A previously undiscovered role for clade III TGA basic leucine zipper transcription factors in BLADE-ON-PETIOLE dependent regulation of plant development

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ABSTRACT

Boundaries that join lateral organs to the plant body are an important determinant of plant architecture. These specialized junctions originate within the shoot apex and control leaf shape, branching architecture, and abscission. Boundary patterning relies on a pair of BTB-ankyrin co-transcription factors encoded by *BLADE-ON-PETIOLE 1* and *2* (*BOP1/2*). Members of this class bind to DNA indirectly, by interacting with other types of transcription factors, especially TGACG-motif binding (TGA) basic leucine zipper proteins. My works shows a previously undiscovered role for clade III TGA factors, previously associated with plant defense, in BOP-dependent plant development. This work uncovers a dual role for clade III TGAs in development and defense.

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PREFACE

My thesis explores the role of clade III TGA transcription factors in plant development. I completed most of the work in my thesis, under the supervision of Dr. Shelley Hepworth, at Carleton University. Chris Bergin assisted me with the qRT-PCR experiment. Ying Wang helped me with construction of *TGA3* and *TGA7* promoter *GUS* reporter genes and created the *BOP1* and *BOP2* binding domain constructs used in the yeast two-hybrid experiment.

None of the work described in my thesis has been submitted for publication.

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Protein fusion: *BOP1-GR*

LIST OF ABBREVIATIONS

AG - AGAMOUS

AS - ASYMMETRIC LEAVES

ATH - ARABIDOPSIS THALIANA HOMEOBOX GENE

BTB - broad complex tramtrack

BOP - BLADE-ON-PETIOLE

BOP-OE - BOP-overexpressing

BR - brassinosteroids

BZR - BRASSINOZOLE RESISTANT

bZIP - basic leucine zipper

CaMV 35S - Cauliflower mosaic virus 35S promoter

cDNA - complementary DNA

CDS - coding sequence

CmYLCV - cestrum yellow leaf curling virus

Col - Columbia

CUC - CUP-SHAPED COTYLEDON

CUL - CULLIN

Cys - cysteine

FEA - FASCIATED EAR

Flg - flagellin peptide

GA - gibberellins

GRAS - GIBBERELLIN-ACID INSENSITIVE (GAI), REPRESSOR of GA1 (RGA), SCARECROW (SCR)

GUS - β-Glucuronidase

KNOX - KNOTTED-LIKE HOMEOBOX

LFY - LEAFY

LOB - LATERAL ORGAN BOUNDARIES

NPR1 - NON-EXPRESSER OF PATHOGENESIS RELATED GENE

PCR - Polymerase chain reaction

PIF - PHYTOCHROME INTERACTING FACTOR

PDF - PLANT DEFENSIN

PIN - PIN-FORMED

POZ - POX virus and zinc finger

PR - PATHOGENESIS-RELATED

qRT-PCR - quantitative reverse transcriptase PCR reaction

RAM - root apical meristem

ROS - reactive oxygen species

SA - salicylic acid

SAM - shoot apical meristem

SAR - systemic acquired resistance

SCL - SCARECROW-LIKE

TB - TEOSINTE BRANCHED

TGA - TGACG

TRU - TASSELS REPLACE UPPER EARS

WT - wild-type

WRKY - WRKY domain-containing

WUS - WUSCHEL

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CHAPTER 1: INTRODUCTION

1.1 Thesis overview

The body plan (architecture) of a plant is determined by activity of meristems, which are dividing stem cell populations from which organs are derived. Lateral organs (leaves, branches, flowers) formed by the shoot apical meristem (SAM) are separated from surrounding stem cells by low growth regions called boundaries (Aida and Tsaka, 2006ab). Boundaries ultimately connect organs to the plant body and control diverse traits such as leaf patterning, branching complexity and abscission, which are important determinants of crop yield (Hepworth and Pautot, 2015). *BLADE-ON-PETIOLE1/2* (*BOP1/2*) in *Arabidopsis thaliana* (*Arabidopsis*) represent a class of genes that are important for boundary patterning in land plants. The expression of these genes is enriched at organ boundaries (Hepworth *et al.,* 2005; Khan *et al.,* 2014). Loss-of-function *bop1 bop2* mutations disrupt boundaries resulting in fused organs and changes in growth at the base of leaves and flowers that alter morphology and prevent abscission whereas up-regulation of *BOP1* or *BOP2* inhibits growth (Hepworth *et al.*, 2005; McKim *et al.*, 2008). BTB-ankyrin proteins like BOP1 and BOP2 have a transcriptional activation domain but no DNA-binding domain and interact with TGACG-motif binding (TGA) basic leucine zipper (bZIP) transcription factors for recruitment to DNA (reviewed in Khan *et al.*, 2014; Wang *et al.,* 2019). My thesis identifies a previously undiscovered role for clade III TGA factors (TGA3 and TGA7) in BOP-dependent regulation of plant development. This work expands the number of TGA factors known to interact with BOPs for regulation of plant development, illustrating a surprisingly large degree of functional redundancy.

1.2 Modern agriculture

Plant architecture is a major determinant of crop yield. Features such as plant height, leaf architecture, branching complexity, and abscission are commonly optimized in crop plants to improve yield. Ideal architecture for row crops consists of short, compact plants with few branches. These traits reduce overcrowding and competition for resources (Holalu and Finlayson, 2017). A reduction in branching also means that more energy can be invested into fruit and seed production (Dong *et al.,* 2017). Limitation of natural seed dispersal mechanisms like abscission and pod shattering are also desirable (Dong and Wang, 2015).

Early farmers who domesticated crops like wheat, maize, and legumes selected for natural variants that improved their performance as row crops (Ross-Ibarra *et al.,* 2007; Dong *et al.,* 2017). For example, maize was cultivated from a wild grass called teosinte through selection of the *tb1* allele (Doebley *et al.*, 1997). The *tb1* variant produced shorter plants and reduced the number of branches, resulting in larger, more compact female cobs of corn (Dong *et al.,* 2017). As another example, wild wheat has dehiscent ears, which easily shatter. Early farmers preferentially chose strains of wheat that resisted abscission, with seeds that remain attached to the ear during harvest (Dubcovsky and Dvorak, 2007). Remarkably, only a handful of domestication genes have been identified. Uncovering genetic routes to ideal plant architecture is therefore relevant to modern agriculture. However, directly working with crops tends to be difficult because of complex genomes. Experimentation in a plant model organism is one approach that can be used to facilitate the discovery of gene regulatory networks.

1.3 *Arabidopsis thaliana* as a plant model organism

Arabidopsis thaliana (Arabidopsis) is a small flowering plant in the mustard family that is widely used as a genetic model for plants (Provart *et al.,* 2016). *Arabidopsis* became selected among plant biologists in the mid-1970s to be used as a plant model organism because it was easier to work with compared to traditional crop plants (Meinke *et al.*, 1998; Koornneef and Meinke, 2010; Provart *et al.,* 2016). *Arabidopsis* plants have simple growth requirements and reach maturity in six to eight weeks. The plants are self-fertilizing and produce many seeds (Meinke *et al.*, 1998; Provart *et al.,* 2016). The *Arabidopsis* genome is diploid and small by plant standards, about 135 mega base pairs (Meinke *et al.*, 1998). The genome is also fully sequenced and annotated, with many online databases and tools for studying gene expression, genetic interactions, and metabolic pathways (Meinke *et al.*, 1998; *Arabidopsis* Genome Initiative, 2000; Papatheodorou *et al.,* 2018). Extensive mutant knockout libraries have been created using T-DNA and transposon insertional mutagenesis to facilitate reverse genetic approaches. *Arabidopsis* is also easy to mutagenize, which is ideal for forward genetic screens (Alonso *et al.,* 2003; Kuromori *et al.,* 2004; O'Malley *et al.,* 2015). *Arabidopsis* shares homologues of genes with many important agricultural species. All of these traits make *Arabidopsis* an ideal genetic model for plant research.

Many important discoveries in plant biology including the mechanisms of hormone signaling were first identified in *Arabidopsis*. For example, mutant screens using *Arabidopsis* plants elucidated many key components of ethylene, cytokinin, and auxin hormone signaling pathways in plants (McCourt, 1999; Swarup *et al.,* 2002; Moubayidin

et al., 2009; Muday *et al.,* 2012). Major genetic discoveries in plant development were also made using *Arabidopsis*, including the characterization of *KNOTTED1-LIKE HOMEOBOX (KNOX)* transcription factor genes. *KNOX* genes influence leaf shape and complexity (Hake *et al.,* 2004). *KNOX* genes are also key regulators of meristem function in plants. The study of these genes has led to detailed knowledge about meristems and how they work to regulate plant growth and development (Hake *et al.,* 2004; Scofield and Murray, 2006; Scofield *et al.,* 2007; Ha *et al.,* 2010).

1.4 Shoot apical meristem

In plants, two primary meristems called the shoot apical meristem (SAM) and the root apical meristem (RAM) are established during embryogenesis. The RAM produces the root system whereas the SAM produces future aerial tissues.

In *Arabidopsis* and other plants, the SAM is a layered structure made up of the tunica (latin, meaning "sheath" or "covering") and the corpus (latin, meaning "central mass") which give rise to different cell layers in the plant body. The outer layers (L1 and L2) comprise the tunica, which give rise to the outer layers of the plant. The inner layer (L3) is the corpus, which gives rise to the internal pith tissues of the plant (Murray *et al.,* 2012; Figure 1.1). Super-imposed on the layered structure of the SAM are three main functional zones (Figure 1.1). The central zone contains slowly dividing stem cells. Flanking the central zone is the peripheral zone, where lateral leaves and flowers originate. Finally, located deep in the meristem is the rib zone. The rib zone contains an organizing center that maintains the central zone and produces cells of the corpus including the internal tissue of the stem (Stahl and Simon, 2005; Figure 1.1). A fourth

subdomain of the SAM is a specialized layer of cells called the boundary that forms between undifferentiated stem cells and initiating organ primordia (Figure 1.1). Compared to other parts of the SAM, the boundary is understudied.

1.5 Meristem organ boundary

The boundary is a subdomain of the SAM that keeps meristem cells separate from emerging plant organs and is important for the structural integrity of the SAM as a whole (Hepworth and Pautot, 2015; Figure 1.1). The boundary forms a physical barrier between meristem and organ compartments and an interface between organs. Beyond separation, the boundary contributes to patterning at the base of organs and is the main point of axillary branching and flowering (Figure 1.2).

1.5.1 Boundary formation

Each time the SAM produces a new organ, the organ boundary develops as a region of restricted growth at the organ base that separates it from the surrounding tissue. Cells in the boundary are small and divide infrequently compared to cells in the meristem and cells in the organ. This repressed growth causes a groove to form at the base of the organ (Hepworth and Pautot, 2015).

1.5.2 Boundary genes

Arabidopsis forward and reverse genetic approaches have discovered a number of genes that impact boundary structure and function. Of chief importance are *CUP-SHAPED COTYLEDON* (*CUC*) transcription factor genes that confer boundary identity.

Progressive loss of *CUC* activity causes fused organs, disrupts axillary branching, and in extreme cases prevents establishment of the embryonic SAM (Aida *et al.*, 1997; Aida *et al.*, 1999; Vroeman *et al.*, 2003). Also important are *BOP1* and *BOP2* co-transcription factor genes that function in parallel with or downstream of *CUC*s to maintain and pattern boundaries. Loss of *BOP* activity results in mildy-fused organs, patterning alterations at the base of organs, impaired axillary meristems, and loss of abscission (Hepworth *et al.*, 2005; McKim *et al.*, 2008; Khan *et al.*, 2014; Khan *et al.*, 2015). Both groups of genes play an important role in growth repression.

1.5.3 Boundary hormones

The boundary is a zone of repressed growth. Low levels of the plant hormones auxin, brassinosteroid (BR) and gibberellin (GA) facilitate this repression. One study showed that an auxin minimum is maintained by spatial regulation of PIN-FORMED1 (PIN1) auxin transporters at boundaries. Fluorescence confocal microscopy showed that PIN1 transporters are oriented outwards along the plane of the groove at boundaries in the SAM. This orientation causes auxin to be drained away from boundary cells into surrounding tissue (Heisler *et al.*, 2010). An auxin-depleted state also makes boundary cells competent to form axillary meristems and respond to signals for abscission (Sorefan *et al.*, 2009; Wang *et al.*, 2014; Meir *et al.*, 2015).

Low levels of BR at the boundary are maintained by a feedback loop that begins with transcription factor LATERAL ORGAN BOUNDARIES (LOB) activation of P450 cytochrome gene *BAS1*, which encodes a BR-degrading enzyme (Bell *et al.,* 2012; Gendron *et al.,* 2012). Auxin activities in nearby leaf cells induce *LOB* expression to

complete the loop (Gendron *et al.,* 2012). BOP1 and BOP2 reinforce this loop by promoting *LOB* expression in the boundary domain (Ha *et al.*, 2007). BOP1 and BOP2 also reinforce this loop by inhibiting the accumulation of BR-activated BRASSINOZOLE RESISTANT1 (BZR1) and BZR2 transcription factors in the nucleus (Gendron *et al.,* 2012; Shimada *et al.,* 2015). Low levels of BR and BZR1 stimulate the expression of *CUC* organ boundary identity genes, which inhibit growth and are involved in organ boundary formation (Gendron *et al.,* 2012).

Low levels of GA in stem cells, and presumably the boundary, suppress differentiation and promote indeterminacy. *KNOX* genes expressed in the meristem code for transcription factors that lower GA abundance through inhibition of GA biosynthetic genes and promotion of GA catabolic genes (Sakamoto *et al.,* 2001; Bolduc and Hake, 2009). GA catabolic genes are also expressed at the boundary between the SAM and leaves, which is consistent with low GA levels (Jasinski *et al.,* 2005; Bolduc and Hake, 2009).

1.5.4 Leaf patterning

Boundaries influence the morphology of leaves (Hepworth and Pautot, 2015). Improper differentiation and maintenance of the leaf organ boundaries can result in various defects such as irregular leaf shapes (Rast and Simon, 2012), leafy outgrowths on petioles (Hepworth *et al.,* 2005), and leaf fusions (Rast and Simon, 2012). Generally speaking, these defects are caused by excessive growth and/or extension/re-initiation of meristematic activity in leaves.

Leaf differentiation requires the repression of *KNOX* genes. Down-regulation of *KNOX* genes "switches off" stem cell fate so that differentiation can begin (Hake *et al.,* 2004; Koyama *et al.,* 2007; Hay and Tsiantis, 2010; Koyama *et al.,* 2010). *KNOX* repression involves transcription factors ASYMMETRIC LEAVES1 (AS1) and AS2 that form a trimeric complex with JAGGED LATERAL ORGANS. This complex binds to the promoter of target *KNOX* genes and directs stable repressive changes in the chromatin structure that are inherited by daughter cells (Guo *et al.,* 2008; Rast and Simon, 2012). Stable repression of *KNOX* genes in *Arabidopsis* gives rise to simple leaves with a smooth petiole and an undivided blade. The mutation of boundary genes can increase leaf complexity by extending growth or causing new growth centers to form along the leaf petiole or margin. For example, leaves in *cuc1*, *cuc2*, and *cuc1 cuc2* mutants are fused into a cup-shape because of excess growth between leaves at the boundary (Aida *et al.,* 1997). As another example, *bop1 bop2* leaves have elongated leafy petioles due to extended growth coupled with reactivation of meristem activity in leaves (Ha *et al.*, 2003; Ha *et al.*, 2004; Hepworth *et al.*, 2005). BOP1 is shown to directly activate the expression of *AS2*, which encodes part of the complex required for repression of *KNOX* genes in the leaf (Ha *et al.,* 2007; Jun *et al.,* 2010).

1.5.5 Axillary meristems

Axillary meristems are formed in the axil of leaves. Depending on the phase of development, axillary meristems can develop as secondary inflorescences (side-shoots with many flowers) or as single flowers. Other more specialized axillary meristems form structures like stipules (leafy appendages) at the base of leaves and nectaries (nectar-

producing glands) at the base of floral organs (Schultz and Haughn, 1991; Blázquez *et al.,* 2006; Xu *et al.,* 2010).

Loss of *CUC3* alone or in combination with *CUC2* specifically affects the formation of axillary meristems that form flowering side-branches (Vroeman *et al.*, 2003). *BOP1* and *BOP2* on the other hand are essential for the formation of nectaries and stipules (McKim *et al.,* 2008; Ichihashi *et al.*, 2011). Flower initiation is impaired in *bop1 bop2* mutants, but only partially due to other factors that compensate (Norberg *et al.*, 2005; Xu *et al.*, 2010). However, delayed differentiation in *bop1 bop2* floral meristems results in a bract and extra floral organs compared to wild-type flowers (Hepworth *et al.*, 2005). There is no obvious effect on formation of side-branches in *bop1 bop2* mutants but this defect can be seen in barley where tillers (vegetative branches) are severely reduced (Tavakol *et al.,* 2015; Jost *et al.,* 2016).

1.5.6 Abscission

Abscission is another important function of the boundary. Zones for abscission typically develop at joints where organs connect to the plant body (Estornell *et al.,* 2013). Abscission can be divided into four main steps: establishment of an abscission zone, competence to respond to abscission signals, separation, and trans-differentiation of cells in the abscission zone to seal the "wound" (Kim, 2014). In *Arabidopsis* plants, abscission zones form at the base of cauline leaves (small leaves that form on the inflorescence), floral organs, and seeds (Patterson, 2001; Partharkar and Walker, 2016). *CUCs* have no known role in abscission (Estornell *et al.*, 2013). By contrast, *BOP1* and *BOP2* are essential for abscission zone formation in cauline leaves and floral organs (McKim *et al.*,

2008). *BOP1* and *BOP2* are expressed continuously in abscission zones and may promote later steps of abscission as well (Bergin, 2018; Corrigan, 2018).

1.6 Boundaries determine important crop traits

Our lab studies *Arabidopsis BOP1* and *BOP2* genes and their mode of action at organ boundaries. These genes are about 81% identical at the amino acid level and both genes must be knocked out to see a phenotype (Norberg *et al.,* 2005). The major loss and gain of function phenotypes of *BOP1* and *BOP2* are summarized in Figure 1.3. Leaves of *bop1 bop2* mutants have an extended petiole that is curved and leafy (Ha *et al.*, 2004; Hepworth *et al.*, 2005; Norberg *et al.*, 2005; Figure 1.3A). Flowers of *bop1 bop2* mutants develop bracts and have extra petals and sepals, which are sometimes fused at the base (Figure 1.3B, C). Abscission of cauline leaves and floral organs is blocked in *bop1 bop2* mutants because specialized layers of cells required for separation fail to differentiate (McKim *et al.,* 2008; Lee *et al.,* 2018; Figure 1.3D). By contrast, constitutive expression of *BOP1* or *BOP2* restricts growth resulting in a dwarf phenotype (Norberg *et al.,* 2005; Khan *et al.,* 2012a; Khan *et al.,* 2015; Figure 1.3E).

All land plants with a sequenced genome encode one or more *BOP*-like genes (Khan *et al.*, 2014). Recent investigations of BOP orthologues in diverse species supports a conserved role in boundary patterning. In *Hordeum vulgare* (barley), *BOP1/2* homologues regulate tillering (vegetative branches that develop from axillary buds), grain density, and leaf patterning (Tavakol *et al.,* 2015; Jost *et al.,* 2016). *BOP1/2* homologues in *Pisum sativum* (field pea) and *Medicago truncatula* (barrel clover) regulate leaf and floral patterning (Kumar *et al.,* 2009; Kumar *et al.,* 2011; Sharma *et al.,* 2012; Couzigou

et al., 2015). These BOPs are also necessary for the abscission of petals, leaflets, leaves, and fruits (Couzigou *et al.,* 2015). In addition, they regulate the formation of root symbiotic nodule organs (Couzigou *et al.,* 2012; Couzigou *et al.*, 2017; Magne *et al.,* 2018). More recently, *Oryza sativa* (rice) *BOP1/2* homologues were discovered to play a role in regulating the sheath-to-blade ratio of leaves. This ratio changes as the plant develops to optimize growth (Toriba *et al.*, 2019). Lastly, in *Solanum lycopersicum* (tomato), *BOP1/2* homologues act to regulate inflorescence architecture and complexity which effects fruit number and size (Xu *et al.,* 2016).

Interestingly, domestication studies suggest that early farmers have already taken advantage of local changes in *BOP* expression to select for more desirable crops. *TB1* was discovered to be one of the genes farmers selected for in maize during millennia of human domestication (Doebley *et al.,* 1997). *TB1* encodes an axillary branch repressor, and farmers selected for gain of function *TB1* plants that reduced branching in maize. A *BOP1/2* homologue in maize, *TRU1*, was found to be a direct target of *TB1* (Dong *et al.,* 2017). *TB1* targets the *BOP1/2* homologue *TRU1* to exert changes in axillary branching. This is one example that shows how human domestication resulted in genetic changes that led to the modification of organ boundary related processes such as axillary branching.

A further understanding of how organ boundary genes control patterning is desirable for crop breeding research. Boundaries connect organs to the plant body and control diverse traits such as leaf patterning, branching complexity, and abscission, which are important determinants of crop yield (Figure 1.2 and Figure 1.3). *BOP1/2* genes, which are important for organ boundary function, are part of the BTB-ankyrin protein family.

