The following individuals certify that they have read, and recommend to the Faculty of Graduate and Postdoctoral Studies for acceptance, the dissertation entitled:

Engineering cell wall carbohydrate composition in Arabidopsis thaliana seed mucilage as a means to understand the plant cell wall

submitted by Robert Thomas McGee in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Botany

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Abstract

The seed coat epidermal cells of *Arabidopsis thaliana* synthesize and secrete large quantities of mucilage, a specialized secondary cell wall which is released from mature seeds upon hydration. The goal of this thesis was to study the biological and biochemical consequences of modifying the structure of cell wall carbohydrates using cell wall degrading enzymes as a tool. To develop seed mucilage as a model, promoters that would only drive expression of genes encoding cell wall degrading enzymes within the mucilage-producing epidermal cells were utilized. In order to demonstrate their ability to promote gene expression sufficiently to modify mucilage, the seed coat-specific promoters from the following genes; *TESTA-ABUNDANT2 (TBA2)*, *PEROXIDASE36 (PER36)*, and *MUCILAGE-MODIFIED4 (MUM4)*, were fused upstream of *MUCILAGE-MODIFIED2 (MUM2)*, a gene that encodes a known mucilage modifying enzyme, which were transformed into a *mum2* mutant background (Chapter 3). All three promoters were shown to be able to drive sufficient expression of *MUM2*, in a spatial and temporal pattern to manipulate mucilage composition and complement the *mum2* phenotypes. The strongest of the three promoters, *TBA2p*, was then used to examine the ability of three previously uncharacterized MUM2 homologs; BGAL11, BGAL16 and BGAL17, to complement the *mum2* extrusion and cell wall compositional phenotypes (Chapter 3). It was found that cytological and biochemical complementation of *mum2* varied and correlated with the amino acid sequence similarity of the homologous gene products to MUM2.

Consistent with the fact that the pectin rhamnogalacturonan-I (RG-I) is the major component of seed mucilage, transgenic plants expressing genes encoding RG-I degrading enzymes driven by *TBA2p* produced seeds with very little mucilage and greatly reduced levels of
sugars comprising RG-I (Chapter 4). Unexpectedly, modifications to a minor component of seed mucilage, homogalacturonan (HG), using HG-degrading enzymes driven by TBA2p, resulted in reduced cell adhesion, no mucilage pocket formation and cell death in developing seed coat epidermal cells (Chapter 5), highlighting HG’s essential role in the cell wall. In summary, the data represented in this thesis has demonstrated the feasibility of manipulating cell wall carbohydrate composition using a genetic engineering approach to explore the relationships between structure and function within the plant cell wall.
Lay Summary

Unlike in animals, plant cells are surrounded by a cell wall that provides structural support that is analogous to the concrete walls of a building. As in a concrete wall, removing or modifying components of the cell wall typically result in negative consequences to the plant. However, the cell walls within the outer most (epidermal) cell layer of the seed coat in the model plant, *Arabidopsis thaliana* can be modified without negatively impacting the rest of the plant. In this doctoral research, two components of the plant cell wall, rhamnogalacturonan-I (RG-I) and homogalacturonan (HG), were specifically modified in the seed coat epidermal layer by introducing cell wall degrading enzymes. While the consequences of modifying RG-I was consistent with our prior knowledge, degrading HG unexpectedly resulted in loss of cell adhesion and cell death, pointing towards the importance of this component in the plant cell wall.
Preface

A version of Chapter 3 has been submitted for peer-review. The article was entitled: “Assessing the utility of seed coat-specific promoters to engineer cell wall polysaccharide composition of mucilage”. The authors of this research article are as follows: Robert McGee (RM), Gillian H. Dean (GHD), Shawn D. Mansfield (SDM) and George W. Haughn (GWH). RM designed and conducted research, analyzed data, and wrote the article. GHD designed and conducted research, and analyzed data. GWH designed research, analyzed data, and wrote the article. SDM designed research, analyzed data, provided equipment, developed the analytical methods, and contributed to revisions of the article.

A version of Chapters 4 and 5 will be combined into a research article that will be submitted for peer-review with the following co-authors RM, SDM, GWH. RM designed all experiments, collected the data presented, and wrote the article. GWH supervised the experimental design, oversaw data analysis and edited the article. SDM provided equipment, developed the analytical methods, and contributed to revisions of the article.
Table of Contents

Abstract........................................................................................................................................ iii
Lay Summary .................................................................................................................................. v
Preface........................................................................................................................................... vi
Table of Contents ............................................................................................................................ vii
List of Tables .................................................................................................................................... xii
List of Figures ................................................................................................................................... xiii
List of Symbols and Abbreviations ................................................................................................. xvi
Acknowledgements ......................................................................................................................... xx
Dedication ......................................................................................................................................... xxii

Chapter 1: Introduction ..................................................................................................................1

1.1 The important role of the plant cell wall...................................................................................... 1
1.2 Composition of the cell wall ....................................................................................................... 2
1.3 The complex structure of pectins .............................................................................................. 3
1.4 The essential role of pectin within the cell wall ......................................................................... 6
1.5 Seed mucilage and its adaptive role .......................................................................................... 8
1.6 Advantages of using seed mucilage to study the cell wall ......................................................... 9
1.7 Development of the mucilage-producing seed coat epidermal cells ....................................... 10
1.8 Composition of the seed mucilage: pectin, RG-I ....................................................................... 13
1.9 Composition of the seed mucilage: pectin, HG ......................................................................... 14
1.10 Composition of the seed mucilage: hemicelluloses ................................................................. 15
1.11 Composition of the seed mucilage: cellulose ...................................................... 17
1.12 Composition of the seed mucilage: proteins .................................................... 17
1.13 Research objectives ......................................................................................... 19

Chapter 2: Materials and methods ....................................................................... 23

2.1 Plant materials and growth conditions ............................................................... 23

2.2 Plasmid construction ......................................................................................... 24
  2.2.1 Gateway entry clones ................................................................................ 24
  2.2.2 Gateway destination vectors ..................................................................... 27

2.3 Plant transformation ......................................................................................... 34

2.4 Microscopy ....................................................................................................... 34
  2.4.1 Bright-field microscopy ............................................................................ 34
    2.4.1.1 Seed mucilage analysis ................................................................. 34
    2.4.1.2 Videoming mucilage extrusion ...................................................... 34
    2.4.1.3 Trypan blue staining .................................................................... 35
  2.4.2 Confocal fluorescence microscopy ............................................................ 35

2.5 High-pressure freezing and sectioning ............................................................... 36

2.6 Scanning electron microscopy ........................................................................ 37

2.7 Total RNA extraction and quantitative real-time PCR (qRT-PCR) .................... 37

2.8 Phylogenetic analysis, domain architecture and sequence alignments .......... 39

2.9 Thin layer chromatography (TLC) .................................................................. 40

2.10 Monosaccharide analysis ................................................................................ 41

2.11 Statistical analysis ......................................................................................... 42
Chapter 3: Assessing the utility of seed-coat specific promoters to engineer cell wall carbohydrate composition of mucilage ........................................................................................................43

3.1 Introduction ......................................................................................................................................................... 43

3.2 Results ................................................................................................................................................................... 44

3.2.1 Complementation of the mum2 mucilage extrusion phenotype using different seed coat-specific promoters to drive MUM2-Citrine ........................................................................................................ 44

3.2.2 Confocal fluorescence intensity of MUM2-Citrine correlates with the degree of mum2 complementation ........................................................................................................................................ 49

3.2.3 The TBA2 promoter produces the highest abundance of MUM2-Citrine transcript. ........................................ 51

3.2.4 Seed coat-specific promoters can be used to assess whether candidate BGALs are functionally equivalent to MUM2/BGAL6 ...................................................................................................................................... 54

3.2.5 Complementation of mum2 extrusion using three candidate BGALs .......................................................... 57

3.2.6 Biochemical complementation of mum2 using three candidate BGALs ..................................................... 61

3.3 Discussion ............................................................................................................................................................... 65

3.3.1 Complementation of mum2 depends on the relative strength of seed coat-specific promoters .............................................................................................................................................................. 65

3.3.2 Members of the GH35 enzyme family vary in their ability to complement mum2 .. 67

Chapter 4: Modification of the pectin, RG-I in seed mucilage led to changes in the properties of mucilage .............................................................................................................................................69

4.1 Introduction .............................................................................................................................................................. 69

4.2 Results ................................................................................................................................................................... 71

4.2.1 Secretion of RG-I degrading enzymes to the mucilage pocket resulted in loss of the adherent mucilage layer ........................................................................................................................................... 71
4.2.2 Degradation of RG-I by RglA or RglB result in small mucilage pockets ............ 73
4.2.3 Unusual non-uniform, striped pattern of fluorescence in the small mucilage pockets found in RglA-Citrine and RglB-Citrine transgenic seeds...................................................... 75
4.2.4 Rha and GalA levels are greatly reduced in extruded mucilage and whole seeds from RglA-Citrine and RglB-Citrine transgenic plants ................................................................. 81
4.2.5 Detection of Gal-Rha disaccharides in the extruded mucilage................................. 82
4.3 Discussion................................................................................................................. 86
  4.3.1 Targeted degradation of RG-I in the mucilage pocket led to unexpected decreases in total Rha and GalA................................................................. 86
  4.3.2 Potential salvaging of degraded RG-I fragments from the mucilage pocket......... 87
  4.3.3 Could cell wall components be unevenly distributed within the mucilage pocket... 88

Chapter 5: Investigating the structural role of mucilage HG by secreting HG-degrading enzymes to the mucilage pocket................................................................. 91
  5.1 Introduction........................................................................................................... 91
  5.2 Results................................................................................................................ 93
    5.2.1 Expression of genes encoding HG-degrading enzymes in transgenic seeds resulted in little to no adherent mucilage layer.................................................. 93
    5.2.2 Citrine fluorescence was localized to the radial cell walls and sometimes also in the cytosol in seed coat epidermal cells expressing Citrine-ADPG2 or PelA-Citrine......... 96
    5.2.3 Secretion of ADPG2 and PelA in the cell wall coincided with the separation of seed coat epidermal cells.......................................................... 98
    5.2.4 Compromised membranes and irregular shaped nuclei suggests the occurrence of cell death in 7 DPA developing seeds expressing Citrine-ADPG2 or PelA-Citrine .......... 99
5.3 Discussion.................................................................................................................................................. 103

5.3.1 Cell adhesion was disrupted when expressing genes encoding HG-degrading enzymes.................................................................................................................................................. 104

5.3.2 Possible explanations for why cell death appears to be induced in the Citrine-ADPG2 or PelA-Citrine transgenic seeds .................................................................................................................................................. 104

Chapter 6: Conclusions .................................................................................................................................. 108

6.1 Summary of major findings ...................................................................................................................... 108

6.1.1 Objective 1: Determine if seed coat-specific promoters can be used for modifying mucilage, by testing their ability to complement the mum2 phenotype when driving MUM2 expression (Chapter 3) .................................................................................................................................................. 108

6.1.2 Objective 2: Are all BGALs in the GH35 enzyme family functionally equivalent to MUM2 (BGAL6) in their ability to modify the structure of mucilage? (Chapter 3) .......... 109

6.1.3 Objective 3: Modify the structure of the well-known, abundant mucilage component RG-I and assess the resulting changes to the properties of mucilage (Chapter 4) ................. 109

6.1.4 Objective 4: Modify the structure of the less abundant, less well-known mucilage component HG and assess how these modifications impact the properties of mucilage (Chapter 5) .................................................................................................................................................. 110

6.2 Perspectives and future directions ........................................................................................................ 111

6.2.1 Using the seed coat-specific expression system to investigate other mucilage components and the predicted linkages between them .................................................................................. 114

6.2.2 Agricultural and industrial benefits to reducing the levels of mucilage .......... 115

References ...................................................................................................................................................... 117
List of Tables

Table 2-1: Primer sequences. ................................................................. 27
Table 2-2: Summary of plasmids and chimeric constructs. ................................ 31
Table 2-3: qRT-PCR primer sequences. ......................................................... 39
Table 3-1: Summary of transcript abundance, window of expression, inclusion of a 5’ UTR, and complementation efficiency between different seed coat-specific promoters. ................................. 54
List of Figures

Figure 1-1: Structure of the four main pectin types................................................................. 4

Figure 1-2: Differentiation of the seed coat epidermal cells...................................................... 11

Figure 3-1: Complementation of the mum2 mucilage phenotype using three different promoters
   driving MUM2-Citrine expression in seed coat epidermal cells. ........................................ 46

Figure 3-2: Patchy mucilage extrusion observed in a small number of transgenic seeds where
   MUM2-Citrine expression was driven by either PER36p or PER36modp.................................. 47

Figure 3-3: Mucilage extrusion from seeds of plants transformed with different seed coat-specific
   promoters driving MUM2-Citrine............................................................................................. 47

Figure 3-4: Apoplastic localization of MUM2-Citrine in the seed coat epidermal cells at the
   beginning (4 DPA) and end of mucilage synthesis and secretion (10 DPA).............................. 50

Figure 3-5: MUM2-Citrine transcript abundance in 7 DPA transgenic seeds............................... 53

Figure 3-6: Phylogenetic analysis and predicted domain architecture of Arabidopsis BGALs
   from the GH35 enzyme family. ............................................................................................... 57

Figure 3-7: Mucilage phenotypes of mum2 plants transformed with the TBA2 promoter
   expressing different candidate BGALs either untagged or fused in-frame to a C-terminal Citrine
   yellow fluorescent protein........................................................................................................ 58

Figure 3-8: Complementation of the mum2 mucilage phenotype using candidate BGALs driven
   by the TBA2 promoter............................................................................................................... 59

Figure 3-9: Mucilage monosaccharide composition of wild-type, mum2, and mum2
   complemented with BGAL6-Citrine, BGAL11-Citrine, BGAL16-Citrine or BGAL17-Citrine.... 63
Figure 3-10: Mucilage monosaccharide composition of a second biological replicate of wild-type, mum2, and mum2 complemented with MUM2-Citrine, BGAL11-Citrine, BGAL16-Citrine, or BGAL17-Citrine.......................................................... 64

Figure 4-1: Seed coat mucilage phenotypes and Citrine fluorescence localization during seed development.................................................................................................................................... 72

Figure 4-2: Seed coat mucilage phenotypes of WT (Col-2) transformed with untagged RG-I degrading enzymes........................................................................................................................................ 73

Figure 4-3: Sections of developing epidermal cells and seed surface morphology of dried mature seeds.................................................................................................................................................. 74

Figure 4-4: Localization of the plasma membrane by crossing VAMP721p::mRFP-VAMP721 (VAMP721). ........................................................................................................................................ 76

Figure 4-5: Seed coat mucilage phenotypes and Citrine fluorescence in doubly transgenic plants containing TBA2p::MUM2sp-RglB and TBA2p::MUM2sp-Citrine. ...................................................................................... 78

Figure 4-6: FM 4-64 staining of the plasma membrane in RglB-Citrine transgenic seeds (g-l) and mum2 transformed with TBA2p::MUM2-Citrine (a-f). ...................................................................................... 79

Figure 4-7: Schematic diagram of plasma membrane and Citrine fluorescence localization in 7 DPA seed coat epidermal cells in WT (a) compared to RglB-Citrine transgenic lines (b). .... 80

Figure 4-8: Mucilage and whole seed monosaccharide composition from WT (Col-2) plants transformed with RglA-Citrine (a, c, e) or RglB-Citrine (b, d, f). ......................................................... 82

Figure 4-9: Separation of sugars using thin layer chromatography of soluble sugars extracted from whole seeds and extruded mucilage. .......................................................................................... 85

Figure 4-10: Thin layer chromatography of extruded mucilage from inactivated RglB lines..... 86
Figure 4-11: Two models to explain how the complex mucilage pockets in the *RglB-Citrine* transgenic epidermal cells might have arisen. .............................................................. 90

Figure 5-1: Seed coat mucilage phenotypes and Citrine fluorescence in transgenic seeds expressing HG-degrading enzymes. ............................................................ 94

Figure 5-2: Transgenic seeds containing untagged HG-degrading enzymes.......................... 95

Figure 5-3: Video recording of mucilage extrusion in WT (Col-2) compared to plants expressing HG-degrading enzymes (ADPG2 or PelA)...................................................... 95

Figure 5-4: FM 4-64 staining of the plasma membrane in transgenic *Citrine-ADPG2* or *PelA-Citrine* lines compared to *mum2* complemented with *TBA2p::MUM2-Citrine* as a control....... 97

Figure 5-5: Ruptured outer primary cell walls suggest cell wall weakening in *Citrine-ADPG2* or *PelA-Citrine* transgenic lines. ................................................................. 99

Figure 5-6: Propidium iodide staining of 4 and 7 DPA seed coat epidermal cells in WT (Col-2) compared to *Citrine-ADPG2* and *PelA-Citrine* transgenic lines. ................................. 101

Figure 5-7: Trypan blue staining in 4 and 7 DPA developing seeds from WT (Col-2) compared to *Citrine-ADPG2* and *PelA-Citrine* transgenic seeds.............................................. 101

Figure 5-8: DAPI (nuclear) staining of 7 DPA developing seed coat epidermal cells in WT (Col-2) seeds compared to *Citrine-ADPG2* and *PelA-Citrine* transgenic lines. ......................... 102

Figure 5-9: Scanning electron micrographs of mature seeds from WT (Col-2) and transgenic lines containing *Citrine-ADPG2* or *PelA-Citrine*. ..................................................... 102
### List of Symbols and Abbreviations

<table>
<thead>
<tr>
<th>Symbol</th>
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<tbody>
<tr>
<td>35S</td>
<td>35S RNA promoter from the Cauliflower mosaic virus</td>
</tr>
<tr>
<td>aa</td>
<td>amino acids</td>
</tr>
<tr>
<td>ADPG2</td>
<td><em>ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE2</em></td>
</tr>
<tr>
<td>Ara</td>
<td>arabinose</td>
</tr>
<tr>
<td>BGAL</td>
<td>β-galactosidase</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BXL1</td>
<td>β-<em>XYLOSIDASE1</em></td>
</tr>
<tr>
<td>C</td>
<td>carbon</td>
</tr>
<tr>
<td>C-</td>
<td>carboxyl</td>
</tr>
<tr>
<td>CAZy</td>
<td>carbohydrate-active enzyme</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>Citrine</td>
<td>An acidic stable variant of the yellow fluorescent protein</td>
</tr>
<tr>
<td>Col</td>
<td>Columbia <em>Arabidopsis thaliana</em> ecotype</td>
</tr>
<tr>
<td>DAPI</td>
<td>4′,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DM</td>
<td>degree of methyl-esterification</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DPA</td>
<td>days post anthesis</td>
</tr>
<tr>
<td>EC</td>
<td>Enzyme Commission</td>
</tr>
<tr>
<td>ECH</td>
<td><em>ECHIDNA</em></td>
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<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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EXO70A1  EXOCYST SUBUNIT EXO70 FAMILY PROTEIN A1

Gal  galactose
GalA  galacturonic acid
GAPC  glyceraldehyde-3-phosphate dehydrogenase C subunit 1
GH  glycoside hydrolase
Gluc  glucose
Glucp  glucopyranose
GM  glucomannan
GGM  galactoglucomannan
HG  homogalacturonan
HIS  histidine
IRX  IRREGULAR XYLEM
LUT  look up table
M  Mutagenized
Man  mannose
MCA1  Mid1-Complementing Activity protein
mRFP  monomeric red fluorescence protein
MUM  MUCILAGE-MODIFIED
MUM2sp  MUM2 signal peptide
N  amino-terminus
O-  carbon position
OGA  oligogalacturonide
p  promoter
<table>
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<tr>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PER36</td>
<td><em>PEROXIDASE36</em></td>
</tr>
<tr>
<td>PG</td>
<td>polygalacturonase</td>
</tr>
<tr>
<td>PL4</td>
<td>polysaccharide lyase 4</td>
</tr>
<tr>
<td>PME</td>
<td>pectin methylesterase</td>
</tr>
<tr>
<td>PMEI</td>
<td>PME inhibitor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>quantitative real-time PCR</td>
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<tr>
<td>QUA</td>
<td><em>QUASIMODO</em></td>
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<tr>
<td>Rf</td>
<td>retardation factor</td>
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<tr>
<td>RG-I</td>
<td>rhamnogalacturonan-I</td>
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<td>rpm</td>
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<td><em>SUBTILISIN-LIKE SERINE PROTEASE 1.7</em></td>
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<td>SEC8</td>
<td><em>secretion mutant 8</em></td>
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<tr>
<td>SNARE</td>
<td>N-ethylmaleimide sensitive factor attachment protein receptor</td>
</tr>
<tr>
<td>SUEL</td>
<td>sea urchin egg lectin</td>
</tr>
<tr>
<td>t-</td>
<td>terminal</td>
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<tr>
<td>Acronym</td>
<td>Definition</td>
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<td>------------</td>
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<td>TBA2</td>
<td>TESTA-ABUNDANT2</td>
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<tr>
<td>TUNEL</td>
<td>deoxynucleotidyl transferase dUTP nick-end labeling</td>
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<td>VESICLE-ASSOCIATED MEMBRANE PROTEIN</td>
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<td>Wall-associated kinase</td>
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<td>WT</td>
<td>wild-type</td>
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To the memory of my late grandfather, Dr. Robert Nigel Gourlay.
Chapter 1: Introduction

1.1 The important role of the plant cell wall

One of the defining features of the plant kingdom is the cell wall that surrounds every plant cell (Zamil and Geitmann 2017). The cell wall is essential for a plant’s survival by playing a key role in maintaining the cell’s integrity by withstanding the internal positive osmotic pressure (Somerville et al. 2004; Cosgrove 2005). In addition, in multicellular land plants, the cell wall provides physical support to allow the plant to remain upright (Cosgrove 2005; Cosgrove 2018). While the cell wall typically remains rigid, it can also be selectively loosened by cell wall modifying enzymes which enable the cell to expand and elongate during expansive growth (Jarvis et al. 2003; Somerville et al. 2004; Cosgrove 2005). Cell walls are also the interface between adjacent cells where they are important for intercellular communication and play a critical role in maintaining cell adhesion (Jarvis et al. 2003; Keegstra 2010; Wolf et al. 2012; Osorio et al. 2014; Long et al. 2015; Julius et al. 2017; Zamil and Geitmann 2017). Changes to cell adhesion are also required to allow organs to separate in a controlled manner, for example during shedding of floral organs as they ripen and mature (Rose and Bennett 1999; Brummell 2006; Ogawa et al. 2009; Daher and Braybrook 2015). The cell wall also acts as a protective barrier against abiotic and biotic stresses, such as the invasion of pathogens (Humphrey et al. 2007; Sarkar et al. 2009; Liepman et al. 2010; Ferrari et al. 2013; Bellincampi et al. 2014).
1.2 Composition of the cell wall

The primary cell wall encloses growing and dividing cells (Keegstra 2010; Anderson 2015; Cosgrove 2018) and can be modified to become extensible to allow for cellular expansion or strengthened to maintain cell shape and cell adhesion (Bouton et al. 2002; Cosgrove 2005; Cosgrove and Jarvis 2012). On the other hand, secondary cell walls are typically synthesized after the cessation of cell growth and have specialized compositions, depending on their role (Scheller and Ulvskov 2010; Cosgrove and Jarvis 2012). For example, the secondary cell walls in vascular cells provide tensile and compressive strength that among other things enable the plant to remain rigid and upright (Cosgrove and Jarvis 2012; Zhong and Ye 2014).

The primary cell wall is composed of a complex network of interconnected polysaccharides, glycoproteins, and structural proteins (O’Neill and York 2003; Zamil and Geitmann 2017). The three main groups of polysaccharides are cellulose, hemicellulose, and pectin (Smith 2001). Cellulose is a β-(1→4) chain of glucose (glucan) that forms on average ~15-40 % of the dry mass of the primary cell wall (O’Neill and York 2003; Liepman et al. 2010; Cosgrove and Jarvis 2012). Glucan chains typically cluster together through hydrogen bonding and form long crystalline microfibrils of cellulose, which serve as the main load-bearing component of the primary cell wall (Somerville 2006; Liepman et al. 2010; Cosgrove 2014).

Hemicellulose is another group of cell wall polysaccharides, which consist of β-(1→4)-linked backbones of glucose (Gluc), mannose (Man) or xylose (Xyl), which can also contain side-chains of galactose (Gal), arabinose (Ara) and Xyl (Scheller and Ulvskov 2010). The most common hemicelluloses are xylan, xyloglucan (XG), arabinoxylan, mannan, glucomannan (GM) and galactoglucomannan (GGM, Scheller and Ulvskov 2010). Hemicelluloses are typically less abundant than cellulose, making up ~10-30 % of the dry mass of the primary cell wall (O’Neill
and York 2003; Cosgrove and Jarvis 2012). They are able to cross-link to cellulose microfibrils through hydrogen bonding, which serve as an important connection within a complex network of interconnected polysaccharides thought to exist within the primary cell wall (Hayashi 1989; Cosgrove 2005; Scheller and Ulvskov 2010; Ralet et al. 2016; Cosgrove 2016).

