Enhancers of *mucilage-modified 4* Affecting Seed Coat Mucilage Production in *Arabidopsis thaliana*

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Abstract

Mucilage is a hydrophilic polysaccharide produced in the epidermal cells of the seed coat in various plant families such as the *Brassicaceae*, *Solanaceae*, *Linaceae*, and *Plantaginaceae*. Its release upon imbibition provides a gel-like coating surrounding the seed, which facilitates its dispersal, hydration, and germination. *MUCILAGE MODIFIED4 (MUM4)* encodes a UDP-$_{L}$-rhamnose synthase required for mucilage production. To further investigate the genes involved in mucilage biosynthesis, an enhancer/suppressor analysis of the *mum4-1* phenotype was performed, resulting in the identification of new alleles of the *MUM2* and *MYB61* genes and six new lines that lead to further reductions in mucilage production compared to the *mum4-1* single mutant, named *mum* enhancers (*men1* to *men6*). Characterization of these mutants revealed reduced dormancy in the *men5-1 mum4-1* double mutant, and altered seed coat cell structure, germination patterns, and root growth in the *men2-1 mum4-1* double mutant. The *MEN2* locus was mapped to a 4.4 Mbp region on the top arm of chromosome one.

Acknowledgements

I would like to express my gratitude to my supervisors Dr. Gopal Subramaniam and Dr. Owen Rowland, for giving me the opportunity to be a part of their research labs, for their expertise, continuous support, kindness, encouragement, and patience at all times. It was a real pleasure, an honour, and a life-enriching experience to work with you and your teams. Special thanks to Dr. Tamara Western for allowing me to join her project, for her guidance, and for all the help and support that she and her students, especially Natalie Martin and Andrej Arsovski gave me to successfully complete this thesis. My gratitude and appreciation also go to Charles Nasmith, Li Wang, Winnie Leung, Sean Walkowiak, and Christina Kirkham not only for all their help with my lab work but also, and more importantly, for all their love, invaluable friendship, and for making my time at the lab absolutely delightful. Thank you also to Iva Kurtelova for her countless hours as a volunteer working on my project, which made my life much easier at a time when I really needed it. Finally I would like to dedicate this thesis to my husband Luis, whose unconditional support made this thesis possible, and to my children Andres Felipe and Sofia, who are the love of my life, the light of my eyes, and the inspiration of my soul.

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List of Abbreviations

AG-I: Arabinogalactans

ABA: Absisic acid

AP: APETALA

Col: Columbia

DAH: Days after harvest

DPA: Days post anthesis

EDM: Edward's modified buffer

EDTA: Ethylenediaminetetraacetic acid

EGL: ENHANCER OF GLABRA

GalpA: Galacturonic acid

GA: Gibberellin

HG: homogalacturonan

Ler: Landsberg

InDel: Insertion or deletion mutation

Men: Mum4 enhancer genes

MUM: MUCILAGE MODIFIED

PCR: Polymerase chain reaction

PEG: Polyethyleneglycol

PO: Propylene oxide

RHM: RHAMNOSE SYNTHASE

RG: Rhamnogalacturonan

RF: Recombination frequency

TAIR: *Arabidopsis* Information Resource

TTG: TRANSPARENT TESTA GLABRA

UDP: Uridine diphosphate

WAH: Weeks after harvest

XGA: xylogalacturonan

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1. Introduction

For most cultures and from ancient history, seed products have been very important dietary sources of energy. While cereal and legume seeds constitute the main source of protein worldwide, supplying two-thirds of the protein intake of human beings, oilseeds such as soybean, cottonseed, rapeseed, sunflower seed, and peanut are the largest source of vegetable oils for human consumption and are also widely used in animal feed because of their high protein content (Casey and Wrigley, 1982; McKevith, 2005).

The study of seed crops and seed nutritional content is of increasing importance, given the potential of seeds to cope with the food requirements of the ever growing world population. In fact, it has been predicted that world production of grain would satisfy the protein requirements of the world population, if it were all used for human consumption rather than for animal feed (Casey and Wrigley, 1992). As a consequence, there is a continuous need for plant breeding programs that permit the optimization of growing conditions, seed crops yield, and nutritional value of the harvested seeds.

In angiosperms, the seed is enclosed by a seed coat that serves a variety of functions during seed development and germination, such as the transport of nutrients from the funiculus to the embryo, the protection of the embryo, and the regulation of the dormancy period (Debeaujon *et al.*, 2000; Penfield *et al.*, 2001). The seed coat is of significant economic importance, for example as the source of cotton fibers and also in the processing of seed products such as coffee and cocoa, where it needs to be removed. In addition, the study of seed coat biology and genetics is an important tool for the identification and characterization of metabolic pathways that would allow the

development of novel products based on the seed coat contents such as edible oils, tannins, and antioxidants (Moise *et al.*, 2005).

1.1. Plant fertilization

An important characteristic of flowering plants, such as *Arabidopsis thaliana,* is reproduction by double fertilization. Pollen grain development starts with the meiotic production of four haploid microspores, each of which undergoes two rounds of mitosis to produce a pollen grain. Each pollen grain consists of a uninucleate vegetative cell, which upon germination forms the pollen tube, and a pair of haploid sperm cells. During fertilization, the two male gametes (sperm) travel through the pollen tube to reach the embryo sac where they encounter two female gametes, the egg cell and the central cell. Fertilization of the egg cell by one of the sperm produces the zygote, while fertilization of the central cell by the second sperm produces the endosperm. The endosperm accumulates nutrients that are delivered to the developing embryo and also participates in signaling among seed components (Rotman *et al.* 2003; Scott *et al.*, 2008).

1.2. *Arabidopsis* seed coat development

The development of the *Arabidopsis* seed coat from the outer and inner integuments of the ripened ovule coincides with the onset of embryogenesis, starting immediately after fertilization and lasting for a period of two to three weeks (Beeckman *et al.*, 2000). At the beginning of its development, the seed coat is composed of five cell layers at the curving zone and of four cell layers at the micropylar and chalazal ends (Figure 1). The cells of these five initial layers of the seed coat have very different

characteristics, and undergo growth, cell division, expansion, and various morphological changes, that culminate in the death of all cells in the mature seed coat.