1.7 BTB-ankyrin family

BTB-ankyrin genes are a family in land plants that encode transcriptional co-activator proteins. Members of this group are characterized by two highly conserved protein-protein interaction motifs: a BTB/POZ domain (Broad Complex Tramtrack, Bric-a-brac/POX virus and zinc finger) at the N-terminus and four ankyrin repeats adjacent to the C-terminus (Khan *et al.,* 2014; Backer *et al.*, 2019; Figure 1.4A). The BTB/POZ domain is required for the formation of dimers/heterodimers between family members and recruits E3 ubiquitin ligases to target proteins for degradation (Mukhtar *et al.,* 2009; Spoel *et al.,* 2009; Jun *et al.,* 2010; Zhang *et al.,* 2017; Chahtane *et al.*, 2018). The ankyrin domain interacts with TGA bZIP proteins for recruitment to DNA (Zhang *et al.,* 1999; Després *et al.*, 2000; Zhou *et al.,* 2000; Hepworth *et al.*, 2005; Wang *et al.*, 2019).

The BTB-ankyrin family in *Arabidopsis* contains six gene members that are subdivided into two main clades (Figure 1.4B). The *NPR1* clade (*NPR1, NPR2, NPR3* and *NPR4*) is involved in the regulation of plant defense (Khan *et al.*, 2014; Backer *et al.*, 2019; Figure 1.4B). *NPR1* is a master regulator of the salicylic acid (SA) mediated systemic acquired resistance (SAR) plant defense pathway (Cao *et al.*, 1994; Cao *et al.*, 1997). The other clade is comprised of *BOP1* and *BOP2,* which control plant development and have a recently discovered role in innate immunity that is distinct from *NPR1* (Ha *et al.,* 2003; Hepworth *et al.,* 2005; Norberg *et al.,* 2005; Bergin, 2018; Wang *et al.,* 2019; Figure 1.4B).

1.7.1 BLADE-ON-PETIOLE (BOP1/2)

BOP1/2 proteins are present in both the cytoplasm and nucleus where they might have different roles (Hepworth *et al.,* 2005; Shimada *et al.,* 2015; Zhang *et al.,* 2017). In the cytosol, BOP1 can form complexes with other proteins to regulate plant development (Shimada *et al.,* 2015). For example, BOP1 and BOP2 can bind to BZR1, which is a master regulator of BR response. The formation of a complex between BOP1/2 and BZR1 inhibits transport of BZR1 into the nucleus, which results in the negative regulation of BR responsive gene expression (Shimada *et al.,* 2015). BOP1/2 can also act as a substrate adaptor in an CULLIN3 (CUL3) E3 ubiquitin ligase complex to target and degrade PHYTOCHROME INTERACTING FACTOR4 (PIF4) to control hypocotyl elongation and LEAFY (LFY) to regulate flowering (Zhang *et al.,* 2017; Chahtane *et al.,* 2018). In the nucleus, BOPs bind to DNA via TGA transcription factors to regulate gene expression (Hepworth *et al.,* 2005; Khan *et al.,* 2015; Wang *et al.,* 2019). Only a handful of direct target genes have been identified to date, including *AS2* and *ATH1* involved in boundary patterning (Jun *et al.*, 2010; Khan *et al.*, 2015) and a pair of transcription factor genes involved in innate immunity (Bergin, 2018; Ying Wang, unpublished data).

1.8 TGA bZIP transcriptions factors

bZIP (basic leucine zipper) transcription factors are a group of proteins that contain a highly conserved basic leucine zipper domain. The basic region binds to DNA and the leucine zipper mediates dimerization (Figure 1.5A). There are approximately 75 bZIP transcription factors found in *Arabidopsis,* with many different groups (Jakoby *et al.,* 2002). TGA bZIP transcription factors are a subclade of bZIP transcription factors (Jakoby *et al.*, 2002). They contain a TGACG sequence-specific DNA-binding domain

and bind to specific palindromic half-sites on DNA. At the N-terminus, there is a transcriptional activation or repression domain and a basic leucine zipper domain for DNA binding. At the C-terminus, there are glutamine rich regions (Gatz, 2013; Figure 1.5A). TGA transcription factors can form homodimers and heterodimers with other TGAs (Zhou *et al.,* 2000; Despres *et al.,* 2003).

The *Arabidopsis* genome encodes ten TGA transcription factors grouped into five phylogenetic subclades (Gatz, 2013, Figure 1.5B). Clade I contains TGA1 and TGA4, clade II contains TGA2, TGA5, and TGA6, clade III contains TGA3 and TGA7, clade IV contains TGA9 and TGA10, and clade V contains TGA8 (also known as PERIANTHIA/PAN).

TGA factors can function independently but also form complexes with other transcription factors to exert activity (Gatz, 2013). Some of these roles involve interactions with BTB-ankyrin proteins (Gatz, 2013). TGAs are particularly known for their roles in plant transcriptional response to abiotic stress and pathogen defense. Numerous clades of TGAs have been reported to be involved in plant defense responses, some involving interactions with *NPR1* (Zhang *et al.*, 2003; Kesarwani *et al.,* 2007; Shearer *et al.,* 2012; Gatz, 2013). Developmental roles for TGAs have been described, but far less is known about this aspect of TGA function (Hepworth *et al.,* 2005; Murmu *et al.,* 2010; Wang *et al.,* 2019).

1.8.1 Clade I TGAs (TGA1 and TGA4)

Clade I TGAs are broadly expressed in the plant (Waese *et al.*, 2017). Promoter fusions to a GUS reporter gene depict enrichment of expression in the vasculature of

roots, leaves, and stems. Expression is also enriched in young leaf petioles and boundary regions of the inflorescence such as the base of pedicels and abscission zones (Wang *et al.,* 2019).

Clade I TGAs were initially implicated in plant defense since *tga1 tga4* double mutants are compromised in resistance to *Pseudomonas* infection but otherwise normal (Kesarwani *et al.,* 2007). This susceptibility is mainly due to deficiencies in plant innate immunity (Shearer *et al.,* 2012; Wang and Fobert 2013; Sun *et al.,* 2018). Clade I TGAs also contribute to SAR and co-regulate a subset of SA-induced defense genes, presumably through interaction with NPR1 (Shearer *et al.,* 2012). The interaction of clade I TGAs with NPR1 is under redox regulation (Després *et al.*, 2003; Lindermayr *et al.,* 2010). In the presence of SA, Cys residues are reduced in TGA1, which stimulates interaction with NPR1. Clade I TGAs directly regulate two transcription factor genes, *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT1* and *CALMODULIN-BINDING PROTEIN 60g,* that promote defense-induced SA biosynthesis There is strong evidence that *TGA1* and *TGA4* are required for the full induction of these two genes (Sun *et al.*, 2018).

Clade I TGAs also interact with BOPs to regulate plant development (Wang *et al.,* 2019). Transgenic plants overexpressing *BOP1* or *BOP2* are dwarfed (Khan *et al.,* 2015). This dwarf phenotype is corrected in mutants lacking *TGA1* and *TGA4*, indicating that BOPs require clade I TGAs to exert changes in inflorescence architecture. *TGA1* and *TGA4* were also shown to function in the same genetic pathways as *BOP1* and *BOP2* to regulate SAM maintenance and flowering (Wang *et al.,* 2019). Clade I TGAs and BOPs interact constitutively and directly activate the expression of homeobox gene *ATH1*, which is needed for boundary establishment (Khan *et al.,* 2015; Wang *et al.,* 2019).

TGA4 was also identified biochemically as a direct regulator of the "florigen" gene *FLOWERING LOCUS T* (Song *et al.,* 2008). In addition, *TGA1* and *TGA4* were identified in a screen as regulators of primary and lateral growth of roots in response to nitrogen availability (Alvarez *et al.,* 2014). No BTB-ankyrin partner for these roles has been identified.

1.8.2 Clade II TGAs (TGA2, TGA5 and TGA6)

Clade II TGAs are broadly expressed in the stems, leaves, and flowers of plants (Waese *et al.,* 2017).

Knockout studies show that clade II TGAs play a central role with *NPR1* in SAR (Zhang *et al.*, 2003). SAR is severely disrupted in the triple mutant *tga2 tga5 tga6*. Like *NPR1*, the triple mutant *tga2 tga5 tga6* is unable to induce *PATHOGENESIS-RELATED GENE 1 (PR1)*, a hallmark gene of SAR under SA-mediated regulation (Zhang *et al.*, 2003). It was later shown that NPR1 is able to form a complex with TGA2 to enhance the transcription of *PR1* (Boyle *et al.*, 2009).

A role for clade II TGAs in jasmonic acid/ethylene-induced defense responses has also been discovered (Zander *et al.,* 2010). Under fungal pathogen *Botrytis cinerea* infection, clade II TGAs regulate the expression of jasmonic acid-inducible plant defensin gene *PDF1.2,* but only under conditions of increased ethylene (Zander *et al.,* 2010).

Interestingly, clade II TGAs have been shown to interact with SCARECROW-LIKE 14 (SCL14). SCL14 is a protein belonging to the GRAS family of transcription factors. TGA2, TGA5 and TGA6 are required by SCL14 for recruitment to promoters of target genes (Fode *et al.,* 2008). SCL14-dependent genes are responsible for stress

tolerance and detoxification. Both *scl14* and *tga2 tga5 tga6* mutants are more susceptible to toxic chemicals such as isonicotinic acid (Fode *et al.,* 2008).

1.8.3 Clade IV TGAs (TGA9 and TGA10)

Clade IV TGAs are strongly expressed in flowers and at lower levels in the rest of the plant (Waese *et al.*, 2017).

Double knockout of *TGA9* and *TGA10* was used to reveal redundant roles for these genes in anther development (Murmu *et al.,* 2010). In *tga9 tga10* double mutants, the anther locules are collapsed and pollen are inviable (Murmu *et al.*, 2010). This study also showed that TGA9 and TGA10 can interact with a pair of CC-type glutaredoxins ROXY1 and ROXY2 (Murmu *et al.,* 2010). Glutaredoxins are enzymes that can add or remove glutathione from cysteine residues to regulate protein activity (Li and Zachgo, 2008). In *roxy1 roxy2* mutants, the anthers are sterile and lack pollen because of defects in tapetum development (Li and Zachgo, 2008). Similar anther defects in *tga9 tga10* and *roxy1 roxy2* double mutants coupled with a complex formed by clade IV TGAs and ROXYs suggests that clade IV TGA activity is redox-regulated similar to clade I TGA1 and TGA4 (Despres *et al.*, 2003; Murmu *et al.*, 2010).

Interestingly, clade IV TGAs are also involved in plant defense (Noshi *et al.,* 2016). Bacterial flagellin flg22 peptide is strong elicitor of plant immunity (Zipfel, 2009). Knockout mutants lacking *TGA10* are more sensitive to flg22 treatment than wild-type plants (Noshi *et al.,* 2016). Although knockout mutants lacking *TGA9* do not show any flg22 response defects, the double mutant *tga9 tga10* shows reduced growth indicating increased pathogen susceptibility compared to either single mutant. This indicates a role

for both *TGA9* and *TGA10* in sensitivity to flg22 (Noshi *et al.,* 2016). Reactive oxygen species (ROS) are produced in response to flg22 treatment, and act as signaling molecules (O'Brien *et al.,* 2012). The expression of both genes was induced by ROS and *TGA10* was required for the full induction of several defense genes under flg22 treatment (Noshi *et al.*, 2016).

Clade IV members in other species are implicated in a number of boundary-related processes including leaf patterning and axillary branching. In maize, the mutation of *TGA9* homologue *LIGULELESS2* results in delayed flowering and improper formation of the blade-sheath boundary in leaves (Walsh *et al.,* 1998; Walsh *et al.,* 1999). In tobacco, overexpression of the *TGA10* homologue *NtTGA10* results in defense signalling defects and de-repression of lateral branching (Schiermeyer *et al.*, 2003). Redundancy may be hiding similar roles for clade IV TGAs in *Arabidopsis* plants.

1.8.4 Clade V TGAs (TGA8/PAN)

TGA8, also known as *PAN*/*PERIANTHIA*, is broadly expressed in flowers during all stages. Interestingly, expression was not found in the stems, leaves, or roots indicating a specific role in flower development (Chuang *et al.,* 1999; Jackoby *et al.,* 2001).

Mutation of *PAN* results in flowers with an extra petal and stamen compared to wild-type (Chuang *et al.,* 1999; Running and Hake, 2001). Similar defects are observed in *bop1 bop2* flowers, suggesting that PAN interacts with BOPs to regulate petal organ number (Hepworth *et al.,* 2005). BOP1/2 and PAN proteins interact in yeast and in the nucleus of leaf protoplasts, supporting their role as a unit to regulate floral patterning (Hepworth *et al.,* 2005; Xu *et al.,* 2010). PAN is another TGA protein that interacts with

the floral glutaredoxin, ROXY1. Mutant *roxy1* flowers have fewer petals compared to wild-type (opposite of *pan* flowers) suggesting that PAN is hyperactive if not redox modified (Li *et al.,* 2009).

PAN is also involved in terminating the floral meristem by direct regulation of the *AGAMOUS (AG)* gene (Das *et al.,* 2009; Maier *et al.,* 2009). AG is a transcription factor that represses stem cell maintenance gene *WUSCHEL (WUS).* AG repression of *WUS* terminates floral stem cells allowing for proper flower development (Lenhard *et al.,* 2001). It is not known if this function of PAN requires the activity of BOP1 or BOP2.

A homologue of *PAN* found in maize, *FASCIATED EAR4 (FEA4)*, has been identified as a regulator of shoot meristem size (Pautler *et al.,* 2015). Mutations in *FEA4* results in plants that are dwarfed and have enlarged inflorescence and vegetative meristems. Results from this study suggest that *FEA4* promotes differentiation in the meristem periphery by regulating auxin-based responses and genes associated with leaf differentiation and polarity, potentially in opposition to factors such as *KNOTTED1* and *WUS* (Pautler *et al.,* 2015). These findings have yet been translated to *Arabidopsis*.

1.8.5 Clade III TGAs (TGA3 and TGA7)

Clade III TGAs are broadly expressed in plant tissues (Waese *et al.,* 2017; Papatheodorou *et al.,* 2018; Supplemental Figure S11; Supplemental Figure S12; Supplemental Figure S13).

Both TGA3 and TGA7 interact with NPR1 (Despres *et al.*, 2000; Hepworth *et al.*, 2005). Pathogen assays showed no discernable phenotype in *tga7* mutants but *tga3* mutants were more susceptible to *Pseudomonas* infection compared to the wild-type. This

susceptibility was further increased in *tga3 npr1* double mutants, indicating that TGA3 function is partially dependent on NPR1 (Kesarwani *et al.,* 2007).

Another two studies showed that *TGA3* is involved in defense against necrotrophic pathogens (Windram *et al.,* 2012) and modulates plant defense by cytokinins (Choi *et al.*, 2010). TGA3 recruits cytokinin-activated transcription factor ARR2 and NPR1 to the promoter of defense genes including *PR1* to enhance resistance to *Pseudomonas syringae* (Choi *et al.,* 2010). Cytokinin induces the phosphorylation of ARR2, activating it (Kim *et al.,* 2006). ARR2 will then form a complex with TGA3, and bind to the promoter of *PR1,* however this binding is dependent on *NPR1* (Choi *et al.,* 2010). Similarly, TGA3 can form a complex with WRKY53 to bind to the SA-inducible promoter of Cestrum yellow leaf curling virus (CmYLCV). *NPR1*-dependent SA signaling is required for TGA3/WRKY53 complex binding to the CmYLCV promoter (Sarkar *et al.,* 2017).

TGA3 also contributes to plant heavy metal resistance (Fang *et al.,* 2017). Hydrogen sulfide is produced by plants to reduce the accumulation of heavy metals in vascular tissues. TGA3 was shown to bind to the promoter of the *LCD* gene. *LCD* is a gene that increases hydrogen sulfide and plant tolerance to heavy metals (Fang *et al.,* 2017).

1.9 Thesis rationale

A major goal of our work is to understand the interplay between TGA transcription factors and BTB-ankyrin proteins. A triple knockout of clade II TGAs significantly recreates *npr1*-like defects in SAR, suggesting that the majority of NPR1 functions in plant defense involve a TGA partner (Zhang *et al.*, 2003). If the majority of

BOP1/2 functions also involve a TGA partner, then knockout of the significant TGAs should recreate *bop1 bop2*-like defects in plant development. Yet, identification of these partners has been difficult since BOPs interact with several TGAs and knockout mutants of TGAs in clades I, II, III, and IV have no obvious *bop1 bop2*-related defects in development. Knockout of *PAN*, the sole member of clade V, partially copies *bop1 bop2* defects in flower development leading to speculation that BOPs interact with other TGAs to control leaf patterning, axillary meristems, and abscission. To find these TGAs, we used the transcriptome of *BOP1*-overexpressing (*BOP1-OE*) plants to find co-regulated *TGA* bZIP genes (Khan *et al*., 2015; Wang *et al.,* 2019). Genes from clade I, clade III and clade IV TGAs were up-regulated in *BOP1-OE* plants compared to wild-type. Further characterization of clade I TGAs showed that both members of this clade are expressed at organ boundaries and function in the same genetic pathways as *BOP1* and *BOP2* to regulate SAM activity, flowering, and inflorescence architecture. It was also demonstrated that clade I TGA proteins interact with BOP1 and BOP2 and co-localize to TGA-binding sites in the promoter of boundary gene *ATH1* to activate expression (Wang *et al.,* 2019). By extension, clade III and clade IV TGAs might also interact with BOPs to regulate plant development. My project focused on clade III TGAs for which little is known.

Hypothesis:

Clade III TGA transcription factors and BOPs co-regulate plant development (Figure 1.6).

Objectives:

(1) Test for expression overlap between clade III TGAs and BOPs

(2) Test for a biochemical interaction between clade III TGA and BOP proteins

(3) Test if BOPs require clade III TGAs to regulate inflorescence architecture

(4) Characterize *tga3* and *tga7* single mutants, *tga3 tga7* double mutants, and higher order *tga* mutants (as required) to uncover phenotypic evidence of BOP-like functions in plant development

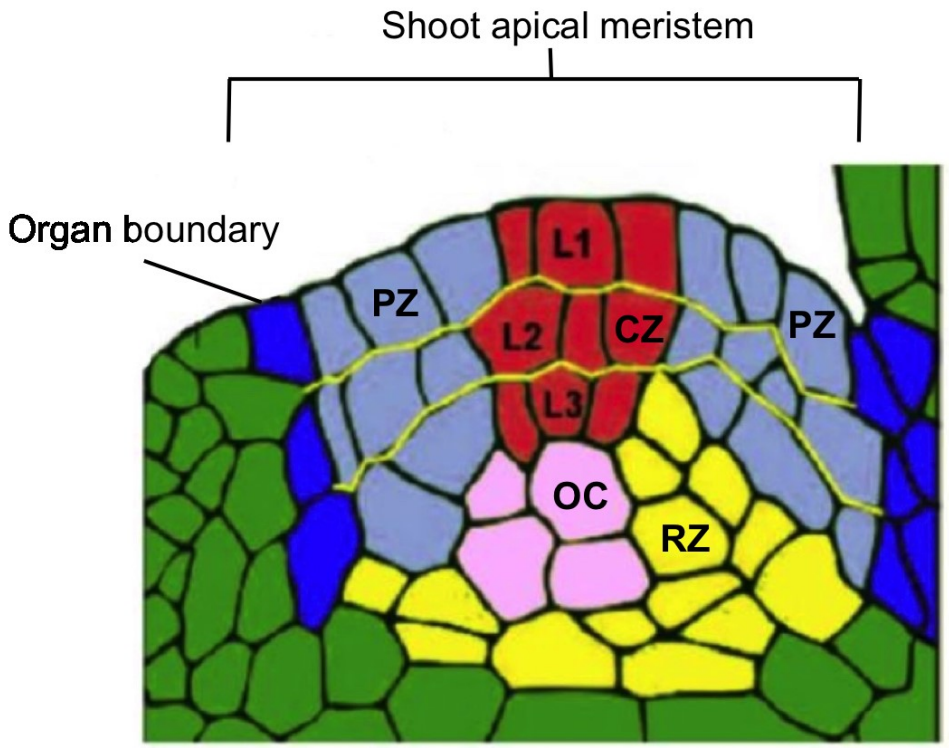

Figure 1.1 Schematic visualizing lateral organ boundaries around the shoot apical meristem.

In the shoot apical meristem, the central zone (CZ, red) is flanked by the peripheral zone (PZ, grey), with an organising center (OC, pink) and rib zone (RZ, yellow) underneath. The meristem organ boundary (blue) separates the meristem cells (red, grey, pink, yellow) from differentiating organ cells (green). Proper maintenance of boundaries is required for normal plant development. Figure modified from Wolpert (2007).

