

**THE BROMODOMAIN PROTEINS GTE9 AND GTE11 ASSOCIATE WITH
BT2-BASED E3 LIGASE COMPLEX AND MEDIATE RESPONSES TO
MULTIPLE SIGNALS IN *ARABIDOPSIS THALIANA***

A Dissertation

by

ANJALI MISRA

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

December 2011

Major Subject: Molecular and Environmental Plant Sciences

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Ligase Complex and Mediate Responses to Multiple Signals in *Arabidopsis*

Thaliana

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December 2011

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ABSTRACT

The Bromodomain Proteins GTE9 and GTE11 Associate with BT2-based E3 Ligase Complex and Mediate Responses to Multiple Signals in *Arabidopsis*

thaliana. (December 2011)

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BT2 is an *Arabidopsis thaliana* protein with N-terminal BTB, central TAZ and a C-terminal calmodulin binding domain and associates with Cullin3 to form an E3 ubiquitin ligase. It has been shown previously that BT2 regulates telomerase activity in mature vegetative organs and controls a variety of hormone, stress and metabolic responses in *Arabidopsis thaliana*. Loss of BT2 results in plants that are hypersensitive to inhibition of germination by ABA and sugars. Conversely, overexpression of BT2 results in resistance to ABA and sugars, suggesting that *BT2* is a negative regulator of ABA and sugar responses. Here, it is shown that the roles of BT2-interacting partners GTE9 and GTE11, bromodomain and extraterminal-domain proteins of Global Transcription Factor Group E, in BT2-mediated responses to sugars and hormones. Loss-of-function mutants *gte9-1* and *gte11-1* phenocopy the *bt2-1*-null mutant responses; germination in all three mutants is hypersensitive to inhibition by

glucose and ABA. Loss of either GTE9 or GTE11 in a BT2 over-expressing background blocks resistance to sugars and ABA, indicating that both GTE9 and GTE11 are required for BT2 function. Additionally, loss of GTE9 or GTE11, similar to loss of BT2, suppresses transcriptional gene activation mediated by CaMV 35S enhancers in Arabidopsis. The suppressed phenotype is accompanied by decreased transcription and hypermethylation of the 35S enhancers in the activation-tagged lines. This study shows that BT2 and GTE9 co-immunoprecipitate and physically interact *in vivo* to mediate diverse responses to biotic and abiotic signals and 35S enhancer activity. This working model shows that the GTE9 and GTE11 function as chromatin adaptors that localize the BT2-CULLIN3 E3 ubiquitin ligase complex to acetylated chromatin on transcriptionally competent promoters in response to calcium signals detected by BT2's calmodulin-binding domain.

DEDICATION

To my parents and parents-in-law: Shri. A. K. Misra and Smt. Vijay Misra, Nandkishore Sharma and Smt. Amrit Sharma for their love, encouragement and blessings.

To my husband Arun Sharma and daughter Arundhati Sharma. Without their constant support and encouragement this would have not been possible. Also to my brother and friends for their support, love and encouragement.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The overall goal of my research is to increase our understanding of how the BT2-based ubiquitin ligase mediates responses to diverse physiological and environmental signals in *Arabidopsis thaliana*. More specifically, I have focused on the roles of GTE9 and GTE11, two proteins in the bromodomain and extraterminal (BET) family that associate with the BT2-Cullin E3 ligase through the BTB domain on BT2. I present genetic and biochemical evidence supporting functional roles for GTE9 and GTE11 in BT2-mediated hormone and energy signaling pathway and in 35S-enhancer mediated activation. Our working model is that the GTE9 and GTE11 recognize acetyl histone marks on transcriptionally competent promoters and localize the BT2-Cullin3 E3 ligase complex to recognize and deliver sequence-specific factors for ubiquitination to modulate the various signaling pathways. In this model, the GTE proteins serve as general recognition factors that mark transcriptionally competent regions of the genome, and the BT protein provides specific recognition of individual promoters. The *Arabidopsis* genome encodes 12 GTE proteins and approximately 80 BTB proteins, suggesting that the regulatory model proposed here could mediate a broad range of transcriptional responses.

This dissertation follows the style and format of Plant Physiology.

Plants have evolved a variety of mechanisms to sense and respond to endogenous and exogenous cues to create cell-specific and/or stimulus specific gene expression patterns (Pfluger and Wagner, 2007). Many of these responses occur at the transcriptional level, where specific suites of genes are induced in response to specific internal and external signals. Three most important regulatory factors orchestrate this controlled gene expression: (1) transcription factors, (2) chromatin remodelers and (3) DNA methyltransferases. Among these, bromodomain-containing transcription factors regulate gene expression in three different ways: (1) activating transcription, (2) maintaining the transcription memory and (3) anti-silencing of the maintained chromosomal regions (Loyola and Almouzni, 2004). The bromodomain proteins carry out these functions by binding to acetyl histones and anchoring sequence specific factors on the chromatin of target promoters. The bromodomain-containing transcription factors in *Arabidopsis* studied here, GTE9 and GTE11, recruit specific factors like BT2 to regulate gene expression. BT2 is a BTB domain containing protein with a central TAZ-zinc finger domain and C-terminal calmodulin binding domain that responds to various physiological and metabolic responses in *Arabidopsis* (Mandadi et al., 2009). Overall, the ability of bromodomain proteins, GTE9 and GTE11 in the BT2 complex, to recognize acetyl lysines on chromatin underscores the fact that gene expression is highly dynamic and relies on the sophisticated histone modifications.

Recent molecular and biochemical advances have identified epigenetic modifications that are mediated by chromatin remodeling and small interfering (si-RNA)-mediated transcriptional silencing (Chinnusamy and Zhu, 2010). In the following sections I will focus on the aspects of chromatin remodeling and factors that determine the fate of gene expression, with emphasis on bromodomain and extra-terminal (BET) class of proteins.

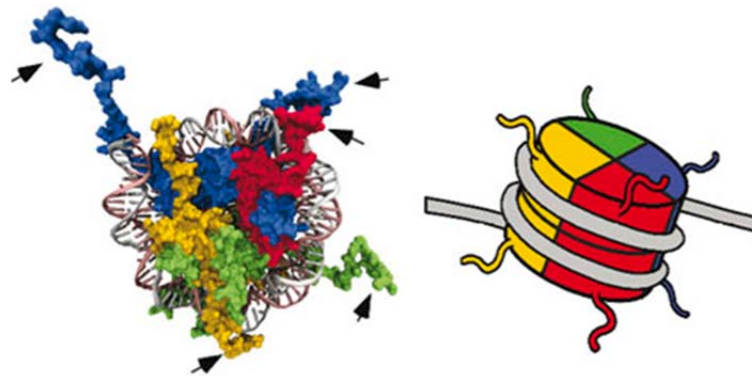
Chromatin Remodeling

The structure of the nucleosome, the basic unit of chromatin, was first elucidated by crystallography studies of Luger et al., in 1997. Nucleosomes consist of 146bp of DNA wrapped around a core complex of histones comprising H2A, H2B, H3 and H4 (Fig. 1). The core complex is an octamer made of a central (H3–H4)₂ tetramer and two flanking histones, H2A and H2B (Richmond et al., 1988). The core complex histones interact with DNA via the alpha helices in the histone fold-domain. The basic N-terminal histone tails (20-35 residues) on H2A, H2B, H3 and H4 interact with ATP-dependent chromatin remodeling factors and undergo post-translation modifications. These modifications include: acetylation, methylation, glycosylation, phosphorylation, ADP-ribosylation, carbonylation, sumoylation and ubiquitination (Fig. 1) (Reviewed in Fuchs et al., 2006). These histone modifications enable the formation of open (euchromatin) or closed (heterochromatin) chromatin to either permit or restrict the access of

the underlying DNA code by the general transcription factors and often referred to as “Histone code” (Strahl and Allis, 2000; Fischle et al., 2003).

The “Histone Code” applies universally to all eukaryotes because the sites and types of post-translation modifications and the amino acid sequence on histones, are highly conserved (DeLange et al., 1969; Patthy et al., 1973). Although there is a high sequence similarity among the majority of histones, there are differences in the N-terminal tails of histone H2B from plants and animals, suggesting there exist different side chain modifications and pathways of signal perception in plants and animals.

Chromatin modifications such as acetylation and phosphorylation stimulate transcription by creating open, transcriptionally competent chromatin (Strahl and Allis, 2000; Oki et al., 2007). In the following section I will discuss histone acetylation, factors that carry out acetylation and recognize acetylation marks on chromatin.



<u>Histone</u>	<u>Variants</u>
H2A	H2A.X H2A.Z H2A.Bbd MacroH2A

H2B *TestisH2B (mammals)*

H3 H3.1 (mammals)
H3.2, H3.3
CENP-A

H4 *No known variants*

H1 *many, especially embryonic isoforms*

Post-translational modifications

Ser/Thr/Tyr phosphorylation

Lys/Arg methylation

Lys acetylation

Lys ubiquitylation

Lys sumoylation

Glu ADP-ribosylation

Figure 1. Crystal structure of the nucleosome core particle. The nucleosome core particle at 2.5 Å resolution. 146 bp of DNA wrapped around histone octamer forms the nucleosome core particle. Coils protruding from the nucleosome (Blue, red, yellow and green color) represent the N-terminal tails of histones (Shechter et al., 2007).

Histone Acetylation

The concept of histone acetylation first emerged in the early 1960's (Alfrey et al., 1964) and is most extensively studied in the area of gene regulation. Histone acetylation regulates transcriptional activity at a specific locus by creating permissive or open chromatin (Deckert and Struhl, 2001; Ricci et al., 2002) and is carried out in two different ways: first, by neutralizing the positive charge of the N-terminal tails that reduces the histone-DNA (Cary et al., 1982; Puig et al., 1998; Georghiou and Ababneh, 2005) and histone-histone interaction (Hansen et al., 1998; Ren and Gorovsky, 2003) and second, by serving as docking site for the recruitment of transcriptional activators such as bromodomain proteins (further detailed in next section). Interestingly, acetylation also occurs on lysine 56 of histone 3 (H3K56) in the histone core by the human bromodomain protein GCN5, causing protrusion of the side chain towards the major groove in DNA (Tjeertes et al., 2009), thus weakening the histone/DNA interaction.

Acetylation of the lysine residues on the N-terminal tails of the H2A (K4, 5, 7), H2B (K5, 11, 12, 15, 16, 20), H3 (K4, 9, 14, 18, 23, 27) and H4 (K5, 8, 12, 16) is often seen and well characterized (Peterson and Laniel, 2004). Histone acetylation is mediated by a specific class of enzymes called histone acetyltransferase (HATs) that transfer an acetyl group from acetyl-coenzyme A (acetyl-CoA) to the ϵ -amino group of lysine side chains of N-terminal histone regions (Sterner and Berger, 2000). The Arabidopsis genome encodes 12 HATs

belonging to three major families:(1) the GCN5-related N-terminal acetyltransferase (GNAT)-MYST class,(2) the p300/CREB-binding protein (CBP) co-activator class, and (3) the mammalian TAFII250 related HATs (Pandey et al., 2002). HATs are also classified as type-A and type-B based on their subcellular localization. Type-B HATs are involved in acetylating free histones in the cytoplasm to allow appropriate localization of newly formed histones (Brownell and Allis, 1996; Bannister and Kouzarides, 2011). Type-A HATs include GNAT, MYST and p300/CBP family enzymes that modify more than one site on the N-terminal histone tails in the nucleus and function as transcriptional co-activators (Yang and Seto, 2000).

Bromodomain: An Acetyl-Lysine Binding Domain

The unifying feature of chromatin associated proteins such as HATs, ATP dependent remodeling complexes (e.g. SWI/SNF) and bromodomain and extraterminal domain (BET) proteins (e.g. human Brd2, Brd4, Bdf1), is the ability to recognize acetyl histones in a bromodomain dependent manner (Kanno et al., 2004; Yang, 2004; de la Cruz et al., 2005; Mujtaba et al., 2007). The bromodomain (from here on referred to as BRD) is a 110-amino acid long motif that recognizes N ϵ -acetyl-lysine in histone tails and was first described in the *Drosophila melanogaster* protein *brahma* (*brm*), from which the name bromodomain was derived (Haynes et al., 1992; Tamkun et al., 1992; Jeanmougin et al., 1997). *brm* encodes a 1638 residue-long protein and shares

properties with Swi2/Snf2 chromatin remodelers. *brm* interacts with *trithoax* (*trx*) genes to overcome *Polycomb* (*pc*) suppression of homeotic genes (Tamkun et al., 1992).

Structural studies of BRD from p300/CBP and GCN5 revealed that the BRD domain in these proteins specifically binds to acetyl lysines on histone (H3) (Dhalluin et al., 1999; Ornaghi et al., 1999; Hudson et al., 2000; Owen et al., 2000). Interestingly, recognition of acetyl lysine by BRD is not limited to histones. BRD can also interact with acetyl lysine in non-histone proteins such as: HIV Tat, p53, c-Myb or MyoD (Yang, 2004). The BRD structurally folds into four alpha helices (α_Z , α_A , α_B , α_C) and two loops, BC and ZA, of varying lengths (Fig. 2). The BC loop in particular stabilizes the BRD structure and forms a hydrophobic pocket for acetyl histone recognition (Dhalluin et al., 1999; Owen et al., 2000).

Apart from a few variations in the ZA and BC loops, the overall BRD structure of various BRD-containing proteins is similar (Jeanmougin et al., 1997). Interestingly, the acetyl-lysine recognizing residues, Tyr760, Tyr802 and Asn803, first discovered in human transcriptional coactivator PCAF (p300/CBP-associated factor) (Dhalluin et al., 1999) are highly conserved in the BRD across species. The acetyl histone interaction is mediated by hydrogen bond formation between the acetyl carbonyl group in the acetyl lysine and the amide nitrogen in asparagine (Asn803), plus other water-mediated hydrogen bonds with the protein backbone. An exception to this rule is transcriptional intermediate factor

1β (TIF1 β), which lacks the conserved Asn803, suggesting an alternate mode of acetyl histone recognition for this subgroup of proteins. Additionally, not all BRD-containing proteins can recognize the acetylation marks with the same affinity, as the affinity (K_d) values for well-known BRD proteins range from 10-100 μ M (Owen et al., 2000; Mujtaba et al., 2002; Mujtaba et al., 2004)

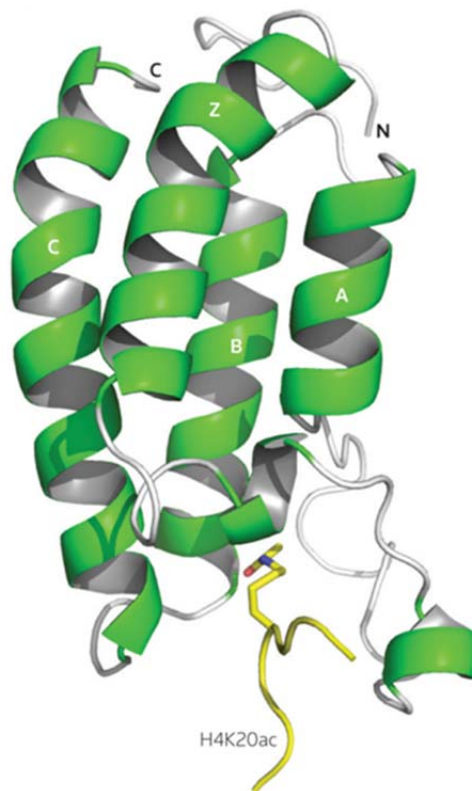


Figure 2. 3-D crystal structure of CREB binding protein.Bromodomain (BRD) bound to a H4K20ac peptide (PDB code: 2RNY) (From: Sanchez and Zhou, 2009).

So far the structure and function of BRD is well established from the characterization of several chromatin assembly factors such as transcription co-activators, transcription factors and HATs. However, an important question emerges here: how does acetyl histone recognition affect the function of BRD-containing protein?

Biological studies carried out using BRD deletion mutants suggest that the BRD is vital for the *in vivo* function of these proteins. For example, in yeast, expression of a Gcn5p histone acetyl transferase lacking the BRD partially complements the *gcn⁻⁵* mutant phenotypes such as reduced toxicity effects of the chimeric activator VP16 and GCN4, slow growth on minimal media etc. (Marcus et al., 1994). Interestingly, Candéau et al., (1997) demonstrated that this partial complementation was attributed to failure of the truncated Gcn5p to target the HAT activity of Gcn5p in the *gcn⁻⁵* mutant at the targeted promoter. A similar explanation has been proposed for the diminished recruitment of the Swi/Snf complex to the yeast *PHO5* promoter-driving *LacZ* (*gPHO5-LacZ*) expression in *Gcn5* BRD deletion mutants, in spite of increased acetylation at the promoter, a prerequisite for Swi/Snf based remodelling (Syntichaki et al., 2000). Based on these and other similar findings, BRD-containing transcriptional complexes are now described as a scaffold that anchors and localizes its interacting partners to their cognate promoters (Denis, 2001). Further evidence for the proposed significance of BRD function comes from the characterization of BET class BRD proteins. In the following section I will review the function of two

mammalian BET proteins to explain this model and eventually draw parallels for the *Arabidopsis* BET proteins GTE9 and GTE11, which are the focus of this study.

Bromodomain Extra-terminal Proteins

The Bromodomain and Extra-Terminal domain (BET) proteins form a separate class of BRD proteins featuring N-terminal BRD (single in plants and tandem BRD in fungi and animals) and a C-terminal extra-terminal ET domain (Haynes et al., 1992; Lygerou et al., 1994; Jeanmougin et al., 1997; Pandey et al., 2002). The best characterized metazoan BET protein, Brd2, carries tandem BRD I and II in the N-terminus and binds acetyl histones in biochemical assays (Kanno et al., 2004) and structural studies (Nakamura et al., 2007; Umehara et al., 2010). A similar functional requirement has been predicted for Brd2 homologs Brd3 (Thorpe et al., 1997), Brd4 (Dey et al., 2003; Lee and Chiang, 2009), Brd6 (Brdt) (Jones et al., 1997), and orthologs in other species: *Saccharomyces* BDF1 (Lygerou et al., 1994), *Arabidopsis* GTE4 (Airoldi et al., 2010), *Drosophila* female sterile (1) homeotic (fs(1)h) (Digan et al., 1986) and *Danio* and *Xenopus* Brd4 (Toyama et al., 2008).

The similarity in acetyl histone recognition, via bromodomain, across species is not surprising. For example, the BRDI in Brd4 has high similarity to the corresponding BRD in Brd2 (80%) and to *Drosophila* fsh (75%) (Nakamura et al., 2007; Wu and Chiang, 2007). Given the high sequence similarity between

the BRDs of various BET proteins, it is reasonable to predict that BET proteins across species can perform similar functions, perhaps using similar mechanisms. Supporting evidence for this comes from the role of yeast (BDF1 and BDF2) and mammalian (BRD2 and BRD4) BET proteins in cell growth and proliferation (Chua and Roeder, 1995; Dey et al., 2000; Houzelstein et al., 2002; Maruyama et al., 2002). Both BDF1 and BRD4 regulate these processes by staying physically associated with condensed chromosomes during the mitotic cell cycle (Chua and Roeder, 1995; Dey et al., 2000). Based on this property it is hypothesized that BET proteins contribute to the transcriptional memory of the daughter cell chromatin (Dey et al., 2003; Kanno et al., 2004). Additionally, inability of other BRD proteins such as p300, CBP, GCN5 and hBrg1/Hsnf2 β to remain associated with condensed chromosomes during mitosis (Kruhlak et al., 2001) also suggests that BET proteins function are not shared by all BRD-containing proteins.

Apart from regulating transcriptional memory, BET proteins may also have an anti-silencing effect on chromatin. For example Bdf1 localizes at the heterochromatin boundaries near telomeres and competes with the silencing proteins (SIRs), histone deacetylases, to maintain transcription in sub-telomeric regions (Ladurner et al., 2003). Loss of Bdf1 results in spread of SIR proteins into the actively transcribing sub-telomeric region and transcriptional silencing of this locus. Chromatin immunoprecipitation (ChIP) and *in vitro* competition assays localized Bdf1 to the heterochromatin boundaries by competing with SIR

proteins for acetylated histone recognition sites on chromatin (Ladurner et al., 2003).

BET proteins also function as an adaptor or scaffold to recruit cellular factors such as sequence-specific DNA-binding or chromatin-remodelling proteins to activate transcription (Weidner-Glunde et al., 2010). Characterization of Brd2's role in mitogenesis by Sinha et al., (2005) clearly demonstrates the 'scaffolding' or bridging function of BET proteins. Sinha and co-workers showed that loss of BRD in Brd2 limits the recruitment of E2F transcription factor and HAT activity to the *cyclin A* promoter, which in turn prevents the transactivation of the *cyclin A* locus, necessary for G1 to S phase transition in cell cycle (Denis et al., 2000; Sinha et al., 2005). Additionally, Brd2 also recruits the TATA box Binding Protein (TBP) in the Brd2-E2F complex to stabilize the interaction between TBP and E2F. Recruitment of TBP by BRD2 bridges the gap between TBP and E2F and allows formation of active TBP-E2F Brd2 scaffold, necessary for transcriptional activation of cell cycle promoters (Peng et al., 2007).

