Contribution of boundary genes to fruit patterning and dehiscence in *Arabidopsis thaliana*

By: Adina Popescu

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ABSTRACT

The production of fruit is an evolutionary innovation that allows plants to distribute seeds. *Brassicaceae* members including the model plant species *Arabidopsis thaliana* (*Arabidopsis*) produce dry fruits that shatter when ripe, a process known as dehiscence. The *Arabidopsis* fruit is an elongated pod composed of two fused valves whose margins are joined to a central seed-bearing replum. The valve margins are specialized zones for dehiscence related to boundaries that separate leaves from the shoot apical meristem. BLADE-ON-PETIOLE1/2 (BOP1/2) are transcriptional co-activators that interact with Clade I TGA transcription factors to promote the activation of three-amino-acid-loop-extension (TALE) homeobox genes, important for boundary development in land plants. How boundary genes contribute to fruit patterning has been unclear. My data show that boundary genes contribute to valve margin identity by enhancing the expression of valve margin identity genes: *SHATTERPROOF1/2*, *INDEHISCENT*, and *ALCATRAZ*. Further, boundary genes are constrained to the valve margin by mechanisms similar in part to valve margin identity genes. Finally, loss and gain-of-function boundary gene mutations modulate replum width and generate shatter-resistant fruits. Manipulation of this pathway provides a new potential strategy for optimizing pod shatter in crop plants.

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PREFACE

This thesis explores the role of organ boundary genes in fruit patterning and dehiscence in a model plant species. None of this work has been submitted for publication.

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GENETIC NOMENCLATURE IN *ARABIDOPSIS THALIANA*

Wild type gene: *BOP1*

Wild type protein: BOP1

Loss-of-function mutant (homozygous): *bop1*

Loss-of-function mutant (hemizygous): *bop1/+*

Gain-of-function mutant (dominant): *bop1-6D*

Double mutant: *bop1 bop2*

Promoter fusion to a gene coding region: *35S:BOP1*

Protein fusion: *BOP1-GR*

GLOSSARY OF GENETIC TERMS

Loss-of-function: loss or reduction of activity

Gain-of-function: ectopic or increased activity

Phenotypic suppression: shift toward wild type phenotype

Phenotypic enhancement: worsening a mutant phenotype

Redundancy: when there is genetic compensation in the event of a gene loss-of-function

Homolog: genes sharing a common ancestor in evolution

Ortholog: genes in different organisms that descend from a common ancestor (often with the same function)

Paralog: genes that are related by a duplication event within an organism

LIST OF ABBREVIATIONS

ADPG – ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE

AG - AGAMOUS

ALC – ALCATRAZ

ATAF- ARABIDOPSIS TRANSCRIPTION ACTIVATION FACTOR

ATH1 – ARABIDOPSIS THALIANA HOMEBOX GENE1

AS – ASYMMETRIC LEAVES

AZ – abscission zone

BELL – BELL1-like homeodomain

bHLH – basic helix-loop-helix

BLR - BELLRINGER

BOP - BLADE-ON-PETIOLE

BP – BREVIPEDICELLUS

CAD-D – CINNAMYL ALCOHOL DEHYDROGENASE-D

CK - cytokinin

CLV – CLAVATA

CMM – carpel margin meristem

Col - Columbia

CUC – CUP-SHAPED COTYLEDON

CZ – central zone

DZ – dehiscence zone

FAA – formaldehyde-acetic acid-alcohol

FUL – FRUITFULL

GA – gibberellic acid

GUS – β -Glucuronidase

IM – inflorescence meristem

IND – INDEHISCENT

IRX – IRREGULAR XYLEM

KNAT – KNOTTED-LIKE FROM ARABIDOPSIS THALIANA

KNOX – KNOTTED1-like homeodomain

Ler - Landsberg

LL – lignified layer

LOB – LATERAL ORGAN BOUNDARY

NAC – NAM, ATAF, and CUC

NAM – NO APICAL MERISTEM

NST – NAC SECONDARY WALL THICKENING PROMOTING FACTOR

OC – organizing centre

PIN – PIN-FORMED

PLL – pectate lyase-like

PNF - POUNDFOOLISH

PNY – PENNYWISE

PZ – peripheral zone

RAM – root apical meristem

RIT – random impact test

RPL – REPLUMLESS

RZ – rib zone

SAM – shoot apical meristem

SHP – SHATTERPROOF

SL – separation layer

SP - SPATULA

STM - SHOOTMERISTEMLESS

TALE – three-amino-acid-loop-extension

TGA – TGACG

Ws – Wassilewskija

WT – wild type

WUS - WUSCHEL

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Chapter 1 : INTRODUCTION

1.1 Thesis overview

The model plant species *Arabidopsis thaliana* (Arabidopsis) is a member of the *Brassicaceae* family. Members of this family include economically important oilseeds, such as *Brassica napus* (canola) and *Camelina sativa* (false flax). *Brassicaceae* species produce dry fruits in which seed dispersal takes place through pod shattering, also known as dehiscence. In nature, this process ensures reproductive success, but poses a problem for domesticated crops because it leads to loss of yield. Premature shedding of seeds translates into lower harvests and loss of revenue for farmers. Thus, breeders seek to generate cultivars that are resistant to pod shatter. An understanding of the molecular basis of fruit patterning and pod shatter provides an important foundation for this process (reviewed in Dong and Wang, 2015).

The *Arabidopsis* fruit is an elongated pod called a silique. Fruits and all aerial organs in a plant derive from the shoot apical meristem (SAM). The pod is composed of two carpel valves whose margins are fused to a central seed-bearing replum. Valve margins in the fruit are specialized domains for dehiscence, related to low-growth zones called boundaries that separate leaves from the SAM (reviewed in Roeder and Yanofsky, 2006; Hepworth and Pautot, 2015). BLADE-ON-PETIOLE (BOP) BTB-ankyrin proteins and three amino acid loop-extension (TALE) homeodomain transcription factors jointly maintain and pattern developmental boundaries during the life cycle (reviewed in Hepworth and Pautot, 2015). My project investigates how boundary genes contribute to *Arabidopsis* fruit patterning and dehiscence.

1.2 *Arabidopsis thaliana* as a model species for plant biology studies

Arabidopsis thaliana (mouse ear cress) is a model plant species that is genetically related to cabbage, mustard, and oilseed crops such as canola. Although classified as a weed, *Arabidopsis*

became widely adopted for plant genetic studies in the early 1980's. The plant is relatively small in size, typically less than 30 cm in height, with a generation time as short as 5-6 weeks. It is self-fertilizing and each plant can produce thousands of seeds. Importantly, *Arabidopsis* has a compact diploid genome comprised of 5 chromosomes with minimum interspersed repetitive DNA (Meyerowitz, 1989). Before the development of molecular cloning methods, most plant studies were based on genetics or physiology and used plants with agricultural or horticultural value, such as pea, maize, tomato, barley, and petunia. Tobacco was also widely used as a plant model for *in vitro* studies on regeneration. The implementation of *Arabidopsis* as a plant model allowed for the shift towards studies in genetics and molecular biology (Somerville and Koorneef, 2002). Gene mutations in *Arabidopsis* can be easily introduced with chemical mutagens or by using *Agrobacterium*-mediated approaches. To date, *Arabidopsis* has a fully annotated genome (Arabidopsis Genome Initiative, 2000) along with many publicly-accessible genomic resources that include characterized mutations, transgenic lines, DNA microarray and next-generation RNA-sequencing data (Somerville and Koorneef, 2002; Lavagi et al., 2012). *Arabidopsis* research has greatly facilitated genomic analyses on other plant systems, serving as an important discovery tool for the improvement of crop traits including drought tolerance, pest resistance, water-use efficiency, and yield among others (Ferrier et al., 2011).

1.3 Arabidopsis life cycle and body plan

The life cycle of a flowering plant can be divided into three main developmental phases: embryonic, vegetative and reproductive. Embryonic development begins at fertilization and ends with the production of a seed. The mature embryo inside the seed contains the first set of basic plant structures: one or two rudimentary leaves (cotyledons), an embryonic stem (hypocotyl) and a root (Wolpert et al., 2011). Two populations of stem cells are formed during embryo

development: the shoot apical meristem (SAM) and the root apical meristem (RAM). During post-embryonic development, the SAM produces all of the above-ground structures in a plant: leaves, stems, branches, and flowers. By contrast, the RAM produces all of the below-ground structures in a plant: primary and lateral roots (Barton, 2010; Wolpert et al., 2011).

1.4 Organization of the SAM

All aerial tissues in a plant derive from the SAM (Figure 1.1). In *Arabidopsis*, the SAM is a dome-like mound of cells with three histologically-defined layers, termed L1, L2, and L3 (Evans and Barton, 1997; Barton, 2010). The outer layers (L1 epidermal and L2 sub-epidermal) are the tunica and the inner layer (L3) is the corpus. The tunica layers are one cell thick and divide in a single plane creating sheets of tissue. By contrast, cells in the corpus divide in three dimensions creating a block of tissue (Fletcher, 2002; Barton, 2010). Each layer in the meristem contributes to the development of specific tissue types: the L1 layer contributes to development of the epidermis, the L2 layer contributes to mesoderm tissue and germ cell production, and the L3 layer contributes to stem vascular tissue and the internal tissue of flowers and leaves (Fletcher, 2002; Barton, 2010).

The SAM is further organized into functional zones (Figure 1.1). The central zone (CZ) at the meristem apex contains slowly-dividing pluripotent stem cells. The peripheral zone (PZ) on the outskirts of the CZ is a transitional zone where cell division is more rapid and organogenesis takes place (Barton, 2010; Aichinger et al., 2012). Cells entering the PZ are incorporated into lateral organs (leaves, branches, or flowers). As lateral organs emerge on the flanks of the meristem, they are rapidly partitioned from the meristem by boundaries. The rib zone (RZ) provides cells for upward growth of the stem (Barton, 2010; Aichinger et al., 2012). Differential regulation of activities in the CZ, PZ, boundaries, and RZ control the changing production of

leaves, stems, branches, and flowers throughout the life cycle (Barton, 2010; Aichinger et al., 2012; Bencivenga et al., 2016).

1.5 Meristem-organ boundaries

Boundaries are specialized domains of low growth that encircle the base of lateral organs (Rast and Simon, 2008; Hepworth and Pautot, 2015) (Figure 1.1). Boundaries contain cells with a low rate of division. These domains originate as regions of repressed growth that separate differentiating lateral organs and the meristem (Aida and Tasaka, 2006; Hepworth and Pautot, 2015). Beyond separation, boundaries are zones for the initiation of axillary meristems, important for the production of branches and flowers. Boundaries are also sites for the regulated detachment of organs from the plant body. Abscission is the mechanism that allows dead or diseased organs to be shed from the plant body. Dehiscence is the mechanism that allows the opening of anthers to release pollen or the opening of dried fruit pods to release seeds (Hepworth and Pautot, 2015).

1.6 Plant shoot development

The SAM directs plant shoot development by producing repeating units called “phytomers”. Each phytomer has three independent parts: a leaf, a leaf attachment site (node) whose cells are capable of forming an axillary meristem, and a subtending stem segment (internode). During the vegetative phase of development, the Arabidopsis SAM forms leaves whose axillary meristems are quiescent (Figure 1.2). Stem elongation is suppressed during this phase. During the transition to flowering, the SAM is converted into an inflorescence meristem (IM), resulting in new patterns of aerial development (Figure 1.2). During the first stage of reproductive development, leaf development is partially repressed. Cauline leaves produce axillary meristems that form secondary inflorescences. During the second stage of reproductive

development, leaf development is fully repressed. Axillary meristems formed during this phase are floral meristems that produce flowers (Schultz and Haughn, 1991; Sablowski, 2007; Barton, 2010). Activation of internode elongation during the reproductive phase results in the even distribution of lateral branches and flowers along the axis of the primary inflorescence. The SAM and IM are indeterminate meristems whereas floral meristems are determinate: the stem cell population terminates after the production of four whorls of floral organs: sepals, petals, stamens, and carpels (Fletcher, 2002; Wolpert et al., 2011).

A pair of carpels in the center of the flower are congenitally fused to form the gynoecium (the female part of the flower). The gynoecium contains three tissues from top to bottom: the stigma, style, and ovary which gives rise to ovules internally. Pollen landing on the stigma fertilize the ovules, which mature into seeds, thus completing the life cycle (Dinnyeny and Yanofsky, 2005; Roeder and Yanofsky, 2006; Arnaud and Pautot, 2014).

1.7 Conserved patterns in plant development

The German poet Wolfgang Goethe famously wrote an essay entitled “The Metamorphosis of Plants” published in 1790. He began by describing the typical life of a plant. After germination of the seed, leaves are successively produced around the axis of the stem. He proposed that the different parts of a flower were the equivalent to leaves clustered together instead of spaced out along a stem (Coen, 2000). Similar comparisons can be made between boundaries in the shoot apex that separate leaves from the SAM and valve margins in the fruit that separate carpels from the replum (Girin et al., 2009; Arnaud and Pautot, 2014).

