber, H., and Hellmann, H. (2009). *Arabidopsis* thaliana BTB/ POZ-MATH proteins interact with members of the ERF/AP2 transcription factor family. FEBS J. **276:** 6624-6635.

Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.-Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F., and Chory, J. (2000). Activation tagging in *Arabidopsis*. Plant Physiol. **122:** 1003-1014.

Weiher, H., Konig, M., and Gruss, P. (1983). Multiple point mutations affecting the simian virus 40 enhancer. Science **219:** 626-631.

Wendehenne, D., Durner, J., and Klessig, D.F. (2004). Nitric oxide: a new player in plant signalling and defence responses. Curr. Opin. Plant Biol. **7:** 449-455.

Xiong, L., Lee, H., Ishitani, M., Tanaka, Y., Stevenson, B., Koiwa, H., Bressan, R.A, Hasegawa, P.M, and Zhu, J-K (2002). Repression of stress-responsive genes by *FIERY2,* a novel transcriptional regulator in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **99:** 10899–10904.

Yu, S.M. (1999). Cellular and genetic responses of plants to sugar starvation. Plant Physiol. **121:** 687-693.

Zhang, Y., Fan, W., Kinkema, M., Li, X., and Dong, X. (1999). Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the *PR-1* gene. Proc. Natl. Acad. Sci. USA **96:** 6523-6528.

Zhao, Y., Christensen, S.K., Fankhauser, C., Cashman, J.R., Cohen, J.D., Weigel, D., and Chory, J (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. Science **291:** 306-309.

Zhou, L., Jang, J-C., Jones, T.L., and Sheen, J (1998). Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. Proc. Natl. Acad. Sci. USA **95:** 10294-10299.

VITA

NAME: Kranthi Kiran Mandadi

ADDRESS: 3258 TAMU, Department of Biology, BSBE 206
College Station, TX-77843
Phone: 1-979-319-1746
Email: kmandadi@mail.bio.tamu.edu

EDUCATION: Ph.D., Molecular and Environmental Plant Sciences,
Texas A&M University, 2010

M.S., Plant and Soil Sciences,
Texas A&M University, Kingsville, 2005

B.S., Agricultural Sciences,
ANGR Agricultural University, India, 2002