The inner integument forms three cell layers at the curving zone of the seed, which become two cell layers at the micropylar and chalazal ends (Beeckman *et al.*, 2000; Haughn and Chaudhury, 2005). The ii1 layer surrounds the embryo and is composed of vacuolated cells, in which the inner cell wall is composed of lipids derived from the cuticle of the inner integument. From the two cell stage of the embryo, these cells synthesize proanthocyanidins (condensed tannins) in their central vacuole, forming a yellow pigment that is later found filling most of the cells at the time when the embryo reaches the torpedo stage (Beeckman *et al.*, 2000). The cells of the ii1 layer become empty and die before the dessication stage, in which the proanthocyanidins become oxidized and are incorporated into the brown pigment layer. The accumulation of proanthocyanidins in these cells has three main roles: (i) strengthening of the cell walls by cross-linking of their components, (ii) protection of the embryo from UV radiation, and (iii) incorporation into the hard, brown, mature seed coat (Beeckman *et al.*, 2000; Haughn and Chaudhury, 2005). The ii1' and ii2 layers of the seed coat are highly vacuolated from the one cell stage of the embryo, undergo rapid expansion during the octant and torpedo stages, start shrinking at the time of the bent cotyledon stage, and become crushed at the desiccation stage, when they are also incorporated into the brown pigment layer of the mature seed coat (Beeckman *et al.*, 2000).

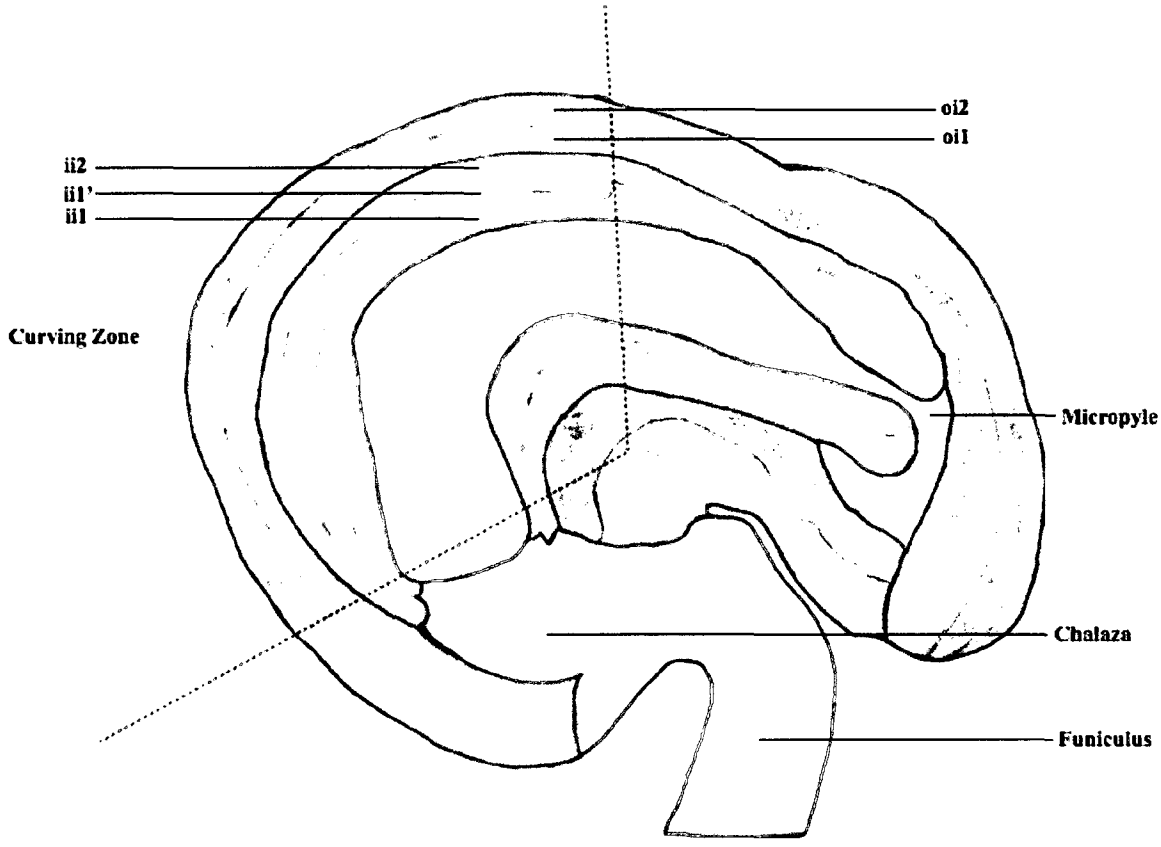

Figure 1. Cell layers of the *Arabidopsis* seed coat. **oi1**, **oi2** are the layers of the outer integument; **ii1**, **ii'1**, and **ii2** are the layers of the inner integument. (Modified from Beeckman *et al.*, 2000).

The two layers of the outer integument (oi1 and oi2) are composed of large, vacuolated cells that become smaller at the curving zone. These two layers start synthesizing starch granules from the dermatogen[1] stage of the embryo, which are the primary source of carbon for the production of mucilage and the precursors to the polymers that reinforce cell walls (Moise *et al.*, 2005; Windsor *et al.*, 2000). At the torpedo stage, the cells of oi1 have thick, lignified cell walls that provide a physical boundary that aids the development of the curved shape of the embryo. The cells of oi1 die at the desiccation stage and their debris is incorporated into the brown pigment layer.

The most external layer of the outer integument (oi2) becomes the seed coat epidermal layer in the mature seed. The differentiation process of these cells (Figure 2) starts with a change in shape from rectangular to hexagonal, due to the expansion of the vacuole that causes the cells to grow. By the fourth day after pollination, the large vacuole occupies the bottom two-thirds of the cell causing a 4-fold increase in its size and restricting the cytoplasm to the center of the cell over the large basal vacuole (Western *et al.*, 2000). During the torpedo stage of embryo development, these cells synthesize pectin compounds and secrete mucilage into the apoplast (Windsor *et al.*, 2000). The accumulation of the secreted mucilage in the apoplast compresses the vacuole and forces the cytoplasm into a volcano-shaped column that becomes lined by a secondary cell wall interior to the mucilage, called the columella. During this phase, the cell accumulates an increased amount of endoplasmic reticulum that facilitates the production of the large

[1] Outer layer of epidermal precursor cells that become separated from the inner cells mass.

number of cellulose biosynthetic enzymes required for cell wall synthesis (Western, 2006). Mature epidermal seed coat cells have no cytoplasm and become desiccated.

