Investigating the multiple roles of boundary genes in abscission in

Arabidopsis thaliana

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B.Sc. University of Toronto, 2018

**A thesis submitted to the Faculty of Graduate and Postdoctoral Affairs in
partial fulfillment of the requirements for the degree of**

Master of Science

in

Biology

Carleton University

Ottawa, Ontario, Canada

Abstract

Plants shed organs through abscission. This process scatters seeds and removes foliage but can reduce crop yield if unmanaged. In the model plant species, *Arabidopsis thaliana*, the outer parts of the flower are shed after pollination. *BLADE-ON-PETIOLE* boundary genes initiate abscission zones (AZ) where separation occurs. This work focused on the abscission role of boundary Three-Amino-Acid-Loop-Extension (TALE) homeodomain transcription factor genes *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA 2 (KNAT2)*, *KNOTTED1-LIKE FROM ARABIDOPSIS THALIANA 6 (KNAT6)*, and functional partner *ARABIDOPSIS THALIANA HOMEODOMAIN GENE 1 (ATH1)*.

I show that TALE boundary genes are required for the correct formation of two distinct layers that develop after specification of the AZ. Collective loss of TALE activity disrupts the proper formation of both layers.

I also show that TALE boundary genes are active in the signalling pathway that activates abscission, and promote the production of hydrolytic enzymes. I propose that TALE boundary genes contribute to multiple steps of the abscission process. 150/150

Acknowledgements

I would like to thank my supervisor, Shelley Hepworth for her teaching and support throughout this project. I am very grateful to have benefitted from her wisdom and warmth. Thank you for giving me the space to get things wrong, and the guidance to get them right. I would also like to thank my committee advisors, Dr. Allyson McLean and Dr. Martha Mullally for their advice and perspectives on this project.

The best thing about the Hepworth lab is its people, and I'd like to thank Chris Bergin and Laura Corrigan for welcoming me aboard and showing me the ropes. I am incredibly grateful to Ying Wang for all of his help, guidance, and never once saying no to a "quick question". I also need to thank Kevin Xiong for being a true friend as well as an amazing lab-mate. I really could not have done it without your help and humor. I'm also grateful to Dr. Jhadeswar Murmu for his guidance and help. Thanks as well to all the Rowland members, Becky Kalinger, Kyle Tapp, and Tim Garant for filling our shared lab with so much fun.

I would also like to say thank you to my mum and brother, thank you for always listening, supporting, and talking me out of my tree.

Preface

My thesis explores the role of the TALE boundary genes in controlling the abscission process. I completed most of the work in my thesis, under the supervision of Dr. Shelley Hepworth, at Carleton University. SEM images of the TALE mutant AZs were taken from the thesis of Laura Corrigan. Images of the *ATH1::GUS* reporter line were provided by Véronique Pautot, taken at the Institute Jean-Pierre Bourgin, France. Technical training and support for the SEM was provided by Dr. Jianqun Wang. Dr. Jhadeswar Murmu assisted with initial qPCR experiments.

None of the work described my thesis 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*

Gain-of-function mutant: *BOP1-oe*

List of Abbreviations

Abbreviation	Full name	Role
IDA	INFLORESENCE-DEFICIENT-IN-ABSCISSION	Small signalling peptide, initiates abscission signalling pathway
HAE/HSL2	HAESA/HAESA-LIKE	Functionally redundant partners, part of receptor complex, binds IDA
BOP1	BLADE-ON-PETIOLE-1	Boundary gene, transcription co-factor, works with BOP2
BOP2	BLADE-ON-PETIOLE-2	Boundary gene, transcription co-factor, works with BOP1
TALE genes	Three-amino-acid-loop genes	A family of transcription factors grouped by their similar structure
BP	BREVIPEDICELLUS	TALE gene, KNOX member, suppresses activity of KNAT2 and KNAT6
KNAT2	KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 2	TALE gene, KNOX member, works with KNAT6 and ATH1
KNAT6	KNOTTED-LIKE FROM ARABIDOPSIS THALIANA 6	TALE gene, KNOX member, works with KNAT2 and ATH1
ATH1	ARABIDOPSIS-THALIANA-HOMEODOMAIN GENE	TALE gene, BEL1 member, works with KNAT2 and KNAT6
NEV	NEVERSHED	ADP-ribosylation factor GTPase-activating protein,

		required for vesicular transport during abscission
RBOH	RESPIRATORY BURST OXIDASE HOMOLOG	A protein class which produces reactive oxygen species necessary for lignin synthesis
BAK1/SERK3	BRI1-ASSOCIATED RECEPTOR KINASE	Receptor-like kinase, one part of the membrane receptor complex for IDA signalling
MAP kinase	MITOGEN-ACITVATED PROTEIN Kinase	Signalling cascade triggered by IDA binding
DAB	3,3'-diaminobenzidine	Stain used to detect hydrogen peroxide (type of ROS)
ROS	Reactive oxygen species	Produced by enzymes as apoplastic signal or for polymerization of lignin
AZ	Abscission Zone	Specialized area for cell separation

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1. Introduction

1.1. Thesis overview

Plants have the ability to shed organs through a process called abscission. This ability allows a plant to adapt its structure to new stresses or phases of the life cycle. This feature is relevant to agriculture, since the fruits, seeds, and leaves that can be shed are often harvested for food. In different crop species, either delaying or inducing abscission can be beneficial (Patterson et al. 2016). A specially formed abscission zone (AZ) allows the organ to separate without causing damage to the adjacent tissue. In many cases, AZs are initiated as an organ develops, in a boundary region of low growth that separates an organ from the meristem. The abscission process can be broken into four steps: 1) The initial formation of the AZ, 2) further structural development of the AZ into two distinct functional layers, 3) initiation of separation by a signalling pathway, and 4) the development of a sealing layer after the organ is lost (Bleecker and Patterson 1997; Roberts, Elliott, and Gonzalez-Carranza 2002). The genetic control of abscission has been widely studied in the model plant species, *Arabidopsis thaliana*, where floral organs are released a few days after the pollination of flowers. Two BLADE-ON-PETIOLE (BOP) transcription co-factors that pattern organ boundaries in the plant are required in forming AZs. Without BOP function, floral AZs do not form and abscission is blocked (McKim et al. 2008). Members of another family of transcription factors, the three-amino-acid-loop-extension (TALE) homeodomain proteins, form heterodimer pairs and are also involved in boundary regulation (Hamant and Pautot 2010). Mutations in these genes can result in defective abscission (Hepworth et al. 2005; Corrigan 2018; McKim et al. 2008; Gómez-Mena and Sablowski 2008). In the AZ, BOPs require the downstream activity of TALE partners ARABIDOPSIS THALIANA HOMEODOMAIN GENE 1 (ATH1) and KNOTTED LIKE FROM ARABIDOPSIS THALIANA 2 and 6 (KNAT2/KNAT6), indicating a role for a boundary module of genes in regulating abscission (Belles-Boix et al. 2006; Rutjens et al. 2009; Khan, Tabb, and Hepworth 2012; Khan et al. 2012). My thesis clarifies the multiple roles that this conserved group of boundary genes play in abscission, adding to our understanding of this essential process in plants.

1.2. Abscission

Abscission allows plants to shed organs when they are damaged, ripe, or no longer useful. As one of the few instances in which a plant's living cells must separate, abscission is a highly regulated part of developmental life. The actual separation is carried out through a carefully coordinated release of hydrolytic enzymes that degrade the middle lamella that binds the walls of neighbouring AZ cells together (Bleecker and Patterson 1997). The precision of this process ensures that the plant body is not damaged. Both structure and signalling in the AZ coordinate abscission.

1.2.1. Abscission in the plant

By abscising old or damaged organs, and forming new ones, a plant's architecture changes throughout its life cycle. Organ shedding can be a response to wounding, infection, or stress, and happens during reproduction to disperse fruits or seeds. Abscission exists in simple land plants like mosses, a sign that it is an ancient and evolutionarily conserved process (Duckett and Ligrone 1992). In grasses and other monocot lineages, the ability to abscise organs may have been lost and then regained (Yu et al. 2020). Abscission can also be engaged selectively, as some species can shed leaves in response to drought, using abscission to respond to external stress (Pang et al. 1973; Patharkar and Walker 2016). Infected organs can also be abscised to limit the spread of a pathogen, adding another layer to the defense response (Patharkar, Gassmann, and Walker 2017). There are also developmental stages where a flexible anatomy is needed. After fertilization, many plants shed the floral organs, which are no longer necessary. Later, once a seed set is fully developed, these can either be shed, or another form of precise separation, called dehiscence, shatters the pod of dry fruits to disperse the seeds inside (Patharkar and Walker 2018). In all of these examples, abscission allows plant architecture to be flexible.

1.2.2. Relevance in agriculture

Abscission often poses a problem in agriculture since early abscission can reduce harvests. In cereals and many other crops, resistance to abscission can help avoid yield loss before harvest. Abscission is not always undesirable though, as it can be beneficial to induce abscission in fruit trees as way to thin buds, or in macadamia trees as a way to collect the hard-to-reach nuts (Bangerth 2000; Richardson and Dawson 1993). Currently, growers apply hormones to manipulate abscission. Early in the season, fruit tree farmers apply ethylene

precursors to induce a round of bud abscission, to ensure that the remaining fruit grow to size (Jonkers 1979). Later, to avoid premature fruit drop, auxin and ethylene blocking agents are applied to delay abscission until the fruits can be harvested (Cooper et al. 1968). These treatments can be harmful to the environment and costly for farmers. A genetic approach could improve on these practices. The underlying genetics which regulate abscission are not fully understood. Some naturally occurring mutations have been embraced for their altered abscission. In tomatoes, a mutation in the *JOINTLESS* gene prevents the formation of the AZ connecting the pedicel to the stem, making harvest easier (Mao et al. 2000). Mutations affecting this trait also likely motivated the natural selection of grain crops which do not easily shed their seeds in the early domestication of staple crops (Harlan, De Wet, and Stemler 2000). Overall, a genetic approach can improve the way we farm.

1.2.3. Diversity in abscission

Abscission is both an ancient and varied process in plants. Though evidence suggests that mosses, some of the earliest land plants, use abscission to liberate spores, the physiology, process, and governing genes of abscission are starkly different across different species (Duckett and Ligrone 1992). Although the AZ generally forms in well-defined organ boundaries, there are also examples of secondary AZs which do not follow boundary lines, such as the pedicel AZs in tomato and tobacco, which allow flowers on individual stems to separate off the main branch (Roberts, Schindler, and Tucker 1984; Sexton and Roberts 1982; Tabuchi, Ito, and Arai 2001). AZ placement is also variable in monocots, and can fall on the rachis, pedicel, or below each separate floret (Yu et al. 2020). The morphology of the AZ itself is different across plant species. While layers of small cells are a hallmark of the AZ in many species, including *Arabidopsis*, there are also grasses in which the AZ cells are no smaller than neighbouring cells (Bleecker and Patterson 1997; Yu et al. 2020). The placement or even the presence of a lignified AZ layer, which is often seen in eudicots, is another point of variation across AZ morphology, the AZs of some grasses do not contain cells with lignified walls (Lee et al. 2018; Yu et al. 2020).

The genes involved in the hormonal control of abscission are not ubiquitous either. The *ETR1/2/3* genes which mediate ethylene to delay of abscission in *Arabidopsis* are not involved in abscission in tomatoes (Klee 2002; Lanahan et al. 1994). Diversity in the gene families which govern abscission can be found even within the same species. In *Arabidopsis*, the abscission of

leaves and the floral organs are regulated by a module of boundary genes, while a different family, the MADS-box transcription factors control the dehiscence and seed AZs within the developed fruit (Patharkar and Walker 2018; Liljegren et al. 2000). For many fruit trees and tomatoes, which undergo abscission of fruit at the pedicel, the MADS-box transcription factors appear to be the main regulators of abscission (Ito and Nakano 2015; Nakano et al. 2015; Heo and Chung 2019). The mechanisms of separation also vary among species. While cell expansion on the proximal side of the AZ and the release of cell wall remodelling enzymes is common to many species, the involvement of programmed cell death in abscission appears variable (Shi et al. 2011; Estornell et al. 2013; Merelo et al. 2017). *Arabidopsis*, tomato, and citrus show transcriptional activity which suggests that the leaving organ may undergo senescence as it is shed (Bar-Dror et al. 2011; Lee et al. 2018; Zhang et al. 2014). In contrast, no cell death is observed in the detached cells during seed abscission in oat (Yeung, Chinnappa, and Chen 1987; Yu et al. 2020). Despite the very different profiles of abscission across the plant world, many diverse crops share points of overlap in either morphology, process, or genetics. Diverse crop species with unique modes of abscission can still be approached by first searching for overlap with what has been learned about abscission in model species (Patterson et al. 2016). In this way, study in *Arabidopsis* provides a framework in which to expand our models of the abscission process.