1.8 Arabidopsis fruit development

Arabidopsis plants produce a dry dehiscent fruit typical of *Brassicaceae* called a silique. Seed dispersal occurs through a process known as pod shatter. This dispersal mechanism relies on the precise patterning of tissues in the fruit, including the valve margin, whose properties enable the fruit to open at maturity in scattering seeds. Siliques develop from two fused carpels (modified leaves) produced at the center of the floral meristem. At an early stage, the carpels become fused along their margins to form a tubular structure called the gynoecium (Figure 1.3). The gynoecium is divided into three regions from top to bottom: the stigma, style, and ovary, whose internal surface forms ovules that are fertilized and become seeds. After fertilization, the gynoecium becomes a fruit and the carpels become valves. The valves rapidly increase in size, enclosing and protecting the seeds as they develop (Roeder and Yanofsky, 2006; Ferrándiz et al., 2010; Ballester and Ferrándiz, 2017).

The *Arabidopsis* fruit in cross-section has bilateral symmetry. Three distinct tissue types are present on the exterior: valves, replum and valve margins (Figure 1.3). The replum forms at the place where the edges of two carpels are fused in the gynoecium (Roeder and Yanofsky, 2006). The inner surface of the replum is called the carpel margin meristem (CMM). The CMM produces two central ridges of tissue that grow out and fuse to form a septum that divides the fruit into two halves. Tissues flanking the septum, termed the placenta, produce primordia that develop into ovules. The replum displays itself as narrow raised files of elongated cells on the exterior surface of the fruit (Reyes-Olalde et al., 2013). An anatomically distinct domain known as the valve margin separates the replum and the valves on either side along the length of the fruit. Each valve margin constitutes an organ boundary (Roeder and Yanofsky, 2006; Hepworth and Pautot, 2015).

Twenty landmark stages have been defined for *Arabidopsis* fruit development (Ferrándiz et al., 1999). Floral development completes at stage 13. Fertilization at stage 14 initiates

development of the fruit. At stage 17, the valve margins undergo enzymatic and mechanical changes in forming a dehiscence zone (DZ). This specialized zone allows the fruit to open at maturity and scatter the seeds (Ferrándiz et al., 1999; Ballester and Ferrándiz, 2017). The DZ is composed of a separation layer (SL) closest to the replum made of small parenchyma cells and a lignified layer (LL) (Spence et al., 1996; Ferrándiz et al., 1999) (Figure 1.3). At stage 17A, hydrolytic enzymes contribute to breakdown of the middle lamella in the separation layer (Ferrándiz et al., 1999; Roeder and Yanofsky, 2006). At stage 17B, lignification of the endocarp *b* layer (inner lining of the valves) continuous with the lignified layer creates tension in the DZ as the fruit becomes dry due to water loss. At stage 19, these forces lead to spring-like opening of the fruit. At this point, the exposed seeds abscise from the funiculus by which they were attached to the ovary wall. Seed dispersal at stage 20 completes the cycle (Ferrándiz et al., 1999; Ferrándiz, 2002; Roeder and Yanofsky, 2006).

1.9 Genetic network for SAM initiation

1.9.1 *CUP-SHAPED COTYLEDON* genes

The SAM is initiated at the boundary formed by two cotyledons during late heart stage of embryogenesis (Barton, 2010). This mechanism relies on NAC (NAM, ATAF, and CUC) domain-containing transcription factors, which confer boundary identity in all species of plants that have been tested. In *Arabidopsis*, the combined activities of three *CUP-SHAPED COTYLEDON* (*CUC*) genes provide this function (Maugarny et al., 2016). During the late globular stage of embryo development, *CUC1* and *CUC2* genes are activated in overlapping domains in the emerging shoot apex (Takeda and Aida, 2011). *CUC1* directly promotes the expression of *SHOOT MERISTEMLESS* (*STM*) (Aida et al., 1999; Spinelli et al., 2011). Progressive loss of *CUC1* and *CUC2* activity prevents establishment of the SAM and causes the fusion of cotyledons (Aida et

al., 1997; Hibara et al., 2006; Takeda and Aida, 2011). During the reproductive phase, progressive loss of *CUC2* and *CUC3* activity impairs separation between leaves and the stem and impairs the formation of axillary meristems that give rise to lateral branches and flowers (Hibara et al., 2006). CUC activity in flowers is also essential for the development and separation of ovules (Ishida et al., 2000; Gonçalves et al., 2015).

1.10 Genetic network for SAM maintenance

Maintenance of the SAM relies on two interconnecting mechanisms: a WUSCHEL-CLAVATA (WUS-CLV) feedback loop and the overlapping activities of KNOX and BELL three-amino-acid-loop-extension (TALE) homeodomain transcription factors (Aichinger et al., 2012; Tsuda and Hake, 2016). WUS acts primarily to renew the stem cell population whereas KNOX-BELL factors inhibit differentiation of the stem cell population. These two modules play distinct and complementary roles in meristem maintenance (Gallois et al., 2002; Lenhard et al., 2002).

1.10.1 WUSCHEL-CLAVATA feedback loop

A WUS-CLV negative feedback loop maintains the stem cell niche and controls its size. WUS is expressed in the Organizing Center (OC), a small subdomain of the RZ underlying the CZ (Figure 1.1). WUS transcription factor moves from the OC into overlying stem cells where it promotes stem cell fate and activates its own negative regulator, *CLV3* (Schoof et al., 2000; Müller et al., 2006; Yadav et al., 2011). *CLV3* is a small, secreted peptide that binds to leucine-rich receptor kinases integral to the plasma membrane of cells in the RZ (Clark et al., 1997; Fletcher et al., 1999; Jeong et al., 1999; DeYoung et al., 2006; Kondo et al., 2006; Müller et al., 2008; Kinoshita et al., 2010; Shinohara and Matsubayashi, 2015). Activation of these receptors represses

WUS transcription thereby confining its expression to the OC (Schoof et al., 2000; Yadav et al., 2011).

WUS function in plants is similar to animal morphogens: the protein forms a gradient and has concentration-dependent effects on gene expression (Yadav et al., 2011; Rodriguez et al., 2016). *WUS* movement from the OC into surface layers of the CZ and PZ creates an activity gradient (Yadav et al., 2011). Biochemical studies show that *WUS* activates *CLV3* at low levels and represses *CLV3* at high levels, by binding to the same regulatory elements as monomers and as dimers, respectively (Yadav et al., 2011; Perales et al., 2016). Inactivation of *WUS* or experimentally restricting its movement causes meristem termination (Laux et al., 1996; Mayer et al., 1998; Yadav et al., 2011; Daum et al., 2014). Conversely, experimentally boosting *WUS* expression results in a larger meristem (Brand et al., 2000; Yadav et al., 2010).

1.10.2 TALE homeodomain transcription factors

TALE homeodomain transcription factors are distinguished by a unique three amino acid extension in the loop that joins helices 1 and 2 in the homeodomain (Bertolino et al., 1995; Bürglin, 1997). Plant TALEs are classified into two subfamilies: KNOTTED1-like homeobox (KNOX) and BELL1-like homeobox (BELL or BLH) whose members function as heterodimers (Hamant and Pautot, 2010; Tsuda and Hake, 2016). Heterodimer formation is a mechanism that promotes nuclear localization and determines binding site selection (Smith et al., 2002; Cole et al., 2006; Lee et al., 2008)

Four class I *KNOX* genes with overlapping functions are involved in SAM maintenance: *STM*, *BREVIPEDICELLUS* (*BP*, also called *KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1/KNAT1*), *KNAT2*, and *KNAT6* (Scofield and Murray, 2006; Hamant and Pautot, 2010; Tsuda and Hake, 2016). *STM* is expressed in the CZ, PZ, and RZ. *STM* maintains cells in

the SAM and prevents their differentiation. Some strong *stm* mutants lack a SAM and fail to develop post-embryonic vegetative tissues (Long et al., 1996). STM directly and indirectly promotes the expression of other class I *KNOX* genes: *BP*, *KNAT2*, and *KNAT6* in specific subdomains of the SAM (Belles-Boix et al., 2006; Scofield et al., 2013; Scofield et al., 2014). *BP* is expressed in the PZ and RZ (Lincoln et al., 1994), *KNAT2* is expressed in the RZ and organ boundaries (Dockx et al., 1995; Pautot et al., 2001) and *KNAT6* is expressed at organ boundaries (Belles-Boix et al., 2006). Mutation of *BP* or *KNAT6* enhance the meristem defect of weak *stm* mutants indicating their involvement (Byrne et al., 2002; Belles-Boix et al., 2006). The *knat6 stm-2* double mutant lacks a meristem and shows a strong fusion between cotyledons demonstrating a role in boundary formation and SAM maintenance (Belles-Boix et al., 2006). Genetic assays have so far failed to identify a role for *KNAT2* in the SAM.

KNOX and *BELL* transcription factors function as heterodimers. *ARABIDOPSIS THALIANA* *HOMEBOX GENE1* (*ATH1*), *PENNYWISE* (*PNY*), and *POUND-FOOLISH* (*PNF*), encode three *BELL* transcription factors that interact with STM to maintain SAM activity (Rutjens et al., 2009). *ATH1*, which contributes to boundary establishment, is expressed in the SAM during vegetative development and is downregulated at the transition to flowering (Proveniers et al., 2007; Gómez-Mena and Sablowski, 2008). A specific role for *ATH1* in establishing boundaries at the base of shoot organs and the stem was demonstrated by *ath1* mutants showing fusion defects at these locations (Gómez-Mena and Sablowski, 2008). *PNY* and *PNF* are expressed in an overlapping domain with *STM* (Smith et al., 2004). The SAM is narrower in *pny pnf* double mutants and often terminates after producing 5-6 leaves (Rutjens et al., 2009; Ung et al., 2011). The double mutant is unable to promote stem elongation or initiate flowers (Smith et al., 2004). This is because *PNY* and *PNF* prevent boundary genes including *KNAT6* and *ATH1* from

expressing in the meristem thereby preserving continuous organized division of cells for the production of stem and flowers (Andrés et al., 2015; Khan et al., 2015; Bencivenga et al., 2016). Inactivation of boundary genes in *pny pnf* mutants restores meristem activity including stem elongation and flower initiation highlighting the remarkable functional plasticity of stem cell populations in plants (Khan et al., 2015). Further, KNOX and BELL homeodomain proteins in meristem and boundary domains function antagonistically. This mode of interaction preserves boundaries for maintenance of the stem cell population and continuous production of lateral organs throughout the life cycle.

1.10.3 *BLADE-ON-PETIOLE* genes

Two closely-related BTB-ankyrin co-transcription factors called BLADE-ON-PETIOLE1 (BOP1) and BOP2 are important regulators of boundary development. These genes appear to function downstream from or in parallel with *CUC* genes based on genetic interaction studies. Loss-of-function mutations in *bop1 bop2* have little or no impact on *CUC* expression but enhance the organ fusion defects of *cuc* mutants during the reproductive stage (Khan et al., 2014). *BOP1/2* have a nearly identical expression pattern and function redundantly (Khan et al., 2014; Hepworth and Pautot, 2015). BOP1/2 activities are not essential during embryogenesis but loss-of-function mutations disrupt leaf patterning, the initiation and differentiation of axillary meristems, and the differentiation of abscission zones (Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008; Xu et al., 2010; Khan et al., 2014).

BOP1/2 proteins are found in both the cytoplasm and the nucleus (Hepworth et al., 2005; Wu et al., 2012). In the nucleus, BOP1/2 function as transcriptional co-activators (Hepworth et al., 2005; Jun et al., 2010; Khan et al., 2014). Members of this family lack a DNA binding domain, interacting with TGACG-motif binding (TGA) bZIP transcription factors for recruitment to DNA

(Hepworth et al., 2005; Xu et al., 2010; Khan et al., 2014). BOP1/2 activity at shoot and organ boundaries is recently shown to depend on two Clade I TGA transcription factors: TGA1 and TGA4 (Wang et al., 2018)

BOP1/2 exert their function in part by promoting expression of boundary genes. This was first shown in leaves. Prolonged meristematic activity at the base of *bop1 bop2* leaves results in the initiation of leaflets, a so-called “blade-on-petiole” phenotype, similar to the development of compound leaves (Ha et al., 2003; Ha et al., 2004; Ha et al., 2007; Ha et al., 2010). Further analysis revealed that BOP1 directly activates the LATERAL ORGAN BOUNDARY domain transcription factor gene *ASYMMETRIC LEAVES2* during boundary initiation (Jun et al., 2010). Loss of *AS2* expression during these early stages disrupts the formation of a complex that directs stable repressive changes in the chromatin structure required for permanent downregulation of meristematic *KNOX* genes in leaves, a determinant of simple leaf shape (Di Giacomo et al., 2013; Lodha et al., 2013).