The third major type of cell wall polysaccharide is pectin, which encompasses a group of acidic polysaccharides that are rich in galacturonic acid (GalA) residues (Ridley et al. 2001; Broxterman and Schols 2018). The primary cell walls in dicots, gymnosperms, and non-Poales monocots, is typically composed of ~25-50 % pectin, by dry mass (Zablackis et al. 1995; Darley et al. 2001; O’Neill and York 2003; Mohnen 2008; Liepman et al. 2010; Cosgrove and Jarvis 2012). Pectins can be covalently connected to hemicelluloses (Thompson and Fry 2000; Popper and Fry 2005; Popper and Fry 2008; Zykwinska et al. 2008; Tan et al. 2013) and are believed to be hydrogen bonded to cellulose, through neutral sugar side-chains (Zykwinska et al. 2005; Zykwinska et al. 2008; Wang et al. 2015). Pectins are therefore an important component of the primary cell wall because they form a flexible network to which the other major types of cell wall components are embedded (Cosgrove 2005; Cosgrove and Jarvis 2012; Cosgrove 2016).

1.3 The complex structure of pectins

Pectins are a complex and heterogeneous group of acidic polysaccharides consisting of four main types (Cosgrove 2005; Bonnin et al. 2014). The simplest, and most common pectin is homogalacturonan (HG), which is a linear chain of \( \alpha-(1\rightarrow4) \)-linked GalA residues (Figure 1-1; Harholt et al. 2010), that typically constitute ~65 % of the total pectin content (O’Neill et al. 1990; Mohnen 2008; Atmodjo et al. 2013). Through ionic interactions with calcium, chains of
HG can cross-link and form strong complexes that provide support to the cell wall and are important in maintaining adhesion between neighbouring cells (Grant et al. 1973; Jarvis et al. 2003; Caffall and Mohnen 2009). The second most common pectin is rhamnogalacturonan-I (RG-I), which is a repeating disaccharide of α-(1→2)-linked rhamnose (Rha) and α-(1→4)-linked GalA residues, that typically comprises ~20-30 % of total pectin content (Mohnen 2008; Caffall and Mohnen 2009). Rha residues within the RG-I backbone can be decorated in position (O)-4 with linear or branched chains of Ara (arabinans), Gal (galactans), or a combination of

Figure 1-1: Structure of the four main pectin types.
both, which are referred to as type I or II arabinogalactans (Figure 1-1; O’Neill et al. 1990; Ridley et al. 2001; Harholt et al. 2010; Yapo 2011; Bonnin et al. 2014). The third and minor pectin component is xylogalacturonan (XGA), which like HG is composed of a α-(1→4)-linked GalA backbone, but is decorated in the O-3 position with Xyl residues (Figure 1-1; Voragen et al. 2009). XGA is reported to make up <10 % of pectin (Zandleven et al. 2007; Mohnen 2008; Harholt et al. 2010). The fourth and most structurally complex type of pectin is rhamnogalacturonan-II (RG-II), which is composed of a α-(1→4)-linked GalA backbone, and is highly decorated in the O-2 and O-3 positions with side-chains comprised of 12 different sugar residues linked together by multiple glycosidic bonds (Figure 1-1; O’Neill et al. 1990; Mohnen 2008; Caffall and Mohnen 2009; Harholt et al. 2010; Atmodjo et al. 2013). Similar to XGA, RG-II is typically a minor pectin component in dicots and gymnosperms, which on average makes up ~1-4 % of the primary cell wall pectin content (O’Neill et al. 1990; Matoh et al. 1996), although this percentage varies depending on the species (Darvill et al. 1978; Zablackis et al. 1995). Uniquely, RG-II side-chains have the capacity to dimerize through borate-mediated diester bonds, which are thought to be important for maintaining cell adhesion (O’Neill et al. 2001; Iwai et al. 2002; O’Neill et al. 2004; Cosgrove 2005). All four types of pectins are thought to be covalently linked to one another, resulting in the formation of a complex polysaccharide network. Within this network, RG-I is thought to act as a scaffold to which HG, XGA, and RG-II are covalently attached at the ends of the RG-I backbone (Nakamura et al. 2002; Cosgrove 2005; Tan et al. 2013; Bonnin et al. 2014). HG, XGA and RG-II might also be connected directly to the RG-I backbone as side-chains, similar to the neutral sugar side-chains attached to RG-I (Vincken et al. 2003; Ralet and Thibault 2009; Voragen et al. 2009).
1.4 The essential role of pectin within the cell wall

The interconnected network of pectins within the cell wall fulfills a number of important functions within the plant. One of these is enabling the primary cell wall to provide both the support and elasticity required during growth (Wolf and Greiner 2012). Increasing the content of the pectin, RG-I, and increasing the number and length of arabinan and arabinogalactan side-chains on RG-I, have both been associated with elevated elasticity or extensibility of the cell wall (Moore et al. 2008). Similarly, the presence or absence of calcium-mediated ionic bonds between HG chains is also closely associated with cell wall extensibility (Stolle-Smits et al. 1999; Caffall and Mohnen 2009; Wolf and Greiner 2012). The formation of HG complexes is modulated by the degree of methyl-esterification (DM) of HG. This is controlled by pectin methylesterases (PME) that remove methyl esters from HG and by the action of PME inhibitors (PMEI; Micheli 2001). As an example, mutations in the pollen tube PME, VANGUARD1, result in HG with high DM, which halts the formation of HG complexes and leads to a more elastic cell wall (Jiang et al. 2005). As a result of this more elastic cell wall, the pollen tubes in vdgl mutants are unable to penetrate female tissue, and are more likely to burst compared to wild-type (WT) pollen tubes (Jiang et al. 2005). Conversely, overexpression of VANGUARD1, leads to HG with low levels of DM, increased HG cross-linking, which impede normal growth and results in a dwarf phenotype (Wolf and Greiner 2012).

Pectins also play a key role in maintaining cell adhesion between neighbouring cells within the region known as the middle lamella, which is predominately composed of HG but also contains low levels of RG-I and RG-II (Jarvis et al. 2003). As an example, the colourless non-ripening mutant in tomato that contains less cross-linked HG, possess large intercellular spaces, and have deficiencies in cell adhesion (Thompson et al. 1999; Orfila et al. 2001). Lower levels of
HG within the middle lamella caused by mutations in \textit{QUASIMODO (QUA)} 1 or \textit{QUA2}, also result in the formation of large intercellular spaces and reduced cell adhesion (Bouton et al. 2002; Mouille et al. 2007). Furthermore, as a result of the reduced cell adhesion in the \textit{qua1} and \textit{qua2} mutants, individual cells fall off during physical manipulation of the hypocotyl, roots, and leaves, in addition to the mutant plants that are dwarf in size and their morphology misshapen (Bouton et al. 2002; Mouille et al. 2007). While HG’s role in cell adhesion is well known, changes to RG-I within the middle lamella have also resulted in defects to adhesion. Changes to the number of arabinan and galactan side-chains of RG-I result in losses in cell adhesion in potato tubers (Ulvskov et al. 2005) and in \textit{Nicotiana plumbaginifolia} mutant callus (Iwai et al. 2001). Similar losses in adhesion were also associated with the inability of RG-II side-chains to dimerize via borate ions in another \textit{Nicotiana plumbaginifolia} mutant callus line (Iwai et al. 2002). These studies clearly serve to demonstrate the important role that pectin plays in maintaining cell adhesion.

During the course of growth and development, specific organs of the plant such as leaves, petals and seed pods, are shed in a controlled fashion (Gulfishan et al. 2019). This process requires the targeted degradation of pectins within specific cell layers or regions known as abscission zones (Gulfishan et al. 2019) by carbohydrate-active enzymes (CAZy), such as polygalacturonases (PGs), which specifically degrade HG (Ogawa et al. 2009; Kim 2014; Merelo et al. 2017). In one such example, degradation of HG by the \textit{A. thaliana} endo-PG, \textit{ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE2} (ADPG2) is required to enable the separation of the siliquic valves from the replum, to allow the siliquic pods to shatter upon maturity (Ogawa et al. 2009). Similarly, degradation of HG by the putative PG,
QUARTET3, within the pollen mother cell wall is necessary to separate individual microspores during the tetrad phase of pollen development (Rhee et al. 2003).

Cell wall degrading enzymes, such as PGs, are also secreted by invading pathogens in order to break down the cell wall to gain entry to their host for infection or to use the degraded cell wall as an energy source (Ridley et al. 2001; Choi et al. 2013; Mäkelä et al. 2014). During this process, HG is broken down to oligogalacturonides (OGAs), which are detected by wall-associated kinases (WAKs) localized to the plasma membrane (Ridley et al. 2001; Decreux and Messiaen 2005; Decreux et al. 2006; Ferrari et al. 2013). Once activated, WAKs can initiate a signal cascade that leads to a defense response and resistance to an invading pathogen (Ferrari et al. 2007; Ferrari et al. 2008; Galletti et al. 2008).

It is clear that the network of pectin within the primary cell wall plays a number of essential roles in the plant. However, as a result of the essential nature of pectin, mutants with abnormal pectin typically have impaired growth and development, reduced cell adhesion, and defects in fertility (Somerville et al. 2004). These types of negative pleiotropic effects not only make it challenging to work with pectin-deficient mutants, but can also complicate the interpretation of the data. This has led to increasing calls to explore new model systems to study cell walls. One such system is seed mucilage.

1.5 Seed mucilage and its adaptive role

Seed mucilage is a pectin-rich mixture of cell wall components which has a distinct structure and function. Hydration of seeds from myxodiasporic (mucilage-producing) species leads to the extrusion of mucilage from the epidermal cells of the seed coat which then forms a thick gelatinous capsule around the seed (Voiniciuc et al. 2015c; Phan and Burton 2018; Šola et
al. 2019b). While the functional role of the mucilage capsule in *Arabidopsis thaliana* is not fully understood, increasing evidence of its importance in other myxodiasporic species is emerging (Phan and Burton 2018). One such role is in seed dispersal. For example, the mucilage-extruding seeds from the desert plant *Anastatica hierochuntica* adhere to the soil and sand near the mother plant, thereby inhibiting wind dispersal to potentially unfavourable environmental conditions (Yang et al. 2012c). Conversely, seeds that extrude mucilage can be dispersed over long distances by adhering to the outside of other organisms (Lobova et al. 2003; Yang et al. 2012c). Following dispersal, the ability of mucilage-extruding seeds to retain water has been associated with higher rates of germination especially within environments with low levels of water or high levels of salinity (Penfield et al. 2001; Yang et al. 2010; Yang et al. 2012c). After germination, the biodegradation of mucilage is also correlated with improved establishment of seedling which is thought to be due to the uptake of sugars from degraded mucilage or due to the increases in the biomass of soil microbes that supply resources to the seedling (Yang et al. 2012a; Yang et al. 2012c). Seedling establishment might also be improved by the mucilage capsule acting as a physical barrier which is thought to be resistant to enzymatic attacks instigated by pathogens (Macquet et al. 2007a; Van Craeyveld et al. 2009; Phan et al. 2016). The mucilage capsule in some species has also been found to contain enzymatically active anti-microbial and anti-fungal proteins that have been shown to reduce growth and germination of the gram-positive bacteria and pathogenic fungi (Raviv et al. 2017).

### 1.6 Advantages of using seed mucilage to study the cell wall

Since the late 1990s, the seed mucilage from *Arabidopsis thaliana* has been used as a model system to study cell walls (Arsovski et al. 2010; Haughn and Western 2012; Western
2012; North et al. 2014; Voiniciuc et al. 2015c; Griffiths and North 2017; Francoz et al. 2018; Phan and Burton 2018; Šola et al. 2019b). Since *A. thaliana* seeds extrude mucilage simply by adding water, it can be easily observed cytologically and collected for biochemical analysis (Arsovski et al. 2010; Haughn and Western 2012). In addition, alternations in the amount or composition of mucilage have little impact on the viability of the seed or health of the plant, making mucilage amenable to genetic analysis (Western et al. 2001; Arsovski et al. 2010; Haughn and Western 2012). As a result, a number of mutants with defective mucilage have been isolated and characterized, which have contributed to our understanding of the plant cell wall (most recently reviewed in: Voiniciuc et al. 2015c; Phan and Burton 2018; Šola et al. 2019b).

### 1.7 Development of the mucilage-producing seed coat epidermal cells

During seed development, seed mucilage is synthesized and secreted to the cell wall or apoplast of the seed coat epidermal cell layer (Western et al. 2000; Haughn and Chaudhury 2005; North et al. 2014). Starting at ~1-4 days post anthesis (DPA), seed coat epidermal cells increase in size through expansion of their vacuole (Figure 1-2a). At the same time, starch-containing amyloplasts accumulate (Figure 1-2a).

At ~5 DPA, when the embryo has reached the heart stage, mucilage is synthesized and secreted in a polar manner to the apoplast specifically at the junction between the outer tangential and radial primary cell walls (Figure 1-2b, labelled ‘O’ and ‘R’). Little is known about how secretion of mucilage is targeted to these regions of the cell wall. It is however thought that the ECHIDNA (ECH) protein is most likely involved since secretion of mucilage is mistargeted to the vacuole in *ech* mutants (McFarlane et al. 2008; Gendre et al. 2013). Similarly, mutations in two genes encoding subunits of the exocyst complex; EXO70A1 and SEC8, which are known to
be involved in directing polar secretion in other cell types (Cole et al. 2005; Sekereš et al. 2017),
are known to cause reduced secretion to the mucilage pocket (Kulich et al. 2010).

Figure 1-2: Differentiation of the seed coat epidermal cells.
Seed coat epidermal cell development between 2 DPA to maturity. R = Radial primary cell wall, 
O = Outer tangential primary cell wall, M = mucilage pocket, CC = cytoplasmic column, C = 
columella.

By 7 DPA, at the bent cotyledon or mid-torpedo stage of embryo development, ongoing
polar secretion of mucilage results in the formation of a large mucilage pocket between the
plasma membrane and the outer primary cell wall (Figure 1-2c). Expansion of the mucilage pocket displaces the underlying vacuole, which contracts to the bottom of the cell, and forces the cytoplasm into a column, known as the cytoplasmic column (Figure 1-2c, labelled ‘CC’).

Mucilage secretion is complete by ~8-9 DPA, corresponding to the late torpedo stage of the embryo, after which a cellulosic secondary cell wall is deposited at the surface of the cytoplasmic column. Over time, cellulose deposition moves inwards and replaces all the cytoplasm with a secondary cell wall, forming a volcano-shaped structure known as the columella (Figure 1-2d, e).

By ~13 DPA the cell has undergone programmed cell death (apoptosis), leaving a donut-shaped pocket of mucilage compressed between the outer primary cell wall and the columella (Figure 1-2e). When mature dehydrated seeds are rehydrated by exposure to water, the hydrophilic mucilage expands (Stephen and Churms 1995) rupturing the outer primary cell wall (Figure 1-2f) and surrounding the seed in a gel-like mucilage capsule (Western et al. 2000). The mucilage capsule is easily visualized by staining with the dye ruthenium red that stains acidic polysaccharides (Haughn and Western 2012). The mucilage capsule is composed of two distinct layers, an outer non-adherent layer that can be easily removed by shaking the seeds and an inner adherent layer that remains firmly attached to the seed surface, which can only be removed by harsh chemical or physical treatments (Western et al. 2000; Western et al. 2001; Macquet et al. 2007a). The adherent mucilage layer is thought to remain attached to the seed by being connected indirectly to long microfibrils of cellulose, known as rays, that unwind during extrusion and remain affixed to the columella (Griffiths and North 2017).
1.8 Composition of the seed mucilage: pectin, RG-I

The seed mucilage of *A. thaliana* is composed of cellulose, hemicelluloses, and pectins (Voiniciuc et al. 2015c). The most abundant component in extruded mucilage is the pectin RG-I, which makes up between ~80-90 % of total extractable sugars (Voiniciuc et al. 2015c). RG-I has been confirmed to be present within the mucilage pocket and extruded mucilage, via a number of anti-RG-I specific antibodies (Young et al. 2008; Ralet et al. 2010; Sullivan et al. 2011; Voiniciuc et al. 2015b; Hu et al. 2016a). Since mucilage is predominately composed of RG-I, changes to its structure and composition substantially alter the properties of mucilage. This is particularly apparent in a number of RG-I biosynthetic mutants, where mutant seeds contain less RG-I, and as a result have greatly reduced mucilage capsules (Western et al. 2004; Usadel et al. 2004; Oka et al. 2007; Caffall et al. 2009; Kong et al. 2013; Saez-Aguayo et al. 2017; Takenaka et al. 2018; Voiniciuc et al. 2018). Similarly, the adherent mucilage layer was lost when RG-I degrading enzymes were exogenously applied to extruded WT seeds (Macquet et al. 2007a), pointing towards the importance of RG-I in maintaining the structure of the adherent mucilage layer. While linkage analysis of extruded mucilage has revealed that the RG-I polysaccharide is predominantly unbranched, it can also be infrequently decorated with side-chains of single terminal (t)-β-Gal and Ara residues (Dean et al. 2007; Arsovski et al. 2009). The length of these side-chains has been shown to impact the properties of the mucilage. Mutations in the *MUCILAGE-MODIFIED2 (MUM2)* gene, which encodes a β-galactosidase (BGAL), increases the number of Gal side-chains on RG-I, resulting in seeds which are unable to extrude mucilage (Macquet et al. 2007b; Dean et al. 2007). Similarly, longer arabinan side-chains on RG-I, caused by mutations in the bifunctional β-D-xylosidase / α-L-arabinofuranosidase encoding gene β-XYLOSIDASE1 (*BXLI*), result in slow incomplete mucilage extrusion in *bxl1* mutants (Arsovski
et al. 2009). In summary, due to the fact that seed mucilage is predominately composed of RG-I, changes to its structure and amount have a major impact on the properties of mucilage, which include its ability to extrude and form a WT-like adherent mucilage layer.

1.9 Composition of the seed mucilage: pectin, HG

Extruded mucilage also contains the pectin HG, which accounts for ~10 % of total extractable sugars, making it the second most abundant cell wall component in extruded mucilage (Macquet et al. 2007a; Dean et al. 2007; Voiniciuc et al. 2015a). HG has been confirmed to be present in the adherent mucilage layer based on binding with HG specific monoclonal antibodies (Willats et al. 2000; Willats et al. 2001; Macquet et al. 2007a; Rautengarten et al. 2008; Hu et al. 2016a). Even though HG is a minor component of extruded mucilage, changes to its structure impact the properties of mucilage. For example, mutants containing HG with lower DM than WT have more cohesive mucilage that extrudes more poorly (Rautengarten et al. 2008; Saez-Aguayo et al. 2013; Voiniciuc et al. 2013; Shi et al. 2018). Conversely, mutant seeds containing HG with higher DM and also when WT seeds are treated with divalent cation chelators that remove calcium, both result in less cohesive mucilage (Willats et al. 2001; Saez-Aguayo et al. 2013; Turbant et al. 2016). These data have established a clear relationship between the DM of HG, the cohesiveness of mucilage and the ability of mucilage to extrude when hydrated in water. The structural role of HG within the mucilage pocket is not however fully understood. This is because, while mutations in GALACTURONOSYLTRANSFERASE 11 result in a ~30 % decrease in mucilage GalA levels there is also a corresponding decrease in Rha levels, making it possible that RG-I rather than HG has been affected in these mutants (Caffall et al. 2009). Therefore, without mucilage mutants that
are specifically deficient in HG, it is currently unknown what the structural role of HG might be in seed mucilage.

1.10 Composition of the seed mucilage: hemicelluloses

The most abundant hemicellulose detected in extruded mucilage is xylan, a β-(1→4)-linked backbone of Xyl (Scheller and Ulvskov 2010) that is thought to be decorated in the O-2 position with a single Xyl residue (Voiniciuc et al. 2015a; Ralet et al. 2016). Xylan is a minor component of mucilage, making up < 3% of the monosaccharide composition of extruded mucilage (Macquet et al. 2007a; Huang et al. 2011) and it has been detected in extruded mucilage using a number of xylan-specific antibodies (Hu et al. 2016a; Hu et al. 2016b; Ralet et al. 2016; Voiniciuc et al. 2018). The 4-xylopyranose (Xylp) linkages required to form xylan have also been detected in extruded mucilage through linkage analysis (Yu et al. 2014). The role of xylan within mucilage has been studied using mutants that are unable to synthesize normal xylan due to mutations in the genes encoding xylan biosynthetic enzymes. Such genes include IRREGULAR XYLEM (IRX) 7, IRX14, and MUM5 (Voiniciuc et al. 2015a; Hu et al. 2016b; Hu et al. 2016a; Ralet et al. 2016). irx7, irx14, and mum5 mutants produce seeds that lack the adherent mucilage normally found in WT (Voiniciuc et al. 2015a; Hu et al. 2016b; Hu et al. 2016a; Ralet et al. 2016). In addition in vitro binding assays showed that the binding of RG-I to mucilage cellulose was dependent on xylan (Ralet et al. 2016). These data suggest a hypothesis for mucilage adherence where the mucilage RG-I has xylan side-chains which bind the mucilage cellulose anchored to the seed surface (Voiniciuc et al. 2015a; Ralet et al. 2016).
The hemicelluloses GM and GGM have also been detected in extruded mucilage through staining with heteromannan-specific antibodies (Hu et al. 2016a; Yu et al. 2014; Voiniciuc et al. 2015a). The glycosidic linkages necessary to form GM and GGM have also been detected within extruded mucilage (Dean et al. 2007; Huang et al. 2011; Yu et al. 2014). Even though Man and Gluc make up <2 % of total monosaccharides in WT mucilage (Macquet et al. 2007a; Huang et al. 2011), mutations that lead to alternations in the levels of GM and GGM have been shown to impact the properties of mucilage. Mutations in CELLULOSE SYNTHASE-LIKE A2, which results in less GM synthesis and mutations in MUCILAGE-RELATED10, which results in less GGM, both lead to a thinner, more compact adherent mucilage layer (Yu et al. 2014; Voiniciuc et al. 2015b). In these mutants, the adherent mucilage layer is thought to be thinner because less GM and GGM is available to bind to the cellulosic rays, which are important for forming the adherent mucilage layer (Whitney et al. 1998; Eronen et al. 2011; Voiniciuc et al. 2015b; Griffiths and North 2017).

It has been hypothesized that seed mucilage might contain another hemicellulose XG (Western 2012; Haughn and Western 2012; Voiniciuc et al. 2015c). While the correct polysaccharide linkages that make up XG have indeed been detected in extruded mucilage (4,6-glucopyranose (Glucp), t-Xylp and 2-Xylp) these are however not specific to XG (Arsovski et al. 2009; Huang et al. 2011). A polyclonal anti-XG antibody was also able to bind to mucilage and the columella in sections of developing epidermal cells. However it is unclear whether this antibody is specific to XG (Moore et al. 1986). Therefore, the presence of XG in mucilage is still doubtful.
1.11 Composition of the seed mucilage: cellulose

Extruded mucilage also contains cellulose, which has been visualized using an array of glucan and cellulose-specific stains (Hu et al. 2016b; Macquet et al. 2007a; Voiniciuc et al. 2015b; Voiniciuc et al. 2015a; Voiniciuc et al. 2013; North et al. 2014; Griffiths et al. 2015; Griffiths and North 2017; Ben-Tov et al. 2018). Staining of extruded mucilage has also revealed that cellulose forms long microfibrils or rays where one end is anchored to the columella and the other at the outer edge of the adherent mucilage layer (Griffiths et al. 2015; Griffiths and North 2017). The cellulosic rays are important for anchoring the mucilage to the seed-surface, which was discovered by characterizing a number of cellulose deficient mutants (Harpaz-Saad et al. 2011; Mendu et al. 2011; Sullivan et al. 2011; Griffiths et al. 2014; Griffiths et al. 2015; Ben-Tov et al. 2015; Zhang et al. 2016; Griffiths and North 2017; Ben-Tov et al. 2018).