1.3 *Arabidopsis* as a model organism

Arabidopsis thaliana is widely used as the model organism of the plant world. As a small, annual member of the Brassica family, its size and short generation time make it a manageable plant to grow in lab settings. It produces many flowers that self-pollinate to create a generous seed set, another advantage for research (Woodward and Bartel 2018). Scientific interest in *Arabidopsis* began with Friedrich Laibach's Ph.D. thesis, which centered on the species, in 1907. He noted that despite having only five chromosomes, the plant showed high phenotypic variety. From there, focused study found that X-ray treatment, and later, *Agrobacterium* transformation could be used to easily induce mutations in this species. As plant biology shifted towards a molecular and genetic approach, the *Arabidopsis* genome was sequenced, and collaborative projects created cDNA libraries and transcriptomes (Somerville

and Koornneef 2002; Koornneef and Meinke 2010). My project makes use of these databases, as well as the extensive library of single mutants created by the Salk SIGnAL project (Alonso et al. 2003).

Arabidopsis sheds its floral organs a few days after pollination and fertilization have occurred. The stamen, petals, and sepals detach from the base of the pistil, the central part of the flower formed from two fused carpels, which elongates into a seed pod (Figure 1.1.). This regularly programmed abscission provides a model of the process that shares overlap in hormonal and genetic control with many other vascular plants. The use of *Arabidopsis* to study abscission was first presented by Bleeker and Patterson (Bleeker and Patterson 1997). Subsequent investigation has proved that many of the components of abscission discovered in *Arabidopsis* apply to a range of crop species. Current research is looking to translate what has been learned about abscission into crops such as sesame, cotton, cranberries, grapes, and traditional cereals like fonio, where abscission still poses a problem (Patterson et al. 2016).

1.4 Steps of abscission

The abscission process is generally considered as four phases (Figure 1.3). For abscission to occur, an AZ, made up of dense cells at the organ boundary, must first be formed. This zone further develops into two distinct layers (Figure 1.2), and gains competence to respond to abscission signals. Once this signaling is activated, enzymes are induced to break down the cell walls and middle lamella between cells, allowing the organ to separate from the plant body. Finally, newly exposed cells on the plant body form a new epidermis that secretes a protective coating (Kim 2014; Kim, Chun, and Tucker 2019; Patterson 2001).

1.4.1. The initiation of AZs

Abscission usually occurs at the base of the organ, where it meets the plant body. In the *Arabidopsis* flower, each separate floral organ attaches to its own AZ around the base of the fruit, which is called a silique (Patterson 2001). The AZ is derived from a subdomain of the meristem called the boundary. The boundary is a region of low growth that separates emerging organs from the meristem. This region maintains a balance between the growth of the new organ and the continued proliferation of the stem cell core (Hepworth and Pautot 2015). Only certain

organ boundaries in a plant will form an active AZ. Because this junction gives rise to the AZ (Figure 1.1), boundary genes are an implicit part of this first step of abscission.

1.4.2. Secondary development of AZ

Although AZs are specified during organ formation, competence to respond to abscission signalling is influenced by hormones (Patharkar, Gassmann, and Walker 2017). Ethylene has been shown to accelerate abscission. Lowered auxin levels before abscission are thought to increase sensitivity to ethylene in the cells of the AZ (Taylor and Whitelaw 2001). The AZ also undergoes further structural development. After the initial AZ formation, two specialized cell layers differentiate: a separation layer on the plant body (Figure 1.2. A), and a lignified layer at the base of the leaving organs (Figure 1.2. B) (Lee et al. 2018). The lignin forms a highly patterned, honeycomb structure of cells at the organ border. This structure is suggested to act as a mechanical brace that limits diffusion of hydrolytic enzymes released by the separation layer. The organ detaches cleanly at the edge of the lignified layer and the plant body (Lee et al. 2018). Lignification is preceded by an accumulation of reactive oxygen species (ROS), produced by a number of NADPH oxidases and peroxidases. In the AZ, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD) and RBOHF are involved in the lignification. In the double *rbohD rbohF* mutant, ROS is reduced in the AZ, and the lignin brace does not form, suggesting these NADPH oxidases play a key role in the secondary development of the AZ (Olsson et al. 2019). Despite this brace defect, abscission occurs normally in this mutant. The exact role and fate of the cells of the separation layer is controversial. In dehiscence, a related process that allows mature seed pods to shatter, the separation layer is directly adjacent to the lignified layer, and produces the hydrolytic enzymes to allow these layers to separate (Ferrándiz, Pelaz, and Yanofsky 1999). Classic studies of limited resolution suggest the same to be true of AZs (Addicott, 1982). Newer evidence suggests that cells of the separation layer become loose and slough away from the plant body (Lee et al., 2018).

1.4.3. Signalling and separation

The signal for abscission is a small secreted peptide ligand called INFLORESCENCE DEFICIENT IN ABSCISSION (IDA). IDA forms a complex with two receptors at the cell membrane called HAESA (HAE) and HAESA-LIKE 2 (HSL2) (Santiago et al. 2016). Loss of function mutations in IDA or HAE/HSL2 block abscission (Butenko et al. 2003; Jinn, Stone, and

Walker 2000; Cho et al. 2008). Meanwhile, over-expression of IDA causes early and ectopic abscission (Stenvik et al. 2006). HAE/HSL2 act as one partner in a receptor complex with SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE 1/2/3/4 (SERK1/2/3/4) (Santiago et al. 2016). Activation of this complex induces a mitogen-activated protein kinase (MAPK) cascade. MKK4/5 and MKK3/6 function in a signalling cascade downstream of IDA binding. Constitutively active forms of MKK4/5 can rescue the abscission defect in *hae hsl2* mutants (Cho et al. 2008). A screen for other mutations that can restore abscission in the *ida* mutant identified BREVIPEDILLUS (BP) as a TALE protein that functions downstream of the MAPK cascade. Phosphorylation of BP removes its repression of the boundary genes *KNOTTED LIKE FROM ARABIDOPSIS THALIANA 2* (*KNAT2*) and *KNAT6*. *KNAT2/6* expression is increased during abscission, suggesting they act as positive regulators of the abscission process (Shi et al. 2011). From here, the signal transduction is not fully understood, but eventually leads to the downstream production of enzymes that degrade connections between cells in the AZ (Figure 1.4) (Estornell et al. 2013).

1.4.4. Sealing of the AZ

Abscission exposes a layer of formerly internal cells on the receptacle surface. A waxy coating is secreted by these cells to complete the abscission process. This layer is thought to include cuticle and, in some species, suberin, which would suggest that the newly exposed cells differentiate to resemble the cells that make up the rest of the plant's epidermis (Lee et al. 2018; Pollard et al. 2008; Clements and Atkins 2001). Though the creation of a sealing layer is treated as a fourth and separate step, there is evidence that the waxy compounds that make up this layer begin to be synthesized during abscission signalling. The *IDA* over-expressor forms an enlarged scar layer. Analysis of this layer indicates that it includes higher levels of arabinogalactan proteins (AGPs), which are part of the cuticle layer in wild-type AZs (Shi et al. 2011; Kim et al. 2015; Stenvik et al. 2006). Other early abscising mutants also show an exaggerated scar, suggesting that this step begins as signalling initiates abscission (Lewis et al. 2010; Shi et al. 2011).

1.5. Boundary genes

1.5.1. *BLADE-ON-PETIOLE* genes

BLADE-ON-PETIOLE genes (*BOP1* and *BOP2*) were the first boundary genes discovered to have a role in the abscission process. These genes maintain regions of low growth in creating borders that separate meristem cells from the differentiating cells of a new organ. In *bop1 bop2* mutants, no AZ is specified, and all downstream processes are blocked (McKim et al. 2008). *BOP1/2* encode transcription co-factors characterized by a BTB domain and ankyrin repeats, which allow *BOP1/2* to interact with other proteins. As transcription co-factors, *BOP1/2* do not directly bind to DNA, but interact with TGA bZIP transcription factors to be recruited to DNA. *BOP1/2* are members of a larger family of conserved NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) proteins. Members of this larger family are involved in the regulation of salicylic acid-mediated defense responses (Khan, Xu, and Hepworth 2014). The *BOP* genes are grouped into their own subclade and appear to fulfill a distinct role in development. Loss of *BOP* function results in patterning changes at the base of organs that change the shape and complexity of leaves and flowers (Hepworth et al. 2005). *BOP1/2* expression also locates to the base of forming flowers. Expression in this region continues after the flower has matured, suggesting that *BOP1/2* play additional roles in the later steps of abscission (Hepworth et al. 2005; McKim et al. 2008; Xu et al. 2010).

1.5.2. *TALE* genes

To function in boundaries, *BOP1/2* require the downstream activity of a family of transcription factors containing a three-amino-acid-loop-extension homeodomain (TALE) (Khan et al. 2012; Khan, Tabb, and Hepworth 2012). Members of the TALE family are widely involved in the regulation of organ development and meristem activity (Hamant and Pautot 2010). The TALE family is split into KNOTTED1-like (KNOX) and BELL1-like (BELL) subclasses that function as heterodimer pairs (Hamant and Pautot 2010; Bhatt et al. 2004). In their role in regulating inflorescence architecture, *BOP1/2* requires the downstream activity of the KNOX member *KNAT6* and its functional BELL partner *ATH1* (Khan et al. 2012; Khan, Tabb, and Hepworth 2012). *KNAT6* activity is partially redundant with *KNAT2*, another KNOX member (Belles-Boix et al. 2006). Loss of *KNAT2/6* function does not result in obvious patterning

defects, but the double mutant *knat2 knat6* shows a slight delay in abscission (Shi et al. 2011). Similarly, abscission is delayed in *ath1* mutants. Investigations showed that this delay differentially affects stamen AZs which take longer to develop than normal (Gómez-Mena and Sablowski 2008). The basis of delayed abscission in these mutants is only partly understood.

1.6. Boundary genes in abscission

Traditional studies on abscission can overlook that sites of abscission are defined by organ boundaries. Our knowledge of how boundary genes function in abscission is fragmented. For this reason, my thesis investigates the role of boundary genes in abscission.

1.6.1. Structure

The proper formation of the AZ is essential to abscission. In the *bop1 bop2* mutant, which does not form an AZ, abscission is blocked completely (McKim et al. 2008). *BOP1/2* regulate inflorescence architecture through *KNAT6* and *ATH1*, suggesting that the TALE boundary genes could also be involved in regulating the formation of the AZ (Khan et al. 2012; Khan, Tabb, and Hepworth 2012). Supporting this, mutations in *ATH1* lead to fused floral organs, a sign that *ATH1* works to maintain organ boundaries in this area (Gómez-Mena and Sablowski 2008). Another potential role for boundary genes is in regulating the development of the AZ structure, in which the distinct separation and lignified layers form. As *bop1 bop2* mutants lack a lignified layer, this secondary stage of structural development may be dependent on boundary gene function (Lee et al. 2018). The structure of the AZ changes again following abscission, when a waxy layer forms to cover the scar, and the exposed AZ cells take on an epidermal identity. Over-expression of *IDA*, or loss of the downstream suppressor *BP* lead to an overgrown scar with a high accumulation of components found in the scar of mature wild-type AZs (Shi et al. 2011; Stenvik et al. 2006). Since the boundary module genes are predicted to function downstream of these genes to promote abscission, they may also play a role in the final structural development of the AZ.

1.6.2. Signalling

Beyond the confirmed role of *BOP1/2* in initiating the AZ, *BOP1/2* expression is maintained at the base of mature flowers throughout the abscission process. (Hepworth et al.

2005; McKim et al. 2008; Xu et al. 2010). *KNAT2/6* expression also locates to the base of mature flowers (Ragni et al. 2008). Inactivation of *KNAT2/6* rescues the early abscission phenotype of the *bp* loss of function mutant, and the *35S:IDA* over-expression mutant, supporting a second role for boundary genes during the signalling phase of abscission (Shi et al. 2011).

1.6.3. Separation

Inactivation of *IDA* or *HAE/HSL2* blocks abscission (Butenko et al. 2003; Cho et al. 2008; Jinn, Stone, and Walker 2000). Transcriptome analysis of these mutants revealed the lowered expression of cell wall remodelling enzyme genes presumed to be important for the separation phase of abscission (Niederhuth, Patharkar, et al. 2013). As downstream effectors of the signalling pathway, boundary genes play a possible role in promoting the production of these enzymes. This involvement would link the boundary gene module to a third role, in the separation step of abscission.

1.7. Enzymes

IDA signalling promotes abscission by triggering the release of hydrolytic enzymes from the separation layer. This step relies on delivery of vesicles carrying cell wall modification enzymes to the plasma membrane (Niederhuth, Cho, et al. 2013). There are a variety of enzyme families that are proposed to be important for cellular separation in abscission. The exact enzymes responsible for dissolving the middle lamella which connects neighbouring cells have not been confirmed through loss of function studies, but transcript analyses of the AZ suggest that hydrolase, pectinase, cellulase, polygalacturonase, extensin, and expansin families are active in this process (Lashbrook and Cai 2008; Niederhuth, Cho, et al. 2013). A genetic study shows that the TALE genes *KNAT2* and *KNAT6* function downstream in the IDA signalling pathway in activating abscission (Shi et al. 2011). Since *KNAT2* and *KNAT6* are downstream targets of the IDA signaling pathway and promote abscission, it is suggested that they contribute to the expression of hydrolytic enzymes required for abscission. Roles for *BOP1/2* and *ATH1* in completing the IDA signaling pathway are also possible (Figure 1.3).