Overexpression of *BOP1/2* dramatically inhibits plant growth resulting in late flowering and dwarfing (Norberg et al., 2005; Khan et al., 2012b; Khan et al., 2015). Analysis of these plants led to the discovery that BOP1/2 require the downstream activity of boundary TALE homeodomain transcription factors: a BELL-like member *ATH1* and its *KNOX* binding partner *KNAT6* to exert changes in plant architecture (Rutjens et al., 2009; Li et al., 2012; Khan et al., 2012a; Khan et al., 2012b). BOP1 directly activates *ATH1* whereas activation of *KNAT6* is indirect (Khan et al., 2015). PNY repression of this module maintains boundaries flanking the apical stem cell population throughout the life cycle (Hepworth and Pautot, 2015).

1.11 Genetic network for fruit development

Molecular genetic studies in *Arabidopsis* plants have identified a core network of MADS-box, bHLH, and TALE transcription factors that control fruit patterning (Figure 1.4; reviewed in (Ferrándiz et al., 2010; Arnaud and Pautot, 2014)). Part of this network is borrowed from leaves, which are evolutionarily related to carpels (Ferrándiz et al., 2010; Arnaud and Pautot, 2014). Formation of the valve margins is controlled by a set of regulatory genes that confer valve margin identity (Liljegren et al., 2000; Rajani and Sundaresan, 2001; Liljegren et al., 2004). Two MADS-box genes *SHATTERPROOF1* and 2 (*SHP1/2*) are essential for valve margin differentiation (Liljegren et al., 2000). *SHP1/2* MADS-domain transcription factors activate the expression of *INDEHISCENT* (*IND*) (Liljegren et al., 2004) and *ALCATRAZ* (*ALC*) genes (Rajani and Sundaresan, 2001). These genes encode two bHLH transcription factors. *IND* specifies the lignified layer of the valve margin, whereas the combined activities of *IND* and *ALC* specify the separation layer (Rajani and Sundaresan, 2001; Liljegren et al., 2004). Mutation of these genes results in shatterproof fruits (Liljegren et al., 2000; Rajani and Sundaresan, 2001; Liljegren et al., 2004).

Valve margin identity genes are restricted to the valve margin by the MADS domain transcription factor *FRUITFULL* (*FUL*) which patterns valves (Gu et al., 1998; Ferrándiz et al., 2000; Liljegren et al., 2004) and by the *BELL* homeodomain transcription factor *PNY* (also known as *REPLUMLESS*) which patterns repla (Roeder et al., 2003). Misexpression of valve margin genes in the valves and repla of *ful* and *pnv* fruits, respectively, causes these tissues to adopt characteristics of the valve margin (Roeder et al., 2003; Liljegren et al., 2004; Ripoll et al., 2011). In *ful pnv* double mutants, the entire outer surface of the ovary takes on valve margin identity. A suppressor screen identified *APETALA2* (*AP2*) as a negative regulator of replum and valve margin

identity genes (Ripoll et al., 2011). Consistent with this interaction, *ap2* mutants have enlarged repla and valve margins (Ripoll et al., 2011).

FUL promotes the elongation and differentiation of the valves (Gu et al., 1998). Overexpression of *FUL* in fruits converts valve margin cells into valve cells thereby rendering the silique indehiscent (Ferrándiz et al., 2000). Loss-of-function mutants have short compact fruits that rupture prematurely (Gu et al., 1998). This phenotype is attributed to misexpression of valve margin identity genes and can be partially rescued by the inactivation of *IND* or *SHP1/SH2/ALC* genes (Ferrándiz et al., 2000; Liljegren et al., 2004; Roeder and Yanofsky, 2006).

PNY and BP are in combination important for replum development. Overexpression of *BP* produces fruit with enlarged repla (Alonso-Cantabrana et al., 2007). Conversely, mutations in *pny* and *bp pny* produce fruits with progressively stronger defects in replum development (Roeder et al., 2003; Alonso-Cantabrana et al., 2007). Roeder et al. (2003) isolated an allelic series of *rpl* mutants. Weak alleles have short compact fruits with repla made of narrow cells mimicking valve margin cells. Stronger alleles have a more severe phenotype in which the replum has fewer cell layers. In the strong *rpl-3* allele, the lignified layers are completely connected making a “lignin bridge” and fruits are partially indehiscent (Roeder et al., 2003). Remarkably, formation of the replum is restored in *shp1 shp2 rpl* and *ind rpl* mutants revealing that replum development is strongly dependent on the dosage and localization of valve margin identity genes (Roeder and Yanofsky, 2006). Evidently, other factors compensate for loss of PNY/RPL to form the replum (Arnaud and Pautot, 2014). One such candidate is STM, which is functionally redundant with BP and interacts with PNY (Byrne et al., 2002; Cole et al., 2006). *STM* is expressed in the central portion of the gynoecium at stage 7 (Long et al., 1996) and becomes restricted to the replum with BP and PNY (Ragni et al., 2008). STM is essential for ovule formation (Endrizzi et al., 1996;

Scofield et al., 2007). CUC transcription factors were found to activate *STM* to initiate the CMM similar to their role in the SAM (Kamiuchi et al., 2014).

BP is repressed in valves by the combined activities of the MYB transcription factor ASYMMETRIC LEAVES1 (AS1) and AS2. This mechanism also applies to leaves (Alonso-Cantabrana et al., 2007). Mutation of *AS1* or *AS2* produces fruit with enlarged repla and reduced valves (Alonso-Cantabrana et al., 2007). The *JAGGED* (*JAG*) gene encodes a C₂H₂ zinc finger transcription factor and the *YABBY* (*YAB*) *FILAMENTOUS FLOWER* (*FIL*) and *YAB3* genes control leaf pattern formation. These genes are also expressed in the valves of the fruit (Dinneny et al., 2005; González-Reig et al., 2012). The combined activities of JAG/FIL and AS1/2 are proposed to form a patterning gradient across the fruit (Dinneny et al., 2005; Alonso-Cantabrana et al., 2007; González-Reig et al., 2012). High and medium levels of activity promote *FUL* expression in the valves and activation of valve margin identity genes in the valve margin. Low levels of activity are required for expression of *BP* and *PNY* in the replum (Dinneny et al., 2005; Dinneny and Yanofsky, 2005). Consistent with this model, *fil yab* double mutants have tiny fruits with small valves and enlarged repla similar to *ful* mutants (Dinneny et al., 2005).

1.12 Hormonal signaling at the valve margin

Hormones including auxin, gibberellic acid (GA), and cytokinin (CK) play important roles in plant development including fruit patterning and dehiscence zone formation. Formation of the dehiscence zone begins at fertilization (stage 14) and is associated with an increase in auxin levels at the valve margin which promotes cell division. IND leads to reduction in the levels of PIN-FORMED3 (PIN3), an auxin efflux transporter. During this phase, auxin accumulates in the valve margin due to increased auxin influx resulting from a reduction of PIN3 plasma membrane abundance at the valve margin. The *ind-2* mutant fruit characterized by the lack of DZ, has low

levels of auxin in the area between replum and valves from stages 13-17 and persistently high PIN3 abundance in the valve, valve margin, and replum. This suggested that IND activity at stage 14, leads to a decrease in PIN3, resulting in a net influx of auxin into the dehiscence zone. Thus at earlier stages of fruit development, IND promotes auxin-triggered division to form the DZ (van Gelderen et al., 2016). In contrast, at late stage of fruit development (stage 17B), the specification of the separation layer requires the creation of a local auxin minimum at the valve margin mediated by IND (Sorefan et al., 2009). An attractive model is that auxin accumulation at earlier stages promotes initiation of the dehiscence zone while at stage 17B depletion of auxin promotes cell separation just prior to pod shattering (Ballester and Ferrándiz, 2017).

Depletion of auxin is similarly associated with cell separation at abscission zones in tomato during abscission (Meir et al. 2010), a process related to dehiscence (Estornell et al., 2013; Hepworth and Pautot, 2015).

GA is another important hormone that regulates the growth and function of reproductive tissues (Swain and Singh, 2005). In fruits, the proper differentiation of valve margins requires a local increase of GA in valve margins which involves both IND and ALC transcription factors (Arnaud et al., 2010). The increase in GA promoted by IND leads to the destabilization of DELLA proteins and the release of ALC that can activate its targets to specify the separation layer (Arnaud et al., 2010). The release of ALC also leads to the negative feedback of *IND* expression and may delimit the separate differentiation of lignified and separation layers (Ballester and Ferrándiz, 2017). Interestingly, an auxin minimum at the valve margin is preserved in GA deficient mutants, suggesting that the GA and auxin pathways function independently (Hepworth and Pautot, 2015).

CK also plays a critical role in dehiscence zone formation. In support of this, Marsch-Martinez et al. (2012) found that valve margin formation and dehiscence are restored in *shp1/2*

and *ind* mutants through the local application of CK, and that the synthetic CK marker *TCS::GFP* localizes to the valve margins in a *SHP1/2* and *IND*-dependent manner. This confirms that CK regulation is a downstream target of core genetic networks in the gynoecium (Marsch-Martínez et al., 2012).

1.13 Secondary cell wall development at the valve margin

Lignin biosynthesis genes involved in secondary cell wall formation are further implicated in differentiation and dehiscence at the valve margin after the establishment of tissue identity. Secondary cell walls rich in lignin and cellulose are deposited between the plasma membrane and primary cell walls. In tissues like xylem, lignified walls provide mechanical support whereas in siliques, lignified walls provide tension for dehiscence (Spence et al., 1996; Mitsuda and Ohme-Takagi, 2008). NAC SECONDARY WALL THICKENING PROMOTING FACTOR1 (NST1) and NST3 are two transcription factors involved in lignin deposition. These factors promote pod shattering in a partially redundant manner. *NST1* and *NST3* genes are expressed in the replum and valve endocarp *b* lignified regions. *NST1* is also expressed in the valve margin. Loss-of-function *nst1* fruits are moderately indehiscent due to the absence of the lignification layer in the dehiscence zone while *nst3* single mutations have no effect on lignification layer formation. The *nst1 nst3* double mutant shows indehiscent fruits and lacks secondary cell walls except in the vascular vessels (Mitsuda and Ohme-Takagi, 2008). NST1 and NST3 are master regulators of secondary xylem fiber differentiation in the stem. Their involvement in valve margin suggests evolution of regulatory elements allowing adoption into the SHP1/2 network (Dong and Wang, 2015). A cellulose synthase gene, *IRREGULAR XYLEM 3 (IRX3)*, has an expression pattern similar to *NST1* (Mitsuda and Ohme-Takagi, 2008). *IRX3* and the lignin biosynthesis gene *CINNAMYL ALCOHOL DEHYDROGENASE-D (CAD-D)* seem to be targets of the NST transcription factors (Mitsuda and

Ohme-Takagi, 2008). Peroxidases and laccases are another set of factors that participate in the oxidative steps of lignin synthesis (Boerjan et al., 2003). Indeed, inhibition of class III peroxidases delays pod dehiscence (Cosio and Dunand, 2010). In the separation layer, enzymes are required for structural breakdown of the connective middle lamella which joins cell walls together in an adhesive layer. Degradation of pectin in the middle lamella of separation layer cells leads to breakdown of the layer just prior to dehiscence through the action of polygalacturonases such as ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE 1 (ADPG1) and ADPG2 (Ogawa et al., 2009; Swain et al., 2011). Other potential regulators include pectin methyltransferases although their specific role in the dehiscence zone has not been fully elucidated (Ballester and Ferrándiz, 2017). Studies by Mitsuda and Ohme-Takagi (2008) showed that *ADPG1* activity was retained in the *nst1-1 nst3-1* double mutant, suggesting that the processes of lignification and cell separation may be distinct. *ADPG1* and *ADPG2* are also expressed in flower organ separation layers (Ogawa et al., 2009). Sun and van Nocker (2010) investigated pectate lyase-like (*PLL*) genes potentially involved in pectin de-polymerization and discovered that 17 of the genes were expressed in the valve margin, and most of them also showed activity in the floral organ abscission zones. Not surprisingly, the *ind* mutant lacks expression of *NST1* and *ADPG1/2* genes, pointing to a downstream pathway under the direct or indirect regulation of *IND* (Sun and van Nocker, 2010; Ballester and Ferrándiz, 2017).

FUL required for valve identity contributes to lignification of the endocarp *b* layer, as a mutation in *ful* eliminates lignification of this layer in *ind alc shp1 shp2* mutants (Mitsuda and Ohme-Takagi, 2008). Conversely, the *nst1-1 nst3-1* mutant maintained *IND* and *SHP2* expression in the dehiscence zone, and shows no apparent defects in valve margin formation (Mitsuda and Ohme-Takagi, 2008). Intriguingly, siliques with defects only in valve margin secondary cell wall

formation showed the same resistance to shattering as those with further secondary wall inhibition at the replum and endocarp *b* layer (Mitsuda and Ohme-Takagi, 2008).