A similar model has been described for the Brd4's role in E2-dependent activation and repression of papilloma virus (PV) promoters. Brd4 is unique among all metazoan BET proteins because in addition to carrying BRD and ET domains, it also carries an extended C-terminal domain (CTD) (Fig. 3), which mediates E2 protein interaction. E2 is a viral protein that tethers viral DNA to the host chromosome by directly binding to the viral DNA on the E2-binding sites (Lee and Chiang, 2009). Using a tandem affinity approach, it was demonstrated

that Brd4 is the host protein that recruits E2 protein to its binding sites on the viral DNA (You et al., 2004). Lee and Chiang (2009) further showed that a functional Brd4 bromodomain is necessary for the recruitment of E2 on the E2-BS on human papillomavirus (HPV-11) chromatin to regulate transcription activation (McPhillips et al., 2006; Wu et al., 2006; Schweiger et al., 2007; Sénéchal et al., 2007; Lee and Chiang, 2009). Using HPV-11 chromatin in a DNase I footprinting assay, the authors showed that full-length Brd4 enhances protection of E2 binding sites. However, loss of Brd4 eliminates the protection of these sites. The loss of E2 binding was specifically associated with the loss of functional bromodomains in BRD4 and was not related to other domains such as the ET and CTD.

Another study using BRD4-NUT fusion oncoproteins underscores that the general role of the BRD is to localize or anchor sequence specific factors on the acetylated chromatin (Reynoird et al., 2010). BRD4-NUT is a naturally occurring in frame fusion between BRD4 and NUclear protein in Testis (NUT) gene responsible for NUT midline carcinoma (NMC), a malignant and lethal form of cancer in adults and children. Here, the NUT protein strongly interacts with p300 to create acetylated chromatin foci and chromatin compaction. Loss of BRD in the BRD4-NUT protein limits the formation of these foci, indicating that targeting of p300 to the chromatin and HAT activity relies on the functional BRD in Brd4-NUT fusion oncoprotein.

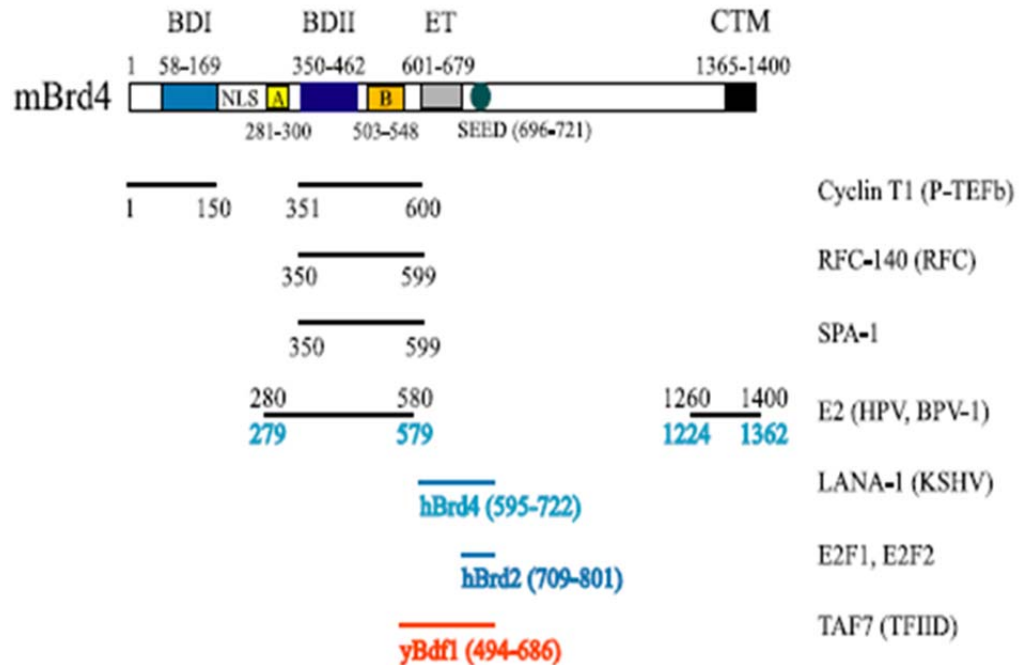


Figure 3. BET family of proteins. Domain map showing regions of interaction and interacting partners. Bromodomain I, II (BDI and II); Extraterminal Domain (ET); The region beyond the seed motif in BRD4 represents its unique carboxy terminal domain (CTD) (Wu and Chiang et al., 2007).

Despite extensive studies on the interactions and functions of the BRD in bromodomains in BET proteins, very little is known about the ET domain in

these proteins. The ET domain (60 amino acids long) is predicted to function as protein-protein interaction domain (Lygerou et al., 1994) and shares >80% identity among the BET proteins. Interestingly, a recent proteomics study by Rahman et al., (2011) identified cellular factors such as NSD3, a histone methyltransferase, that specifically interact with the Brd4 ET domain. They further demonstrated that ET domain interactions in other BET proteins, Brd2 and Brd3, were conserved for multiple candidates identified in this screen. The novel interactions thus identified are suggestive of an alternate mechanism of transcriptional regulation from the BET proteins lacking the CTD and other variable regions beyond the ET domain as seen in Fig. 3 (Rahman et al., 2011). This finding may help uncover the function of Arabidopsis BET proteins that carry an ET domain similar to its fungal and metazoan orthologs.

Here, I will present a brief overview of Arabidopsis BET proteins and their function in plants. Following this I will describe in detail two of its family members, GTE9 and GTE11, which are required to mediate multiple BT2 responses in plants (Mandadi et al., 2009).

BET proteins in Arabidopsis are characterized by a single BRD at the N-terminus followed by an ET domain and a variable region at the C-terminus (Florence and Faller, 2001). The ET domain in plant BET proteins is homologous to only the N-terminal portion of ET domain (NET) in yeast and mammals and potentially functions as protein-protein interaction domain (Lygerou et al., 1994; Florence and Faller, 2001; Rahman et al., 2011). The BRD

in Arabidopsis BET proteins shares homology with the second BRD in mammals. However, a plant-specific amphipathic domain (PAD) replaces the first BRD commonly found in mammalian BET proteins (Chapter II). There are 12 members in this family that belong to the Global Transcription Factor Group E (hence name GTE) family in Arabidopsis (Florence and Faller, 2001; Pandey et al., 2002), and they are distributed in two separate clades, GTE1-7 and GTE8-12 (Table 1; Chapter II). So far, only three members in this family have been characterized. *GTE1*, also referred to as *IMBIBITION INDUCIBLE1 (IMB1)* negatively regulates ABA-mediated inhibition of seed germination and regulates expression of a wide variety of genes (Duque and Chua, 2003). *GTE4* has been shown to regulate cell cycle activation and maintenance (Airoldi et al., 2010). Another BET protein, *GTE6* functions to regulate leaf morphology and shape in developing plants by activating the myb-domain gene *ASYMMETRIC LEAF 1 (AS1)* (Chua et al., 2005).

Table 1. *Arabidopsis thaliana* GTE family

GTE	BET	Alias	Reference	TAIR locus
1	4	IMB1	Duque et al., (2003)	At2g34900
2	6			At5g0550
3	1			At1g73150
4	3	GTE4	Airoldi et al., (2010)	At1g06230
5	2			At1g17790
6	12	GTE6	Chua et al., (2006)	At3g52280
7	5			At5g65630
8	7			At3g27260
9	9	BET9	Du et al., (2004)	At5g14270
10	8	NPX1	Kim et al., (2009)	At5g62230
11	10	BET10	Du et al., (2004)	At3g01770
12	11			At5g46550

GTE6, like other BET proteins, recognizes histone H3 and H4 acetylation marks on the *AS1* promoter and first exon to up-regulate its expression (Chua et al., 2005). However, there is no apparent HAT activity or DNA binding domain in the GTE6 protein. Thus, an outstanding question remains; how do GTE6 and other GTE proteins carry out the transcriptional activation of the target promoter? GTE6 may do so by forming a scaffold that will direct the recruitment of either HAT activity or sequence specific transcription factors at the target promoters to activate transcription. Presumably, these factors are recognized by the ET domain that provides specificity to the complex, and the BRD provides general recognition of acetyl histones at the promoters.

Two members of the GTE family, GTE9 and GTE11, have been reported to interact with a BTB domain containing protein, BT2, in a yeast-two hybrid assay (Du and Poovaiah, 2004). However, BT2 is not a typical chromatin remodeling protein or transcription factor. Rather it functions as part of a CULLIN3-based E3 ubiquitin ligase complex (Figueroa et al., 2005) and regulates diverse signaling responses in *Arabidopsis* (Mandadi et al., 2009). Interestingly, the BT2 interaction is not mediated at the ET domain in GTE9 and GTE11. Instead, it interacts at the C-terminal region of GTE11 that functions as transcriptional activation domain when fused to Gal 4 DNA-binding domain (Du and Poovaiah, 2004). Based, on this interaction we propose that the mechanism of transcriptional regulation by *Arabidopsis* BET proteins is distinct from that reported for fungal or mammalian BET proteins (as reviewed in previous

sections). In the following section I will elaborate on the functions of GTE9 and GTE11 in Arabidopsis.

Arabidopsis GTE9 and GTE11 Regulate BT2 Responses

BT2 belongs to the five-member BT family in Arabidopsis. It features an N-terminal BTB domain, a central TAZ-zinc finger protein-protein interaction domain and a C-terminal domain that interacts with calmodulin in a Ca^{++} dependent manner (Du and Poovaiah, 2004). BT2 was initially characterized as direct target of the zinc-finger transcription factor TELOMERASE ACTIVATOR1 (TAC1) that regulates telomerase activity in mature leaves and vegetative organs (Ren et al., 2004; Ren et al., 2007). Additionally, BT2 also perceives and responds to various physiological and metabolic responses in plants (Mandadi et al., 2009) and is required for 35S enhancer-mediated gene activation (Mandadi et al., 2011). The primary aim of my dissertation research project was to characterize the function of Arabidopsis BET proteins GTE9 and GTE11 in the BT2 signaling pathway. I present genetic and biochemical evidence demonstrating that GTE9 and GTE11 associate with BT2 *in vivo* to regulate responses to nutrients and hormones (Chapter II). In the third chapter, I present evidence for the role of BT2-GTE9/GTE11 complex in 35S enhancer activity (Chapter III), followed by the results from biochemical characterization of the GTE9 protein (Chapter IV).

Based on the genetic and biochemical evidence for the role of GTE9 and GTE11 in BT2 pathway we propose a working model for this complex. We believe that GTE9 and GTE11, like other BET proteins, function as chromatin adaptors to localize specific factors such as the BT2-Cullin3 E3 ligase complex, to target gene promoters and the 35S enhancer in response to multiple signals that results in changes in intracellular Ca^{++} levels sensed by the BT2's calmodulin binding domain (CAM) (Fig. 4).

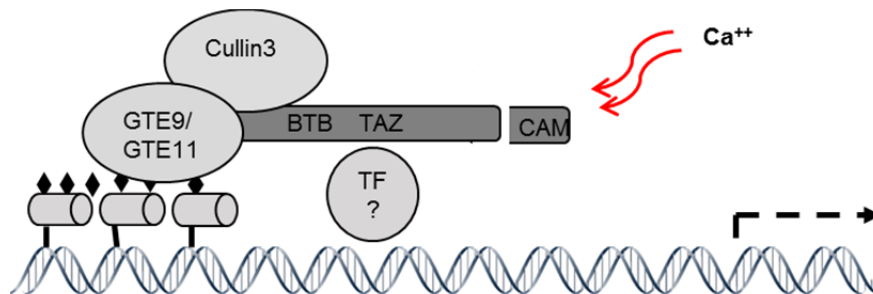


Figure 4. Working model for GTE9/GTE11/BT2 complex. Bromodomain in the GTE proteins recognize acetyl lysines (black diamonds) on histones. The transcriptional activation domain (TAD) of GTE proteins interacts with BT2's BTB domain to localize it on the chromatin in response to multiple signals and changes in intracellular Ca^{++} levels.

CHAPTER II
BROMODOMAIN PROTEINS GTE9 AND GTE11 ARE ESSENTIAL FOR BT2-
MEDIATED RESPONSES TO SUGARS AND ABA IN *ARABIDOPSIS*
THALIANA

Summary

BT2 is a BTB-domain protein that regulates responses to various hormone, stress and metabolic conditions in *Arabidopsis thaliana*. Loss of BT2 results in plants that are resistant to auxin and hypersensitive to inhibition of germination by abscisic acid (ABA) and sugars. Conversely, overexpression of BT2 results in resistance to ABA and sugars. Here, we report the roles of BT2-interacting partners GTE9 and GTE11, bromodomain and extraterminal-domain proteins of Global Transcription Factor Group E, in BT2-mediated responses to sugars and hormones. Loss-of-function mutants *gte9-1* and *gte11-1* phenocopied the *bt2-1*-null mutant responses; germination in all three mutants was hypersensitive to inhibition by glucose and ABA. Loss of either GTE9 or GTE11 in a BT2 over-expressing background blocked resistance to sugars and ABA, indicating that both GTE9 and GTE11 were required for BT2 function. Additionally, loss of GTE9 or GTE11, similar to loss of BT2, suppressed the high-auxin phenotype of *YUCCA1* -overexpressing mutants. Co-immunoprecipitation of BT2 and GTE9 suggested that these BTB-domain and

bromodomain proteins physically interact *in vivo* to mediate responses to diverse environmental and physiological signals.

Introduction

Abscisic acid (ABA) and sugars regulate many important plant processes including seed dormancy, germination, and seedling growth (Rolland et al., 2006). High concentrations of ABA or sugar during *Arabidopsis* (*Arabidopsis thaliana*) seedling development result in an overall arrest of growth, marked by repression of important events, including hypocotyl elongation, cotyledon greening, cotyledon expansion, and shoot growth (Moore et al., 2003). Mutant screens using this growth-arrested phenotype enabled identification of sugar-insensitive and hypersensitive mutants in *Arabidopsis* (Zhou et al., 1998; Pego et al., 2000; Rolland et al., 2002). Characterization of several sugar-response mutants demonstrated that they are allelic to ABA insensitive (*abi*) mutants, thereby providing evidence for crosstalk between sugar and hormone signaling pathways. Among sucrose and glucose insensitive mutants allelic to *abi4* are *sun6*, *sis5* and *isi3* (Huijser et al., 2000; Laby et al., 2000; Rook et al., 2001) and *gin6* (Arenas-Huertero et al., 2000). Crosstalk between ABA and sugar signaling pathways is further supported by activation of several ABA biosynthetic genes, such as *ABA1*, *ABA3*, *NCED3* and *AAO3*, in response to glucose and sucrose (Cheng et al., 2002; Price et al., 2003).

Molecular and genetic screens have revealed a complex network of protein interactions encompassing phosphatases, calcium-dependent protein

kinases and snf1-related protein kinases (e.g. SnRK1) that control various events of hormone and sugar signaling in Arabidopsis (Smeekens, 2000; Rolland et al., 2002; Rolland et al., 2006). Notably, a gene encoding the BTB-domain protein BT2 (AT3g48360) was identified as a potential downstream target of two SnRK1 kinases, KIN10 and KIN11, using a large collection of microarray profiles and was defined as a 'multi-stress' responsive gene (Baena-Gonzalez et al., 2007). We also demonstrated that BT2 regulates multiple stress responses (Mandadi et al., 2009). Loss of BT2 leads to sensitivity to sugar, ABA and H₂O₂. Conversely, constitutive expression of BT2 imparts resistance to sugars, ABA, and H₂O₂. These results suggest that BT2 acts as a negative regulator of sugar and ABA signaling.

Interestingly, BT2 appears to regulate positively regulate some auxin responses. Null mutants of BT2 are resistant to inhibition of root elongation in presence of high auxin (Ren et al., 2007). Furthermore, loss of BT2 abolishes the high-auxin phenotype of the *yucca1D* mutant, which has increased apical dominance, delayed flowering, long hypocotyls and epinastic leaves with long petioles (Mandadi et al., 2009).

BT2 belongs to a family of five closely related proteins that have an N-terminal BTB domain, a central TAZ zinc finger protein-protein interaction domain, and a C-terminal domain that binds to calmodulin in a calcium-dependent manner (Du and Poovaiah, 2004; Robert et al., 2009). BTB domains are typically associated with CULLIN 3 and other proteins to form an E3 ubiquitin

ligase (Pintard et al., 2003; Xu et al., 2003), and some reports have confirmed this interaction for BT2 (Figuroa et al., 2005; Gingerich et al., 2005). However, the N-terminal region of BT2, including the BTB domain, also interacts with two bromodomain proteins, GTE9 and GTE11 in a yeast two-hybrid system (Du and Poovaiah, 2004). GTE9 and GTE11 share 63.2% identity at the amino acid level and belong to the Global Transcription Factor group E (GTE) family, sometimes referred to as the Bromodomain and Extra-Terminal (BET) protein family (Pandey et al., 2002). Most metazoan homologs of these proteins contain two bromodomains and the extra-terminal domain, which interacts with other transcription factors (Matangkasombut et al., 2000; Ottinger et al., 2006; You et al., 2006). Bromodomains bind to acetylated lysine residues, and in many cases form a bridge between acetylated histones and transcription factors to activate the transcription of target genes (Dhalluin et al., 1999; Owen et al., 2000; Dey et al., 2003). Although GTE proteins in *Arabidopsis* have only one bromodomain, they also possess a plant-specific amphipathic domain (Fig. 5A), which is thought to allow dimerization with other GTE proteins (Florence and Faller, 2001). The *Arabidopsis* genome encodes 12 GTE proteins (Fig. 5B), but few have been examined so far. GTE1 (also known as IMB1) plays an important role in seed germination (Duque and Chua, 2003); GTE6, regulates leaf morphology (Chua et al., 2005), and GTE4 is required for proper control of the mitotic cell cycle (Airoidi et al., 2010).

Here, we characterize the role of GTE9 and GTE11 in the BT2 signal transduction pathway. Although steady-state levels of *BT2* transcript respond to light, sugars, fixed nitrogen, H₂O₂, and other stimuli (Mandadi et al., 2009), we found no change in *GTE9* or *GTE11* mRNA under these conditions. However, genetic evidence implicated GTE9 and GTE11 in the BT2 signaling pathway. Both *gte9-1* and *gte11-1* null mutants phenocopied *bt2-1* responses for seed dormancy, inhibition of seedling growth by glucose and ABA, and positively regulated auxin responses. Furthermore, both GTE9 and GTE11 were required for BT2-mediated resistance to glucose and ABA. Neither GTE9 nor GTE11 affected steady-state levels of *BT2* message, consistent with reports that BT2 and GTE9 and GTE11 interact at the protein level (Du and Poovaiah, 2004). We used co-immunoprecipitations to confirm this interaction *in vivo*. Our working model is that these two bromodomain proteins provide general activation of transcription by binding to transcriptionally competent chromatin, and BT2 provides specificity by interacting with calcium signaling pathways and sequence-specific transcription factors. The BT2 complex, including GTE 9 and GTE11, may represent a final junction between calcium signaling and transcription.