1.8. Vesicles

Several stages of abscission occur at the cell membrane, or in the extracellular space beyond it that is referred to as the apoplast. The deposition of lignin, cycling of membrane receptors, and the release of hydrolytic enzymes all rely on vesicles for secretion. One regulator of the endomembrane system has been shown to be necessary for abscission. Mutations in *NEVERSHED*, encoding an ADP-ribosylation factor-GTPase-activating protein (ARF-GAP), block abscission. Cells in the AZ of the *nev* mutant show an irregular golgi formation, indicating that this mutation interferes with the normal system of vesicles which traffic newly produced proteins to the cell membrane (Liljegren et al. 2009). It has been proposed that vesicles are required to bring hydrolytic enzymes to the middle lamella, in order for cell separation to proceed. The *nev* mutant lacks a lignin brace at the edge of the separating organ, indicating a role for vesicle transport in the lignification step (Lee et al. 2018). Additional mutations in *CASTAWAY* or *EVERSHED*, which encode receptor-like protein kinases, can restore normal abscission in the *nev* mutant (Leslie et al. 2010; Burr et al. 2011). Mutations in *SERK1*, which functions as part the HAE/HSL2 receptor complex also rescue the *nev* mutant (Lewis et al. 2010). The basis of this rescue is not yet understood. The *nev evr*, *nev cst*, and *nev serk1* (Burr et al. 2011; Lewis et al. 2010; Leslie et al. 2010) mutants also have enlarged AZ scars following abscission, suggesting the involvement of vesicle transport in the final sealing step of abscission. All together, careful regulation of the endomembrane system appears to be important in several different aspects of the abscission process.

1.9. Hypothesis

How boundary genes contribute to abscission is only partly known. Mutations in boundary genes cause delayed or blocked abscission. A role for *BOP1/2* in the initial formation of the AZ has been demonstrated. We propose that BOPs exert their activity through a conserved module of TALE genes defined by *ATH1/KNAT2/KNAT6* that work together in other areas of boundary regulation.

My experiments were designed to test if boundary genes contribute to abscission by structuring the AZ and completing the IDA signaling pathway (Figure 1.3). Overall, my project deepens our understanding of how boundary genes contribute to steps of abscission.

Figure 1.1. Abscission zones in *Arabidopsis thaliana*. In *Arabidopsis*, the floral organs abscise shortly after flowers are pollinated. Stamen, petals and sepals detach at specialized zones at the base of the developing fruit. AZs are also present at the base of cauline leaves, which grow from the inflorescence. These AZs can become activated by drought or pathogen stresses.

Figure 1.2. Structure of the abscission zone. Morphology of the receptacle and lignified layer of the AZ. A) separation exposes a smooth layer of small, densely packed cells on the plant body, shown here at position 10. B) the lignified layer forms at the edge of the organ to be shed. Here, a honeycomb of lignified cells forms a rigid brace.

Figure 1.3. The four steps of abscission. In this simplified model, abscission begins with the initial formation of the AZ, which is established by the boundary as the organ forms. Next, the two functionally distinct layers of the AZ form, a separation layer which produces hydrolyzing enzymes, and the lignified layer on the organ edge. The third step is the activation of abscission by IDA signalling, which eventually promotes the release of hydrolytic enzymes. In the fourth step, a waxy layer seals exposed cells on the plant body after an organ has detached. Image modified from Corrigan, 2018.

Figure 1.4. IDA signaling pathway. Abscission signalling begins with the receptor kinase component BAK1/SERK3, which partners with HAE/HSL2 subunits at the cell surface (reviewed in Patharkar & Walker, 2018). Activation of these receptor kinases by peptide IDA binding leads to a MAP kinase cascade, which inhibits the homeodomain transcription factor KNAT1/BP and removes repression from boundary-associated homeobox genes *KNAT2/6* and presumably *ATH1* (Shi et al. 2011; Corrigan 2018). In parallel, transcription co-factors BOP1 and BOP2 are recruited to DNA and directly promote *ATH1* gene expression (Khan et al. 2015) whose products are predicted to function with TALE partners KNAT2/6 (Rutjens et al. 2009). We propose that an increase in ATH1/KNAT2/KNAT6 activity drives the expression of cell wall modifying enzymes and/or lignin deposition required for separation.

2. Methods

2.1. Plants

The Columbia-0 ecotype of *Arabidopsis thaliana* was used as wild type. The genetic materials used in this study are summarized in Table 2.1. The following loss of function mutant alleles were used: *ath1-3* (Gómez-Mena and Sablowski 2008), *knat2-5*, *knat6-1* (Belles-Boix et al. 2006), *bp-2* (Venglat et al. 2002), *bop1-3*, *bop2-1* (Hepworth et al. 2005), *ida-2* (Cho et al. 2008), *hae-3*, *hsl2-3* (Niederhuth, Cho, et al. 2013), *nev-7* (Alonso et al. 2003), and *atrbohD atrbohF-F3* (Torres, Dangel, and Jones 2002). The *bop1-6D* mutant (*BOP1 oe*) contains four viral 35S promoter copies in the promoter region of *BOP1*, causing constitutively high expression of *BOP1* (Norberg, Holmlund, and Nilsson 2005). The *35S:BOP2* over-expression line (*BOP2-oe*) contains a viral 35S promoter upstream of *BOP2* leading to constitutively high expression of *BOP2* (Norberg, Holmlund, and Nilsson 2005). *alliKNAT6* (*KNAT6-oe*) is an over-expression line with a double 35S promoter directing *KNAT6*, causing constitutively high expression of *KNAT6* (Shi et al. 2011). In the *D35S:ATH1* line, *ATH1* over-expression (*ATH1-oe*) is driven by a double 35S promoter (Khan et al. 2015). The *35S:IDA* line contains a viral 35S promoter, which drives over-expression of *IDA* (Stenvik et al. 2006). *BOP1:GUS* and *BOP2:GUS* reporter lines were used as previously described (McKim et al. 2008; Xu et al. 2010). The *KNAT2:GUS* and *KNAT6:GUS* lines were also used as previously described (Belles-Boix et al. 2006; Pautot et al. 2001). The *ATH1:GUS* line was constructed using an ~4.0-kb fragment of the *ATH1* promoter (Khan et al. 2015). All mutant combinations were constructed by crossing and confirmed by PCR genotyping.

Seeds were surface sterilized with bleach prior to sowing. In brief, seeds were rinsed with 100% ethanol and transferred to a solution of 5% hypochlorite and 0.5% sodium dodecyl sulphate for one minute. Seeds were rinsed three times with sterile water, then distributed on minimal media agar plates (Haughn and Somerville 1986). Plates were kept in the dark at 4°C for two days to break dormancy. Seeds were then germinated in a growth chamber under long days (16 hrs of light/8 hrs of dark, light intensity ~115 $\mu\text{mol m}^{-2} \text{sec}$) for seven days, then transplanted to sterilized soil (ProMix BX, Premier Horticulture) supplemented with 20-20-20 plant fertilizer (Plant-Prod Inc.). Plants were grown to maturity, with assays performed on adult plants after at least 16 flower positions had developed along the primary inflorescence.

Floral positions were scored according to the usual convention (Bleecker and Patterson 1997). At the top of the inflorescence, the first flower with visible white petals is position 1, with all other positions down the stem numbered consecutively. Based on this numbering system, abscission in wild-type plants occurs at positions 6-7 (Corrigan, 2018).

2.2. Extraction of DNA and genotyping

Genomic DNA was extracted for genotyping using the method described by (Edwards, Johnstone, and Thompson 1991) with several changes. A leaf tissue sample roughly half the size of a dime was collected in a 1.5 ml microcentrifuge tube. The tissue was ground with a blue pellet pestle (Fisherbrand). 400 μ L of extraction buffer (200 mM Tris-HCl pH7.5, 250 mM NaCl, 25 mM EDTA pH 8.0, 0.5% w/v sodium dodecyl sulphate) was then added. The tubes were vortexed and then centrifuged at 13 000 g for 10 minutes. 350 μ L of the supernatant was transferred to a new tube. 400 μ L of isopropanol was added to precipitate the genomic DNA. The tube was inverted to mix, then spun again at 13 000 g for 10 minutes. The supernatant was discarded, then 800 μ L of 70% ethanol was added to wash the pellet. The tube was centrifuged at 13 000 g for 5 minutes, then the supernatant was discarded and the sample was left to dry completely. The genomic DNA was resuspended in 100 μ L TE buffer pH 7.0 and stored at 4°C. 2 μ L of the DNA prep were used for a standard 20 μ L PCR-genotyping reaction.

2.3. Lipids staining

Fluorol yellow was used to stain for the presence of aliphatic polymers in the AZ, proposed to make up the sealing cuticle, which forms post-abscission. Position 10 siliques were incubated in fluorol yellow (0.01% w/v, in lactic acid) at 70°C for 15 min. The samples were then rinsed with distilled water and moved to a counter stain of aniline blue (0.5% w/v, in water) and incubated in darkness for 30 minutes. Counter-staining was used to reduce auto-fluorescence of cell wall components such as lignin. The samples were then rinsed and photographed immediately using an EVOS M5000 microscope (ThermoFisher) with a GFP filter.

2.4. Cuticle permeability assay

To assess AZ permeability, siliques were taken from positions 2 to 14. Any remaining floral organs were removed under a dissecting microscope. Samples were immersed in an 0.25% aqueous solution of toluidine blue for 30 seconds and then rinsed in water. Samples were

photographed with a digital camera on a stereomicroscope (SteRIO Discovery V20 with Axiocam, Carl Zeiss).

2.5. ROS staining

Diaminobenzadine (DAB) staining was performed to visualize H₂O₂ accumulation in the AZ. For each genotype, 24 plants were sampled. Whole flowers at position 5 (post fertilization but before abscission) were treated with 0.1% DAB in water overnight, then moved to a 70% ethanol solution until all chlorophyll was removed (Daudi and O'Brien 2012). Samples were imaged on a digital camera on a stereomicroscope.

2.6. Lignin autofluorescence

To visualize lignin structure at sepal edges, position 5 flowers (a time point just before abscission in the wild type) were collected and cleared in 70% ethanol. For each genotype, 24 plants were included in the sample. Sepals were removed under a dissecting microscope. The samples were first incubated in 0.24N HCl in 20% methanol for 90 minutes at 57°C. Samples were transferred to 7% NaOH in 60% ethanol for 15 mins. Samples were dehydrated in a series of ethanol dilutions for 5 minutes (40%, 20%, 10%). Samples were finally moved to 5% ethanol with 25% glycerol (Malamy and Benfey 1997). Samples were mounted in 50% glycerol on slides with coverslips and imaged on a compound fluorescent microscope with a GFP filter.

2.7. Scanning electron microscopy (SEM)

Samples were prepped for SEM following procedures as previously detailed, omitting the incubation in osmium tetroxide (Modrusan et al. 1994). Floral organs were removed to expose the AZ as required. Samples were fixed overnight at 4°C in 3% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.0). A drop of Triton-X was added to break surface tension. After incubation, the fixative was removed, and samples were rinsed twice with 0.1 M sodium phosphate buffer. The samples were then dehydrated through 30 minute incubations in a series of ethanol solutions (30%, 50%, 70%). Just before critical point drying, the samples were dehydrated further in 90% and then anhydrous 100% ethanol solution (stored over molecular sieves), each for 30 minutes. The samples were dried in a critical point dryer (EMITECH, K850). The dried samples were then coated in gold-palladium using a turbo-pumped sputter coater

(Quorum tech, Q150T ES). For each genotype, siliques were harvested from 12 plants. Samples were photographed using an SEM (Vega-II XMU, Tescan) at an accelerating voltage of 15kV.

2.8. GUS staining

GUS staining was carried out as previously described (Sieburth and Meyerowitz 1997), with modifications. Siliques were collected and stored in cold 90% acetone and kept on ice for 15 minutes. After this time, the acetone was removed, and samples were covered by a solution of 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 2 mM 5-bromo-4-chloro-3-indoxyl- β -D-glucuronide (X-Gluc). Samples were incubated in the staining solution overnight at 37°C. The presence of a blue precipitate indicates where GUS enzyme activity is localized in specific tissues. The staining solution was then removed, and 70% ethanol was used to clear the tissue. The samples were left overnight, then changed to fresh ethanol for storing. GUS stained siliques were photographed with a digital camera on a stereomicroscope (SteRIO Discovery V20 with AxioCam, Carl Zeiss).

2.9. RT-qPCR

AZs were dissected from position 5 flowers (a timepoint just before abscission in wild-type plants). Three biological replicates were collected per genotype, each consisting of 40 AZs from individual plants. Tissues were frozen and ground, then RNA was extracted using an RNA Mini Plant Kit (Geneaid) according to the manufacturer's protocol. Samples were treated with DNase I (Froggabio) to remove residual genomic DNA. RNA concentration was measured according to A260 values using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific). RNA quality was assessed using the A260/A280 ratio, with a ratio of approximately 2 indicating a pure quality. RNA integrity was also checked by running 2 μ L of sample through a 1% agarose gel. A reverse transcription reaction using Superscript III polymerase (Thermo Fisher Scientific) and 1 μ g of RNA was performed to produce cDNA, according to manufacturer's protocol. The cDNA was diluted 10 fold. 2 μ L of the diluted cDNA was used in 10 μ L RT-qPCR reactions.