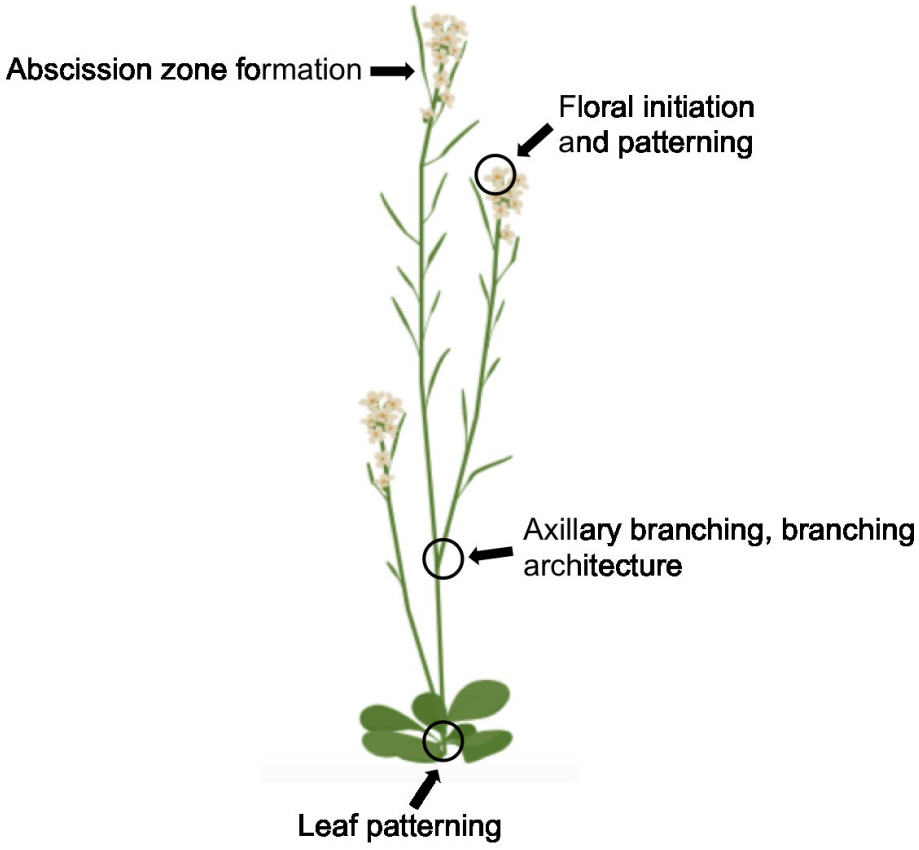

Figure 1.2 Schematic visualizing how organ boundaries determine important developmental traits.

Organ boundaries pattern joints in the plant body. Leaf boundaries regulate leaf patterning. Axillary meristems formed at boundaries determine floral initiation and branching patterns. In *Arabidopsis*, axillary meristems at the bottom of the inflorescence produce side branches and axillary meristems at the top of the inflorescence produce flowers. Abscission zones also form at boundary regions. In *Arabidopsis*, cauline leaves abscise as a defense mechanism, floral organs abscise during fruit development, and seeds abscise for dispersal.

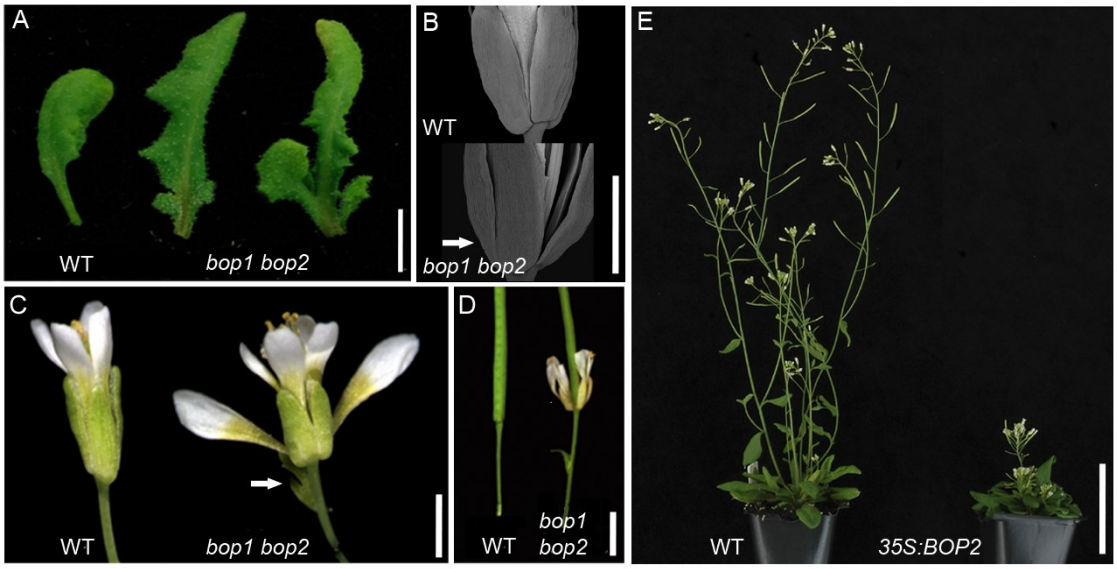

Figure 1.3 Summary of *BOP* loss- and gain-of-function mutant phenotypes.

Characteristic *bop1 bop2* loss-of-function (A-D) and *BOP1/2* gain-of-function (E) mutant phenotypes. Loss-of-function phenotypes: (A) Leafy protrusions along the petiole (Khan *et al.,* 2014). (B) Fusions (arrow) at the base of sepal floral organs (Corrigan, 2018). (C) Flowers with abnormal patterning: an extra bract (arrow) and pair of abaxial sepal-whorl organs that often developed as wing-like petals (Hepworth *et al.,* 2005). (D) Loss of floral organ abscission (Hepworth *et al.,* 2005). Gain-of-function phenotypes: (E) Stem inhibition leading to dwarfism. Scale bar represents 0.1 cm (B, C), 0.25 cm (D), 0.5 cm (A) and 3 cm (E).

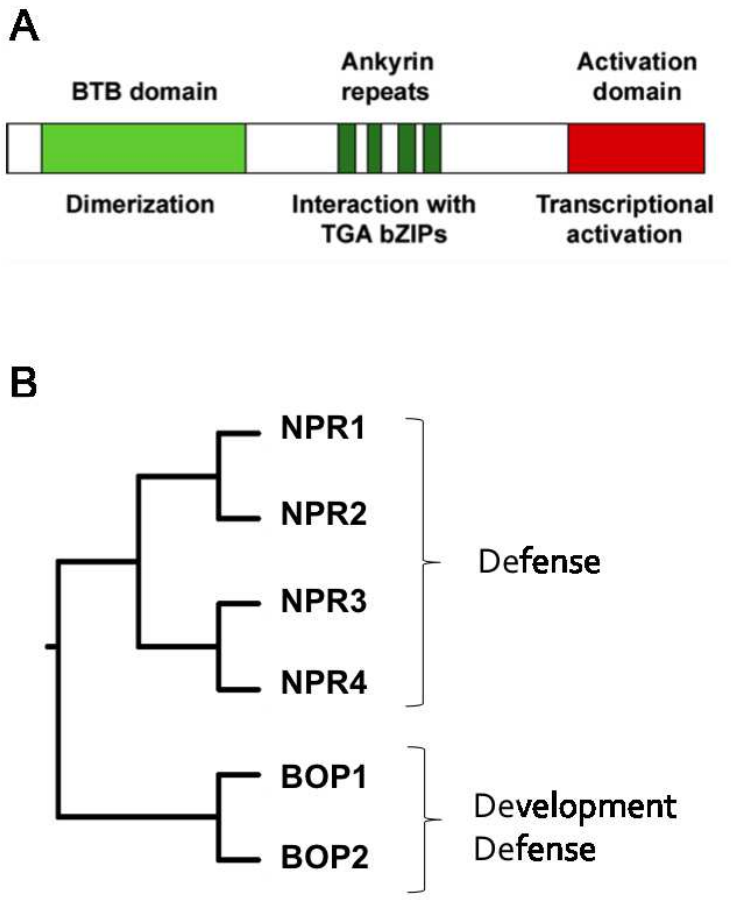

Figure 1.4 Schematic illustrating members of the BTB-ankyrin gene family and showing the BTB-ankyrin protein-protein interaction motifs.

(A) Domain structure of BTB-ankyrin co-transcription factors. The BTB/POZ domain mediates dimerization, the ankyrin repeat domain mediates interactions with TGA bZIP transcription factors, and the C-terminus has a domain for transcriptional activation. (B) Phylogenetic unrooted tree of *Arabidopsis* BTB-ankyrin proteins. Protein sequences were aligned with Clustal W and drawn using Mega 6.0 software (Thompson *et al.,* 1994; Tamura *et al.,* 2013). There are two main clades: *NPR1, NPR2, NPR3* and *NPR4* are receptors for salicylic acid-mediated plant defense whereas *BOP1* and *BOP2* regulate plant development and contribute to innate immunity.

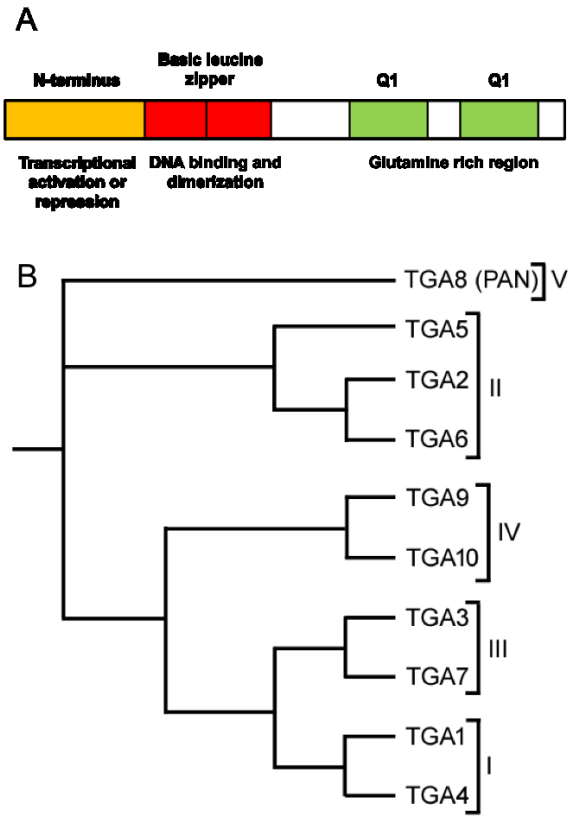

Figure 1.5 Domain structure of TGA bZIP transcription factors and phylogenetic tree.

(A) Domain structure of TGA bZIP transcription factors. The N-terminus contains a transcriptional activation or repression domain and a basic leucine zipper domain that mediates dimerization and DNA binding. There are two glutamine rich regions at the C-terminus. (B) Phylogenetic unrooted tree of *Arabidopsis* TGA bZIP transcription factors. Protein sequences were aligned with Clustal W and drawn using Mega 6.0 software (Thompson *et al.,* 1994; Tamura *et al.,* 2013). The family contains ten members grouped into five subclades.

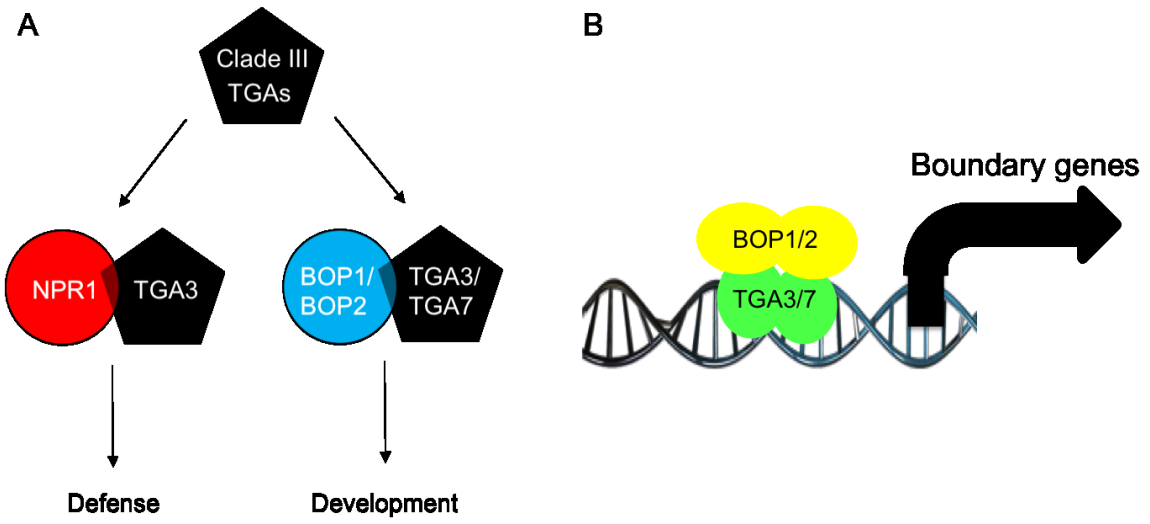

Figure 1.6 Proposed model for clade III TGA interactions with BTB-ankyrin transcriptional co-activator proteins.

(A) We hypothesize multi-functions of clade III TGAs in development and defense via interactions with NPR1 and BOP1/2. TGA3 interacts with NPR1 to regulate plant defense (Choi *et al.*, 2010; Sarkar *et al.,* 2017). We hypothesize clade III TGAs interact with BOP1/2 to regulate plant development. (B) We further hypothesize that clade III TGAs recruit BOP1 and BOP2 to bind to the promoter of target genes to regulate transcription. Potential target genes would include genes involved in organ boundary maintenance.

CHAPTER 2: MATERIALS AND METHODS

2.1 Plant material and growth conditions

The Columbia-0 (Col-0) ecotype of *Arabidopsis* was used as wild-type. Genetic materials used in this study are summarized in Table 2.1. Loss of function mutant alleles used were: *tga3-1* (Kesarwani *et al.*, 2007), *tga7-1* (GABI-KAT 434F04; Kesarwani *et al.,* 2007)*, pan-3* (Running and Hake, 2001)*, tga1-1* (SALK_028212; Kesarwani *et al.*, 2007)*, tga4-1* (SALK_127923; Kesarwani *et al.*, 2007)*, tga9-1* (SALK_091349; Murmu *et al*., 2010), *bop1-3* (SALK_012994; Hepworth *et al*., 2005)*,* and *bop2-1* (SALK_075879; Hepworth *et al*., 2005). The majority of these mutants are T-DNA insertion lines from the SALK collection (Alonso *et al.,* 2003). *tga3-1* was isolated from Thomas Jack's T-DNA-tagged population (Kesarwani *et al.*, 2007), *tga7-1* is a T-DNA insertion line from the GABI-KAT collection (Kesarwani *et al.*, 2007) and *pan-3* is an ethyl methanesulfonate mutant with a single nucleotide polymorphism (Running and Hake, 2001). *pan-3* was originally in a *Landsberg erecta* background and was backcrossed to Col-0. The *BOP1*-overexpressing line used was *bop1-6D*, which contains an insertion of four Cauliflower Mosaic Virus *35S* (CaMV 35S) enhancer sequences in the *BOP1* promoter (Norberg *et al.,* 2005). The *35S:BOP2* overexpressing line contains the CaMV *35S* promoter, which directs high levels of constitutive expression (Norberg *et al.,* 2005). *BOP1:GUS* and *BOP2:GUS* reporter lines were used as previously described (McKim *et al.,* 2008; Xu *et al.,* 2010).

Seeds were surface-sterilized with bleach before sowing. Briefly, seeds were rinsed with 100% ethanol and then transferred to a solution of 5% hypochlorite (bleach)

and 0.5% (w/v) sodium dodecyl sulphate for a one minute incubation. Following this, seeds were rinsed four times with sterile distilled water and sown on agar plates containing minimal media (Haughn and Somerville, 1986). The plates were incubated in the dark at 4ºC for two days to break dormancy, after which, the seeds were germinated under 24 hour continuous light (~115 μmol m^{-2} sec). After 7-10 days of light exposure, the seedlings were transplanted onto sterilized soil (ProMix BX, Premier Horticulture) supplemented with a 1 g L^{-1} solution of 20-20-20 plant fertilizer (Plant-Prod Inc.). Plants were grown in cabinets under continuous 24-hour light (~115 μmol m^{-2} sec) or under long days (16 hour light/8 hour dark) at 21ºC depending on the experiment.

Crossing of *Arabidopsis* was carried out as previously described (Weigel and Glazebrook, 2002). Crosses were used to generate multi loss of function *tga* mutants, shown in Table 2.3. *35S:BOP2* plants were also crossed with *tga3*, *tga7*, and *tga3 tga7* mutants to create hemizygous offspring (Table 2.3). To confirm the crosses were successful, the F1 plants were genotyped using a polymerase chain reaction (PCR) assay that tested for wild-type and mutant alleles for each gene of interest (Table 2.2A). The cross was successful if the F1 plants were heterozygous for both parental mutant alleles. To make homozygous mutant lines, the F1 generation plants were allowed to self. The resulting F2 seeds were sown and the desired combinations of mutants were identified from the segregating population. Presence of desired mutations was identified by phenotype and/or by PCR genotyping. For unlinked genes, triple mutants were expected at a frequency of 1:64 and quadruple mutants were expected at a frequency of 1:256. The homozygous mutant plants were carefully examined for defects similar to *bop1 bop2* loss of function mutants. The triple mutant *tga3 tga7 pan* has not been isolated yet, because

tga7 and *pan* were located close to each other on chromosome 1. This linkage made it difficult to obtain homozygous mutants in the time-frame of my thesis.

2.2 Genomic DNA extraction and genotyping

Plant genomic DNA for PCR genotyping was extracted as described by Edwards *et al.* (1991) with slight modifications. Young leaf tissue around the size of a dime was collected from each plant into a 1.5 ml eppendorf tube. Tissue was then ground uniformly using a blue pellet pestle homogenizer. 400 µL of extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% w/v sodium dodecyl sulphate) was added to each tube. The tubes were vortexed for 5 seconds, and then centrifuged for 10 minutes at 13,000 g. 350 µL of the supernatant was transferred to a new tube, and 350 µL of isopropanol was added to precipitate the DNA. The tubes were inverted 10 times, and left at room temperature for 5 minutes. Tubes were then centrifuged for 10 minutes at 13,000 g to pellet the DNA, and the supernatant was discarded. 800 µL of 70% ethanol was added, and the tubes were centrifuged for 5 minutes at 13,000 g. The supernatant was removed, and the tubes were left to air dry. The samples were re-suspended in 100 µL of TE pH 8.0, and stored at 4°C. 2 µL was used for a standard 20 µL PCR-genotyping reaction.

PCR genotyping was carried out using primer pairs in Table 2.2A. SALK T-DNA insertion mutants were genotyped as described (Alonso *et al.,* 2003). PCR band fragments that matched the expected size confirmed the genotype (Table 2.2A). Single nucleotide polymorphism mutant *pan-3* was genotyped using a Derived Cleaved Amplified Polymorphic Sequence (dCAPS) assay (Neff *et al.,* 1998). PCR amplified fragments

(Table 2.2A) were cut with *Sau*I to distinguish between the wild-type and mutant. Fragments with a *pan-3* mutation do not cut.

2.3 RNA extraction and qRT-PCR

Total RNA was isolated from the stem internode tissue of flowering plants. Three biological replicates were used, with each replicate containing pooled tissue from 10 or more plants. RNA was extracted from frozen, ground tissue using the Plant Total RNA Mini Kit (Geneaid). DNase I (Froggabio) was used to remove any residual DNA from the sample, following the manufacturer's protocol. The concentration of RNA was measured using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific). The sample was read at A260, and the quality was measured using the 260nm/280nm ratio. A 260nm/280nm ratio of around 2 was used to indicate good quality RNA. 2 µL of the sample was also run on a 1% agarose gel to confirm integrity. 1 µg of RNA was reversed transcribed using Superscript III polymerase (Thermo Fisher Scientific), following the manufacturer's protocol. The complementary DNA (cDNA) produced was diluted 10-fold and 2 µL was used in a 10 µL quantitative reverse transcriptase PCR reaction (qRT-PCR). Power SYBER Green PCR Master Mix (Thermo Fisher Scientific) was used to prepare the qRT-PCR reaction. The reaction was run on the Applied Biosystems StepOnePlus thermocycler (Thermo Fisher Scientific). Gene-specific primers used are listed in Table 2.2C. The primers were designed to span an intron-exon junction where possible. Reactions were carried out in triplicate. Differences in gene expression were expressed as a fold-change relative to the wild-type and calculated as described (Pfaffl, 2001). The results were normalized to *GLYCERALDE-3-PHOSPHATE DEHYDROGENASEC* and

ACTIN1 used as reference genes (Khan *et al.,* 2015; Sun *et al.,* 2018). Data shown are the mean ± standard deviation (SD) for three biological replicates.