1.14 Hypothesis: a role for boundary genes in the fruit?

My thesis examines molecular homology between boundaries in the SAM and boundaries in the fruit. Previous studies have described expression of *KNAT2*, *KNAT6*, and *BOP1/2* genes in the gynoecium of flowers and the valve margins of fruit but their functions are mostly unknown (Pautot et al., 2001; Ragni et al., 2008; Khan et al., 2012b). Mutations in *KNAT2/6* and *BOP1/2* have no obvious impact on valve margin differentiation nor do they block dehiscence (Ragni et al., 2008; Khan et al., 2012b). However, inactivation of *KNAT2/6* or *BOP1/2* corrects the narrow replum defect of *pny* and/or *bp pny* mutants suggesting that PNY repression of boundary genes is important for replum formation (Ragni et al., 2008; Khan et al., 2012b; Hepworth and Pautot, 2015). Further, *BOP1/2* and *KNAT2/6* are positive regulators of lignin deposition, which is also required for pod shatter (Khan et al., 2012a; Khan et al., 2012b; Hepworth and Pautot, 2015).

My thesis tests for an underlying role of boundary genes in fruit development. We hypothesize that *BOP1/2* and downstream genes in this pathway contribute to fruit patterning and/or valve margin differentiation in parallel with *SHP/IND/ALC* valve-margin identity genes. We further hypothesize that regulatory mechanisms governing the expression of valve-margin identity genes apply to boundary genes in the fruit (Figure 1.4).

Figure 1.1 Organization of the SAM in *Arabidopsis thaliana*

The SAM is composed of three cell layers (solid yellow lines). Cells in the L1 epidermal and L2 sub-epidermal layers divide in one plane contributing to daughter cells incorporated into lateral organ primordia. Cells in the underlying L3 layer divide in all planes contributing mainly to the stem. The central zone (red) comprises a pool of undifferentiated stem cells. The peripheral zone (light blue) is where organs initiate. As new organs form, boundaries (dark blue) separate them from the meristem. Boundaries also give rise to axillary meristems that form branches and flowers. The rib zone (yellow) contributes to upward growth of the stem. This region contains an organizing center (pink) important for maintaining the central zone. Differential regulation of activities in the central zone, peripheral zone, boundaries, and rib zone govern the architecture of a plant. Figure adapted from Wolpert (2011).

Figure 1.2 Aerial architecture of the model plant *Arabidopsis thaliana*

During the vegetative stage, a rosette of leaves is formed without internode elongation and axillary meristems are dormant. During the first stage of reproductive development, leaf development is partially repressed. Cauline leaves are formed and axillary meristems develop as secondary inflorescences. During the second stage of reproductive development, leaf development is fully repressed. Axillary meristems form flowers that mature into fruits called siliques. Stem elongation during the reproductive phase governs the distribution of lateral shoots (branches and flowers) along the primary inflorescence. Scale bar, 1.5 cm. Figure adapted from Devi (2014).

Figure 1.4 Simplified genetic framework for fruit patterning and hypothesis

The Arabidopsis fruit contains three external tissues: valves, valve margin, and replum. The valve margin has two layers: a separation layer (SL) and a lignified layer (LL). SHATTERPROOF1/2 (SHP1/2) promote the expression of *INDEHISCENT* (*IND*) and *ALCATRAZ* (*ALC*) whose combined activities confer valve margin identity. FRUITFULL (*FUL*) patterns the valves in part by repressing valve-margin identity genes. PENNYWISE (*PNY*) and BREVIPEDICELLUS (*BP*) maintain the replum by repressing valve-margin identity genes. STM also in the replum provides meristem activity for ovule development. Boundary genes are shown in red. BLADE-ON-PETIOLE1/2 (*BOP1/2*) are transcriptional co-activators that require clade I TGA bZIP transcription factors (*TGA1* and *TGA4*) for recruitment to DNA. *BOP1/2* directly activate the BELL homeobox gene *ARABIDOPSIS THALIANA HOMEBOX GENE1* (*ATH1*) whose functional partners are KNOTTED-LIKE FROM *ARABIDOPSIS THALIANA*2 and 6 (*KNAT2/6*). We propose that boundary genes function in parallel with valve-margin identity genes to regulate fruit patterning and dehiscence.

Chapter 2: MATERIALS AND METHODS

2.1 Plant material and growth conditions

The Columbia-0 (Col-0) ecotype of *Arabidopsis thaliana* was used as wild type (WT). Single, double, and triple mutants for boundary genes used the following alleles: *ath1-3* (Gómez-Mena and Sablowski, 2008), *tga1-1* and *tga4-1* (Kesarwani et al., 2007), *bop1-3* and *bop2-1* (Hepworth et al., 2005), *kna2-5* and *kna6-1* or *kna6-2* (Belles-Boix et al., 2006). Gain-of-function lines were as previously described: *bop1-6D/+* and *35S:BOP2* (Norberg et al., 2005), *35S:KNAT6* (Shi et al., 2011), and *35S:ATH1* (Khan et al., 2015). The steroid-inducible line *35S:KNAT2-GR* was made by fusing the coding region of *KNAT2* to the steroid binding domain of the rat glucocorticoid receptor (Pautot et al., 2001). GUS reporter lines used as markers for different carpel domains in the fruit were as published. The marker for valves was *ful:GUS* (Gu et al., 1998). Markers for the replum were *PNY:GUS* (Byrne et al., 2003) and *BP:GUS* (Ori et al., 2000). Markers for the valve margin were *SHP1:GUS* (Baum et al., 2001), *SHP2:GUS* (Savidge et al., 1995), *IND:IND:GUS* (Sorefan et al., 2009; shortened as *IND:GUS* from here on) and *alc:GUS* (Rajani and Sundaresan, 2001). Reporter genes *ful:GUS*, *PNY:GUS*, *SHP1:GUS*, *SHP2:GUS*, and *alc:GUS* are in the Landsberg *erecta* (Ler) ecotype and were backcrossed once to Col-0 (except *SHP1:GUS* due to time constraints). GUS reporter lines marking development boundaries: *BOP1:GUS* (McKim et al., 2008), *BOP2:GUS* (Khan et al., 2012b), *KNAT2:GUS* (Dockx et al., 1995), *KNAT6:GUS* (Belles-Boix et al., 2006), *ATH1:GUS* (Khan et al., 2015), *TGAI:GUS* and *TGA4:GUS* (Devi, 2014) were as previously described. These reporters were crossed with fruit developmental mutants marking each carpel domain: *ful-2*, which is disrupted in valve formation (Ferrándiz et al., 2000), *pnv_40126* (Smith and Hake, 2003), which is disrupted in replum formation, and *shp1-1 shp2-1* (Liljegren et al., 2000), *ind-2* (Liljegren et al., 2004) or

ind-13 (Kay et al., 2013), which are disrupted in valve margin differentiation. F2 progenies were used to monitor GUS expression in loss-of-function mutants, and F1 progenies were used to monitor GUS expression in gain-of-function and transgenic overexpression lines. Table 2.1 provides a detailed description of genetic materials used in this study.

Seeds were surface-sterilized by rinsing in 100% ethanol followed by an aqueous solution of 5% sodium hypochlorite (bleach) and 0.5% (w/v) sodium dodecyl sulphate (SDS). The bleach was removed by rinsing four times in sterile distilled water. The seeds were sown on agar plates containing *Arabidopsis thaliana* minimal medium (Haughn and Somerville, 1986). The plated seeds were incubated at 4°C in the dark for 2-3 days to break dormancy. Plates were transferred to continuous light for 9-10 days for seed germination. Seedlings were transplanted to soil (ProMix BX, Premier Horticulture, Rivière-du-Loup, QC) sterilized by autoclaving and supplemented with a 1 g per liter solution of 20-20-20 plant fertilizer (Plant-Prod Inc., Brampton, ON). Plants were grown to maturity in growth chambers at 21°C under continuous light ($\sim 115 \mu\text{mol m}^{-2} \text{sec}^{-1}$). *35S:KNAT2-GR* steroid-inducible lines were sprayed with a 20 μM solution of dexamethasone daily following transplanting for induction of KNAT2 activity (similar to Khan et al., 2015).

2.2 Extraction of genomic DNA and genotyping

Genomic DNA was extracted from young rosette leaves according to Edwards et al. 1991 with minor modifications. From each plant, one rosette leaf ($\sim 1 \text{ cm}^2$) was added to a clean 1.5 ml microcentrifuge tube and hand-ground using a pellet pestle. 400 μL of DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% w/v SDS) was added to the tube and the mixture was vortexed for 5 sec and then centrifuged for 10 min at 13,000 g. A 350 μl aliquot of the resulting supernatant was transferred to a new tube, ensuring that the pellet was not disturbed. An equal volume of isopropanol was added to each supernatant. The tubes were mixed

by inversion and stored at -20°C for 15-20 min to precipitate the DNA. Upon removal from the freezer, the tubes were centrifuged for 10 min to collect the DNA. The supernatant was discarded and the pellet was washed with 800 µL of 70% ethanol. The tubes were centrifuged once again for 5 min and inverted to remove residual ethanol. The tubes containing the final pellet of genomic DNA were either air-dried at room temperature overnight or incubated at 37°C for at least 30 min to evaporate the ethanol. The pellet was dissolved in 100 µL of molecular-biology grade filtered water and the tubes were stored at -20°C. Two µL of the DNA prep was used as template in a standard 20 µL PCR-genotyping reaction.

The strategy for genotyping T-DNA insertion mutants from the SALK collection was as previously described (www.signal.salk.edu). The genotyping test for *ind-2* was based on loss of an *AluI* restriction site in the mutant (Liljegren et al., 2004). Table 2.2 lists primers used for genotyping.

2.3 Scanning electron microscopy and replum width measurements

Mature stage 17B silique samples for scanning electron microscopy (SEM) were fixed in a solution of 3% glutaraldehyde and 0.1 M sodium phosphate buffer pH 7.0 in glass scintillation vials. One or two drops of Triton X-100 were added (for every 5 mL of buffer) to each vial to reduce surface tension and vials were placed under vacuum overnight at 4°C. The next day, the fixative was removed and samples were washed twice in 0.1 M sodium phosphate buffer pH 7.0. The samples were post-fixed for two hrs in a solution of 1% OsO₄ and 0.05 M sodium phosphate buffer pH 7.0, after which they were washed at least three times with 0.1 M sodium phosphate buffer pH 7.0 and dehydrated in a 30%, 50%, and 70% ethanol series for 30 min each. Samples were stored in 70% ethanol for up to three weeks before critical point drying. Immediately prior to the critical point dry, the samples were further dehydrated in 90% and twice in cold anhydrous

ethanol, which was stored over molecular sieves. The samples were critical point dried in carbon dioxide and then mounted on stubs and sputter coated with gold (Quorum, Q150T ES). The final prepared samples were viewed under a scanning electron microscope at an accelerating voltage of 15 kV (Tescan Vega-II XMU).

Images were taken at 500X, 600X or 1000X magnification. Replum width measurements were taken from the top, middle, and bottom sections of the silique from images at 500X magnification. Measurements were made from the inner side of one valve margin to the inner side of the other, and a minimum of 11 fruits were analyzed per genotype. The width quantification was performed using an ImageJ Java program, and statistical analyses were conducted using a Student's t-test ($p < 0.05$).

2.4 Lignin staining

Lignin staining of stage 17B silique cross-sections was carried out as previously described (Khan et al., 2012b) with minor changes. In detail, siliques were initially collected and fixed in formaldehyde-acetic acid-alcohol (FAA: 50% ethanol, 3.7% formaldehyde, 5% glacial acetic acid) solution at room temperature for ~3 hrs. Fixation was followed by a dehydration series of 50%, 60%, and 70% ethanol for 30 min per step. Samples were either stored in 70% ethanol at this point, or further dehydrated in 85% ethanol for another 30 min to continue tissue processing. Samples were then incubated in 95% ethanol containing 0.5% eosin for 1-2 days to stain the tissue red. The tissues were subsequently cleared in 100% ethanol for 1 hr and slowly exchanged for xylenes using a series of 25%, 50%, 75% and 100% xylenes for 30 min per step. Samples were infiltrated with a final solution of 50% xylene/50% wax (Paraplast Plus, Sigma-Aldrich, St. Louis, Missouri, USA) overnight at 60°C before switching to 100% wax. Two wax changes were performed at least 6 hrs apart on three consecutive days. Samples were poured into petri plates and the wax was allowed

to harden. Blocks were cut from the wax and mounted on wooden stubs. Tissue sections (25 μm thick) were prepared using a microtome and fixed onto Superfrost Plus glass slides (Thermo Fisher) by resting on a slide warmer set at 42°C. The tissue sections were dewaxed in 100% xylenes for 20 min and dehydrated in 100% and 95% ethanol for 30 min each. At this point, lignin was stained by treating slides with a solution of 2% phloroglucinol in 95% ethanol for 5 min. Drops of concentrated HCl were added across the entire surface of slides for colour development. After one min, a cover slip was added and images were immediately collected using a compound microscope with digital camera (Axio Imager M2, Carl Zeiss, North York, ON, Canada).