RT-qPCR reactions were performed on an Applied Biosystems StepOnePlus thermocycler. A list of the primers used for target genes can be found in Table 2.4. Primers were designed to span intron-exon junctions when possible to produce amplicons between 70 and 150 base pairs in length. Primers for *QRT2* and *PGAZAT* were based those used by (I. Taylor and

Walker 2018). *IDA* and *RBOHD* primers were taken from a previous study in the Hepworth lab (Corrigan 2018; Bergin 2018). All other primers were designed using PrimerQuest. PCR reactions were performed in triplicate. A melt curve was performed following each reaction to verify primer specificity. Transcript levels expressed as a relative fold-change compared to the wild type and calculated as described (Pfaffl 2001). For normalization, *GLYCERALDE-3-PHOSPHATE DEHYDROGENASE C (GAPC)* and *ELONGATION FACTOR 1 ALPHA* were used as housekeeping genes (Khan et al. 2015; Czechowski et al. 2005). Target RQ was normalized against the geometric mean of both housekeeping genes. Data shown are the mean $\pm$ standard error (SE) for three biological replicates.

Table 2.1. List of genetic materials

Plant line	Description	Annotation	Reference
Loss of function mutants			
<i>ath1-3</i>	T-DNA insertion	SALK_113353	(Gómez-Mena and Sablowski 2008)
<i>knat2-5</i>	T-DNA insertion	SALK_099837	(Belles-Boix et al. 2006)
<i>knat6-1</i>	T-DNA insertion	SALK_047931	(Belles-Boix et al. 2006)
<i>bop1-3</i>	T-DNA insertion	SALK_012994	(Hepworth et al. 2005)
<i>bop2-1</i>	T-DNA insertion	SALK_075879	(Hepworth et al. 2005)
<i>bp-2</i>	C to T transition, creates stop codon at position 540	RLD ecotype, backcrossed 3X to Col-0	(Venglat et al. 2002)
<i>ida-2</i>	T-DNA insertion	SALK_133209	(Cho et al. 2008)
<i>hae-3</i>	EMS C to T at nt 665		(Niederhuth, Cho, et al. 2013)
<i>hsl2-3</i>	EMS G to A at nt 1078		(Niederhuth, Cho, et al. 2013)
<i>nev-7</i>	T-DNA insertion	SALK_079928C	(Alonso et al. 2003)
<i>atrbohD</i>	Random dSpm D3 insertion into fifth exon after codon P535		(Torres, Dangl, and Jones 2002)
<i>atrbohF</i>	Random dSpm F3 insertion into first exon in codon Q177		(Torres, Dangl, and Jones 2002)

Gain of function mutants			
<i>bop1-6D</i>	Activation tagged line, 4X CaMV 35S enhancer in <i>BOP1</i> promoter	Glufosinate-ammonium selection in plants (active)	(Norberg, Holmlund, and Nilsson 2005)
<i>35S:BOP2</i>	Transgenic line, CaMV 35S enhancer in <i>BOP2</i> promoter		(Norberg, Holmlund, and Nilsson 2005)
<i>alliKNAT6</i>	Double CaMV 35S enhancer in <i>KNAT6</i> promoter		(Shi et al. 2011)
<i>D35S:ATH1</i>	Double CaMV 35S enhancer in <i>ATH1</i> promoter		(Khan et al. 2015)
<i>35S:IDA</i>	Transgenic line, CaMV 35S enhancer in promoter of <i>IDA</i>		(Stenvik et al. 2006)
Reporter lines			
<i>BOP1:GUS</i>	4-kb promoter fragment fused in-frame to GUS	Kanamycin selection in plants (inactive)	(McKim et al. 2008)
<i>BOP2:GUS</i>	4-kb promoter fragment fused in-frame to GUS	Glufosinate ammonium selection in plants (active)	(Xu et al. 2010)
<i>KNAT2:GUS</i>		Kanamycin selection in plants (inactive)	(Dockx et al. 1995)
<i>KNAT6:GUS</i>		Kanamycin selection in plants (inactive)	(Belles-Boix et al. 2006)

<i>ATH1:GUS</i>	4-kb promoter fragment fused in-frame to GUS	Glufosinate ammonium selection in plants (active)	(Khan et al. 2015)
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Table 2.2. List of primers

Description	Primer	Sequence 5'-3'	Amplicon size (base pairs)
Genotyping			
<i>bop1-3</i> (SALK_012994)	bop1-3 SALK_012994 (RP)	TGACATCGGAGAAAGCTTGAC	RP + LP fragment ~900 bp
	bop1-3 SALK_012994 LP	TGCACAATCTTTCGACTTCATC	RP+LB1.3 fragment ~500 bp
	LBb1.3	ATTTTGCCGATTTTCGGAAC	
<i>bop2-1</i> (SALK_075879)	bop2-1 SALK_075879 RP	ATTTGGCCACCTTTGTATTC	RP+LP fragment ~900 bp
	bop2-1 SALK_075879 LP	AAAGAGAGAACCTGGGTGAGC	RP+LB1.3 fragment ~500 bp
<i>knat2-5</i>	knat2-5 Salk 099837 RP	CAAAAGGTGATCTCGCTGCTTTCGT	1300 bp
	knat2-5 Salk 099837 LP	AATCTCTAGCGCAAAAGTTTTTGCT	500 bp
<i>knat6-1</i>	KNAT6-1 04b	ATATCAGTAAACCACAAAGAAAGTC	1125 bp
	KNAT6-1 03	GAAGATAAACCTAGCTACAAG	500 bp
<i>ath1-3</i>	ATH1-RP	GGCGGGTTTCGGATCTACATT	870 bp
	ATH1-LP	CCAATACCGTTTTTCAGACATGA	750 bp
<i>ida-2</i>	ida-2 Forward	GTTCTTTGATCAGGAGAGAGCTTTG	1900 bp
	ida-2 Reverse	CTT CTC ACG CAA AAG ATA GAG TTG G	1400 bp
RT-qPCR			
GAPC	GAPCp1	TCAGACTCGAGAAAGCTGCTA	244 bp
	GAPCp2	GATCAAGTCGACCACACGG	
EF1a	EF1a-RT-F	CAGGCTGATTGTGCTGTTCTTATCAT	204 bp
	EF1a-RT-R	CTTGTAAGACATCCTGAAGTGGAAGA	
qPCR QRT2	QRT2-qF	AGGGAGGACATGGAATGGCAAAGA	107 bp
	QRT2-qR	GCATGCTTCAACACGATCGCAGTA	
qPCR PGAZAT	PGAZAT-F	ACCAAGACTACTGCGACAAGAGCA	81 bp

	PGAZAT-R	TTATGTCCCGGTACACCACGTTCT	
qPCR IDA	IDA-FWD-06/18	CAATGGCTCCGTGTCGT	234 bp
	IDA-REV-06/18	TCAATGAGGAAGAGTTAACAAAAGAG	
qPCR CEL3	qPCR-CEL3 F2	TCTGGTTACAAGGACGAGCTAATG	70 bp
	qPCR-CEL3 R2	CGTAATACGGGTATTTCGTTGCT	
qPCR XTH18	qPCR-XTH18 F	GCAAAGCCGAGGTTCAAATG	78 bp
	qPCR-XTH18 R	CCGGAGACTTAAGATAGAATGTTGTTAC	
qPCR XTH28	qPCR-XTH28 F	CCTTTGGTCTCTATCTCACATCATATTTTAT	74 bp
	qPCR-XTH28 R	TCGACGCCGTACGTTTGA	
qPCR RBOHF	qPCR-RBOHF F	CCCAGTTTGCAGAAACACCATTA	103 bp
	qPCR-RBOHF R	GGCTCCAGCAATTGTCTTGTG	
qPCR RBOHD	RBOHDFwd	CATGGTATCAAGCTCTACCTCAC	150 bp
	RBOHDFwd	AACAGCCACCTTGATCATCTTA	
qPCR PRXR9	AtPRXR9GE-F1	GCGAAGAGCTGTCCAAACGCAGAG	131 bp
	PRXR9-qPCR-R	CCGTCACATCCCCTAACGAAGCAAT	

Table 2.3. List of crosses made

Female parent x Male parent	Resulting progeny	Generation
<i>ida</i> x <i>bop1-6D</i>	<i>ida bop1-6D</i>	F2
<i>ida</i> x 35S: <i>BOP2</i>	<i>ida 35S:BOP2</i>	F2
<i>ida</i> x <i>alliKNAT6</i>	<i>ida alliKNAT6</i>	F2
<i>ida</i> x <i>d35S:ATH1</i>	<i>ida d35S:ATH1</i>	F2
<i>hae hsl2</i> x <i>bop1-6D</i>	<i>hae hsl2 bop1-6D</i>	F2
<i>hae hsl2</i> x <i>alliKNAT6</i>	<i>hae hsl2 alliKNAT6</i>	F2
<i>hae hsl2</i> x <i>D35S:ATH1</i>	<i>hae hsl2 D35S:ATH1</i>	F2
<i>bop1 bop2</i> x 35S: <i>IDA</i>	<i>bop1 bop2 35S:IDA</i>	F2
<i>knat2 knat6</i> x 35S: <i>IDA</i>	<i>knat2 knat6 35S:IDA</i>	F2
<i>ath1</i> x 35S: <i>IDA</i>	<i>ath1 35S:IDA</i>	F2

3. Results

3.1. Boundary genes are required for AZ differentiation

AZ differentiation involves two steps. After AZ initiation at a boundary, two adjacent cell layers with different contributions to abscission are formed. On the side closest to the plant body, which contains the “separation layer”, ordered rows of cells specialized for the secretion of hydrolytic enzymes are formed. Juxtaposed, on the edge of the separating organ, a “lignified layer” is formed. Lignin is deposited in the cell walls to create a honeycomb-structured rigid brace that defines the plane of separation (Lee et al. 2018).

Loss of boundary gene function leads to disordered or absent AZs, indicating a role for these genes in structural development of the AZ (McKim et al. 2008; Gómez-Mena and Sablowski 2008; Shi et al. 2011). The first part of my thesis investigates the contribution of boundary-associated TALE transcription factors *ATH1/KNAT2/KNAT6* in AZ patterning. This module was found to have important roles in development of both the separation and lignified layers, thereby influencing final structural changes, which seal the exposed AZ after abscission.

3.1.1. Separation layer

A) Cell morphology

Previous work in the Hepworth lab investigated the effect of boundary gene mutations on AZ morphology at the base of the receptacle using SEM (Corrigan 2018). Expression studies had indicated that *BOP1*, *BOP2* and TALE genes *ATH1*, *KNAT2* and *KNAT6* are expressed in this region before and after abscission. Accordingly, AZs were examined in wild-type and *bop1 bop2* control plants, single mutants *knat2*, *knat6*, and *ath1*, double mutants *ath1 knat2* and *ath1 knat6* and the triple mutant *ath1 knat2 knat6* (Corrigan 2018). In wild type, residual cells on surface of the receptacle at position 10, which is shortly after abscission, form a smooth, unbroken surface indicating that dissolution of the middle lamella is complete (Figure 3.1A). In the *bop1 bop2* mutant, which does not form a specified AZ, the detachment of organs exposes ruptured cells at the break plane, indicating that no separation layer is formed (Figure 3.1B). A wild-type morphology was observed in single mutants *knat2* and *knat6* (Figure 3.1CD). In the *knat2 knat6* mutant, exposed cells on the receptacle were slightly overgrown and irregularly sized, indicating

a subtle defect in the separation layer (Figure 3.1E). In the *ath1* mutant, where abscission is substantially delayed, the removal of organs exposed a visibly expanded and uneven layer of surface cells. These defects were enhanced in *ath1 knat2* and more so in *ath1 knat6* double mutants. In the *ath1 knat2 knat6* triple where organs can only be removed with strong force, the AZ was uneven and expanded, with many broken cells exposed in the break plane (Figure 3.1I). Altogether, these data show that organization of the AZ depends on TALE boundary genes. While ATH1 makes the largest contribution, the analysis of combination mutants reveals that *KNAT6* and *KNAT2* are also involved.

B) Protective layer

Following abscission, exposed cells on the plant body secrete a waxy layer (Estornell et al. 2013). Classic studies indicated the presence of lignin and suberin in these layers (Addicot 1982; Clements and Atkins 2001; Pollard et al. 2008). However, these studies were mainly conducted in trees and other plants with a woody stem. Further investigations in *Arabidopsis* show a protective layer of cuticle, a material secreted by epidermal cells, which is mixture of cutin and embedded waxes (Kim et al. 2015; Lee et al. 2018). Since the mutation of TALE genes impairs AZ structure, cuticle formation might also be disrupted.

To test for the presence of a lipid layer, I first stained the AZ of wild-type and mutants with fluorol yellow, which can be used to stain aliphatic polymers (Brundrett, Kendrick, and Peterson 1991). In all genotypes, the nectaries, which are located in the area of the AZ, between the separate points of attachment, showed strong staining (Figure 3.2, second row). The AZ did not show much staining in any genotype.