Imbibition[2] of the seeds causes the swelling of the mucilage and the consequent breaking of the cell walls. Once the mucilage is released, it forms a gelatinous layer surrounding the seed that facilitates seed dispersal and germination (Haughn and Chaudhury, 2005). Although the cells of oi2 die at the desiccation stage, their structure is maintained by columella and mucilage, while all the other layers are crushed together forming the brown pigment layer (Haughn and Chaudhury, 2005).

Death of the seed coat layers occurs in a determined sequence at specific times of embryo development, indicating that these are programmed events with specific objectives. For example, it is believed that the disappearance of the yellow pigment layer (ii1) is required for the development of chloroplasts in the embryo, as it occurs at the same time of embryo greening which begins at the torpedo stage (Beeckman *et al.*, 2000). A protease with caspase-like activity called δVPE, appears in layers ii1' and ii2 four to five days after fertilization, and is associated with programmed cell death (Haughn and Chaudhury, 2005).

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[2] Imbibition: uptake of water by a substance that does not dissolve in water resulting in the swelling of the absorbing matter .

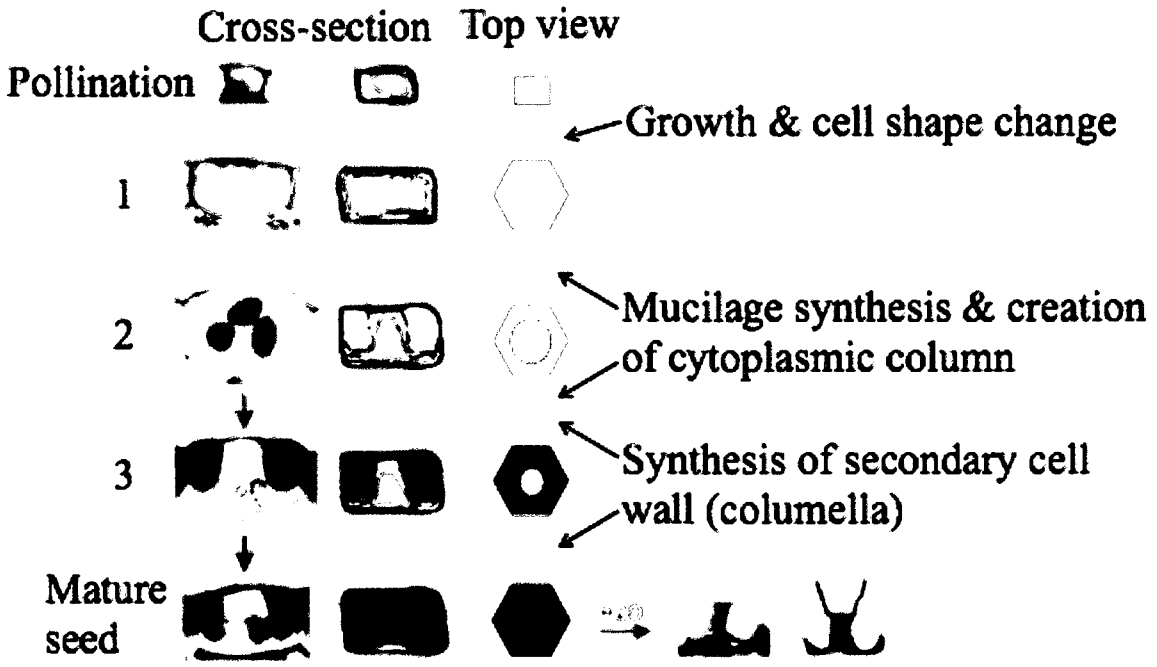


Figure 2. Differentiation of mucilage secretory cells of the *Arabidopsis* seed coat. (1) cell and vacuolar expansion; (2) Mucilage biosynthesis and secretion; (3) Secondary cell wall synthesis. Toluidine blue staining shows cellular structures in different colours: Purple: cell wall; Grey: cytoplasm; Pale yellow: vacuole; Pink: mucilage. In the mature seed the columella (secondary cell wall) is found in the centre of the cell with mucilage on either side. (Modified from: Western, 2006).

Seed development includes growth and differentiation of the embryo, the endosperm and the seed coat. The events of these three processes are highly coordinated. Haughn and Chaudhury (2005) studied mutants of the *HAIKU* gene, which have limited endosperm growth, and demonstrated that an abnormal endosperm causes restricted cell elongation affecting seed coat structural integrity. Mutants of *TRANSPARENT TESTA GLABRA2* (*TTG2*), which present limited elongation of the seed coat cells, are affected in endosperm development (Johnson *et al.*, 2002). These experiments demonstrated that coordination between differentiation of the endosperm and the seed coat ultimately establishes seed size, which physically restricts embryo development.