1.12 Composition of the seed mucilage: proteins

Trace amounts of proteins are present in extruded mucilage (Macquet et al. 2007a). Proteomic analysis of the extruded mucilage from *A. thaliana* identified 28 different proteins (Tsai et al. 2017). These have been categorized into five broad groups based on their function. They include proteins involved in lipid metabolism, proteases, oxidoreductases, structural proteins, and CAZy enzymes (Tsai et al. 2017). However, only a few of these have been characterized, one of which is the protease, SUBTILISIN-LIKE SERINE PROTEASE 1.7 (SBT1.7) that is thought to negatively regulating PME activity (Rautengarten et al. 2008; Saez-Aguayo et al. 2013). This is based on the phenotype of *sbt1.7* mutants, which have higher levels of PME activity and more cohesive mucilage that is unable to extrude in water (Rautengarten et al. 2008; Saez-Aguayo et al. 2013). The role of the oxidoreductase PEROXIDASE36 (PER36),
identified in the proteome of extruded mucilage (Tsai et al. 2017), has also been studied. PER36 is thought to generate reactive oxygen species that degrade and weaken the outer primary cell wall to assist mucilage extrusion during hydration of the seed (Kunieda et al. 2013). Structural proteins identified in the mucilage proteome such as arabinogalactan-proteins, appear to play a role in adherence of the mucilage to the seeds. However how this occurs has yet to be clearly defined (Griffiths et al. 2014; Griffiths and North 2017).

Extruded mucilage also contains a number of CAZy enzymes that are involved in the construction (anabolic) or degradation (catabolic) of complex carbohydrates (Cantarel et al. 2009). Of the six CAZy enzymes detected within the proteome of extruded mucilage (Tsai et al. 2017) only two have been studied in depth. One of these is BXL1, which is a dual β-xylosidase and α-arabinosidase that is thought to degrade long arabinan side-chains on RG-I within the mucilage pocket and primary cell wall, prior to extrusion (Arsovski et al. 2009). Mutations in BXL1 result in slower and patchy mucilage extrusion (Arsovski et al. 2009), demonstrating that specific modification to the polysaccharides in mucilage are required to enable wild type-like mucilage extrusion.

The other well documented catabolic CAZy enzyme found in the proteome of extruded mucilage is BGAL6 also known as MUM2 (Enzyme Commission (EC) 3.2.1.23; Macquet et al. 2007b; Dean et al. 2007). This enzyme serves as an important tool in this thesis. MUM2/BGAL6 is part of the glycoside hydrolase 35 (GH35) enzyme family in A. thaliana, which consists of 17 BGALs, half of which have been enzymatically characterized (Ahn et al. 2007; Chandrasekar and van der Hoorn 2016). GH35 BGALs catalyze the hydrolysis of t-β-Gal residues using a retaining mechanism (Bhalla and Dalling 1984; Zinin et al. 2002; Gamauf et al. 2007; Ohto et al. 2012). This two-step reaction is initiated by a glutamic acid residue within the active site (Rojas
et al. 2004) which mediates a nucleophilic attack on the anomeric carbon, where the glycosidic bond between two monosaccharides is located (Yip and Withers 2004; Brumer 2010; Vuong and Wilson 2010). A second glutamic acid residue within the active site then donates a proton causing the formation of a glycosyl enzyme intermediate, in which the substrate is covalently bound to the active site of the enzyme (Yip and Withers 2004; Brumer 2010; Vuong and Wilson 2010). In the second and final step of the reaction, the glycosyl enzyme intermediate is hydrolyzed by water and a proton is removed from water by the second glutamic residue (Yip and Withers 2004; Brumer 2010; Vuong and Wilson 2010). Within seed coat epidermal cells, MUM2 is secreted to the mucilage pocket during mucilage synthesis, where it is thought to remove the \( \alpha-\beta\)-Gal found on long galactan side-chains on RG-I (Macquet et al. 2007b; Dean et al. 2007). When the galactan side-chains are not removed in the \textit{mum2} mutant (Macquet et al. 2007b; Dean et al. 2007), the putative galactose oxidase RUBY PARTICLES IN MUCILAGE, oxidizes the galactan side-chains (Šola et al. 2019a). This is then thought to promote covalent cross-links to adjacent polysaccharides within the cell wall, preventing mucilage from expanding during hydration (Šola et al. 2019a). As a result, the \textit{mum2} mutant possesses a characteristic phenotype in which mucilage fails to extrude upon contact with water (Macquet et al. 2007b; Western et al. 2001; Dean et al. 2007), even when the outer primary cell wall is removed (Dean et al., 2007). The \textit{mum2} phenotype can be restored to WT-like mucilage extrusion through complementation with a genomic clone of \textit{MUM2} (Dean et al. 2007).

1.13 Research objectives

Characterization of mutants with defective mucilage, such as \textit{bxl1} and \textit{mum2} has demonstrated that changes to the structure of mucilage can directly impact its properties. These
changes can be visualized by staining seeds with the dye ruthenium red and structural changes can be characterized biochemically by collecting and analyzing extruded mucilage. Therefore, mucilage is a useful model system that can be used to study how the structure of the cell wall supports different functional properties. The overarching goal of this thesis was to investigate how the structure of mucilage informs its unique properties and functions. This question was addressed using a genetic engineering approach to express and secrete CAZy enzymes to the mucilage pocket and analyze the consequences of degrading the structure of mucilage on its function and properties.

An important consideration when adopting this type of genetic engineering approach is to avoid expressing genes encoding cell wall degrading enzymes throughout the plant. As the studies described below have demonstrated, negative pleiotropic effects commonly arise when constitutive promoters are used to express genes encoding cell wall degrading enzymes throughout the plant. For instance, using the constitutive 35S promoter to overexpress a gene encoding a HG-degrading enzyme in apple resulted in malfunctioning stomata, brittle leaves, and reduced cell adhesion (Atkinson et al. 2002). Similarly, transgenic Nicotiana tabacum and A. thaliana were dwarf and their stem anatomy was altered when using the 35S promoter to drive expression of another gene encoding a HG-degrading enzyme (Capodicasa et al. 2004). Therefore, to avoid these types of pleiotropic effects and to modify mucilage composition specifically, seed coat-specific promoters were required.
Objective 1: Determine if seed coat-specific promoters can be used for modifying mucilage, by testing their ability to complement the mum2 phenotype when driving MUM2 expression (Chapter 3)

The first objective of this thesis was to test if three previously-identified seed coat-specific promoters could be used for modifying mucilage. Each promoter was tested by determining if it could drive sufficient gene expression of MUM2, which encodes a known mucilage modifying enzyme, to complement the mum2 mutant phenotype. This would serve as a proof of concept that these promoters could be used to engineer the composition of mucilage within the seed coat epidermal cells during mucilage secretion (5-9 DPA) at a sufficient level to alter the mucilage phenotype.

Objective 2: Are all BGALs in the GH35 enzyme family functionally equivalent to MUM2 (BGAL6) in their ability to modify the structure of mucilage? (Chapter 3)

MUM2 or BGAL6 encodes a BGAL that is one of 17 BGALs within the GH35 enzyme family. While all members of this family contain the GH35 catalytic domain necessary for BGAL activity, it is unknown if they are functionally equivalent to MUM2 in their ability to modify the cell wall. Three candidate BGALs from the GH35 enzyme family, which had not been enzymatically characterized, were tested to see if they could complement the mum2 mutant phenotype. To do this, one of the seed coat-specific promoters tested in Chapter 3 was selected to drive transcription of the candidate BGALs within the seed coat epidermal cells during mucilage synthesis and secretion. The seeds from mum2 plants transformed with the candidate BGALs were screened both cytologically and biochemically for complementation.
Objective 3: Modify the structure of the well-known, abundant mucilage component RG-I and assess the resulting changes to the properties of mucilage (Chapter 4)

RG-I was selected for degradation because it is the most abundant component of mucilage, and therefore changes to its structure were expected to have a major impact on the properties of mucilage. To degrade mucilage RG-I, one of seed coat-specific promoters tested in Chapter 3 was used to drive expression of genes encoding RG-degrading enzymes within the seed coat epidermal cells, which was secreted to the mucilage pocket using an appropriate signal peptide sequence. If RG-I composition is altered and there is also a change to the properties of mucilage, this would serve as a proof of concept that the composition of mucilage can be modified using a targeted genetic engineering approach.

Objective 4: Modify the structure of the less abundant, less well-known mucilage component HG and assess how these modifications impact the properties of mucilage (Chapter 5)

HG was chosen to be degraded within the mucilage pocket in order to investigate the structural role HG might play in interconnecting different cell wall components and how degrading these connections might alter the properties of mucilage. Using the same genetic engineering approach as in Objective 3, HG was targeted for degradation by secreting HG-degrading enzymes to the mucilage pocket using an appropriate signal peptide sequence, and specifically expressed in the seed coat using one of the promoters tested in Objective 1.
Chapter 2: Materials and methods

2.1 Plant materials and growth conditions

*Arabidopsis thaliana* Columbia (Col-2) ecotype was used as the WT in this thesis. Col-2 was originally isolated from Col-0 seeds through five generations of single seed decent by Shauna Somerville. The *mum2-1* mutant was originally isolated from an ethyl methanesulfonate mutagenized population of Col-2 seeds (Western et al. 2001). Yi-Chen Lee kindly provided *mum4-1* plants (Western et al. 2001) crossed with lines carrying the following chimeric construct: the *MUM4_1.5* promoter (*p*; Esfandiari 2012) fused upstream of the sequence encoding the basic endochitinase B (At3g12500) signal peptide fused in-frame to the sequence encoding Citrine, a dimeric acidic stable variant of yellow fluorescent protein (Griesbeck et al. 2001; Zacharias et al. 2002). Col-0 plants transformed with *VESICLE-ASSOCIATED MEMBRANE PROTEIN 721* (*VAMP721*) *p::monomeric red fluorescence protein* (*mRFP*)-*VAMP721* were kindly provided by Dr. Masa H. Sato (Ichikawa et al. 2014).

Seeds were germinated on 6.25 % (w/v) agar (BD-Difco) plates containing *Arabidopsis thaliana* minimal medium (Haughn and Somerville 1986), under continuous light (80-120 μmol m⁻² s⁻¹ photosynthetically active radiation) at 20°C. Ten-day-old seedlings were transferred to soil (Sunshine Mix #4; SunGro, sungro.com) enriched once with 35 mL of liquid *Arabidopsis thaliana* minimal medium at the time transplanting and grown under the same conditions as described above. Plants grown for ribonucleic acid (RNA) extraction and monosaccharide analysis were grown in a growth chamber (E7/2, Conviron) under continuous light (80-120 μmol m⁻² s⁻¹ photosynthetically active radiation) at 20-22 °C.
Developing flowers were staged according to Western et al. (2000), with the pedicel of newly opened flowers, defined as 0 DPA, marked with water-soluble, non-toxic paint. Using this approach, developing siliques and seeds of specific ages could be harvested. Seeds from completely desiccated plants were harvested and stored in paper envelopes (S-7798, U-line) then sealed in plastic boxes containing silica gel desiccant (Fisher Scientific) and stored at 7 °C.

2.2 Plasmid construction

As a means of rapidly assembling different combinations of promoters, genes and sequences encoding fluorescence tags, the MultiSite Gateway™ cloning system (Invitrogen) was used and gateway entry clones containing promoter and complementary deoxyribonucleic acid (cDNA) sequences were constructed, as described below.

2.2.1 Gateway entry clones

All promoter sequences were recombined into the Gateway pDONRG_P4P1R (Invitrogen) promoter entry vector (Oshima et al. 2011). The previously generated PER36 promoter entry clone used in this thesis contained 2,635 bp of DNA sequence immediately upstream of the PER36 ATG, which includes the predicted PER36 5' UTR and two upstream genes, Xero1 (At3g50980.1) and Xero2 (At3g50970.1). The PER36_0.2 promoter entry clone contained 289 bp of DNA sequence upstream of the PER36 ATG, which included the predicted PER36 5' UTR and all upstream sequence to, but not including, the predicted 3' UTR of Xero1 (At3g50980.1).

To generate the PER36_mod promoter entry clone, premature stop codons were introduced into the Xero1 (cytosine to thymine mutation at 1,560 bp) and Xero2 (guanine to
thymine mutation at 345 bp) genes through site-directed mutagenesis of the pDONR P4-P1R/ProPER36 promoter entry clone (Kunieda et al. 2013). The TBA2 promoter entry clone generated in this thesis contained the same 1,346 bp of TBA2 promoter region previously characterized by Tsai et al. (2017) and includes the predicted TBA2 5′ UTR. The previously generated MUM4_0.3Pro_35S promoter entry clone (Dean et al. 2017) contains 307 bp of the MUM4 promoter region fused to the 54 bp 35S minimal promoter sequence (Benfey et al. 1990).

All cDNA sequences were recombined into the Gateway pDONR207 (Invitrogen) entry vector. The MUM2 cDNA entry clone contained the MUM2 coding sequence (2,155 bp) starting from the ATG, in which the stop codon was replaced with a single thymine to keep the gene fusion in-frame with the C-terminal Citrine tag in the destination vector. Similarly, the start codons were included and the stop codons were replaced with a single thymine in the cDNA entry clones containing BGAL11 (At4g35010; 2,535 bp), BGAL16 (At1g77410.1; 2,446 bp), and BGAL17 (At1g72990.1; 2,092 bp). The ADPG2 (At2g41850.1, Q8RY29) cDNA sequence, amplified from Col-2 cDNA, was 1,277 bp in length, which excluded the start codon, the sequence encoding the predicted ADPG2 signal peptide (72 bp), and a stop codon. The sequence encoding the predicted signal peptide of ADPG2 and all subsequently described proteins were identified using Phobuis (Käll et al. 2007), SignalP (Nielsen 2017), Signal-BLAST (Frank and Sippl 2008), and cross-referenced with the Uniprot database (Bateman et al. 2017). Amino-terminus (N)-terminal tagging of ADPG2 was chosen because N-terminal tagged ADPG2 had previously been shown to possess polygalacturonase activity in vitro (Ogawa et al. 2009). To add an N-terminal tag to ADPG2 while still using the Gateway cloning method a new pDONR207 (Invitrogen) entry clone was constructed which contained a 922 bp sequence encoding the MUM2 signal peptide (MUM2sp) fused in-frame to Citrine amplified from the
MUM4_1.5p::MUM2sp-Citrine plasmid and followed downstream by two restriction sites (SpeI and AatII). The aforementioned ADPG2 cDNA sequence was then ligated between the SpeI and AatII restriction sites to form an N-terminal tagged MUM2sp-Citrine-ADPG2 cDNA entry clone.

To ensure successful secretion of the fungal-derived RG-I degrading enzymes; Rhamnogalacturonate lyase A (RglA), Rhamnogalacturonate lyase B (RglB), and Pectin Lyase A (PelA) to the apoplast, it was important to replace the sequences encoding their respective signal peptides with the sequence encoding MUM2sp. First, the 84 bp sequence encoding the predicted MUM2sp, which includes a start codon, followed downstream by two restriction sites (SpeI and AatII), was amplified and recombined to make the MUM2sp-pDONR207 entry clone. These two restriction sites were designed for use as cloning sites for the cDNA sequences of the three previously mentioned Aspergillus nidulans genes downstream of the MUM2sp sequence within the MUM2sp-pDONR207 entry clone. The three fungal-derived cDNA sequences consisted of 1,521 bp of RglA (Q5AX45, AN7135.2), 1,926 bp of RglB (Q5AZ85, AN6395.2), and 1,077 bp of PelA (Q5BAU9, AN2331.2), which excluded both the start and stop codons as well as the sequences encoding their respective predicted signal peptide (0-60 bp in RglA and PelA and 0-54 bp in RglB). The stop codons were also replaced with a single thymine to keep the gene fusions in-frame with the sequence encoding C-terminal tags within the destination vector. These gene sequences were amplified from recombinant Pichia pastoris strains containing RglA, RglB, PelA kindly provided by Chris Somerville (Bauer et al. 2006).

To inactivate the enzymatic activity of RglB, the two catalytic sites necessary for RGL activity (Jensen et al. 2010) were first identified by aligning the sequences of three well characterized RGLs (McDonough et al. 2004) with RglB using MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle). Once the two catalytic sites had been identified, site-
directed mutagenesis was performed on the *MUM2sp-RglB* entry clone to mutant the catalytic lysine 228 to alanine (AA to GC mutation at 682 bp) and the catalytic histidine 290 to alanine (CAC to GCT mutation at 868 bp).

### 2.2.2 Gateway destination vectors

The R4pGWB401 destination vector (Nakagawa et al. 2008) was modified to include the sequence encoding Citrine, a dimeric acid stable variant of the yellow fluorescent protein (Griesbeck et al. 2001; Zacharias et al. 2002). The 768 bp Citrine sequence, which had no ATG but included a stop codon was amplified from the pAD binary vector (DeBono 2011). This sequence was ligated into R4pGWB401 downstream of the Gateway recombination sites using the AfeI and SacI restriction enzyme sites to generate R4pGWB401+Citrine. Finally, entry clones containing promoters or cDNA sequences were recombined into R4pGWB401 or R4pGWB401+Citrine using Gateway cloning technology (Thermo-Fisher Scientific; thermofisher.com) to generate chimeric constructs with tagged or untagged versions of each protein. A complete list of all entry clones, destination vectors, and primer sequence used in this thesis can be found in Table 2-1.

Table 2-1: Primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>PER36_0.2_ for</td>
<td>GGGGACAACCTTTGTATAGAA AAGTTGcgggtggtttcagatttaatcg</td>
<td>Amplify the short PER36 promoter sequence (289 bp). Gateway adaptors (underlined).</td>
</tr>
<tr>
<td>PER36_0.2 rev</td>
<td>GGGGACTGCTTTTTTGTACAA ACTTCGttgacttcagcattgtttggg</td>
<td></td>
</tr>
<tr>
<td><strong>PER36_Xer o1_for</strong></td>
<td>CTCACCAATAGCCTTGACCAA TTTGGAAATC</td>
<td>Overlapping primers for introducing a premature stop codon (underlined bp to be modified) in <em>Xero 1</em> within the <em>PER36</em> promoter region.</td>
</tr>
<tr>
<td><strong>PER36_Xer o1_rev</strong></td>
<td>GTCAAGCTATTGGTGAGTCTCTGCTGC</td>
<td></td>
</tr>
<tr>
<td><strong>PER36_Xer o2_for</strong></td>
<td>CAAAAGAAGGCTGATAACCGG GAGAAATCATGG</td>
<td>Overlapping primers for introducing a premature stop codon (underlined bp to be modified) in <em>Xero 2</em> within the <em>PER36</em> promoter region.</td>
</tr>
<tr>
<td><strong>PER36_Xer o2_rev</strong></td>
<td>CGTTATCTCCTTTTGCACTCCG</td>
<td></td>
</tr>
<tr>
<td><strong>TBA2p_for</strong></td>
<td>GGGGACAACCTTTGTATAGAA AAGTTGgtttttgcttcctacat</td>
<td>Amplify the <em>TBA2</em> promoter sequence (1,346 bp). Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>TBA2p_rev</strong></td>
<td>GGGGACTGCTTTTTTGTACA AACTTGctttttgctttttttcttttgg</td>
<td></td>
</tr>
<tr>
<td><strong>MUM2_for</strong></td>
<td>GGGGACCCACTTTGTACAAGA AAGCTGCGTATGGAGATGGG TCGTCTGGTCTTGGGTT</td>
<td>Amplify the <em>MUM2</em> coding sequence (2,155 bp), including the ATG. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>MUM2_rev</strong></td>
<td>GGGGACCCACTTTGTACAAGA AAGCTGCGTATGGAGATGGG TCGTCTGGTCTTGGGTT</td>
<td><em>MUM2</em> coding sequence (2,155 bp), stop codon was replaced by a single thymine base (green) to keep in-frame with C-terminal tags. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>BGAL11_for</strong></td>
<td>GGGGACAAGTTTGTACAAAA AAGCAGGCTATGGAGATGGG TCGTCTGGTCTTGGG</td>
<td>Amplify the <em>BGAL11</em> coding sequence (2,535 bp). Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>BGAL11_rev</strong></td>
<td>GGGGACAAGTTTGTACAAAA AAGCAGGCTATGGAGATGGG TCGTCTGGTCTTGGG</td>
<td>Amplify the <em>BGAL11</em> coding sequence (2,535 bp), stop codon was replaced by a single thymine base (green) to keep in-frame with C-terminal tags. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>BGAL16_for</strong></td>
<td>GGGGACAAGTTTGTACAAAA AAGCAGGCTATGGAGATGGG TCGTCTGGTCTTGGG</td>
<td>Amplify the <em>BGAL16</em> coding sequence (2,446 bp). Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>BGAL16_rev</strong></td>
<td>GGGGACAAGTTTGTACAAAA AAGCAGGCTATGGAGATGGG TCGTCTGGTCTTGGG</td>
<td>Amplify the <em>BGAL16</em> coding sequence (2,446 bp), stop codon was replaced by a single thymine base (green) to keep in-frame with C-terminal tags. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>BGAL17_for</strong></td>
<td>GGGGACAAGTTTGTACAAAA AAGCAGGCTATGGAGATGGG TCGTCTGGTCTTGGG</td>
<td>Amplify the <em>BGAL17</em> coding sequence (2,092 bp), including the gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>BGAL17_rev</strong></td>
<td>GGGGACAAGTTTGTACAAAA AAGCAGGCTATGGAGATGGG TCGTCTGGTCTTGGG</td>
<td>Amplify the <em>BGAL17</em> coding sequence (2,092 bp), stop codon was replaced by a single thymine base (green) to keep in-frame with C-terminal tags. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>Citrine_for</strong></td>
<td>ATAAAGCGCTCCTGGAGGTTGG\nAGGTGGGAGC</td>
<td>Citrine-encoding sequence (768 bp) amplified from pAD, includes an AfeI adaptor (underlined) for ligation into R4pGWB401.</td>
</tr>
<tr>
<td><strong>Citrine_rev</strong></td>
<td>TATGAGCTCTTTATGCTAGAG\nGCGCAGCAGCACCAGC</td>
<td>Citrine-encoding sequence (768 bp) amplified from pAD, includes a stop codon and a SacI adaptor (underlined) for ligation into R4pGWB401.</td>
</tr>
<tr>
<td><strong>MUM2sp_for</strong></td>
<td>GGGGACAAGTTTTGTACAAAA\nAAGCAGGCTatggagatgggtcgtctg\ngcttccggg</td>
<td>Amplify the 84 bp sequence encoding the <em>MUM2sp</em>, ATG of <em>MUM2</em> included. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>MUM2sp_rev</strong></td>
<td>GGGGACACCTTTGTACAAGA\nAAGCTGGGTatggaaattcaat\ngcaatcaaaaac</td>
<td>Amplify the 84 bp sequence encoding the <em>MUM2sp</em>, including the SpeI (red) and AatII sites (blue) flanked by additional bps to keep in-frame. The stop codon was also replaced with a single thymine base (green) to keep the gene-fusions in-frame with C-terminal tags. Gateway adaptors (underlined).</td>
</tr>
<tr>
<td><strong>RglA_for</strong></td>
<td>tatgcACTAGTggcctttggcattaccga\naac</td>
<td>Amplify the 1,521 bp <em>RglA</em> cDNA sequence, does not include the start codon or the 60 bp sequence encoding the predicted RglA signal peptide sequence, contains a SpeI site (red) for ligation into the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>RglA_rev</strong></td>
<td>acgatgGACGTCcAtggaaaagtcaat\ngcaatccaaaac</td>
<td>Amplify the 1,521 bp <em>RglA</em> cDNA sequence, stop codon replaced with a thymine (green) to keep gene fusions in-frame with C-terminal tags, contains an AatII site (blue) for ligation into the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>RglB_for</strong></td>
<td>tatgcACTAGTgcccctaactgcaacaga\ngaatg</td>
<td>Amplify the 1,926 bp <em>RglB</em> cDNA sequence, does not contain a start codon or the 54 bp sequence encoding the predicted RglB signal peptide sequence, contains a SpeI site (red) for ligation into the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>RglB_rev</strong></td>
<td>acgatgGACGTCcAttcaactcatactct\naaagcactc</td>
<td>Amplify the 1,926 bp <em>RglB</em> cDNA sequence, stop codon replaced with a thymine base (green) to keep gene fusions in-frame with C-terminal tags, contains an AatII site (Blue) for ligation into the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>RglB_K228A_f1</strong></td>
<td>GTACTTTACCGCTACACCTT\nCTCGAAACCACTG</td>
<td>Overlapping primers for mutating the catalytic cysteine 228 to alanine (underlined) in <em>RglB</em> within the <em>MUM2sp-RglB</em> entry clone.</td>
</tr>
<tr>
<td><strong>RglB_K228A_r1</strong></td>
<td>GAAGGTGTACCGGTAAAAGT\nACTCTGAAAACCTGCTG</td>
<td>Overlapping primers for mutating the catalytic cysteine 228 to alanine (underlined) in <em>RglB</em> within the <em>MUM2sp-RglB</em> entry clone.</td>
</tr>
<tr>
<td>Primer Name</td>
<td>Sequence</td>
<td>Description</td>
</tr>
<tr>
<td>-------------</td>
<td>----------</td>
<td>-------------</td>
</tr>
<tr>
<td><em>RglB_H290_A_f1</em></td>
<td>GTTTCCAACGCTCACGGCGAGGCCACTCC</td>
<td>Overlapping primers for mutating the catalytic histidine 290 to alanine (underlined) in <em>RglB</em> within the <em>MUM2sp-RglB</em> entry clone.</td>
</tr>
<tr>
<td><em>RglB_H290_A_r1</em></td>
<td>TCGCCGTACGTTTGGAACGATGATT</td>
<td>Overlapping primers for mutating the catalytic histidine 290 to alanine (underlined) in <em>RglB</em> within the <em>MUM2sp-RglB</em> entry clone.</td>
</tr>
<tr>
<td><em>MUM2sp_for</em></td>
<td>GGGGACAAGTTTTGTACAAAAAAGCAGGCTaatggagatgggtcgtctggtcttcggg</td>
<td>Amplify the 922 bp sequence encoding the MUM2 signal peptide sequence (84 bp), followed downstream by a PstI site and the Citrine encoding sequence (765 bp) amplified from the <em>MUM4_1.5p:MUM2sp-Citrine</em> plasmid, ATG included. Gateway adaptors (underlined)</td>
</tr>
<tr>
<td><em>MUM2sp-Citrine_rev</em></td>
<td>GGGGACCACCTTTGTACAAAGACTGGGGTTagGACGTCccACATGTgcggtaggggagcagcaccag</td>
<td>Amplify the 922 bp sequence encoding the MUM2 signal peptide sequence (84 bp), followed downstream by a PstI site and the Citrine encoding sequence (765 bp) amplified from the <em>MUM4_1.5p:MUM2sp-Citrine</em> plasmid. SpeI (red) and AatII sites (blue) flanked by additional bp to keep in-frame. Does not contain a stop codon. Gateway adaptors (underlined)</td>
</tr>
<tr>
<td><em>ADPG2_for</em></td>
<td>tatgcACTAGTgtcaagaatcagccctaatgtatagcc</td>
<td>Amplify the 1,227 bp <em>ADPG2</em> cDNA sequence, does not include a start codon or the 72 bp sequence encoding the predicted <em>ADPG2</em> signal peptide sequence, contains a SpeI site (red) for ligation into the <em>MUM2sp</em> entry clone or the <em>MUM2sp-Citrine</em> entry clone.</td>
</tr>
<tr>
<td><em>ADPG2_rev</em></td>
<td>tcgatgGACGTCcctaatgggtgccctacgagc</td>
<td>Amplify the 1,227 bp <em>ADPG2</em> cDNA sequence, contains a stop codon and an AatII site (blue) for ligation into the <em>MUM2sp</em> entry clone or the <em>MUM2sp-Citrine</em> entry clone.</td>
</tr>
<tr>
<td><em>PelA_for</em></td>
<td>tatgcACTAGTggtctccgcagggcctctcg</td>
<td>Amplify the 1,077 bp <em>PelA</em> cDNA sequence that excludes the start codon and the 60 bp sequence encoding the predicted <em>ADPG2</em> signal peptide, includes a SpeI site (red) for ligation into the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><em>PelA_rev</em></td>
<td>acgatgGACGTCeAaggttacgccagaagagg</td>
<td>Amplify the 1,077 bp <em>PelA</em> cDNA sequence where the stop codon was replaced by a thymine base (green) to keep the gene in-frame with C-terminal tags, includes an AatII site (blue) for ligation into the <em>MUM2sp</em> entry clone.</td>
</tr>
</tbody>
</table>
Table 2-2: Summary of plasmids and chimeric constructs.