Waxy compounds are difficult to stain due to their hydrophobic nature, so I used a negative staining method to assess the extent of the sealing layer in our various boundary mutants. Siliques from every second position from 2 to 14 were submerged in toluidine blue, which stains most plant tissue but cannot permeate the cuticle layer (Figure 3.2, bottom row and Supplemental Table S1). In non-abscising genotypes, the floral organs were removed before staining, to assess whether any sealing exists beyond the physical barrier that the remaining organs provide. In wild-type siliques (Figure 3.2A), the AZ was completely impermeable by position 8, indicated by an absence of staining in the AZ. The *bop1 bop2* double mutant remained fully permeable through all positions (Figure 3.2B), indicating a block before this final

step. The single mutants *knat2* and *knat6* developed an impermeable layer at the same position as wild-type siliques, which is in line with the fact that abscission is not delayed in these mutants (Supplementary Figure S3.1). The *knat2 knat6* double mutant (Figure 3.2C) showed a slight delay in the formation of a cuticle layer. The *ath1* mutant, which does not abscise, also did not form a cohesive sealing layer and remained permeable through all later positions (Figure 3.2D). The double mutants *ath1 knat2* and *ath1 knat6*, as well as the triple mutant *ath1 knat2 knat6* also remained permeable following abscission (Figure 3.2EFG). Taken together, these results indicate that the block in abscission caused by loss of boundary gene function also interferes with structural changes associated with the final steps of abscission.

3.1.2. Lignified layer

I next tested the role of the boundary genes on formation of the lignified layer on the edge of the floral organs. Formation of lignin polymers requires ROS (Freudenberg 1959; C.-J. Liu 2012). Expression studies had indicated that the boundary genes *BOP1*, *BOP2*, *ATH1* and *KNAT6*, are expressed at the organ edge. Only *KNAT2* is not expressed in this region (Corrigan 2018).

A) ROS signaling

During lignification, monolignols are coupled into a polymer through an oxidation reaction (Freudenberg 1959; C.-J. Liu 2012). Respiratory burst oxidases, peroxidases, and laccases, provide the necessary ROS for this reaction to occur (Yahong Li et al. 2003; Berthet et al. 2011; Lee et al. 2018). In the AZ, ROS accumulates in a localized pattern at the organ edge, where it is likely to act as a precursor to the lignin brace. The inactivation of ROS-producing NADPH oxidase genes *RBOHD* and *RBOHF* dramatically reduces the accumulation of ROS at the organ edge. A normally-patterned AZ develops, but no lignin brace is formed (Lee et al. 2018)(Supplementary Figure S3.2BD). The inactivation of *BOP1* and *BOP2* also reduce the accumulation of ROS at the organ edge but no AZ develops and no lignin brace is formed on the organ edge (Lee et al. 2018). These observations suggest a link between boundary genes and the proper formation of the lignified layer, however the role of the TALE genes in this layer has not been determined.

To assess whether TALE genes contribute to the set-up of these two layers in the AZ, I first analyzed the ROS accumulation in TALE boundary gene mutants at the organ edge. Wild

type and the *nevershed* mutant were used as positive controls and *bop1 bop2* and *rbohD rbohF* double mutants were used as negative controls. Diaminobenzidine (DAB) staining was used to detect ROS abundance at the sepal edges of position 5 flowers, the stage just before abscission normally occurs (Figure 3.3, top rows; Supplemental Table S2). The wild-type control showed an accumulation of ROS that was tightly localized to the organ edge (Figure 3.3A). ROS levels were reduced in the *bop1 bop2* double mutant (Figure 3.3B). ROS levels in the TALE mutants, *knat2* and *knat6*, and the *knat2 knat6* double mutant were comparable to the wild type (Figure 3.3C and Supplemental Figure S3.3). In the *ath1* mutant, the ROS was less specifically localized to the organ edge and appeared more diffuse than in the wild type (Figure 3.3D). The pattern of ROS became increasingly faint and mislocalized with the addition of *knat2* and *knat6* mutations (Figure 3.3EFG). The triple mutant *ath1 knat2 knat6* showed a disordered, patchy diffusion of ROS (Figure 3.3G). Overall, these data indicate that loss of boundary genes interferes with the normal accumulation of ROS, suggesting that boundary genes might contribute to ROS production during abscission.

B) Deposition of lignin

ROS accumulation triggers the deposition of lignin in the cell wall (Barros et al. 2015; Freudenberg 1959). Since ROS patterning is disrupted by boundary gene mutations, I next investigated what effect this has on the lignified layer. Sepals were dissected from position 5 flowers, a stage just before abscission normally occurs. Lignin structure, which is auto-fluorescent, was visualized under UV light on a compound microscope (Figure 3.3, middle and bottom rows; Supplemental Table S3). Wild-type sepals had a tightly ordered honeycomb structure of lignin, approximately three cell layers deep at the organ edge (Figure 3.3A). The *bop1 bop2* double mutant had no lignin brace and the organ edge was ripped and undefined (Figure 3.3B). In *knat2* and *knat6* single mutants, cells were ordered and the lignin brace was present and intact similar to the wild type (Supplemental Figure S3.3). In the *knat2 knat6* double mutant, cells of the AZ were enlarged and disordered (Figure 3.3C). Though normal levels of lignin could be seen in this mutant, the irregularly shaped cells caused the brace to have a disorganized structure, rather than a uniform honeycomb. This disorder also occurred in the *ath1* single mutant (Figure 3.3D). The lignin patterning became increasingly disrupted with the addition of *knat2* and *knat6* mutations (Figure 3.3EF). In the triple *ath1 knat2 knat6* mutant, though lignin was still deposited, the lignin pattern followed the mislocalized patches seen in

ROS staining. In the triple mutant AZ, enlarged cells abolished the neat rows that normally form, so while lignin was present, a cohesive brace could not be formed (Figure 3.3G). These collective findings indicate that loss of boundary gene function leads to reduced ROS signalling and loss of an ordered, lignified layer at the organ edge.

While the *bop1 bop2* mutant does not abscise, previous work revealed that the BOP1 over-expression (*BOP1-oe*) line undergoes accelerated abscission. In this line, the rounding of cells in the AZ and the subsequent shedding of floral organs occurs early, indicating a role for BOP1/2 in the activation step of abscission (Corrigan 2018). No AZ is formed in the *bop1 bop2* mutant, which points to a role for the BOPs in the set-up step as well. Further analysis of the *BOP1-oe* AZ showed high and expanded accumulation of ROS, and a uniform but shallow lignin brace. The brace included fewer cell layers than the wild type (Supplementary Figure S3.4A). The cuticle layer was also investigated in this line, and showed that, following the early abscission of *BOP1-oe*, the cuticle becomes impermeable 1 position earlier than wild-type AZs (Supplementary Figure 3.4B, bottom row). Overall, these results show that the BOPs are involved in both the structural set-up and the activation of abscission.

C) Transcript analysis

To further investigate the lignin defects seen in boundary mutants, the expression levels of genes related to ROS and lignification genes were also measured. AZs were harvested at position 5, a stage before the floral organs are abscised. These samples captured both the separation and lignified layers of the AZ. Transcript levels of genes involved in ROS production and lignification were measured using RT-qPCR and normalized to the expression levels in wild-type control samples.

Transcripts of two members of the NADPH oxidase family, *RBOHD* and *RBOHF*, were measured (Figure 3.4). This class of enzyme contributes to ROS accumulation, which acts as a precursor to the lignification of the separation layer in the AZ (Morales et al. 2016). While the expression of *RBOHD* was reduced in both mutants, *RBOHF* expression was not significantly altered in the *ath1 knat2 knat6* mutant or the *bop1 bop2* mutant, suggesting that these related genes are under differential regulation in the AZ. Peroxidases also contribute to lignification (Boerjan, Ralph, and Baucher 2003). Among these, *PRXR9* expressed in AZs is an immediate target of BOP1 activation (Khan et al. 2012; Khan 2013). Measurement showed that *PRXR9*

transcripts were significantly reduced in both the *bop1 bop2* and the *ath1 knat2 knat6* mutants compared to wild-type AZs.

3.2. A downstream role for boundary genes in the IDA signaling pathway

3.2.1. Boundary genes are under repression by BP

Boundary genes including *BOP1*, *BOP2*, *ATH1*, *KNAT2* and *KNAT6* are spatially repressed in the stem and root-hypocotyl by KNAT1/BP (Ragni et al. 2008; Khan et al. 2012; Khan, Tabb, and Hepworth 2012; Woerlen et al. 2017). Genetic tests suggested a similar dynamic during abscission. A model was proposed whereby IDA signaling decreases BP repression of *KNAT2* and *KNAT6* in AZs thereby allowing these genes to promote organ separation (Shi et al. 2011).

ATH1 acts genetically with *KNAT2* and *KNAT6* in patterning boundaries but this relationship is untested within abscission signaling (Yang Li et al. 2012; Hamant and Pautot 2010; Khan et al. 2015). *BOP1* and *BOP2* are positive regulators of *ATH1* and *KNAT6* gene expression and might contribute as well (Khan et al. 2012; Khan, Tabb, and Hepworth 2012; Khan et al. 2015).

A) Boundary genes are under repression by BP

To ascertain a role for *ATH1* and *BOP1/2* within the IDA signaling network, I used reporter GUS lines to compare expression patterns of these boundary genes in the AZ of *bp* mutants compared to the wild type (Figure 3.5). *KNAT2* and *KNAT6* reporter lines were used as controls. As expected, *KNAT2* and *KNAT6* reporter expression was expanded and prolonged in *bp* AZs compared to the wild type (Figure 3.5ABFG). A similar pattern of expanded expression was seen for *ATH1*, *BOP1*, and *BOP2* reporter genes. Staining was prolonged in the AZ and extended into the pedicel region of *bp* developing fruits (Figure 3.5C-E,H-J). The increased expression of these genes in mutants lacking *BP* indicates that this group of boundary genes is under common regulation in the AZ.

B) TALE homeobox genes maintain cell order in AZ downstream of BP

The *bp* mutant abscises early and has an expanded AZ similar to *35S:IDA* mutants (Shi et al. 2011). If boundary TALE genes *KNAT2*, *KNAT6*, and *ATH1* are repressed by BP until IDA signaling, then inactivation of these boundary genes should rescue the *bp* phenotype. It has been suggested that inactivation of boundary genes downstream of *BP* restores normal morphology to the AZ (Shi et al. 2011). To confirm this relationship, *bp* AZ morphology was compared to *bp knat2*, *bp knat6*, and *bp knat2 knat6* morphology using SEM imaging (Figure 3.6). The *bp* AZ was expanded towards the pedicel, as has previously been shown (Figure 3.6B). Microscopic analysis of *bp knat2* and *bp knat6* double mutants did not show a recovery of normal morphology. The triple mutant, *bp knat2 knat6*, showed a partially recovered morphology when compared to *bp*, though the AZ remained slightly expanded compared with wild-type AZs (Figure 3.6C). The *bp bop1 bop2* triple mutant AZ resembled the disordered and undefined morphology of *bop1 bop2* (Figure 3.6D). Similarly, the *bp ath1* double mutant AZ resembled the disordered phenotype of the *ath1* mutant (Figure 3.6E). Thus, *knat2 knat6*, *ath1*, and *bop1 bop2* mutations mask *bp* characteristic defects in AZ morphology. Surprisingly, some *bp bop1 bop2* samples in the population showed the partial formation of an AZ, possibly indicating that BP hinders AZ initiation in the *bop1 bop2* mutant (Figure 3.6D).

3.2.2. Boundary genes function downstream of IDA

A) *ida* mutant showed varying rescue by over-expression of single boundary genes

Abscission is activated when secreted IDA peptides bind to the extracellular domain of HAE/SHL2 receptor like kinases and initiate signaling (Jinn, Stone, and Walker 2000; Butenko et al. 2003; Stenvik et al. 2008). Mutation of these components blocks abscission. Consistent with a downstream function for boundary genes in this pathway, over-expression of *KNAT2* and *KNAT6* restores abscission in an *ida* mutant (Shi et al. 2011). To further explore a downstream function for boundary genes in completing the IDA signaling pathway, crosses with *ida* were generated using over-expression transgenic lines for *BOP1*, *BOP2*, and *ATH1*, using *KNAT6* as a control. Individuals homozygous for *ida* with at least one copy of the transgene were isolated from each F2 population.

As expected, *ida* plants retained partial floral organs at all positions (Figure 3.7BG) (Butenko et al. 2003). Also as expected, *ida KNAT6-oe* mutants showed a full rescue of the *ida*

abscission defect. In these plants, abscission occurred by position 6, slightly earlier than wild-type (Figure 3.7DI). This timing aligns with the slightly early abscission observed in plants carrying the *KNAT6-oe* transgene (Shi et al. 2011). By contrast, *ida ATH1-oe* mutants did not show rescue. In these plants, abscission was blocked (Figure 3.7EJ). *ida BOP1-oe* mutants showed a partial rescue of the *ida* abscission defect, with occasional later positions losing their floral organs. *ida BOP2-oe* plants showed a complete rescue of *ida* abscission defects. Similar to *BOP1-oe* plants, this mutant underwent abscission slightly earlier than in wild-type plants, with abscission mostly complete by position 6 (Figure 3.7CH).