2.4 Construction of GUS reporter gene lines

Putative promoters of *TGA3* and *TGA7* were cloned and fused in-frame with the start codon of the *GUS* gene. A 2-kb promoter region is considered to be sufficient for most genes to capture the full-length promoter, based on empirical evidence (Benhamad *et al.*, 2008). To ensure no promoter elements were missing, two different promoter lengths (2-kb and 4-kb) were fused with the GUS gene. For *TGA3*, one construct containing a 2-kb fragment of genomic DNA with sequences upstream of and including the start codon (nucleotides -1757 to +3) was created. Two constructs were created for *TGA7*: one containing a 2-kb genomic region (nucleotides -2492 to +3) and one containing a 4-kb (nucleotides -4207 to +3) genomic region. A 4-kb promoter region construct for *TGA3* was attempted, but was unsuccessful. There was non-specific binding, and amplification of the target fragment was never obtained. The 2-kb and 4-kb promoter fragments of *TGA3/TGA7* were first amplified from bacterial artificial chromosome (BAC) DNA template obtained from the ABRC (https://abrc.osu.edu). A high fidelity DNA polymerase "iProof" (Biorad) was used for amplification to reduce chance of mutations. BAC "F2E2" was used as a template for *TGA3* and BAC "F28K19" was used as a template for *TGA7*. The primers (Table 2.2B) used for template amplification included restriction sites that have the same sticky ends as the GUS vector (*Bam*HI and *Nco*I). The resulting DNA fragments were cloned into zero blunt TOPO vector (Thermo Fisher Scientific) following manufacturer's recommendations. The samples were then

sequenced with primers shown in Table 2.2B. After sequencing to verify the correct sequence of the insert in the TOPO vector, the 2-kb and 4-kb promoter fragments were cut out with the appropriate restriction enzymes, *Bam*HI and *Nco*I. The vector containing GUS (*pGCO:GUS*, which is a modified version of pGreen 0229 containing a translational fusion of *CO:GUS* with the NOS terminator) was also cut with restriction enzymes, and the sticky ends were ligated with the 2-kb and 4-kb DNA promoter fragments into the GUS vector (Hepworth *et al.,* 2002). Ligation was performed using T4 DNA ligase (New England Biolabs inc). The resulting construct was used to transform *Arabidopsis* plants using *Agrobacterium tumefaciens (Agrobacterium)* mediated gene insertion (Zhang *et al.,* 2006). Six constructs in total were used to transform *Arabidopsis,* with around 1000 seeds screened per construct. For each of the unique promoter construct fusions, two different *Agrobacterium* transformant strains were used for *Arabidopsis* infection (Table 2.4 and Table 2.5). Primary transformants were identified using the selectable marker glufosinate-ammonium (a component of the herbicide Finale™ developed by Bayer CropScience, Germany). Seedlings were sprayed with a solution comprised of 250 µm of Finale™ herbicide (5.78% glufosinate-ammonium formulation) and 10% Silwett L-77 (v/v). For each construct line, I obtained around 10 primary transformants (T1) plants that were screened by GUS staining (Table 2.5). Two to three of the strong T1 plant lines were carried forward to the T2 generation (Table 2.5). These T2 plants were further analyzed by GUS staining.

2.5 β-glucuronidase (GUS) staining and sectioning

GUS staining was performed as described (Sieburth and Meyerowitz, 1997), with minor modifications. Plant tissue samples were collected in vials containing 90% chilled acetone on ice. The samples were then incubated for 15 minutes at room temperature. After incubation, the acetone was removed, and a staining solution containing 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-Gluc) was added. Samples were incubated overnight at 37°C to allow for a blue precipitate to form. A blue precipitate indicates GUS enzyme activity, which indicates the active locations of promoter expression. The staining solution was removed and 70% ethanol was added to clear the tissue. After leaving the sample overnight, the 70% ethanol solution was changed to a fresh 70% ethanol solution and the samples were stored at room temperature.

Wax embedding and sectioning was done following our lab protocol (Popescu, 2018). The samples embedded in wax were cut into blocks around 8-12 microns thick using a microtome. Samples were de-waxed using tert-butanol, with a quick xylene dip at the end to clear the remaining wax.

2.6 Yeast two-hybrid assay

Yeast two-hybrid constructs were created using the Matchmaker ® Gold Yeast Two-Hybrid System (Takara Bio Inc), and Gateway™ compatible plasmids pGBKT7-DEST (bait) and pGADT7-DEST (prey). pGBKT7-DEST and pGADT7-DEST were modified from pGBKT7 and pGADT7-Res vectors (Lu *et al.*, 2010). The full-length coding sequence (CDS) of *TGA3* and *TGA7* were amplified from cloned cDNA template

using high fidelity DNA polymerase "iProof" (Biorad) to reduce chance of mutations. Primers used are shown in Table 2.2D. The templates used for CDS amplification were as described (Hepworth *et al.,* 2005). The resulting PCR amplified fragments were cloned into pENTR™/D-TOPO™ plasmid system (Thermo Fisher Scientific). The inserts were sequenced to verify that there were no mutations introduced from PCR. An LR reaction was performed using Gateway™ LR Clonase™ II (Thermo Fisher Scientific) between the pENTR™ plasmid containing *TGA3* or *TGA7* CDS and the pGADT7-DEST gateway™-cloning vector. The resulting plasmid was the final expression vector used for the yeast two-hybrid assay, and contains the *TGA3* or *TGA7* CDS in frame with the activation domain of yeast GAL4 protein (Table 2.4).

The resulting plasmids were isolated from *E. coli* host cells, and transferred into yeast (*Saccaryomyces cerevisiae*) as described previously (Gietz and Schiestl, 2007). The successfully transformed yeast cells were used to inoculate a 5mL culture of SD selection medium (-TRP, -LEU) and grown overnight (Gietz and Schiestl, 2007). The next day, the OD600 of the cells was recorded. The cells were pelleted and washed with sterile water to remove any media. The amount of water added to re-suspend the yeast cells was calculated using the OD600 (normalized to treatment with the highest cell density) to normalize the cell density between treatments. For each yeast treatment, a serial dilution of 10X was done 3 times (10^0, 10^{-1}, 10^{-2}, 10^{-3}). 2 µl of each treatment and dilution was plated on regular SD selection media (-TRP, -LEU) as the control (Gietz and Schiestl, 2007). 2 µl of each treatment and dilution was also plated on SD selection media lacking histidine (-TRP, -LEU, -HIS) and included 10 mM of the histidine competitive inhibitor 3-amino-1,2,4-triazole (3-AT; Sigma-Aldrich; Gietz and Schiestl, 2007). The cells were

incubated at 30°C for 2-4 days to determine if an interaction was occurring. Plates were photographed on the third and fourth day of incubation (Alpha Imager HP, Protein Simple).

2.7 Statistical analysis

Statistical analysis was done using R software (www.R-project.org). For qRT-PCR data analysis, an unpaired Student's t-test was used. For analysis of *35S:BOP2* crosses to clade III TGAs, a one-way ANOVA was used with Tukey's post-hoc test.

Table 2.1 List of genetic material used in this study

Plant line	Description	Annotation	Reference
Loss of function mutants			
bop1-3	T-DNA insertion	SALK_012994	Hepworth *et al.,* 2005
bop2-1	T-DNA insertion	SALK_075879	Hepworth *et al.,* 2005
tga3-1	Jack Thomas enhancer trap insertion		Kesarwani *et al.,* 2007
tga7-1	T-DNA insertion	GABI-KAT 434F04	Keswarwani *et al.,* 2007
tga1-1	T-DNA insertion	SALK_028212	Kesarwani *et al.,* 2007
tga4-1	T-DNA insertion	SALK_127923	Kesarwani *et al.,* 2007
pan-3	Single nucleotide polymorphism		Running and Hake, 2001
tga9-1	T-DNA insertion	SALK_091349	Murmu *et al*., 2010
Gain of function mutants			
bop1-6D	Activation tagged line, 4X CaMV 35S enhancer in *BOP1* promoter	Glufosinate-ammonium selection in plants (active)	Norberg *et al.*, 2005
35S:BOP2	Trangenic line, CaMV 35S enhancer in *BOP2* promoter	Kanamycin in plants (inactive)	Norberg *et al.,* 2005
GUS reporter line			
BOP1:GUS	4-kb promoter fragment fused in-frame to GUS	Glufosinate-ammonium selection in plants (active)	McKim *et al.,* 2008
BOP2:GUS	4-kb promoter fragment fused in-frame to GUS	Glufosinate-ammonium selection in plants (active)	Xu *et al.,* 2010
TGA3:GUS	2-kb promoter fragment fused in-frame to GUS	Glufosinate-ammonium selection in plants (active)	This study
TGA7:GUS	2-kb promoter fragment fused in-frame to GUS	Glufosinate-ammonium selection in plants (active)	This study
TGA7:GUS	4-kb promoter fragment fused in-frame to GUS	Glufosinate-ammonium selection in plants (active)	This study

Table 2.2A Genotyping primers used this study

Description	Primer	Sequence 5'-3'	Band Length
Genotyping			
bop1-3 (SALK_012994)	bop1-3 SALK_012994 RP	TGACATCGGAGAAAGCTTGAC	RP+LP= ~900 bp
	bop1-3 SALK_012994 LP	TGCACAATCTTTCGACTTCATC	RP+LB1.3= ~500 bp
	Lb1.3	ATTTTGCCGATTTCGGAAC	
bop2-1 (SALK_075879)	bop2-1 SALK_075879 RP	ATTTGGCCCACCTTTGTATTC	RP+LP= ~900 bp
	bop2-1 SALK_075879 LP	AAAGAGAGAACCTGGGTGAGC	RP+LB1.3= ~500 bp
tga3-1 (Jack Thomas Enhancer Trap)	MBtga3-1_G_RB	GCTTCTGGTCTAGCATCAACC	RB+LB= ~1100 bp
	MBtga3-1_G_LB	TCTAACAAAATCATAAACAAATCACATC	RB+T3LB= ~600 bp
	MBtga3-1_T_LB (T3LB)	GGCAATCAGCTGTTGCCCGTCTCACTGGT	
tga7-1 (GABI-KAT 434F04)	MBtga7_GSP_LP (RP)	GCATCTTTAAGAGACATGGGAATC	RP+LP= ~1000 bp
	MBtga7-1_R (LP)	TCCATCTCAAATGATGCAATCCCTGTGTTA	RP+T7LB= ~1000 bp
	Tga7-o8474 (T7LB)	ATAATAACGCTGCGGACATCTACATTTT	
tga1-1 (SALK_028212)	TGA1 SALK_028212 RP	GCTCTGCTGAAGTTTTCCACATTCC	RP+LP= ~1000 bp
	TGA1 SALK_028212 LP	TTCAAAACCTGGATTCATGGTTTCC	RP+LB1.3= ~800 bp
tga4-1 (SALK_127923)	TGA4 SALK_127923 RP	GAAGGTTTGAAGTTTACGAGCCTCT	RP+LP= ~1100 bp
	TGA4 SALK_127923 LP	GCTCTGCTGAAGTTTTCCACATTCC	RP+LB1.3= ~800 bp
pan-3 (Single nucleotide polymorphism)	pan-1-dCAPS-F1	CTAACTTACACTACACGACGTTTGATACG	PCR Band= 210 bp
	pan-3 dCAPS2-R2	GTGACAATGCATCCTCTGCTTGTTG	WT cuts with *Eco81*I/*Sau*I to produce 180 bp and 30 bp fragments, *pan-3* mutant doesn't cut
tga9-1 (SALK_091349) (bZIP21-255)	bZIP21_SALK_057609/570 RP2	TCATGATCACTCCAAGTACGTACCATGC	RP+LP= ~1100 bp
	bZIP21_SALK_057609/570 LP2	GGTGATGATACGGAGGCAAAAGAGAGCC	RP+LB1.3= ~500 bp

Table 2.2B GUS cloning primers used in this study

Description	Primer	Sequence 5'-3'
GUS Cloning		
TGA7 2-kb (upstream of start codon) forward promoter primer (has *Bam*HI)	TGA7-2KB-BamHI-Forward	ATAGGATCCCGTGCACAAGAATCTCATCAGG
TGA7 4-kb (upstream of start codon) forward promoter primer (has *Bam*HI)	TGA7-4KB-BamHI-Forward	ATAGGATCCTTGACGTTGTTAGCCTTTCTTCTCT G
TGA7 reverse promoter primer (has NcoI)	TGA7-2/4KB-NcoI-Reverse	TCACCATGGTTGGAGAAGAAGAACTCATCAT CTC
TGA3 2-kb (upstream of start codon) forward promoter primer (has *Bam*HI)	TGA3-1.7KB-BmH-F+	ATAGGATCCCCAGTTCTATTGAGCCGGTTTT
TGA3 reverse promoter primer (has *Nco*I)	TGA3-2/4KB-NcoI-Reverse	TCACCATGGTAGAAGAAGAAGAGCTCATCAT CTG
TGA7 2-kb promoter sequencing primer	TGA7-2KB-P1-Seq	TCGGTGTCTTCGTGATGAAT
TGA7 2-kb promoter sequencing primer	TGA7-2KB-P2-Seq	TGGTCCCCTCAAAATAAACC
TGA7 2-kb promoter sequencing primer	TGA7-2KB-P3-Seq	ACCACCGGAATAATCGACTG
TGA7 4-kb promoter sequencing primer	TGA7-4KB-P1-Seq	CTCAGCCAACAACGGTATGA
TGA7 4-kb promoter sequencing primer	TGA7-4KB-P2-Seq	TGAGAATATCCTCCGGCTTG
TGA7 4-kb promoter sequencing primer	TGA7-4KB-P3-Seq	GCGTGCACAAGAATCTCATC
TGA7 4-kb promoter sequencing primer	TGA7-4KB-P4-Seq	CCAAGGCTTAGTTAGCTCGGTA
TGA7 4-kb promoter sequencing primer	TGA7-4KB-P5-Seq	TTCTTTTGCAGACCCATCAG
TGA7 4-kb promoter sequencing primer	TGA7-4KB-P6-Seq	AAAGCGCACGATAAGGTCAC
TGA3 2-kb promoter sequencing primer	TGA3-2kb-Seq-P1-F	TCTCTCTTGTGGTCACCGTTTG
TGA3 2-kb promoter sequencing primer (anneals to GUS gene)	GUS-1R	CCACCAACGCTGATCAATTCCACAG

Table 2.2C qPCR primers used in this study

Description	Primer	Sequence 5'-3'
qRT-PCR		
TGA3 forward primer	TGA3-FWD	GAAGAAAGCCGGTTGAAGTTG
TGA3 reverse primer	TGA3-REV	TGCAGCAATACCTGAGTTCA
TGA7 forward primer	TGA7-FWD	CGTTTAGCGCAGAACCGAGAA
TGA7 reverse primer	TGA7-REV	CCTAAATGGCCCTGCTGCTTA
Reference genes		
ACTIN1 forward primer	ACTIN-F	CGATGAAGCTCAATCCAAACGA
ACTIN1 reverse primer	ACTIN-R	CAGAGTCGAGCACAATACCG
GLYCERALDEHYDE-3-PHOSPHATE forward primer	GAPCp1	TCAGACTCGAGAAAGCTGCTA
GLYCERALDEHYDE-3-PHOSPHATE reverse primer	GAPCp2	GATCAAGTCGACCACACGG

Table 2.2D Yeast two-hybrid cloning primers used in this study

Description	Primer	Sequence 5'-3'
Yeast two-hybrid		
TGA3 forward CDS cloning primer (CACC sequence for TOPO vector)	TGA3-SYFP-F	CACCATGGAGATGATGAGCTCT TCTTC
TGA3 reverse CDS cloning primer (no stop codon)	TGA3-SYFP-ns-R	AGTGTGTTCTCGTGGACGAG
TGA7 forward CDS cloning primer (CACC sequence for TOPO vector)	TGA7-SYFP-F	CACCATGATGAGTTCTTCTTCTC CAAC
TGA7 reverse CDS cloning primer (no stop codon)	TGA7-SYFP-ns-R	AGTTGGTTCTTGTGGACGAGCT

Table 2.3 Crosses done in this study

Female Parent ♀	X	Male Parent ♂	Resulting Progeny	Generation
35S:BOP2	X	Col-0	*35S:BOP2*/+	F1
35S:BOP2	X	*tga3*	*35S:BOP2*/+ *tga3*/+	F1
35S:BOP2	X	*tga7*	*35S:BOP2*/+ *tga7*/+	F1
35S:BOP2	X	*tga3 tga7*	*35S:BOP2*/+ *tga3*/+ *tga7*/+	F1
tga3 tga7	X	*tga1 tga4*	*tga3 tga7 tga1 tga4*	F2
tga3 tga7	X	*tga9*	*tga3 tga7 tga9*	F2
tga3 tga7	X	*pan*	*tga3 tga7 pan*	F2

Table 2.4 Constructs created in this study

Construct	Description	Strain	Resistance	Strain collection number
GUS assay				
TGA3$_{2kb\text{-}promoter}$ in PCR blunt II TOPO	2-kb upstream of start codon promoter fragment cloned into Zero Blunt pCR-Blunt II-TOPO Vector	DH5α (*E. coli*)	Kan^r	B700
TGA7$_{2kb\text{-}promoter}$ in PCR blunt II TOPO	2-kb upstream of start codon promoter fragment cloned into Zero Blunt pCR-Blunt II-TOPO Vector	DH5α (*E. coli*)	Kan^r	B683
TGA7$_{4kb\text{-}promoter}$ in PCR blunt II TOPO	4-kb upstream of start codon promoter fragment cloned into Zero Blunt pCR-Blunt II-TOPO Vector	DH5α (*E. coli*)	Kan^r	B684
TGA3$_{2kb\text{-}promoter}$*:GUS*	2-kb promoter fragment ligated in frame with GUS (pGREEN)	DH5α (*E. coli*)	Kan^r	B701, B702
TGA7$_{2kb\text{-}promoter}$*:GUS*	2-kb promoter fragment ligated in frame with GUS (pGREEN)	DH5α (*E. coli*)	Kan^r	B696, B697
TGA7$_{4kb\text{-}promoter}$*:GUS*	4-kb promoter fragment ligated in frame with GUS (pGREEN)	DH5α (*E. coli*)	Kan^r	B698, B699
TGA3$_{2kb\text{-}promoter}$*:GUS*	2-kb promoter GUS fusion transformed into *A. tumefaciens*	GV3101 (*A. tumefaciens*)	Kan^r, Rif^r, Gen^r	B708, B709
TGA7$_{2kb\text{-}promoter}$*:GUS*	2-kb promoter GUS fusion transformed into *A. tumefaciens*	GV3101 (*A. tumefaciens*)	Kan^r, Rif^r, Gen^r	B704, B705
TGA7$_{4kb\text{-}promoter}$*:GUS*	4-kb promoter GUS fusion transformed into *A. tumefaciens*	GV3101 (*A. tumefaciens*)	Kan^r, Rif^r, Gen^r	B706, B707
Yeast two-hybrid				
TGA3 CDS in pENTR/D-TOPO	*TGA3* CDS cloned into pENTR vector	DH5α (*E. coli*)	Kan^r	B779
TGA7 CDS in pENTR/D-TOPO	*TGA7* CDS cloned into pENTR vector	DH5α (*E. coli*)	Kan^r	B778
TGA3 CDS in pGADT7-DEST (AD)	*TGA3* CDS cloned into pGADT7-DEST via LR reaction	DH5α (*E. coli*)	Amp^r (leu in yeast)	B781
TGA7 CDS in pGADT7-DEST (AD)	*TGA7* CDS cloned into pGADT7-DEST via LR reaction	DH5α (*E. coli*)	Amp^r (leu in yeast)	B782
BOP1 CDS in pGBKT7-DEST (BD)	*BOP1* CDS cloned into pGBKT7-DEST via LR reaction	DH5α (*E. coli*)	Kan^r (trp in yeast)	B715
BOP2 CDS in pGBKT7-DEST (BD)	*BOP2* CDS cloned into pGBKT7-DEST via LR reaction	DH5α (*E. coli*)	Kan^r (trp in yeast)	B716

Table 2.5 Summary of *TGA3* and *TGA7* GUS lines

Generation		Number of lines	Chosen lines for next generation
Primary transformants (T_0)	Strain number		
$TGA3_{2kb\text{-}promoter}$, agro #1	B708 #C; 5C	N/A	Screened ~1000
$TGA3_{2kb\text{-}promoter}$, agro #2	B709 #A; 6A	N/A	Screened ~1000
$TGA7_{2kb\text{-}promoter}$, agro #1	B704 #A; 1A	N/A	Screened ~1000
$TGA7_{2kb\text{-}promoter}$, agro #2	B705 #B; 2B	N/A	Screened ~1000
$TGA7_{4kb\text{-}promoter}$, agro #1	B706 #A: 3A	N/A	Screened ~1000
$TGA7_{4kb\text{-}promoter}$, agro #2	B707 #C: 4C	N/A	Screened ~1000
First generation (T_1)	Strain number		
$TGA3_{2kb\text{-}promoter}$, agro #1	B708 #C; 5C	13	3 (TrayC-5C-6, TrayC-5C-10, TrayA-5C-1)
$TGA3_{2kb\text{-}promoter}$, agro #2	B709 #A; 6A	13	2 (TrayC-6A-12, TrayC-6A-2,)
$TGA7_{2kb\text{-}promoter}$, agro #1	B704 #A; 1A	13	3 (TrayB-1A-12, TrayB-1A-6, TrayB-1A-4)
$TGA7_{2kb\text{-}promoter}$, agro #2	B705 #B; 2B	16	2 (TrayB-2B-12, TrayB-2B-10)
$TGA7_{4kb\text{-}promoter}$, agro #1	B706 #A: 3A	7	2 (TrayA-3A-4, TrayA-3A-3)
$TGA7_{4kb\text{-}promoter}$, agro #2	B707 #C: 4C	6	2 (TrayA-4C-5, TrayA-4C-3)
Second generation (T_2)	Strain number		
$TGA3_{2kb\text{-}promoter}$, agro #1	B708 #C; 5C	20	To be determined
$TGA3_{2kb\text{-}promoter}$, agro #2	B709 #A; 6A	21	To be determined
$TGA7_{2kb\text{-}promoter}$, agro #1	B704 #A; 1A	26	To be determined
$TGA7_{2kb\text{-}promoter}$, agro #2	B705 #B; 2B	17	To be determined
$TGA7_{4kb\text{-}promoter}$, agro #1	B706 #A: 3A	14	To be determined
$TGA7_{4kb\text{-}promoter}$, agro #2	B707 #C: 4C	9	To be determined

CHAPTER 3: RESULTS

3.1 Transcript profiling identifies *TGA3* as up-regulated in *BOP1*-overexpressing plants

Arabidopsis has ten TGA factors grouped into five clades (Gatz, 2013). To identify potential TGA partners of BOP1 and BOP2, the transcriptome of *BOP1*-overexpressing plants (*BOP1-OE*) was used to find co-regulated *TGA* bZIP genes (Khan *et al.*, 2015). Transcripts from the stems of wild-type and dwarf *BOP1-OE* plants were compared by microarray. *TGA* bZIP genes from three different clades were significantly up-regulated in *BOP1-OE* stems (Wang *et al.,* 2019). In descending order, *TGA4* (clade I) was up-regulated 3.1 fold, *TGA3* (clade III) was up-regulated 1.5-fold, and *TGA9* (clade IV) was up-regulated 1.4-fold in *BOP1-OE* stems compared to wild-type (Figure 3.1). Clade I TGAs were confirmed to interact with BOP1/2 in regulating meristem function and inflorescence architecture (Wang *et al.,* 2019). Clade IV TGAs have a role in anther development (Murmu *et al.*, 2010). My project focused on clade III TGAs, which have no known role in plant development.