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Plasmid type</th>
<th>Plasmid backbone</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>PER36p entry clone</td>
<td>Gateway promoter entry clone</td>
<td>pDONR P4-P1R</td>
<td>Gift from Dr. Tadashi Kunieda, see Kunieda et al. 2013 for details.</td>
</tr>
<tr>
<td>PER36_0.2p entry clone</td>
<td>Gateway promoter entry clone</td>
<td>pDONRG_P4-P1R</td>
<td>Contains 289 bp of upstream 5’ DNA sequence from PER36 ATG to the predicted 3’ UTR of Xero1 (At3g50980.1).</td>
</tr>
<tr>
<td>PER36_modp entry clone</td>
<td>Gateway promoter entry clone</td>
<td>pDONR_P4P1R</td>
<td>Premature stop codons were introduced into the Xero1 (cytosine to a thymine mutation at 1,560 bp) and Xero2 (guanine to thymine mutation at 345 bp) genes in the PER36p entry clone.</td>
</tr>
<tr>
<td>TBA2p entry clone</td>
<td>Gateway promoter entry clone</td>
<td>pDONRG_P4-P1R</td>
<td>Contains the TBA2 promoter sequence (1,346 bp), previously identified in Tsai et al. 2017.</td>
</tr>
<tr>
<td>MUM40.3Pro_35S entry clone</td>
<td>Gateway promoter entry clone</td>
<td>pDONR207</td>
<td>Gift from Gillian H. Dean, see Dean et al. 2017 for details.</td>
</tr>
<tr>
<td>MUM2 entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains the MUM2 coding sequence (2,155 bp), includes the ATG, and no stop codon.</td>
</tr>
<tr>
<td>BGAL11 entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains the BGAL11 coding sequence (2,535 bp), includes the ATG, and no stop codon.</td>
</tr>
<tr>
<td>BGAL16 entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains the BGAL16 coding sequence (2,446 bp), includes the ATG, and no stop codon.</td>
</tr>
<tr>
<td>BGAL17 entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains the BGAL17 coding sequence (2,092 bp), includes the ATG, and no stop codon.</td>
</tr>
<tr>
<td>MUM2sp entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains the 84 bp sequence encoding the predicted MUM2sp sequence, including an ATG, followed downstream by the SpeI, AatII sites.</td>
</tr>
<tr>
<td>MUM2sp-RglA entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains 1,521 bp RglA sequence without the sequence encoding the predicted RglA signal peptide sequence, ligated between</td>
</tr>
<tr>
<td>Clone Name</td>
<td>Gateway cDNA Entry Clone</td>
<td>Destination Vector</td>
<td>Description</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>--------------------------</td>
<td>--------------------</td>
<td>-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td><strong>MUM2sp-RglB</strong> entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains 1,926 bp RglB sequence without the sequence encoding the predicted RglB signal peptide sequence, ligated between the SpeI and AatII sites in the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>MUM2sp-RglB(K228A,H290A)</strong> entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Site-directed mutagenesis of both RglB predicted catalytic sites, lysine 228 to alanine and histidine 290 to alanine, within the <em>MUM2sp-RglB</em> entry clone.</td>
</tr>
<tr>
<td><strong>MUM2sp-Citrine</strong> entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains the 84 bp sequence encoding the predicted <em>MUM2sp</em> (84 bp), PstI site and Citrine encoding sequence (765 bp). Also contains an ATG, followed downstream by the SpeI, AatII sites.</td>
</tr>
<tr>
<td><strong>MUM2sp-ADPG2</strong> entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains 1,227 bp <em>ADPG2</em> sequence (without the sequence encoding the predicted ADPG2 signal peptide) ligated between the SpeI and AatII sites in the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>MUM2sp-Citrine-ADPG2</strong> entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains 1,227 bp <em>ADPG2</em> sequence (without the sequence encoding the predicted ADPG2 signal peptide) ligated between the SpeI and AatII sites in the <em>MUM2sp-Citrine</em> entry clone.</td>
</tr>
<tr>
<td><strong>MUM2sp-PelA</strong> entry clone</td>
<td>Gateway cDNA entry clone</td>
<td>pDONR207</td>
<td>Contains 1,077 bp PelA sequence (without the sequence encoding the predicted ADPG2 signal peptide) ligated between the SpeI and AatII sites in the <em>MUM2sp</em> entry clone.</td>
</tr>
<tr>
<td><strong>R4pGWB401</strong></td>
<td>Gateway R4pGWB destination vector</td>
<td>R4pGWB401</td>
<td>Gift from Tsuyoshi Nakagawa, see Nakagawa et al. 2008.</td>
</tr>
<tr>
<td><strong>R4pGWB401+Citrine</strong></td>
<td>Gateway R4pGWB destination vector</td>
<td>R4pGWB401</td>
<td>R4pGWB401 destination vector modified to include the sequence encoding the Citrine yellow fluorescent protein.</td>
</tr>
<tr>
<td><strong>TBA2p::MUM2-Citrine</strong></td>
<td>Gateway R4pGWB destination vector</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td><strong>PER36p::MUM2-Citrine</strong></td>
<td>Gateway R4pGWB destination vector</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td><strong>MUM4a3Pro_35S::MUM2-Citrine</strong></td>
<td>Gateway R4pGWB destination vector</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>Construct Name</td>
<td>Gateway Vector</td>
<td>Destination Vector</td>
<td>Status</td>
</tr>
<tr>
<td>----------------</td>
<td>----------------</td>
<td>---------------------</td>
<td>--------</td>
</tr>
<tr>
<td>PER36_0.2p::MUM2-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>PER36_modp::MUM2-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::BGAL11</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::BGAL11-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::BGAL16</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::BGAL16-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::BGAL17</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::BGAL17-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-RglA</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-RglA-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-RglB</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-RglB-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-RglB(K228A, H290A)-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-ADPG2</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-Citrine-ADPG2</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-PelA</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401 +Citrine</td>
<td>Completed chimeric construct</td>
</tr>
<tr>
<td>TBA2p::MUM2sp-PelA-Citrine</td>
<td>Gateway R4pGWB</td>
<td>R4pGWB401</td>
<td>Completed chimeric construct</td>
</tr>
</tbody>
</table>
2.3 Plant transformation

*Agrobacterium*-mediated plant transformation was carried out using the floral spray method (Weigel and Glazebrook 2006) with modifications in Dean et al. (2017). All transgenic plants were genotyped for transgenes and confirmed to be homozygous for the *mum2-l* mutation (guanine to alanine mutation at 1,537 bp; Dean et al. 2007) by restriction digestion analysis using the TatI restriction enzyme (Thermo Scientific), which cannot digest the WT copy of the *MUM2* gene.

2.4 Microscopy

2.4.1 Bright-field microscopy

2.4.1.1 Seed mucilage analysis

Mucilage was extruded by hydrating mature seeds in distilled water in combination with shaking at 130 revolutions per minute (rpm) for 1 h, or for 2 h when hydrating seeds with 0.05 M ethylenediaminetetraacetic acid (EDTA), after which seeds were washed twice with distilled water. Seeds were then immersed in 0.02 % (w/v) ruthenium red (Sigma-Aldrich) and shaken at 130 rpm for 15 min. After staining, the ruthenium red solution was replaced with fresh distilled water before bright-field images were obtained using a Zeiss Axioskop 2 light microscope (Carl Zeiss) equipped with a CCD Camera (DFC450 C; Leica) and the LAS software (v4.2.0, Leica).

2.4.1.2 Videoing mucilage extrusion

Video recording of mucilage extrusion was performed using single seeds held in place between a microscope slide and a cover slip. Immediately after filming had commenced, 100 µl of ruthenium red, 0.02 % working solution, was added between the coverslip and the microscope
slide, as described in Šola et al. (2019a). Video recording was performed using the Zeiss Axioskop 2 light microscope (Carl Zeiss) and the LAS software (v4.2.0, Leica) as described previously. Mucilage extrusion from three seeds, each from three separate independent transformants was videoed for each of the two chimeric constructs which were analyzed.

2.4.1.3 Trypan blue staining

A working solution of trypan blue (0.4 %, Gibco, Fisher Scientific) 1:1 in water was added directly to developing siliques, in which one silique valve was removed to allow exposure of the stain to developing seeds attached to the silique. After 30 mins of staining, trypan blue was removed and siliques were washed twice in 100 % ethanol. The siliques with the seeds still attached were destained for a further 72 hrs in fresh 100 % ethanol. They tissue was then imaged using a digital camera (Canon EOS Rebel T5) mounted to a dissecting scope (Stemi 2000-C, Zeiss) and illuminated with a light box (Ace Eke Lamp, Schott-Fostec).

2.4.2 Confocal fluorescence microscopy

Fluorescence microscopy was performed on developing seeds mounted in water and viewed through a 63x objective lens using 100 % glycerol as an immersion medium on an UltraView VoX spinning disk confocal microscope (PerkinElmer) equipped with an EMCCD camera (C9100-02; Hamamatsu) and the Volocity software (Improvision). Citrine was excited using a 514 nm argon laser and emission was detected using a 540 nm filter. All 7 DPA images in Chapter 3 were acquired using identical microscope and laser intensity settings. The FM 4-64 plasma membrane dye (5 μg/ml, Molecular Probes, Invitrogen), and propidium iodide (2 mg/ml) were added directly to developing seeds and then imaged after 5-10 mins of staining at room
Developing seeds were kept in the dark when stained with DAPI (10 μg/ml) for 5-10 minutes. FM 4-64 was excited at 514 nm, propidium iodide at 561 nm and DAPI at 405 nm. Emission was detected at 650 nm for both FM 4-64 and propidium iodide and at 460 nm for DAPI. ImageJ was used to add scale bars and apply the Fire LUT filter for pseudo-colouring images based on fluorescence intensity (Schindelin et al. 2015). Figures were produced using Inkscape (https://inkscape.org).

Average gray values for each chimeric construct (BGAL11-Citrine, BGAL16-Citrine or BGAL17-Citrine) were calculated from 12 representative images (taken using identical microscope settings), three images each from four independent transformants. Initially, the Threshold function in ImageJ (Rasband 2011) was performed on each representative micrograph, using the Default ImageJ algorithm, followed by the Analyze Particle function (pixel size set to 0-Infinity and 0-1.0 circularity) to create a list of regions of interest. The Measure function was then used to calculate the mean gray value (sum of all pixel gray values divided by number of pixels) per regions of interest in the threshold micrographs. The average gray value and standard deviation was calculated from these mean gray values.

2.5 High-pressure freezing and sectioning

In order to obtain high resolution sections with high-quality preservation of developing seed coat epidermal cells, high pressure freezing was employed. Initially, 7 DPA seeds (staging as described previously) were pricked using an insect pin and transferred in-between two Type A hats (copper-gold, 3.0 x 0.5 mm, Leica) filled with 1-hexadecene. After high-pressure freezing (EM HPM 100, Leica) samples were transferred under liquid nitrogen to cryovials containing 2 % w/v osmium tetroxide in 8 % v/v 2-dimethoxypropane as the freeze-substitution medium.
Cryovials were placed in a dry ice and acetone slurry within a sealed freeze-substitution box, which was stored at -80°C for four days, then opened and transferred to -20°C for one day. The following day, samples were moved to 4°C, and then to room temperature before being embedded in Spurr’s epoxy resin (Spurr 1969) over the course of four days (Quilichini et al. 2014). Spurr’s epoxy resin blocks were then cut into 0.5 μm sections using an EM UC7 (Leica), stained with 1% toluidine blue in 1% sodium borate, and then mounted in Permount (Fisher-Chemical). Sections were imaged using a VHX-5000 digital microscope (Keyence Corp.).

2.6 Scanning electron microscopy

To visualize the seed surface, dried mature seeds were affixed to stubs using double-sided tape then sputter coated (208HR, Cressington) with 10 nm of gold-palladium and examined using a scanning electron microscopy (S-2600N, Hitachi High Technologies) at an acceleration voltage of 8 or 20 kV.

2.7 Total RNA extraction and quantitative real-time PCR (qRT-PCR)

Two independent transformants for each construct were selected, except in the case of lines transformed with MUM4_{0.3Pro_{35S}} constructs where two representative independent transformants for each of the three mucilage extrusion phenotypes were selected. Three sets of each genotype plus controls were grown independently. Tissue was harvested from these sets to create a total of 4 biological replicates (1 replicate each from sets 1 and 2, and 2 replicates from set 3). Each biological replicate consisted of two 7 DPA siliques, which after harvesting were immediately flash frozen in liquid nitrogen and stored at -80°C. Frozen siliques were ground to a fine powder in a pre-chilled mortar and pestle on dry ice followed by RNA extraction (Meisel et
al. 2005). RNA was dissolved in Milli-Q water and stored at -80°C. The RNA quality and quantity were assessed spectrophotometrically using a NanoDrop 8000 (Thermo Scientific). RNA was assessed for potential genomic DNA contamination using the ACT2_for and ACT2_rev primers (Table 2-3). Prior to cDNA synthesis, 500 ng of RNA of each genotype was treated with DNase I (Amplification Grade; Invitrogen) according to the manufacturer’s protocol. First strand cDNA synthesis was then carried out using the iScript cDNA synthesis Kit (Bio-Rad) and oligo(dT)20 primers.

Initially, PCR amplification efficiency of the gene-specific primers (MUM2-Citrine_for and MUM2-Citrine_rev, Table 2-3) was validated by constructing a standard calibration curve using cDNA from a single transgenic line (PCR efficiency was 92.7 %, R^2 = 0.9935). The qRT-PCR reaction volumes and PCR conditions were as described in Dean et al. (2017), and four technical replicates were performed for each cDNA. MUM2-Citrine transcript abundance was calculated relative to Glyceraldehyde-3-phosphate dehydrogenase C subunit 1 (GAPC, At3g04120) using the GAPC_for and GAPC_rev primers (Table 2-3), and the ΔCT method (Livak and Schmittgen 2001). All four biological replicates showed similar trends. Primers sequences used for qRT-PCR are listed in Table 2-3.
Table 2-3: qRT-PCR primer sequences.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence (5′→3′)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACT2_for</td>
<td>GTATTGCTCCTGAAGAGCACC</td>
<td>Assessing genomic DNA contamination in extracted RNA.</td>
</tr>
<tr>
<td>ACT2_rev</td>
<td>GACGGAGGATGGCATGAGG</td>
<td></td>
</tr>
<tr>
<td>MUM2-Citrine_for</td>
<td>GGTGAATGGTGAAAGCATTGGTCG</td>
<td>Amplification of the MUM2-Citrine transcript.</td>
</tr>
<tr>
<td>MUM2-Citrine_rev</td>
<td>AGGAGCGCTGTTATCAACCAC</td>
<td></td>
</tr>
<tr>
<td>GAPC_for</td>
<td>TCAGACTCGAGAAAGCTGCTAC</td>
<td>Amplification of the GAPC transcript as an internal control, previously used in Dean et al. 2017.</td>
</tr>
<tr>
<td>GAPC_rev</td>
<td>GATCAAAGTCGACCACACCG</td>
<td></td>
</tr>
</tbody>
</table>

2.8 Phylogenetic analysis, domain architecture and sequence alignments

Full-length protein sequences of the 17 *A. thaliana* GH35 BGALs and the *Escherichia coli* BGAL *lacZ* (P00722) were retrieved from the UniProt database (Bateman et al. 2017). Sequences were aligned using MAFFT version 7 (Katoh and Standley 2013, https://mafft.cbrc.jp/alignment/server) using default parameters. Model Selection analysis in MEGA v7.026 (Kumar et al. 2016) was used to determine that the amino acid substitution model with the highest likelihood for the MAFFT-generated alignment was the WAG+G+I model, which is typically associated with globular proteins such as BGALs (Whelan and Goldman 2001). A maximum likelihood phylogenetic tree was then generated using the WAG+G+I protein substitution model, partial deletion for gaps/missing data, 95 % site coverage cut-off for gap penalties, default tree interference options, and 1,000 bootstrap replicates using MEGA v7.026.
The predicted protein domains for each of the 17 GH35 BGAL were obtained from the Pfam database (Finn et al. 2016) and predicted domains with e-values > 0.04 were omitted.

Percentage protein similarity between full-length protein sequences of different BGALs were calculated using BLASTP pairwise alignment (version 2.7.0, Altschul et al. 1990; Altschul and Gish 1996) using the default general parameters, substitution BLOSUM-62 matrix, and conditional compositional score matrix adjustment.

Alignment of the GH35 catalytic domains in BGAL6 (32-345 amino acids (aa)), BGAL11 (39-357 aa), BGAL16 (31-336 aa), and BGAL17 (69-394 aa) was performed using MAFFT version 7, as described above. To visualize conserved amino acids, JalView 2.10.3b1 (Waterhouse et al. 2009) was used to overlay protein identity by colour onto the alignment. Location of the glutamic acid catalytic residues within the GH35 domain is based on crystal structural analysis of a *Pencillium* sp. BGAL (Rojas et al. 2004).

### 2.9 Thin layer chromatography (TLC)

For each genotype, ~20 mg of seeds was weighed out from two biological replicates. Seeds were vortexed for 2 hrs (Vortex Mixer VX100, Labnet International) in 1.4 mL 0.022 M sodium carbonate to release mucilage, which was then transferred to a new tube. The remaining seeds (with mucilage removed) were pulverized using ~four 2 mm zirconium oxide beads (Next Advance, Inc.) and the Precellys 24 tissue homogenizer (Bertin Technologies) for 20 s at 6,000 rpm. The resulting tissue was vortexed briefly in 0.5 mL 70 % ethanol to release soluble sugars, and transferred to a fresh tube. The soluble sugars aliquots were then vortexed for 30 min (Vortex Mixer VX100, Labnet International) and centrifuged at 16,000 × g for 10 min to pellet the debris. Silica-Gel 60 aluminum TLC plates (EMD Millipore) were spotted with 20 μL of
extruded mucilage or 2 μL of soluble sugars from the seeds where mucilage was extruded and removed. TLC plates were also spotted with 0.5 μL of 25-35 mM monosaccharide standards of D-(+)-GalA, L-Rha, Sucrose, D-(+)-Gluc, L-(+)-Ara and D-(+)-Gal. Once dried, TLC plates were submerged in solution of n-Butanol, glacial acetic acid and water (3:2:1 of, v/v/v) within a glass chamber at room temperature for 2 hrs. After sugars were sufficiently separated, the TLC plate was removed from the glass chamber, air dried, sprayed with 0.5 % thymol in ethanol/sulfuric acid (95:5, v/v), and sugars were visualized by heating the TLC plate at 120 °C.

The isolation and hydrolysis of specific compounds of interest was carried out by first spotting extruded mucilage from RglA-Citrine and RglB-Citrine transgenic seeds onto fresh TLC plates. Sugars were separated as described above, and ultraviolet fluorescence was used to identify the region on the TLC plate containing the compound of interest. This region was removed using a spatula, washed in distilled water, centrifuged to pellet the debris and the resulting aqueous layer was removed. The aqueous layer containing the compounds of interest was hydrolyzed in 0.1 M acetic acid at 120 °C for 1 hr. The hydrolyzed solution was spotted onto a new TLC plate and sugars were separated and visualized as previously described.

2.10 Monosaccharide analysis

Three or four independent transformants were selected expressing each chimeric BGAL construct. One biological replicate for monosaccharide analysis consisted of pooled seed from eight plants for each line. All lines were grown at the same time in the same growth chamber. A second biological replicate was grown in the same way in a separate chamber.

Mucilage monosaccharide composition was analyzed by first weighing four batches of seeds for each line (~20 mg, exact weights were recorded). All subsequent steps were as
described in Dean et al. (2007) and Šola et al. (2019a). Whole seed monosaccharide composition was analyzed by preparing alcohol insoluble residue. Again, four separate batches of ~20 mg seed for each line were weighed out and exact weights recorded. Seeds were pulverized by ~four 2 mm zirconium oxide beads (Next Advance, Inc.) using a Precellys 24 tissue homogenizer (Bertin Technologies) for 20 s at 6,000 rpm, before 1 mL 70 % ethanol was added. All subsequent steps were as described in Dean et al. (2007) and Šola et al. (2019a).

Monosaccharides were analyzed by converting peak area to molar amounts as described in Dean et al. 2007, using Excel (2007; Microsoft). A total of three outliers from three genotypes were identified using the Grubbs outlier test (Grubbs 1950) alpha = 0.05 (www.graphpad.com/quickcalcs/grubbs1). All monosaccharide data generated from that batch of seeds was subsequently removed from the analysis.