B) *hae hsl2* abscission defect is not rescued by over-expression of boundary genes

HAE and HSL2 work as functional partners and serve as the receptor to IDA signalling at the cell surface. Like the *ida* mutant, *hae hsl2* mutants also fail to abscise, with floral organs remaining attached at all positions. To test if the over-expression of boundary genes can also rescue *hae hsl2* abscission defects, crosses were generated using over-expression transgenic lines for *BOP1* and *ATH1*, using *KNAT6* as a control. Individuals homozygous for *hae hsl2* with at least one copy of the transgene were isolated from each F2 population.

Contrasting with *ida* crosses, *hae hsl2* crosses to *BOP1-oe* showed no rescue of the abscission defect (Figure 3.8BF). Similarly, *hae hsl2 KNAT6-oe* and *hae hsl2 ATH1-oe* mutants retained floral organs at all positions, indicating that the *hae hsl2* abscission defect was not rescued (Figure 3.8CDGH).

C) Over-expression of IDA does not rescue abscission defect in boundary mutants

While *ida* mutants fail to abscise, the *35S:IDA* mutant, which constitutively over expresses *IDA*, has an early abscission phenotype and shows overgrowth of the cells in the AZ (Stenvik et al. 2006). If TALE genes contribute to abscission downstream of IDA signalling, then the loss of TALE activity should block the *35S:IDA* early abscission phenotype. This outcome has already been shown for *35S:IDA knat2 knat6* mutants (Shi et al. 2011). To further test this hypothesis, *35S:IDA* plants were crossed with *ath1*, *knat2 knat6* and *bop1 bop2* mutants, all of which have AZ structural defects that impair abscission to different extents.

The *35S:IDA bop1 bop2* mutant had an abscission phenotype similar to the *bop1 bop2* mutant (Figure 3.9CH). In these mutants as expected, floral organs remained firmly attached at all positions due to lack of an AZ (McKim et al. 2008). *35S:IDA knat2 knat6* mutants showed a

return to a wild-type abscission phenotype, with abscission occurring by positions 6-7 (Figure 3.9DI). This result is also in line previous findings (Shi et al. 2011). The *35S:IDA ath1* mutant showed a severe abscission defect, similar to that of the *ath1* mutant (Figure 3.9EJ). In these plants, the attachment of floral organs was prolonged. These results show the phenotype of boundary gene mutants is epistatic to the early abscission phenotype of *35S:IDA* plants.

D) Boundary genes promote hydrolytic enzymes

Cell separation relies on the degradation of bonds that connect cells together in the separation layer (Patharkar and Walker 2018; Bleecker and Patterson 1997; Kim, Chun, and Tucker 2019). A host of hydrolytic enzymes are proposed to be involved, though the specific enzymes which are needed have not been confirmed (Kim et al. 2015; Taylor and Walker 2018; Patterson 2001). Loss of the IDA peptide or its receptors, HAE/HSL2, results in lowered expression levels of cell wall modifying enzymes that are normally upregulated during abscission in the AZ (Niederhuth, Patharkar, et al. 2013). This reduction links the IDA activation pathway to the release of hydrolytic enzymes. Hence, boundary genes may function downstream of *IDA* to promote the expression of hydrolytic enzyme genes.

RT-qPCR was used to measure the transcripts of *IDA* and selected hydrolytic enzymes that may be released upon IDA signaling. In both the *bop1 bop2* and *ath1 knat2 knat6* mutants, the expression of *IDA*, the signalling peptide which initiates abscission, was not significantly altered in comparison to the wild type (Figure 3.10).

The expression levels of two polygalacturonases linked to cell separation, *POLYGALACTURONASE ABSCISSION ZONE ARABIDOPSIS THALIANA* (*PGAZAT*) and *QUARTET 2* (*QRT2*), were measured in each background (Ogawa et al. 2009). Additionally, three other genes encoding hydrolytic enzymes, *CELLULASE 3* (*CEL3*), *XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 18* (*XTH18*) and *XTH28* were selected on the basis of their presence in other AZ transcript analyses (Lashbrook and Cai 2008; Cai and Lashbrook 2008; Taylor and Walker 2018; Niederhuth, Patharkar, et al. 2013). All five hydrolytic enzymes had significantly reduced expression in the both the *bop1 bop2* and *ath1 knat2 knat6* mutants (Figure 3.10). This result indicates these boundary genes play a role in the promotion of cell wall remodelling proteins which are likely required during abscission.

Figure 3.1. SEM images of receptacle layer after abscission. Wild-type and mutant AZs were depicted at position 10 of the inflorescence. A, wild-type AZ showing a smooth layer of small, ordered cells on the plant body. B, *bop1 bop2* mutants have no specialized AZ and contain ruptured cells. C, D, receptacle layers in *knat2* and *knat6* mutants are comparable to the wild type. E, *knat2 knat6* double mutants have an enlarged, disordered residual layer. F, *ath1* mutants show an expanded and ruptured receptacle layer. G, H, I, the *ath1 knat2*, *ath1 knat6*, and *ath1 knat2 knat6* mutants show increasingly disordered and expanded receptacle layers. Scale bars: 100 μ m. This work has been taken from the thesis of Laura Corrigan, 2018.

Figure 3.2. Analysis of cuticle formation in wild-type and boundary mutant AZs. Top row, SEM images of AZ at position 10. Middle row, lipid stain using fluorol yellow, photographed at 405 nm wavelength with GFP filter. For all genotypes, the nectaries show strong staining. Bottom row, cuticle permeability assay, representative images of position 10 siliques after immersion in aqueous toluidine blue. For non-abscising mutants, floral organs were removed prior to staining. A, wild-type silique showing a completely impermeable cuticle layer at position 10. B, *bop1 bop2* mutant where the AZ remains permeable at all positions. C, *kmat2 kmat6* mutant where the AZ remains partially permeable at position 10. D, *ath1* mutant in which the AZ remains permeable at all positions. E, F, G, *ath1 kmat2*, *ath1 kmat6*, and *ath1 kmat2 kmat6* mutants in which AZs remain permeable at all positions. For SEM, at least 6 siliques from individual plants were imaged. For lipid and cuticle assays, at least 12 siliques from individual plants were imaged. Representative images are shown, see Supplementary Table S1 for numerical scoring. Scale bars: 100 μm (SEM) and 250 μm (lipid and cuticle assays).

Figure 3.3. ROS and lignin at organ edge in wild-type and boundary mutants.

Representative images showing edge of sepal from position 5 flowers. Brightfield images show ROS stained with DAB (top rows), fluorescent microscopy shows lignin autofluorescence (lower rows). A, wild-type sepal showing strong, localized DAB staining and uniform rows of honeycomb lignified cells at the organ edge. B, *bop1 bop2* mutant showing reduced DAB staining and no lignin brace. C, *knat2 knat6* mutant showing normal levels of DAB staining and a lignin brace similar to the wild type except slightly enlarged and irregular cell shape. D, E, *ath1* and *ath1 knat2* mutants showing expanded DAB staining and a disordered but complete lignin brace. F, G, *ath1 knat6* and *ath1 knat2 knat6* mutants showing severely altered DAB staining and an incomplete, disordered lignin brace. Lignin was visualized at 405 nm wavelength with a GFP filter. For ROS and lignin staining, sepals were dissected from position 5 flowers on at least 20 different plants. At least 60 sepals were scored per genotype. Representative images were chosen, see Supplementary Table S2, S3 for numerical scoring. Scale bars: 200 μm (bright field compound microscope images) and 100 μm (fluorescent compound microscope images).

Figure 3.4. Transcript analysis of genes involved in lignin polymer formation. Normalized transcript levels in AZ tissues of position 5 flowers were measured using RT-qPCR. Data represents the mean $\pm$ SE for three biological replicates of tissue harvested from different plants. Three technical replicates were performed for each biological replicate. Asterisks, significantly different than the wild type (one-way ANOVA with Tukey's post-hoc test, $p < 0.05$). Genes listed: *RBOHF* (AT1G64060), *RBOHD* (AT5G47910), and *PRXR9* (AT3G21770).

Figure 3.5. Boundary gene GUS expression in wild-type and *bp* mutant AZs.

Representative images of stained tissue showing position 10 siliques. A-E, GUS expression of indicated boundary genes in a wild-type background showing localized expression in the AZ. F-J, GUS expression indicated boundary genes in a *bp* mutant background showing ectopic expression extending into the pedicel. For each genotype, at least twelve siliques from individual plants were imaged. Representative images are shown. Scale bars: 0.5 mm.

Figure 3.6. SEM micrographs showing AZ morphology in wild-type, *bp*, *bp knat2 knat6*, and *bp ath1* mutants. Representative SEM micrographs of position 10 AZs. Remaining floral organs removed prior to fixing. A, wild-type AZ showing ordered morphology with rounded cells. B, *bp* mutant showing an expanded AZ with overgrowth extending towards pedicel. C, *bp knat2 knat6* mutant showing a reduction in overgrowth at the lower edge which partially resembles a wild-type AZ. D, *bp bop1 bop2* showing an undefined AZ typical of a *bop1 bop2* mutant phenotype. E, *bp ath1* mutant showing a disordered and expanded AZ resembling the *ath1* mutant phenotype. For each genotype, at least 6 siliques from individual plants were imaged. Representative images are shown. Scale bars: 100 μ m.

Figure 3.7. Crosses of *ida-2* mutant to boundary gene over-expression lines. A and G, wild type plants undergo abscission at position 6-7. B and H, *ida* mutants showing an abscission defect, organs remain loosely attached at later positions. C and I the *ida BOP1-oe* double mutant showing partial rescue of the *ida* abscission defect. D and J the *ida BOP2-oe* double mutant showing early abscission, around position 5. E and K, the *ida KNAT6-oe* double mutant showing early abscission, around position 5. F and L, the *ida ATH1-oe* double mutant showing an abscission phenotype similar to *ida-2*. For each genotype, at least 4 plants were photographed to assess abscission timing. Representative images are shown. Scale bars: 1.5 cm.

3.8 Crosses of *hae hsl2* mutant to boundary gene over-expression lines. A and E, wild-type plants undergo abscission at position 6-7. B and F, *hae hsl2 BOP1-oe* mutants do not abscise. C and G, *hae hsl2 KNAT6-oe* mutants do not abscise. D and H, *hae hsl2 ATH1-oe* mutants do not abscise. For each genotype, at least 4 plants were photographed to assess abscission timing. Representative images are shown. Scale bars 1.5 cm.

Figure 3.9. Crosses of boundary mutants to 35S:IDA plants. A and F, wild-type plants undergo abscission at position 6-7. B and G, 35S:IDA plants undergo early abscission at position 3-4. C and H, 35S:IDA *bop1 bop2* plants do not undergo abscission at any position. D and I, 35S:IDA *kn2 kn6* plants undergo abscission at about position 7, which is comparable to the wild type. E and J, 35S:IDA *ath1* plants showing an abscission defect similar to *ath1*. For each genotype, at least 4 plants were photographed to assess abscission timing. Representative images are shown. Scale bars: 1.5 cm.

Figure 3.10. Transcript analysis of enzyme genes in the AZ. Transcript levels in AZ tissues of position 5 flowers were measured using qPCR and normalized to the wild type. Data represent the mean $\pm$ SE for three biological replicates of tissue harvested from different plants. Three technical replicates were performed for each biological replicate. Asterisks, significant difference compared to the wild type (one-way ANOVA with Tukey's post hoc test, $p < 0.05$). Genes listed: *IDA* (AT1G68765), *PGAZAT* (AT2G41850), *QRT2* (AT3G07970), *CEL3* (AT1G71380), *XTH18* (AT4G30280), *XTH28* (AT1G14720).

4. Discussion

The initiation of the AZ is linked to organ boundaries that form as a new organ differentiates (Bleecker and Patterson 1997; McKim et al. 2008). Organ boundary patterning relies on transcription co-factors BOP1/2 and a set of TALE homeodomain proteins defined by the BELL1-like member ATH1 and its KNOX functional partners KNAT2 and KNAT6. (Hepworth and Pautot 2015; Khan et al. 2015). Individually, these genes were shown to be involved in various steps of the abscission process (McKim et al. 2008; Gómez-Mena and Sablowski 2008; Shi et al. 2011). My thesis further explores TALE members of this module. In particular, I show that TALE genes contribute to: 1) the structural changes that follow the initial specification of the AZ (Step 2 in Fig. 4.1), 2) the signalling pathway that activates abscission (Step 3 in Fig. 4.1), and 3) the production of enzymes that achieve separation (Step 3 in Fig. 4.1).

4.1 Structure

4.1.1. Boundary genes maintain structure of the abscission zone

Arabidopsis floral organ AZs undergo two phases of development (Lee et al. 2018). In the first phase, selection of the AZ takes place at sites of restricted growth that define the boundary between a floral organ and the meristem. These cells have the potential to form an active AZ. In the second phase, the AZ differentiates lignified and separation layers of cells that are responsive to IDA signaling (Bleecker and Patterson 1997; Kim 2014).