2.5 β -Glucuronidase staining assay

Staining of flowers and fruits for β -glucuronidase (GUS) activity was carried out as previously described (Khan et al., 2012b) with minor modifications. Tissue was collected and fixed in chilled 90% acetone on ice. Samples were warmed by placing at room temperature for 15 mins and then submerged in GUS staining solution containing 0.25 to 3 mM $\text{K}_3\text{Fe}(\text{CN})_6$, 0.25 to 3 mM $\text{K}_4\text{Fe}(\text{CN})_6$ and 2 mM of 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide (X-Gluc). Samples were incubated at 37°C until a localized blue precipitate of suitable intensity formed (3 to 24 hrs). Whole tissue samples were cleared in 70% ethanol and imaged using an Axiocam digital camera mounted on a stereomicroscope (SteRIO Discovery V20, Carl Zeiss, North York, ON, Canada). Alternatively, thin tissue sections bound to glass slides were dehydrated for 30 min each in 30% and 50% ethanol solutions and then fixed in FAA for 2-3 hrs before transfer to 70% ethanol. Samples were further dehydrated and stained with eosin as described above. However, tissues were further processed using *tert*-butanol instead of xylenes because the X-Gluc precipitate is soluble in xylenes. During the final round of 100% *tert*-butanol, the tissues were kept overnight at 60 °C. The next day, the 100% *tert*-butanol was replaced with a 1:1 (v/v) solution of *tert*-butanol/wax at

60°C for 24 hours. Finally, the samples were embedded in wax for 3 days as described above. Sections (10-12 µm thick) were cut from wax blocks using a microtome and fixed onto glass slides. For dewaxing, the slides were placed in *tert*-butanol at 60°C for 20 min with occasional shaking and then dipped into 100% xylenes for 1-3 minutes to remove all traces of wax. Slides were put through a hydration series of 100%, 95%, 85%, 70%, 50%, and 30% ethanol and then distilled water for 3 min per step before mounting in 50% glycerol. A coverslip was added and sections were observed using a compound microscope.

2.6 Random impact test for dehiscence

Quantification of resistance to pod shatter was measured using a random impact test (RIT) (Arnaud et al., 2010). Each replicate consisted of n=20 stage 19 siliques randomly collected from at least 6 plants per genotype. Three biological replicates were analyzed unless otherwise stated. Prior to testing, siliques were moisture-equilibrated in a chamber at 25°C and 50% relative humidity for a minimum of three days. The equilibrated siliques were placed in a 1.25 cm x 4.25 cm glass dram vial with screw-cap lid containing five 3 mm diameter stainless steel ball bearings (FOX Industries; Fairfield, NJ, USA). The vials were taped onto an orbital shaker (Lab-Line Model SHK E4000, Barnstead International) and agitated at 250 rpm. The number of shattered siliques, where either one or both valves were fully detached from the replum, was counted every 10 sec until 150 sec were elapsed. Any replicates that did not reach 100% shattering at 150 sec were subjected to another 150 sec of continuous shaking. A binomial regression curve was appropriately fitted to the data to calculate the time point at which 50% of the pods were shattered (RIT₅₀) as a comparative measure of shattering between different lines.

Table 2.1 List of genetic materials used in this study

Plant line	Description	Reference	Stock Notes
<u>Loss-of-function mutants</u>			
<i>Boundary</i>			
<i>bop1-3</i>	T-DNA insertion	Hepworth et al., 2005	SALK_012994
<i>bop2-1</i>	T-DNA insertion	Hepworth et al., 2005	SALK_075879
<i>tga1-1</i>	T-DNA insertion	Kesarwani et al., 2007	SALK_028212
<i>tga4-1</i>	T-DNA insertion	Kesarwani et al., 2007	SALK_127923
<i>ath1-3</i>	T-DNA insertion	Gómez-Mena and Sablowski, 2008	SALK_113353
<i>knat2-5</i>	T-DNA insertion	Belles-Boix et al., 2006	SALK_099837
<i>knat6-1</i>	T-DNA insertion	Belles-Boix et al., 2006	SALK_047931
<i>Replum</i>			
<i>pny_40126</i>	T-DNA insertion	Smith and Hake, 2003	SALK_40126
<i>Valves</i>			
<i>ful-2</i>	Nonsense mutation in exon 3 (W91 to STOP)	Ferrándiz et al., 2000a	
<i>Valve margin</i>			
<i>shp1-1 shp2-1</i>	T-DNA insertion	Liljegren et al., 2000	CS3844, <i>shp1-1</i> in Ws and <i>shp2-1</i> in Col, double mutant backcrossed 3X to Col
<i>ind-13</i>	T-DNA insertion	Kay et al., 2013	SALK_058083
<i>ind-2</i>	Single nt deletion in codon 26	Liljegren et al., 2004	Ler ecotype
<u>Gain-of-function mutants</u>			
<i>Boundary</i>			
<i>bop1-6D</i>	Activation tagged line, 4X CaMV 35S enhancer in <i>BOP1</i> promoter	Norberg et al., 2005	
<i>35S:BOP2</i>	CaMV 35S promoter upstream of <i>BOP2</i> cDNA	Norberg et al., 2005	
<i>35S:KNAT2-GR</i>	CaMV 35S promoter upstream of <i>KNAT2</i> cDNA fused to GR	Pautot et al., 2001	Dexamethasone-inducible
<i>35S:KNAT6</i>	2X CaMV 35S promoter upstream of <i>KNAT6</i> cDNA	Shi et al., 2011	
<i>35S:ATH1</i>	2X CaMV 35S promoter upstream of <i>ATH1</i> cDNA	Khan et al., 2015	
<u>GUS reporter lines</u>			
<i>Boundary</i>			
<i>BOP1:GUS</i>	4-kb of <i>BOP1</i> genomic sequence upstream of the translational start site fused in-frame to GUS	McKim et al., 2008	
<i>BOP2:GUS</i>	4-kb of <i>BOP2</i> genomic sequence upstream of the translational start site fused in-frame to GUS	Xu et al., 2010	

<i>TGA1:GUS</i>	4-kb of <i>TGA1</i> genomic upstream region including the translational start site fused in-frame to GUS	Devi, 2014	
<i>TGA4:GUS</i>	4-kb of <i>TGA1</i> genomic upstream region including the translational start site fused in-frame to GUS	Devi, 2014	
<i>ATH1:GUS</i>	3.3-kb of <i>ATH1</i> genomic sequence upstream of the translational start site fused in-frame to GUS	Khan et al., 2015	
<i>KNAT2:GUS</i>	A 3.5-kb fragment of <i>KNAT2</i> genomic sequence and 0.25 kb of the coding region upstream of GUS	Dockx et al., 1995	
<i>KNAT6:GUS</i>	3.8-kb of <i>KNAT6</i> genomic sequence containing the promoter fused upstream of GUS	Belles-Boix et al., 2006	
<i>Replum</i> <i>BP:GUS</i>	5-kb of <i>KNAT1/BP</i> genomic upstream region including translational start site fused to GUS	Ori et al., 2000	
<i>PNY:GUS</i>	3.9 kb genomic sequence containing the <i>BLR</i> promoter fused in-frame to GUS	Byrne et al., 2003	Ler ecotype; backcrossed 1X to Col
<i>Valves</i> <i>ful:GUS</i>	<i>ful-1</i> bears an enhancer trap in the 5'-UTR in creating a null allele. GUS activity faithfully mimics <i>FUL</i> expression pattern and can be used as a reporter for <i>FUL</i> in <i>ful-1</i> heterozygous backgrounds	Gu et al., 1998	Ler ecotype; backcrossed 1X to Col
<i>Valve margin</i> <i>SHP1:GUS</i>	3.5-kb of <i>SHP1</i> genomic sequence upstream of the translational start to GUS	Baum et al., 2001	Ler ecotype
<i>SHP2:GUS</i>	2.1-kb of <i>SHP2</i> genomic sequence upstream of the translational start fused to GUS	Savidge et al., 1995	Ler ecotype; backcrossed 1X to Col
<i>IND-IND:GUS</i>	2.7-kb of <i>IND</i> genomic sequence upstream of the translational start and 0.5 kb of the coding region fused to GUS	Sorefan et al., 2009	
<i>alc:GUS</i>	GUS cloned as part of a Ds gene trap in third exon of <i>ALC</i>	Rajani and Sundaresan, 2001	Ler ecotype; backcrossed 1X to Col

Table 2.2 List of primers used for genotyping

Allele	Primers	Sequence (5' to 3')	Reference
<i>bop1-3</i>	bop1-3 SALK_012994 RP	TGACATCGGAGAAAGCTTGAC	This study
	bop1-3 SALK_012994 LP	TGCACAATCTTTCGACTTCATC	
<i>bop2-1</i>	bop2-1 SALK_075879 RP	ATTTGGCCCACCTTTGTATTC	This study
	bop2-1 SALK_075879 LP	AAAGAGAGAAACCTGGGTGAGC	
<i>tga1-1</i>	TGA1 Salk_028212 RP	GCTCTGCTGAAGTTTTCCACATTCC	Devi, 2014
	TGA1 Salk_028212 LP	TTCAAAACCTGGATTCATGGTTTCC	
<i>tga4-1</i>	TGA4-1 SALK_127923 RP	GAAGGTTTGAAGTTTACGAGCCTCT	Devi, 2014
	TGA4-1 SALK_127923 LP	GCTCTGCTGAAGTTTTCCACATTCC	
<i>ath1-3</i>	ath1 RP	GGCGGGTTTCGGATCTACATT	Gómez-Mena and
	ath1 LP	CCAATACCGGTTTTTCAGACATGA	Sablowski, 2008
<i>knat2-5</i>	knat2-5 RP-2k	TTCAACCACCGGAGACAATCAAAGA	This study
	Knat2-5 LP-2	TGTAGCAGACGCTGGACCAGTGCAC	
<i>knat6-1</i>	knat6-1 LP	GAAGATAAACCCCTAGCTACAAG	This study
	knat6-1 RP	ATATCAGTAAACCACAAAGAAAGTC	
<i>ind-2</i>	ind-2 Fwd	CAGCCTCACCATCTCCTCATGGATTGGAA	This study
		CAAGCCTAATG	
	ind-2 Rev	CTTGAGAATTCGGATCTTCTCGCTG	
<i>ind-13</i>	ind SALK_058083 RP	TGTTCTGGTTGGGTTCAAAAG	Khan et al., 2012
	ind SALK_058083 LP	CACCCATACAATCTTGGATCG	
<i>pnv-40126</i>	PNY SALK_40126 RP	TTGGAATTGGAGACAAAATGTGTTA	Smith and Hake,
	PNY SALK_40126 LP	GGAACCAAGTTCAAACCTCGAATCCA	2003

Chapter 3: RESULTS

3.1 Boundary and valve margin identity genes are co-expressed in developing fruit

Valve margins in fruits are specialized organ boundaries for dehiscence. To test the hypothesis that boundary and valve-margin identity genes jointly pattern the valve margin, I first compared the expression patterns of boundary genes (*BOP1*, *BOP2*, *ATH1*, *TGAI* and *TGA4*) with previously characterized boundary (*KNAT2* and *KNAT6*) and valve-margin identity (*SHPI*, *SHP2*, *IND*, and *ALC*) genes in developing flowers and the fruit. For this, reporter *GUS* lines were used (Materials and Methods). Whole mount expression patterns were recorded at three different developmental stages: young flowers (stages 10-11), mature flowers (stage 12-13), and mature fruits (stage 17). I also examined expression in cross-sections of mature fruits (stage 17). As expected, *SHP* genes were broadly expressed in young carpels, becoming localized to valve margins in young flowers around stage 10 ((Roeder et al., 2003; Roeder and Yanofsky, 2006); Figure 3.1A). *IND* expression was immediately specific for valve margins ((Liljegren et al., 2004; Roeder and Yanofsky, 2006); Figure 3.1B)). *ALC* was expressed at low levels in the carpels and at high levels in the valve margins, gradually becoming exclusive to the dehiscence zone in mature fruits ((Rajani and Sundaresan, 2001); Figure 3.1C)). Expression of the boundary genes *BOP1/2* was specific to the valve margins in young flowers prior to fertilization (Figure 3.2AB). *KNAT2* and *KNAT6* showed a similar pattern ((Ragni et al., 2008); Figure 3.1DE)). *ATH1* and Clade I *TGAI/4* expression in fruit boundaries have not been previously investigated. Moderate *ATH1:GUS* expression was detected in the carpels of flowers and valves of fruit, with enrichment in the valve margin. This expression was difficult to detect in older flowers and fruits (Figure 3.2C). Cross-sections showed that *ATH1* expression in valves was mainly associated with the endocarp *b* layer (Figures 3.2C). *TGAI* and *TGA4* reporters showed no specificity for the valve

margin. *TGAI* expression was concentrated in the endocarp *b* layer of valves, the septum, and inner vascular bundles of the replum. *TGA4* expression dominated in vascular bundles of the replum (Figure 3.2DE). Based on other work in our lab, these two reporters might be missing certain genetic control sequences, thus potentially not giving an accurate depiction of their expression patterns in the fruit. Overall, these data show that boundary and valve margin identity genes are co-expressed in the valve margin during flower and fruit development, consistent with potentially overlapping roles.