To test if clade III *TGA* up-regulation in *BOP1-OE* plants was reproducible, quantitative reverse transcriptase PCR (qRT-PCR) was performed to measure *TGA3* and *TGA7* transcript levels in a separate batch of plants. RNA was extracted from the stem of wild-type and *BOP1-OE* flowering plants. This RNA was used as template to make complementary DNA (cDNA). qRT-PCR was performed using gene-specific primers for *TGA3* and *TGA7* (Table 2.2C).

Using an unpaired Student's t-test, I found that *TGA3* transcripts (Figure 3.2) were significantly up-regulated 1.3-fold in *BOP1-OE* compared to wild-type plants ($p<0.05$). *TGA7* transcripts showed no statistically significant change ($p>0.05$). These data agree with the microarray, which showed differential up-regulation of *TGA3* in *BOP1-OE* lines. This experiment confirmed co-regulation of *BOP1* and *TGA3* in plants and prompted further analyses.

3.2 *TGA3* and *TGA7* expression is enriched at organ boundaries

Genes whose products work together are expected to be co-expressed in some or all of the same tissues. I therefore monitored the spatial pattern of *TGA3* and *TGA7* expression in plants relative to *BOP1* and *BOP2*, using promoter fusions to a *GUS* reporter gene (Jefferson *et al.,* 1987). For the *TGA3* promoter, a 2-kb (nucleotides -1757 to +3) fragment of genomic DNA was isolated, which included the sequence immediately upstream of and including the translation start codon for *TGA3*. For *TGA7* promoters, 2-kb (nucleotides -2492 to +3) and 4-kb (nucleotides -4207 to +3) fragments of genomic DNA were isolated. These sequences were used to make translational fusions with a bacterial *GUS* gene, where activation of the *TGA3* or *TGA7* putative promoters would trigger the expression of *GUS*, which codes for β-glucuronidase. The GUS enzyme acts on a colorless substrate to produce a blue precipitate allowing the visualization of gene expression in different tissues when expressed in plants (Jefferson *et al*., 1997).

The transgenic *TGA3:GUS* and *TGA7:GUS* reporter lines selected for analysis were representative of gene expression that mostly matched with information in gene expression databases. Expression atlas (https://www.ebi.ac.uk/gxa) and BAR *Arabidopsis*

eFP browser (https://www.bar.utoronto.ca) both indicate that *TGA3* is broadly expressed in stems, leaves, and flowers, at relatively high transcript levels (Waese *et al.,* 2017; Papatheodorou *et al.,* 2018; Supplemental Figure S11; Supplemental Figure S12). The promoter activity of my *TGA3:GUS* lines mostly agree with these results, although expression in the leaves was lower than expected (Figure 3.3A; Supplemental Figure S11; Supplemental Figure S12). This could indicate that there are some promoter elements not captured in the 2-kb *TGA3* promoter region.

Expression databases show that transcript levels of *TGA7* are found broadly throughout plant tissues, although the levels are relatively low (Waese *et al.,* 2017; Papatheodorou *et al.,* 2018; Supplemental Figure S11; Supplemental Figure S13). The 2-kb *TGA7:GUS* line did not have a lot of promoter expression, which contradicts information in expression databases. Therefore, the 4-kb promoter *TGA7:GUS* line was chosen over the 2-kb promoter *TGA7:GUS* line to be most representative, as its expression more closely matched expression databases. Staining of 4-kb *TGA7:GUS* tissues indicated promoter activity throughout the stems, leaves and flowers, which is consistent with expression databases (Figure 3.3B; Supplemental Figure S11; Supplemental Figure S13).

Figure 3.3 shows GUS stained plants expressing *TGA3:GUS* and *TGA7:GUS* reporter genes. Based on these data and previous studies characterizing *BOP1*:*GUS* and *BOP2:GUS* expression, expression of *TGA3* and *TGA7* partially overlaps with *BOP1* and *BOP2* expression (McKim *et al.,* 2008; Xu *et al.,* 2010). *TGA3* and *TGA7* expression are enriched at boundaries in the inflorescence; where the flower pedicel meets the stem and at floral organ boundaries (Figure 3.3A, B). This expression pattern is similar to *BOP1*

and *BOP2*, although *BOP1/2* expression seems to be stronger and more localized (Figure 3.3C, D). *TGA3* and *TGA7* are both also strongly expressed in abscission zones, leaf vascular tissues, and in the vasculature of young seedlings, which overlaps with the expression of *BOP1* and *BOP2* (Figure 3.3A, B, C, D). There were also some notable differences in expression between *TGA3/7* and *BOP1/2*. *BOP1* and *BOP2* are mainly expressed at nodes in the stem, whereas *TGA3* is broadly expressed throughout the stem (Figure 3.3A, C, D). *TGA7* is also expressed in the stem, but mainly in the phloem (Figure 3.3B). From these data, I concluded that *TGA3* and *TGA7* expression significantly overlaps with *BOP1* and *BOP2* at the proximal base of lateral organs where boundaries are located consistent with possible roles together in plant development.

3.3 Clade III TGA loss of function mutants have no obvious developmental defect

A developmental role for clade III TGAs was first assessed by analyzing the phenotype of *tga3*, *tga7*, and *tga3 tga7* loss of function mutants compared to *bop1 bop2* and wild-type control plants. Leaves of the *bop1 bop2* mutant had elongated leafy petioles that were curved and asymmetric flowers with floral organs that did not detach during fruit development (Figure 3.4B, G) as previously described (Hepworth *et al.,* 2005). The vegetative and reproductive development of *tga3* and *tga7* single mutants and the *tga3 tga7* double mutant was similar to wild-type (Figure 3.4A, C-E and Figure 3.4F, H-J). Thus, inactivation of clade III TGAs in a wild-type background has no obvious impact on plant architecture.

3.4 *TGA3* is required by *BOP1/2* to exert changes in inflorescence architecture

To further assess if clade III TGAs have a developmental role, I studied the impact of *TGA3* and *TGA7* loss of function in a *35S:BOP2* genetic background. Constitutive overexpression of *BOP1* or *BOP2* inhibits stem elongation resulting in short plants (Norberg *et al.,* 2005; Khan *et al.*, 2012ab, Khan *et al.,* 2015). Previously, it has been shown that knocking out boundary genes including *TGA1* and *TGA4* can reverse this dwarf phenotype (Khan *et al.,* 2012a; Wang *et al.,* 2019). To test if clade III TGAs also have this function, homozygous *35S:BOP2* plants were crossed with the wild-type or *tga3*, t*ga7*, and *tga3 tga7* mutants and the phenotype of F1 offspring was observed. Since BOPs interact with TGAs to regulate gene expression, we predicted that depletion of *TGA3* and/or *TGA7* might reverse the dwarf phenotype of *35S:BOP2* plants.

Figure 3.5A shows representative photos of F1 offspring of *35S:BOP2* plants crossed with Col-0, *tga3*, *tga7* or *tga3 tga7* and Figure 3.5B shows the average height of these plants. As expected, homozygous *35S:BOP2* plants crossed with wild type (*35S:BOP2/+*) were short. Crosses to *tga7 (35S:BOP2/+ tga7/+)* resulted in a non-significant increase in plant height ($p>0.05$). Crosses to *tga3 (35S:BOP2/+ tga3/+)* and *tga3 tga7 (35S:BOP2/+ tga3/+ tga7/+)* resulted in a statistically significant 6-fold recovery of height compared to the control ($p<0.05$). The height of Col-0 wild-type plants were not measured in this experiment. These data suggest that BOPs require the activity of *TGA3* and perhaps *TGA7* to exert changes in plant architecture.

3.5 BOP1/2 interacts with TGA3 and TGA7

Our model proposes that BOP1/2 interact with TGA3/7 to bind to DNA and exert function (Figure 1.6). This recruitment is through physical interaction and formation of a complex between BOP1/2 and TGA3/7. To test if TGA3 and TGA7 physically interact with BOP1/2, a yeast two-hybrid assay was used (Field and Song, 1989; Gietz *et al.,* 1997). The yeast two-hybrid assay was used because it is a highly sensitive method for detecting protein-protein interactions and has been used previously to demonstrate complex formation between PAN, TGA1, and TGA4 with BOP1 and BOP2 (Hepworth *et al.,* 2005; Wang *et al.,* 2019). To this end, BOP1 and BOP2 proteins fused to the DNA-binding domain of yeast GAL4 were used as bait. TGA3, TGA7, and PAN proteins fused to the transactivation domain of yeast GAL4 were used as prey. PAN was used as a positive control since it interacts strongly with BOP1 and BOP2 in yeast two-hybrid assays (Hepworth *et al*., 2005; Wang *et al.,* 2019). Bait and prey constructs were used to co-transform yeast. If the bait and prey interact and reconstitute GAL4 function, the result is activation of a histidine (*HIS3)* reporter gene thereby allowing the yeast to grow on solid media lacking histidine (Field and Song, 1989; Gietz *et al.,* 1997). 3-amino-1,2,4-triazole (3-AT) was used in the selection media to reduce background expression of the *HIS3* reporter. Some baits can activate the *HIS3* reporter without binding to a prey, so a negative control was also used. The negative control was a empty prey vector.

Figure 3.6 shows that there was very little growth on media lacking histidine for BOP1 or BOP2 baits paired with an empty vector prey. Strong growth on media lacking histidine showed that PAN interacted with both BOP1 and BOP2, which matches previous reports (Hepworth *et al*., 2005; Wang *et al.*, 2019). TGA3 and TGA7 also

interacted with BOP1 and BOP2. These data indicate that TGA3 and TGA7 can physically interact with BOP1/2 in yeast cells. Presumably, TGA3 and TGA7 can recruit BOP1 and BOP2 to the promoter of target genes important for development.

3.6 Functional redundancy of clade III TGAs in plant development

The above results suggest that clade III TGAs interact with BOP1/2 to regulate inflorescence architecture, despite no obvious boundary-related defects observed in *tga3 tga7* double mutants. TGA members of clade I (*TGA1* and *TGA4*) and clade V (*PAN*) are also required for *BOP* function and overlap in expression pattern with clade III TGAs (*TGA3* and *TGA7*) (Hepworth *et al.*, 2005; Wang *et al.*, 2019). Preliminary evidence suggests that a clade IV TGA (*TGA9*) is also weakly expressed at boundaries in the flower and involved in abscission (unpublished work by Ying Wang, Michael Bush, and Omar Al-Juboori). To break down functional redundancy between TGAs from different clades, higher order mutants were generated. Crossing was done to construct *tga3 tga7 tga1 tga4* quadruple mutants and *tga3 tga7 tga9* triple mutants. A *tga3 tga7 pan* triple mutant was partially constructed. There was difficulty isolating the *tga3 tga7 pan* triple mutant because *tga7* and *pan* were linked. *TGA7* and *PAN* are located very closely on chromosome 1: *PAN* is located at nucleotide position 68640 (AT1G68640), while *TGA7* is located at nucleotide position 77920 (AT1G77920). The close proximity of these genes on the same chromosome resulted in non-independent sorting during crossing.

Figure 3.7A-L shows the rosettes and inflorescences of the higher order *TGA* loss of function mutants compared to wild-type and parental control plants. *bop1 bop1* mutants showed usual hallmark defects including leafy petioles, pinwheel curvature of leaves,

abnormal flowers, and loss of floral organ abscission (Figure 3.4B, G; see also Hepworth *et al.*, 2005). The *tga3 tga7* mutants were similar to wild-type (Figure 3.7A, B and Figure 3.7G, H). The *tga1 tga4* mutants were similar to wild-type except for slight defects in petiole curvature (Figure 3.7C; see also Wang *et al.,* 2019). In the higher order *tga3 tga7 tga1 tga4* quadruple mutant, the rosette appearance was disordered. Leaves were initiated with uneven phyllotaxy and petioles were more curved compared to parental control lines (Figure 3.7D). The *tga3 tga7 tga1 tga4* quadruple mutant had no obvious defect in the inflorescence (Figure 3.7J). The *tga9* single mutant (Figure 3.7E, K) and the *tga3 tga7 tga9* triple mutant (Figure 3.7F, L) had no obvious developmental defects during vegetative or reproductive phases. Taken together, clade I and clade III TGAs function redundantly to regulate phyllotaxy and leaf curvature.

TGA Clade	Gene	Fold Increase in *BOP1-OE* compared to WT
I	*TGA4*	3.1
III	*TGA3*	1.5
IV	*TGA9*	1.4

Figure 3.1 Microarray data showing differentially expressed *TGA* bZIP genes in *BOP1-OE* plants.

A microarray approach was used to compare transcripts in the stem of wild-type (Col-0) and *BOP1*-overexpressing *(BOP1-OE)* transgenic plants (Khan *et al.*, 2015). Three *TGA* genes were significantly up-regulated in *BOP1-OE* stem tissue: *TGA4* was up-regulated 3.1-fold, *TGA3* was up-regulated 1.5-fold, and *TGA9* was up-regulated 1.4-fold relative to the wild-type.

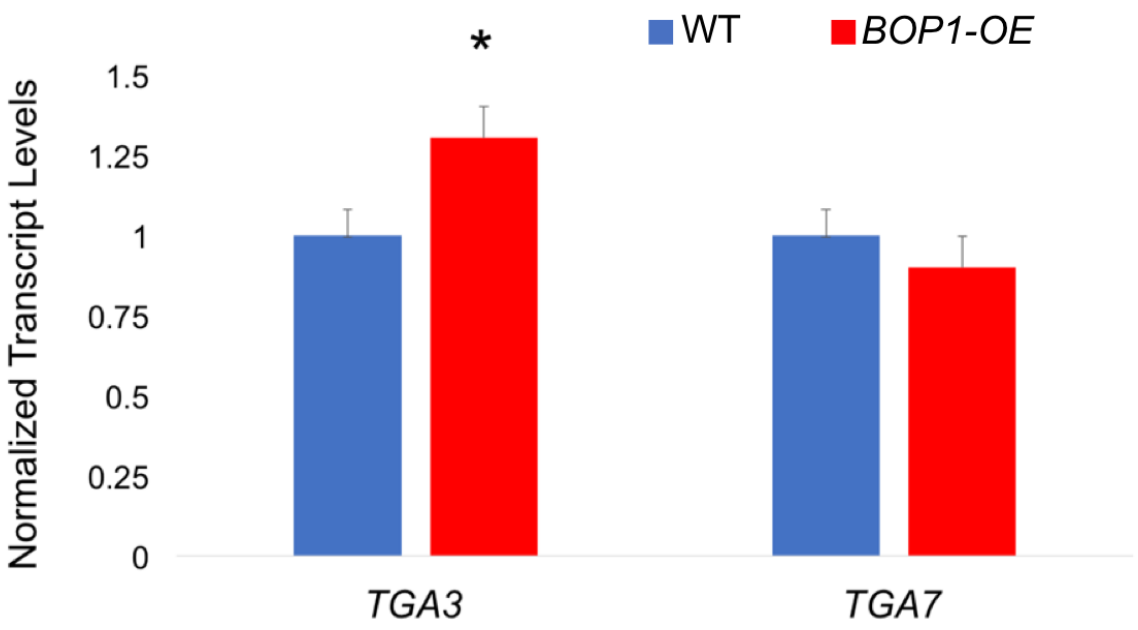

Figure 3.2 Transcript analysis of *TGA3* and *TGA7* in *BOP1-OE* plants.

Clade III *TGA* transcript levels were measured by qRT-PCR in stems of transgenic *BOP1-OE* plants normalized to wild type. Data shown are mean ± SD for three biological replicates, each consisting of pooled tissue from three different plants. Three technical replicates were performed per replicate. The results were statistically analyzed using an unpaired Student's t-test. *TGA3* is significantly 1.3-fold higher in *BOP1-OE* stems than wild type ($p<0.05$). *TGA7* is not statistically different from wild-type ($p>0.05$). The results show that *TGA3* is up regulated in *BOP1-OE* lines but *TGA7* is not.

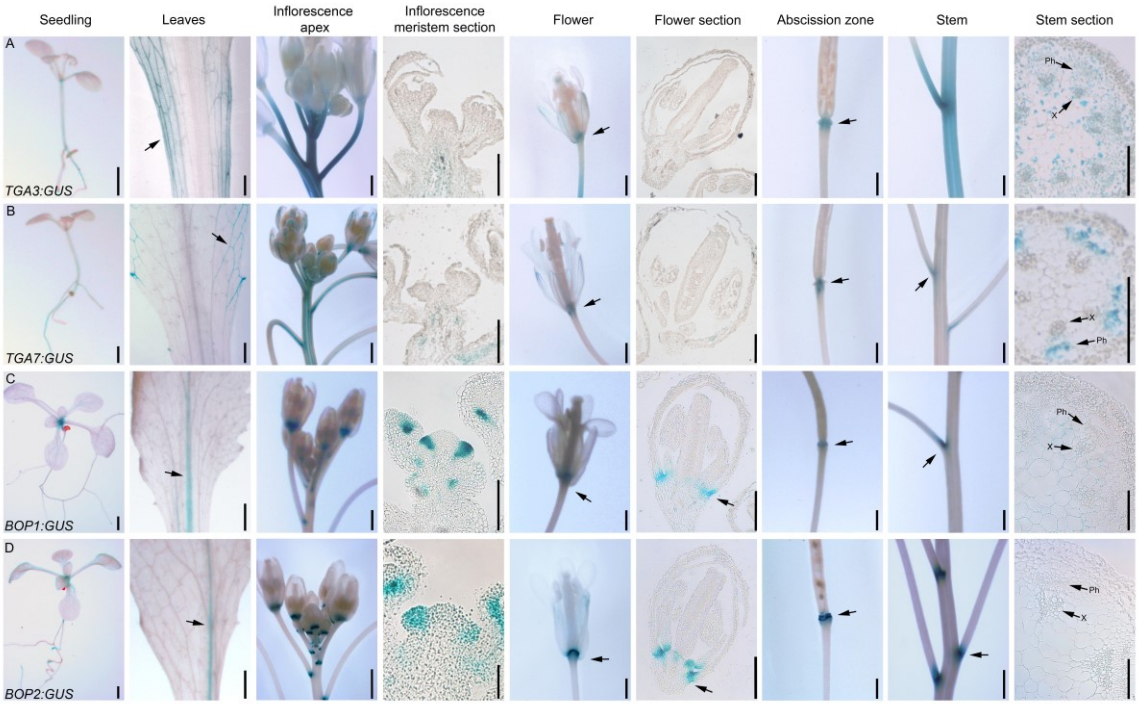
Seedling
Leaves
Inflorescence apex
Inflorescence meristem section
Flower
Flower section
Abscission zone
Stem
Stem section
A
TGA3:GUS
B
TGA7:GUS
C
BOP1:GUS
D
BOP2:GUS
Ph
X

Figure 3.3 Expression patterns of *TGA3* and *TGA7* using GUS reporter.

Promoter fusions to a reporter gene encoding β-glucuronidase (GUS) were used to visualize the expression patterns of *TGA3* and *TGA7* in plants. 15 plants from 2-kb promoter *TGA3:GUS* line 6A12 and 15 plants from 4-kb promoter *TGA7*:GUS line 4C5 were stained in the T2 generation. Representative images taken are shown. *BOP1/2* expression is shown for comparison using images from previous publications (Khan *et al.,* 2015; Wang *et al.,* 2019). Seedlings shown are 10-days-old and leaves shown are from 5-week-old flowering plants. Wax cross sections were done on inflorescence meristems, flowers, and stems. *TGA3* and *TGA7* expression is enriched at boundaries in the inflorescence and strongly expressed in abscission zones (A, B). Expression is also observed in the vascular tissue of young seedlings, leaves and stems (A, B). *TGA3* expression in the stem is concentrated in the cortex, while *TGA7* is expressed in the phloem (A, B). *BOP1* and *BOP2* are enriched in the vasculature of young seedlings and leaves, and boundaries in the inflorescence including the junction where the base of the pedicel meets the stem and abscission zones (C, D). *BOP1* and *BOP2* are also highly expressed at organ boundaries in the inflorescence meristem and flowers (C, D). Scale bar represents 1 mm (seedling, leaves, inflorescence apex, flower, abscission zone, stem) and 0.1 mm (inflorescence meristem section, flower section, stem section). X, xylem; Ph, phloem.