1.3. Mucilage properties

Mucilage is hydrophilic complex polysaccharide composed of pectins, cellulose[3], and hemicellulose. It is produced in epidermal cells of the seed coat, the transmitting tract of the pistil, and the outer layer of the root cap, in various plant families such as *Brassicaceae*, *Solanaceae*, *Linaceae*, and *Plantaginaceae* (Western *et al.*, 2000). The release of mucilage upon imbibition provides a gelatin-like coating surrounding the mature seed that is thought to facilitate seed dispersal, hydration, germination, prevention of gas exchange, and attachment to soil and animal vectors, as well as to protect the seedling in its early developmental stages (Windsor *et al.*, 2000; Western *et al.*, 2004; Arsovski *et al.*, 2009*a*). Although mucilage is not essential for seed germination, as shown by the viability of loss-of-function *apetala2* alleles (Western *et al.*, 2000), mutants

[3] Hemicellulose contains many different sugar monomers while cellulose contains only anhydrous glucose.

that release less mucilage than wild type Col-0 seeds have altered germination patterns (Rautengarten *et al.*, 2008; Arsovski *et al.*, 2009*b,* Panikashvili *et al.*, 2009). Low germination rates under water limiting conditions have been observed in seeds carrying a mutation in the *AtSBT1.7* gene, which encodes a subtilisin-like Ser protease involved in the regulation of cell wall modifying enzymes in mucilage secretory cells (Rautengarten *et al.* 2008), as well as in seeds carrying a mutation in the *DCR* gene, which codes for an acyltranserase involved in cutin polyester formation, epidermal cell differentiation, and seed hydration properties (Panikashvili, *et al.*, 2009). In addition, seeds carrying a mutation in the *AtBXL1* gene, which encodes a bifunctional β-δ-xylosidase/α-L-arabinofuranosidase and have a slow and patchy release of mucilage, have a 40% delay in germination three days after planting (Arsovski *et al.*, 2009*b*). This delay in germination is most likely a consequence of a reduced ability to attract and hold the water around the seed to facilitate germination (Arsovski *et al.*, 2009*b*).

1.4. Structural pectins

The mucilage found in the seed coat of *Arabidopsis* is primarily composed of an unbranched form of the pectin rhamnogalacturonan I (RG-I), with some pectic side chains such as arabinans, galactans, homogalacturonan, hemicellulose, and cellulose (Goto, 1985). Pectins are structural polysaccharides in the cell walls of plants composed of up to 17 different monossaccharides. The most common pectins found in plants are galacturonans, which are composed of a linear backbone of α-(1-4) linked D-galacturonic acid (α -D-Gal*p*A) residues, and are subdivided into four groups: unbranched homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II),

and xylogalacturonan (XGA) (Vincken *et al.*, 2003). RG-I (Figure 3) is formed of the repeating disaccharide unit [α-1,2-$_{L}$-Rhamnose-α-1,4-$_{D}$-Gal*p*A]$_{n}$ where *n* can be larger than 100. The Gal*p*A residues can be linked to acetyl groups on O-2 and O-3 and the rhamnose residues can be substituted at O-4 with neutral sugars, creating branched molecules. The side chains can be single unit β-1,4-$_{D}$-Gal*p*A or polymers such as arabinogalactans I (AG-I), which have a 1,4-linked β-$_{D}$-Gal*p*A backbone, and arabinans which have a 1,5-linked α-$_{L}$-Araf backbone (Vincken *et al.*, 2003; Western, 2006). RG-II are groups of approximately eight Gal*p*A residues connected to side chains of sugar residues. The difference between RG-I and RG-II is that RG-II contains significantly less rhamnose residues on its backbone and these rhamnose residues are located mainly in the side chains (Vincken *et al.*, 2003). XGA is a branched galacturonan with β-1,3-$_{D}$-Xylp side chains. The Gal*p*A residues of XGA can be methyl-esterified at C-6. Homogalacturonans can also be methyl-esterified at C-6 and can carry acetyl groups on O-2 and O-3 (Vincken *et al.*, 2003).

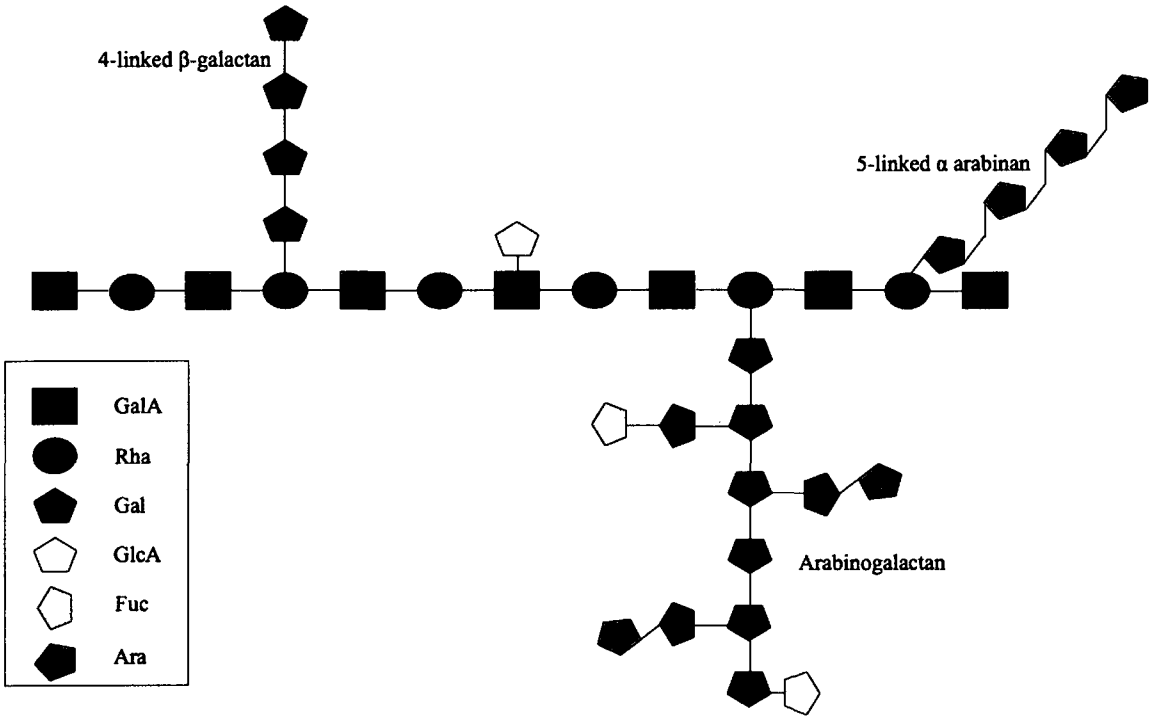


Figure 3. Structures of the main constituent polysaccharides of pectin (Modified from Vincken *et al.*, 2003).