3.2 *ATH1* is important for *KNAT2* and *KNAT6* expression in the valve margin

ATH1 is a predicted upstream regulator of *KNAT6* and *KNAT2* (Khan et al., 2015). To further test this interaction, I examined the expression of *KNAT2* and *KNAT6* reporter genes in wild type and *ath1-3* flowers. This analysis showed a marked reduction for *KNAT2* and absence of *KNAT6* expression in *ath1-3* flowers (Figure 3.3; see also Bao, 2009). Overall, these data support the model that *ATH1* directs expression of *KNAT6* and *KNAT2* in the valve margin.

3.3 *PNY* repression of Clade I *TGAs* and *ATH1* boundary genes is important for replum formation

Interactions with *PNY* have previously defined a role for boundary genes in the fruit (Ragni et al., 2008; Khan et al., 2012b). *PNY* is important for replum identity (Roeder et al., 2003). Mutant fruits are compact with a narrow replum, whose outer layers are missing in severe cases (Roeder et al., 2003). This phenotype is caused by misexpression of valve-margin identity genes (Roeder et al., 2003; Roeder and Yanofsky, 2006; Ripoll et al., 2011; Bencivenga et al., 2016). However, repression of boundary genes is also important since *pnv* replum defects are corrected in *pnv knat6* and *pnv bop1 bop2* mutant combinations (Ragni et al., 2008; Khan et al., 2012b). The inactivation of *KNAT2* has no such rescue effect, showing distinct contributions of *KNAT6* and *KNAT2* in fruits

(Ragni et al., 2008). BOP1/2 form a complex with Clade I TGAs transcription factors that activates *ATH1* expression at boundaries in shoot apices (Khan et al., 2015; Wang et al., 2018). To test if *ATH1* and Clade I *TGA* genes are part of this boundary module in fruits, I examined genetic interactions with *PNY*.

If *ATH1* and Clade I *TGA1/4* genes function in the same genetic pathway(s) as *BOP1/2* and *KNAT6* genes in the fruit, loss of these genes is expected to restore replum formation in *pnY* mutants. I therefore examined the morphology of *ath1 pnY*, *tgal pnY*, *tga4 pnY* and *tgal tga4 pnY* double and triple mutant fruits compared to wild type and *pnY* controls. The exteriors of stage 17B fruits were examined by SEM. The interiors of stage 17B fruits were examined by staining lignin in tissue cross-sections with phloroglucinol. These analyses showed that inactivation of *ATH1*, *TGA1*, and *TGA4* corrects *pnY* replum defects (Figure 3.4). Quantification showed that this recovery was partial. Measurements showed that the replum in *ath1 pnY*, *tgal pnY*, *tga4 pnY*, and *tgal tga4 pnY* mutants was significantly wider than in *pnY* mutants, but narrower than wild type (Figure 3.5). The replum width for *tga4 pnY* mutants was highly variable according to SEM quantification, indicating a weaker rescue compared to *ath1* or *tgal* mutants in a *pnY* background. Cross-sections of *ath1 pnY*, *tgal pnY*, *tga4 pnY*, and *tgal tga4 pnY* siliques showed a lignification pattern similar to wild type (Figure 3.4).

To further monitor rescue, a random impact test (RIT) was used to measure pod shatter resistance. Pods of the *pnY* mutant are short and compact, showing strong resistance to pod shatter compared to wild type (Table 3.1; Figure 3.6). Some pods showed partial shattering early on, but full separation of valves from the replum in *pnY* mutants was delayed, as previously reported (Roeder et al., 2003). In double and triple mutant combinations, pod length and shatter characteristics were significantly restored (Table 3.1; Figure 3.6). These data suggest that *ATH1*

and Clade I TGAs function in the same genetic pathway as *BOP1/2* and *KNAT2/6* in controlling boundaries in the fruit. A higher impact for *TGA1* is observed when compared to *TGA4*. These data suggest that boundary genes play an underlying role in dehiscence, either directly or indirectly by controlling parameters that potentially influence pod shatter, such as pod length and replum width.

3.4 Mechanisms governing spatial regulation of boundary genes in the fruit

Next, I tested mechanisms that constrain boundary genes to the valve margin of fruits. *SHP1/2* promote the expression of downstream genes, *IND* and *ALC*, whose combined activities define the valve margin. Expression of these genes is confined to the valve margin by *PNY* in the replum and *FUL* in the valves (Roeder and Yanofsky, 2006). To test if these mechanisms apply to boundary genes, *BOP1:GUS*, *BOP2:GUS*, *ATH1:GUS*, *KNAT2:GUS*, and *KNAT6:GUS* reporter genes were crossed to mutants marking each of the domains in a fruit: valve margins (*shp1 shp2* and *ind-2*), replum (*pnv*), and valves (*ful-2*). Boundary genes showed no change of expression in *shp1 shp2* or *ind-2* mutants (Supplemental Figure 1). By contrast, *KNAT2:GUS* and *KNAT6:GUS* expression domains were coalesced in *pnv* siliques (Ragni et al., 2008). *BOP1:GUS*, *BOP2:GUS*, and *ATH1:GUS* were likewise expressed in the medial domain of *pnv* siliques where the valve margins are converged (Figure 3.7).

Valve margin identity genes *SHP1/2* are expressed in both layers of the valve margin (Figure 1.1 and Roeder et al., 2003). By contrast, cross-sections show that *BOP1/2* are expressed in a subdomain of the valve margin: at the base of the valve margin towards the inner side of the replum (Figure 3.8). *KNAT2* and *KNAT6* are expressed in a similar subdomain (Ragni et al., 2008). *ATH1* is also expressed in this subdomain, but shows additional expression in the endocarp *b* cell layer that lines the inside of valves (Figure 3.2). In the absence of *PNY*, *BOP1* and *BOP2*

expression invade the inner replum domain, similar to *SHP1/2* expression pattern in the *rpl* mutant (Figure 3.8; Roeder et al. 2003). The inner replum is still present in the *pnv* mutant (see cross-section in Figure 3.3) used in this study (see cross-section in Figure 3.4) and in an *rpl1* strong mutant (Roeder et al., 2003).

FUL patterns the valves in part by repressing valve-margin identity genes. My data show that this mechanism does not necessarily apply to all boundary genes. Boundary genes were expressed normally in *ful-2* fruits, with the exception of *KNAT6::GUS*, which was misexpressed in the valves (Figure 3.3 and Supplemental Figure 1). Interestingly, misexpression was limited to post-fertilization stages of fruit development, which is characterized by a rapid elongation of the valves. Overall, the repression of boundary genes in the valves appears to be under different genetic control compared to *SHP/IND/ALC*. These data suggest a specific role for *KNAT6* apart from other boundary genes that I investigated.

3.5 Inactivation of boundary genes narrows the replum and inhibits pod shatter

A role for boundary genes in the fruit was also investigated by analyzing loss-of-function mutants for changes in fruit structure and/or pod shatter. Previous data show that *knat2*, *knat6*, *knat2 knat6*, *bop1*, *bop2* and *bop1 bop2* have wild type fruit morphology and wild type lignin deposition (Ragni et al., 2008; Khan et al., 2012b). Here, I also characterized *ath1*, *ath1 knat2*, *ath1 knat6* and *ath1 knat2 knat6* mutant fruits. SEM analysis of the external morphology of *bop1 bop2*, *ath1*, *knat2*, *knat6*, *knat2 knat6*, *ath1 knat2*, *ath1 knat6*, and *ath1 knat2 knat6* fruits showed no obvious defects (Figure 3.9). Nevertheless, measurements showed a small but significant decrease of replum width for several genotypes, including *bop1 bop2*, *knat2 knat6*, *ath1 knat2*, *ath1 knat6*, and *ath1 knat2 knat6* (Figure 3.10). Cross-sections were used to assess fruit internal morphology and lignin deposition, a prerequisite for shattering. No obvious changes were detected

(Figure 3.9; see also Ragni et al., 2008; Khan et al., 2012). However, RIT testing showed that *bop1 bop2* siliques were strongly resistant to shattering (Figure 3.11). A small but significant increase in pod shatter resistance was also observed for *knat6*, *knat2 knat6*, *ath1 knat2*, and *ath1 knat2 knat6* mutants (Figure 3.11). In all of these boundary mutants, average pod lengths were similar to wild type. Siliques shattered completely along the entire length of valve margin. Thus, reasons for delayed shattering in *pny* and boundary mutants are likely different.

3.6 Overexpression of boundary genes widens the replum and inhibits fruit elongation

I next tested the role of boundary genes by monitoring gain-of-function mutants and transgenic overexpressor lines for changes in fruit morphology and/or pod shatter resistance. For these experiments, I used characterized transgenic lines, in which *BOPI*, *BOP2*, *ATH1*, *KNAT2-GR*, or *KNAT6* were expressed under the control of a strong constitutive viral 35S promoter (Pautot et al., 2001; Norberg et al., 2005; Shi et al., 2011). The *KNAT2-GR* line contains a steroid-inducible version of *KNAT2* fused to the steroid binding domain of the rat glucocorticoid receptor (Pautot et al., 2001). SEM imaging and quantification showed that the *bop1-6D/+* fruits have large variability in replum width (Figures 3.12 and 3.13). Enlarged repla were most reliably detected at the base of the fruit (data not shown). The majority of *35S:BOP2*, *35S:ATH1*, *35S:KNAT2-GR*, and *35S:KNAT6* siliques showed enlarged repla (Figures 3.12 and 3.13). *35S:BOP2*, *35S:ATH1*, and *35S:KNAT6* siliques showed a small but significant increase in replum width (25% increase when compared to wild type) whereas the replum width in DEX-induced *35S:KNAT2-GR* fruits was twice than that of wild type. In *35S:KNAT2-GR* fruits, the entire carpel surface was composed of small uniform cells, and there was little or no distinction between different tissues. Strong *35S:KNAT6* lines also had short, distorted fruits with altered cell morphology. In both genotypes, the distinctions between valve, valve margin, and replum domains were obscured (Figure 3.12IL).

In rare cases, these genotypes formed an additional partial carpel in the replum region (Supplemental Figure 2). Despite dramatic changes in replum and valve margin positioning, the valve margins and the endocarp *b* layer in *35S:KNAT2:GR* and *35S:KNAT6* fruits became lignified. Lignification in transgenic lines overexpressing *BOP1*, *BOP2*, or *ATH1* was also similar to wild type (Figure 3.12). Measurements of pod size and shatter indicated that *bop1-6D/+*, *35S:BOP2*, *35S:KNAT2-GR*, and *35S:KNAT6* fruits were shorter than wild type and less prone to shattering (Table 3.1 and Figure 3.14). The very stunted growth of fruits in some of these mutants probably affects their resistance to pod shatter.

3.7 Boundary genes are positive regulators of valve margin identity genes

Finally, I tested if boundary genes control the expression of genes conferring valve, valve-margin, or replum formation. Loss-of-function *bop1 bop2* and gain-of-function *bop1-6D*, *35S:BOP2*, *35S:KNAT2-GR* and *35S:KNAT6* lines were crossed to characterized *GUS* lines marking each carpel domain: valve margins (*SHPI*, *IND*, *ALC*), valves (*FUL*), and replum (*PNY*, *BP*). The resulting progenies were monitored for *GUS* expression. In *bop1 bop2* mutants, there were no obvious changes (Supplemental Figure 3). By contrast, positive regulation of valve-margin identity genes was detected in boundary gain-of-function / overexpressor lines. In *35S:BOP2* fruits, *IND* was misexpressed in the valves (Figure 3.15). In *35S:KNAT2-GR* and *35S:KNAT6* fruits, *IND* and *ALC* were misexpressed in the valves. The *35S:KNAT6* line also misexpressed *SHPI* in the valves (Figure 3.15). Replum (*BP*, *PNY*) and valve (*FUL*) markers showed a wild type pattern (Figure 3.16) with the caveat that *35S:KNAT2-GR* lines expressing *BP:GUS* do not appear to have the normal induction phenotype (panel I). Collectively, these findings reveal that boundary genes promote valve margin identity. These analyses also underline that *KNAT6* has a higher impact in promotion of valve margin identity. *KNAT6* is also the only

gene regulated by FUL. This implies a central role for *KNAT6* with other genes involved in fruit patterning.