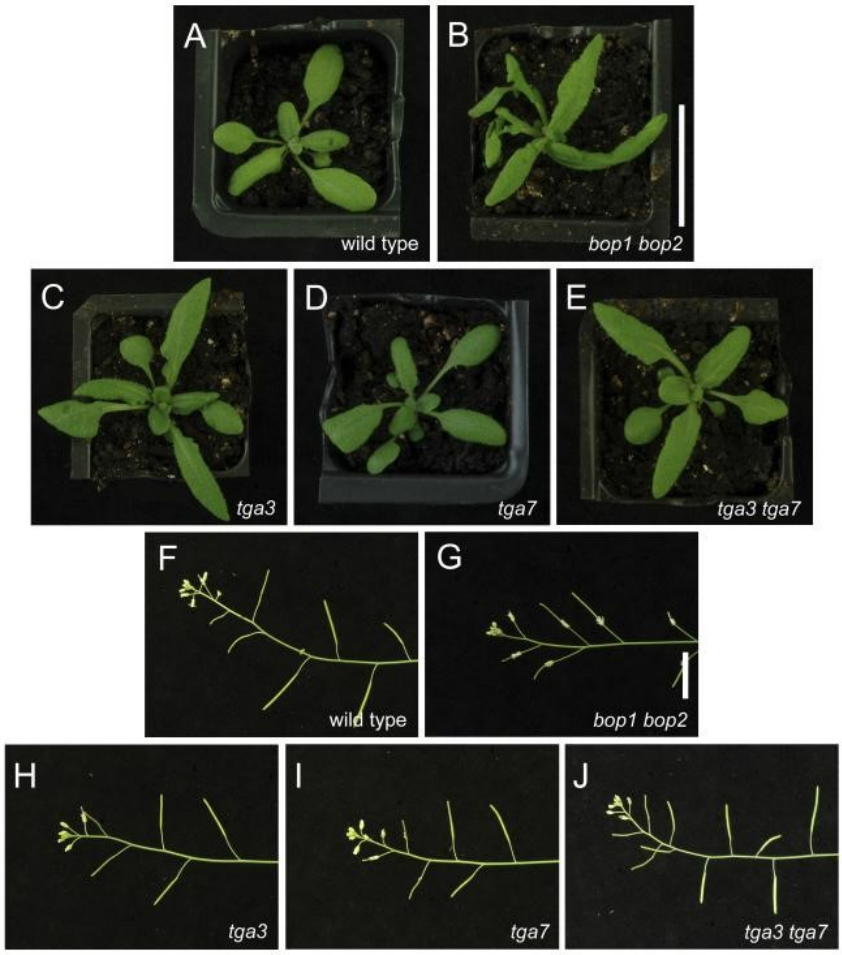

Figure 3.4 Comparing rosette and inflorescence of *bop1 bop2* and *tga3 tga7*.

For each genotype, 12 plants were grown in continuous light. Representative pictures taken of 3-week-old plant rosettes (A-E) and 5-week-old plant inflorescences (F-J) are shown. Wild type (A, F) was used as the control. Loss of function *bop1 bop2* mutants (B, G) had characteristic pinwheel leaf curvature, leafy petioles (B), and loss of floral abscission (G). Single mutants *tga3* (C, H), *tga7* (D, I), and double mutant *tga3 tga7* (E, J) had no obvious developmental defects in the vegetative or reproductive phase. Scale bar represents 3 cm (A-E), 1.5 cm (F-J).

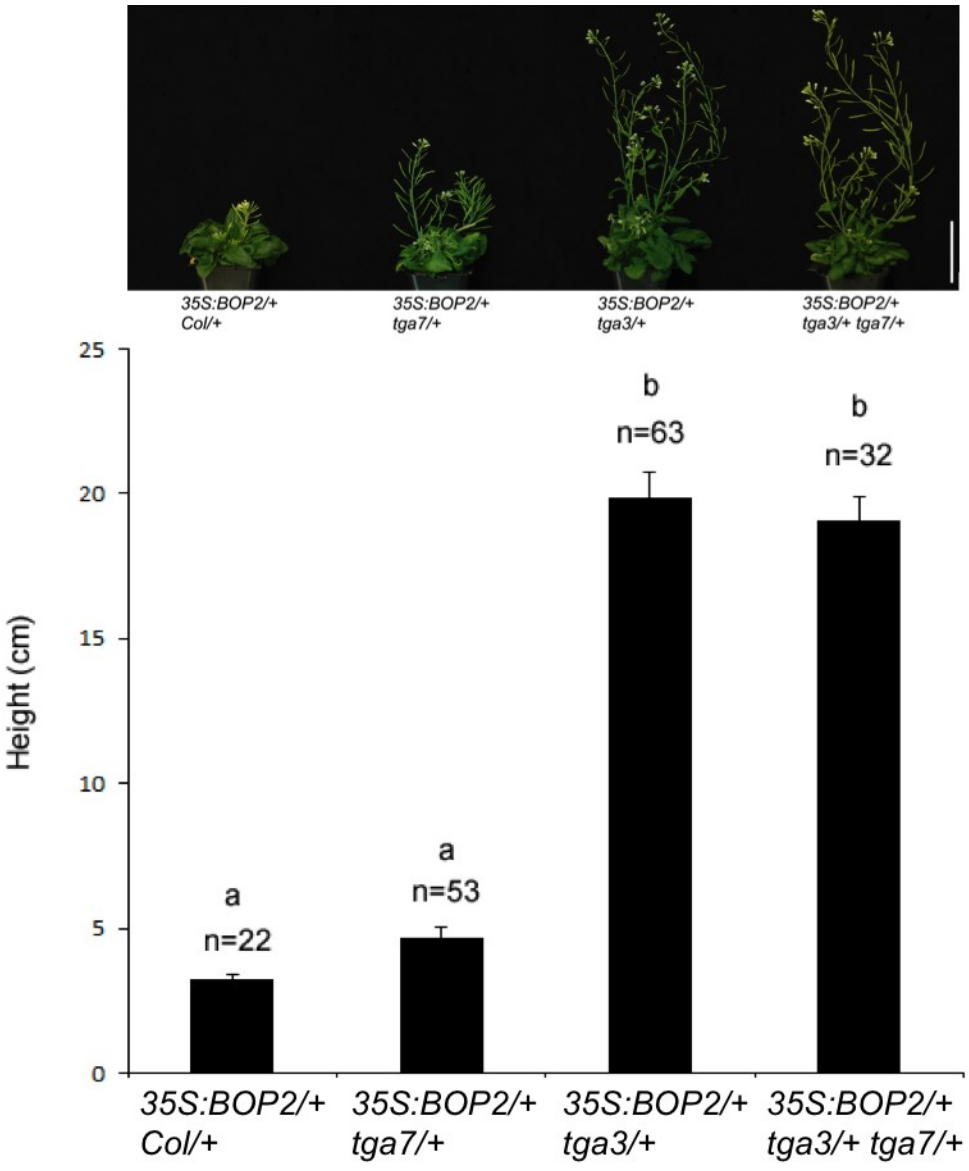
35S:BOP2/+ Col/+
35S:BOP2/+ tga7/+
35S:BOP2/+ tga3/+
35S:BOP2/+ tga3/+ tga7/+
Height (cm)
25
20
15
10
5
0
a
n=22
a
n=53
b
n=63
b
n=32
35S:BOP2/+ Col/+
35S:BOP2/+ tga7/+
35S:BOP2/+ tga3/+
35S:BOP2/+ tga3/+ tga7/+

Figure 3.5 Height recovery of *BOP2*-overexpressing plants hemizygous for *TGA3* and/or *TGA7*.

Plants homozygous for a *35S:BOP2* transgene were crossed to wild-type Col-0 plants or homozygous *tga3*, *tga7*, or *tga3 tga7* mutants. (A) Phenotype of representative F1 offspring. Scale bar = 4.5 cm. (B) Quantitative analysis of plant height. The height of F1 7-week-old plant progenies grown under long day conditions (16 hour light/8 hour dark) were measured. Data represent the mean ± standard error and n indicates the number of plants that were measured. The data were statistically analyzed using a one-way ANOVA with Tukey's post-hoc test ($p<0.05$). Based on this analysis, *35S:BOP2* plants crossed to *tga3* and *tga3 tga7* were significantly taller than control crosses to Col-0 ($p<0.05$). *35S:BOP2* plants crossed to *tga7* were slighter taller but this increase did not reach statistical significance ($p>0.05$).

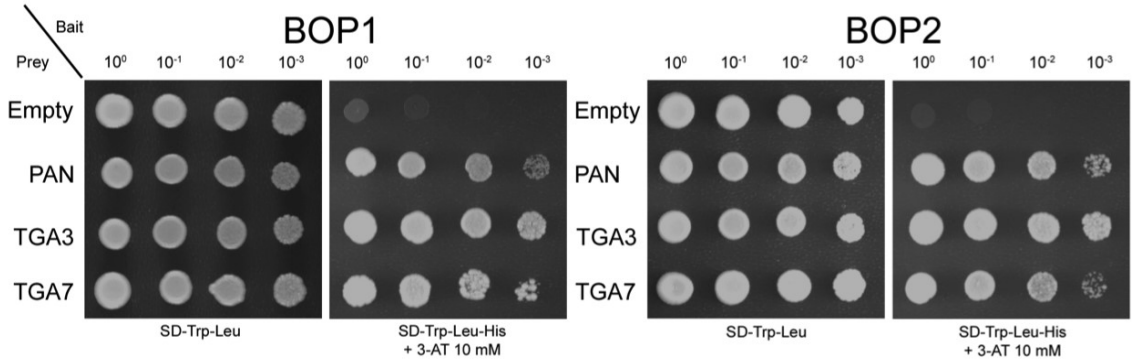

Figure 3.6 Pair-wise yeast two-hybrid assays showing BOP1 and BOP2 interaction with TGA3 and TGA7.

BOP1 and BOP2 baits were fused to the DNA-binding domain of yeast GAL4. TGA preys were fused to the transactivation domain of GAL4. Yeast strain AH109 was co-transformed with bait and prey plasmid pairs. Serial dilutions (10^{0}, 10^{-1}, 10^{-2}, 10^{-3}) were plated onto SD/-Trp/-Leu medium or SD/-Trp/-Leu/-His medium with 10 mM 3-AT (a competitive inhibitor of histidine). Interactions were detected based on expression of a *HIS3* reporter gene. Growth on medium lacking histidine above background indicates an interaction. *PAN* was used as a positive control. Empty prey vector was used as a negative control. The results show that BOP1 and BOP2 interact with TGA3 and TGA7.

Figure 3.7 Phenotype of TGA multi-clade loss of function mutants.

Functional redundancy between clade III TGAs and TGAs from clade I (TGA1 and TGA4) or clade IV (TGA9) was assessed by higher-order mutant analysis. For each genotype, 18 plants were grown under continuous light. Shown are representative pictures taken of 3-week-old rosettes (A-F) and 5-week-old inflorescences (G-L) for wild-type and mutant plants. Wild-type (A, G) was used as the control. Higher order *tga* mutant combinations were characterized. *tga3 tga7* (B), *tga9* (E), and *tga3 tga7 tga9* (F) mutants had no obvious rosette defects. *tga1 tga4* mutants (C) have a pinwheel-like leaf curvature defect. In the quadruple mutant *tga3 tga7 tga1 tga4* (D) this leaf curvature is enhanced and phyllotaxy is more disordered compared to the *tga1 tga4* double mutant alone (C). No defects were visible in the inflorescences (G-L). Scale bar represents 3 cm (A-F), 1.5 cm (G-L).

CHAPTER 4: DISCUSSION

The SAM is ultimately responsible for determining the aerial body plan of plants. Organ boundaries are an important subdomain of the meristem that control diverse aspects of plant development. Leaf patterning, flower development, inflorescence architecture, and abscission are all determined at boundaries.

BOP1 and *BOP2*, first discovered in *Arabidopsis*, represent a class of genes important for boundary patterning in land plants (Ha *et al.,* 2003; Ha *et al.,* 2004; Hepworth *et al.,* 2005; Norberg *et al.,* 2005). BOP1/2 interact with TGA transcription factors for attachment to DNA to exert function (Hepworth *et al.,* 2005; Wang *et al*., 2019). It has been shown that BOP1/2 interact physically and genetically with TGA1 and TGA4 from clade I to regulate boundary maintenance and inflorescence architecture and with PAN from clade V to regulate flower development (Hepworth *et al.,* 2005; Wang *et al.,* 2019). However, inactivation of clade I and clade V factors alone or in combination fails to recreate a *bop1 bop2* mutant phenotype suggesting that other TGAs might be involved.

My thesis identifies clade III TGAs as a third group involved in BOP-mediated regulation of plant development. Expression of *TGA3* and *TGA7* is enriched at organ boundaries in the plant, and TGA3 and TGA7 can physically interact with BOP1 and BOP2 to form a complex in yeast cells. BOPs require the activity of *TGA3* and possibly *TGA7* to regulate inflorescence architecture. Double mutant analysis shows that clade III and clade I TGAs function redundantly to regulate leaf patterning. Taken together, these results indicate a previously undiscovered role for clade III TGAs in plant development.

4.1 A dual role for TGA3 in defense and development

Clade III TGAs interact with NPR1 (Despres *et al.*, 2000). While no defense role has been identified for *TGA7*, several studies show *NPR1*-dependent defense roles for *TGA3* (Johnson *et al.,* 2003; Choi *et al.,* 2010; Sarkar *et al.,* 2017). These studies show that TGA3 can interact with cytokinin activated transcription factor ARR2 to induce plant defense genes in an *NPR1*-dependent manner (Choi *et al.,* 2010). TGA3 also interacts with WRKY53 to enhance SA-inducible CmYLCV promoter activity under *NPR1*-dependent SA signalling (Sarkar *et al.,* 2017). In addition, loss of function *tga3* mutants have increased susceptibility to diverse pathogens (Kesarwani *et al.,* 2007; Windram *et al.,* 2012).

My work suggests that clade III TGAs also interact with BOPs and function in plant development. To summarize, use of reporter genes showed that *TGA3* and *TGA7* expression patterns overlap with *BOP1* and *BOP2* in the petiole region of leaves and at boundaries in the inflorescence, including the abscission zones in flowers. Although loss-of-function *tga3*, *tga7*, and *tga3 tga7* mutants have no obvious developmental phenotype, crosses showed that *35S:BOP2* plants require *TGA3* and potentially *TGA7* activity to inhibit growth. *35S:BOP2* plants are dwarfed. However, stem inhibition is abolished in *35S:BOP2* plants hemizygous for *TGA3* indicating its requirement by *BOP2* to regulate plant height (Figure 3.5).

Taken together, *TGA3* has dual functions in development and defense. The mechanism of TGA3 selection of BOP1/2 and NPR1 for its different roles is unknown, but might depend on the presence of other development or defense-specific third parties like ARR2. Clade I TGAs also interact with BOP1/2 and NPR1 and have resulting dual

functions in plant development and defense (Shearer *et al.,* 2012; Bergin, 2018; Wang *et al.,* 2019). Interestingly, clade I TGA activity is under redox regulation (Després *et al.,* 2003; Sun *et al.,* 2018). The reduction of Cys residues in TGA1 in defense-triggered plants promotes an interaction with NPR1 (Despres *et al.*, 2003) and hinders interactions with BOP1 and BOP2 (Wang *et al.*, 2019). TGA3 selectivity may be under similar redox regulation. Another potential regulatory mechanism for TGA3 may be proteolysis. TGA3 abundance appears to be developmentally regulated by proteolysis (Pontier *et al.,* 2002). These finding raises the possibility that TGA3 interactions with BOP1/2 and NPR1 could be gated by controlling protein abundance. In addition, TGA3 has been shown to bind to Ca^{2+} as a result of Ca^{2+}/calmodulin2 defense signaling to regulate gene expression (Fang *et al.,* 2017). The binding of Ca^{2+} to TGA3 could change the binding affinity of TGA3 for BOP1/2 or NPR1 to differentially regulate gene expression. The mechanism by which TGA3 selectivity interacts with BTB-ankyrin proteins should be further explored.

4.2 TGAs are very functionally redundant

TGA transcription factors function with a high degree of redundancy (Gatz, 2013). Most single loss of function *tga* mutants show no obvious phenotype (Jakoby *et al.*, 2001; Gatz, 2013; Khan *et al.*, 2014). This study addresses functional redundancy between clade I and clade III members by analyzing higher order mutants. However, a quadruple knock out of clade I and clade III TGAs did not substantially result in *bop1 bop2*-like defects in development. Loss of *TGA3* and *TGA7* in a *tga1 tga4* double mutant background merely enhanced *tga1 tga4* defects in petiole curvature and disrupted the phyllotaxy of leaves (Figure 3.7D). A petiole curvature defect is also seen in *bop1 bop2* leaves, but other

characteristic defects of *BOP1* and *BOP2* mutation that affect floral patterning and abscission were absent. We speculate that knocking out clade I, clade III, clade V, clade II or clade IV TGAs together might expose additional *bop1 bop2*-like defects. BOPs interact with all these clades of TGAs, so knocking out their functions should result in defects more severe than the single or double clade *tga* mutants.

4.3 BOPs and other co-factors

Given that knocking out clade I and clade III TGAs produces plants with surprisingly few developmental defects, we must consider the possibility that TGAs are but one class of proteins that BOPs interact with to regulate plant development. BOPs have also been shown to act as substrate adaptors for CUL3 E3 ubiquitin ligase-dependent targeting of proteins for ubiquitination and degradation (Zhang *et al.,* 2017; Chahtane *et al.,* 2018). The first study showed that BOP proteins act in a CUL3-based E3 ubiquitin ligase complex to regulate PIF4 protein abundance. This regulation controls light and temperature-dependent hypocotyl elongation in seedlings (Zhang *et al.*, 2017). Through similar mechanisms, BOP1/2 also modulate the abundance of LFY transcription factor to regulate *APETALA1* expression and floral meristem development (Chahtane *et al.,* 2018). Based on these two studies, BOP1/2 could potentially also regulate boundary patterning as part of an E3 ubiquitin ligase complex that regulates transcription factor abundance. Thus, BOP1/2 may only exert part of its boundary-related functions through TGA transcription factors. Future studies should explore the possibility of the BOP1/2-CUL3 complex targeting other transcription factors that regulate boundaries.

4.4 BOP1/2 and TGA3/7 target genes

Clade III TGAs presumably recruit BOP1 and BOP2 to the promoter of target genes to activate transcription. The recovery of plant height in *35S:BOP2* plants hemizygous for *TGA3* and *TGA7* suggests that these target genes inhibit growth (Figure 3.5). At the moment, these target genes are unknown.

Interestingly, dwarf *BOP1-OE* plants constitutively express defense genes (Bergin, 2018). Activation of plant immunity is tightly linked to growth repression (van Wersch *et al.*, 2016). For example, seedlings exposed to bacterial flagellin for one week exhibit a >75% reduction in growth compared to non-exposed seedlings (Gomez-Gomez *et al.,* 1999). ROS generated during defense contribute to growth inhibition. One way that ROS inhibits growth is by stiffening the cell wall through cross-linking of proteins and polymerization of lignin (O'Brien *et al.,* 2012). *BOP1-OE* plants express higher levels of an ROS-generating NADPH oxidase gene and show higher levels of hydrogen peroxide staining in leaves (Bergin, 2018). Direct targets of TGA3 and BOP1/2 might include genes involved in ROS metabolism.

Repression of BR and GA signaling are additional mechanisms of growth inhibition that link boundaries and plant defense. BR and GA are major growth-promoting hormones in plants. Boundaries are a minimum for BR and GA (Hepworth and Pautot, 2015). BR and GA signaling are repressed during plant defense (Huot *et al.*, 2014; Lozano-Duran and Zipfel, 2015).

BR stimulates growth by binding to receptors in the plasma membrane. Activation of these receptors leads to de-phosphorylation of BZR1 and BZR2 transcription factors and activation of BR-responsive genes (Lozano-Duran and Zipfel, 2015). Similar to

BOP1-OE plants, BR-deficient or BR-insensitive mutants are dwarf (Clouse *et al.,* 1996; Li *et al.*, 1996). One way that BOPs repress BR is through induction of *LOB* (Ha *et al.,* 2007). LOB activates *BAS1,* which encodes a BR-inactivating enzyme (Neff *et al.,* 1999; Bell *et al.,* 2012). Several BR biosynthetic genes are down-regulated in *BOP1-OE* plants (data not shown) suggesting that BOP1/2 and TGA3 possibly target BR regulators that inhibit BR, resulting in decreased stem elongation.

GA-insensitive mutants also show inhibition of stem elongation and growth (Hedden and Phillips, 2000). GA promotes plant growth through the repression of DELLA proteins. DELLA proteins inhibit growth and flowering, and GA reverses these effects through DELLA degradation (Achard and Genschik, 2009). The BOP1/2 complex with TGA3 could promote the expression of DELLA genes to repress GA signaling and reduce growth. Several DELLA genes are up-regulated in *BOP1-OE* plants but simple addition of exogenous GA to *BOP1-OE* does not restore plant height suggesting that other mechanisms are involved (Khan *et al.*, 2015).