Only some of the enzymes involved in the biosynthesis of HG and RG-I have been identified. The production of pectins occurs in the Golgi apparatus as follows (Young *et al.*, 2008; Western, 2006): (i) Nucleotide sugar interconversion enzymes synthesize monosaccharides from nucleotide sugar substrates, (ii) nucleotide sugar transporters move these monosaccharides into the lumen of the Golgi apparatus, (iii) glycosyltransferase enzymes add the monosaccharides to the growing polysaccharide chains producing the pectins RGI, RGII, and HG, (iv) the pectins are methylated by pectin methyl transferases, (v) the end product pectins are transported by secretory vesicles to the plasma membrane and secreted to the extracellular space via exocytosis, where (vi) pectin methylesterases remove methyl groups and glycosyl hydrolases break glycosydic bonds between carbohydrate molecules in HG linear molecules. The *Arabidopsis* genome includes 412 putative glycosyltransferases and nine families of genes involved in the synthesis of nucleotide sugars and nucleotide sugar transporters; however, most of the glycosyltransferases and biosynthetic genes required for the production of nucleotide sugars, nucleotide sugar transporters, and pectic polysaccharides present in mucilage, are yet to be characterized (Usadel *et al.*, 2004; Western, 2006).

1.5. Genes involved in mucilage biosynthesis and release

Arabidopsis mutants *mum1* to *mum5* were identified in a screen for plants defective in seed coat mucilage. Western *et al.* (2001) showed that these genes are involved in different steps of seed coat differentiation and mucilage biosynthesis, as follows: (i) *MUM1* encodes a β-galactosidase that regulates the post-synthesis chemical modifications in the cell wall and mucilage that facilitate mucilage extrusion upon seed

hydration, (ii) *MUM2* also encodes a β-galactosidase (localized to the cell wall) and is involved in the modifications that allow swelling of mucilage upon hydration (Dean *et al.*, 2007). *Mum2* mutants produces a normal amount of mucilage but fail to extrude it upon hydration (Dean *et al.*, 2007). (iii) *MUM3* and *MUM5* affect the polysaccharide structure of the mucilage acting most likely as regulators or biosynthetic enzymes.

MUM4 encodes a UDP-L-rhamnose synthase protein required for the production of the primary mucilage pectin RG I, and contains two domains: (i) a N-terminal domain similar to bacterial dTDP-D-Glucose-4,6-dehydratases, and (ii) a C-terminal domain similar to bacterial 4-reductases. The *MUM4* gene is part of a three member gene family encoding putative nucleotide sugar interconversion factors: *RHM1*, *RHM2* (*MUM4*), and *RHM3*. *RHM1* and *RHM2* were mapped to chromosome I, while *RHM3* was mapped to chromosome III. *RHM1* and *RHM3* encode UDP-L-rhamnose synthases that catalyse the conversion of UDP-D-glucose to UDP-L-rhamnose (Wang *et al.*, 2008).

Mum4 mutants have reduced mucilage accumulation in the extracellular space and the columella appear flattened (Figure 4) as a consequence of reduced cytoplasmic and vacuolar constriction (Western *et al.*, 2004). By contrast, *mum1*, *mum2*, *Atsbt1.7* (subtilisin-like Ser protease), *Atbxl1* (β-δ-Xylosidase/α-L-Arabinofuranosidase), and *dcr* (acyltransferase) mutants are defective in mucilage release, and *mum3* and *mum5* mutants have a reduced and altered mucilage composition (Dean *et al.*, 2007; Arsovski e*t al.*, 2009*a,* 2009*b*; Rautengarten *et al.*, 2008; Panikashvili *et al.*, 2009). The production of residual amounts of mucilage as well as the formation of a normal primary cell wall in *mum4-1* mutants may be a consequence of the ubiquitous expression of *RHM1* and *RHM3* in the seed coat (Western *et al.*, 2004).