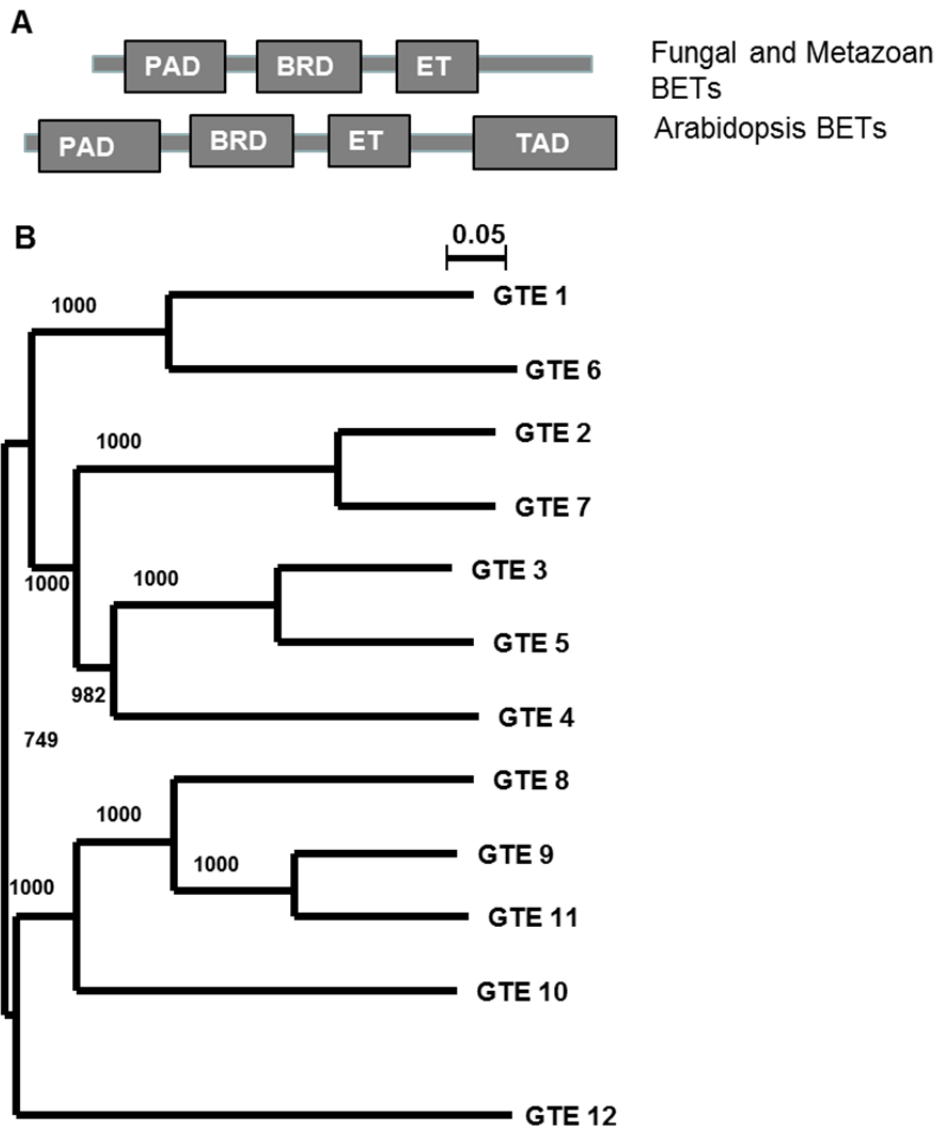


Figure 5. The GTE family of Arabidopsis. A, Domain structure of GTE proteins. PAD, plant amphipathic domain; BRD, bromodomain; ET, extraterminal domain; TAD, transcriptional activation domain. In metazoan and fungal homologs, the PAD domain is typically replaced by another bromodomain (Florence and Faller, 2001). The function of the transcriptional activation domain shown here has been empirically demonstrated only for GTE9 and GTE11, so far (Du and Poovaiah, 2004). B, Phylogenetic relationship of Arabidopsis GTE family members. Protein sequences were retrieved from GenBank, aligned with Clustal W (Chenna et al., 2003), and displayed with NJplot, (Perriere and Gouy, 1996). Bootstrap values (from 1,000 replicates) are shown at the nodes. Arabidopsis Genome Initiative locus numbers are as follows: *GTE1*, At2g34900; *GTE2*, At5g10550; *GTE3*, At1g73150; *GTE4*, At1g06230; *GTE5*, At1g17790; *GTE6*, At3g52280; *GTE7*, At5g65630; *GTE8*, At3g27260; *GTE9*, At5g14270; *GTE10*, At5g63320; *GTE11*, At3g01770; *GTE12*, At5g46550.

Results

GTE9 and GTE11 suppress sugar and ABA signaling and are required for BT2-mediated resistance to sugar and ABA

To investigate the potential roles of BT2 interacting partners GTE9 and GTE11 in BT2-mediated responses to sugar and ABA signaling pathways, we obtained T-DNA insertion lines for *GTE9* and *GTE11* (Fig. 6A) from the Salk T-DNA collection (Alonso et al., 2003) and identified *gte9-1* and *gte11-1* homozygous mutants by PCR screening. RT-PCR analysis confirmed that expression of both *GTE9* and *GTE11* mRNA was abolished in these lines (Fig. 6B).

Because BT2 affects germination in the presence of sugars and ABA (Mandadi et al., 2009), we wanted to determine whether its partners, GTE9 and GTE11, also affected germination under these conditions. We plated seeds from *gte9-1* and *gte11-1* mutants on medium with 4% glucose or 4% mannitol. To minimize differences in seed maturation and storage conditions among different genotypes, seeds used were from plants of each genotype grown and harvested under identical conditions and at the same time. The germination frequency for all genotypes on mannitol was 90-100%. Both *gte9-1* and *gte11-1* were hypersensitive to high glucose concentrations, similar to *bt2-1* (Fig. 7A).

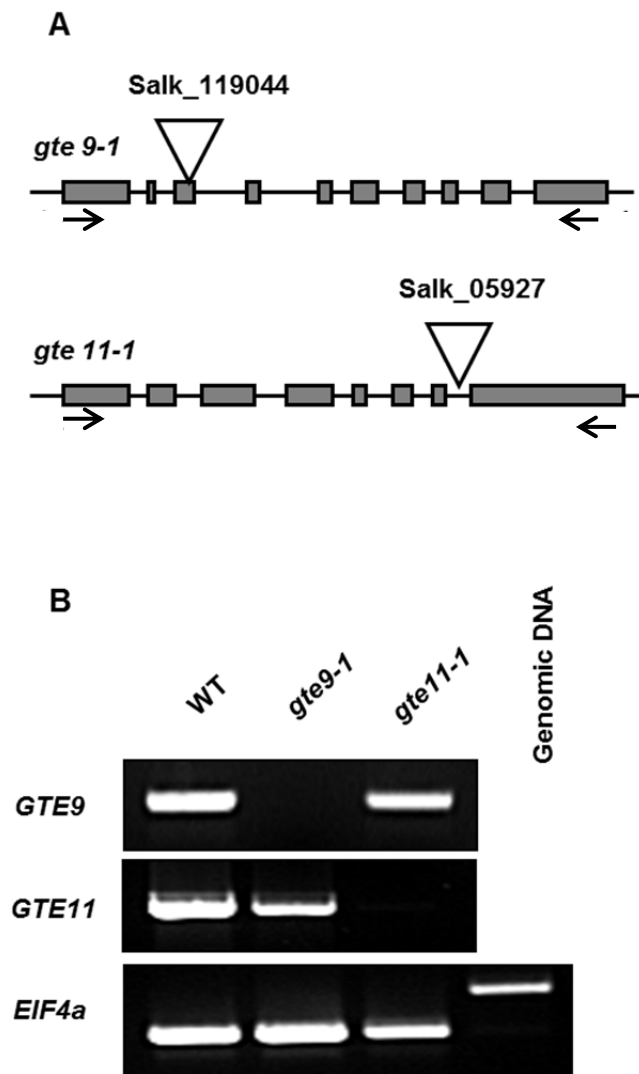


Figure 6. Null alleles of *GTE9* and *GTE11*. A, Diagram of *GTE9* and *GTE11* showing position of T-DNA insertions. B, RT-PCR analysis with *GTE9*, *GTE11*, and Elongation factor (*EIF4-A2*) primers using total RNA extracted from seedlings of the wild type ecotype (Col-0), *gte9-1* and *gte11-1* mutants as template. Arrows indicate the position of primers used for amplifying *GTE9* and *GTE11* mRNA.

To confirm that *gte9-1* and *gte11-1* phenotypes are due to the T-DNA disruption of the respective genes and not an unrelated mutation in these lines, we transformed *gte9-1* and *gte11-1* single mutants with full-length, wild-type cDNA of *GTE9* and *GTE11*, respectively, expressed under control of the constitutive CaMV 35S promoter. T1 transformants were analyzed for transcript abundance relative to wild type, and three lines were identified where *GTE9* or *GTE11* mRNA was at least as abundant as it was in wild type (data not shown). These complementation lines were further assayed for sensitivity on 4% glucose. All lines tested showed resistance to glucose through increased germination at approximately twice the level of wild type (Fig. 7B). Overexpression of *GTE9* and *GTE11* will result in overall increase of *GTE9* *GTE11* protein levels and will often bind to the BT2-CULLIN3 complex and will trigger a resistance response to glucose stress.

Thus, complementation experiments verify that loss of *GTE9* or *GTE11* was responsible for glucose sensitivity in *gte*-null mutants and confirm that the mutant phenotypes are not due to potential uncharacterized T-DNA insertions or other mutations in the respective mutant backgrounds.

To further elucidate the relationship between *GTE9*, *GTE11*, and *BT2*, we independently crossed null mutants *gte9-1* and *gte11-1* into the *35S:BT2* over-expression background. *35S:BT2 gte9-1* and *35S:BT2 gte11-1* double mutants were identified by PCR screens and analyzed for response to glucose. Over-expression of *BT2* alone resulted in resistance to glucose (Mandadi et al., 2009). However, loss of *GTE9* or *GTE11* in the *35S:BT2* background blocked *BT2*'s ability to confer resistance to glucose (Fig. 7B). Importantly, the loss of *GTE9* or *GTE11* had no effect on the expression from the *35S:BT2* transgene, indicating that the interaction between the bromodomain proteins and *BT2* does not occur at the transcriptional level.

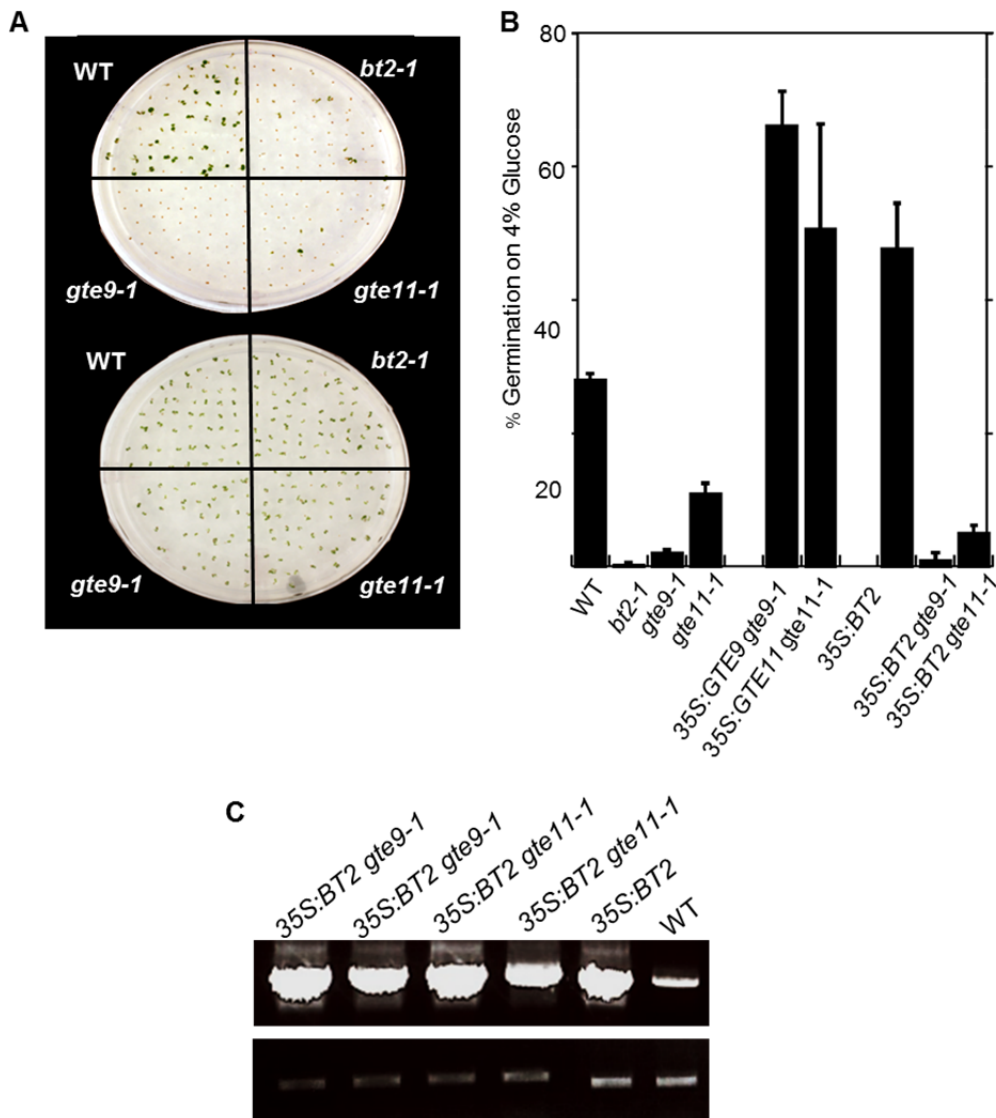


Figure 7. Inhibition of germination by glucose. A, Seeds and seedlings after 7 days on MS medium with 4% glucose (top) or 4% mannitol (bottom). B, Quantification of germination for wild type (WT), *bt2-1*, *gte9-1*, *gte11-1*, 35S:BT2, 35S:GTE9, 35S:GTE11, 35S:BT2 *gte9-1*, 35S:BT2 *gte11-1* on 4% glucose. Data show the mean percent germination \pm SD; n = 120 in three independent trials. C, Expression analysis of *BT2* in double mutants of 35S:BT2 *gte9-1*, 35S:BT2 *gte11-1*, 35S:BT2 and WT using RNA extracted from three-week-old plants.

ABA, similar to sugars, affects seed germination and dormancy, and we observed increased seed dormancy in *bt2-1* in response to ABA (Mandadi et al., 2009). Because GTE9 and GTE11 were involved in BT2-mediated responses to glucose, we asked whether they were also involved in responses to ABA. Germination of both *gte9-1* and *gte11-1* null mutant seed was more sensitive to inhibition by 1 μ M ABA than wild-type seed, and this sensitivity was reversed by constitutive expression of the corresponding GTE cDNAs (Fig. 8 A and B). Furthermore, loss of either GTE9 or GTE11 blocked BT2-mediated resistance to inhibition of germination by ABA (Fig. 8B). Together, these results indicate that both GTE9 and GTE11 function in the sugar and ABA signaling pathways, and both of these bromodomain proteins are required for BT2-mediated responses to glucose and ABA.

GTE9 and GTE11 phenocopy BT2's effect on high-auxin phenotype in yucca1D

We previously demonstrated that BT2 potentiates some responses to

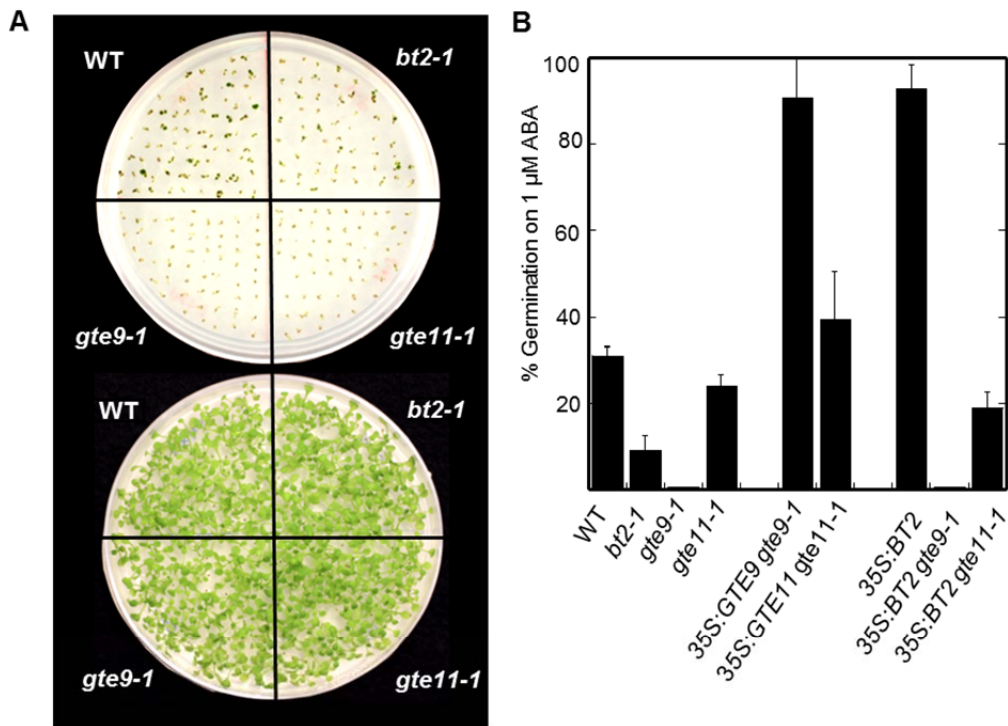


Figure 8. Inhibition of germination by ABA. A, Seeds and seedlings after 10 days on MS medium with (top) and without (bottom) 1 μ M ABA. B, Quantification of germination for wild type (WT), *bt2-1*, *gte9-1*, *gte11-1*, 35S:BT2, 35S:GTE9 *gte9-1*, 35S:GTE11 *gte11-1*, 35S:BT2 *gte9-1*, 35S:BT2 *gte11-1* on 1 μ M ABA. Data show the mean percent germination \pm SD; n = 120 in three independent trials.

auxin, while suppressing ABA and sugar responses in plants (Ren et al., 2007; Mandadi et al., 2009). One interesting phenotype is the effect of BT2 on the high-auxin phenotype of *yucca1D*, an activation tagged line that overexpresses a flavin monooxygenase in the tryptophan-dependent pathway for IAA biosynthesis (Zhao et al., 2001). IAA concentrations are approximately 50% higher in *yucca1D* than in wild type, leading to a characteristic phenotype of epinastic cotyledons and leaves, narrow leaf blades, long hypocotyls, delayed flowering, and reduced fertility (Zhao et al., 2001). Overexpression of BT2 in *yucca1D* exacerbates this high-auxin phenotype (Ren et al., 2007). Conversely, loss of BT2 suppresses the characteristic *yucca1D* phenotype (Mandadi et al., 2009). Because *gte9-1* and *gte11-1* null mutants phenocopy the effect of *bt2-1* on ABA and sugar responses, we also analyzed their affect on the *yucca1D* phenotype. Loss of *GTE9* or *GTE11* suppressed the high-auxin phenotype in a *yucca1D* background, similar to the effect of *bt2-1* (Fig. 9).

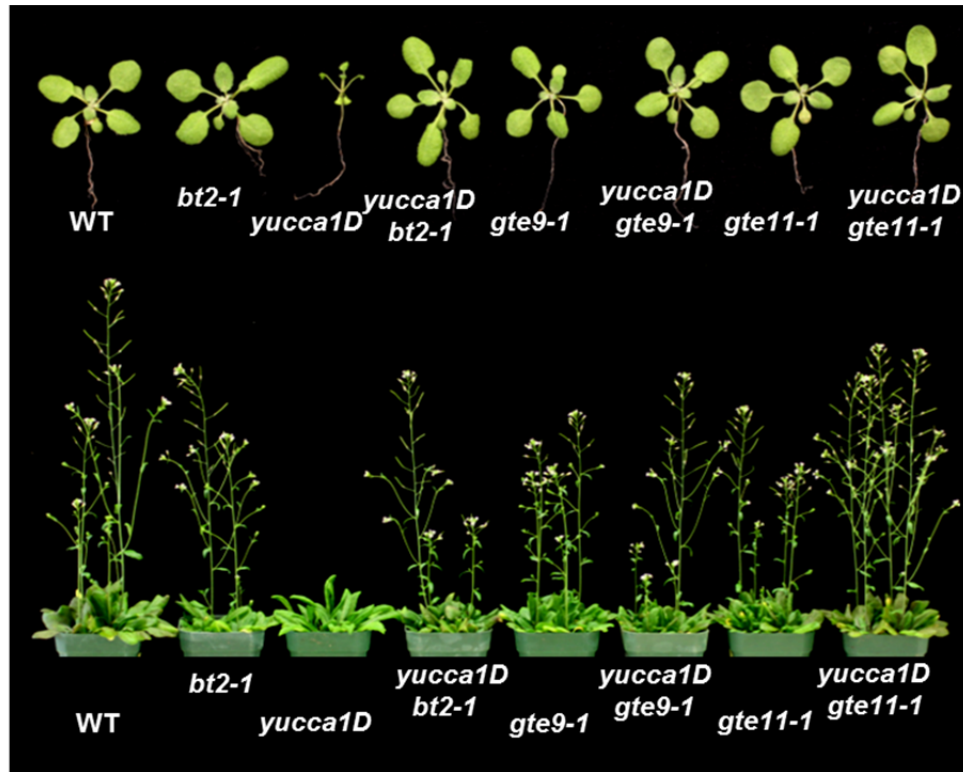


Figure 9. *GTE9* and *GTE11* are required for the high-auxin phenotype of *yucca1D*. Loss of *GTE9* and *GTE11* suppress seedling morphology (above), and delayed flowering phenotype (below) of *yucca1D*, similar to loss of *BT2*.

BT2 may be regulated post-transcriptionally through GTE9 and GTE11

The phenotypes of *gte9-1* and *gte11-1* single mutants and *35S:BT2 gte9-1* and *35S:BT2 gte11-1* double mutants provided strong genetic evidence that all three genes function in the same pathway, and that both GTE proteins are required for BT2 function. Unlike *BT2*, mRNA abundance for *GTE9* and *GTE11* was relatively stable and does not fluctuate in responses to exogenous sugars or ABA (Fig. 10). To investigate the mechanism of interaction between BT2 and

GTE9 and GTE11, we analyzed expression of *BT2* in the *gte*-null-mutants (Fig. 10). Abundance of *BT2* mRNA was unaltered in *gte9-1* and *gte11-1* compared to wild type, suggesting that the interaction occurs post-transcriptionally, which is consistent with the initial identification of GTE9 and GTE11 as proteins that interact with BT2 in a yeast two-hybrid screen (Du and Poovaiah, 2004).