4.5 Summary and future direction

In summary, my findings show that clade III TGAs have a previously undiscovered role in plant development. This finding underscores that TGA bZIP transcription factors are involved in a host of plant processes, including defense and development (Zhang *et al.*, 2003; Hepworth *et al.,* 2005; Choi *et al.,* 2010; Shearer *et al.,* 2012; Wang *et al.,* 2019).

A future challenge is to identify how the interaction of clade III TGAs with BTB-ankyrin proteins is regulated and how components of this complex influence the selection

of target genes. Future work should focus on identifying and characterizing target genes of BOP1/2 and TGA3/7. The redundancy of clade III TGAs with TGAs in other clades and their function with BOPs should also be explored.

Closing the loop on agriculture, domestication has taken advantage of regulators of *BOP* expression to improve branching in maize (Dong *et al.,* 2017) and seed dispersal in rice (Konishi *et al.,* 2006). It will be interesting to discover if changes in TGA regulation have also been exploited through domestication to optimize architecture.

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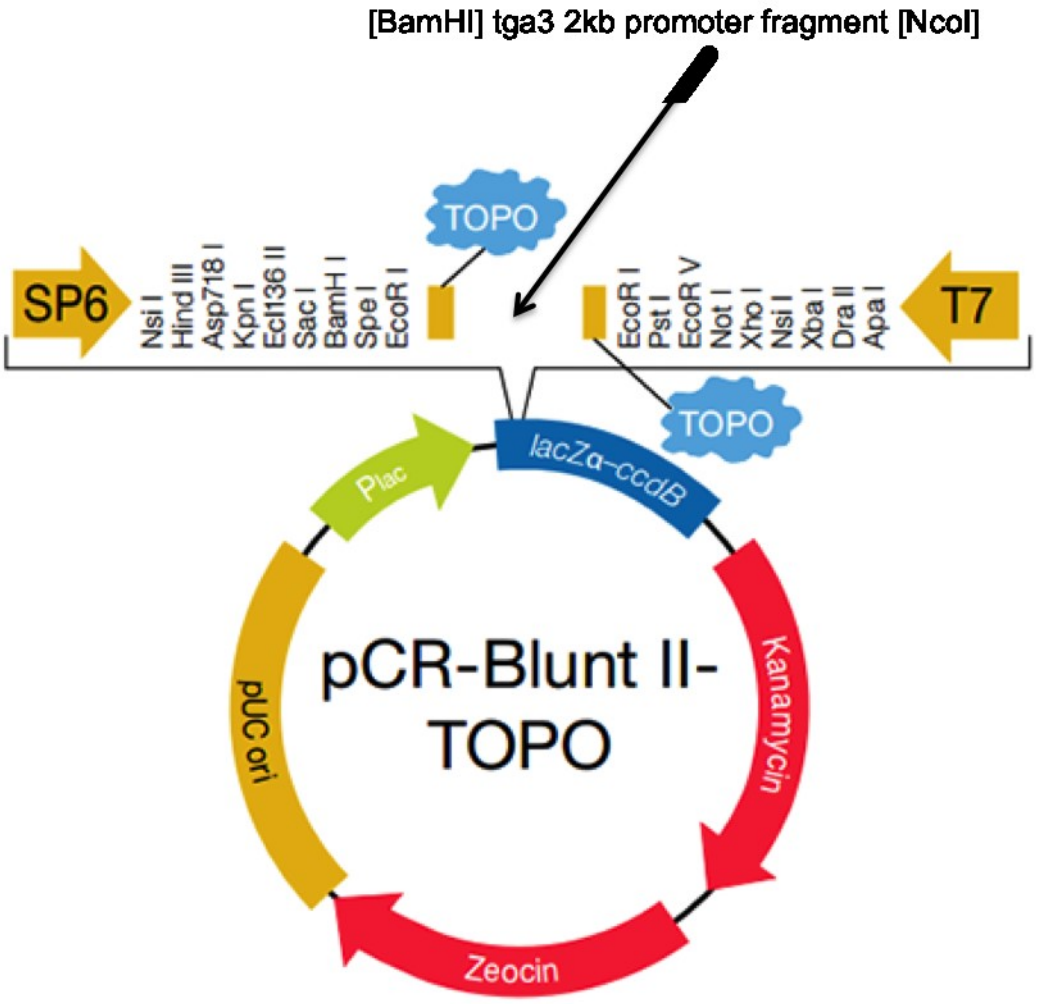
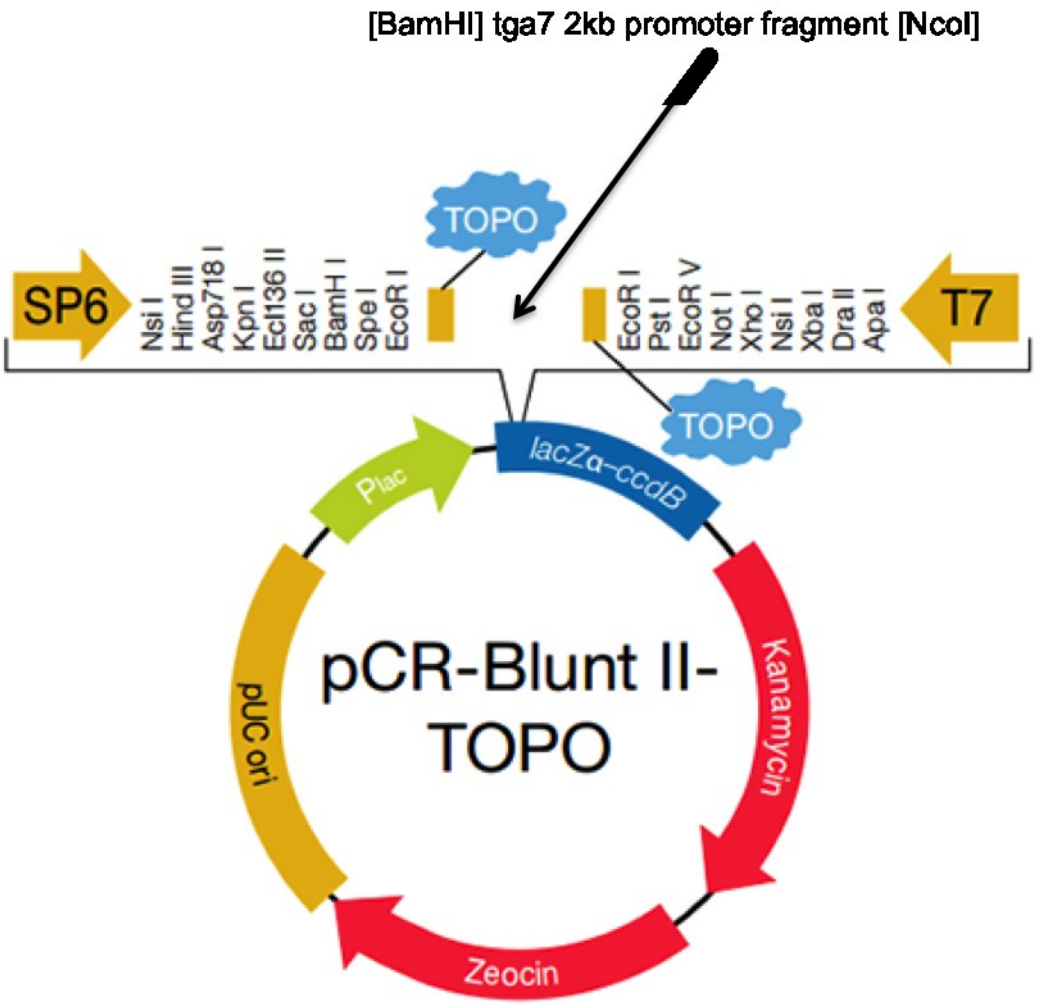
SUPPLEMENTAL MATERIALS



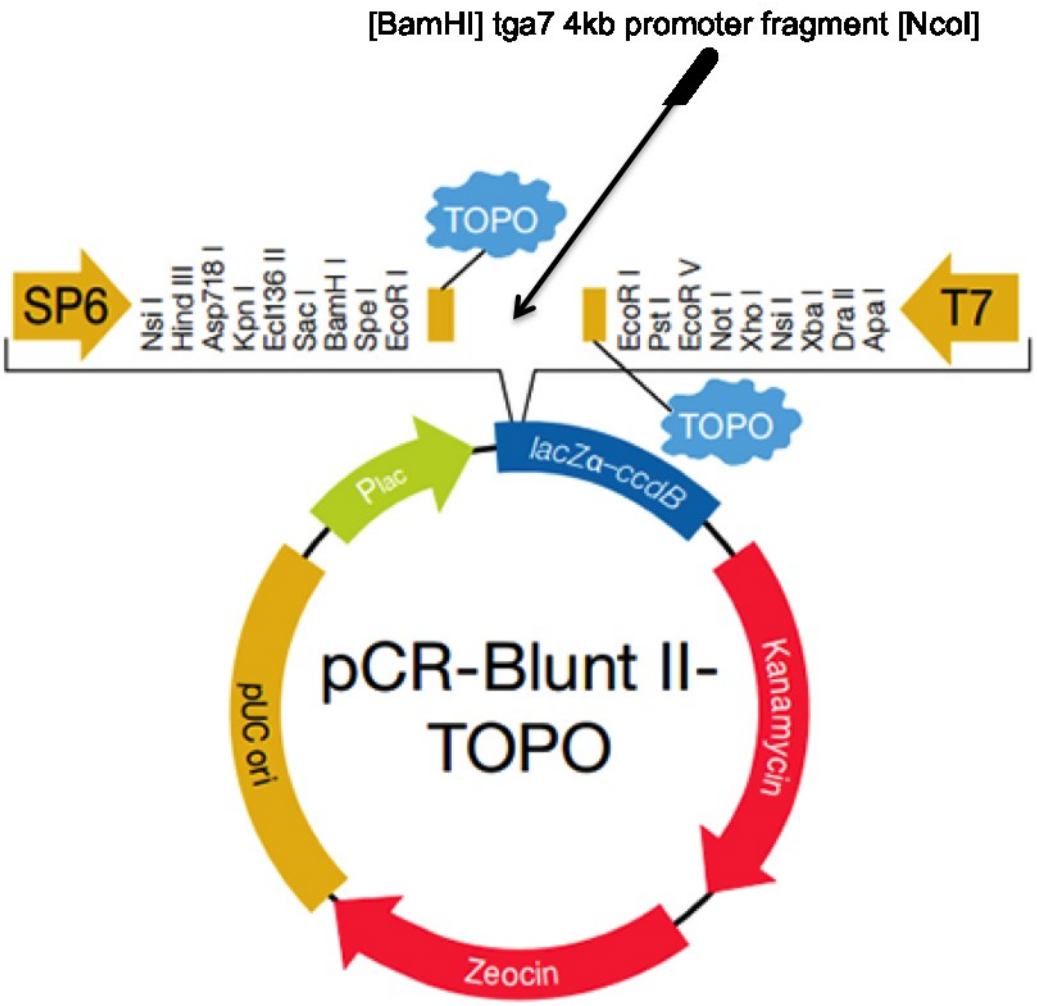
Supplemental Figure S1. Plasmid map of *TGA3* 2-kb promoter in TOPO vector.

TGA3 2-kb promoter (2-kb upstream of and including ATG, nucleotides -1757 to +3) cloned into PCR-BLUNT-II-TOPO vector (strain number B700). Restriction sites (*Bam*HI and *Nco*I) were added at each end using primers during PCR amplification.



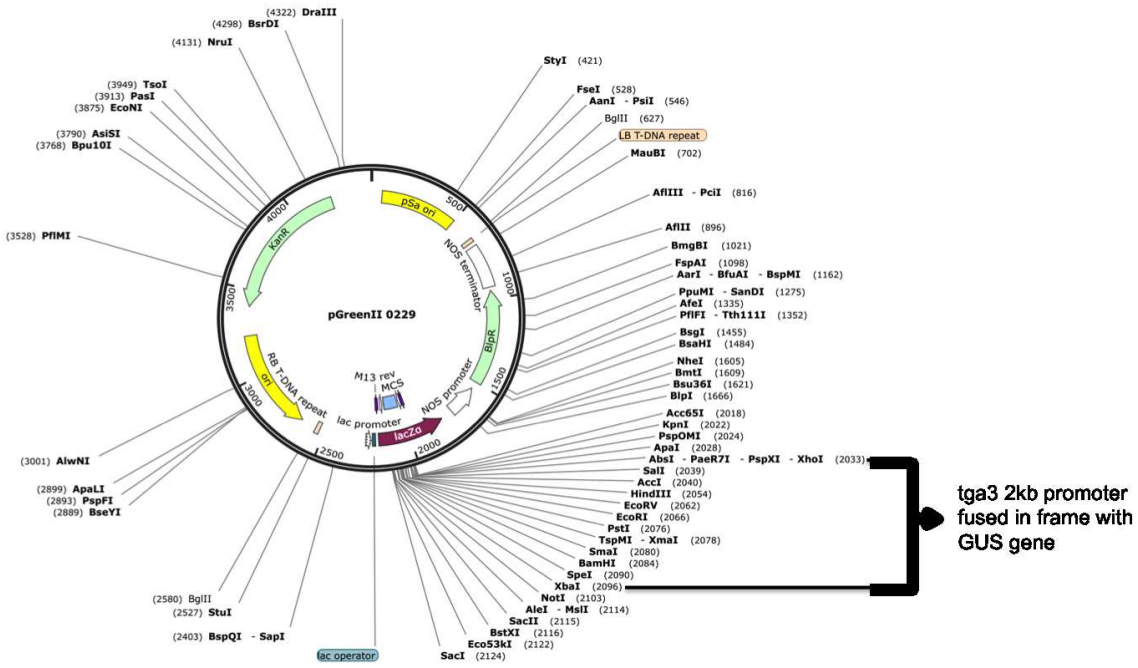
Supplemental Figure S2. Plasmid map of *TGA7* 2-kb promoter in TOPO vector.

TGA7 2-kb promoter (2-kb upstream of and including ATG, nucleotides -2492 to +3) cloned into PCR-BLUNT-II-TOPO vector (strain number B683). Restriction sites (*Bam*HI and *Nco*I) were added at each end using primers during PCR amplification.



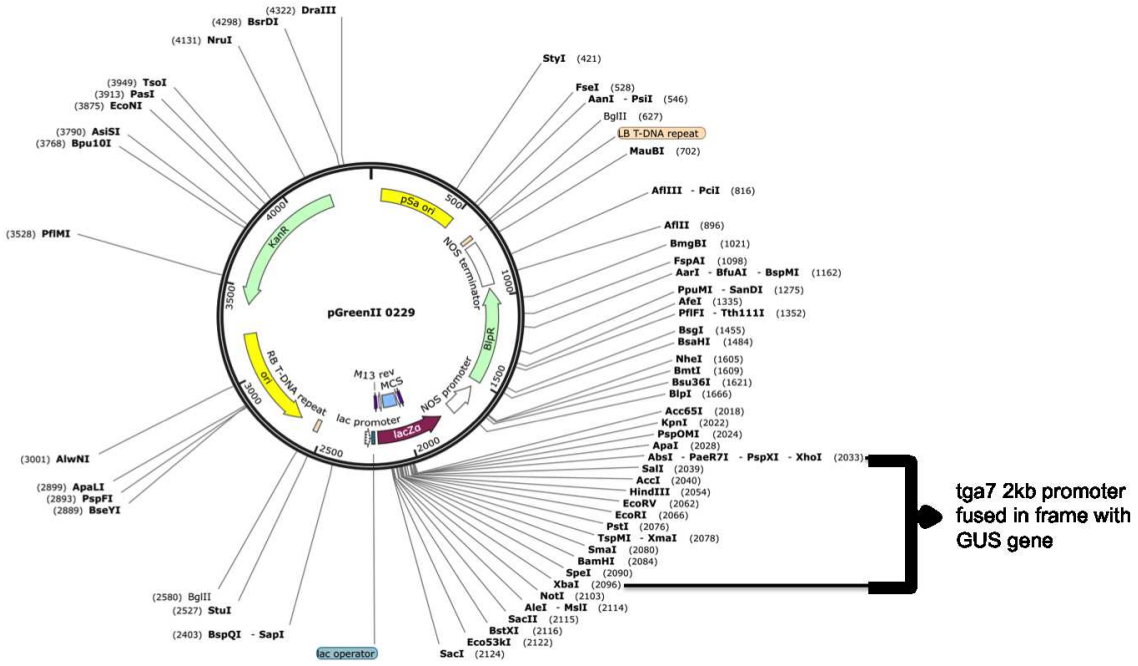
Supplemental Figure S3. Plasmid map of *TGA7* 4-kb promoter in TOPO vector.

TGA7 4-kb promoter (4-kb upstream of and including ATG, nucleotides -4207 to +3) cloned into PCR-BLUNT-II-TOPO vector (strain number B684). Restriction sites (*Bam*HI and *Nco*I) were added at each end using primers during PCR amplification.



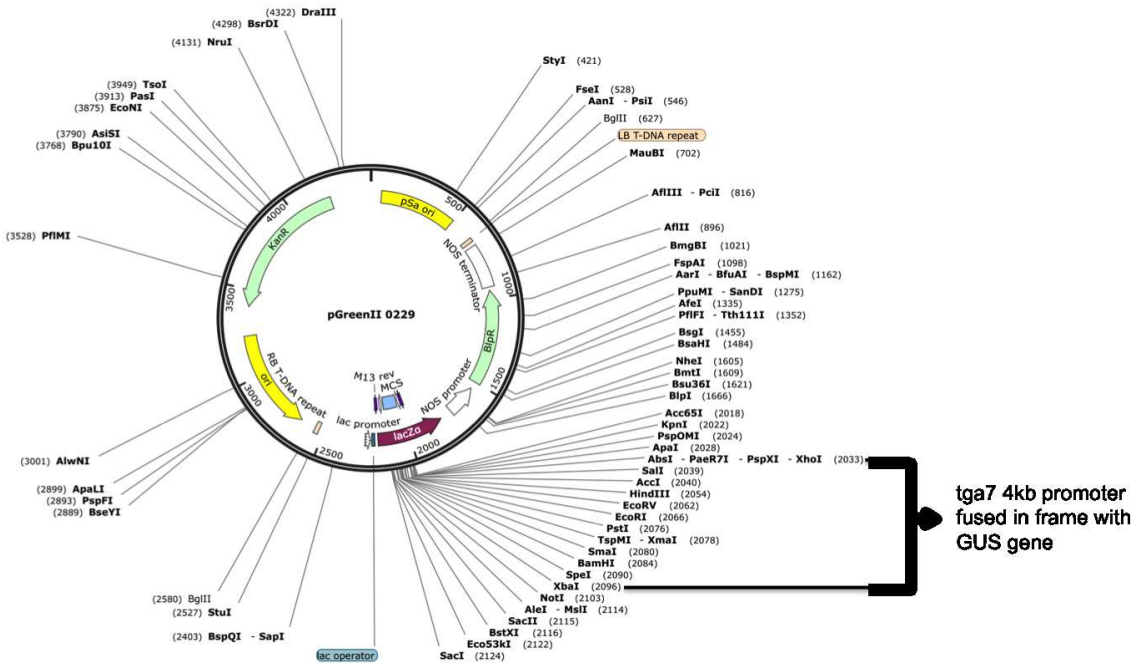
Supplemental Figure S4. Plasmid map of *TGA3* 2-kb promoter fusion with *GUS* gene in the pGreen 0229 vector.

The vector used had previously been modified to contain a translational fusion of *CO:GUS* with the NOS terminator between the *Xba*I and *Xho*I sites (Hepworth *et al.,* 2002). The *CO* promoter was excised through restriction sites *Bam*HI and *Nco*I and *TGA3* 2-kb promoter was ligated in frame with GUS (strain number B701, B702).



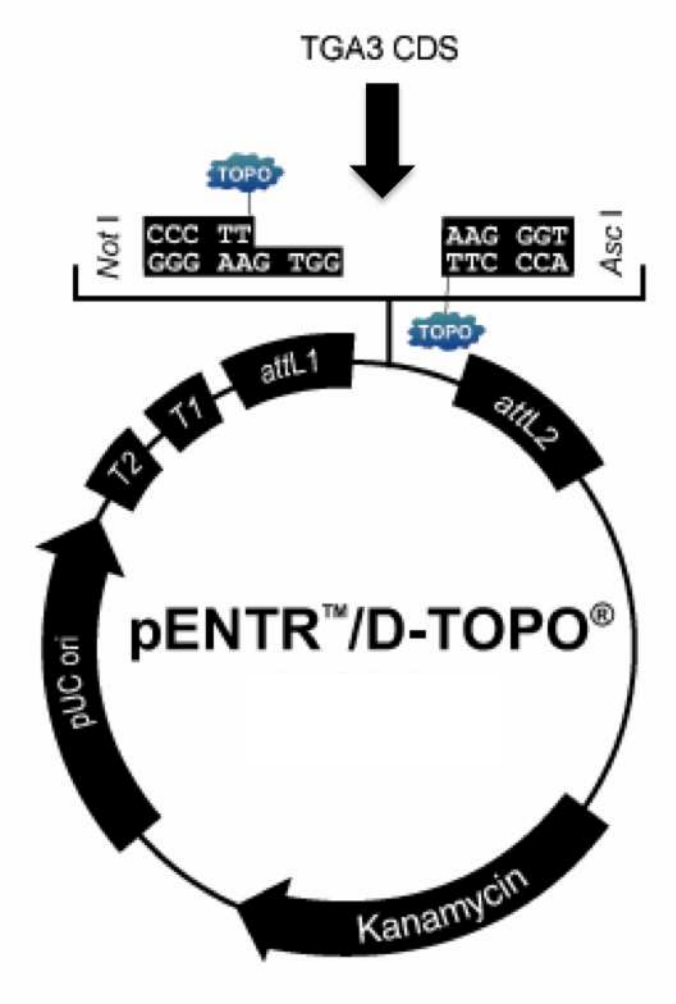
Supplemental Figure S5. Plasmid map of *TGA7* 2-kb promoter fusion with *GUS* gene in the pGreen 0229 vector.