Table 3.1 Comparison of silique lengths between different Arabidopsis genotypes

Genotype	Sample number	Silique length (mm)	Standard deviation (mm)	Statistical significance
WT (Col)	60	13.1	1.2	
<i>shp1 shp2</i>	60	13.7	1.6	
<i>pny</i>	60	9.7	1.2	a
<i>ath1-3 pny</i>	60	11.0	1.2	a, c
<i>tga1 pny</i>	59	10.3	1.1	a, c
<i>tga4 pny</i>	60	10.4	1.3	a, c
<i>tga1 tga4 pny</i>	60	11.9	1.3	a, c
<i>bop1 bop2</i>	60	13.3	1.4	
<i>tga1</i>	60	12.5	1.2	b
<i>tga4</i>	60	12.4	2.2	b
<i>ath1-3</i>	60	12.3	1.6	b
<i>knat2-5</i>	60	12.1	1.3	b
<i>knat6-1</i>	60	12.4	1.6	b
<i>knat2-5 knat6-2</i>	60	14.1	2.1	b
<i>ath1-3 knat2-5</i>	60	11.5	1.1	b
<i>ath1-3 knat6-2</i>	60	11.2	1.3	b
<i>ath1-3 knat2-5 knat6-2</i>	60	12.1	1.2	b
<i>bop1-6D</i>	60	6.1	0.8	a
<i>35S:BOP2</i>	60	6.8	1.0	a
<i>35S: ATH1</i>	60	12.6	1.3	b
<i>35S:KNAT2-GR</i>	40	1.9	0.6	a
<i>35S:KNAT6</i>	60	12.7	1.3	b

a, very significantly different from wild type (student's t-test, $p \leq 0.001$)

b, significantly different from wild type (student's t-test, $p \leq 0.05$)

c, very significantly different from *pny* (student's t-test, $p \leq 0.001$)

Figure 3.1 Expression of valve-margin identity and boundary genes *KNAT2* and *KNAT6* during fruit development

GUS reporters were used to monitor gene expression. Left to right: inflorescence apex, stage 10-11 flowers, stage 12-13 mature flowers, stage 17B mature fruits, and cross-sections of stage 17B mature fruits. (A) *SHP2:GUS*, intense expression in the valve margins of flowers and dehiscence zones of fruits. (B) *IND:GUS*, similar to *SHP2:GUS*. (C) *ALC:GUS*, intense expression in the valve margins of flowers; weak expression in the carpel valves. (D) *KNAT2:GUS*, expression the valve margins of flowers and dehiscence zones of fruits. (E) *KNAT6:GUS*, expression in the valve margin of flowers and dehiscence zones of fruits; Scale bars, 1 mm; except 50 μ m in cross-sections.

Figure 3.2 Expression of *BOP1/2*, *ATH1*, *TGA1*, and *TGA4* during fruit development

GUS reporters were used to monitor gene expression. Left to right: inflorescence apex, stage 10-11 flowers, stage 12-13 mature flowers, stage 17B mature fruits, and cross-sections of stage 17B mature fruits. (A) *BOP1:GUS*, expression the valve margins of flowers and dehiscence zones of fruits. (B) *BOP2:GUS*, similar to *BOP1:GUS*. (C) *ATH1:GUS*, broad expression in flowers, including endocarp *b* layer of carpels, valve margins of flowers, dehiscence zones of fruits. (D) *TGA1:GUS*, broad expression in flowers, including vasculature, septum, and endocarp *b* layer of carpels. (E) *TGA4:GUS*, broad expression in young carpels. In older flowers and fruit, diffuse expression is concentrated in replum vasculature. Arrows point to staining in the endocarp *b* layer of the valves. Scale bars, 1 mm; except 50 μ m in cross-sections

Figure 3.3 Expression of *KNAT2* and *KNAT6* in wild type, *ath1-3*, and *ful-2* carpels

Representative images showing GUS staining in flowers (stage 13). (A) *KNAT2:GUS*, GUS expression in valve margins and receptacle. (B) *KNAT2:GUS ath1-3*, diminished expression compared to wild type (denoted by asterisk). (C) *KNAT2:GUS ful-2*, similar to wild type. (D) *KNAT6:GUS*, expression in valve margins and receptacle. (E) *KNAT6:GUS ath1-3*, little or no expression compared to wild type (denoted by asterisk). (F) *KNAT6:GUS ful-2*, misexpression in the valves (arrow). Scale bars, 0.25 mm.

Figure 3.4 Inactivation of *ATH1* and genes encoding Clade I TGA factors restores replum formation in *pny* mutants

Images are representative. (A-C, G-I) SEM micrographs showing exterior replum region of late stage 17B fruits. Scale bars, 20 μm . (D-F, J-L) Cross-sections stained with phloroglucinol (pink) showing lignification of valve margins, inner replum, and endocarp b layer of valves. Scale bars, 50 μm . (A, D) Col, showing a wild type replum. (B, E) *pny*, showing a narrow replum. (C, F) *ath1 pny*, showing rescue of replum formation. (G, J) *tga1 pny*, showing rescue of replum formation. (H, K) *tga4 pny* showing rescue of replum formation. (I, L) *tga1 tga4 pny*, showing rescue of replum formation. Arrows denote the valve margins.

Figure 3.5 Quantification of *pny* replum phenotypic rescue by *ath1*, *tga1*, *tga4*, and *tga1 tga4* mutations

Image J software was used to measure the replum width in SEM images. Data represent means $\pm$ standard deviation (n=11 fruits per genotype). Asterisk, significantly different from *pny* (student's t-test, $p \leq 0.05$).

Figure 3.6 Quantification of *pny* pod shatter phenotypic rescue by *ath1*, *tga1*, *tga4*, and *tga1 tga4* mutations

A random impact test (RIT) was used to measure resistance to pod shatter. RIT50 denotes the time point at which 50% of pods were shattered. Data represent the mean $\pm$ standard deviation, $n \geq 3$ biological replicates per genotype. Each replicate was a pool of $n=20$ siliques from at least six different plants. Asterisks, significantly different from *pny* (student's t-test, $p \leq 0.05$).

Figure 3.7 Expression of boundary genes in *pny* mutants

Representative images of flowers showing GUS staining in the carpel. (A) *BOP1:GUS*, expression in valve margins. (B) *BOP1:GUS pny*, expression in converged valve margins; arrow denotes area of interest between valve margins. (C) *BOP2:GUS*, expression in valve margins. (D) *BOP2:GUS pny*, expression in converged valve margins; arrow denotes area of interest between valve margins. (E) *ATH1:GUS*, broad expression in carpels. (F) *ATH1:GUS*, expression in converged valve margins. Scale bars, 0.25 mm.

Figure 3.8 *BOP1/2* expression in wild type and *pny* replum cross-sections

Representative images using stage 12 flowers. (A) *BOP1:GUS*, expression in inner subdomain of valve margins. (B) *BOP1:GUS pny*, expression in the converged valve margins and inner replum. (C) *BOP2:GUS*, expression in inner subdomain of valve margins. (D) *BOP2:GUS pny*, expression in the converged valve margins and inner replum. Scale bars, 0.25 mm.

Figure 3.9 Boundary mutants show a wild type replum and lignification pattern

Images are representative. (A-C, G-I, and M-O) SEM micrographs showing the exterior of the replum region of late stage 17B fruits. Scale bars, 20 μm . (D-F, J-L, P-R) Cross-sections stained with phloroglucinol (pink) to show lignification of valve margins, inner replum, and endocarp *b* layer in the valves. Scale bars, 50 μm . (A, D) Col. (B, E) *bop1 bop2*. (C, F) *ath1*. (G, J) *knat2*. (H, K) *knat6*. (I, L) *knat2 knat6*. (M, P) *ath1 knat2*. (N, Q) *ath1 knat6*. (O, R) *ath1 knat2 knat6*. Arrows denote the valve margins.

Figure 3.10 Quantification of replum width in boundary mutants

Image J software was used to measure the replum width in SEM images. Data represent means $\pm$ standard deviation (n=11 fruits per genotype). Asterisk, significantly different from Col control plants (student's t-test, $p \leq 0.05$).

Figure 3.11 Quantification of resistance to pod shatter in boundary mutants

A random impact test (RIT) was used to measure resistance to pod shatter. RIT50 denotes time point at which 50% of pods were shattered. The *shp1 shp2* double mutant served as an indehiscent control. Data represent means $\pm$ standard deviation, $n \geq 3$ biological replicates per genotype. Each replicate was a pool of $n=20$ siliques from at least six different plants. Asterisks, significantly different from Col (student's t-test, $p \leq 0.05$).

Figure 3.12 Replum morphology of wild-type and transgenic plants overexpressing boundary genes

Representative images are shown. (A-C, G-I) SEM micrographs showing the exterior of the replum region of late stage 17B fruits. Scale bars, 20 μm . (D-F, J-L) Cross-sections stained with phloroglucinol (pink) showing lignification of valve margins, inner replum, and endocarp b layer in the valves. Scale bars, 50 μm . (A, D) Col. (B, E) *bop1-6D/+*, showing a normal to wide replum. (C, F) *35S:BOP2*, showing a wide replum. (G, J) *35S:ATH1*, showing a wide replum. (H, K) *35S:KNAT2-GR*, showing a wide replum, altered cell morphology, and an indistinct valve margin (I, L) *35S:KNAT6*, showing a wide replum and altered cell morphology. Arrows denote the valve margins.

Figure 3.13 Quantification of replum width in wild type and transgenic plants overexpressing boundary genes

SEM images was measured using Image J software. Data represents means $\pm$ standard deviation, $n=11$ fruits per genotype except *35S:KNAT6*, $n=3$. Siliques were pooled from at least three different plants and imaged when they were still green but close to their yellowing stage. Asterisk, significantly different from Col control plants (student's t-test, $p \leq 0.05$).

Figure 3.14 Quantification of resistance to pod shatter in wild type and transgenic plants overexpressing boundary genes

Pod shatter was measured in stage 19 fruits using a random impact test (RIT). RIT50 denotes time point at which 50% of pods were shattered. The *shp1 shp2* double mutant served as an indehiscent control. Data represent the mean $\pm$ standard deviation of 2-4 biological replicates per genotype. Each replicate was a pool of $n=20$ siliques from six different plants. Asterisks, significantly different than Col wild type control (student's t-test, $p \leq 0.05$). *35S:KNAT2-GR* was indehiscent like the *shp1 shp2* control.

Figure 3.15 Expression pattern of valve-margin marker genes in wild-type and transgenic plants overexpressing boundary genes

Representative images of flowers at or near anthesis (stage 13). (A-E) *SHP1:GUS*, arrows denote ectopic expression in valves for (E) *35S:KNAT6* line compared to (A) wild type. (F-J) *IND:GUS*, arrows denote ectopic expression in valves for (H) *35S:BOP2*, (I) *35S:KNAT2-GR*, and (J) *35S:KNAT6* lines relative to (F) wild type. (K-O) *ALC:GUS*, arrows denote expanded expression in valves for (N) *35S:KNAT2-GR* and (O) *35S:KNAT6* lines relative to (K) wild type. Scale bars, 0.5 mm.

Figure 3.16 Expression pattern of valve and replum marker genes in wild-type and transgenic plants overexpressing boundary genes

Representative images of flowers at anthesis (stage 13). (A-E) *FUL:GUS*, no reproducible changes. (F-J) *BP:GUS*, no reproducible changes, although flower in panel I appears to lack the *35S:KNAT2-GR* phenotype. (K-O) *PNY:GUS*, no reproducible changes. Scale bars, 0.5 mm.

Chapter 4: DISCUSSION

The *Arabidopsis* fruit is characterized by three tissue types: valves, valve margins, and replum. The valve margins are specialized zones for dehiscence that allow the fruit to open at maturity. These margins constitute a boundary (Roeder and Yanofsky, 2006; Hepworth and Pautot, 2015). *BOP1/2* co-activators and downstream TALE homeodomain transcription factors *ATH1/KNAT6* regulate the maintenance and patterning of boundaries in shoot apices (Hepworth and Pautot, 2015).

Boundary genes including *KNAT2*, *KNAT6* and *BOP1/2* are expressed in valve margins of the fruit and are predicted to play a role in patterning despite mutants having no obvious defects (Ragni et al., 2008; Khan et al., 2012). Close examination of genes in this pathway reveals an underlying role for boundary genes in the fruit. As part of this study, I show a previously undiscovered role for *ATH1* and Clade I TGA transcription factors as part of this module in fruits. Clade I TGAs are co-factors required by *BOP1/2* for activation of *ATH1*, which is a functional partner for *KNAT2* and *KNAT6*. The major findings are: 1) boundary genes promote valve-margin identity in fruits thereby regulating fruit patterning and dehiscence with *KNAT6* playing a central role; and 2) partially conserved mechanisms govern the expression of boundary and valve-margin identity genes in the fruit (Figure 4.1).

4.1 Integration of Clade I TGAs and *ATH1* into the fruit boundary module

BOP1/2 interact with Clade I TGA factors to activate the expression of boundary genes including *ATH1* (Khan et al., 2012b; Wang et al., 2018). The significance of this interaction was tested in fruits. My data using GUS reporter lines show that *ATH1*, *TGAI*, and *TGA4* are expressed in maturing flowers and fruits, albeit with lower specificity for the valve margin compared to *BOP1/2* and *KNAT2/6*. Low levels of *ATH1:GUS* were detected throughout the valves, especially

the endocarp *b* layer, which undergoes lignification (Roeder and Yanofsky, 2006). *TGAI* was also detected in this layer. Low levels of *TGAI* and *TGA4* were similarly detected throughout the fruit, especially around vascular bundles in the replum interior. However, the expression patterns for *TGAI:GUS* and *TGA4:GUS* should be checked by *in situ* hybridization since these reporters fail to express in meristematic tissues and may be missing some control sequences (Wang et al., 2018).