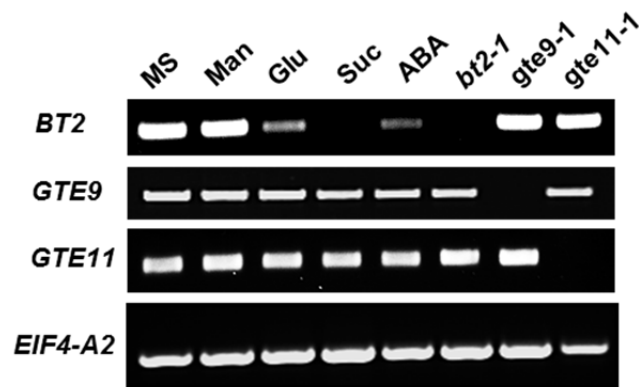


Figure 10. Expression analysis of *BT2*, *GTE9*, and *GTE11*. Analysis of *BT2*, *GTE9*, and *GTE11* expression was performed by RT-PCR using RNA extracted from three-week-old plants. *EIF4-A2* expression was used as the RNA loading control. Treatments with mannitol, glucose, and sucrose (100 mM) were carried out for 3 h. Treatment with 10 μ M ABA was carried for 4h.

GTE9 and BT2 Interact in vivo

Based on *GTE9* and *GTE11* expression analysis (Fig. 10) and the previously reported interaction between BT2 and GTE9 in yeast two-hybrid assays (Du and Poovaiah, 2004), we proposed that these two proteins physically interact with each other *in vivo*. We prepared antiserum against GTE9 and used it to investigate potential interactions *in vivo*. Immunoblots demonstrated the specificity of the antiserum, but they also showed that loss of BT2 or GTE11 has no effect on GTE9 protein levels (Fig. 11A).

To further examine interactions between GTE9 and BT2 *in vivo*, we used antiserum against BT2 to immunoprecipitate proteins from nuclear extracts of wild-type and mutant plants. Precipitated proteins were then analyzed by immunoblots with antiserum against GTE9. BT2 antiserum co-precipitated GTE9 in extracts from wild-type plants, but not from mutants lacking either BT2 or GTE9 (Fig. 11B), thereby confirming that these two proteins associate *in vivo*.

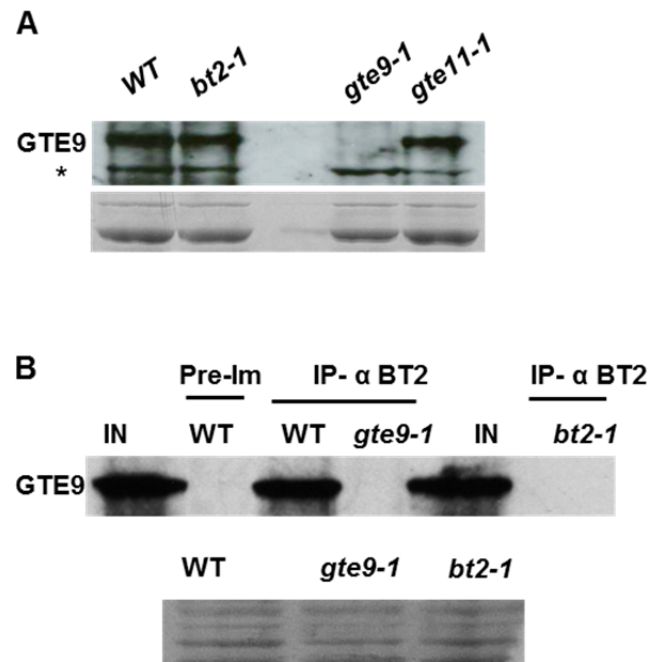


Figure 11. BT2 and GTE9 associate *in vivo*. A, Immunoblot analysis of GTE9 protein in nuclear extracts from wild type, *bt2-1*, *gte9-1*, *gte11-1*. Coomassie stained gel was used for loading control (bottom panel). A non-specific cross-reacting band in the immunoblot (asterisk) also indicates uniform loading. B, Co-immunoprecipitation of GTE9 by BT2 antiserum. GTE9 antiserum detects GTE9 protein in wild-type nuclear extracts used as input (In) for the reactions (one-tenth of the total extract used in immunoprecipitation) and in proteins precipitated by BT2 antiserum, but not by pre-immune serum. Specificity of the interaction is shown by absence of GTE9 in proteins precipitated by BT2 antiserum in both *gte9-1* and *bt2-1* null mutants. The lower panel shows a portion of the Coomassie stained gel used to monitor loading.

Discussion

We initially characterized the BTB-domain protein BT2 as a direct target of the TELOMERASE ACTIVATOR1 transcription factor (Ren et al., 2004). Constitutive expression of BT2 itself was sufficient to induce telomerase expression in mature, vegetative organs (Ren et al., 2007). Subsequently, we described a broader role for BT2 as an integrator of diverse stress and hormone responses in Arabidopsis (Mandadi et al., 2009). In addition, results from other researchers implicate BT2 and the other four members of the BT family in female gametophyte development (Robert et al., 2009). Overall, these findings suggest that BT2 regulates a wide array of physiological and developmental processes.

Identification and characterization of interacting proteins is important to understand mechanistic basis of BT2-mediated responses. Du and Poovaiah (2004) used two-hybrid screens in yeast to identify the bromodomain proteins GTE9 and GTE11 as interacting with the N-terminal region (including the BTB domain) of BT1 and BT2. They also reported that the last 175 amino acids of GTE11 (residues 445 to 620) functions as a transcriptional activation domain when fused to the DNA-binding domain of Gal4. This C-terminal region of GTE11, which is also the region that interacts with BT1 and BT2, activated transcription from four different yeast promoters and increased reporter gene expression up to 100 fold (Du and Poovaiah, 2004).

Here, we analyzed *GTE9* and *GTE11* mutant phenotypes and characterized their genetic interactions with *BT2* in response to sugars and hormones. Germination of both *gte9-1* and *gte11-1* null-mutants were hypersensitive to glucose and ABA, similar to the *bt2*-null mutant (Fig. 7 and 8). In contrast, constitutive over-expression of *GTE9* imparted resistance to glucose- and ABA-mediated inhibition of germination and development, similar to results from *BT2* overexpression (Fig. 7 and 8). These results demonstrate that *GTE9* is a negative regulator of sugar and ABA signaling, and its loss phenocopies loss of *BT2* (Mandadi et al., 2009). Although loss of *GTE11* conferred sensitivity to glucose and ABA, its overexpression in the *gte11-1* background imparted resistance above wild-type levels only for glucose and not for ABA. However, the role of *GTE11* in ABA signaling was confirmed by its requirement for *BT2*-mediated ABA-resistance (Fig. 8).

To further explore parallels between the *GTE* bromodomain proteins and *BT2*, we examined their affect on the high-auxin phenotype of *yucca1D*. Loss of *BT2* in this mutant background results in suppression of characteristic epinastic cotyledons and epinastic leaves, shorter primary roots, excess root hairs, and delayed flowering (Mandadi et al., 2009). As predicted, loss of either *GTE9* or *GTE11* also suppressed the high-auxin phenotype of *yucca1D* (Fig. 9), similar to loss of *BT2* (Mandadi et al., 2009), thereby indicating that *GTE9* and *GTE11* also positively regulate some auxin responses while negatively regulating ABA

responses. These results provide an additional example of the classical antagonism between ABA and auxin (Fedoroff, 2002).

The *Arabidopsis* genome encodes 12 proteins in the GTE family, which are characterized by possessing both a single bromodomain and an extra-terminal domain (Florence and Faller, 2001; Pandey et al., 2002). These proteins fall into two distinct clades (GTE1-7 and GTE 8-12) when their entire protein sequence is aligned (Fig. 5B). Previously characterized proteins GTE1 (Duque and Chua, 2003), GTE4 (Airoldi et al., 2010), and GTE6 (Chua et al., 2005) which affect gene expression during germination, cell cycle control, and leaf development, respectively, all group in the first clade. The last five family members (GTE8-12) cluster together in the second clade. In addition to GTE9 and GTE11, the only other member of this second group that has been characterized is GTE10, also known as NPX1, a nuclear protein that affects ABA signaling (Kim et al., 2009).

Although *Arabidopsis* GTE bromodomain proteins affect a broad range of important physiological and developmental processes, their exact mode of action is not yet clear. Bromodomains are protein-protein interaction domains that bind acetylated lysines. Many bromodomain proteins from fungi and animals bind acetylated lysines in histones to help mark genomic regions that are primed for transcription (Hassan et al., 2001; Hassan et al., 2002), but bromodomains also interact with acetylated lysines in other proteins (Mujtaba et al., 2002; Mujtaba et al., 2004; Huang et al., 2009). GTE9 interacts with BT2

physically *in vivo* (Fig. 11), although pull-down assays using recombinant proteins indicate that this interaction is not through the bromodomain (Du and Poovaiah, 2004).

In fungi and animals, GTE proteins regulate transcription by affecting transcriptional initiation (Florence and Faller, 2001). GTE11 activates transcription from yeast promoters (Du and Poovaiah, 2004), indicating that it, too, has the capability to broadly promote transcription. Because there was no difference in *BT2* transcript levels in either *gte9-1* or *gte11-1* mutant backgrounds compared to wild type (Fig. 10), any interaction with BT2 must be post-transcriptional.

Interestingly, the C-terminal region of GTE11 that activates transcription in yeast is the same region that interacts with N-terminus, including the BTB domain, of BT proteins (Du and Poovaiah, 2004). One possible mechanism for transcriptional activation by GTE and BT protein complexes is that the GTE component provides a general recognition of the chromatin state near a promoter, and the BT component provides a more specific recognition of regions to be transcribed, possibly by binding to transcription factors through its TAZ domain. A similar model has been proposed for the human Brd4 bromodomain protein, which facilitates binding of papillomavirus E2 transcription factors (Lee and Chiang, 2009). The proposed general nature of transcription activation by GTE9 and GTE11 is consistent with the relatively stable expression pattern of their transcripts (Fig. 10). Similarly, the proposed specific role of BT2 is

consistent with changes in its steady-state transcript concentrations upon exposure to multiple biotic and abiotic conditions (Du and Poovaiah, 2004; Mandadi et al., 2009). However, this model raises a question about how increased expression of GTE9 or GTE11, the stable partners in this complex, can affect physiological changes. If the interaction between the GTE proteins and BT2 is relatively weak, then at equilibrium, little of the protein will be in a productive GTE-BT2 complex. Because formation of this complex depends on second order kinetics, increasing concentration of either partner will drive more of the BT2 into the active complex.

Because there is no apparent DNA binding site in the BT2 protein, the basis for its specificity remains unresolved. However, recent descriptions of NPR1, another Arabidopsis BTB protein that shares a similar overall structure to BT2, may be informative. NPR1, like BT2, has a BTB domain near its N-terminus, a protein-protein interaction domain in the center (ankyrin repeats for NPR1; TAZ for BT2), and a signal sensing domain at the C-terminus (redox-sensitive disulfide bonds for NPR1; calmodulin-binding domain for BT2). Changes in redox potential upon pathogen infection trigger transit of NPR1 from the cytoplasm to the nucleus. There, NPR1 interacts through its ankyrin-repeat domain with the transcriptional repressor TGA2.2, which is bound to the promoter of *PR1* and other pathogen response genes. Binding of NPR1 negates the ability of TGA2.2 ability to repress transcription and results in induction of *PR1* and other components of systemic acquired response (Rochon

et al., 2006; Boyle et al., 2009). We postulate that BT2 and other members of the BT family may act in a similar fashion, except that they would respond to calcium/calmodulin signals rather than changes in redox potential. Identification of protein partners that interact with the BT2 TAZ domain and genes that are regulated by GTE9, GTE11, and BT2 should confirm or refute this model.

Materials and Methods

Plant materials and growth conditions

Arabidopsis Genome Initiative locus numbers for the genes used in this article are as follows: *GTE9*, At5g14270; *GTE11*, At3g01770; *BT2*, At3g48360; *EIF4-A2*, At1g54270. Seeds from wild-type *Arabidopsis thaliana* (Col-0), *gte9-1* (Salk_0119044), *gte11-1* (Salk_059327) T-DNA insertion and other mutant lines were grown in soil in 14-h photoperiod under a light intensity of ~120-130 $\mu\text{mol}/\text{m}^2\text{s}$ at 21 °C. T-DNA insertion lines were obtained from ABRC stock center (Alonso et al., 2003). Location of the T-DNA was confirmed by amplifying the genomic DNA using gene specific primers: *GTE9*, 5'-AGACTCTGAGAATGTCGTAGA-3' and 5'-ATCCAACATAGGTTCAAGATC-3'; and for *GTE11*, 5'-CTTTTTCTGTTCTGACAGTTGA-3' and 5'-CTTAAACGATTGATGTCGCAG-3' and left border T-DNA primer 5'-TTTTCGCCCTTTGACGTTGGA-3'. Suppression of high-auxin *yucca1D* phenotype (Zhao et al., 2001), was examined in *gte9-1 yucca1D* and *gte11-1 yucca1D* double mutants. The *yucca1D* allele was identified by PCR with

primers for the 35S enhancer (5'-ATGGTGGAGCACGACACTC-3') and *YUCCA1* (5'-TCTTGATGGATGATGGAAAATG-3') primers.

Phylogenetic analysis

GTE proteins sequences from Arabidopsis were retrieved from GenBank, aligned in Clustal W (Chenna et al., 2003), and the resulting neighbor-joining tree was displayed with NJplot (Perriere and Gouy, 1996).

Seed germination assays

For germination assays, seeds from wild-type Arabidopsis (Columbia), *gte9-1*, *gte11-1*, *bt2-1*, *35S:BT2*, *35S:BT2 gte9-1*, *35S:BT2 gte11-1*, *35S:GTE9*, and *35S:GTE11* were surface sterilized with 50% (v/v) bleach and 0.1% (v/v) Triton X-100 for seven minutes and then plated on Murashige and Skoog (MS) medium or MS medium containing 4% glucose or 1 μ M ABA. The plates were subsequently kept at 4°C for 4 d for seed stratification before they were moved under continuous low light ($\sim 30 \mu\text{mol}/\text{m}^2\text{s}$) at 25°C. Germination was monitored for 7 days (4% glucose-MS plates) or 10 days (1 μ M ABA-MS plates). Each plate was then scored for the presence of green cotyledons. Data from three replicates for each treatment was averaged to perform statistical analysis (standard deviation and Student's t test).

RT-PCR and gene expression analysis

Total RNA was isolated from 3-week-old leaves using TRI reagent following manufacturer's protocol (Ambion). The total RNA was dissolved in 100 μ L of RNase-free water followed by DNase treatment. Reverse transcription reactions were performed using 5 μ g of total RNA and Superscript III reverse transcriptase (Invitrogen). PCR was then performed for *EIF4-2A*, *GTE9*, *GTE11* and *BT2*. Primers used for *EIF4-2A* and *BT2* were the same as used in Mandadi et al (2009). 24 cycles for PCR of *BT2* and *EIF4-2A* were used compared to 32 cycles for *GTE9* and *GTE11*. Sugar and ABA treatment for expression analysis was performed as described previously (Mandadi et al., 2009).

Complementation of gte9-1 and gte11-1 mutants

The *GTE9* and *GTE11* full-length cDNAs were PCR amplified and cloned into pGem-T-Easy vector (Promega) using *BamHI* and *SacI* sites engineered into the primers and re-cloned in the binary vector pCBK05 (Riha et al., 2002). Binary vectors carrying either *GTE9* or *GTE11* full-length cDNA was transformed into *Agrobacterium tumefaciens* GV3101 and then into *gte9-1* and *gte11-1* plants using the floral dip method (Clough and Bent, 1998). Transformants were selected by spraying *Basta* on two-week-old plants. The overexpression lines were identified by RT-PCR analysis. At least two lines per genotype were obtained and analyzed for each genotype.

GTE9 antibody generation and protein expression analysis

GTE9 cDNA was subcloned into the *Bam*HI and *Sac*I sites of pET32 (a) to obtain a fusion of thioredoxin tag, 6X His tag, and S tag at the 5' end of the coding region. Recombinant *GTE9* was expressed in *E. coli* Rosetta2 (DE3)/*lysS* (Novagen) and induced with 1 mM isopropylthio- β -galactoside (IPTG) for 2 h at 37°C. *GTE9* protein was insoluble at 37°C and solubilized with 1M urea. The solubilized protein was subjected to overnight enterokinase (rEK, Novagen) cleavage to remove the tags at the N-terminus. The digested protein was acetone precipitated overnight and centrifuged at 10,000 X g to pellet the protein. The pellet was resuspended in 2X Laemmli sample buffer, and then resolved on by SDS-PAGE using 7.5% gel. Following electrophoresis, G250 Commassie dye was used to stain the gel. The *GTE9* protein was excised from the stained gel and sent to a commercial immunology service (Covance Inc., Princeton, New Jersey) for polyclonal antibody generation in rabbits. Detection of *GTE9* protein in wild type, *bt2-1*, *gte9-1*, and *gte11-1* lines was performed by immunoblot analysis. Three-week-old rosettes (2 g each) were harvested and frozen directly in liquid nitrogen to prepare nuclear extracts using nuclei isolation buffer (NIB) containing 5 mM EDTA, 50 mM Tris pH 8.0, 10 mM KCL, 250 mM sucrose, 1.5 mM MgCl₂, 0.3% Triton X-100, 1 mM PMSF, 5 mM B-ME, 1 mM spermine, 1 mM spermidine with protease inhibitors (Sigma). The nuclear pellet from each genotype was resuspended in 2X Laemmli sample buffer. The nuclear extracts were boiled for 10 min then centrifuged at 13,000 g for 10 min.

Proteins in the supernatant were resolved on 7.5% SDS-PAGE gels and blotted onto nitrocellulose membranes (Amersham). Blots were first incubated with primary anti-GTE9 antibodies (1:1000 dilution), followed by peroxidase-conjugated light chain-specific mouse anti-rabbit secondary antibodies (Jackson ImmunoResearch) at a 1:10,000 dilution. Protein visualization was performed by chemiluminescence using Super Signal West Pico detection reagents (Pierce) and Hyperfilm-ECL films (Amersham). Equal loading was confirmed by Coomassie staining the nuclear extracts from each genotype.

Co-immunoprecipitation assay

Nuclear extracts from ~20 g of three-week-old plants (wild type, *gte9-1* and *bt2-1*), were prepared using the nuclear extraction protocol described above. Nuclear protein extract was diluted 1:5 in immunoprecipitation buffer W100 (20 mM TrisOAc pH7.5, 10% glycerol, 1 mM EDTA, 5 mM MgCl₂, 0.2 M NaCl, 0.1 M KGlu, 1% NP40, 0.5 mM NaDeoxycholate, 1 mM DTT and precleared for 3h. Two reactions were prepared; one for use with pre-immune serum, and one for use with antiserum. Ten µl of serum was added to 500 µl of diluted nuclear extract and incubated overnight at 4°C. Immunocomplexes were collected using BSA-blocked Protein A beads (Pierce). After the final wash beads were resuspended in 2X Lamelli buffer and boiled for 3 m. Proteins in the supernatant were resolved on 7.5% SDS-PAGE and characterized by immunoblot analysis.

CHAPTER III

GTE9/GTE11-BT2 COMPLEX IS REQUIRED FOR CAULIFLOWER MOSAIC VIRUS 35S ENHANCER-MEDIATED ACTIVATION OF GENE EXPRESSION

Summary

The Arabidopsis proteins GTE9 and GTE11, belong to the Bromodomain and Extra-terminal (BET) class of proteins which have a single bromodomain, a short extra-terminal (ET) domain, and a variable C-terminal domain. In the previous chapter we demonstrated that both GTE9 and GTE11 are required for the BT2-CULLIN3 ubiquitin ligase to confer appropriate responses to nutrients, hormones, abiotic and biotic stresses in Arabidopsis. In addition to mediating diverse cellular responses to a variety of signals, we have found that BT2 is also required for increased transcriptional activity directed by the Cauliflower Mosaic Virus 35S enhancers in transgenic plants (Mandadi et al., 2011). Here, we report that GTE9 and GTE11 are also required for this aspect of BT2 function. Loss of either GTE9 or GTE11 in 35S enhancer activation-tagged lines, such as *yucca1d* and *jaw1d*, resulted in suppression of the activation tagged phenotypes in both the lines. Suppression of the phenotypes in the activation lines was due to reduced concentrations of mRNA from the tagged gene and hypermethylation of the enhancers at the cytosines. Our working model is that acetyl histone recognition by the bromodomain proteins GTE9 and GTE11 increases the affinity of the BT2-CULLIN3 ubiquitin ligase complex for the acetylated

chromatin at the 35S enhancers to either destroy repressors or activate factors necessary for enhanced transcription from the activated promoters.

Introduction

Plant breeding and basic plant science research has been revolutionized in the last 25 years by the advent of genetically engineered plants. Most of the genetically engineered plants result from transfer of DNA from *Agrobacterium tumefaciens* into the plant genome. The transferred DNA (T-DNA) can be manipulated to express a variety of new genes in the plant, but many of the commercial transgenic crops make use of the strong constitutive 35S promoter from Cauliflower Mosaic Virus (CaMV) to express genes encoding resistance to insects or herbicides (Mitsuhara et al., 1996).

Basic plant research has also benefited from the *A. tumefaciens* T-DNA transfer system. In addition to being able to add genes of interest into a plant's genome to study their effect, there is a large collection of T-DNA insertion lines available for the model plant *Arabidopsis thaliana*. Because the *Arabidopsis* genome is so compact, many of the T-DNAs are inserted into the coding region of genes and disrupt their function. These T-DNA insertion lines can be studied for the effects of loss of function for a particular gene (Krysan et al., 1999; Alonso et al., 2003).