The initial step of AZ selection requires the boundary genes *BOP1* and *BOP2*. In *bop1 bop2* mutants, the AZ is never defined, blocking all further steps of abscission (McKim et al. 2008). Here, the analysis of mutants shows that *ATH1* and *KNAT2/6* are required in structuring the AZ. In the *ath1 knat2 knat6* triple mutant, an AZ is specified, but organization of the separation and lignified layers is disrupted. Electron microscopy of the receptacle shows irregular, broken, and disorganized cells at the silique base. These triple mutants are unable to shed their floral organs, suggesting that the cell loosening that normally takes place is inhibited. The organization of cells in the lignin brace is also disrupted. The analysis of TALE single mutants revealed differential roles for *ATH1* and *KNAT2/KNAT6* in structuring the AZ. In the *ath1* mutant, the receptacle and lignified layers were noticeably expanded and disorganized. These defects correlate with a strong delay in abscission (Gómez-Mena and Sablowski 2008; this

study). By contrast, structural and abscission defects in the *knat2 knat6* double mutant were slight (Shi et al. 2011; Corrigan 2018; this study). Consistent with a stronger requirement for ATH1 in structuring the AZ, *ATH1* is a direct transcriptional target of BOP1, which is involved in AZ initiation (Khan et al. 2015; McKim et al. 2008).

4.1.2. Boundary genes promote lignin deposition

A lignin brace is formed at the lower edge of separating floral organs. This rigid barrier defines the break plane and prevents the over diffusion of hydrolytic enzymes from the separation layer. Lignin is added to this border as a secondary cell wall modification, meaning that lignin deposition follows the existing shape of the cell wall (Boerjan, Ralph, and Baucher 2003; Freudenberg 1959; C.-J. Liu 2012).

In stems, mutants that mis-express *BOP1/2* or *KNAT6* in combination with *ATH1* or *KNAT2* genes show altered vascular bundling and abnormal deposition of lignin in epidermal and interfascicular tissues (Khan, Tabb, and Hepworth 2012). Over-expression of *BOP1* or *BOP2* expands the lignification of stem tissue (Khan et al. 2012). This lignification is associated with the higher expression of lignin biosynthesis genes, including the cell-wall peroxidase *PRXR9*, which has a proposed role in polymerization of monolignol subunits (Khan et al. 2012). Also expressed in AZs, BOP1 directly activates the *PRXR9* gene (Khan et al. 2012).

As previously reported, little or no lignification of petal or sepal edge cells takes place in the *bop1 bop2* mutant (Lee et al. 2018; this study). The analysis of TALE mutants *ath1*, *ath1 knat2*, *ath1 knat6* and *ath1 knat2 knat6* mutants showed an uneven, disordered lignified layer of cells at the organ border. While lignin was deposited, a cohesive brace was not formed. Interlocking cells in the break plane may resist separation or lack the optimal strength provided by a honeycomb structure (Mackenzie 1999; Lee et al. 2018). Hence, TALE genes, by maintaining the neat rows and uniform size of the cells in the AZ, establish a structural framework for cells that eventually become lignified.

The general mechanism of lignin polymerization involves the joining of monomers in between cellulose fibrils in the cell wall. A large number of enzymes that use hydrogen peroxide can polymerize these subunits via an oxidative coupling reaction (Boerjan, Ralph, and Baucher

2003; Freudenberg 1959; C.-J. Liu 2012). The two main classes of enzymes for coupling are peroxidases and laccases (Berthet et al. 2011; Yahong Li et al. 2003; Barros et al. 2015). These enzymes use hydrogen peroxide generated by NADPH oxidases in the cell wall. Two NADPH oxidase genes, *RBOHD* and *RBOHF*, expressed in the AZ have been implicated (Lee et al. 2018).

The absence of a lignin brace in the *bop1 bop2* mutant might indicate that AZ cells in this region lack the identity required for ROS production. Staining methods in this mutant showed very low production of ROS coupled with transcript analysis showing significantly lower levels of *PRXR9* and *RBOHD* transcripts in *bop1 bop2* and *ath1 knat2 knat6* triple mutants compared to the wild type. By directly comparing the spatial patterns of ROS to lignin structure in the TALE mutants *ath1 knat2*, *ath1 knat6*, and *ath1 knat2 knat6*, the shape of lignification appears to follow the distribution of ROS accumulation. This correlation underlines that ROS patterning relates directly to lignin formation, consistent with the role of ROS as a trigger for lignification in the AZ (Lee et al. 2018).

Interestingly, a lignin brace is not a universal feature among plant species. In some monocots, the AZ does not include a lignified layer, an indication that a lignin brace is not essential for abscission (Lee et al. 2018; Yu et al. 2020). There are also *Arabidopsis* mutants which show that the lignin brace is not a determining factor for normal abscission. For example, the *rbohD rbohF* mutant shows little or no lignin at the sepal edge, yet undergoes normal abscission (Olsson et al. 2019; Supplementary Figure 3.3). Other studies demonstrate the opposite: the non-abscising mutants *ida* and *hae hsl2* form normal lignin braces (Lee et al. 2018). These cases illustrate that the absence or presence of the lignin brace does not directly correspond to competent abscission, meaning a lignin brace alone does not ensure normal abscission, nor does the lack of a brace prevent abscission.

4.1.3. Blocks in abscission prevent cuticle development

The final structural change in the AZ is the formation of a cuticle over newly exposed cells on the plant surface post-abscission (Bleecker and Patterson 1997; Estornell et al. 2013; Kim 2014). Consistent with disordered cells on this surface, the production of a cuticle is impaired in boundary mutants. Wild-type siliques form a sealed layer by position 10, with abscission occurring at about position 6. By contrast, *bop1 bop2* siliques do not form a cuticle

layer even at late positions. The *ath1* single mutant and higher order TALE mutants also failed to form a seal in the AZ. This failure is not surprising, given that abscission is blocked in these mutants and the floral organs cannot be detached without tearing. In the *kmat2 kmat6* mutant, where abscission is almost normal, the AZ remains partially permeable at position 10. This permeability indicates a delay in the sealing step, with the layer becoming impermeable by position 14. What this tells us is that even when the AZ is not physically disrupted in the process of organ removal, timely formation of the cuticle layer is impaired by the loss of TALE gene function.

The lignin brace is suggested to contribute to formation of the cuticle layer. Here, the brace may leave behind a smooth surface for the cuticle to coat. The lipid polymers that make up the epidermal coat are secreted on the outside of AZ cells following abscission, and eventually fill in to create a completely sealed layer (Lee et al. 2018). The lack of a cohesive lignin brace in TALE mutants may interfere with formation of a continuous waxy layer.

Transcriptome analysis of the AZ suggests that cutin is a main component of this sealing layer (Lee et al. 2018). Throughout the plant, cutinous epidermal layers regulate the exchange of water and solutes. Beyond this function, the cutin layer has been shown to prevent infection as more than a passive barrier. When broken down by pathogen-associated cutinases, cutin monomers may act as signals that activate plant defense programs (Underwood 2012; Malinovsky, Fangel, and Willats 2014). The cutin barrier is also dynamic, since pathogen attack can trigger increased lipid synthesis and deposition in the epidermal cells (Marques et al. 2016). As an alternative, the presence of the cuticle layer could be viewed simply as an inherent trait of epidermal cells. Cutin is specific to epidermal cells, and epidermal identity is defined by a cell's position at the surface (Takada and Iida 2014; Stewart and Dermen 1975). In woody species such as citrus, where epidermal identity involves different components for the secondary layer, lignin is more highly represented in the sealing layer, supporting the theory that the cuticle forms as a default characteristic of epidermal cell fate (Agustí et al. 2008; Addicot 1982).

While cuticle formation might be temporally dependent on the completion of the separation step, other evidence ties its formation to the same signalling pathway that triggers abscission. The *35S:IDA* over-expressor shows an overgrown AZ which secretes a white substance identified as arabinogalactan proteins (AGPs). AGPs are a component of epidermal

cell walls and are deposited in low levels after abscission in wild type plants. AGPs are absent in the non-abscising *ida* mutant (Stenvik et al. 2006; Shi et al. 2011). AGPs are proposed to function in a wide range of cellular processes, and are also linked to cell expansion and growth regulation (Seifert and Roberts 2007; Ellis et al. 2010; Addicot 1982). It is possible that AGPs are present in the AZ not as a functional component of the cuticle layer, but as a by-product of the expansion that occurs as the AZ cells become enlarged and rounded.

Inactivation of inhibitors of abscission, such as the *bp* mutant, or the *nev serk1* double mutant also show an over-accumulation of this protein, while over-expression of *IDA*, the initiating signal for the beginning of abscission, also results in increased production of AGPs (Shi et al. 2011; Lewis et al. 2010; Stenvik et al. 2006). This could indicate that beyond their role in the first step of abscission, components of the signalling network which triggers abscission may continue to act in the final step, when the AZ cuticle is formed.

4.2. Signalling

4.2.1. Boundary genes act downstream of *BP*

The KNOX gene *BREVIPEDICELLUS* is one of two TALE homeodomain transcription factors that maintain cells in an undifferentiated state. In performing this role, BP represses boundary genes (Ragni et al. 2008; Khan et al. 2012; Khan, Tabb, and Hepworth 2012).

In abscission, BP acts downstream of *IDA* as a negative regulator of cell separation. The *KNAT2/6* expression domain is expanded and prolonged in the *bp* mutant, indicating that *BP* normally restricts these patterns. Abscission occurs one position early in the *bp* mutant and the AZ is expanded and overgrown (Shi et al. 2011). Introducing a *knat2 knat6* mutation corrects *bp* early abscission showing that KNAT2 and KNAT6 mis-regulation is responsible for *bp* defects (Shi et al. 2011).

As a functional partner to KNAT2/6, ATH1 was predicted to function downstream of BP in the IDA signaling pathway. I used GUS reporter lines to show that *ATH1* is under repression by *BP*. As has already been shown for *KNAT2/6*, the expression of these boundary genes is ectopic and prolonged in the *bp* mutant, indicating that *BP* normally restricts their expression patterns. BP binds to the promoter of *KNAT2* and *KNAT6* as a repressor (Zhao et al. 2015) or may downregulate the activity of these similar KNOX proteins by competing for functional

partners or DNA binding sites on target genes (Hamant and Pautot 2010; Shi et al. 2011). It is not known if BP also directly represses *ATH1* transcription.

Rescue of abscission in *bp knat2 knat6* triple mutants was presumed to correlate with restored cellular structure of the AZ (Shi et al. 2011). I confirmed this prediction using electron microscopy. The AZ overgrowth of the *bp* mutant is clearly reduced by the inactivation of *KNAT2/6*. Taken alone, this might suggest that *KNAT2/6* are directly responsible for the cell-rounding and expansion of the AZ that precedes organ separation. But inactivation of the TALE genes also results in disordered and expanded AZs, and ectopic expression of *KNAT2/6* or *ATH1* does not cause overgrowth of the AZ (Shi et al. 2011; Corrigan 2018). While the TALE genes have a role in AZ expansion, it must be partial. These conflicting effects may also point to separate timing of two phases of AZ development: the cell-division step, in which the AZ grows as the organ forms, and then a separate phase of cell expansion that causes rounding and loosening of the cells in this area just before abscission. The *bp* AZ is overgrown, but more specifically, has more cells in the AZ (Shi et al. 2011). The TALE boundary genes may both promote cell division, which is important as the AZ develops to maintain small and orderly cells, and additionally promote cell expansion in the form of cell rounding just before abscission occurs.

4.2.2. Boundary genes act downstream of IDA signalling

The binding of the IDA ligand to HAESA and HAESA-LIKE2 receptors begins the signalling pathway for separation (Butenko et al. 2003; Jinn, Stone, and Walker 2000). Genetic experiments placed *KNAT2/6* downstream of IDA by showing that inactivation of this gene pair blocks the early abscission phenotype of *35S:IDA* plants (Shi et al. 2011). Mutation of *ATH1* also blocks *35S:IDA* effects but structural defects in the AZ caused by *ath1* mutation are severe and cells may not appropriately respond to IDA signals. Since *ATH1* contributes with *BOP1/2* to initial formation of the AZ, it makes sense that abscission defects in *bop1 bop2* and *ath1* mutants cannot be rescued by *IDA* over-expression (McKim et al. 2008; this study).

In reciprocal experiments, an *ida* mutant crossed to *KNAT6-oe* showed rescue of the *ida* abscission defect (Shi et al. 2011; this study). Abscission is also restored in *ida BOP1-oe* and *ida BOP2-oe* plants, but *ida ATH1-oe* mutants showed an abscission defect similar to *ida* alone. *KNAT6* might hold an independent or more central role in the IDA signaling pathway compared

to ATH1. Interestingly, *KNAT2:GUS* and *KNAT6:GUS* reporter gene expression was reduced in the abscission and dehiscence zones of *ath1* mutant fruits compared to the wild type, showing that ATH1 can promote the expression of these genes (Popescu 2015). Thus, ATH1 activity in the IDA signaling pathway might depend on the availability of functional partners.