Unpublished data identify *ATH1* as a potential direct regulator of *KNAT6* (Khan et al., 2015; personal communication by Marcel Proveniers). Accordingly, there is a marked reduction of *KNAT2:GUS* and *KNAT6:GUS* expression in *ath1-1* mutant inflorescences (Bao, 2009). My data confirm this interaction, showing little or no expression of *KNAT6:GUS* in *ath1-3* flowers. Expression of *KNAT2:GUS* was also diminished. Unpublished work (Dubreucq and Pautot) has identified *BLH5* as a second BELL-like homeobox gene expressed in the valve margin. This gene is predicted to have overlapping functions with *ATH1* and might potentially contribute to the activation of *KNAT2* expression in the valve margin.

Genetic interactions with *PNY* confirm a functional role for *TGAI/4* and *ATH1* similar to *KNAT6* and *BOP1/2* genes in the fruit. Formation of the replum was restored in *ath1 pny*, *tgal pny*, *tga4 pny*, and *tgal tga4 pny* fruits. Formation of the replum is likewise restored in *shp1 shp2 pny* and *ind pny* double mutants (Roeder et al., 2003; Ripoll et al., 2011) and in *knat6 pny* and *bop1 bop2 pny* mutants (Ragni et al., 2008; Khan et al., 2012b). Besides having a narrow replum, *pny* fruits are short and partially indehiscent. Quantitative analyses of *ath1 pny*, *tgal pny*, *tga4 pny*, and *tgal tga4 pny* fruits showed that *pny* defects in pod size and shatter are also rescued. How these traits are linked is unknown. It remains unclear whether pod shatter is restored by widening of the replum or restoring activity in the DZ or both.

4.2 Boundary genes promote valve margin identity

A comparative analysis of boundary and valve-margin identity gene expression showed strong co-expression in maturing flowers and fruits. *BOP1/2* similar to *KNAT2* and *KNAT6* began expression in the valve margin at stage 9 or 10. Around this same time or slightly earlier *SHP/IND/ALC* expression are also localized to the valve margin (Roeder and Yanofsky, 2006). This pattern is consistent with regulatory interactions uncovered using boundary gain-of-function lines and valve margin reporters. Overexpression of *BOP2* caused ectopic *IND* expression in valves. Overexpression of *KNAT2* led to ectopic *IND* and *ALC* expression in the valves. Overexpression of *KNAT6* had the greatest impact with ectopic expression of *SHP1*, *IND*, and *ALC* in the valves. These data suggest distinct roles for these factors and underline a stronger impact for *KNAT6*. Promoter analysis coupled with chromatin immunoprecipitation assays are approaches that can be used to test if valve margin identity genes are direct transcriptional targets of *KNAT2* or *KNAT6* in fruit.

Siliques from transgenic lines overexpressing boundary genes were typically short with a wider replum compared to wild type. The majority of *35S:BOP2*, *35S:KNAT2-GR*, *35S:KNAT6* and *35S:ATH1* siliques showed significant increases in replum width with *35S:KNAT2-GR* showing the greatest increase. The increased repla width did not correlate with misexpression of *BP:GUS* or *PNY:GUS* outside the replum (data not shown). *35S:KNAT2-GR* fruits had additional strong defects including indistinct valve margins and severe inhibition of cell elongation. These phenotypes have been described previously (Pautot et al., 2001). RIT testing indicated that transgenic lines overexpressing *BOP1/2*, *KNAT2*, or *KNAT6* are less prone to shattering than wild type. This may be due in part to shorter fruits. In *35S:KNAT2-GR*, valves failed to elongate resulting in part from ovules being unfertilized in this strong transgenic line. Homeotic conversion

of ovule to carpel and inhibition of stamen elongation occurs in this line. Stamens are unable to reach the pistil causing defects in fertilization (Pautot et al., 2001).

The phenotype of boundary gain-of-function lines partially resembles *ful* mutants in which repla are enlarged and valves fail to expand after fertilization (Gu et al., 1998). The *yab3 fil* double mutant with lower levels of *FUL* shows a similar phenotype (Dinneney et al., 2005). Despite this phenotype similarity, *35S:KNAT2-GR* and *35S:KNAT6* fruits showed no obvious change in *FUL:GUS* expression suggesting that *FUL* is not a prime target. Valve margin identity genes are ectopically expressed in *ful* valves causing lignified valve-margin cell identity. Inactivation of *IND* or *SHPI/2/ALC* can partially rescue this phenotype (Ferrándiz et al., 2000; Roeder and Yanofsky, 2006). It will be interesting to test whether inactivation of *BOPI/2* alone or in combination with *KNAT2* or *KNAT6* also rescues *ful* defects in lignification or valve identity.

4.3 Divergent mechanisms for repression of boundary genes in the valves

The expression of valve-margin identity genes is repressed to valve margins by PNY in the replum and *FUL* in the valves (Roeder and Yanofsky, 2006). Only part of this mechanism applies to boundary genes. PNY repression of boundary genes is conserved. Whole mounts and sectioning show converged domains of expression for *BOPI*, *BOP2*, and *ATH1* in the replum core of *pnv* fruits. Previous studies show that PNY represses *KNAT2*, *KNAT6*, and *BOP1/2* in inflorescences and whole mounts showed convergence of expression domains for *KNAT2* and *KNAT6* in *pnv* fruits (Ragni et al. 2008, Khan et al. 2012a). Consistent with this, PNY is a direct repressor of *BOPI/2* and is important for preventing expression in apical meristems (Andrés et al., 2015; Bencivenga et al., 2016). Genome-wide analysis of PNY binding sites also shows a direct interaction with *ATH1* and *KNAT6* genomic DNA (Bencivenga et al., 2016).

FUL repression of *SHP1/2*, *IND*, and *ALC* is required for proper differentiation of valves (Ferrándiz et al., 2000; Liljegren et al., 2004; Roeder and Yanofsky, 2006). Boundary genes appear to be under different negative regulation in valves: only *KNAT6* is misexpressed in *ful* fruits, limited to valves at post-fertilization stages of development. The repression of boundary genes in valves may be under the major control of networks functioning in leaves. For example, *BP* repression in valves relies on the same regulation as in leaves (Alonso-Cantabrana et al., 2007). In leaves, *KNAT2* and other *KNOX* class I members are silenced in leaves by repressive complexes and histone regulatory proteins (Sluis and Hake, 2015; Tsuda and Hake, 2016). Mechanisms for repression of *BOP1/2* in leaves are not yet identified.

4.4 Summary model

A summary model integrates the role of boundary genes in the fruit (Figure 4.1). *BOP1/2* and Clade I TGAs are established to function upstream of *ATH1/KNAT6/KNAT2* in boundaries (Khan et al., 2015; Wang et al., 2018). This hierarchy applies in fruits where boundary genes are demonstrated to promote valve margin identity. Use of transgenic overexpression lines pinpoints distinct roles for boundary genes and a central role for *KNAT6* as evidenced by promotion of *SHP/IND/ALC* valve-margin identity genes in valve tissue. *PNY* in the replum is a negative regulator of boundary and valve-margin identity genes. *FUL* in the valves is a negative regulator of valve-margin identity genes and *KNAT6*. Other repressors for boundary genes in the valves are currently unknown.

4.5 Boundary genes as novel regulators of pod shatter resistance

Quantitative analysis of fruit morphology in boundary loss-of-function and gain-of-function mutants provides evidence that boundary genes regulate fruit length, replum width, and

pod shatter. In particular, quantitative analyses demonstrate that loss-of-function *bop1 bop2* or *knat2 knat6*, *ath1 knat2*, and *ath1 knat2 knat6* mutations lead to narrowing of the replum and improved resistance to pod shatter. The mechanism may involve weakening of *IND* expression in the valve margin. *IND* plays a crucial role in pod shatter, being required for differentiation of both separation and lignified layers of the DZ. Downstream targets of *IND* include lignin regulators such as *NST1* and cell separation regulators such as *ADPG1/2* at the valve margin (Mitsuda and Ohme-Takagi, 2008; Ogawa et al., 2009; Ballester and Ferrándiz, 2017). Given that boundary loss-of-function mutants show no obvious defects in lignification, shatter resistance may result from changes in the expression of hydrolytic or cell wall remodeling enzymes in the separation layer. Both *alc* mutants and the weak *ind-1* allele produce indehiscent siliques but maintain a wild type lignification pattern (Rajani et al., 2001; Liljegren et al., 2004).

4.6 Conclusion and future directions

In summary, boundary genes play an underlying role in patterning the fruit. These genes promote valve margin identity and modulate pod shatter. Results depict negative regulation of boundary genes by valve and replum factors. Whether these interactions are direct will be determined in future studies. Direct binding to promoter regions can be determined through chromatin immunoprecipitation. Transcription factor binding sites on promoters can be identified. Other next steps should look at the possible regulation of *KNAT2* by valve factors other than *FUL* since no misexpression was present for *KNAT2* in the *ful* mutant. Good candidates are the leaf factors *AS1/2*, *JAG*, and *FIL*. Regulation of *IND* by boundary genes is a promising aspect of this project worth pursuing. Novel positive regulators of *IND* are of potential interest to oilseed breeders. Researchers at Bayer CropScience have recently engineered a new variety of canola with a more robust pod that is resistant to shatter (Bayer, 2014). This new genetically engineered variety

is based on weak alleles of *ind* that are partially indehiscent (Bayer, 2014). This is more desirable than traditional loss-of-function mutations in *ind* that are completely indehiscent and strongly resist opening using conventional harvesting techniques (Bayer, 2014). Brassica crops contain unexplored orthologs of boundary genes, rendering these excellent candidates for the production of shatter resistant cultivars. Future steps will be to identify these boundary gene orthologs and determine whether they have similar expression patterns and functions to those described here for *Arabidopsis*. There is also strong overlap in the molecular networks at play in dehiscence and abscission. Both processes rely on cell separation and lignification (Ballester and Ferrándiz, 2017). Abscission studies have identified a clear role for boundary genes including *BOP1/2*, *ATH1*, *KNAT2/6* in abscission zone initiation and as downstream effectors of organ separation (Gómez-Mena and Sablowski, 2008; McKim et al., 2008; Shi et al., 2011; Corrigan, 2018). Thus, it is unsurprising that these factors also play a role in dehiscence, through as yet unknown mechanisms. Alteration in cell separation at the dehiscence zone due to enzymatic activity is a possibility. More detailed analyses of cell morphology during dehiscence zone differentiation in boundary gene mutants can be performed by Transmission Electron Microscopy (TEM) to probe for changes specifically in the separation layer. Polygalacturonases expressed in the valve margin are active in the seed abscission zone along with orthologs of *PNY*, *FUL*, *SHP1/2*, and *IND* (Dong and Wang, 2015). Future work will test if boundary genes are desirable for modulating pod or seed shatter-resistance in field crops such as *Brassica napus* (canola) and *Camelina sativa* (camelina).

Figure 4.1 Integrative model for boundary genes in fruit development

Boundary genes are proposed to function upstream and/or in parallel with *SHP1/2* contributing to valve margin identity and DZ formation. BOP1/2 interact with Clade I TGAs to promote *ATH1* expression in boundary zones (data not shown). The data show that *ATH1* is important for expression of *KNAT6* and to a lesser extent *KNAT2* in the valve margin, whose collective activities upregulate the valve margin identity genes *SHP1/2*, *IND*, and *ALC*. Boundary and valve-margin identity genes are spatially localized to the valve margin by PNY, which is important for replum formation. STM provides meristematic activity for septum and ovule formation in the inner central domain of the replum.

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SUPPLEMENTAL MATERIALS

Supplemental Figure 1. Expression pattern of boundary gene markers in carpel mutant lines

Representative images of flowers and fruits. Expression of *BOP1:GUS*, *ATH1:GUS*, *KNAT2:GUS*, and *KNAT6:GUS* in *ful* (B, F, J, N), *shp1 shp2*, (C, G, K, O) and *ind* (D, H, L) resemble that in Col wild type (A, E, I, M) with the exception of *KNAT6:GUS* in *ful* (panel N). Expression of *ATH1:GUS* in *shp1 shp2* (panel G) is very weak. Scale bars, 0.5 mm.

Supplemental Figure 2. Extra partial valve in 35S:KNAT2-GR and 35S:KNAT6 fruits

FUL:GUS reporter gene expression detects the formation of potentially an extra partial valve domain in the enlarged repla of rare 35S:KNAT2-GR and 35S:KNAT6 fruits. Scale bar, 0.5 mm.

Supplemental Figure 3. Expression of carpel marker identity genes in wild type and *bop1 bop2* mutant plants

Representative images of flowers at or near anthesis. Expression of *FUL:GUS*, *SHP1:GUS*, *SHP2:GUS*, *IND:GUS*, *ALC:GUS*, *BP:GUS*, and *PNY:GUS* in wild type (A-G) remains unchanged in the *bop1 bop2* mutant background (H-N). Scale bars, 0.5 mm.