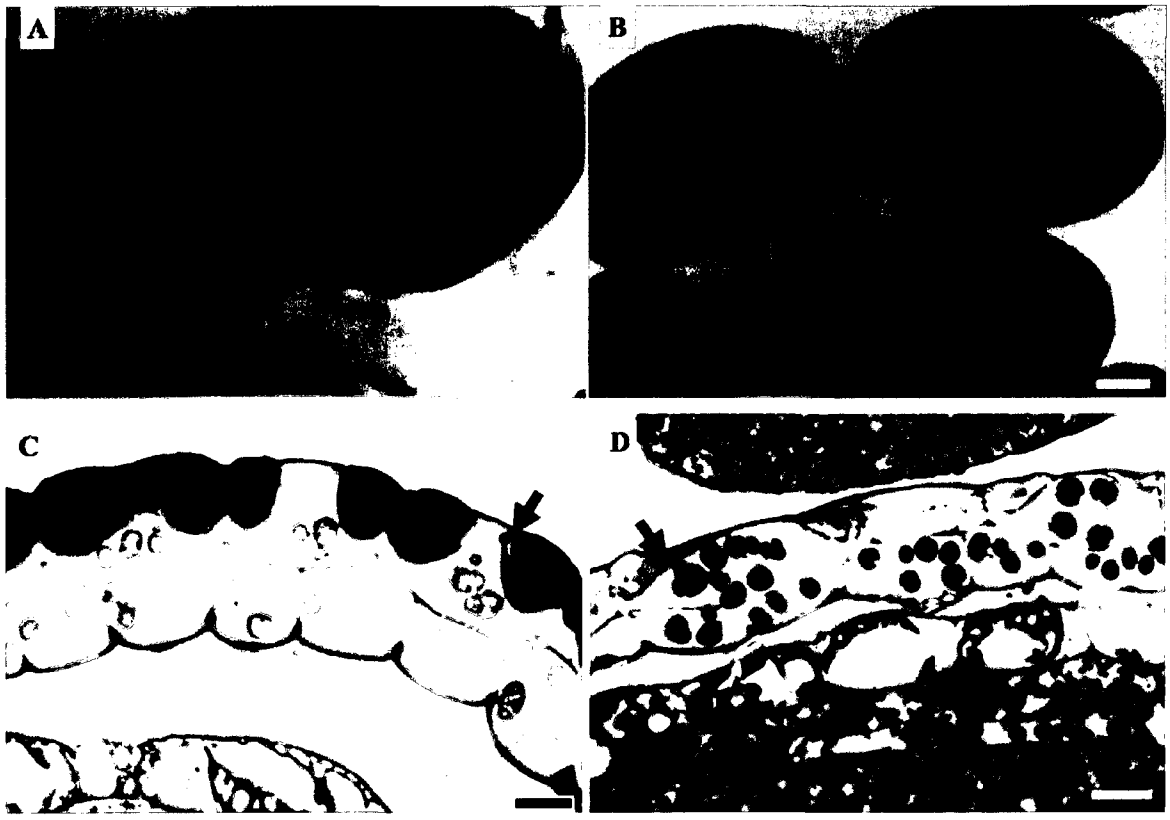


Figure 4. Seed coat mucilage and structure of columella in wild-type and *mum4-1* seeds. Top panel: Ruthenium red staining of mucilage in: **A**, wild-type Col-2 seeds; and **B**, *mum4-1* seeds. Bottom panel: Toluidine blue staining of mucilage (pink) and secondary cell walls (purple) in **C**, wild-type Col-2 seeds; and **D**, *mum4-1* seeds. In wild-type seeds the centre of the cell contains cytoplasm surrounded by a secondary cell wall forming a volcano-shaped columella with a large amount of mucilage around, while in *mum4-1* seeds the cytoplasm fills most of the cell in a dome shape surrounded by a thin secondary cell wall, with a very small amount of mucilage limited to the corners of the cells (Modified from Western *et al.*, 2004). Scale bars: A and B: 100 μm, C and D: 10 μm.

It has been proposed that the biosynthesis of mucilage in the seed coat occurs through two pathways (Figure 5) that depend on *AP2* and a third alternative pathway that is dependent on *MYB61* (Western *et al.*, 2004). In the first two pathways the *TTG1* seed coat-specific complex (*MYB5* and the bHLH proteins EGL3, TT8, and TT2) acts downstream of *AP2* and activates either *GL2*, which in turn activates *MUM4*, or *TTG2* for *MUM4*-independent production of mucilage. In the *AP2*-independent pathway, *MYB61* regulates mucilage production most likely by having a role in the allocation of sugars required for RG I synthesis.

AP2 encodes a transcription factor that is required for the differentiation of the outer layers (epidermis) of the seed coat. Accordingly, *ap2* mutants fail to produce the mucilage and form the columella (Western *et al.*, 2004). Although *AP2* is required for optimal *GL2* and *TTG2* transcript levels, transcription of *TTG1* and *MYB61* is independent of AP2 activity, demonstrating that AP2 (Table 1) works in parallel with TTG1 for the activation of *GL2* (then subsequently *MUM4*) and *TTG2* during mucilage production (Western *et al.*, 2004).

TTG1 (WD40 repeat protein) and *GL2* (homeodomain transcription factor), in addition to *AP2*, are implicated in *Arabidopsis* seed coat development (Penfield *et al.*, 2001). These two genes have important roles in trichome and root trichoblast differentiation and are essential for columella development and mucilage release upon imbibition (Koornneef, 1981). The TT2 transcription factor is expressed during the development of the outer epidermal layer of the seed coat and interacts with *TT8, EGL3,*

and *TTG2* for the regulation of mucilage production (Gonzalez *et al.*, 2009; Zhang *et al.*, 2003).

MYB-related proteins comprise a large family of transcription factors in higher plants, identified by their number of helix-loop-helix repeats in the MYB domain (Penfield *et al.*, 2001). Their various functions in plant development include control of organ and cell morphology and regulation of metabolism. In the seed coat, MYB61 promotes accumulation of linear rhamnogalacturonans for mucilage production, and MYB5 is required for the proper differentiation of the mucilage-secreting cells. MYB5 is also involved in trichome development and pranthocyanidins accumulation. *Myb61* and *myb5* mutant seeds fail to extrude mucilage upon imbibition (Penfield *et al.*, 2001; Gonzalez *et al.*, 2009).

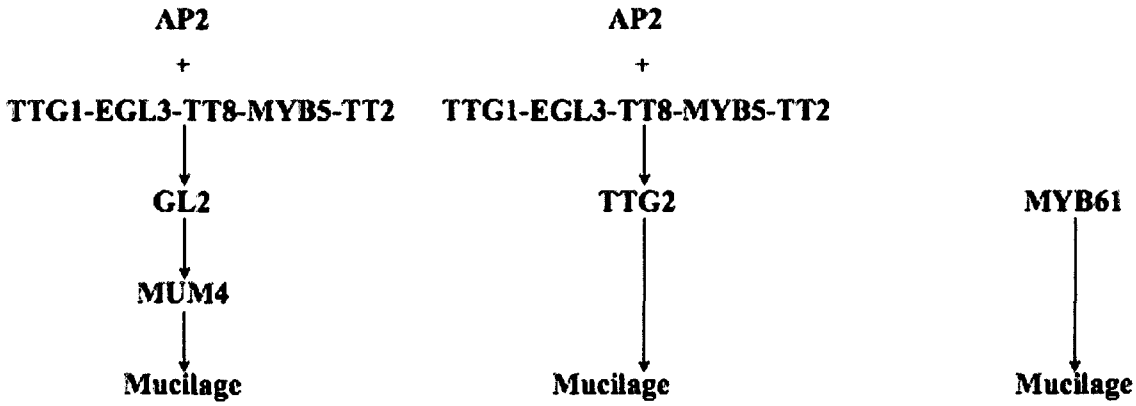


Figure 5. Proposed genetic pathway for the regulation of mucilage production during differentiation of mucilage secretory cells in the seed coat of *Arabidopsis*. AP2 and a TTG1 complex with a bHLH protein (candidates include *EGL3* and/or *TT8*) and a tissue-specific MYB transcription factor activate *GL2* and *TTG2*. GL2 acts upstream of *MUM4*. In contrast, both TTG2 and MYB61 appear to affect aspects of mucilage production independent from *MUM4* (Western *et al.*, 2004).

Table 1. Genes affecting production of mucilage and morphology of the mucilage secretory cells in the seed coat of *Arabidopsis*. (Modified from Western, 2006). **TF**, Transcription factor.