Surprisingly, the over-expression of *BOP1/2* or *ATH1/KNAT6* genes failed to restore abscission in a *hae hsl2* mutant. This could mean that HAE/HSL2 have independent roles in abscission, beyond their interaction with IDA ligands. While IDA and HAE HSL2 have been found generally to exert control over similar genes promoting abscission, transcriptome analysis indicates that a larger number of genes are differentially regulated by *HAE/HSL2* (B. Liu et al. 2013). Interestingly, HAE phosphorylation triggers a positive feedback loop, prompting its own expression after binding to IDA. The expression levels of *HAE* were shown to increase 27 fold in the stages just before abscission through this amplifying loop (Patharkar and Walker 2015). Inactivation of the *HAE/HSL2* complex may inhibit additional functions, which are not addressed by increasing the expression of boundary genes.

The production of additional receptors by a *HAE* positive feedback loop is likely to involve the vesicle network that brings proteins to the cell membrane during abscission (Burr et al. 2011; Patharkar and Walker 2018). *NEVERSHED* encodes an ARF-GAP protein associated with regulating vesicular transport between the ER and the cell membrane (Liljegren et al. 2009). Inactivation of *NEV* leads to defective abscission which appears to be independent of the *IDA* signalling pathway (B. Liu et al. 2013). There are several key points during abscission in which components must be moved to the cell membrane, such as the transport of new HAESA receptors to the cell surface, and the exocytosis of hydrolytic enzymes for cell separation. *NEV* is presumed to play a role in this cellular transport (Liljegren et al. 2009; Patharkar and Walker 2018). While the network which regulates vesicular transport during abscission is not fully understood, three receptor-like kinases have been shown to negatively regulate *NEV* function in order to inhibit premature abscission. Mutations in *CASTAWAY*, *EVERSHED*, or *SERK1* all restore abscission in the *nev* mutant (Leslie et al. 2010; Lewis et al. 2010; Groner et al. 2016). There is also evidence that *SERK1* may be a negative regulator of HAESA abundance at the protein level, by sequestering HAESA receptors away from the cell membrane prior to IDA binding (Lewis et al. 2010).

Functional redundancy may also explain why the *ida* abscission defect is more easily complemented by over-expression of boundary genes compared to the *hae hsl2* defect. The *Arabidopsis* genome includes four other IDA-like genes (IDLs), which code for peptides with similar structures to IDA. Three of these IDLs are normally expressed at low levels in the AZ. Over expression of these IDLs results in a phenotype similar to the *35S:IDA* phenotype, with early and ectopic expression, as well as an overgrown AZ. Over-expression of IDLs also resulted in partial rescue of the *ida* mutant abscission defect (Stenvik et al. 2008). This implies that though they do not normally appear to play a role in abscission signalling, these peptides are capable of standing in for IDA. Though this does not fill in the gaps as to what other roles *HAESA* and *HSL2* may have in abscission, the partial functional redundancy between *IDA* and *IDLs* supports that missing the ligand may be more repairable than missing the receptor complex.

4.3. Separation

4.3.1. Boundary genes promote hydrolytic enzymes

Functional studies suggest a final role for boundary genes in the promotion of hydrolytic genes for separation. Plants that overexpress *BOP1* shed their organs about one position earlier than wild type, suggesting that BOP1 promotes separation (Corrigan 2018). The force required to remove floral organs in *KNAT6-oe* was lower than in wild type, indicating that *KNAT6* can also promote cell loosening (Shi et al. 2011).

The middle lamella that binds the cell walls of neighbouring cells in the AZ is rich in proteins and pectin. A wide range of hydrolyzing enzymes promotes its dissolution (Roberts, Elliott, and Gonzalez-Carranza 2002). Previous studies have investigated the transcriptional profiles of *ida* and *hae hsl2* mutants to determine hydrolytic enzyme genes targeted by the IDA signaling pathway. Compared to wild type, these backgrounds show reduced expression of many enzyme genes that encode polygalacturonases, cellulases, extensins, and xyloglucanases. Two key polygalacturonases activated in abscission are PGAZAT and QRT2, which degrade pectin (Ogawa et al. 2009). The double mutant *pgazat qrt2* has an abscission defect, underscoring that these genes play an important role in organ separation (Ogawa et al. 2009). Both of these enzyme genes are downregulated in *ida* or *hae hsl2* mutants, suggesting that the IDA signalling pathway, which boundary genes act in, promotes their expression (Niederhuth, Patharkar, et al. 2013).

The expression of *IDA* is not significantly altered in either the *bop1 bop2* or *ath1 knat2 knat6* mutants suggesting that abscission signaling is initiated in these mutants. A reduction in transcripts for *PGAZAT*, *QRT2*, *CEL3* and xyloglucanase genes *XTH18* and *XTH28* in *bop1 bop2* and *ath1 knat2 knat6* compared to wild-type AZ could indicate that signal transduction is blocked or that boundary genes play a direct role in promoting the expression of hydrolytic genes required for separation. Future experiments will test if the regulation of these enzyme genes is direct.

4.4. Conclusion and future directions

In summary, the function of boundary genes goes well beyond basic AZ architecture. The initial step of AZ selection requires the boundary genes *BOP1* and *BOP2*. A conserved module of TALE boundary genes works to pattern the AZ, specify two functionally distinct layers, and promote the production of separation enzymes as a part of the signalling pathway that triggers abscission. Many components of the abscission process are conserved across crop species, meaning that molecular genetic approaches could allow breeders to modify abscission for improved crops.

The study of abscission offers multiple avenues to crop improvement. Besides the abscission of shoot organs, cell separation plays a less-studied role in root architecture (Shi et al. 2019). Roots grow outwards from a pool of stem cells, called the root apical meristem (RAM), near the tip of the root. The root cap covers these stem cells and undergoes constant sloughing as a new layer of root cap cells forms below the older ones (Kumpf and Nowack 2015). The emergence of lateral roots also relies on cell separation (Péret et al. 2009). It is important to note that this process is distinct from abscission, in that no specific zone delineates the point of separation in the roots, but overlap exists (Addicot 1982). Recent study has shown that in this less explored example of cell separation, abscission signalling components are conserved. A member of the IDA-like family of peptides, IDL1, and the HSL2 receptor have been shown to regulate the shedding of root cap cells, mediating the balance between stem cells and differentiated organs (Shi et al. 2018). Lateral root emergence shows even greater overlap, the IDA signalling peptide and the HAE/HSL2 receptor pair are required for normal lateral root formation in *Arabidopsis* (Kumpf et al. 2013). If boundary genes are also conserved in this

separation event as is suggested by their expression in roots, root architecture may represent a new avenue for research.

Figure 4.1. Boundary genes have multiple roles in abscission. In step 1, the BOPs and TALE genes contribute to the cellular identity and structure of the AZ. Later, in step 3, after IDA signalling initiates abscission, the BOPs and TALE genes promote the gene expression of hydrolytic enzymes, which carry out the cell separation. It is not known if promotion of hydrolytic enzyme genes is direct or indirect, as consequence of defects in cell identity.

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6. Supplementary Material

Supplementary Table S1. Quantitative scoring of cuticle permeability

Genotype	First impermeable position	Scoring (n)
Wild type	8	14/14
<i>knat2-5</i>	8	12/12
<i>knat6-1</i>	8	12/12
<i>bop1 bop2</i>	permeable throughout	12/12
<i>knat2 knat6</i>	10	11/12
<i>ath1-3</i>	permeable throughout	12/12
<i>ath1 knat2</i>	permeable throughout	12/12
<i>ath1 knat6</i>	permeable throughout	12/12
<i>ath1 knat2 knat6</i>	permeable throughout	12/12
<i>rbohD rbohF</i>	8	12/12
<i>nev-7</i>	8	12/12
<i>bop1-6D</i>	6	10/12

Supplementary Table S2. Quantitative scoring of DAB staining

Genotype	Scoring (n)	Phenotype description
Wild type	153/161	strong staining
<i>knat2-5</i>	158/168	strong staining
<i>knat6-1</i>	169/181	strong staining
<i>bop1 bop2</i>	163/175	absent staining
<i>knat2 knat6</i>	168/171	strong staining
<i>ath1-3</i>	164/170	strong staining
<i>ath1 knat2</i>	159/170	strong staining
<i>ath1 knat6</i>	136/156	reduced and mis-localized staining
<i>ath1 knat2 knat6</i>	140/163	highly reduced and mislocalized staining
<i>rbohD rbohF</i>	143/162	reduced staining
<i>nev-7</i>	150/155	diffuse, strong staining
<i>bop1-6D</i>	158/158	strong staining

Supplementary Table S3. Quantitative scoring of lignin brace (on sepal edge)

Genotype	Scoring (n)	Phenotype description
Wild type	154/158	normal
<i>knat2-5</i>	157/160	normal
<i>knat6-1</i>	156/158	normal
<i>bop1 bop2</i>	162/162	absent
<i>knat2 knat6</i>	172/173	present but disordered
<i>ath1-3</i>	165/171	present but disordered
<i>ath1 knat2</i>	158/163	present but disordered
<i>ath1 knat6</i>	140/145	present but disordered
<i>ath1 knat2 knat6</i>	148/166	present but disordered
<i>rbohD rbohF</i>	159/165	absent
<i>nev-7</i>	155/159	absent
<i>bop1-6D</i>	155/158	normal (slightly fewer rows of cells)

Supplementary Figure 3.1. Receptacle AZ layer in *knat2* and *knat6* mutants. Top row, SEM images of AZ on receptacle at position 10. Middle row, lipid stain using fluorol yellow, photographed at 405 nm wavelength with GFP filter. Nectaries are brightly stained with fluorol yellow. Bottom row, cuticle permeability assay showing representative images of position 10 siliques after immersion in aqueous toluidine blue. A-B, AZ morphology and cuticle permeability in *knat2* and *knat6* mutants is comparable to wild type (see Figure 3.2). For SEM, at least 6 siliques from individual plants were imaged. For lipid and cuticle assays, at least 12 siliques from individual plants were imaged. Representative photos are shown. Scale bars: 100 μm (SEM) and 250 μm (lipid and cuticle assays).

Supplementary Figure 3.2. AZ morphology in the *nevershed* and *rbohD rbohF* mutant lines.

A,B, lignified layer. A, *nev* shows higher, mis-localized ROS accumulation, and no lignin brace when compared to the wild type. B, *rbohD rbohF* mutant shows slightly reduced ROS and no lignified layer. Lignin autofluorescence was visualized at 405 nm wavelength with a GFP filter. For ROS and lignin staining, sepals were dissected from position 5 flowers on at least 20 different plants. At least 60 sepals were scored per genotype. Representative images were chosen. Scale bars: 200 μ m (bright field compound microscope images) and 100 μ m (fluorescent compound microscope images). C,D, Analysis of cuticle formation in *nev* and *rbohD rbohF* mutants AZs. Top row, SEM images of receptacles at position 10. Middle row, lipid stain using fluorol yellow, photographed at 405 nm wavelength with GFP filter. Nectaries are brightly stained with fluorol yellow. Bottom row, cuticle permeability assay. Representative images of position 10 siliques after immersion in aqueous toluidine blue. C, *nev* exposed cells on the receptacle are expanded and disordered, cuticle permeability is comparable to wild type. D, *rbohD rbohF* mutant shows wild-type AZ morphology on the receptacle and cuticle permeability. For SEM, at least 6 siliques from individual plants were imaged. For lipid and cuticle assays, at least 12 siliques from individual plants were imaged. Representative photos are shown. Scale bars: 100 μ m (SEM) and 250 μ m (lipid and cuticle assays).

Supplementary Figure 3.3. Lignified layer in *knat2* and *knat6* mutants. ROS distribution and lignin in the lignified layer of *knat2* and *knat6* mutants is comparable to wild type. Lignin autofluorescence was visualized at 405 nm wavelength with a GFP filter. For ROS and lignin staining, sepals were dissected from position 5 flowers on at least 20 different plants. At least 60 sepals were scored per genotype. Representative images were chosen. Scale bars: 200 μm (bright field compound microscope images) and 100 μm (fluorescence compound microscope images).

Supplementary Figure 3.4. Morphology of wild-type and *BOP1-oe* mutant lignified layer and receptacle AZ. A, sepal lignified layer of *bop1-6D* mutant showing higher ROS accumulation (top row) , and uniform but shallow lignin brace compared to the wild type (Col) (middle and bottom images). Lignin autofluorescence was visualized at 405 nm wavelength with a GFP filter. For ROS and lignin staining, sepals were dissected from position 5 flowers on at least 20 different plants. At least 60 sepals were scored per genotype. Representative images were chosen. Scale bars: 200 μ m (bright field compound microscope images) and 100 μ m (fluorescence compound microscope images). B, Analysis of cuticle formation in wild type and *bop1-6D* mutant AZs. Top row, SEM images of wild-type and *bop1-6D* receptacle at position 10. Middle row, lipid stain using fluorol yellow, photographed at 405 nm wavelength with GFP filter. Nectaries are brightly stained with fluorol yellow. Bottom row, cuticle permeability assay. Representative images of position 10 siliques after immersion in aqueous toluidine blue. *bop1-6D* mutant showing an impermeable barrier to toluidine blue at position 6, which is slightly earlier than the wild type. For SEM, at least 6 siliques from individual plants were imaged. For lipid and cuticle assays, at least 12 siliques from individual plants were imaged. Representative photos are shown. Scale bars: 100 μ m (SEM) and 250 μ m (lipid and cuticle assays).