2.11 Statistical analysis

One-way ANOVA with a Bonferroni post-hoc test was performed by comparing nmol of Gal/mg seed values in mum2 to the transgenic lines within a biological replicate using GraphPad Prism version 6 (GraphPad Software La Jolla California USA, www.graphpad.com).
Chapter 3: Assessing the utility of seed-coat specific promoters to engineer cell wall carbohydrate composition of mucilage

3.1 Introduction

The usefulness of seed coat epidermal cells as a genetic model can be widened using genetic engineering. For example, if the polysaccharide composition of mucilage is modified in a defined manner by introducing genes that encode anabolic or catabolic CAZy enzymes, and the consequences of such changes impart structural or biochemical changes, then structure-function relationships could be established \textit{in vivo}. In the past, most attempts to modify cell wall composition have relied on constitutive promoters that drive expression of genes encoding cell wall modifying enzymes throughout the organism. These approaches have often resulted in negative effects on plant growth and physiology, complicating interpretation of the data (reviewed in Wang et al. 2016). An alternative approach is to use tissue-specific promoters. In the case of the Arabidopsis seed coat, a number of tissue-specific promoters have been identified in recent years (reviewed in Francoz et al. 2018). Promoters from at least three genes can drive transcription specifically in seed coat epidermal cells during the peak of mucilage synthesis and secretion (7 DPA). These are \textit{TBA2} (Tsai et al. 2017), \textit{PER36} (Kunieda et al. 2013), and a chimeric \textit{MUM4} promoter (\textit{MUM4}_{0.3Pro}_{35S}; Dean et al. 2017).

In this chapter I sought to determine if three seed coat-specific promoters could be used as tools for engineering mucilage composition, by testing their ability to express a gene encoding a known mucilage modifying enzyme at levels sufficient to complement the corresponding mucilage mutant phenotype. I tested and compared the ability of the \textit{TBA2}, \textit{PER36}, and \textit{MUM4}_{0.3Pro}_{35S} promoters to express the \textit{MUM2} gene. \textit{MUM2} encodes a BGAL (EC 3.2.1.23)
from the GH35 enzyme family which removes Gal side-chains from the seed mucilage pectin RG-I (Macquet et al. 2007b; Dean et al. 2007). In loss-of-function mum2 mutants, seed mucilage contains RG-I with increased side-chains that cannot expand and extrude when seeds are hydrated (Western et al. 2001; Macquet et al. 2007b; Dean et al. 2007). Promoter function was evaluated by direct measurements of mRNA abundance and their ability to rescue the mum2 mucilage extrusion phenotype. It was shown that all of the tested promoters could be used as tools to modify mucilage, even though expression levels varied among them. Using this knowledge, the utility of this seed coat-specific expression system was demonstrated by showing that three previously uncharacterized enzymes from the GH35 enzyme family had BGAL activity, by complementation of the mum2 mucilage extrusion and cell wall composition phenotypes.

3.2 Results

3.2.1 Complementation of the mum2 mucilage extrusion phenotype using different seed coat-specific promoters to drive MUM2-Citrine

In order to deliver cell wall-modifying enzymes to the apoplast of seed coat epidermal cells without disrupting the function of other cell types, seed coat-specific promoters are required. Of the Arabidopsis seed coat epidermal-specific promoters that have been described, TBA2p, PER36p, and MUM4.3Pro.35S were selected as they can drive transcription at 7 DPA during the peak of mucilage secretion (Kunieda et al. 2013; Dean et al. 2017; Tsai et al. 2017). The sequences of each promoter as well as the cDNA sequence of MUM2 were recombined into separate Gateway entry vectors and assembled into promoter::MUM2-Citrine chimeric constructs using three-way Gateway cloning. By using this approach, the only differences
between each chimeric construct were the promoter sequences, in which TBA2p and PER36p contained native 5' UTRs and MUM4_0.3Pro_35S lacked a native 5' UTR. The promoter::MUM2-Citrine chimeric constructs were transformed into plants homozygous for the mum2 mutation and seeds from the resulting transgenic plants were examined for their ability to extrude mucilage.

All 22 independent mum2 transformants expressing TBA2p::MUM2-Citrine produced seeds with mucilage extrusion equivalent to WT (Figure 3-1c), demonstrating that TBA2p can promote transcription of MUM2 sufficiently to consistently complement the mum2 extrusion phenotype. Of the 16 independent transformants expressing PER36p::MUM2-Citrine in mum2, 14 produced seeds with wild type-like mucilage extrusion (Figure 3-1g) while the two remaining lines produced seeds with patchy mucilage extrusion, suggesting partial complementation (Figure 3-2a). In contrast, of the 32 independent transformants expressing MUM4_0.3Pro_35S::MUM2-Citrine in the mum2 background, only 15 produced seeds that had wild type-like extrusion (Figure 3-1m). Of the remaining transgenic lines, nine showed patchy mucilage extrusion and displayed a reduced mucilage adherent layer (Figure 3-1n) and eight appeared indistinguishable from mum2 with no extrusion (Figure 3-1o). Due to the distinct differences in complementation, representative lines from each of these three mucilage extrusion phenotypes were analyzed separately. While hydration of seeds is typically achieved with water, EDTA can also be used, which is thought to chelate calcium ions causing the mucilage to loosen (Western et al. 2001; Macquet et al. 2007a; Dean et al. 2007). This enables some mucilage to be released from mum2 seeds (Figure 3-3b). Of the eight MUM4_0.3Pro_35S::MUM2-Citrine transgenic lines that did not extrude mucilage in water, hydration with EDTA caused more mucilage to be extruded than in untransformed mum2 (Figure 3-3i). These results suggest that although the no
Figure 3-1: Complementation of the mum2 mucilage phenotype using three different promoters driving MUM2-Citrine expression in seed coat epidermal cells.

a-c, g-i, m-o Mature seeds from WT, mum2 and mum2 mutants carrying the indicated chimeric constructs were hydrated in water then stained with ruthenium red to detect extruded mucilage.

d-f, j-l, p-r Seed coat epidermal cells of developing 7 DPA Arabidopsis seeds from plants of WT, mum2 and mum2 mutants carrying the indicated chimeric constructs were examined for Citrine fluorescence using identical microscope settings. The Fire LUT Filter was applied to all micrographs to visualize differences in fluorescence intensity as indicated by the intensity bar. M = mucilage pocket, CC = cytoplasmic column. Scale bar = 200 μm (a-c, g-i, m-o) and 20 μm (d-f, j-l, p-r).
Mature Arabidopsis seeds from WT, mum2 and mum2 mutants carrying the indicated transgenic construct were hydrated in 0.05 M EDTA to release mucilage and then stained with ruthenium red to detect extruded mucilage. Scale bar = 200 μm.

Figure 3-2: Patchy mucilage extrusion observed in a small number of transgenic seeds where MUM2-Citrine expression was driven by either PER36p or PER36modp.

Figure 3-3: Mucilage extrusion from seeds of plants transformed with different seed coat-specific promoters driving MUM2-Citrine.

Mature seeds hydrated in 0.05 M EDTA to release mucilage and then stained with ruthenium red to observe extruded mucilage. Scale bar = 200 μm.
extrusion MUM4::MUM2-Citrine transgenic lines did not extrude mucilage in water, there was partial complementation of the mum2 extrusion phenotype.

The PER36 promoter sequence used above was first described by (Kunieda et al. 2013). The sequence includes two small genes, Xero1 (At3g50980.1) and Xero2 (At3g50970.1; Rouse et al. 1996), which are located approximately 289 base pairs (bp) upstream of the PER36 start codon. Problematically, both genes are also expressed in the seed coat cell layer during mucilage secretion (Dean et al. 2011). To assess whether translation of these gene products influences the ability of this promoter to complement, a truncated version of the sequence (PER36_0.2p) that contains only the 289 bp immediately upstream of the PER36 start codon and excludes the predicted transcribed regions of Xero1 and Xero2 was generated. Analysis using the PLACE database (Higo et al. 1999) predicted that the PER36_0.2p sequence contains a number of cis-acting DNA elements including a TATA box (-51), two CAAT boxes (-17 and -129), and three GATA boxes (-47, -120 and -148) that may contribute to transcriptional activity. PER36_0.2p was fused upstream of MUM2-Citrine and transformed into mum2. None of the 17 independent transformants expressing PER36_0.2p::MUM2-Citrine produced seeds that extruded mucilage in water (Figure 3-1h). However, when these transgenic seeds were hydrated with EDTA some mucilage was released, suggesting weak complementation of the mum2 extrusion phenotype (Figure 3-3e). In contrast to PER36p, these data suggest that the PER36_0.2 promoter sequence was transcriptionally active during seed development, but not at a level that was sufficient to completely complement the mum2 extrusion phenotype. Therefore, as an alternative to removing the sequences encoding Xero1 and Xero2, premature stop codons were introduced into the coding regions of both Xero1 and Xero2 gens in the PER36p sequence. This change ensured that Xero1 and Xero2 would not be translated while leaving the remainder of the DNA sequence of
PER36p unchanged. The new sequence, PER36_modp was fused upstream of MUM2-Citrine and transformed into mum2 homozygous plants. Wild type-like mucilage extrusion was observed in 16 of the 18 independent transformants expressing PER36_modp::MUM2-Citrine (Figure 3-1i). The remaining two transgenic lines produced seeds with patchy mucilage extrusion, indicating partial complementation of the mum2 phenotype (Figure 3-2b). Therefore, PER36_modp was comparable to PER36p in its ability to complement mum2.

3.2.2 Confocal fluorescence intensity of MUM2-Citrine correlates with the degree of mum2 complementation

Like native MUM2, chimeric MUM2-Citrine contains a signal peptide that should target the fusion protein to the mucilage pocket. MUM2 is thought to function in the apoplast, where it processes RG-I to allow mucilage to expand when hydrated (Macquet et al. 2007b; Dean et al. 2007; Šola et al. 2019a). Since each of the promoter::MUM2-Citrine chimeric constructs was able to complement the mum2 extrusion phenotype (Figure 3-1c, g, i, m, n, Figure 3-3c-i), it was expected that the MUM2-Citrine fusion protein would also be localized to the apoplast of seed coat epidermal cells. Confocal microscopy verified that Citrine fluorescence was detected in the apoplast of the seed coat epidermal cells at 4, 7 and 10 DPA in transgenic lines driven by TBA2p, PER36p, PER36modp, and MUM40.3Pro_35 (Figure 3-1f, j, l, p-r, Figure 3-4). Even when the sensitivity of the confocal microscope was increased no fluorescence signal was detected in transgenic lines driven by PER36_0.2p (Figure 3-1k), which very weakly complemented mum2 and only when hydrated in EDTA (Figure 3-3e) and was therefore excluded from further analysis.
Identical confocal microscope settings were used in the \textit{PER36p}, \textit{PER36modp} and \textit{MUM4}0.3Pro_35S panels. The \textit{MUM4}0.3Pro_35S transgenic line shown here exhibited complete \textit{mum2} complementation when hydrated in water (Figure 3-1m). R = radial primary cell wall, M = mucilage pocket, C = columella. Scale bar = 20 μm.

When using identical microscope settings, Citrine fluorescence intensity was similar across all analyzed lines driven by the same promoter (\textit{TBA2p}, \textit{PER36p} and \textit{PER36modp}). On the other hand, Citrine fluorescence intensity was highly variable between transgenic lines expressing \textit{MUM4}0.3Pro_35S::\textit{MUM2}-Citrine. In these transgenic plants, fluorescence intensity correlated with the degree of complementation, in which the highest fluorescence intensity was observed in lines classified into the group with wild type-like extrusion signifying complete complementation (Figure 3-1m, p). Similarly, weaker fluorescence was observed in the group of lines with patchy extrusion (partial complementation; Figure 3-1n, q), and almost no fluorescence in lines displaying no extrusion (no complementation; Figure 3-1o, r).

In order to compare fluorescence intensity between lines driven by different promoters, two representative lines from each of the four promoters were examined using identical
microscope settings in 7 DPA epidermal cells. The fluorescence intensity varied considerably between lines driven by different promoters, as visualized by applying the ImageJ Fire look up table (LUT) filter to each micrograph, which pseudo-colors regions based on fluorescence intensity (see intensity bar in Figure 3-1). The highest intensity of fluorescence was observed in TBA2p::MUM2-Citrine lines (Figure 3-1f), which had wild type-like mucilage extrusion indicating complete complementation (Figure 3-1c). The second highest intensity of fluorescence was observed in transgenic lines expressing PER36p::MUM2-Citrine or PER36modp::MUM2-Citrine (Figure 3-1j, l), which had complete complementation in most transgenic lines (Figure 3-1g, i). The population of transgenic lines expressing MUM40.3Pro_35::MUM2-Citrine had the lowest fluorescence intensity (Figure 3-1p-r). In summary, these data show clear differences in the fluorescence intensity between transgenic lines driven by different promoters, which correlated with the extent of complementation.

3.2.3 The TBA2 promoter produces the highest abundance of MUM2-Citrine transcript

The TBA2p, PER36p, PER36modp, and MUM40.3Pro_35 promoters used in the mum2 complementation experiments differ in both their promoter and 5’ UTR sequences. Therefore, the observed differences in complementation could be due to variation in promoter strength, mRNA stability or mRNA translation. To determine the extent to which transcript abundance correlated with the level of complementation, qRT-PCR analysis was performed on 7 DPA developing seeds to quantify differences in MUM2-Citrine transcript abundance using the same transgenic lines previously used for the fluorescence intensity analysis. No MUM2-Citrine transcript was detected within WT or mum2 (Figure 3-5). On the other hand, the average transcript abundance of MUM2-Citrine was found to be at least 250 times higher in transgenic
lines expressing *TBA2p::MUM2-Citrine* compared to lines expressing the three other chimeric constructs (Figure 3-5). As may be expected, higher transcript abundance were correlated with higher fluorescence intensity (e.g. in *TBA2p::MUM2-Citrine* lines), whereas lower transcript abundance was correlated with lower fluorescence intensity (e.g. in the *PER36p::MUM2-Citrine* transgenic lines; Figure 3-1j, l, p-r, Figure 3-5). Such variation in expression between independent transformants is not uncommon. The location of the transgene within the genome is distinct for each independent transformant which often influences transcription (position effect), due to the proximity to centromeres, telomeres, repeat-rich regions and retroelement remnants and regulatory regions of adjacent genes (reviewed in Matzke and Matzke 1998).

Within the population of *MUM40.3Pro_35S::MUM2-Citrine* transgenic lines, transcript abundance could also partially predict the fluorescence intensity and the degree of mum2 complementation, which were characterized into three distinct phenotypic groups, as described earlier. In this case almost no *MUM2-Citrine* transcript was apparent in those transgenic lines (#7 and #10) with very low fluorescence intensity and no mucilage extrusion (Figure 3-1o, Figure 3-5). On the other hand, higher abundance of *MUM2-Citrine* transcript was observed in the *MUM40.3Pro_35S::MUM2-Citrine* transgenic lines that showed either patchy (#3 and #11) or wild type-like extrusion (#4 and #8; Figure 3-5). Somewhat surprisingly, the transcript abundance was similar among the patchy and wild type-like extrusion lines, implying that transcript abundance may not be the only factor influencing complementation in these transgenic lines.

It was clear that all four promoters could be used to express MUM2 in seed coat epidermal cells at a range of expression levels, any one of which might be useful depending on the application. As a guide for selecting promoters, a summary of transcript abundance, window
of expression, inclusion of 5’ UTRs, and complementation efficiency are summarized in Table 3-1.

Figure 3-5: *MUM2-Citrine* transcript abundance in 7 DPA transgenic seeds.

Relative quantification was based on four technical replicates performed on each cDNA sample and normalized to an internal *GAPC* control. Error bars indicate standard deviation. qRT-PCR analysis was also performed on three additional biological replicates, all showing similar trends (data not shown).
Table 3-1: Summary of transcript abundance, window of expression, inclusion of a 5’ UTR, and complementation efficiency between different seed coat-specific promoters.

For each promoter, MUM2-Citrine transcript abundance from one biological replicate was averaged among all transgenic lines. ±represents standard deviation.

<table>
<thead>
<tr>
<th>Seed coat-specific promoter</th>
<th>Average transcript abundance relative to GAPC</th>
<th>Window of expression</th>
<th>Inclusion of a 5’ UTR</th>
<th>Complete complementation of mum2 extrusion phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>TBA2p</td>
<td>7.38 ± 1.06</td>
<td>4 - 10</td>
<td>Yes</td>
<td>22/22 lines - 100%</td>
</tr>
<tr>
<td>PER36p</td>
<td>0.04 ± 0.01</td>
<td>DPA</td>
<td>Yes</td>
<td>14/16 lines - 88%</td>
</tr>
<tr>
<td>PER36_modp</td>
<td>0.06 ± 0.05</td>
<td></td>
<td>Yes</td>
<td>16/18 lines - 89%</td>
</tr>
<tr>
<td>MUM40.3Pro_35S</td>
<td>0.02 ± 0.01</td>
<td></td>
<td>No</td>
<td>15/32 lines - 47%</td>
</tr>
</tbody>
</table>

3.2.4 Seed coat-specific promoters can be used to assess whether candidate BGALs are functionally equivalent to MUM2/BGAL6

MUM2/BGAL6 encodes a member of the GH35 enzyme family that has been shown to have BGAL activity (Macquet et al. 2007b; Dean et al. 2007). The GH35 family consists of 17 BGALs, all of which contain the GH35 catalytic domain necessary for BGAL activity (Rojas et al. 2004; Ahn et al. 2007). A limited number of GH35 proteins have been shown to have BGAL activity, while the remainder are uncharacterized (Chandrasekar and van der Hoorn 2016). To test the seed coat-specific expression system developed earlier in this chapter, I examined whether putative GH35 BGALs could functionally replace BGAL6 in Arabidopsis. Candidate BGALs were selected using the following criteria. First, the level of divergence between all GH35 BGALs was assessed by constructing a maximum likelihood phylogenetic tree (Figure 3-6a). Using this tree, candidate BGALs were assessed based on their sequence similarity relative to BGAL6, which might impact functional equivalency. Second, the predicted protein domains for each BGAL obtained from the Pfam database (Finn et al., 2016) were reviewed to
identify potential structural differences or similarities that might impact functional equivalency (Figure 3-6b). In agreement with a number of previous phylogenetic studies (Perez Almeida 2004; Ahn et al. 2007; Chandrasekar and van der Hoorn 2016) the amino acid sequence of BGAL16 (At1g77410.1) was found to be the most similar to BGAL6 (73 %; Figure 3-6a, b) and was therefore selected as a candidate for the complementation analysis. Interestingly, BGAL16 was predicted to contain a galactose/rhamnose binding SUEL-type lectin domain that is not present in BGAL6 (Figure 3-6b; Ozeki et al. 1995; Hosono et al. 1999). While it has been suggested that this domain anchors BGALs to their substrate, there is no evidence that this domain mediates binding of galactose in recombinant BGAL1 and BGAL3 (Gantulga et al. 2009), therefore the function of this domain within GH35 BGALs remains to be determined. Within the phylogenetic tree (Figure 3-6b), the next sub-clade most similar to BGAL6 contained BGAL11, BGAL13 and BGAL14, all of which are also predicted to contain the aforementioned galactose/rhamnose binding domain (Figure 3-6a, b). BGAL11 (At4g35010, 67 % amino acid sequence similarity to BGAL6) was selected as a representative of this sub-clade for the complementation assay. Of the remaining GH35 BGALs, BGAL17 (At1g72990.1, 44 % amino acid sequence similarity to BGAL6) was the most distantly related to BGAL6 (Figure 3-6a) and is more closely related to animal than plant BGALs (Ahn et al. 2007). Therefore, BGAL17 is predicted to represent an ancestral BGAL that was present in the common ancestor of plants and animals (Ahn et al. 2007), which might therefore be functionally different from BGAL6. For this reason, BGAL17 was select as a third candidate for the complementation assay. The protein sequences of the three candidate BGALs were aligned with BGAL6 to confirm that both catalytic glutamic acids residues required for BGAL activity were present within their respective GH35 domains (Figure 3-6c; Rojas et al. 2004).
3.2.5 Complementation of mum2 extrusion using three candidate BGALs

The functional equivalency of BGAL11, BGAL16 and BGAL17 to MUM2/BGAL6 was determined by fusing the TBA2 promoter upstream of the genomic sequence encoding each candidate BGAL and transforming these constructs into a mum2 background to assess complementation of the mum2 phenotypes.

All 14 independent transformants expressing TBA2p::BGAL16-Citrine (BGAL16-Citrine) in mum2 produced seeds with wild type-like extrusion when hydrated with water (Figure 3-7a) or EDTA (Figure 3-8d). Thus, BGAL16 is able to fully complement the mum2 extrusion phenotype suggesting that BGAL16, which is most closely related to MUM2/BGAL6, is also functionally similar. In contrast, when TBA2p::BGAL11-Citrine (BGAL11-Citrine) was transformed into mum2, seeds from 11 independent transformants failed to extrude mucilage when hydrated in water (Figure 3-7b). However, when these seeds were treated with EDTA, patchy extrusion was observed in 10 of 11 transgenic lines (Figure 3-8e) demonstrating that BGAL11 can partially replace MUM2/BGAL6 function. Similarly, none of the 11 independent transformants
expressing $TBA2p::BGAL17$-Citrine ($BGAL17$-Citrine) extruded mucilage when hydrated in water (Figure 3-7c), and only patches of mucilage were released when seeds were hydrated with

Mature seeds were hydrated in water (a-f) or in 0.05 M EDTA (g-i) before being stained with ruthenium red to observe extruded mucilage. Scale bar = 200 μm.

**Figure 3-7:** Mucilage phenotypes of $mum2$ plants transformed with the $TBA2$ promoter expressing different candidate $BGALs$ either untagged or fused in-frame to a C-terminal Citrine yellow fluorescent protein.
**Figure 3-8: Complementation of the mum2 mucilage phenotype using candidate BGALs driven by the TBA2 promoter.**

**a-f** Mature seeds were hydrated in 0.05 M EDTA and then stained with ruthenium red to observe extruded mucilage. Percentage protein similarity was calculated using BLASTP pairwise alignment of full-length MUM2 to each full-length BGAL protein sequence. **g-i** Citrine fluorescence from each tagged-BGAL within the apoplast of 7 DPA seed coat epidermal cells was imaged using identical microscope settings. The Fire LUT Filter was applied to all micrographs to help visualize differences in fluorescence intensity as indicated by the intensity bar. M = mucilage pocket, CC = cytoplasmic column. Using identical microscope settings, average pixel intensity (average gray values) was calculated from a total of 12 representative micrographs (3 micrographs each from four different independent transformants). ± represents standard deviation. Scale bar = 200 μm (a-f) and 20 μm (g-i).
EDTA (Figure 3-8f). Although BGAL17-Citrine transformants released even less mucilage than BGAL11-Citrine lines after hydration with EDTA, this was still substantially more than untransformed mum2 seed under similar conditions (Figure 3-8b), which suggests that BGAL17 can very weakly functional complement MUM2/BGAL6. Mucilage extrusion was complemented to the same degree when mum2 was transformed with untagged TBA2p::BGAL11, TBA2p::BGAL16, or TBA2p::BGAL17, suggesting that the C-terminal Citrine fluorescent protein tag had no measurable impact on the ability of the enzyme to complement the extrusion phenotype (Figure 3-7d-i).

Confocal microscopy analysis verified that all three C-terminal Citrine tagged BGALs were localized to the mucilage pocket at 7 DPA (Figure 3-8g-i), where they are expected to hydrolyze the galactose side-chains on RG-I. It is worth noting that the differences in fluorescence intensity between cells within a given micrograph are probably due at least in part to the curvature of the seed (Figure 3-8g-i).