Gene Name	Mutant Phenotype	Protein	Reference
Regulation of outer integument differentiation			
AP2 (*APETALA2*)	No mucilage or columella	AP2 TF	Jofuku *et al.*, 1994
Regulation of mucilage synthesis			
TTG1 (*TRANSPARENT TESTA GLABRA1*)	Reduced mucilage and columella	WD40 repeat	Koornneef, 1981
MYB23	Reduced mucilage and columella	MYB TF	Kirik *et al.*, 2005
EGL3 (*ENHANCER OF GLABRA3*)	Reduced mucilage and columella	bHLH TF	Zhang *et al.*, 2003
TT8 (*TRANSPARENT TESTA8*)	Reduced mucilage and columella	bHLH TF	Zhang *et al.*, 2003
GL2 (*GLABRA2*)	Reduced mucilage and columella	Homeobox TF	Rerie *et al.*, 1994
TTG2	Reduced mucilage and columella	WRKY TF	Johnson *et al.*, 2002
MYB61	Reduced mucilage and columella	MYB TF	Penfield *et al.*, 2001
MYB5	Reduced mucilage and columella	MYB TF	Li *et al.*, 2009
Pectin biosynthesis and modification			
MUM4 (*MUCILAGE MODIFIED4*)	Reduced mucilage and columella	UDP-L-rhamnose synthase	Western *et al.*, 2004
RSW3 (*RADIAL SWELLING3*)	Reduced mucilage and columella	Glucosidase II	Burn *et al.*, 2002
MUM3	Altered mucilage sugar composition, staining, and mechanical properties	Unknown	Western *et al.*, 2001

Gene Name	Mutant Phenotype	Protein	Reference
MUM5	Altered mucilage sugar composition, staining, and mechanical properties	Pectin methylesterase	Western *et al.*, 2001
Primary cell wall modification			
MUM2	No mucilage release upon hydration	β-galactosidase	Dean *et al.*, 2007
AtBXL1	Patchy and slow mucilage release at hydration	β-δ-Xylosidase/ α-L-Arabinofuranosidase	Arsovski, *et al.*, 2009b
Unknown function			
PRA (*PRAIRIE*)	Reduced mucilage and columella	Unknown	Western, T.L. (unpublished data)
MUM1	Defective in seed coat mucilage secretory cells. No mucilage release upon hydration	Unknown	Western *et al.*, 2001
ATS (*ABERRANT TESTA SHAPE*)	Abnormal testa development and reduced mucilage staining	Unknown	Leon-Kloosterziel *et al.*, 1994
At-*Ga30x4*	Reduced mucilage staining and abnormal columella shape	GA-3-oxidase	Kim *et al.*, 2005
ABA1 (*ABSISIC ACID1*)	Reduced mucilage staining	Zeaxanthin epoxidase	Karssen *et al.*, 1983
DCR	Altered epidermal cell differentiation and post-genital organ fusion	Acyltransferase	Panikashvili, *et al.*, 2009

1.6. Thesis objectives

A model for the regulation of mucilage production in the *Arabidopsis* seed coat (Figure 5) illustrates that apart from the regulation of *MUM4* expression (rhamnose synthesis), very little is known about the three possible mucilage biosynthetic pathways. The involvement of other biosynthetic and regulatory genes acting upstream, downstream, or in parallel with *TTG2* and *MYB61*, and possibly affecting the *MUM4* pathway, is evident (Arsovski *et al.*, 2009*a*). To further investigate the regulation of *MUM4* and to identify other genes involved in the biosynthesis of mucilage, an enhancer/ suppressor analysis was performed in an EMS mutagenized *mum4-1* Col-2 population (performed by Prof. Tamara Western, McGill University). This resulted in the identification of new alleles of the *MUM2* and *MYB61* genes and six new lines with a further reduction in mucilage production compared to the *mum4-1* parental seeds, named *mum* enhancers (*men1* to *men6*).

The first objective of my thesis was to phenotypically characterize the eight *mum4-1* enhancer lines, including the study of their growth and development characteristics, mucilage production and release, seed coat permeability, and germination rates. The results of these experiments indicated that in addition to the mucilage phenotype, one enhancer line *men2-1 mum4-1* displayed differences to the *mum4-1* parental line in root growth as well as in germination characteristics. Consequently, additional phenotypic analyses were conducted on *men2-1 mum4-1*, including microscopy of the seed coat structure, quantitative and qualitative study of root growth and development, and a germination timeline as well as germination in water-limiting conditions. The second objective of my thesis was to map the genetic location of the *MEN2* locus.

2. Materials and Methods

2.1. *mum4-1* enhancer lines

The eight *mum4-1* enhancer lines were identified from a *mum4-1* (Col-2 ecotype) mutagenized population (performed by Prof. Tamara Western, McGill University). The mutagenesis was performed by treating 0.33 g of *mum4-1* seeds (about 15,000 seeds) with 0.25% (v/v) ethyl methanesulphonate (EMS) for 12 hours. The mutagenized seeds were planted in 10 flats of eight pots each containing 100-150 plants (M1). The seeds of each pot were harvested in bulk and constituted a batch (80 M2 batches). The M2 seeds were planted and their seeds (M3) were individually harvested. The seeds of about 5000 M3 lines were screened for mucilage deposition defects by EDTA treatment followed by ruthenium red staining (see staining protocol numeral 2.4.3), resulting in the isolation of eight *mum4-1* enhancer lines from 10 batches (1500 parental lines).

men2-1 mum4-1 and *men3-1 mum4-1* were isolated from the same parental batch (ie. the same group of 100-150 M1 plants), as were *men6-2 mum4-1* and *myb61-6 mum4-1*, however the other five enhancer lines were each isolated from separate parental batches. The selected mutants were backcrossed at least twice to *mum4-1* to reduce background mutations.

2.2. Plant materials and growth conditions

Arabidopsis thaliana Columbia-2 (Col-2) and Landsberg *erecta* (L*er*) ecotypes were provided by Dr. Gopal Subramaniam (ECORC, Agriculture and Agri-Food Canada, Ottawa, ON), while *mum4-1* (Col-2 ecotype), *ttg1-1* (L*er* ecotype), and all the *mum-4*

enhancer lines were provided by Professor Tamara Western (Biology Department, McGill University, Montreal, QC).