Analysis of *Arabidopsis* T-DNA insertion lines has been very fruitful and has revealed the function of many genes. However, this approach does have

some limitations. First, many plant genes, even in the small Arabidopsis genome, belong to multi-gene families, and their redundancy can often compensate for the loss of one family member. Second, some genes are absolutely essential to plant survival, and their disruption by a T-DNA insertion is lethal.

Activation tagging has emerged as an alternative way to characterize genes belonging to large families and overcomes the issues of redundancy and lethality (Hayashi et al., 1992; Weigel et al., 2000). The approach involves random integration of a T-DNA, carrying a tetramer of the CaMV 35S enhancer, in the plant genome causing an activation of endogenous expression of the nearby genes, up to 70kb from the insertion (Ren et al., 2004). It is proposed that increased expression of the endogenous genes is an outcome of interaction between 35S enhancers and host-encoded transcription factors. The technique has been demonstrated to work effectively both in dicotyledons and monocotyledons (Weigel et al., 2000; Mathews et al., 2003; An et al., 2005; Hsing et al., 2007; Mori et al., 2007) and has been very useful in identifying genes involved in abiotic (Aharoni et al., 2004) and biotic stress tolerance (Xia et al., 2004). Additionally, mutants in metabolic pathways (Borevitz et al., 2000) and plant development and morphogenesis (Zhao et al., 2001; Palatnik et al., 2003; Pogorelko et al., 2008) have also been characterized successfully using this approach.

Despite the utility of the CaMV 35S promoter and enhancer in agriculture and basic research, we know little about how these elements function. The 35S promoter consists of two domains, A and B, where domain A works as a minimal promoter and domain B comprises the enhancers (Fig. 12) (Odell et al., 1985, Kay et al., 1987, Benfey et al., 1990). The CaMV 35S enhancers are derived from the domain B (-343 to -90 of the transcription start site) in the 35S promoter (Benfey et al., 1989) and harbor a binding site (*as-2*) for the ASF-2 transcription factor (Lam and Chua, 1989). The 35S minimal promoter in the domain A (-90 to -8) contains a binding site (*as-1*) for the ASF-1 transcription factor, belonging to the TGA family of basic-leucine-zipper (bZIP) class of transcription factors (Lam and Lam, 1995). 35S enhancers activate transcription independent of the CaMV 35S promoter, and this difference can be attributed to the unique trans-acting factors recognized by the two *cis* elements in the 35S promoter (Benfey et al., 1989, Fang et al., 1989, Lam et al., 1990). This is also evidenced from differential expression patterns rendered by 35S enhancers (active in the shoots) and 35S minimal promoter (active in the roots) in plants (Ohtsuki et al., 1998)

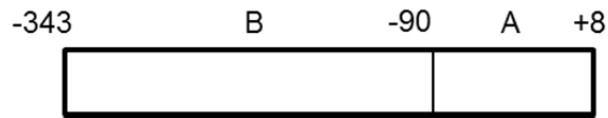


Figure 12. Sub-domains of cauliflower mosaic virus (CaMV 35S) promoter. The two domains in 35S promoter comprise of core promoter and the enhancer. The Domain A, core promoter, spans from -90 to +8 and domain B, enhancer, spans from -343 to -90.

Various models have been proposed to explain the 35S-enhancer-mediated transcriptional activation. According to the current models, the enhancer-promoter interactions are mediated by myriad factors in the transcription machinery, including transcriptional activators and remodeling proteins that allow enhancer looping and/or scanning of the target promoter (Blackwood and Kadonaga, 1998; Bulger and Groudine, 1999; Agalioti et al., 2000; Calhoun et al., 2002; Hatzis and Talianidis, 2002). More recently, enhancers have been implicated in the synthesis of long non-coding RNAs ~200 nt long that can activate expression of neighboring genes by forming a scaffold with proteins necessary for transcription (Ørom et al., 2010). Given a variety of ways an enhancer can communicate *in vivo* with its targets, it is important to characterize the endogenous cellular factors essential for 35S-enhancer activity. Discovery of these novel factors will further our understanding of enhancer-

mediated gene activation in heterologous systems and will allow us to overcome the limitations associated with it.

Here, we report that the GTE9/GTE11/BT2 complex, in addition to regulating multiple responses in plants (Chapter II), is also necessary for maintaining 35S-enhancer-mediated gene activation. Loss of BT2, GTE9 or GTE11 in the *yucca1d* activation line causes suppression of *yucca1d* associated phenotypes throughout the plant life cycle (Chapter II). Suppression of the *yucca1d* phenotype is accompanied with loss of *yucca* transcript in the double mutants of *yucca1d bt2-1*, *yucca1d gte9-1* and *yucca1d gte11-1*. Loss of GTE9 and GTE11, similar to loss of BT2, in other activation tagged lines such as *jaw1d* and *jaw3d*, also suppressed the phenotypes associated with respective lines, suggesting a more general role of the GTE9/GTE11/BT2 complex in maintaining the 35S-enhancer activated phenotype. Suppression of the phenotype in the activation tagged lines was due to loss of transcript from the activated genes and was associated with hypermethylation of cytosines at the 35S enhancers. We propose that GTE9 and GTE11 recognize the acetylated chromatin on 35S enhancers and localize the BT2-CULLIN E3-ligase complex on the 35S enhancers to stimulate transcription by either polyubiquitination or monoubiquitination of factors required for 35S enhancer function.

Results

GTE9 and GTE11 are required to maintain 35S enhancer phenotype

We have shown previously that null mutants of *GTE9* and *GTE11* phenocopy a *BT2* null allele's suppression of the high-auxin phenotype of *yucca1d* (Chapter II). Surprisingly, upon further characterization of *BT2*'s role in auxin signaling we found that *BT2* is not required to mediate auxin responses in *yucca1d*, instead it plays broader and more general role of regulating 35S enhancer-mediated gene activation (Mandadi et al., 2011).

To determine if both *GTE9* and *GTE11*, similar to *BT2*, are essential for 35S-enhancer mediated gene activation we crossed the *gte9-1* and *gte11-1* null alleles (Misra et al., 2011) into the additional 35S-enhancer activation tagged mutants of *jaw1d* and *jaw3d*. The phenotype of the activation lines was suppressed in the double mutants of *yucca1d gte9-1*, *yucca1d gte11-1*, *jaw1d gte9-1* and *jaw1d gte11-1* (Chapter II and Fig. 13 A, B). Overexpression of *BT2* exacerbated the high-auxin phenotype in the *yucca1d* background (Ren et al., 2007).

However, loss of GTE9 or GTE11 prevents this exacerbation *yucca1d* (Fig.13 D), providing additional evidence that both GTE9 and GTE11 are required for 35S enhancer-mediated gene activation. As expected, suppression of the activation-tagged phenotype was accompanied by a loss of *YUCCA1* transcript in the double mutants of *yucca1d gte9-1*, *yucca1d gte11-1*, as seen in *yucca1d bt2-1* (Fig. 14).

One alternative explanation for reduced transcript from the *yucca1d* locus is that because the *gte*-null lines also contain 35S enhancer sequences (from the native 35S promoter in the T-DNA), this arrangement could trigger homology-dependent transgene silencing (Chandler et al., 2002; Matzke et al., 2002). However, we showed that neither crossing of unrelated T-DNA lines (Mandadi et al 2011) nor transfer of 35S promoter-driven *GTE9* or *GTE11* transgenes in *yucca1d* had any effect on the activation phenotype (Mandadi et al., 2011). These data together suggest that suppression of the phenotypes in the activation-tagged lines works independently of the homology dependent transgene silencing.

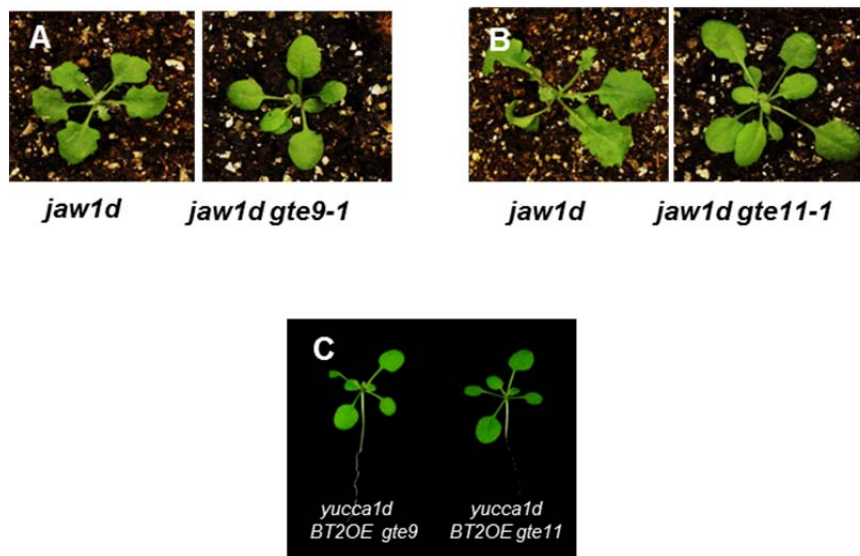


Figure 13. Loss of GTE9 and GET11 abolishes 35S enhancer activation tagged phenotypes. (A and B) Loss of un-even leaf shape phenotype of *jaw1d* in *jaw1d gte9-1* and *jaw1d gte11-1*. (C) Reversal of exacerbated phenotypes of *yucca1d 35S:BT2* in and *yucca1d 35S:BT2 gte9* and *yucca1d 35S:BT2 gte11* lines.

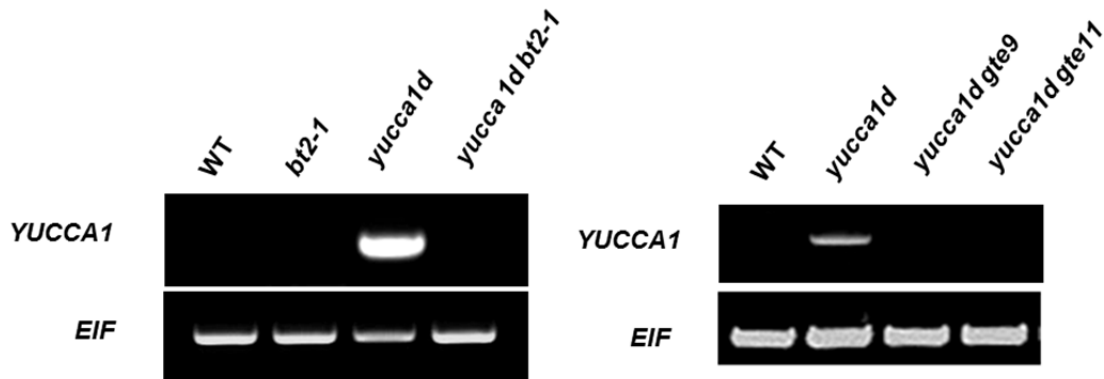


Figure 14. GTE9 and GTE11 are required for BT2 function to maintain expression of 35S enhancer-activated genes. Expression of *YUCCA1* in WT, *bt2-1*, (on left) and *yucca1d gte9*, and *yucca1d gte11* lines, respectively. Total RNA from respective genotypes was extracted and subjected to RT-PCR. *EIF* expression was used as a loading control.

GTE9/GTE11/BT2 complex regulates transcription of 35S enhancer tagged lines

We next examined whether lowered transcript levels of the activated gene in the 35S enhancer lines was due to transcriptional or post-transcriptional gene silencing (TGS or PTGS). Messenger RNA stability assays in the *yucca1d bt2-1* and *bt2-1* mutants eliminated the role for PTGS (Mandadi et al., 2011), so we performed nuclear run-on analysis to examine the role of BT2 in TGS. Our results showed that BT2 is indeed required to activate transcription in *yucca1d* activation line and loss of BT2 triggers TGS in the 35S-enhancer activation tagged lines (Mandadi et al., 2011).

TGS is often accompanied by hypermethylation of the transgene DNA and is independent of the methylation status of the site of transgene integration (Vaucheret and Fagard, 2001). We made use of the methylation-specific restriction endonuclease, McrBC assay to determine the methylation status of *yucca1d* locus (Chinnusamy and Zhu 2010). McrBC is a type IV restriction endonuclease that recognizes R^mC[n40-3000] R^mC in the DNA, here R stands for A and G, and selectively cleaves in this region. Subsequent PCR amplification of the McrBC-digested DNA only enriches for regions that are not methylated, and thus identifies methylated regions (Ishikawa et al., 2010). Using this assay we found that 35S enhancers in *yucca1d bt2-1* and *yucca1d gte11-1* double mutants were hypermethylated compared to *yucca1d* single mutant (Fig. 15). However, loss of neither BT2 nor GTE11 had any effect on the methylation status of the *HELITRONY1D*, an endogenous transposable element in the *YUCCA1* promoter region (-2000). However, the methylation at the *HELITRONY1D* region potentially contributes to the methylation at the -600bp to -2000bp region of the *YUCCA1* promoter, as we found this region to be uniformly methylated in all mutant lines examined (Fig. 15), implying a role for *HELITRONY1D* in creating a restricted *YUCCA1* expression in different parts of the plant (Cheng et al., 2006).

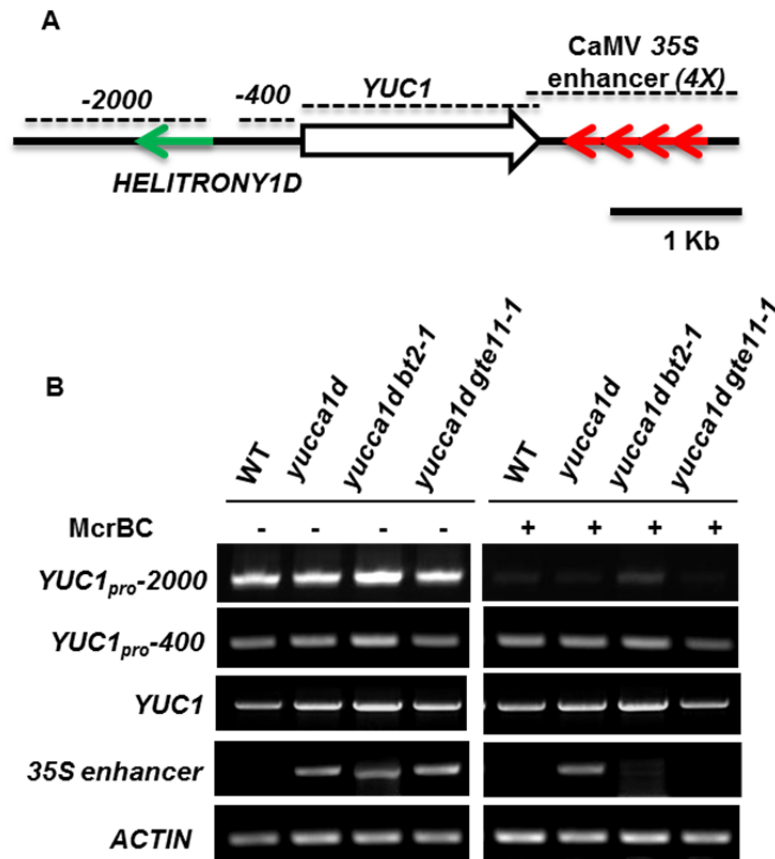


Figure 15. GTE9 and GTE11 suppress methylation at the 35S enhancers. (A) *YUCCA1* locus in *yucca1d*. 35S enhancers are indicated as red arrows. Dotted lines represent regions of DNA subjected to McrBC-PCR analysis. (B) McrBC sensitive PCR analysis of *YUCCA1* locus in WT, *yucca1d*, and *yucca1d bt2-1*. (-) and (+) represent mock and McrBC digestion, respectively. Unmethylated DNA is enriched by PCR amplification and can be visualized as a single fragment; while absence of fragment represents methylated DNA. *ACTIN* genomic region was amplified to serve as a negative control for methylation, respectively.

Bromodomain in GTE9 and GET11 is essential for 35S-enhancer phenotypes

Nearly all BET proteins associate with acetyl histone in a bromodomain-dependent manner. Selective binding of acetyl histone to the bromodomain protein occurs via a hydrophobic binding pocket formed between the two loops of the left handed alpha helices in the bromodomain (Sanchez and Zhou, 2009). Using Clustal W alignment we have shown that GTE9 and GTE11 bromodomains share high homology with well-characterized bromodomain proteins in other organisms (Fig. 16). To investigate if bromodomains in GTE9 and GTE11 are critical for 35S enhancer mediated gene activation, we overexpressed the 110aa bromodomain region from GTE9 and GTE11 in *yucca1d*. Over-expression of bromodomain alone resulted in lowered *YUCCA1* expression from the activated allele and suppression of the *yucca1d* phenotype (Fig. 17 A, B). This result indicates that bromodomain is indeed essential for maintaining 35S enhancer mediated gene activation. To further strengthen this finding we carried out site directed mutagenesis of the residues engaged in acetyl histones recognition (two tyrosines separated by 41 aa and an asparagine, Fig. 16) (Owen et al., 2000; Kanno et al., 2004). The cDNA for this altered gene, designated GTE9^{MUT}, was overexpressed in the *yucca1d* activation line and suppressed the *yucca1d* phenotype as expected (Fig. 17 C, D). These data together suggest that recognition of acetyl lysines is mediated by the conserved tyrosine and asparagine in the 110aa bromodomain module is required for the 35S enhancer function at the *yucca1d* locus.

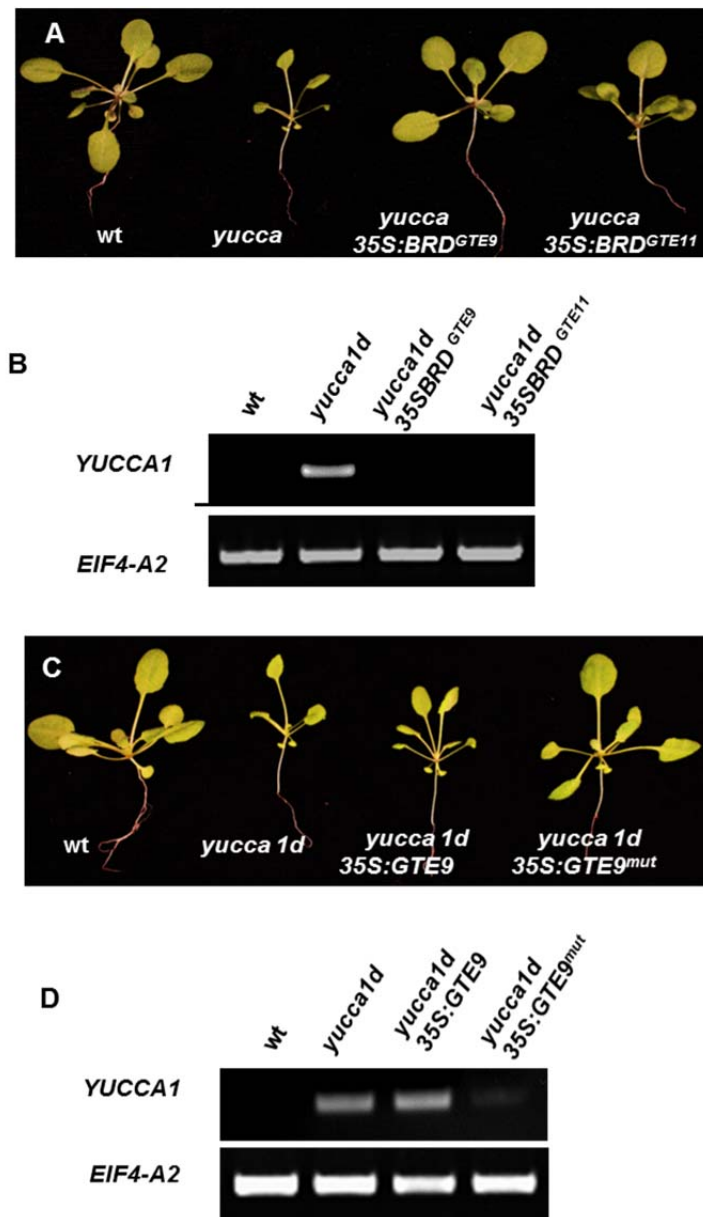


Figure 17. Bromodomain (BRD) of GTE9 and GTE11 is critical for 35S enhancer activity. Suppression of activation phenotypes by (A) over-expression of BRD- GTE9 and GTE11 (35S:BRD^{GTE9}), and 35S:BRD^{GTE11} and (C) over-expression GTE9 with a mutated Tyrosine and Asparagine (35S:GTE9^{mut}) in *yucca1d* lines. (B & D) Suppression of YUCCA1 expression in *yucca1d*, *yucca1d* 35S:BRD^{GTE9}, *yucca1d* 35S:BRD^{GTE9} and 35S:GTE9^{mut}. Total RNA from respective genotypes was extracted and subjected to RT-PCR. EIF expression was used as a loading control.