To determine if the differences in complementation when using different candidate BGALs might be due to differences in the amount of proteins synthesized as measured by fluorescence intensity in the mucilage pocket, the average pixel intensity was calculated from four independent transformants expressing the same BGAL-encoding gene, using the same microscope settings. Although there was some variation in fluorescence intensity observed between lines expressing the same construct and between lines expressing different constructs, these differences did not correlate with the degree of complementation (Figure 3-8g-i). Suggesting that the distinct differences in complementation when expressing different BGALs were not due to substantial variations in the amount of translated protein.
3.2.6 Biochemical complementation of *mum2* using three candidate BGALs

In *mum2*, the loss of MUM2/BGAL6 activity is thought to impact mucilage extrusion due to the presence of longer or increased number of galactose side-chains on RG-I (Dean et al. 2007; Macquet et al. 2007b; Šola et al. 2019a). These differences in side chains are detected as higher levels of galactose when *mum2* mucilage monosaccharide composition is analyzed (Dean et al. 2007; Arsovski et al. 2009). Therefore, I investigated whether the higher mucilage galactose levels present in the *mum2* background were returned to lower wild type-like levels when candidate BGALs were introduced into *mum2*. In order to release seed mucilage for monosaccharide analysis in the BGAL-complemented transgenic lines, Na$_2$CO$_3$ was used to release *mum2* mucilage (Dean et al. 2007). *mum2* seeds complemented with *TBA2p::MUM2-Citrine* were used as a positive control and were found to have low wild type-like levels of galactose (Figure 3-9a). Similarly, when *mum2* extrusion was completely complemented with *BGAL16-Citrine* (Figure 3-7a, Figure 3-8d), wild type-like levels of mucilage galactose that were significantly different from *mum2* were detected in three independent transformants (Figure 3-9a; *p*<0.0001), indicating that *mum2* mucilage galactose levels were fully complemented by BGAL16. Similarly, levels of Rha and GalA were wild type-like in the *BGAL16-Citrine* transgenic lines (Figure 3-9a). When *mum2* extrusion was partially complemented with *BGAL11-Citrine* (Figure 3-7b, Figure 3-8e), three out of four independent transformants had levels of galactose intermediate between WT and *mum2* (Figure 3-9b; *p*<0.004). These data suggest that BGAL11 can partially complement the high galactose levels in *mum2* mucilage. This increase in galactose was observed despite the fact that *BGAL11-Citrine* lines also had low *mum2*-like levels of Rha and GalA (Figure 3-9b), that is thought to occur because mucilage is poorly released in the *BGAL11-Citrine* lines (Figure 3-8e). In the *BGAL17-Citrine* transgenic lines, where *mum2*
extrusion was, at best, weakly complemented (Figure 3-7c, Figure 3-8f), seed mucilage galactose levels in two of three transgenic lines which were most similar to mum2 (Figure 3-9c; p>0.148). This suggests that BGAL17 has a low level of functional equivalency to MUM2/BGAL6. Similar to BGAL11-Citrine transgenic lines, low mum2-like levels of Rha and GalA were measured (Figure 3-9c), indicating a failure to release mucilage as a result of partial mucilage extrusion in the BGAL17-Citrine transgenic seeds (Figure 3-8f). To confirm these data, a complete set of lines were regrown and the experiment was repeated with similar results (Figure 3-10). Taken together, these data show a correlation between the degree of complementation and the galactose level in extruded mucilage.
Three or four independent transformants per construct were analyzed. Values represent mean nmoles monosaccharide normalized to mg of seeds used to extract mucilage. Four replicates were performed for each genotype. Error bars indicate standard deviation. \( p \) values generated by one-way ANOVA by comparison to \( mum2 \), ns = not significant.

Figure 3-9: Mucilage monosaccharide composition of wild-type, \( mum2 \), and \( mum2 \) complemented with \( BGAL6\)-Citrine, \( BGAL11\)-Citrine, \( BGAL16\)-Citrine or \( BGAL17\)-Citrine.
Three or four independent transformants per construct were analyzed. Values represent mean nmols monosaccharide normalized to mg of seeds used to extract mucilage. Four replicates were performed. Error bars indicate standard deviation. \( p \) values generated by one-way ANOVA by comparison to \textit{mum2}, \textit{ns} = not significant.

\textbf{Figure 3-10}: Mucilage monosaccharide composition of a second biological replicate of wild-type, \textit{mum2}, and \textit{mum2} complemented with \textit{MUM2-Citrine}, \textit{BGAL11-Citrine}, \textit{BGAL16-Citrine}, or \textit{BGAL17-Citrine}. 
3.3 Discussion

3.3.1 Complementation of mum2 depends on the relative strength of seed coat-specific promoters

During differentiation, seed coat epidermal cells produce and deposit copious amounts of pectinaceous mucilage to the apoplast (Voiniciuc et al. 2015c). These cell wall components interact to establish a polysaccharide network with distinct properties that allow mucilage to expand and extrude upon exposure to water, while also remaining firmly attached to the seed (Phan and Burton 2018). The structure and amount of mucilage can be altered without impacting normal growth and development of the plant (Western et al. 2001; Haughn and Western 2012). For these reasons, mucilage provides a unique opportunity to investigate structure-function relationships between cell wall polysaccharides by manipulating its composition using genetic engineering. In order to specifically modify mucilage, promoters are required that function spatially and temporally within the seed coat epidermal cells. Towards this goal, I selected three previously isolated seed coat-specific promoters for testing: TBA2p (Tsai et al. 2017), PER36p (Kunieda et al. 2013), and MUM40.3Pro_35S (Dean et al. 2017). Due to the presence of two Xero genes within the PER36 promoter sequence, I also generated a truncated version that excluded the transcribed regions of both these genes (PER36_0.2p) and a version where premature stop codons were introduced into both genes (PER36_modp). In this chapter I compared the ability of all five promoter sequences to drive expression of the BGAL-encoding gene MUM2 (Macquet et al. 2007b; Dean et al. 2007) and complement the mum2 mucilage extrusion phenotype (Western et al. 2001). Tagging MUM2 with the yellow fluorescent protein Citrine allowed verification that the gene products were localized to the mucilage pocket within the seed coat epidermal cells during the peak of mucilage synthesis and secretion (7 DPA; Figure 3-1f, j, l, p-r). MUM2-
Citrine driven by all five promoter sequences were able to complement mum2, but differed in their relative strength and consistency to do so (Figure 3-1c, g, i, m, n, Figure 3-3c-i). Since PER36_0.2p::MUM2-Citrine transgenic lines only very weakly complemented mum2 with the aid of a calcium chelator (Figure 3-3e) they were removed from further analysis. One potential cause for the variation in complementation between different promoters is they might differ in their ability to drive MUM2-Citrine transcription. Transcript analysis indicated that the abundance of the MUM2-Citrine transcript is approximately 250 times higher when driven by the TBA2 promoter than the three other promoters. The transcript abundance in lines driven by PER36p and PER36modp was on average several fold higher than the chimeric promoter MUM4_{0.3Pro_35S} (Figure 3-5). In general, the differences in transcript abundance correlated well with the differences in fluorescence intensity of MUM2-Citrine in the mucilage pocket, and the degree of mum2 complementation (Figure 3-1, Figure 3-5). While this might be caused by differences in the strength of each promoter, I cannot rule out the influences of the 5’ UTR on transcript stability and translation (Wilkie et al. 2003). Indeed, unlike the other three promoters, the MUM4_{0.3Pro_35S} sequence did not include a 5’ UTR (Dean et al. 2017). Without a native UTR, translation rates of MUM2-Citrine might have been lower, which may explain why transgenic lines driven by MUM4_{0.3Pro_35S} have lower Citrine fluorescence than the transgenic lines driven by the PER36p or PER36modp (Figure 3-1j, l, p-r), despite having similar transcript abundance (Figure 3-5). A similar influence of the MUM4 5’ UTR on expression of transgenic genes under the control of the MUM4_{0.3Pro_35S} sequence has been described previously (Dean et al. 2017).

Distinct differences in the transcript abundance, Citrine fluorescence, and mucilage extrusion were also observed between different independent transformants driven by the MUM4_{0.3Pro_35S} promoter. In general, higher abundance of the MUM2-Citrine transcript was
correlated with increased fluorescence intensity and more wild type-like mucilage extrusion (Figure 3-1m-r, Figure 3-5). These differences are likely due to the influence of different sites of genomic insertion on transgene expression of the relatively weak \( MUM4_{0.3Pro_35S} \) (position effect, reviewed in Matzke and Matzke 1998).

### 3.3.2 Members of the GH35 enzyme family vary in their ability to complement \( \text{mum2} \)

One application of the seed coat-specific expression system developed here is to test the functional equivalence between homologous proteins from the same enzyme family. I chose to investigate the GH35 enzyme family that consists of 17 BGALs (Ahn et al. 2007), only half of which have confirmed BGAL activity (Chandrasekar and van der Hoorn 2016). I tested for functional equivalency of three previously uncharacterized GH35 BGALs, BGAL11, BGAL16, and BGAL17, relative to MUM2/BGAL6. This was achieved by determining if these candidate BGALs could rescue the \( \text{mum2} \) extrusion and biochemical phenotypes when driven by the \( TBA2 \) promoter. Complementation of \( \text{mum2} \) was found to vary considerably among the transgenic lines complemented with different candidate BGALs in both the degree of mucilage extrusion (Figure 3-7, Figure 3-8d-f) and the amount of galactose in extruded mucilage (Figure 3-9, Figure 3-10). Additionally, the differences in complementation correlated with the level of amino acid sequence similarity relative to MUM2/BGAL6 (Figure 3-8). The fact that less phenotypic variation was observed between independent transformants expressing the same \( \text{BGAL} \) than between transformants expressing different \( \text{BGALs} \) suggests that differences in the degree of complementation were primarily due to the activity of the different BGAL proteins. In support of this hypothesis, enzyme kinetics between Arabidopsis GH35 BGALs are known to differ greatly, although substrate preference and optimal pH for maximal BGAL activity are highly conserved.
(Ahn et al. 2007; Gantulga et al. 2008; Gantulga et al. 2009), as commonly observed for other plant BGALs (De Alcantara et al. 2006; Rahman et al. 2015; Atrooz et al. 2016). Similarly, the presence or absence of a predicted galactose/rhamnose binding SUEL-type lectin domain in some of the candidate BGALs (Figure 3-6b) did not correlate with the degree of mum2 complementation (Figure 3-8d-f, Figure 3-9, Figure 3-10). Therefore, the role of this domain within GH35 BGALs remains unknown (Trainotti et al. 2001).
Chapter 4: Modification of the pectin, RG-I in seed mucilage led to changes in the properties of mucilage

4.1 Introduction

Through mutation, the seed coat epidermal cells including mucilage can be altered without impacting the viability of the seed or the growth and development of the plant. This is one of the key features that have made seed coat epidermal cells a useful model system for studying cell wall structure and function (Griffiths and North 2017). Since seed mucilage is composed of ~80-90% RG-I (Voiniciuc et al. 2015c; Ralet et al. 2016; Griffiths and North 2017; Phan and Burton 2018), changes to its structure have a major impact on the properties and function of mucilage. Indeed when the biosynthesis of RG-I was disrupted by mutations in RG-I:RHAMNOSYLTRANSFERENCE1, the mucilage capsule becomes much smaller than in WT (Takenaka et al. 2018). Similarly, mutations in the MUM4 gene, which encodes a Rha synthase that supplies Rha for RG-I synthesis, results in a small mucilage capsule (Western et al. 2001; Western et al. 2004; Usadel et al. 2004; Oka et al. 2007). In addition, the size of the mucilage pocket are smaller in mum4 mutants compared to WT (Western et al. 2004). This was thought to occur because less RG-I is synthesized and secreted to the mucilage pocket in mum4, suggesting that the size of the mucilage pocket may be dependent on the amount of secreted cell wall material (Western et al. 2004). Furthermore, the adherent mucilage layer was lost following the application of exogenous RG-I degrading enzymes directly to extruded WT seeds (Macquet et al. 2007a). All these data highlight the importance of RG-I in determining the properties of mucilage.
In this chapter, I tested the impact of synthesizing and secreting RG-I degrading enzymes to the mucilage pocket during the synthesis and secretion of mucilage. To degrade RG-I, rhamnogalacturonan lyase (RGL), classified as a member of the CAZy Polysaccharide Lyase 4 (PL4) family (EC 4.2.2.23), were employed in attempts to hydrolyze $\alpha$-Rha-(1→4)$\alpha$-GalA linkages within the backbone of RG-I through an endo-type $\beta$-elimination mechanism (Azadi et al. 1995; Mutter et al. 1996). This three step enzymatic reaction eliminates the 4-$O$ glycosidic bond between Rha and GalA and causes the formation of a double bond between carbon (C)4 and C5 in GalA, which as a result, becomes a non-reducing or unsaturated end (Gacesa 1987; Yip and Withers 2004; Brumer 2010; Garron and Cygler 2010). In this chapter, I used two genes from the fungus Aspergillus nidulans, Rhamnogalacturonate lyase A (RglA), Rhamnogalacturonate lyase B (RglB) that encode RGLs, and have been shown to degrade linseed RG-I when heterologously expressed and purified from Pichia pastoris (Bauer et al. 2006). Both RGL-encoding genes were transformed into WT plants under the control of the seed coat-specific promoter, TBA2p, and were successfully secreted to the mucilage pocket of seed coat epidermal cells in transgenic plants, where as evidence of enzymatic breakdown of RG-I was detected and as predicted the properties of mucilage were altered. In this chapter, I have therefore demonstrated the feasibility of using cell wall degrading enzymes to manipulate mucilage composition using a genetic engineering approach to facilitate the study of structure-function relationships.
4.2 Results

4.2.1 Secretion of RG-I degrading enzymes to the mucilage pocket resulted in loss of the adherent mucilage layer

Within the mucilage pocket, RG-I is a major cell wall component within a network of interconnected polymers that enables the formation and unique properties of the adherent mucilage layer (Voiniciuc et al. 2015c). The impact of modifying RG-I within the mucilage pocket using RG-I degrading enzymes was tested by constructing two chimeric genes, each encoding one of two Aspergillus nidulans-derived RG-I degrading enzymes, RglA and RglB. Because fungal-derived signal peptides are not always recognized by the plant endomembrane system (Sieber et al. 2000), they are typically replaced with a signal peptide from a well-known plant gene (Coca et al. 2004; Chen et al. 2008; Narvaez et al. 2018). With this in mind, the sequence encoding the predicted signal peptide of both Aspergillus nidulans-derived genes, RglA and RglB, was replaced with the MUM2sp, which was sufficient to direct secretion of the Citrine fluorescence tag to the mucilage pocket (Lee, Gilchrist and Haughn, unpublished results). The seed coat-specific TBA2 promoter was fused upstream of the RglA and RglB genes, which were either untagged or fused in-frame with the sequence encoding a C-terminal Citrine fluorescent tag and transformed into WT plants. In addition, the control, TBA2p::MUM2sp-Citrine (MUM2sp-Citrine) was transformed into WT plants.

Nine different T2 transformants expressing MUM2sp-Citrine possessed a wild type-like adherent mucilage layer (compare Figure 4-1b to a). In contrast, seeds from 24 different T2 transformants expressing TBA2p::MUM2sp-RglA-Citrine (RglA-Citrine) and another 15 different T2 transformants expressing TBA2p::MUM2sp-RglB-Citrine (RglB-Citrine) possessed little to no adherent mucilage layer when hydrated (Figure 4-1j, k). Similarly, transgenic seeds from plants
Figure 4-1: Seed coat mucilage phenotypes and Citrine fluorescence localization during seed development.

a-c, j-l Ruthenium red stained mucilage from hydrated mature seeds. d-i, m-r Confocal fluorescence micrographs of cross-sectional and longitudinal views of 7 DPA developing seed-coat epidermal cells showing Citrine fluorescence within the apoplast. M = mucilage pocket, CC = cytoplasmic column. Scale bar = 200 μm (a-c, j-l), and 20 μm in (d-i, m-r).
transformed with untagged TBA2p::MUM2sp-RglA (RglA) or TBA2p::MUM2sp-RglB (RglB) have almost no adherent mucilage layer (Figure 4-2a, b). These data suggest that RglA and RglB activity could be the cause of the failure to extruded mucilage. To verify this hypothesis both of the predicted catalytic sites necessary for RGL activity (Rojas et al. 2004) in RglB were mutated and the new chimeric gene TBA2p::MUM2sp-RglB(K228A, H290A)-Citrine (RglB(K228A, H290A)-Citrine) was transformed into WT. 11 different T2 transformants expressing RglB(K228A, H290A)-Citrine possessed a wild type-like adherent mucilage layer (compare Figure 4-1l to a), confirming that RGL activity in the RglB, RglB-Citrine transgenic seeds were the cause of the mucilage defects (Figure 4-1j, k, Figure 4-2a, b).

Figure 4-2: Seed coat mucilage phenotypes of WT (Col-2) transformed with untagged RG-I degrading enzymes.

Ruthenium red stained mucilage from hydrated mature seeds. Scale bar = 200 μm.

4.2.2 Degradation of RG-I by RglA or RglB result in small mucilage pockets

Changes to the mucilage extrusion phenotype in WT plants transformed with two RGL-encoding genes (Figure 4-1j, k, Figure 4-2a, b) suggest that both enzymes were secreted to the mucilage pocket. To verify this hypothesis, confocal fluorescence microscopy was used. At 7 DPA, at the peak of mucilage synthesis, fluorescence was localized in the apoplasts of the seed
coat epidermal cells of plants transformed with *MUM2sp-Citrine* (Figure 4-1e, h). Similarly, fluorescence was localized to the apoplast and mucilage pocket in 7 DPA in transgenic plants transformed with *RglA-Citrine* or *RglB-Citrine* (Figure 4-1m, n, p, q). The mucilage pockets were also much smaller in these transgenic seeds (Figure 4-1m, n) compared to that of *MUM2sp-Citrine* (Figure 4-1e). Sectioning of 7 DPA developing seeds from two independent transformants expressing *RglB-Citrine* confirmed that the mucilage pockets were much smaller

**Figure 4-3: Sections of developing epidermal cells and seed surface morphology of dried mature seeds.**

**a-d** Sections of 7 DPA seed coat epidermal cells in WT (Col-2), *mum4-1* and two independent transformants expressing *TBA2p::MUM2sp-RglB-Citrine* in a WT background. **e-l** Scanning electron micrograph of mature seeds. M = mucilage pocket, CC = cytoplasmic column, C = columella, R = radial primary cell wall. Scale bar = 20 μm (a-d, i-l), 50 μm (e-h).
in these transgenic lines (compare Figure 4-3c, d to a). However, wild type-sized pockets were observed in transgenic plants expressing RglB(K228A, H290A)-Citrine (compare Figure 4-1o, r to e, h), which supports the hypothesis that the reduced size of the mucilage pocket was directly due to RGL activity. The small mucilage pockets observed in plants transformed with RglA-Citrine or RglB-Citrine appeared similar to those in the mum4-1 mutant crossed with MUM4-1.5p::secretory(sec)-Citrine (Figure 4-1f, i, Figure 4-3b). The mucilage pockets in the mum4 mutants are thought to fail to expand because less RG-I is synthesized and secreted to the mucilage pocket (Western et al. 2004). As a consequence of having small mucilage pockets, mum4-1 seed coat epidermal cells produce columella which are much broader and flatter than those in WT (compare Figure 4-3j to i; Western et al. 2004). Broader and flatter columella were also observed on the seed surface of RglA-Citrine and RglB-Citrine mature transgenic seeds (Figure 4-3g-l), which again appear more similar to that of mum4-1 (Figure 4-3f, j) than those in WT (Figure 4-3e, i). These data suggest that the RglA-Citrine and RglB-Citrine transgenic seed either produce less mucilage or that the mucilage pocket occupies a smaller volume.

4.2.3 Unusual non-uniform, striped pattern of fluorescence in the small mucilage pockets found in RglA-Citrine and RglB-Citrine transgenic seeds

Surprisingly, a non-uniform, striped pattern of fluorescence was detected when the small mucilage pockets found in transgenic seeds expressing RglB-Citrine were imaged longitudinally (Figure 4-1q). A similar non-uniform pattern of fluorescence was observed occasionally in transgenic seeds expressing RglA-Citrine (Figure 4-1p). The non-uniform fluorescence pattern in RglA-Citrine and RglB-Citrine transgenic seeds was in stark contrast to the uniformly distributed fluorescence in WT plants transformed with sec-Citrine or RglB(K228A, H290A)-Citrine.
Figure 4-4: Localization of the plasma membrane by crossing VAMP721p::mRFP-VAMP721 (VAMP721).

a, b WT (Col-0) lines expressing VAMP721 were crossed to TBA2p::MUM2sp-RglB (c, d) and TBA2p::MUM2sp-RglB-Citrine (e, j) lines. Confocal fluorescence micrographs of cross-sectional and longitudinal views of 7 DPA developing seed-coat epidermal cells showing mRFP-VAMP721 or RglB-Citrine fluorescence. M = mucilage pocket, CC = cytoplasmic column, PM = plasma membrane. Scale bar = 20 μm.
This non-uniform pattern was not simply due to the presence of smaller mucilage pockets, such as those in mum4-1, since a uniform pattern of fluorescence was observed in mum4-1 transformed with MUM4_1.5p:: sec-Citrine (Figure 4-1i). Next I sought to determine if the unusual RglB-Citrine distribution pattern was unique to this protein or was also observed in other fluorescent proteins secreted to the small mucilage pocket found within the RglB transgenic background. This was tested by introducing a secreted Citrine fluorescent tag (MUM2sp-Citrine) into the untagged-RglB transgenic background, which itself has small mucilage pockets (Figure 4-4d), like in the RglA-Citrine and RglB-Citrine transgenic lines (Figure 4-1m, n). Thus, the fluorescence observed in MUM2sp-Citrine, RglB doubly transgenic plants was from secreted Citrine and is therefore independent of the localization of RglB itself. In these doubly transformed plants, a non-uniform pattern of Citrine fluorescence was present (Figure 4-5b), which was similar to that observed in RglB-Citrine transgenic lines (Figure 4-1q). This demonstrated that the non-uniform fluorescence pattern was not specific to the RglB-Citrine fusion protein but must be due to an intrinsic property of the small mucilage pockets within the RglB transgenic background.

Due to the unusual non-uniform pattern of fluorescence, the next step was to determine if the shape of the plasma membrane might explain this pattern of fluorescence within the cell wall. The plasma membrane was visualized by staining developing seeds from RglB and RglB-Citrine transgenic lines with the plasma membrane stain, FM 4-64. In addition, lines expressing a chimeric construct that expresses a fluorescently tagged plasma membrane-localized VAMP721 protein (Ichikawa et al. 2014) were crossed into the RglB and RglB-Citrine transgenic backgrounds. Confocal fluorescence microscopy was then used to visualize the plasma membrane based on FM 4-64 or VAMP721 fluorescence. The plasma membrane was found to
follow the contours of Citrine fluorescence in the mucilage pocket in seeds from doubly transgenic plants expressing *RglB-Citrine* and *VAMP721p::mRFP-VAMP721* (Figure 4-4e-g) and when stained with the FM 4-64 dye (Figure 4-6g-i). The same plasma membrane pattern was observed in seeds of doubly transgenic plants expressing both *RglB* and *VAMP721p::mRFP-VAMP721* (Figure 4-4c), but not in transgenic plants expressing only *VAMP721p::mRFP-VAMP721* (Figure 4-4a), or in *mum2* transformed with *TBA2p::MUM2-Citrine* (Figure 4-6a-c).

Surprisingly, when viewed longitudinally, the mucilage pockets in seeds from *RglB* and *RglB-Citrine* transgenic lines alternated in depth between shallow and deep troughs (Figure 4-4h-j) which contrasted with the wild type-like uniform shape and size of the pockets in *mum2* plants transformed with *TBA2p::MUM2-Citrine* transformed (Figure 4-6d-f). Using these observations, a model was developed to illustrate the complex shape of the mucilage pockets in *RglB* and *RglB-Citrine* transgenic seeds when compared to those in WT (Figure 4-7).

![Figure 4-5: Seed coat mucilage phenotypes and Citrine fluorescence in doubly transgenic plants containing *TBA2p::MUM2sp-RglB* and *TBA2p::MUM2sp-Citrine*.](image)

- **a** Ruthenium red stained mucilage from hydrated mature seeds. **b, c** Confocal fluorescence micrographs of cross-sectional and longitudinal views of 7 DPA developing seed-coat epidermal cells showing localization of Citrine fluorescence within the apoplast. M = mucilage pocket, CC = cytoplasmic column. Scale bar = 200 μm (a), 20 μm (b, c).
Confocal fluorescence micrographs of cross-sectional and longitudinal views of 7 DPA developing seed-coat epidermal cells. M = mucilage pocket, CC = cytoplasmic column, PM = plasma membrane. Scale bar = 20 μm.

Figure 4-6: FM 4-64 staining of the plasma membrane in RglB-Citrine transgenic seeds (g-l) and mum2 transformed with TBA2p::MUM2-Citrine (a-f).
a WT contains a large uniformly sized and shaped mucilage pocket with bright and uniform Citrine fluorescence (dark green) throughout the pocket. b The complex mucilage pockets in the seed coat epidermal cells RglB-Citrine transgenic lines alternate in width and depth, with deeper regions containing brighter Citrine fluorescence (dark green) and shallower regions containing weaker Citrine fluorescence (light green).
4.2.4 Rha and GalA levels are greatly reduced in extruded mucilage and whole seeds from *RglA-Citrine* and *RglB-Citrine* transgenic plants

The lack of an adherent mucilage layer in *RglA-Citrine* and *RglB-Citrine* transgenic seeds (Figure 4-1j, k), and the localization of both Citrine-tagged enzymes to the mucilage pocket (Figure 4-1m, n, p, q) suggest that mucilage RG-I was degraded. To investigate the cell wall composition of *RglA-Citrine* and *RglB-Citrine* transgenic plants, monosaccharide analysis was performed on both extruded mucilage and whole seeds. When compared to WT, the Rha and GalA monosaccharide levels were ~80-90 % lower in the extruded mucilage derived from transgenic seeds expressing *RglA-Citrine* and *RglB-Citrine* and approximately 50 % lower in whole seeds, which was consistent across multiple independent transformants and separate biological replicates (Figure 4-8). This suggests that compared to WT, low levels of RG-I are present in the seed mucilage of transgenic plants expressing *RglA-Citrine* or *RglB-Citrine*, suggesting that RG-I has been degraded due to the enzymatic activity of RglA and RglB.
Values represent mean nmole of a given monosaccharide normalized to mg of seeds used to extract mucilage or mg of dried alcohol insoluble residue. Three to four independent transformants from each construct were analyzed, as well as WT. Error bars indicate average standard deviation.