The vector used had previously been modified to contain a translational fusion of *CO:GUS* with the NOS terminator between the *Xba*I and *Xho*I sites (Hepworth *et al.,* 2002). The *CO* promoter was excised through restriction sites *Bam*HI and *Nco*I and *TGA7* 2-kb promoter was ligated in frame with GUS (strain number B696, B697).



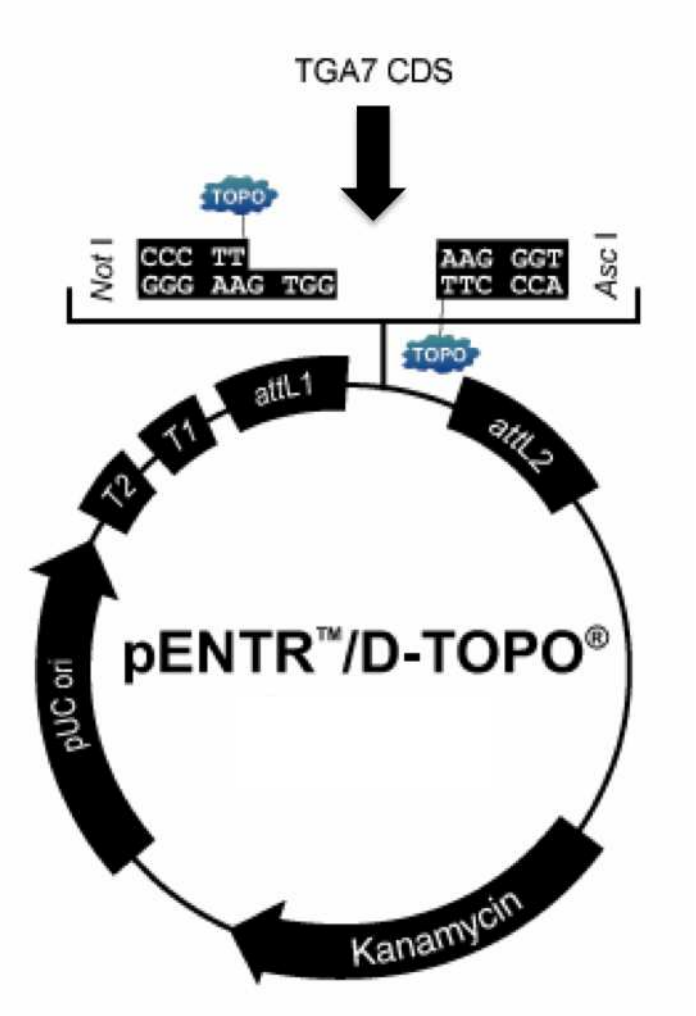
Supplemental Figure S6. Plasmid map of *TGA7* 4-kb promoter fusion with *GUS* gene in the pGreen 0229 vector.

The vector used had previously been modified to contain a translational fusion of *CO:GUS* with the NOS terminator between the *Xba*I and *Xho*I sites (Hepworth *et al.,* 2002). The *CO* promoter was excised through restriction sites *Bam*HI and *Nco*I and *TGA7* 4-kb promoter was ligated in frame with GUS (strain number B698, B699).



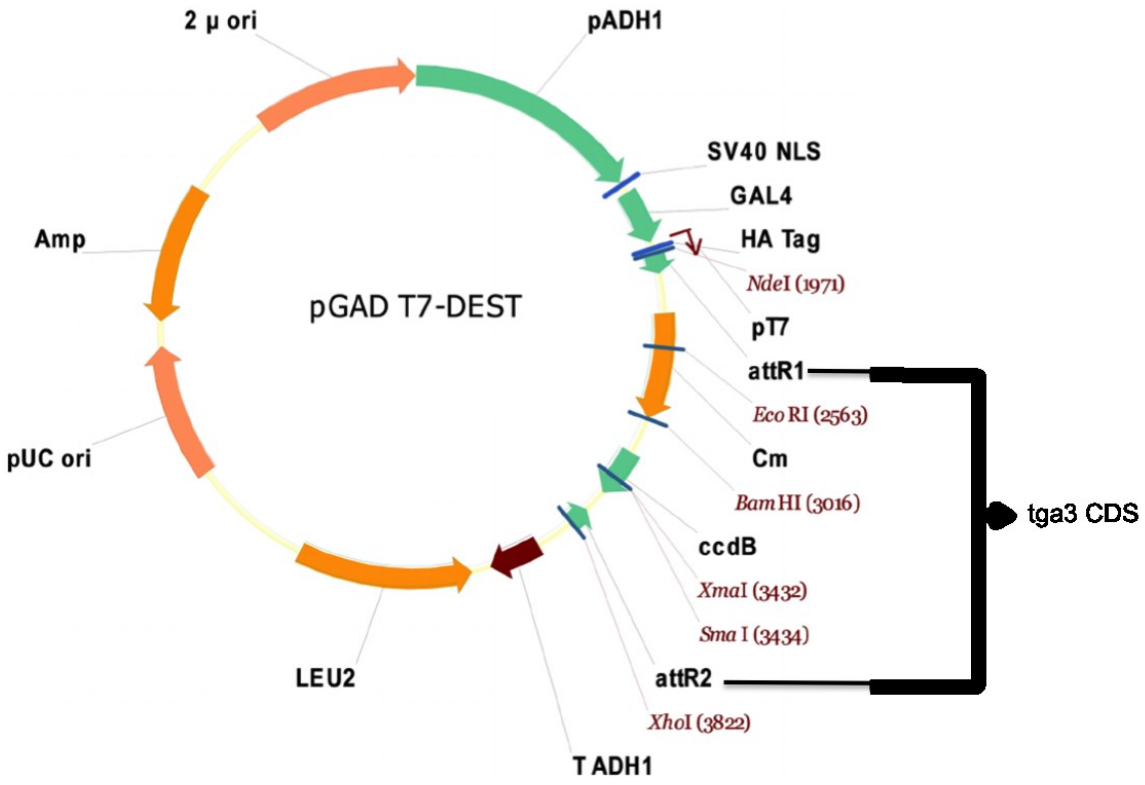
Supplemental Figure S7. Plasmid map of *TGA3* CDS Gateway™ compatible pENTR vector.

TGA3 CDS was amplified out through PCR, and cloned into Gateway™ compatible pENTR vector (strain number B779). CACC was added to the forward CDS primer for directionality, while the reverse primer removed the stop codon.



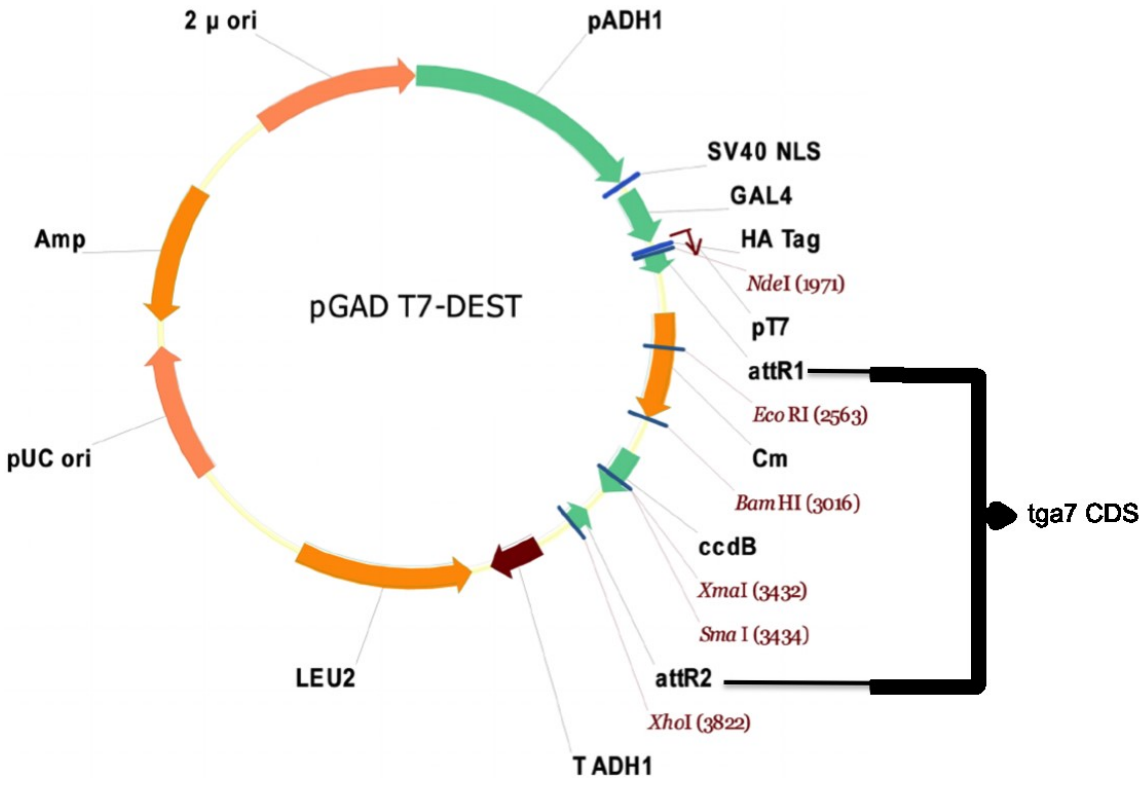
Supplemental Figure S8. Plasmid map of *TGA7* CDS Gateway™ compatible pENTR vector.

TGA7 CDS was amplified out through PCR, and cloned into Gateway™ compatible pENTR vector (strain number B778). CACC was added to the forward CDS primer for directionality, while the reverse primer removed the stop codon.



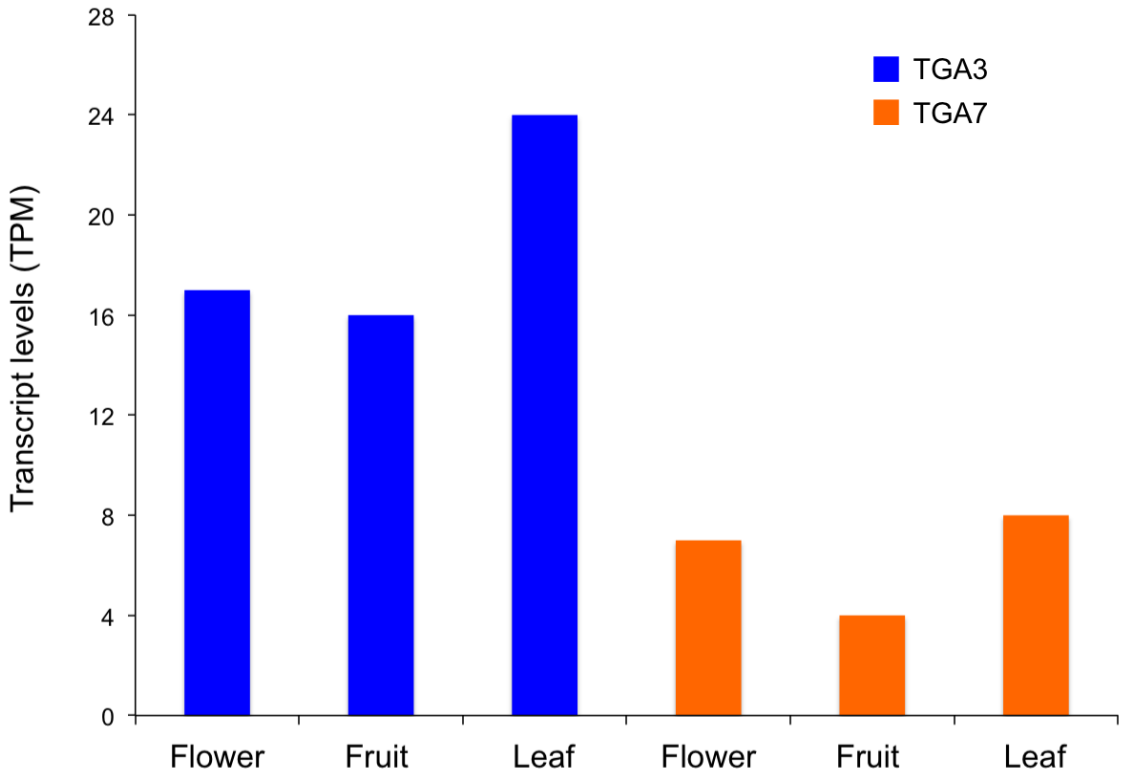
Supplemental Figure S9. Plasmid map of *TGA3* CDS in Gateway™ compatible yeast activation domain vector pGAD T7-DEST.

TGA3 CDS was introduced into the vector through LR reaction with the *TGA3* CDS pENTR vector (strain number B781).



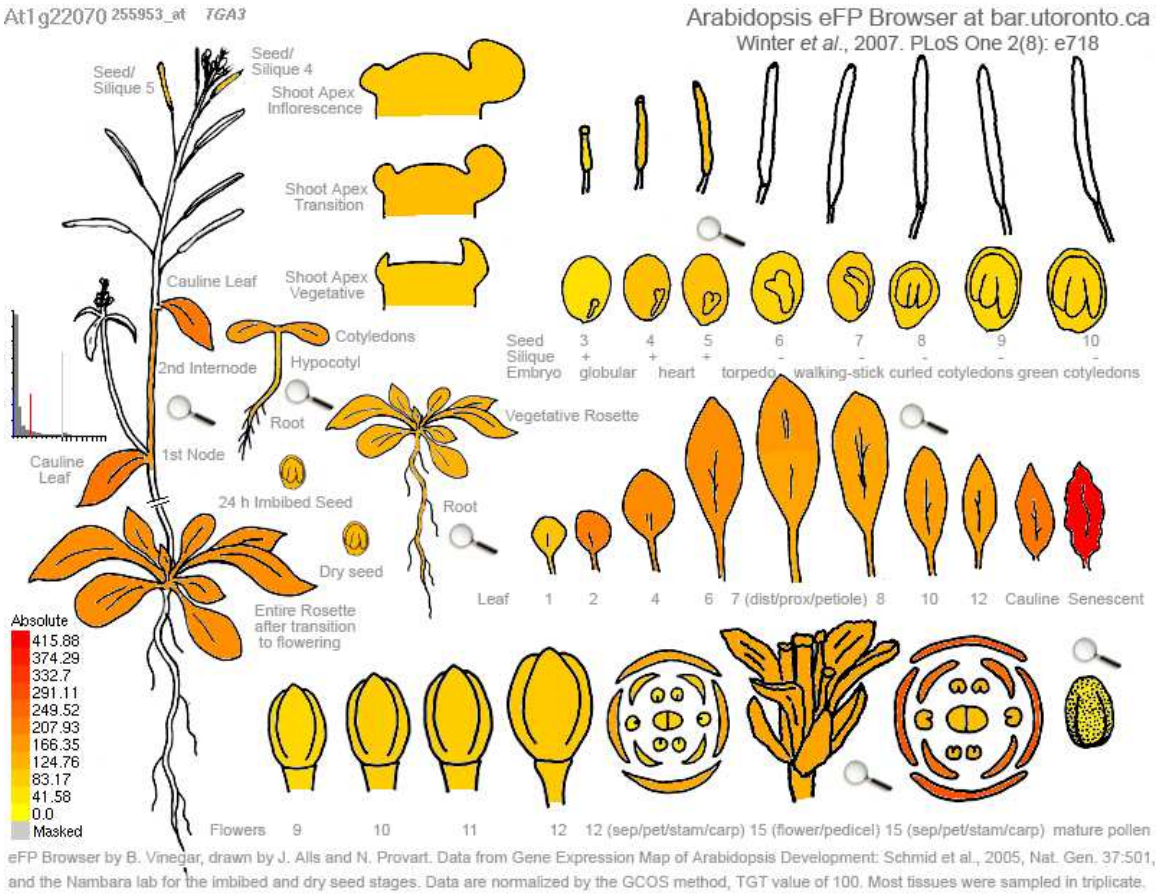
Supplemental Figure S10. Plasmid map of *TGA7* CDS in Gateway™ compatible yeast activation domain vector pGAD T7-DEST.

TGA7 CDS was introduced into the vector through LR reaction with the *TGA7* CDS pENTR vector (strain number B782).



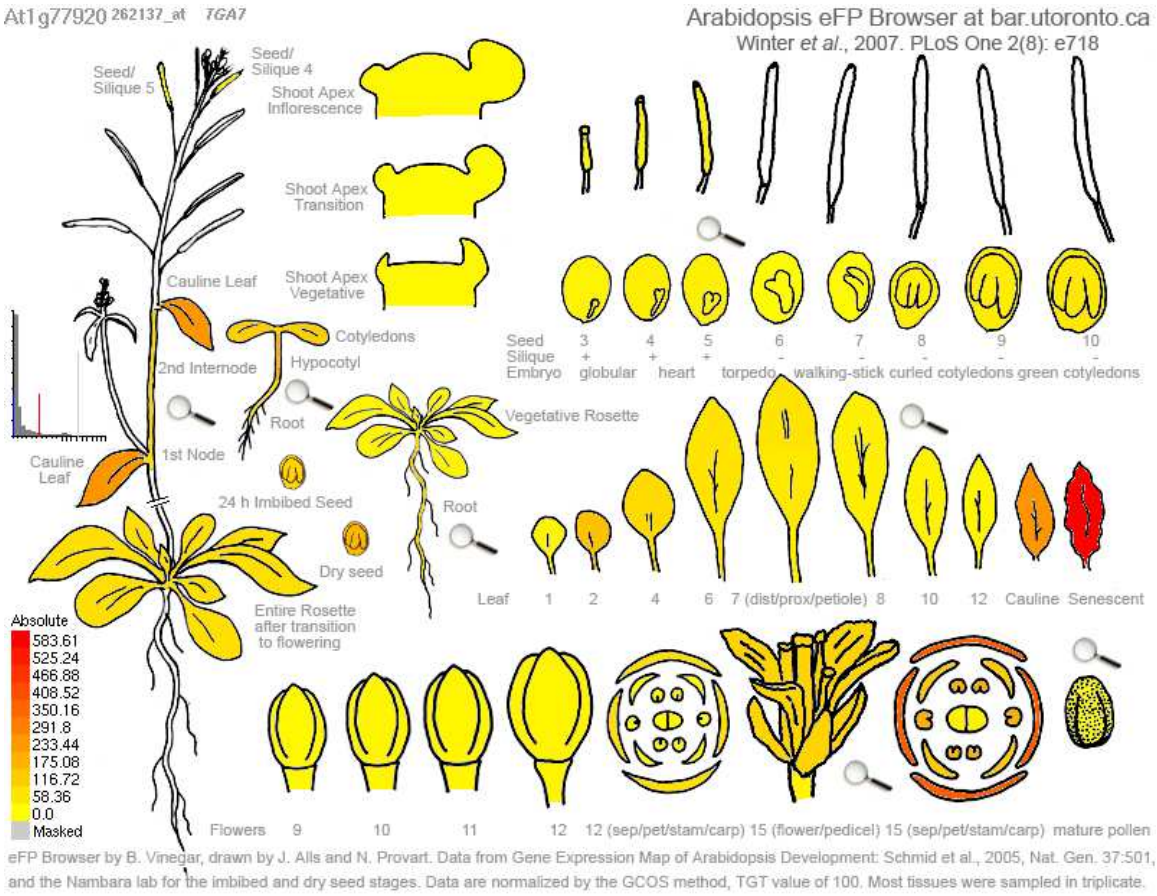
Supplemental Figure S11. Transcript levels of *TGA3* and *TGA7* in various plant tissues.

This figure summarizes the expression levels of clade III TGAs in various plant tissues as transcripts per million (TPM), obtained from expression atlas database (Papatheodorou *et al.,* 2018). Both *TGA3* and *TGA7* are expressed broadly throughout the different plant tissues. *TGA3* transcript levels are higher than *TGA7*.



Supplemental Figure S12. Schematic showing transcript levels of *TGA3* in different plant tissues.

This schematic from eFP browser shows the expression levels of *TGA3* in different tissue types based on publically available transcriptome data (Waese *et al.,* 2017; accessed from https://www.bar.utoronto.ca). *TGA3* is broadly expressed at relatively high transcript levels throughout the plant.



Supplemental Figure S13. Schematic showing transcript levels of *TGA7* in different plant tissues.

This schematic from eFP browser shows the expression levels of *TGA7* in different tissue types based on publically available transcriptome data (Waese *et al.,* 2017; accessed from https://www.bar.utoronto.ca). *TGA7* is broadly expressed at relatively low transcript levels throughout the plant.