Discussion

Activation tagging, in the past few years has emerged as an alternative approach for functional studies in Arabidopsis (Kondou et al., 2010). The approach employs synthetic multimerized enhancers, derived from the 35S promoter in Cauliflower Mosaic Virus, to activate the endogenous expression pattern of the tagged gene (Weigel et al., 2000). Here we show that the GTE9/GTE11/BT2 complex is an essential endogenous factor that regulates 35S enhancer activity. We found that loss of GTE9 and GTE11, similar to loss of BT2, dramatically compromises the 35S enhancer function in two independently activation tagged lines (Fig. 13 A, B, C) and is not necessarily required for mediating auxin responses as originally proposed (Ren et al., 2007). Suppression of the 35S-enhancer phenotype was accompanied by loss of transcript from the activated gene and was due to impaired transcription rather than unstable RNA, suggesting a role for transcriptional gene silencing (TGS). (Mandadi et al., 2011).

Delivery of transgenes bearing homologous promoter sequences by transformation or by sexual crosses triggers transcriptional gene silencing (TGS) in plants (Mette et al., 2000; Vaucheret and Fagard, 2001). Transformation of 35S promoter driven *GTE9* and *GTE11* cDNA (Fig. 17D), and crossing of unlinked T-DNA lines sharing ~330 bp (Weigel et al., 2000) with the enhancers in *yucca1d* background, had no effect on the activation tagged phenotype of *yucca1d* and other lines tested (Mandadi et al., 2011). These data eliminated the

role of homology dependent gene silencing (HDGS) in suppression of *yucca1d* phenotype. This also suggests that loss of function mutants of *gte9-1*, *gte11-1*, and *bt2-1* may not respond to

Transcriptional gene silencing is often accompanied by DNA methylation in the promoter regions (Vaucheret and Fagard, 2001). In accordance with this, extensive cytosine methylation of the enhancers was observed in the *yucca1d bt2-1* and *yucca1d gte11-1* double mutant lines (Fig. 15) suggesting that the BT2 complex suppresses DNA methylation of the 35S enhancers. We also believe that BT2 complex specifically recognizes the chromatin modifications at the multimer of enhancers and thus may not respond to CaMV infection that carries one enhancers.

One of the many ways DNA methylation triggers transcriptional silencing is by excluding H2A.Z, a histone H2A variant, from the transcriptionally active sites on DNA (Zilberman et al., 2008). Interestingly, genome wide studies to map the epigenetic state of various regions in eukaryotic DNA has revealed that enhancers commonly harbor two histone H2A variants, H3.3 and H2A.Z, that contribute to low nucleosome occupancy on the enhancers (Heintzman et al., 2007 Jin et al., 2009). This in turn favors the assembly of an enhanceosome complex comprising general transcription factors and co-activators like CREB-binding protein (CBP) and p300 (Ong and Corces, 2011). Thus, it is likely that loss of BT2-GTE9 complex may limit the recruitment of histone variants (H3.3 and H2A.Z) which are essential for creating dynamic chromatin at the 35S

enhancers. This situation will eventually lead to hypermethylation of the enhancers and silencing of the transgene in the activation lines.

The transcriptional co-activators CBP and p300 (Tijan et al., 1994) in the enhanceosome complex perform dual functions of histone acetylation and recognition via plant homeodomains (PHD) (Bannister and Kouzarides 1996, Ogryzko et al., 1996) and bromodomains, respectively (Dhaullin et al., 1999). The acetyl histone recognition function in CBP and p300 depends on the conserved tyrosine and asparagine residues in the 110aa bromodomain module (Dhaullin et al., 1999). Presence of conserved tyrosine and asparagine residues in the bromodomain module of Arabidopsis GTE9 and GTE11 led us to predict a similar function for these two proteins (Fig. 16). Mutation of the conserved tyrosine and asparagine in the bromodomain of the GTE9, GTE9^{mut}, suppressed the *yucca1d* activation phenotype and was accompanied with lowered *YUCCA1* expression (Fig. 17 C & D). We also found that overexpression of the bromodomain (BRD) from GTE9 and GTE11 suppressed the *yucca1d* phenotype (Fig. 17 A & B). Presumably, overexpression of BRD from GTE9 and GTE11 in *yucca1d* results in copious amounts of GTE9:BRD that sequesters GTE9 recognition sites on the chromatin, thus causing inhibitory effect on 35S-enhancer activity.

These data imply that the BRD module in GTE9 and GTE11 is vital for 35S enhancer activity and overexpression of the acetyl lysine-recognizing domain negatively affects the enhancer function. Apart from recognizing

acetylated lysines on histones, bromodomain also recognizes acetyl lysines on non-histone proteins (Mujtaba et al., 2002; Mujtaba et al., 2004; Huang et al., 2009), and its possible that GTE9 and GTE1 may anchor transcriptional activators (HATs) on the enhancers by selectively binding to acetyl lysines on these proteins.

The above mentioned hypothesis is based on the assumption that GTE9 and GTE11 bind to acetyl histones. However, interaction between acetyl histones and GTE9 has not been confirmed yet, instead we have demonstrated that GTE9 interacts with BT2 *in vivo* (Chapter II). Thus, based on what is known about GTE9 interaction we propose a working model that GTE9/GTE11 /BT2 complex is indispensable for 35S enhancer mediated gene activation. Here the GTE9 and GTE11 bromodomain proteins function as chromatin adaptor that recruits factors, such as the BT2-CULLIN E3 ubiquitin ligase. BT2 provides specificity to the 35S enhancer function by recognizing targets for polyubiquitination or monoubiquitination via its TAZ domain to activate gene expression. Additional specificity at the BT2 complex is achieved by the Ca⁺⁺ sensing calmodulin binding domain at the C-terminus. Thus, future identification and biochemical characterization of proteins that interact with GTE9/GTE11/BT2 complex will allow us to elaborate on the exact mechanism of the 35S enhancer function in plants.

Materials and Methods

Plant growth conditions and materials

Plants were grown in soil in 14 h light/10 h dark at 21 °C, under a light intensity of ~120-130 $\mu\text{mol}/\text{m}^2\text{s}$ 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 $\mu\text{mol}/\text{m}^2\text{s}$). All media contained 1% sucrose.

Transgenic lines, plasmids and constructs

BT2, *GTE9*, *GTE11* overexpression lines, *35S:YUC1*, *yucca1d*, *bt2-1*, *bet9-1*, and *bet10-1* lines were previously described (Zhao et al., 2001; Ren et al., 2007, Chapter II). For overexpression of BRD^{GTE9} and BRD^{GTE11}, the conserved 110aa bromodomain (BRD) sequence corresponding to BRD domain of GTE9 and GTE11 (Florence and Faller, 2001) was cloned in the binary vector pCBK05, under the control of CaMV 35S promoter. The acetyl recognizing residues, tyrosine (Y) and asparagine (A), were identified after sequence alignment of the BRD of GTE9 and GTE11 with the known acetyl recognizing proteins from metazoans (Box plot figure). GTE9^{mut} construct was generated by site directed mutagenesis of the Y (70) and N (71) to alanine (A) in the GTE9 BRD domain (Zheng and Zhou, 2002). QuickChange II mutagenesis kit was

used for site directed mutagenesis using manufacturer's instructions (Stratagene). The mutated PCR products were cloned into pGem-T easy vector and later subcloned into the binary vector pCBK05, under the control of CaMV 35S promoter. The clones thus obtained were sequenced and subsequently used for plant transformation. Transformation was carried out as described in Clough and Bent (2000).

RNA isolation and expression analysis

Total RNA was isolated from three-week-old plants using tri reagent (ambion). cDNA using. First-strand cDNA synthesis was carried out using 5 µg RNA SuperScript III (Invitrogen). The cDNA synthesized was used for PCR amplification using NEB Taq polymerase. *EIF-4A2* (At1g54270) was used as loading control for equal amount of RNA in the PCR reaction. Three biological replicates were used and PCR was repeated twice.

McrBC-based methylation assay

The methylation assay was performed using manufacturer's protocol (New England Biolabs). Approximately 1µg of genomic DNA was isolated from the respective genotypes was used for digestion in a 100 µl reaction volume with 50 units of enzyme. A mock digestion without McrBC was performed alongside for 8hrs. The digested product was subsequently amplified using primers specific enhancers:

YUCCA1 pro (-2000) 5'-AGTCCTTTGCCTCATGGTTC-3',
5'-ATATGGCGCTTCACCTCTTGT-3'

YUCCA1 pro(-400) 5'-AAAGGCATCTCCAACCTATTTTT-3'
5'- TCTTGATGGATGATGGAAAATG-3'

YUCCA1 coding region 5'-ATGGAGTCTCATCCTCACAAC-3',
5'-CAGCGATCTTAACGGCGTCA-3'

HELITRONY1d 5' - GAAGCCGCTACTCCTGTGAC-3'
5' - TGGCGCTTATCTCCTGTTCT-3'

Actin7 5'-CGTTTCGCTTTCCTTAGTGTTAGCT-3'
5'-ACTCCATAGATCTAACACATAAACC-3'

CHAPTER IV

BIOCHEMICAL CHARACTERIZATION OF GTE9

Summary

In the previous chapters we demonstrated that GTE9 physically associates with BT2 to regulate multiple responses, in addition to regulating CaMV 35S enhancer-mediated activation of genes in *Arabidopsis*. GTE9 is constitutively expressed both spatially and temporally, unlike BT2, which responds to environmental and physiological inputs perceived by plants. GTE9 is a nuclear localized protein and loss of BT2 does not alter GTE9 protein levels in the nucleus. We propose that GTE9, like other BET proteins, functions as “chromatin tag” that localizes regulatory complex of BT2-CULLIN E3 ligase to the dynamic chromatin of target promoters and 35S enhancers.

Introduction

Stable heritable genetic and epigenetic states are central to plant growth and developmental programs. The signals that establish and maintain these programs rely on environmental cues, the “epigenetic initiator” (DNA binding proteins and other transcription activators) that respond to the environmental signals and “epigenetic maintainer” (histone and DNA modifiers) that sustain the epigenetic mark on the chromatin (Berger et al., 2009).

The maintainer proteins that bind histones recognize diverse post-translational modifications on the N-terminal tails of histones such as acetylation, methylation, phosphorylation, ubiquitination and sumoylation (de la Cruz et al., 2005). Among these modifications, acetylation is linked with gene activation, whereas methylation is associated with the repression. Histone methylation is recognized by chromodomains, tudor, MBT domains and PHD fingers (Bannister et al., 2001; Lachner et al., 2001), and acetylation is exclusively recognized by bromodomains (BRDs) (Dhalluin et al., 1999; Sanchez and Zhou, 2009).

Bromodomain-containing proteins have been proposed to regulate transcription activation, transcriptional memory and silencing (Ogryzko et al., 1996; Loyola and Almouzni, 2004). The bromodomain, when present in combination with an extra-terminal (ET) domain, forms a special class of proteins called BET proteins, which play important roles in transcription activation and initiation (Lygerou et al., 1994; Florence and Faller, 2001; Wu and Chiang, 2007). Mammalian and yeast BET proteins contain tandem bromodomains (Dey et al., 2003; Kanno et al., 2004), as opposed to the single bromodomain found in Arabidopsis BET proteins (Florence and Faller, 2001). Very few BET proteins have been characterized in Arabidopsis, but they seem to function as general transcription regulators (Duque and Chua, 2003; Chua and Gray, 2007).

GTE9 belongs to a 12 member family of BET proteins in Arabidopsis (Pandey et al., 2002). Characterization of a *gte9-1* null mutant demonstrated that

GTE9 is a negative regulator of sugar and ABA responses, and *GTE9* transcript remains unaltered in response to various environmental and physiological conditions (Chapter II). We showed that GTE9 interacts with BT2-CULLIN3 E3 ligase complex *in vivo* to mediate these diverse responses (Chapter II). We also showed that GTE9 is required to maintain BT2 mediated 35S enhancer activity in Arabidopsis (Chapter III).

Here we show that GTE9 protein is localized to the nucleus, and loss of BT2 or GTE11, GTE9's closest paralog, has no effect on GTE9 protein levels. GTE9 likely functions as a transcription activating scaffold that provides general recognition to target promoters through acetyl histone interaction. Once bound to acetyl histones, GTE9, like other BET proteins, may anchor specific factors like BT2-CULLIN3 ubiquitin ligase at the target promoters to orchestrate specific transcriptional responses.

Results

GTE9 protein is constitutively expressed and nuclear localized

Polyclonal and monoclonal antibodies against full-length GTE9 protein were raised and could detect as low as 50ng of recombinant GTE9 protein (Fig. 18). However, polyclonal antibodies were best suited for immuno-detection of GTE9 in plants (Chapter II).

Using GTE9 polyclonal antibodies, we found that GTE9 protein expression was independent of temporal or spatial regulation in plants (data not shown) compared to BT2 protein, which is diurnally and developmentally regulated (Mandadi et al., 2009). Since BT2 is a component of a CULLIN3-E3 ligase complex we hypothesized that BT2 may play a role in regulating GTE9 levels. However, we found that loss of BT2 or GTE11, interacting partners in the GTE9 complex, had no effect on the GTE9 protein levels suggesting, that these proteins have no effect on the abundance of each other (Chapter II).

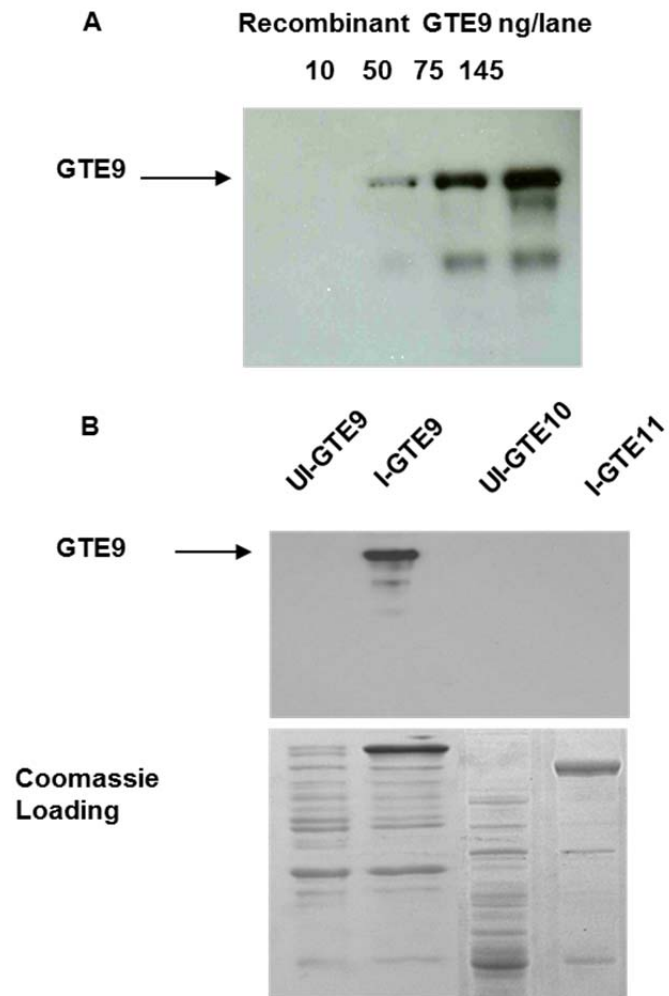


Figure 18. GTE9 polyclonal antibodies are specific to GTE9 protein. (A) Immunoblot analysis of recombinant GTE9 protein using GTE9 polyclonal antibodies. (B) Immunoblot analysis of recombinant GTE9 protein using GTE9 monoclonal antibody. GTE9 and GTE11 recombinant protein expressed from *E. coli*. GTE9 and GTE11. Uninduced and induced GTE9 and GTE11 protein extract from *E. coli* were used as negative and positive controls respectively. Coomassie stained gel was used for loading control.

GTE9 and other family members belong to the GLOBAL TRANSCRIPTION FACTOR GROUP E (GTE) class of transcription factors. The GTE9-interacting protein, BT2 is reported to be nuclear localized (Mandadi 2010; Du and Poovaiah, 2004). Based on this information, we assumed that GTE9 is localized in the nucleus. Total protein from cytoplasmic and nuclear fractions from three-week old plants was used in immunoblot analysis. As expected, we found that GTE9 signal was enriched in the nuclear fraction (Fig. 19) similar to BT2 (Mandadi 2010).

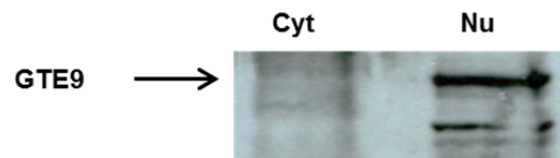


Figure 19. GTE9 protein is primarily localized in nucleus. 3-week-old wild-type was harvested and cytoplasmic (Cyt) and nuclear (Nuc) proteins were extracted and subjected to immunoblot analysis using GTE9 antibody.

Discussion

We previously showed that the GTE9/BT2 complex regulates responses to sugar and ABA stress (Mandadi et al., 2009; also in Chapter II), and is required for 35S enhancer activity (Mandadi et al., 2011; also in Chapter III). In the current study, we found that GTE9 protein is easily detectable in plants (Fig. 19) and is not regulated by diverse environmental or physiological conditions. This data is consistent with constitutive expression of GTE9 mRNA in response to various stimuli (Chapter II) and is in agreement with the ability of GTE9 and GTE11 proteins to activate transcription in a heterologous system (Du and Poovaiah, 2004). Results from the characterization of two other GTE proteins, GTE1 and GTE6, also support the idea that GTE proteins function as general transcriptional activators (Duque and Chua, 2003; Airoidi et al., 2010). Protein fractionation data is also suggestive of GTE9 functions as a transcription factor in the nucleus. However, an important question emerges from the proposed general role of these proteins, i.e. what provides the specificity to the BET protein complex in transcriptional activation.

In the case of GTE9 and GTE11 the BT2-CULLIN3 complex provides the specificity to the transcriptional complex. BT2 protein has a central TAZ domain and C-terminal Ca^{++} calmodulin binding domain. We propose that BT2 senses changes in the calcium levels and undergo a conformational change which may allow the recognition of sequence specific transcription activators by the TAZ domain to confer a more specific response.

Materials and Methods

Antibody generation and immunoblot analysis

Full-length GTE9 cDNA was cloned into pET32a (Novagen) vector and transformed into *E.coli* BL21 (DE3) *lysS* strain. 5ml culture was raised induced with 1 mM IPTG for 3 h at 25 °C in *E.coli* strain Rosetta2 (DE3) *lysS*. The protein was purified on the Hisbind column (Novagen) and purified protein was subjected to sequential rEK cleavage to remove the ~20Kda N-terminal tag on the protein. The protein thus obtained was concentrated by acetone precipitation and resuspended in 1X Laemmli sample buffer, and separated by SDS-PAGE @ 120V. GTE9 protein thus separated was excised from the gel and ~2.5µgm of *E. coli* expressed protein was shipped to Covance immunology services (Covance, Princeton, New Jersey) to obtain polyclonal anti-GTE9 antibodies. For monoclonal antibody generation around 10µg of digested GTE9 protein was given to Biology Department Microbiology (351) undergraduate lab.

For analysis of GTE9 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 MgCl₂, 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 MgCl₂, 0.25 M sucrose, 1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM PMSF, 1 mM spermine, 1 mM spermidine, 5 μl/ml Protease inhibitor cocktail (Sigma), 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.

Immunoblot analysis was performed to study GTE9 protein expression in wild-type, *bt2-1*, *gte9-1*, *gte11-1* and *gte9-1 gte11-1* lines using nuclear extracts from the respective genotypes. Three-week-old plants (2 g) were harvested in the middle of the day and nuclear extracts were prepared using nuclear extraction protocol (Cold Spring Harbor protocol). The nuclear pellet was resuspended directly in 2X Laemmli sample buffer (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 7.5% SDS-PAGE gels and blotted on the nitrocellulose membranes (Amersham). Blots were incubated first with primary anti-GTE9 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. Protein visualization was performed by chemiluminescence using Super Signal West Pico detection reagents (Pierce)

and Hyperfilm-ECL films (Amersham). Equal loading was confirmed by Coomassie staining the nuclear extracts from each genotype.

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

We previously showed that BT2, a BTB domain protein, regulates telomerase activity in mature, vegetative organs (Ren et al., 2007) and responds to diverse hormone and stress signals (Mandadi et al., 2009). BTB-domain proteins interact with CULLIN3 and form E3 ubiquitin ligase complexes *in vivo* (Pintard et al., 2003). Specifically, BT2 interacts with CULLIN3 in *in vitro* pull down assays (Figueroa et al., 2005; Gingerich et al., 2005), and we have demonstrated this interaction genetically as well (Mandadi et al., 2011). BT2 also interacts with GTE9 and GTE11, bromodomain and extra-terminal (BET) proteins, in a yeast-two hybrid assay and *in vitro* pull down assay (Du and Poovaiah, 2004) and in *in vivo* co-immunoprecipitation assay (Chapter II). GTE9 and GTE11 belong to 12 member family in Arabidopsis, also referred as Global Transcription Factor Group E (GTE). Very little is known about the Arabidopsis GTE family and prior to this work only 4 members of the GTE family had been characterized (Table 1.) (Pandey et al., 2002).