4.2.5 Detection of Gal-Rha disaccharides in the extruded mucilage

Confocal fluorescence microscopy confirmed localization of the Citrine-tagged RG-I degrading enzymes to the mucilage pocket (Figure 4-1m, n, p, q), where enzymatic breakdown of the mucilage RG-I was expected to yield GalA→Rha disaccharides or oligosaccharides. To
detect these degraded RG-I fragments, seed mucilage was extracted from \textit{RglA-Citrine} and \textit{RglB-Citrine} transgenic lines and WT, and separated using TLC. Unique compounds were detected in the extruded mucilage from transgenic seeds expressing \textit{RglA-Citrine} (compounds ‘A1’ and ‘A2’) and \textit{RglB-Citrine} (compound ‘B1’) that were not present in extruded mucilage from WT (Figure 4-9a, Lanes 7-12). Similarly, these unique compounds were not present in the soluble sugars from whole seeds expressing \textit{RglA-Citrine} or \textit{RglB-Citrine} in which mucilage had been removed (Figure 4-9a, Lanes 15-18). Of these three unique compounds, the position and colour of ‘B1’ detected in the extruded mucilage from \textit{RglB-Citrine} transgenic lines, appeared similar to GalA→Rha disaccharides previously identified by TLC using the same solvent system, which were also structurally confirmed to be GalA→Rha disaccharides using nuclear magnetic resonance spectroscopy (Iqbal et al. 2016). To confirm the identity of ‘B1’, this compound was removed from the TLC plate and degraded to monosaccharides by acid hydrolysis. After acid hydrolysis, two new compounds were generated, one of which was similar to the Rha standard (Figure 4-9b, dashed arrow). The second new compound’s (Figure 4-9b, solid arrow) \textit{R}\textsubscript{f} value and colour were similar to unsaturated GalA (Dongowski 1996; Iqbal et al. 2016) that is generated as a by-product of RGL activity (Bonnin et al. 2014). To confirm that ‘B1’ might present a putative GalA→Rha disaccharides generated by the activity of RglB, the extruded mucilage from \textit{RglB(K228A, H290A)-Citrine} transgenic seeds was separated using TLC. The ‘B1’ compound (putative GalA→Rha disaccharides) was not detected in the mucilage from \textit{RglB(K228A, H290A)-Citrine} transgenic seeds (Figure 4-10). Taken together, these results suggest that the ‘B1’ compound detected in extruded mucilage from transgenic \textit{RglB-Citrine} lines, most likely contained GalA→Rha disaccharides via the enzymatic breakdown of the RG-I backbone by RglB.
The two unique compounds, ‘A1’ and ‘A2’, detected in the extruded mucilage from *RglA-Citrine* transgenic lines (Figure 4-9a) were isolated and degraded via acid hydrolysis to determine if they might represent GalA→Rha disaccharides tentatively detected in extruded mucilage from *RglB-Citrine* transgenic seeds. Acid hydrolysis of the ‘A1’ compound yielded a single new compound, that possessed a *R*<sub>f</sub> value and colour not corresponding to Rha standard (Figure 4-9c) and therefore it was unclear what this new compound was. Acid hydrolysis of compound ‘A2’ yielded two new compounds ‘Y’ and ‘Z’, which did not correspond to the position or colour of the Rha or GalA standards or the predicted position of unsaturated GalA. The identity of the unique compounds detected in the extruded mucilage of *RglA-Citrine* transgenic lines remains unknown, but could be investigated at a later date using mass spectrometry.
For each genotype, seeds from two separate biological replicates were analyzed. Compounds ‘A1’, ‘A2’, ‘B1’ were only identified in the mucilage of TBA2p::MUM2sp-RglA-Citrine (RglA) and TBA2p::MUM2sp-RglB-Citrine (RglB) transgenic lines. All three compounds were not present in WT (Col-2) mucilage or whole seeds. Monosaccharide standards: Lanes 1-6 = Galacturonic acid (GalA), Rhamnose (Rha), Sucrose (Suc), Glucose (Glc), Arabinose (Ara) and Galactose (Gal).

b Acid hydrolysis of compound ‘B1’ from RglB extruded mucilage (M) yielded two compounds labelled with dashed and solid arrows, Lane 5. Monosaccharide standards: Lanes 1-3 = GalA, Rha and Ara.

c Acid hydrolysis of ‘A1’ and ‘A2’ compounds from RglA extruded mucilage (M) yielded three unknown compounds; ‘X’ in Lane 5 and ‘Y’ and ‘Z’ in Lane 6. Monosaccharide standards: Lanes 1-3 = GalA, Rha and Ara.

AcOH = Acetic acid.
For each genotype, seeds from two separate biological replicates were analyzed. Compound ‘B1’ was only detected in extruded mucilage (M) from WT (Col-2) plants transformed with TBA2p::MUM2sp-RglB-Citrine (RglB) and not in Col-2 or when both the RglB active sites were inactivated in RglB (K228A, H290A)-Citrine transgenic lines. Monosaccharide standards: Lanes 1-6 = Galacturonic acid (GalA), Rhamnose (Rha), Sucrose (Suc), Glucose (Gluc), Arabinose (Ara) and Galactose (Gal).

4.3 Discussion

4.3.1 Targeted degradation of RG-I in the mucilage pocket led to unexpected decreases in total Rha and GalA

The relationship between cell wall polysaccharide structure and function was investigated by modifying seed mucilage composition through the expression of genes encoding RG-I degrading enzymes in the seed coat epidermal cells using the seed coat specific promoter, TBA2p (Tsai et al. 2017), and secreted to the cell wall using the MUM2sp. Secretion of the two fungal-derived RG-I degrading enzymes to the mucilage pocket during mucilage synthesis resulted in
developing seeds with small pockets (Figure 4-1m, n, p, q, Figure 4-3c, d), which was similar to the small mucilage pockets seen in the RG-I biosynthetic mutant, mum4 (Western et al. 2001; Western et al. 2004; Usadel et al. 2004). In addition, the transgenic seeds expressing RglA or RglB failed to extrude mucilage when hydrated (Figure 4-1j, k, Figure 4-2) which again is similar to the mum4 mutants that synthesize much less mucilage RG-I (Western et al. 2001; Western et al. 2004; Usadel et al. 2004). The levels of Rha and GalA monosaccharides, which make up the RG-I backbone, were also drastically reduced in transgenic seeds expressing RglA- Citrine or RglB-Citrine (Figure 4-8) accompanied by preliminary evidence of GalA→Rha disaccharides in the mucilage of RglB-Citrine transformants (Figure 4-9a, b). Taken together these data suggest that the RG-I degrading enzymes RglA and RglB were successfully targeted to the mucilage pocket and resulted in the degradation of the mucilage RG-I.

4.3.2 Potential salvaging of degraded RG-I fragments from the mucilage pocket

The degradation of mucilage RG-I by RglA and RglB was expected to generate GalA→Rha disaccharides. Although such disaccharides were tentatively detected in extruded mucilage (Figure 4-9a, b), the low levels of the Rha and GalA monosaccharides in transgenic RglA-Citrine or RglB-Citrine seeds (Figure 4-8) suggest that the degraded RG-I from the cell wall might have been recycled by the cells as part of the sugar salvage pathway (Barnes and Anderson 2018). The putative GalA→Rha disaccharides might have transported into the cell through facilitated diffusion driven by transporter proteins, as widely documented in apoplastic disaccharides, such as sucrose (Julius et al. 2017). Similarly, putative GalA→Rha disaccharides could also have been degraded further to monosaccharides by hydrogen peroxides generated within the cell wall (Fry 1998) and then absorbed into the cell through monosaccharide
transporter proteins (Sherson et al. 2000). Alternatively, GalA→Rha disaccharides could have been absorbed through endocytosis, as was documented to occur with trisaccharides and disaccharides of Ara (Baluška et al. 2005). Once absorbed through either pathway and broken down to monosaccharides, the resulting free sugars could undergo interconversion to other monosaccharides (Bar-Peled and O’Neill 2011). However, there no substantial increases were observed in the other monosaccharides within these transgenic seeds that could account for the large decreases in total Rha and GalA (Figure 4-8).

4.3.3 Could cell wall components be unevenly distributed within the mucilage pocket

The mucilage pocket within WT A. thaliana is normally donut-shaped and uniform in shape (Haughn and Western 2012). Mutants that synthesize limited amounts of seed mucilage RG-I, such as mum4, have much smaller pockets which are also uniform in width and depth (Western et al. 2001; Western et al. 2004; Usadel et al. 2004). Transgenic seeds expressing RglA-Citrine or RglB-Citrine also contained smaller mucilage pockets (Figure 4-1m, n, Figure 4-3c, d) which are thought to have arisen due to the degradation of RG-I. However, in these transgenic seeds, the shape of the pockets was unusually complex, alternating in depth and width (Figure 4-4h-j, Figure 4-7b). The cause of the complex shape is unknown but could relate to the fact that in these transgenic plants, the RG-I should be secreted to the pocket normally and is then degraded in the mucilage pocket. This is unlike mum4 mutants that synthesis much reduced RG-I and do not have a complex mucilage pocket (Western et al. 2001; Western et al. 2004; Usadel et al. 2004). One possible explanation for the unusual mucilage pocket shape in RglB-Citrine transgenic plants is that certain cell wall components cannot be degraded by RglB or act as a barrier that prevents RglB from gaining access to RG-I (Meng and Ragauskas 2014). For
example, the rays are adjacent to the cytoplasmic column are composed of cellulose and hemicellulose (Voiniciuc et al. 2015c; Griffiths and North 2017; Phan and Burton 2018) which RglB should be unable to degrade, and therefore may have resulted in deeper mucilage pockets in this region. See Figure 4-11a, b for an illustrated model. The deposition of cell wall components to specific domains is also known to occur in secondary cell walls, where lignin is deposited within specific bands in protoxylem tracheary elements (Zhong and Ye 2012).

A further explanation for the unusual and complex shape of the mucilage pockets is that the RG-I degrading enzymes were secreted to specific domains of the plasma membrane lining the mucilage pocket, resulting in localized degradation of RG-I and therefore shallower pockets in those regions. See Figure 4-11c, d for an illustrated model. Targeted secretion of vesicles expressing RglB-Citrine could be achieved by the plasma membrane-anchored exocyst complexes that tether and initiate docking of incoming vesicles to specific domains of the plasma membrane (Vukašinović and Žárský 2016; van de Meene et al. 2017). Two members of the exocyst complex, EXO70A1 and SEC8 are both present in A. thaliana seed coat epidermal cells and when the genes encoding these proteins are knocked out, the adherent mucilage layer becomes much smaller (Kulich et al. 2010). Therefore, it is possible that EXO70A1 and SEC8, are involved in the targeted secretion of proteins, but this has yet to be determined. This hypothesis could be investigated in the future, by determining if fluorescently tagged EXO70A1 and SEC8 are localized to specific domains of the plasma membrane lining the mucilage pocket.
Figure 4-11: Two models to explain how the complex mucilage pockets in the RglB-Citrine transgenic epidermal cells might have arisen.

Model 1 – Uneven Distribution of Cell Wall Components Within the Mucilage Pocket

(A) Shallow Mucilage Pocket

(B) Deeper Mucilage Pocket

Model 2 – Uneven Secretion of RG-I Degrading Enzymes

(C) Shallow Mucilage Pocket

(D) Deeper Mucilage Pocket

a, b Model 1 = uneven distribution of cell wall components within the mucilage pocket in which RG-I rich regions were degraded by RG-I degrading enzymes. c, d Model 2 = RglB-Citrine protein might have been unevenly secreted to the mucilage pocket causing localized degradation of RG-I.
Chapter 5: Investigating the structural role of mucilage HG by secreting HG-degrading enzymes to the mucilage pocket

5.1 Introduction

Homogalacturonan (HG) is the second most abundant cell wall component in *A. thaliana* seed mucilage, comprising ~10 mol% of the total extractable sugars (Voiniciuc et al. 2015c). Long polymers of HG with low DM are thought to cross-link to each other through calcium-mediated ionic bonds, forming strong complexes (Jarvis et al. 2003) that consequently increase the rigidity of the mucilage (Willats et al. 2001; Saez-Aguayo et al. 2013; Turbant et al. 2016). The formation of HG-complexes is prevented through mutations of PMEs that reduce the DM of HG, or through the overexpression of gene encoding a PMEI, which inhibit PME activity (Micheli 2001). In these cases, mucilage becomes more cohesive and results in a larger adherent mucilage layer (Willats et al. 2001; Saez-Aguayo et al. 2013; Turbant et al. 2016). Similar changes to the mucilage phenotype that occur when calcium, which is required to form HG-complexes, is removed with a chelator (Willats et al. 2001; Turbant et al. 2016). While changes to the DM of HG impact the properties of mucilage, is not known if HG plays a structural role in the mucilage pocket and how they might impact the properties of mucilage. (Griffiths and North 2017; Phan and Burton 2018). The structural role of HG is unclear because a number of mucilage biosynthetic mutants have reduced levels of both GalA and Rha, making it unclear if HG or RG-I is being affected in these mutants (Caffall and Mohnen 2009).

In this chapter, I sought to investigate the structural role of HG in establishing the unique properties of mucilage. To achieve this HG was to be degraded using catabolic CAZy enzymes secreted to the mucilage pocket. Based on the existing literature outlined above, degradation of
the HG polysaccharide should prevent the formation of HG complexes in a similar manner to removing calcium using a calcium chelator, and therefore lead to a larger adherent mucilage layer. HG can be specifically degraded using endo-PGs (EC 3.2.1.15), which can hydrolyze the glycosidic linkage between two α-(1→4)-linked GalA residues within the HG backbone (Voragen et al. 2009; Bonnin et al. 2014). PGs are part of either the CAZy PL1 enzyme family that uses the β-elimination mechanism during hydrolysis, as described in Chapter 4, or they can belong to the GH28 enzyme family that use an inverting mechanism to perform hydrolysis (Biely et al. 1996). During the inverting mechanism, an aspartic acid within the active site donates a proton to activate a water molecule for nucleophilic attack (Brumer 2010; Vuong and Wilson 2010). A second aspartic acid within the active site, acts as a base which removes a proton from the anomeric carbon, where the glycosidic bond is located (Brumer 2010; Vuong and Wilson 2010). Similar to the RG-I degrading enzymes described in Chapter 4, some of most well characterized PGs originate from plant pathogens, where they are utilized to break down HG in the cell wall during infection (Choi et al. 2013; Mäkelä et al. 2014). One of the PG-encoding genes used in this chapter was PelA, which is derived from the saprophytic fungus Aspergillus nidulans (Bauer et al. 2006). PG activity of PelA has been confirmed heterologously in Pichia pastoris, where citrus pectin was degraded to methyl-esterified GalA oligosaccharides with unsaturated ends (Bauer et al. 2006). The other well characterized PG-encoding gene used in this chapter is ADPG2 from A. thaliana. ADPG2 weakens the cell walls within the dehiscence zone of anthers and maturing siliques so that pods can shatter during maturity, which enables seeds to disperse (González-Carranza et al. 2002; Kim et al. 2006; Gonzalez-Carranza et al. 2007; Ogawa et al. 2009). In-vitro PG activity of recombinant ADPG2 has also been confirmed in Escherichia coli using commercially available polygalacturonic acid from citrus fruit (Ogawa et al., 2009).
Furthermore, increased expression of \textit{ADPG2} has been shown to reduce HG content and lead to weaker leaves compared to WT (Wang et al. 2017). In this chapter both PG-encoding genes were transformed into WT and seeds from the resulting transgenic lines were examined for changes to the properties of mucilage.

5.2 Results

5.2.1 Expression of genes encoding HG-degrading enzymes in transgenic seeds resulted in little to no adherent mucilage layer

In an attempt to degrade the HG component of mucilage, two HG-degrading enzymes, ADPG2 and PelA, were targeted to the mucilage pocket by fusing the sequence encoding the \textit{MUM2sp} upstream of their coding region. To avoid degrading HG throughout the plant, the seed-coat specific promoter \textit{TBA2p} (tested in Chapter 3) was used to drive transcription of these chimeric genes. The resulting chimeric constructs, \textit{TBA2p::MUM2sp-Citrine-ADPG2} (Citrine-ADPG2) and \textit{TBA2p::MUM2sp-PelA-Citrine} (PelA-Citrine), were transformed into WT plants and the effect(s) on seed mucilage was examined. Degrading HG polysaccharides in the mucilage pocket was expected to result in a larger adherent mucilage layer. However, contrary to this prediction, only small patches of mucilage were observed in seeds isolated from 17 independent transformants expressing Citrine-ADPG2 and from 13 transformants expressing PelA-Citrine (Figure 5-1c, d). Similarly, WT plants transformed with untagged \textit{TBA2p::MUM2sp-ADPG2} or \textit{TBA2p::MUM2sp-PelA} produced transgenic seeds that released small patches of adherent mucilage when hydrated (Figure 5-2). One possible explanation for the lack of mucilage in these transgenic lines is that during extrusion the mucilage was loosely attached to the seed, due to the degradation of HG and was lost during washes carried out prior
to staining with ruthenium red. To test this hypothesis, mature seeds were exposed to a solution of ruthenium red while being videoed. During this process, WT seeds formed a large mucilage capsule within ~10 s after the addition of ruthenium red (Figure 5-3a-d). However, even after 50 s of staining, no mucilage was released in transgenic seeds expressing Citrine-ADPG2 or PelA-Citrine (Figure 5-3e-l). These data show that the lack of mucilage was due to a failure of mucilage extrusion.

![Figure 5-1](image)

**Figure 5-1: Seed coat mucilage phenotypes and Citrine fluorescence in transgenic seeds expressing HG-degrading enzymes.**

**a-d,** Ruthenium red stained mucilage from hydrated mature seeds. **e-h,** Confocal fluorescence micrographs of Citrine fluorescence in 7 DPA developing seed coat epidermal cells. M = mucilage pocket, C = cytosol, R = radial cell wall. Gap = potential gaps between cells. Scale bar = 200 μm (a-d) and 20 μm (e-h).
Figure 5-2: Transgenic seeds containing untagged HG-degrading enzymes.
Ruthenium red stained mucilage from hydrated mature seeds. Scale bar = 200 μm.

Figure 5-3: Video recording of mucilage extrusion in WT (Col-2) compared to plants expressing HG-degrading enzymes (ADPG2 or PelA).

Images represent four different time points extracted from a one minute movie taken before and after the ruthenium red stain. Scale bar = 200 μm.
5.2.2 Citrine fluorescence was localized to the radial cell walls and sometimes also in the cytosol in seed coat epidermal cells expressing Citrine-ADPG2 or PelA-Citrine

Citrine-ADPG2 or PelA-Citrine transgenic lines did not contain large adherent mucilage layers, as predicted (Figure 5-1c, d). Therefore, confocal fluorescence microscopy was used to determine if the fluorescently-tagged enzymes were secreted to the mucilage pocket, as intended. Within 50\% of the epidermal cells surveyed Citrine fluorescence was exclusively localized to the apoplast as predicted, specifically in the radial cell walls (Figure 5-1g, h). The remaining epidermal cells had fluorescence within the cell wall and a highly-disordered cytosolic signal, which was not present in the control (Figure 5-1f, g, h). Additionally, none of the transgenic seed coat epidermal cells had the distinctive ring of fluorescence which represents the mucilage pocket, as seen in mum2 transformed with TBA2p::MUM2-Citrine (Figure 5-1f-h). Similarly, when the plasma membrane was stained with the FM 4-64 dye, no mucilage pocket or cytoplasmic column was observed in the seed coat epidermal cells from transgenic Citrine-ADPG2 or PelA-Citrine lines (Figure 5-4d-i). This was in contrast to mum2 transformed with TBA2p::MUM2-Citrine, where the plasma membrane surrounds the mucilage pocket (Figure 5-4a-c). These data highlight altered cellular and potentially developmental phenotypes within the Citrine-ADPG2 and PelA-Citrine transgenic epidermal cells.
Confocal fluorescence micrographs of 7 DPA developing seed coat epidermal cells. M = mucilage pocket, CC = cytoplasmic column, PM = plasma membrane, R = radial cell wall, C = cytosol. Gap = potential gaps between cells. Scale bar = 20 μm.

Figure 5-4: FM 4-64 staining of the plasma membrane in transgenic Citrine-ADPG2 or PelA-Citrine lines compared to mum2 complemented with TBA2p::MUM2-Citrine as a control.
5.2.3 Secretion of ADPG2 and PelA in the cell wall coincided with the separation of seed coat epidermal cells

As previously described, confocal fluorescence microscopy revealed that Citrine-tagged HG-degrading enzymes were localized to the radial cell walls within 50\% of the surveyed seed coat epidermal cells in the transgenic seeds (Figure 5-1g, h). In the wild type-looking epidermal cells found in mum2 lines transformed with TBA2p::MUM2-Citrine, the radial cell walls of two neighbouring cells were in close contact, and therefore appear as a single line when viewed at the resolution the micrographs were imaged (Figure 5-1f). In contrast, the region between a number of neighbouring epidermal cells in the Citrine-ADPG2 and PelA-Citrine transgenic had no fluorescence which could indicate that the radial cell walls were not physically in contact in these regions (labelled as ‘Gap’ in Figure 5-1g, h). In addition, when viewed longitudinally, the outer primary cell walls were sometimes ruptured at the junctions between the seed coat epidermal cells in transgenic Citrine-ADPG2 or PelA-Citrine lines, which were not observed in WT (Figure 5-5). In these ‘Gap’ regions, the plasma membranes were also separated between neighbouring cells (labelled as ‘PM’ in Figure 5-4d-i). This was to a much greater extent than in mum2 lines transformed with TBA2p::MUM2-Citrine, in which the plasma membranes of two neighbouring epidermal cells were sufficiently close to appear as a single line at the resolution the micrographs were imaged (Figure 5-4b). Thus, the data suggests the presence of large intercellular spaces between the seed coat epidermal cells in the transgenic Citrine-ADPG2 or PelA-Citrine lines.
Figure 5-5: Ruptured outer primary cell walls suggest cell wall weakening in Citrine-ADPG2 or PelA-Citrine transgenic lines.

M = mucilage pocket, CC = cytoplasmic column, R = radial cell wall. Scale bar = 20 μm.

5.2.4 Compromised membranes and irregular shaped nuclei suggests the occurrence of cell death in 7 DPA developing seeds expressing Citrine-ADPG2 or PelA-Citrine

As described previously, the FM4-64 dye was used to stain the plasma membrane in seed coat epidermal cells expressing Citrine-ADPG2, PelA-Citrine or TBA2p::MUM2-Citrine. Intense fluorescence of the dye was also located in the cytosol in Citrine-ADPG2 and PelA-Citrine transgenic epidermal cells (Figure 5-4e, h). This was in contrast to mum2 transformed with TBA2p::MUM2-Citrine, where no fluorescence signal associated with the FM 4-64 dye was observed in the cytosol when stained for the same period of time (Figure 5-4b). Such increased internalization of the FM 4-64 dye would be possible either through increased endocytosis (Rigal et al. 2015) or through loss of plasma membrane integrity (Evans and Cousin 2007). To differentiate between these two possibilities, developing seeds were stained with propidium iodide and trypan blue, both of which can only be internalized if the plasma membranes are compromised (Tran et al. 2011; Zuidema and Korthuis 2015). At 4 DPA, when the transcriptional activity of TBA2p is low (Tsai et al. 2017), propidium iodide remained at the
plasma membrane in epidermal cells and trypan blue was not internalized in seeds from both WT and in those expressing Citrine-ADPG2 or PelA-Citrine (Figure 5-6a-c, Figure 5-7a-c). Therefore at 4 DPA, no loss in plasma membrane integrity was detected. However, by 7 DPA, when TBA2p-driven transcription is known to be high (Tsai et al. 2017), both propidium iodide and trypan blue were internalized in the epidermal cells and seeds expressing Citrine-ADPG2 or PelA-Citrine (Figure 5-6e, f, Figure 5-7e, f), which did not occur WT epidermal cells or seeds (Figure 5-6d, Figure 5-7d). This suggests that the plasma membrane of the epidermal cells in the transgenic seeds was not intact at 7 DPA. The occurrence of compromised membranes is characteristic of both programmed cell death (apoptosis) and necrosis (Zhang et al. 2018) during which chromatin undergoes condensation and the nuclear envelope is broken down (Darzynkiewicz et al. 1992; Toné et al. 2007). As a result, apoptotic-nuclei appear irregularly shaped and are intensely stained with DAPI, a DNA stain. In contrast, non-apoptic-nuclei appear circular in shape and are not as intensely stained with DAPI (Darzynkiewicz et al. 1992; Toné et al. 2007). DAPI-staining of 7 DPA epidermal cells expressing Citrine-ADPG2 or PelA-Citrine revealed that almost all nuclei were irregularly shaped and intensely stained with DAPI, this was in contrast to the circular shaped nuclei in 7 DPA WT epidermal cells (Figure 5-8). The presence of apoptotic-like nuclei and compromised plasma membranes suggests that the seed coat epidermal cells in plants expressing Citrine-ADPG2 or PelA-Citrine are undergoing cell death at 7 DPA. This is consistent with the observation that the seed coat epidermal cells from the transgenic lines appear to fail to differentiate normally and form mucilage pockets when imaged using confocal microscopy (Figure 5-1g, h, Figure 5-4d-i) and that the seed surface was irregular when imaged using SEM (Figure 5-9).
Figure 5-6: Propidium iodide staining of 4 and 7 DPA seed coat epidermal cells in WT (Col-2) compared to Citrine-ADPG2 and PelA-Citrine transgenic lines.