In the current study we characterized GTE9 and GTE11 proteins and presented genetic and biochemical evidence for GTE9/GTE11 interaction with BT2. We showed that both *gte9-1* and *gte11-1* null-mutants phenocopied a *bt2-1* null mutant for germination response to sugar and ABA. Furthermore, loss of GTE9 and GTE11 blocks the BT2-mediated resistance to sugars and ABA (Chapter II). Although GTE9 and GTE11 have a distinct phenotypic response to

sugar and ABA stress, their mRNA levels do not change like BT2 transcript in response to sugars and ABA (Chapter II , Mandadi et al., 2009). The unaltered *GTE9* and *GTE11* transcript levels in response to sugar and ABA are consistent with their function as general transcription factors (Pandey et al., 2002; Du and Poovaiah, 2004). Moreover, the absence of multiple signal responsive *cis*-elements that are found in the BT2 promoter (Mandadi dissertation 2010), from the *GTE9* and *GTE11* promoters also corroborate the observed constitutive expression pattern for both. These data are suggestive of post-transcriptional regulation by GTE9 and GTE11 for sugar and ABA signaling pathways.

However, we did not find any change in the GTE9 protein in response to either sugars or ABA and suspect the same for GTE11 (Chapter II). Additionally, we found steady state levels of *BT2* transcript were unaffected in the single mutants of *gte9-1*, *gte11-1*(Chapter II) and double mutants of *gte9-1 gte11-1* (data not shown), indicating that BT2 interaction with GTE9 and GTE11 is post-transcriptional. Using BT2 antibody, we immunoprecipitated GTE9 protein and demonstrated that this interaction is indeed at the protein level (Chapter II).

Further characterization of BT2's role in ABA signaling pathway indicated that BT2 may regulate the ABA signaling at the posttranscriptional level (Mandadi et al., 2009). Analysis of protein levels of ABA signaling components such as ABI5, in mutant background of *gte9-1*, *gte11-1* and *bt2-1* might uncover the role of this complex in ABA signaling. Alternatively, GTE9/GTE11/BT2 complex may localize at the promoter of ABA signaling/responsive genes to

regulate expression of the downstream effectors in the sugar and ABA signaling pathways. A microarray based expression profiling of *gte9-1 gte11-1* and *bt2-1* mutants treated with sugar or ABA will allow us to identify candidate genes directly regulated by the GTE9/GTE11/BT2 complex.

We also showed that the GTE9/GTE11/BT2 complex regulates CaMV 35S enhancer-mediated activation of gene expression in Arabidopsis (Chapter III, Mandadi et al., 2011). Loss of GTE9 or GTE11, similar to loss of BT2, in two independent activation-tagged lines resulted in suppression of the 35S enhancer-associated activation phenotypes in both the lines (Mandadi et al., 2011; also in Chapter III). The suppression phenotype in activation tagged lines was accompanied by loss of transcript from the activated gene and hypermethylation of the 35S enhancers. The loss of transcript in these lines was attributed to impaired transcription, and was not due to unstable RNA of the activated gene in 35S enhancer lines (Mandadi et al., 2011).

Based on the GTE protein domain structure and known interaction with BT2, we proposed a working model to explain how the GTE9/GTE11/BT2 complex may regulate a variety of phenotypes (Chapter I; Fig. 19). We propose that GTE9 and GTE11, like other BET proteins, can recognize acetyl histones on the transcriptionally competent promoters in a bromodomain (BRD) dependent manner. The GTE proteins then recruit the BT2-CULLIN3 E3 ubiquitin ligase and sequence specific transcription factors. To validate this hypothesis we mutated the acetyl lysine recognizing residues, Y70A, N71A, and overexpressed the

BRD in the *yucca* mutant background. We found that BRD was indeed critical for the GTE9/GTE11 function, because overexpression of the 35S:*GTE9*^{Y70A, N71A} and *GTE9*^{BRD} alone suppressed the activation tagged phenotype in *yucca1d* (Chapter III). Although this is an indirect evidence for recognition of acetylated histones by GTE9/GTE11, we propose to perform *in vivo* co-immunoprecipitation using GTE9 antibody to immunoprecipitate acetyl histones and vice versa. Alternatively, we can express *GTE9*^{Y70A, N71A} in *E. coli* and use it in *in vitro* binding assays to pull down acetyl histones. The wild-type GTE9 should immunoprecipitate the modified histone peptide and the mutant form should not. Biochemical characterization of the interaction between GTE9/GTE11 BRD and acetyl histones should strengthen this model. Here the GTE proteins, GTE9 and GTE11, will serve to recognize the modified chromatin on target promoters. Whereas the BT2-CULLIN3 E3 ligase complex will provide sequence-specific recognition by recruiting transcription factors through the TAZ domain upon sensing changes in Ca⁺⁺ levels through the C-terminal calcium calmodulin binding domain (CaMBD) (Fig. 20).

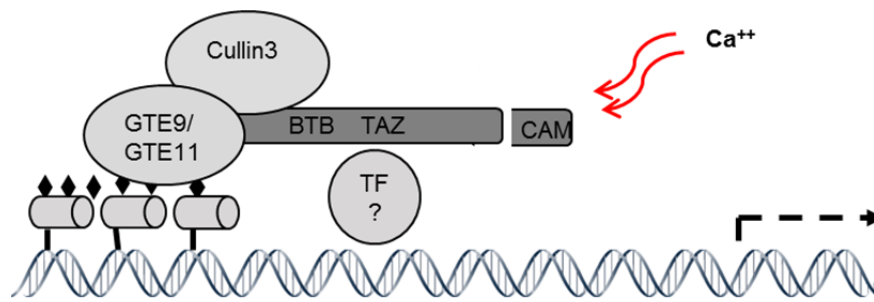


Figure 20. Working model for GTE9/GTE11/BT2 complex. Bromodomain in the GTE proteins recognize acetyl lysines (black diamonds) on histones. The (transcriptional activation domain) TAD of GTE protein interacts with BT2's BTB domain to localize it on the chromatin in response to multiple signals and changes in intracellular Ca^{++} levels.

GTE proteins contain a canonical protein-protein interaction motif, the extra-terminal (ET) domain, in addition to a transcriptional activation domain (TAD) (Chapter I and II). The BT2 interaction is reported at the TAD domain, however, there are no known interacting proteins for plant ET domains. Identification of cellular factors interacting with the ET domain may provide additional ways of transcriptional regulation by GTE proteins. Interestingly, Rahman and co-workers recently showed that human BET protein, Brd4 ET domain, interacts with NSD3, a histone methyltransferase, which adds a trimethyl group, H3K36 to activate transcription. They also showed that this interaction is highly conserved among Brd4 homologs. This is attributed to >80% identity between the ET domain of the Brd4 homologs and orthologs. Since the

ET domain is highly conserved across species it is possible that GTE9 and GTE11 may interact with Arabidopsis homolog(s) of NSD3. Thus, an *in vitro* or *in vivo* interaction between Arabidopsis ortholog of human NSD3 and GTE9 will provide evidence for an additional mode of transcriptional activation by GTE9 and GTE11. However, our model remains the same except, now the GTE9/GTE11 scaffold may recruit additional factors like NSD3 to activate transcription. Upon characterizing the GTE9 and NSD3 interaction we can perform additional assays like ChIP analysis using antibodies specific to histone H3K36me3 to immunoprecipitate 35S enhancer or *yucca* promoter.

We also showed that loss of BT2, GTE9 and GTE11 results in hypermethylation of enhancers (Chapter III). Zilberman and co-workers showed that DNA methylation antagonizes the establishment of H2A.Z, a H2A histone variant often found at enhancers (Chapter III, discussion). It is possible that the GTE9/GTE11/BT2 complex may stabilize the H2A.Z recruitment on the enhancers by targeting DNA methyltransferases (DNMTs), enzymes that carry out DNA methylation, for polyubiquitination and degradation. Once the GTE9/GTE11/BT2 complex is lost the H2A.Z cannot maintain a stable interaction in the nucleosome and thus the DNMTs can methylate cytosines to create a repressive environment at the enhancers. To test this hypothesis we can perform ChIP assays using antibodies against H2A.Z to enrich for 35S enhancers from the *yucca1d* mutant using *yucca1d bt2-1*, *yucca1d gte9-1* and *yucca1d gte11-1* double mutants as controls. Absence of 35S enhancer signal

from these double mutants will support the hypothesis that the GTE9/GTE11/BT2 complex is indeed required to stabilize the H2A.Z association within the nucleosome of the enhancers.

Additionally, using GTE9 polyclonal antibodies we have demonstrated that GTE9 protein is nuclear localized (Chapter IV) and can specifically recognize GTE9 protein in nuclear extracts (Chapter IV). Thus, we propose to use GTE9 antiserum to perform ChIP-sequencing of the whole genome to identify promoter targets of GTE9/GTE11/BT2 complex using wt, *yucca1d*, *yucca1d gte9 gte11* and *gte9 gte11* double mutant chromatin. We expect to confirm localization to 35S enhancers and identify new promoter targets for the GTE/BT2 protein complex in Arabidopsis to better understand the role of this complex in myriad plant signal transduction pathways.

It is also possible that the 12 GTE proteins form multiple transcriptional scaffolds with the other 80 BTB proteins. A preliminary evidence for this comes from the reported interaction between GTE11 and two other BT family members, BT1 and BT4, along with BT2 (Du and Poovaiah, 2004). Thus Arabidopsis GTE proteins and BTB proteins may assemble into multiple scaffolds to confer a variety of transcriptional responses in plants. To test this hypothesis we can perform mass spectrometric analysis of immunoprecipitated complexes from wild-type using GTE9 antibody and determine if additional BT proteins associate with it *in vivo*.

The 12 GTEs and 80 BTB proteins provide many combinatorial possibilities for gene regulation. Further investigation of GTE and BTB proteins will reveal how broadly the regulatory model developed here can be applied to transcriptional control of gene expression in plants.

REFERENCES

- Agalioti T, Lomvardas S, Parekh B, Yie J, Maniatis T, Thanos D (2000)** Ordered recruitment of chromatin modifying and general transcription factors to the IFN- β promoter. *Cell* **103**: 667-678
- Aharoni A, Dixit S, Jetter R, Thoenes E, van Arkel G, Pereira A (2004)** The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle, and confers drought tolerance when overexpressed in *Arabidopsis*. *Plant Cell* **16**: 2463-2480
- Airoldi CA, Della RF, Falasca G, Marino G, Kookier M, Altmura MM, Citterio S, Kater MM (2010)** The *Arabidopsis* BET bromodomain factor GTE4 is involved in maintenance of the mitotic cell cycle during plant development. *Plant Physiol* **152**: 1320-1334
- Allfrey V, Faulkner R, Mirsky A (1964)** Acetylation and methylation of histones and their possible role in the regulation of RNA synthesis. *Proc Natl Acad Sci USA* **51**: 786-794
- Alonso JM, Steponova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmermann J, Barajas P, Cheuk R, et al. (2003)** Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. *Science* **301**: 653-657
- An G, Jeong D-H, Jung K-H, Lee S (2005)** Reverse genetic approaches for functional genomics of rice. *Plant Mol Biol* **59**: 111-123
- Arenas-Huertero F, Arroyo A, Zhou L, Sheen J, León 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, Sheen J (2007)** A central integrator of transcription networks in plant stress and energy signalling. *Nature* **448**: 938-942
- Bannister AJ, Kouzarides T (1996)** The CBP co-activator is a histone acetyltransferase. *Nature* **384**: 641-3
- Bannister AJ, Kouzarides T (2011)** Regulation of chromatin by histone modifications. *Cell Res* **21**: 381-395

- Bannister AJ, Zegerman P, Partridge J, Miska E, Thomas J, Allshire R, Kouzarides T** (2001) Selective recognition of methylated lysine 9 on histone H3 by the HP1 chromo domain. *Nature* **410**: 120-124
- Benfey PN and Chua N-H** (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. *Science* **250**: 959-966
- Benfey PN, 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
- Berger S, Kouzarides T, Shiekhata R, Shilatfard A** (2009) An operational definition of epigenetics. *Genes Dev* **23**: 781-783
- Blackwood E, Kadonaga J** (1998) Going the Distance: A current view of enhancer action. *Science* **281**: 60-63
- Borevitz J, Xia Y, Blount J, Dixon RA, Lamb C** (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* **12**: 2383-2394
- Boyle P, Le Su E, Rochon A, Shearer H, Murmu J, Chu J, Fobert P, Després C** (2009) The BTB/POZ domain of the *Arabidopsis* disease resistance protein NPR1 interacts with the repression domain of TGA2 to negate its function. *Plant Cell* **21**: 3700-3713
- Brownell J, Allis C** (1996) Special HATs for special occasions: Linking histone acetylation to chromatin assembly and gene activation. *Current Opinion in Genetics & Develop.* **6**: 176-184
- Bulger M, Groudine M** (1999) Looping versus linking: Toward a model for long-distance gene activation. *Genes Dev* **13**: 2465-2477
- Calhoun V, Stathopoulos A, Levine M** (2002) Promoter-proximal tethering elements regulate enhancer-promoter specificity in the *Drosophila* Antennapedia complex. *Proc Natl Acad Sci USA* **99**: 9243-9247
- Candeau R, Zhou JX, Allis CD, Berger SL** (1997) Histone acetyltransferase activity and interaction with ADA2 are critical for GCN5 function in vivo. *16*: 555-65
- Cary P, Crane-Robinson C, Bradbury E, Dixon G** (1982) Effect of acetylation on the binding of N-terminal peptides of histone H4 to DNA. *Eur J Biochem* **127**: 137-144

- Chandler VL, Stam L, Sidorenko LV** (2002) Long distance cis and trans interactions mediate pramutations. *Adv Genetics* **46**: 215-34
- Cheng W, Endo A, Zhou L, Penney J, Chen H, Arroyo A, Leon P, Nambara E, Asami T, Seo M, Koshiba T, Sheen J** (2002) A unique short-chain dehydrogenase/reductase in *Arabidopsis* glucose signaling and abscisic acid biosynthesis and functions. *Plant Cell* **14**: 2723-2743
- Cheng Y, Dai X, 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
- Chenna R, Sugawara H, Koike T, Lopez R, Gibson TJ, Higgins DG, Thompson JD** (2003) Multiple sequence alignment with the clustal series of programs. *Nuc. Acid Res.* **31**: 3497-500
- Chinnusamy V, Zhu J-K** (2010) Epigenetic regulation: Chromatin modeling and small RNAs. *In* A Pareek, SK Sopory, HJ Bohnert, eds, *Abiotic Stress Adaptation in Plants*. Springer, The Netherlands, pp 217-241
- Chua P, Roeder G** (1995) Bdf1, a yeast chromosomal protein required for sporulation. *Mol Cell Biol* **15**: 3685-3696
- Chua Y, Channelière S, Mott E, Gray J** (2005) The bromodomain protein GTE6 controls leaf development in *Arabidopsis* by histone acetylation at ASYMMETRIC LEAVES1. *Genes Dev* **19**: 2245-2254
- Chua Y, Gray J** (2007) *Histone modifications and transcription in plants*. Blackwell Publishing Ltd
- Clough S and Bent A** (1998) Floral dip: A simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J* **16**: 735-743
- de la Cruz X, Lois S, Sánchez-Molina S, Martínez-Balbás MA** (2005) Do protein motifs read the histone code? *BioEssays* **27**: 164-175
- Deckert J, Struhl K** (2001) Histone acetylation at promoters is differentially affected by specific activators and repressors. *Mol Cell Biol* **21**: 2726-2735
- DeLange R, Fambrough D, Smith E, Bonner J** (1969) Calf and Pea histone IV. *J Biol Chem* **244**: 319-334

- Denis G, Vaziri C, Guo N, Faller D** (2000) RING3 kinase transactivates promoters of cell cycle regulatory genes through E2F. *Cell Growth Differ* **11**: 417-424
- Denis GV** (2001) Bromodomain motifs and “scaffolding”?. *Front Biosci* **6**: d1065-1068
- Dey A, Chitsaz F, Abbasi A, Misteli T, Ozato K** (2003) The double bromodomain protein Brd4 binds to acetylated chromatin during interphase and mitosis. *Proc Natl Acad Sci USA* **100**: 8758-8763
- Dey A, Ellenberg J, Farina A, Coleman A, Maruyama T, Sciortino S, Lippincott-Schwartz J, Ozato K** (2000) A bromodomain protein, MCAP, associates with mitotic chromosomes and affects G2-to-M transition. *Mol Cell Biol* **20**: 6537-6549
- Dhalluin C, Carlson JE, Zeng L, Aggarwal A, Zhou M** (1999) Structure and ligand of a histone acetyltransferase bromodomain. *Nature* **399**: 491-496
- Digan M, Haynes S, Mozer B, Dawid I, Forquignon F, Gans M** (1986) Genetic and molecular analysis of fs(1)h, a maternal effect homeotic gene in *Drosophila*. *Dev Biol* **114**: 161-169
- Du L, Poovaiah B** (2004) A novel family of Ca²⁺/calmodulin-binding proteins involved in transcriptional regulation: Interaction with fsh/Ring3 class transcription activators. *Plant Mol Biol* **54**: 549-569
- Duque P, Chua N** (2003) IMB1, a bromodomain protein induced during seed imbibition, regulates ABA- and phyA-mediated responses of germination in *Arabidopsis*. *Plant J.* **35**: 787-799
- Fang RX, Nagy F, Sivasubramaniam S and Chua NH** (1989) Multiple *cis* regulatory elements for maximal expression of the cauliflower mosaic virus 35S promoter in transgenic plants. *Plant Cell* **1**: 141-150.
- Fedoroff N** (2002) Cross-talk in abscisic acid signaling. *Sci STKE* **140**: re10-15
- Figueroa P, Gusmaroli G, Serino G, Habashi J, Ma L, Shen Y, Feng S, Bostick M, Callis J, Hellmann H, Deng X** (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

- Fischle W, Wang Y, David Allis C** (2003) Binary switches and modification cassettes in histone biology and beyond. *Nature* **425**: 475-479
- Florence B, Faller DV** (2001) You BET-CHA: A novel family of transcriptional regulators. *Front Biosci* **6**: D1008-D1018
- Fuchs J, Demidov D, Houben A, Schubert I** (2006) Chromosomal histone modification patterns - from conservation to diversity. *Trends in Plant Sci* **11**: 199-208
- Georgiou S and Ababneh AM** (2005) Maintenance of open DNA base pairs through histone acetylated lysine-purine interaction leading to transcriptional activation: a proposed mechanism. *Int J Mol Med* **16(5)**: 911-7
- Gingerich D, Gagne J, Salter D, Hellmann H, Estelle M, Vierstra R** (2005) Cullins 3a and 3b assemble with members of the broad complex/tramtrack/bric-a-brac (BTB) protein family to form essential ubiquitin-protein ligases (E3s) in *Arabidopsis*. *J Biol Chem* **280**: 18810-18821
- Hansen J, Tse C, Wolffe A** (1998) Structure and function of the core histone N-Termini: More than meets the eye. *Biochemistry* **37**: 17637-17641
- Hassan A, Neely K, Workman J** (2001) Histone acetyltransferase complexes stabilize SWI/SNF binding to promoter nucleosomes. *Cell* **104**: 817-827
- Hassan A, Prochasson P, Neely K, Galasinski S, Chandy M, Carrozza M, Workman J** (2002) Function and selectivity of bromodomains in anchoring chromatin-modifying complexes to promoter nucleosomes. *Cell* **111**: 369-379
- Hatzis P, Talianidis I** (2002) Dynamics of enhancer-promoter communication during differentiation-induced gene activation. *Mol Cell* **10**: 1467-1477
- Hayashi H, Czaja I, Lubenow H, Schell J, Walden R** (1992) Activation of a plant gene by T-DNA tagging: Auxin-independent growth in vitro. *Science* **258**: 1350-1353
- Haynes S, Dollard C, Winston F, Beck S, Trowsdale J, Dawid I** (1992) The bromodomain: A conserved sequence found in human, *Drosophila* and yeast proteins. *Nucleic Acids Res* **20**: 2603

- Heintzman ND, Stuart RK, Hon G, Fu Y, Ching CW, Hawkins RD, Barrera LO, Van Calcar S, Qu C, Ching KA, Wang W, Weng Z, Green RD, Crawford GE, Ren B** (2007) Distinctive and predictive chromatin signatures of transcriptional promoters and enhancers in the human genome. *Nat Genet.* **39**: 311-8
- Houzelstein D, Bullock S, Lynch D, Grigorieva E, Wilson V, Beddington R** (2002) Growth and early postimplantation defects in mice deficient for the bromodomain-containing protein Brd4. *Mol Cell Biol* **22**: 3794-3802
- Hsing Y, Chern C, Fan M, Lu P, Chen K, Lo S, Sun P, Ho S, Lee K, Wang Y, Huang W, Ko S, Chen S, Chen J, Chung C, Lin Y, Hour A, Wang Y, Chang Y, Tsai M, Lin Y, Chen Y, Yen H, Li C, Wey C, Tseng C, Lai M, Huang S, Chen L, Yu S** (2007) A rice gene activation/knockout mutant resource for high throughput functional genomics. *Plant Mol Biol* **63**: 351-364
- Huang B, Yang X, Zhou M, Ozato K, Chen L** (2009) Brd4 coactivates transcriptional activation of NF- κ B via specific binding to acetylated RelA. *Mol Cell Biol* **29**: 1375-1387
- Hudson B, Martinez-Yamout MA, Dyson HJ, Wright PE** (2000) Solution structure and acetyl-lysine binding activity of the GCN5 bromodomain. *J Mol Biol* **304**: 355-370
- Huijser C, Kortstee A, Pego J, Weisbeek P, Wisman E, Smeekens S** (2000) The *Arabidopsis* SUCROSE UNCOUPLED-6 gene is identical to ABCISIC ACID INSENSITIVE-4: involvement of abscisic acid in sugar responses. *Plant J* **23**: 577-585
- Ishikawa K, Fukuda E, Kobayashi I** (2010) Conflicts targeting epigenetic systems and their resolution by cell death: Novel concepts for methyl-specific and other restriction systems. *DNA Res* **17**: 325-342
- Jeanmougin F, Wurtz J-M, Le Douarin B, Chambon P, Losson R** (1997) The bromodomain revisited. *Trends Biochem Sci* **22**: 151-153
- Jin C, Zang C, Wei G, Cui K, Peng W, Zhao K, Felsenfeld G** (2009) H3.3/H2A.Z double variant-containing nucleosomes mark 'nucleosome-free' regions of active promoters and other regulatory regions. *Nat Genet.* **41**: 914-5