Confocal fluorescence micrographs of 4 DPA (low TBA2p activity) and 7 DPA (High TBA2p activity) developing seed coat epidermal cells. M = mucilage pocket, CC = cytoplasmic column, R = radial cell wall, C = cytoplasm. Scale bar = 20 μm.

Figure 5-7: Trypan blue staining in 4 and 7 DPA developing seeds from WT (Col-2) compared to Citrine-ADPG2 and PelA-Citrine transgenic seeds. Scale bar = 200 μm.
Figure 5-8: DAPI (nuclear) staining of 7 DPA developing seed coat epidermal cells in WT (Col-2) seeds compared to Citrine-ADPG2 and PelA-Citrine transgenic lines. N = nucleus. Scale bar = 20 μm.

Figure 5-9: Scanning electron micrographs of mature seeds from WT (Col-2) and transgenic lines containing Citrine-ADPG2 or PelA-Citrine. C = columella, R = Radial primary cell wall. Scale bar = 50 μm (in a-c) and 20 μm (in d-f).
5.3 Discussion

Homogalacturonan (HG) comprises approximately 10% of the total sugars in extruded mucilage (Voiniciuc et al. 2015c). Even though HG is a minor component of mucilage, changes to the DM of HG have been found to impact the properties of mucilage, in which low DM enable HGs to cross-link to one another resulting in more cohesive mucilage (Voiniciuc et al. 2015c). However, it is not known if HG plays a structural role in the mucilage pocket, potentially interconnecting different cell wall components to form a network which is thought to be important in establishing the properties of mucilage. To investigate the structural role HG in mucilage, two genes encoding HG-degrading enzymes were expressed in the seed coat epidermal cells during the period of mucilage synthesis and the gene products were targeted to the apoplast. Degradation of HG was expected to result in a large adherent mucilage layer. However, contrary to the expected outcome, almost no mucilage was present in hydrated transgenic seeds expressing Citrine-ADPG2 or PelA-Citrine (Figure 5-1c, d, Figure 5-2). Indeed, almost all 7 DPA developing epidermal cells in these transgenic seeds were found to lack mucilage pockets (Figure 5-1g, h, Figure 5-4d-i) and failed to produce a columella (Figure 5-9b, c, e, f). In addition, transgenic seeds possessed compromised plasma membranes (Figure 5-6e, f, Figure 5-7e, f) irregular shaped nuclei (Figure 5-8b, c), and appeared to have defects in cell adhesion (Figure 5-1g, h, Figure 5-4d-i, Figure 5-5b,c). The sections which follow examine how these cellular defects could be related to the expression of genes encoding HG-degrading enzymes.
5.3.1 Cell adhesion was disrupted when expressing genes encoding HG-degrading enzymes

An examination of Citrine fluorescence in 7 DPA epidermal cells in transgenic seeds expressing Citrine-ADPG2 or PelA-Citrine confirmed that the Citrine-tagged HG-degraded enzymes were secreted to the apoplast, specifically to the radial walls (Figure 5-1g, h). Cell adhesion within these epidermal cells also appeared to be compromised (Figure 5-1g, h, Figure 5-4d-i, Figure 5-5b, c). Since cell adhesion is dependent on the middle lamella, which is mainly composed of HG (Jarvis et al. 2003), the loss of cell adhesion in these epidermal cells is likely due to the weakening of the middle lamella by digestion of HG. Similar cell adhesion defects have been observed in two A. thaliana mutants, quasmido1 and quasmido2, where levels of HG were reduced within the middle lamella (Bouton et al. 2002; Mouille et al. 2007).

5.3.2 Possible explanations for why cell death appears to be induced in the Citrine-ADPG2 or PelA-Citrine transgenic seeds

The compromised plasma membrane and misshapen nuclei of differentiating seed coat epidermal cells in Citrine-ADPG2 or PelA-Citrine transgenic seeds (Figure 5-6e, f, Figure 5-7e, f, Figure 5-8b, c) suggest they are undergoing cell death. If this is true, it is not surprising that the epidermal cells fail to differentiate past an early stage of development, and therefore do not produce mucilage or a columella (Figure 5-1c, d, Figure 5-9b, c, e, f). The fact that expression of two different genes encoding HG-degrading enzymes resulted in the same defects, which were also not observed when introducing RG-I degrading enzymes using a similar approach in Chapter 3, suggests that the induction of cell death is specific to the synthesis of HG-degrading enzymes.
As explained below, several hypotheses could explain how expression of \textit{ADPG2} or \textit{PelA} could induce cell death. First, intercellular communication between neighbouring cells might have been disrupted due to the degradation of HG within the cell wall. Intercellular communication is necessary to coordinate differentiation, development, cell death (Long et al. 2015), and expansion of the cell wall (Wolf et al. 2012). Intercellular communication occurs through the cell wall (apoplastic pathway) or the plasmodesmata that connects neighbouring cells (symplastic pathway), through which plant hormones, peptides and RNA are exchanged (Molnar et al. 2010; Matsubayashi 2014; Lu et al. 2018). As an example, disruption to the apoplastic pathway in the \textit{tumourous shoot development} cell adhesion mutants, was found to result in abnormal development of the shoot apical meristem, caused by impaired cytokinin signalling between neighbouring cells (Frank et al. 2002). In the transgenic seed coat epidermal cells expressing \textit{Citrine-ADPG2} or \textit{PelA-Citrine}, losses in cell adhesion and the formation of large intercellular spaces (Figure 5-1g, h, Figure 5-4d-i, Figure 5-5b,c) may have disrupted the symplastic and apoplastic pathways needed for intercellular communication and possibly triggered cell death.

A second hypothesis to explain cell death is that losses in cell wall integrity brought about by degrading HG were detected by mechanosensors located on the plasma membrane. It is possible that degradation of HG might have been detected by stretch-activated calcium (Ca$^{2+}$) channels, such as the Mid1-Complementing Activity (MCA1) protein, which can detect stretching of the plasma membrane caused by degradation of the cell wall (Wolf et al. 2012; Basu and Haswell 2017). Activation of MCA1 leads to increased cytosolic Ca$^{2+}$, which in turn activates a signal transduction cascade, ultimately leading to a transcriptional response (Monshausen and Gilroy 2009). Similarly, plasma membrane-localized WAKs that directly bind
to HG within the cell wall (Decreux and Messiaen 2005; Decreux et al. 2006) are thought to be involved in sensing cell wall integrity (Monshausen and Gilroy 2009; Wolf et al. 2012; Ferrari et al. 2013). Within the transgenic seeds expressing ADPG2 or PelA, activation of cell wall integrity sensors might have led to a downstream transcription response, potentially leading to the suspension of mucilage pocket formation and cell death.

Degradation of HG within the cell wall releases oligogalacturonides (OGAs), which upon their detection can initiate a number of cellular responses (Ridley et al. 2001; Ferrari et al. 2013). OGAs themselves are also known to act as auxin-antagonists, by directly inhibiting the transcription of auxin-response genes (Ferrari et al. 2013), thereby affecting auxin’s role in growth and development (Leyser 2018). This is based off a number of studies where exogenous application of either HG-degrading enzyme PG or OGAs, inhibited auxin-induced pea stem elongation and root formation in leaf explants (Branca et al. 1988; Bellincampi et al. 1993) and induced stomata and flower formation (Marfà et al. 1991; Altamura et al. 1998). It is therefore possible that OGAs released from the breakdown of HG by ADPG2 or PelA might have directly or indirectly led to disruption in the development of the mucilage pocket in these transgenic seeds.

OGAs are also released from the cell wall by the enzymatic activity of PGs secreted by invading pathogens (Ridley et al. 2001; Ferrari et al. 2013). An example of such a PG is PelA, which is derived from the saprophytic fungus Aspergillus nidulans (Bauer et al. 2006) and was used in this chapter. Following the breakdown of HG within the cell wall by fungal PGs, OGAs can be detected by WAKs that are localized to the plasma membrane (Decreux and Messiaen 2005; Decreux et al. 2006). Activation of WAKs has been shown to upregulate plant defense-related genes, enhance resistance to invading pathogens and increase levels of reaction oxygen.
species (Ferrari et al. 2003; Ferrari et al. 2007; Galletti et al. 2008). Similarly, releasing more OGAs from the cell wall by overexpressing genes encoding PGs, was also found to upregulate expression of defense-related genes, increase resistance to invading pathogens and lead to increased production of reaction oxygen species (Ferrari et al. 2008). Furthermore, increased expression of ADPG2 specifically through the overexpression of its positive regulator, has been found to induce a defense response (Wang et al. 2017). In such cases, exogenous application of OGAs or the overexpression of genes encoding PG tend to lead to rapid increases in reaction oxygen species. This is also known as an oxidative burst, which is part of the plant’s immune response to halt the spread of pathogen, and has been implicated in inducing cell death (Ridley et al. 2001; Galletti et al. 2008; Coll et al. 2011). It is therefore possible that in the transgenic epidermal cells expressing Citrine-ADPG2 or PelA-Citrine, cell death was induced as part of the plant’s immune response upon detection of OGAs generated by degrading HG within the cell wall. This hypothesis could be tested in the future by determining if the transcription of plant defense-related genes such as PATHOGENESIS-RELATED1 or PHYTOALEXIN DEFICIENT3 is increased in these transgenic lines, both of which are upregulated upon exogenous application of OGAs (Ferrari et al. 2007; Ferrari et al. 2008; Galletti et al. 2008).
Chapter 6: Conclusions

6.1 Summary of major findings

6.1.1 Objective 1: Determine if seed coat-specific promoters can be used for modifying mucilage, by testing their ability to complement the mum2 phenotype when driving MUM2 expression (Chapter 3)

To be able to use cell wall degrading enzymes to modify the carbohydrate composition in seed mucilage and not in the cell walls throughout the rest of the plant, promoters were required that could drive transcription specifically in the seed coat epidermal cells. A number of A. thaliana seed coat-specific promoters had been identified in previous studies (Francoz et al. 2018), however, it was unknown if they could drive sufficient levels of expression of genes encoding mucilage modifying enzymes to lead to a measurable change in the properties of mucilage. Towards this goal, three seed coat-specific promoters; TBA2p PER36p, and MUM40.3Pro_35S were used to drive expression of MUM2, a known mucilage modifying gene, within a mum2 mutant background to determine if they could lead to complementation of the mum2 mucilage phenotype. MUM2 expression driven by each of these promoters resulted in complementation of the mum2 phenotype, but the ability to do so varied considerably among different promoters. The fluorescence intensity of MUM2-Citrine and the amount of MUM2-Citrine transcript expressed by each promoter was found to correlate with the degree of mum2 complementation. Complemented lines where MUM2-Citrine expression was driven by TBA2p, were found to have ~250 times higher transcript abundance compared to the other promoters. These differences were taken in account when selecting which promoters would be best suited to achieve the objectives outlined below.
6.1.2 Objective 2: Are all BGALs in the GH35 enzyme family functionally equivalent to \textit{MUM2 (BGAL6)} in their ability to modify the structure of mucilage? (Chapter 3)

Extruded mucilage contains several CAZy enzymes which are required to modify the structure of mucilage in order for extrusion to occur normally. One such enzyme is MUM2 or BGAL6, which encodes a BGAL from the GH35 enzyme family. The objective of this chapter was to determine to what degree other BGALs in the GH35 enzyme family were functionally equivalent to BGAL6 in their respective ability to modify the structure of the cell wall. To pursue this objective, the seed coat-specific promoter \textit{TBA2p}, tested in Chapter 3, was fused upstream of three candidate BGALs; BGAL11, BGAL16 and BGAL17, which had not previously been enzymatically characterized, and transformed into a \textit{mum2} background. Complementation of \textit{mum2} was found to vary depending on which BGAL was transformed into the \textit{mum2} background, and this correlated with their level of amino acid similarity to BGAL6. It was proposed that differences in enzyme kinetics between the three candidate BGALs might explain the differences in \textit{mum2} complementation, which could be investigated in future studies.

6.1.3 Objective 3: Modify the structure of the well-known, abundant mucilage component RG-I and assess the resulting changes to the properties of (Chapter 4)

The objective of this chapter was to secrete RG-I degrading enzymes to the mucilage pocket in an attempt to modify the structure of mucilage RG-I and determine the consequences to the properties of mucilage. The seed coat-specific promoter \textit{TBA2p} tested in Chapter 3 was used to drive expression of two fungal-derived genes, \textit{RglA} and \textit{RglB} that encode RG-I degrading enzymes within a WT background. When hydrated in water, transgenic seeds from
plants transformed with RglA or RglB contained almost no adherent mucilage layer. This result was as predicted based on the role RG-I is thought to play within the mucilage pocket and the phenotype of the mum4 mutants, which synthesize less mucilage RG-I. Extruded mucilage from transgenic lines expressing RglB-Citrine appeared to contain disaccharides of degraded RG-I that would be generated as a by-product from the enzymatic activity of these RG-I degrading enzymes. Furthermore, total levels of the Rha and GalA monosaccharides which make up the RG-I backbone, were greatly reduced in these transgenic seeds suggesting degradation of RG-I. It was also observed that the mucilage pockets in these transgenic seeds were much smaller than WT, a result consistent with the fact that RG-I represents ~80-90% of mucilage polysaccharides. Surprisingly, the structure of these small mucilage pockets was complex, in which the depth and width alternated in a regular manner, creating a ‘spoked’ pattern of Citrine fluorescence within the apoplast. This contrasts with WT where the pocket is uniform and ‘donut-shaped’. A number of possibilities were proposed to explain this phenomenon. The data presented in this chapter indicate that the structure of mucilage can be successful modified, and its properties altered using a genetic engineering approach.

6.1.4 Objective 4: Modify the structure of the less abundant, less well-known mucilage component HG and assess how these modifications impact the properties of mucilage (Chapter 5)

Within the mucilage pocket, HG is thought to connect to other cell wall components, and these connections may be important in enabling some of the properties of mucilage. Using an approach similar to that used in Chapter 4, the objective of this chapter was to investigate the structural role of HG in mucilage by secreting HG-degrading enzymes to the mucilage pocket.
Unexpectedly, plants transformed with two different genes both encoding HG-degrading enzymes produced seeds with large intercellular spaces between the seed coat epidermal cells, suggesting loss of cell adhesion. This is thought to have occurred because HG was degraded within the middle lamella, which is necessary for the maintenance of cell adhesion. In addition to the defects in cell adhesion, developing seed coat epidermal cells in the transgenic seeds appear to be undergoing cell death. Signs of cell death were first detected at 7 DPA when the expression of genes encoding HG-degrading enzymes would be expected to be at its highest. A number of possible mechanisms were proposed to explain why cell death was induced in these transgenic lines.

6.2 Perspectives and future directions

Starting with Chapter 3, the main contribution to this field of research is the identification of the major differences and similarities between the three seed coat-specific promoters examined in this chapter. Using this information, researchers will now be able to select which of these promoter(s) is best suited for a particular research objective. For example, high level of transcription driven by TBA2p, might be desired when synthesizing and secreting high-value compounds to the mucilage pocket for subsequent extraction (Francoz et al. 2018). Examples of such high-value compounds include β-carotene, a precursor to vitamin A which was synthesized and extracted from transgenic rice (Ye et al. 2000; Lucca et al. 2006) and an antibody against the hepatitis B virus extracted from transgenic tobacco (Pujol et al. 2005). In contrast, the high-levels of transcription promoted by TBA2p could be problematic in some cases if it leads to excess accumulation of a protein that leads to cell toxicity. This could be overcome by using one
of the promoters with lower levels of transcription which were identified in this chapter, such as PER36_modp or MUM4_{0.3Pro_35S}.

In Chapter 3, BGAL11, BGAL16 and BGAL17 were able to complement the cytological and biochemical phenotypes of mum2, which is the first evidence of BGAL activity for these specific BGALs. This knowledge should prove useful when attempting to characterize their role within the tissues in which they are natively expressed. The approach undertaken in Chapter 3, could also be extended to other uncharacterized members of the GH35 enzyme family as a relatively simply means of testing for BGAL activity prior to undertaking a more detailed enzymatic characterization. In addition, the approach could be broadly extended to other uncharacterized cell wall modifying enzymes. For example, it could be used to assess the ability of putative xylosidases in the A. thaliana genome, to complement the bxl1 mucilage mutant. Alternatively, the same approach could be used to characterize functional equivalency amongst anabolic enzymes that are required for the biosynthesis of mucilage or more generally pectin. It might also be possible to introduce new types of anabolic enzymes within the seed coat epidermal cells which could synthesize novel polysaccharides to determine how they might alter the properties of mucilage. In the future, by using such approaches to gain a greater understanding of cell wall modifying and biosynthetic enzymes, it might be possible to tailor cell wall composition for specific purposes (Phan and Burton 2018).

In Chapter 4, the RG-I component of mucilage was targeted for modification by introducing genes that encode and secrete RG-I degrading enzymes to the mucilage pocket, which resulted in transgenic seeds with almost no adherent mucilage. This phenotype was predicted and therefore supports the literature surrounding RG-I’s role within mucilage. Transgenic seeds from plants transformed with RglA-Citrine or RglB-Citrine also had small
mucilage pockets, similar to those in the mum4-1 RG-I biosynthetic mutant. However, unlike mum4-1, the smaller mucilage pocket in these transgenic seeds had a complex structure which alternated in depth and width. This was hypothesized to be due to uneven distribution of RG-I within the mucilage pocket which was subsequently degraded in a localized manner by RglA or RglB, leading to the complex shape of the mucilage pockets. In future studies, this could be investigated by sectioning the developing epidermal cells in RglB-Citrine transgenic seeds and probing the mucilage pocket with RG-I specific antibodies, to determine the progression of mucilage deposition through time. Alternatively, it might be possible to introduce sugar analogs of Rha and GalA which are compatible with click chemistry that would be used to assemble the RG-I polysaccharide (Anderson et al. 2012). The labelled RG-I would then be secreted to the mucilage pocket, where its deposition could be followed.

A second possible explanation for the complex shape of mucilage pockets within these transgenic seeds may be due to targeted secretion of RG-I degrading enzymes to a specific domain surrounding the mucilage pocket, leading to localized degradation of RG-I. This hypothesis could be investigated by determining if proteins involved in targeted secretion in other cell types, such as EXO70A1 and SEC8 which also have mucilage phenotypes, might co-localize within specific domains of the plasma membrane surrounding the mucilage pocket in the transgenic lines and in WT. Both possible explanations represent fundamental questions relate to cell wall biology that could be investigated using the transgenic lines generated in Chapter 4.

In Chapter 5, the synthesis and secretion of HG-degrading enzymes to the apoplast of the seed coat epidermal cells resulted in phenotypes typically associated with loss of cell adhesion in addition to the induction of cell death. Loss of cell adhesion associated with degrading of HG is consistent with HG’s known role in cell adhesion. However, the induction of cell death in the
developing ADPG2 or PelA transgenic seeds was unexpected. Cell death might have been induced as part of a defense response from the plant’s immune system, which is known to react when detecting fragments of HG known as OGAs that are released by degrading HG in the cell wall by pathogen-secreted enzymes. In future studies, this hypothesis could be tested by determining if the transcript from plant defense genes had been upregulated in these transgenic seeds, which would be indicative of programmed cell death (apoptosis). Additional phenotypes specific to apoptosis and not necrosis that could be screened for such as laddering of degraded genomic DNA, detection of double stranded breaks in the DNA using a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay, and activation of executioner caspases that degrade cellular components (reviewed in Kabbage et al. 2017). As a summary, it was not possible to establish the role(s) HG plays in mucilage structure and function due to the loss of cell adhesion and the induction of cell death in the transgenic seeds expressing Citrine-ADPG2 or PelA-Citrine that encode HG-degrading enzymes. However, it is possible that these phenotypes were more pronounced due to TBA2p driving high levels of transcription of the HG-degrading enzymes. Therefore, in future studies, it would be advisable to use a weaker promoter, such as PER36modp or MUM40.3Promo_35S, to drive expression of gene encoding HG-degrading enzymes. This may reduce the severity of these phenotypes and make it possible to study the structural role of HG within the mucilage pocket.

6.2.1 Using the seed coat-specific expression system to investigate other mucilage components and the predicted linkages between them

While this thesis focused on modifying one major and one minor component of mucilage, the genetic engineering approaches developed in this thesis could be expanded to investigate
other mucilage components, such as the role(s) of arabinogalactan-proteins, which remain a debated topic (Griffiths and North 2017). Their role could be investigated by degrading the arabinogalactan component of the arabinogalactan-proteins by introducing the appropriate genes that will encode and secrete endo-β-1→3-galactanases or endo-β-1→4-galactanases (Sakamoto and Ishimaru 2013) to the mucilage pocket. Any resulting changes to the structure and properties of mucilage might prove useful in uncovering the role of arabinogalactan-proteins within mucilage, which may also be applicable to other cell walls.

A further application for the seed coat-specific expression system developed in this thesis could be in the validation or confirmation of specific linkages between pectins, hemicelluloses, and cellulose, which are thought to be necessary to form a network of polymers within mucilage (Phan and Burton 2018) and in other cell walls (Thompson and Fry 2000; Vincken et al. 2003; Cosgrove 2005; Tan et al. 2013; Cosgrove 2014). Within mucilage fractions, the hemicellulose xylan, has been found to mediate binding between RG-I and cellulose in vitro (Ralet et al. 2016). It is therefore thought that xylan might be attached to RG-I as a side-chain, as detected in other polysaccharides (Tan et al. 2013) thereby being an important structural connections between mucilage and the cellulosic rays (Ralet et al. 2016). This connection could be verified in vivo by introducing the appropriate genes to synthesize and secrete endoxylanases, which can degrade xylan (EC 3.2.1.8, Vries and Visser 2001) to the mucilage pocket.

6.2.2 Agricultural and industrial benefits to reducing the levels of mucilage

Seed mucilage is known to impede seed processing in a number of oil crops and costly methods have been developed to remove it prior to seed oil extraction from flax (Fabre et al. 2015), white mustard (Balke and Diosady 2000), and chia (Castejon et al. 2017). Using the
genetic engineering approach outlined in Chapter 3, levels of mucilage could theoretically be greatly reduced in these oil-producing seeds, thereby eliminating the need for costly industrial processes to remove mucilage prior to oil extraction. Mucilage released from linseeds are known to have an anti-nutritional effects when used as animal feed (Alzueta et al. 2002; Alzueta et al. 2003), this could be avoided if mucilage was removed using the approaches developed in this thesis. Furthermore, removing mucilage could also provide protection against nematodes, which are not attracted to mutant seeds that do not release mucilage (Tsai et al. 2019).

Within *A. thaliana* seeds, oil is one of the major storage compounds (Aguirre et al. 2018). Seed oil content has been shown to increase when mucilage is no longer synthesized in *glabra2* mutants, which is thought to occur due to increased carbon flux to the embryo instead of the mucilage within the epidermal layer (Shen et al. 2006; Shi et al. 2012). Since transgenic seeds expressing genes encoding RG-I degrading enzymes contain almost no mucilage, it is conceivable that degraded RG-I was absorbed and used to increase seed oil content. If found to be the case, seed oil content in commercial seed oil varieties closely related to *A. thaliana*, such as *Brassica napus* (Canola/rapeseed) or *Camelia sativa*, might be increased by introducing genes encoding mucilage-degrading enzymes within the seed coat epidermal cells using the *TBA2* promoter.
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124


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