- Jones MH, Numata M, Shimane M** (1997) Identification and characterization of BRDT: A testis-specific gene related to the bromodomain genes RING3 and Drosophila fsh. *Genomics* **45**: 529-534
- Kay R, Chan A, Daly M and McPherson J** (1987) Duplication of the CAMV 35S promoter sequences creates a strong enhancer for plant genes. *Science* **236**: 1299-302
- Kanno T, Kanno Y, Siegel RM, Jang MK, Lenardo MJ, Ozato K** (2004) Selective recognition of acetylated histones by bromodomain proteins visualized in living cells. *Mol Cell* **13**: 33-43
- Kim MJ, Shin R, Schachtman DP** (2009) A nuclear factor regulates abscisic acid responses in *Arabidopsis*. *Plant Physiol* **151**: 1433-1445
- Kondou Y, Higuchi M, Matsui M** (2010) High-throughput characterization of plant gene functions by using gain-of-function technology. *Ann Rev Plant Biol* **61**: 373-393
- Kruhlak MJ, Hendzel MJ, Fischle W, Bertos NR, Hameed S, Yang X-J, Verdin E, Bazett-Jones DP** (2001) Regulation of global acetylation in mitosis through loss of histone acetyltransferases and deacetylases from chromatin. *J Biol Chem* **276**: 38307-38319
- Krysan PJ, Young JC, Sussman MR** (1999) T-DNA as an insertional mutagen in *Arabidopsis*. *Plant Cell* **11**: 2283-2290
- Laby RJ, Kincaid MS, Kim D, Gibson SI** (2000) The *Arabidopsis* sugar-insensitive mutants *sis4* and *sis5* are defective in abscisic acid synthesis and response. *Plant J* **23**: 587-596
- Lachner M, O'Carroll D, Rea S, Mechtler K, Jenuwein T** (2001) Methylation of histone H3 lysine 9 creates a binding site for HP1 proteins. *Nature* **410**: 116-120
- Ladurner AG, Inouye C, Jain R, Tjian R** (2003) Bromodomains mediate an acetyl-histone encoded antisilencing function at heterochromatin boundaries. *Mol Cell* **11**: 365-376
- Lam E, Chua NH** (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, Lam YK-P** (1995) Binding site requirements and differential representation of TGA factors in nuclear ASF-1 activity. *Nuc Acids Res* **23**: 3778-3785
- Lam E, Katagiri F and Chua NH** (1990) Plant nuclear factor ASF-1 binds to an essential region of the nopaline synthase promoter. *J. Biol. Chem.* **265**: 9909-9913
- Lee A-Y, Chiang C-M** (2009) Chromatin adaptor Brd4 modulates E2 transcription activity and protein stability. *J Biol Chem* **284**: 2778-2786
- Loyola A, Almouzni G** (2004) Bromodomains in living cells participate in deciphering the histone code. *Trends Cell Biol* **14**: 279-281
- Luger K, Madder W,** (1997) Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature* **389(6648)**: 251-260
- Lygerou Z, Conesa C, Lesage P, Swanson RN, Ruet A, Carlson M, Sentenac A, Séraphin B** (1994) The yeast BDF1 gene encodes a transcription factor involved in the expression of a broad class of genes including snRNAs. *Nuc Acids Res* **22**: 5332-5340
- Mandadi KK, Misra A, McKnight TD** (2011) BT2, is required for cauliflower mosaic virus mediated activation of gene expression in *Arabidopsis*. (In-preparation)
- Mandadi KK, Misra A, Ren SX, McKnight TD** (2009) BT2, a BTB Protein, mediates multiple responses to nutrients, stresses, and hormones in *Arabidopsis*. *Plant Physiol* **150**: 1930-1939
- Marcus GA, Silverman N, Berger SL, Horiuchi J, Guarente L.** (1994) Functional similarity and physical association between GCN5 and ADA2: Putative transcriptional adaptors. *EMBO J* **13**: 4807-15
- Martinez-Garcia JF, Monte E and Quail PH** (1999) A simple, rapid and quantitative method for preparing *Arabidopsis* protein extracts for immunoblot analysis. *Plant J.* **20**: 251–257
- Maruyama T, Farina A, Dey A, Cheong J, Bermudez VP, Tamura T, Sciortino S, Shuman J, Hurwitz J, Ozato K** (2002) A mammalian bromodomain protein, Brd4, interacts with replication factor C and inhibits progression to S phase. *Mol Cell Biol* **22**: 6509-6520

- Matangkasombut O, Buratowski RM, Swilling NW, Buratowski S** (2000) Bromodomain factor 1 corresponds to a missing piece of yeast TFIID. *Genes Dev* **14**: 951-962
- Mathews H, Clendennen SK, Caldwell CG, Liu XL, Connors K, Matheis N, Schuster DK, Menasco DJ, Wagoner W, Lightner J, Wagner DR** (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. *Plant Cell* **15**: 1689-1703
- Matzke MA, Aufsatz W, Kanno T, Mette MF, Matzke AJ** (2002) Homology dependent gene-silencing and host defense in plants. *Adv. Genetics* **46**: 235-75
- McPhillips MG, Oliveira JG, Spindler JE, Mitra R, McBride AA** (2006) Brd4 is required for E2-mediated transcriptional activation but not genome partitioning of all papillomaviruses. *J Virol* **80**: 9530-9543
- Mette MF, Aufsatz W, van der Winden J, Matzke MA, Matzke AJM** (2000) Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *EMBO J* **19**: 5194-5201
- Mitsuhara I, Ugaki M, Hirochika H, Ohshima M, Murakami T, Gotoh Y, Katayose Y, Nakamura S, Honkura R, Nishimiya S, Ueno K, Mochizuki A, Tanimoto H, Tsugawa H, Otsuki Y, Ohashi Y** (1996) Efficient promoter cassettes for enhanced expression of foreign genes in dicotyledonous and monocotyledonous Plants. *Plant Cell Physiol* **37**: 49-59
- Moore B, Zhou L, Rolland F, Hall Q, Cheng W-H, Liu Y-X, Hwang I, Jones T, Sheen J** (2003) Role of the *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science* **300**: 332-336
- Mori M, Tomita C, Sugimoto K, Hasegawa M, Hayashi N, Dubouzet J, Ochiai H, Sekimoto H, Hirochika H, Kikuchi S** (2007) Isolation and molecular characterization of a spotted leaf mutant by modified activation-tagging in rice. *Plant Mol Biol* **63**: 847-860
- Mujtaba S, He Y, Zeng L, Farooq A, Carlson JE, Ott M, Verdin E, Zhou M-M** (2002) Structural basis of lysine-acetylated HIV-1 Tat recognition by PCAF bromodomain. *Mol Cell* **9**: 575-586
- Mujtaba S, He Y, Zeng L, Yan S, Plotnikova O, Sachchidanand, Sanchez R, Zeleznik-Le NJ, Ronai Ze, Zhou M-M** (2004) Structural mechanism of

the bromodomain of the coactivator CBP in p53 transcriptional activation. *Mol Cell* **13**: 251-263

Mujtaba S, Zeng L, Zhou MM (2007) Structure and acetyl-lysine recognition of the bromodomain. *Oncogene* **26**: 5521-5527

Nakamura Y, Umehara T, Nakano K, Jang MK, Shirouzu M, Morita S, Uda-Tochio H, Hamana H, Terada T, Adachi N, Matsumoto T, Tanaka A, Horikoshi M, Ozato K, Padmanabhan B, Yokoyama S (2007) Crystal structure of the human BRD2 bromodomain. *J Biol Chem* **282**: 4193-4201

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

Ogryzko VV, Schiltz RL, Russanova V, Howard BH, Nakatani Y (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferases. *Cell* **87**: 953-959

Ohtsuki S, Levine M, Cai HN (1998) Different core promoters possess distinct regulatory activities in the *Drosophila* embryo. *Genes Dev* **12**: 547-556

Oki M, Aihara H, Ito T (2007) Role of histone phosphorylation in chromatin dynamics and its implications in diseases. TK Kundu, R Bittman, D Dasgupta, H Engelhardt, L Flohe, H Herrmann, A Holzenburg, HP Nasheuer, S Rottem, M Wyss, P Zwickl, eds, *Chromatin and Disease*, Vol 41. Springer, The Netherlands, pp 323-340

Ong C-T, Corces VG (2011) Enhancer function: New insights into the regulation of tissue-specific gene expression. *Nat Rev Genet* **12**: 283-293

Ornaghi P, Ballario P, Lena AM, González A, Filetici P (1999) The bromodomain of Gcn5p interacts in vitro with specific residues in the N terminus of histone H4. *J Mol Biol* **287**: 1-7

Ørom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R (2010) Long noncoding RNAs with enhancer-like function in human Cells. *Cell* **143**: 46-58

Ottinger M, Christalla T, Nathan K, Brinkmann MM, Viejo-Borbolla A, Schulz TF (2006) Kaposi's Sarcoma-Associated Herpesvirus LANA-1 interacts with the short variant of BRD4 and releases cells from a BRD4- and BRD2/RING3-induced G1 cell cycle arrest. *J Virol* **80**: 10772-10786

- Owen DJ, Ornaghi P, Yang J-C, Lowe N, Evans PR, Ballario P, Neuhaus D, Filetici P, Travers AA** (2000) The structural basis for the recognition of acetylated histone H4 by the bromodomain of histone acetyltransferase Gcn5p. *EMBO J* **19**: 6141-6149
- Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D** (2003) Control of leaf morphogenesis by microRNAs. *Nature* **425**: 257-263
- Pandey R, Muller A, Napoli CA, Selinger DA, Pikaard CS, Richards EJ, Bender J, Mount DW, Jorgensen RA** (2002) Analysis of histone acetyltransferase and histone deacetylase families of *Arabidopsis thaliana* suggests functional diversification of chromatin modification among multicellular eukaryotes. *Nuc Acids Res* **30**: 5036-5055
- Patthy L, Smith EL, Johnson J** (1973) Histone III. *J Biol Chem* **248**: 6834-6840
- Pego JV, Kortstee AJ, Huijser C, Smeekens SCM** (2000) Photosynthesis, sugars and the regulation of gene expression. *J Exp Bot* **51**: 407-416
- Peng J, Dong W, Chen L, Zou T, Qi Y, Liu Y** (2007) Brd2 is a TBP-associated protein and recruits TBP into E2F-1 transcriptional complex in response to serum stimulation. *Mol Cell Biochem* **294**: 45-54
- Perriere and Gouy** (1996) WWW-query: An on-line retrieval systems for biological sequence banks. *Biochem* **78**: 364-9
- Peterson CL and Laniel M-A** (2004) Histones and histone modifications. *Curr Biol* **14**: R546-R551
- Pflugger J, Wagner D** (2007) Histone modifications and dynamic regulation of genome accessibility in plants. *Curr Opin Plant Biol* **10**: 645-652
- Pintard L, Willis JH, Willems A, Johnson J-LF, Srayko M, Kurz T, Glaser S, Mains PE, Tyers M, Bowerman B, Peter M** (2003) The BTB protein MEL-26 is a substrate-specific adaptor of the CUL-3 ubiquitin-ligase. *Nature* **425**: 311-316
- Pogorelko GV, Fursova OV, Ogarkova OA, Tarasov VA** (2008) A new technique for activation tagging in *Arabidopsis*. *Gene* **414**: 67-75
- Price J, Li T-C, Kang SG, Na JK, Jang J-C** (2003) Mechanisms of glucose signaling during germination of arabidopsis. *Plant Physiol* **132**: 1424-1438

- Puig OM, Bellés E, López-Rodas G, Sendra R, Tordera V** (1998) Interaction between N-terminal domain of H4 and DNA is regulated by the acetylation degree. *Biochem Biophys Acta (BBA)-Gene Struct Exp* **1397**: 79-90
- Rahman S, Sowa ME, Ottinger M, Smith JA, Shi Y, Harper JW, Howley PM** (2011) The Brd4 extraterminal domain confers transcription activation independent of pTEFb by recruiting multiple proteins including NSD3. *Mol Cell Biol* **13**: 2641-52
- Ren Q and Gorovsky MA** (2003) The nonessential H2A N-terminal tail can function as an essential charge patch on the H2A.Z variant N-terminal tail. *Mol Cell Biol* **23**: 2778-2789
- Ren S, Johnston JS, Shippen DE, McKnight TD** (2004) TELOMERASE ACTIVATOR1 induces telomerase activity and potentiates responses to auxin in *Arabidopsis*. *Plant Cell* **16**: 2910-2922
- Ren S, Mandadi KK, Boedeker AL, Rathore KS, McKnight TD** (2007) Regulation of telomerase in *Arabidopsis* by BT2, an apparent target of TELOMERASE ACTIVATOR1. *Plant Cell* **19**: 23-31
- Reynoird N, Schwartz BE, Delvecchio M, Sadoul K, Meyers D, Mukherjee C, Caron C, Kimura H, Rousseaux S, Cole PA, Panne D, French CA, Khochbin S** (2010) Oncogenesis by sequestration of CBP/p300 in transcriptionally inactive hyperacetylated chromatin domains. *EMBO J* **29**: 2943-2952
- Ricci AR, Genereaux J, Brandl CJ** (2002) Components of the SAGA histone acetyltransferase complex are required for repressed transcription of ARG1 in rich medium. *Mol Cell Biol* **22**: 4033-4042
- Richmond TJ, Searles MA, Simpson RT** (1988) Crystals of a nucleosome core particle containing defined sequence DNA. *J Mol Biol* **199**: 161-170
- Riha K, Watson JM, Parkey J, Shippen DE** (2002) Telomere length deregulation and enhanced sensitivity to genotoxic stress in *Arabidopsis* mutants deficient in Ku70. *EMBO J* **21**: 2819-2826
- Robert HS, Quint A, Brand D, Vivian-Smith A, Offringa R** (2009) BTB and TAZ domain scaffold proteins perform a crucial function in *Arabidopsis* development. *Plant J* **58**: 109-121

- Rochon A, Boyle P, Wignes T, Fobert PR, Després 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
- Rolland F, Baena-Gonzalez E, Sheen J** (2006) Sugar sensing and signaling In plants: Conserved and novel mechanisms. *Ann Rev Plant Biol* **57**: 675-709
- Rolland F, Moore B, Sheen J** (2002) Sugar sensing and signaling in plants. *Plant Cell* **14**: S185-S205
- Rook F, Corke F, Card R, Munz G, Smith C, Bevan MW** (2001) Impaired sucrose-induction mutants reveal the modulation of sugar-induced starch biosynthetic gene expression by abscisic acid signalling. *Plant J* **26**: 421-433
- Sanchez R, Zhou MM** (2009) The role of human bromodomains in chromatin biology and gene transcription. *Curr Opin Drug Disc Deve* **12**: 659-665
- Schweiger M-R, Ottinger M, You J, Howley PM** (2007) Brd4-independent transcriptional repression function of the papillomavirus E2 proteins. *J Virol* **81**: 9612-9622
- Sénéchal H, Poirier GG, Coulombe B, Laimins LA, Archambault J** (2007) Amino acid substitutions that specifically impair the transcriptional activity of papillomavirus E2 affect binding to the long isoform of Brd4. *Virology* **358**: 10-17
- Shechter M-R, Dormann HL, Allis CD, Hake SB** (2007) Extraction, purification and analysis of histones **2(6)**: 1445-47
- Sinha A, Faller DV, Denis GV** (2005) Bromodomain analysis of Brd2-dependent transcriptional activation of cyclin A. *Biochem J* **387**: 257-269
- Smeekens S** (2000) Sugar-induced signal transduction in plants. *Ann Rev Plant Physiol Plant Mol Biol* **51**: 49-81
- Sterner DE, Berger SL** (2000) Acetylation of histones and transcription-related factors. *Micro Mol Biol Rev* **64**: 435-459
- Strahl BD and Allis CD** (2000) The language of covalent histone modifications. *Nature* **403**: 41-45
- Syntichaki P, Topalidou I, Thireos G** (2000) The Gcn5 bromodomain coordinates nucleosome remodelling. *Nature* **404**: 414-417

- Tamkun JW, Deuring R, Scott MP, Kissinger M, Pattatucci AM, Kaufman TC, Kennison JA** (1992) *brahma*: A regulator of *Drosophila* homeotic genes structurally related to the yeast transcriptional activator. *Cell* **68**: 561-572
- Thorpe KL, Gorman P, Thomas C, Sheer D, Trowsdale J, Beck S** (1997) Chromosomal localization, gene structure and transcription pattern of the ORFX gene, a homologue of the MHC-linked RING3 gene. *Gene* **200**: 177-183
- Tjeertes JV, Miller KM, Jackson SP** (2009) Screen for DNA-damage-responsive histone modifications identifies H3K9Ac and H3K56Ac in human cells. *EMBO J* **28**: 1878-1889
- Toyama R, Rebbert ML, Dey A, Ozato K, Dawid IB** (2008) Brd4 associates with mitotic chromosomes throughout early zebrafish embryogenesis. *Develop Dyn* **237**: 1636-1644
- Umehara T, Nakamura Y, Jang MK, Nakano K, Tanaka A, Ozato K, Padmanabhan B, Yokoyama S** (2010) Structural basis for acetylated histone H4 recognition by the human BRD2 bromodomain. *J Biol Chem* **285**: 7610-7618
- Vaucheret H, Fagard M** (2001) Transcriptional gene silencing in plants: Targets, inducers and regulators. *Trends Genet* **17**: 29-35
- Weidner-Glunde M, Ottinger M, Schulz TF** (2010) WHAT do viruses BET on? *Front Biosci* **15**: 537-549
- Weigel D, Ahn JH, Blázquez MA, Borevitz JO, Christensen SK, Fankhauser C, Ferrándiz C, Kardailsky I, Malancharuvil EJ, Neff MM, Nguyen JT, Sato S, Wang Z-Y, Xia Y, Dixon RA, Harrison MJ, Lamb CJ, Yanofsky MF, Chory J** (2000) Activation tagging in *Arabidopsis*. *Plant Physiol* **122**: 1003-1014
- Wu S-Y, Lee A-Y, Hou SY, Kemper JK, Erdjument-Bromage H, Tempst P, Chiang C-M** (2006) Brd4 links chromatin targeting to HPV transcriptional silencing. *Genes Develop* **20**: 2383-2396
- Wu SY, Chiang CM** (2007) The double bromodomain-containing chromatin adaptor Brd4 and transcriptional regulation. *J Biol Chem* **282**: 13141-13145

- Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, Dixon RA, Lamb C** (2004) An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. *EMBO J* **23**: 980-988
- Xu L, Wei Y, Reboul J, Vaglio P, Shin T-H, Vidal M, Elledge SJ, Harper JW** (2003) BTB proteins are substrate-specific adaptors in an SCF-like modular ubiquitin ligase containing CUL-3. *Nature* **425**: 316-321
- Yang X-J** (2004) Lysine acetylation and the bromodomain: A new partnership for signaling. *BioEssays* **26**: 1076-1087
- Yang XJ, Seto E** (2000) HATs and HDACs: From structure, function and regulation to novel strategies for therapy and prevention. *Oncogene* **26**: 5310-5318
- You J, Croyle JL, Nishimura A, Ozato K, Howley PM** (2004) Interaction of the bovine papillomavirus E2 protein with Brd4 tethers the viral DNA to host mitotic chromosomes. *Cell* **117**: 349-360
- You J, Srinivasan V, Denis GV, Harrington WJ, Jr., Ballestas ME, Kaye KM, Howley PM** (2006) Kaposi's Sarcoma-Associated herpesvirus latency-associated nuclear antigen interacts with bromodomain protein Brd4 on host mitotic chromosomes. *J Virol* **80**: 8909-8919
- Zhao Y, Christensen SK, Fankhauser C, Cashman JR, Cohen JD, Weigel D, Chory J** (2001) A role for flavin monooxygenase-like enzymes in auxin biosynthesis. *Science* **291**: 306-309
- Zheng and Zhou** (2002) Bromodomain: An acetyl lysine binding domain. *FEBS letters* **513**: 124-128
- Zhou L, Jang J-c, Jones TL, Sheen J** (1998) Glucose and ethylene signal transduction crosstalk revealed by an *Arabidopsis* glucose-insensitive mutant. *Proc Natl Acad Sci USA* **95**: 10294-10299
- Zilberman D, Coleman-Derr D, Ballinger T, Henikoff S** (2008) Histone H2A.Z and DNA methylation are mutually antagonistic chromatin marks. *Nature* **456**: 125-129

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