Seeds were sown onto Gamborg's B-5 basal medium (pH 5.8) plates containing 150 mg/L $NaH_2PO_4 \cdot H_2O$, 3000 mg/L KNO_3, 134 mg/L $(NH_4)_2SO_4$, 500 mg/L $MgSO_4 \cdot 7 H_2O$, 150 mg/L $CaCl_2 \cdot 2H_2O$, 28 mg/L $FeSO_4$, 1 mg/L Nicotinic acid, 10 mg/L Thiamine·HCl, 1 mg/L Pyridoxine·HCl, 100 mg/L Inositol, 10 mg/L $MnSO_4 \cdot H_2O$, 3 mg/L, H_3BO_3, 2 mg/L $ZnSO_4 \cdot 7 H_2O$, 250 µg/L $Na_2MoO_4 \cdot 2H_2O$, 25 µg/L $CuSO_4$, 25 µg/L $CoCl_2 \cdot 6 H_2O$, 750 µg/L KI, 20 g/L sucrose, 1mL/L Gamborg's Vitamin Solution, and 10 g Agar (A5054, Sigma-Aldrich). Unless otherwise stated, plates were incubated for three days in the dark at 4°C and then transferred to a growth chamber (Model 2015, VWR Scientific Products) at 22°C (16h light : 8h dark). Seedlings were transplanted to soil (Premier Promix) two weeks after germination and kept under the same light and temperature conditions. The plants were fertilized every week (20-20-20 Mix 1 g/L, Plant Products) until flowering was completed. Seeds were collected as soon as the siliques turned brown. Collected siliques were stored in paper bags (Midco Enterprises, Inc.) for two weeks after which the dry seeds were stored in 1.5mL Eppendorf tubes at 4°C. All phenotypic characterization experiments were completed in triplicate using Col-2, *mum4-1*, and *ttg1-1* lines as controls.

2.3. Gantlet analysis of general growth phenotypes

Six control seeds (Col-2 or *mum4-1*) and six mutant seeds (*men1* to *men6*) were sown side by side on the top of square, gridded petri dishes (120 mm x 120 mm x 17 mm) containing 40 mL of Gamborg's B-5 basal medium. The plates were incubated

horizontally for three days at 4°C in the dark (wrapped in 2 layers of aluminum foil and stored in sealed cardboard boxes), followed by 14 days in a vertical orientation at 22°C in 16 hours light per 8 hours dark. After 14 days the seedlings were transferred into soil and the pots were placed in a growth chamber at 22°C in 16 hours light per 8 hours dark. Differences between the control and mutants were recorded in the General Growth Data Sheet, as follows: (=) no difference observed, (+) subtle difference between the control and the mutant, (++) medium difference between the control and the mutant, and (+++) the control and mutant are very different. Notes were made when necessary to describe the difference between the lines.

2.4. Mucilage production and release

2.4.1. Resin embedding of seeds

(Performed by Prof. Tamara Western, McGill University) For fixation, seeds were extracted from the siliques and placed immediately in 3% glutaraldehyde in 0.1 M $NaPO_4$ buffer pH 7.0 for 16 hours at 4°C. After rinsing three times for 10 minutes with 0.05 M $NaPO_4$ buffer, the samples were treated with the post-fixative 1% OsO_4 in 0.05 M $NaPO_4$ buffer for 2 hours, rinsed two times with distilled water, and treated for 10 minutes in 30% ethanol, 1 hour in 50% ethanol, 1 hour in 70% ethanol, and 1 hour in 85% ethanol. Fixed samples washed in 85% ethanol, and treated for 1 hour in 95% ethanol followed by two consecutive 1 hour incubations in 100% ethanol. The samples were then transferred into 30% propylene oxide (PO) for 30 minutes and incubated in a series of increasing concentrations of Spurrs resin, as follows: 3:1 PO:Spurrs for 2 hours, 1:1 PO:Spurrs for 2 hours, and 1:3 PO:Spurrs for two hours. The embedded samples were incubated

overnight in 100% resin. On the following day, the samples were placed in open tubes with 500 µl of fresh Spurrs resin for 24 hours at 60°C for polymerization. 0.6 µm sections were obtained using a microtome (Performed by M. Villota), which were used in the Toluidine Blue assay.

2.4.2. Toluidine Blue assay

Sections of seeds embedded in resin were stained with 1% (w/v) Toluidine Blue O in 1% (w/v) sodium borate (pH 11.0) for three minutes, and observed on a compound microscope (Zeiss Axioplan 2, Carl Zeiss MicroImaging) equipped with a digital camera (Zeiss AxioCam Colour 412-312, Carl Zeiss Microimaging).

2.4.3. Ruthenium Red assay

Approximately 50 seeds in a 1.5 mL Eppendorf tube were placed in a shaker (Labline Incubator Shaker 0993-3300, Leigh Ryckman) at medium speed and treated with 800 µl 0.05 M Ethylenediaminetetraacetic Acid (EDTA) for two hours. After removing the EDTA, the seeds were shaken for one hour in 800 µl 0.01% ruthenium red (Sigma-Aldrich), rinsed in distilled water and placed in a white porcelain depression plate for observation on a dissection microscope (Leica Zoom 2000 Stereozoom) or in a depression slide for observation on a microscope (Zeiss Axioplan 2, Carl Zeiss MicroImaging) equipped with a digital camera (Zeiss AxioCam Colour 412-312, Carl Zeiss Microimaging).

2.4.4. Calcofluor white assay

Approximately 50 seeds were placed in a 1.5mL Eppendorf tube and 1 mL of 1 mg/mL calcofluor white solution (Calcofluor white fluorescent brightner #28 (Sigma Aldrich) in 40mM NaOH) was added. The samples were vortexed for 20 seconds at medium speed and incubated at room temperature for five minutes. The calcofluor white was removed and the samples were washed three times in distilled water vortexing 20 seconds each time at medium speed. The stained seeds were mounted on depression slides and observed on a compound fluorescence microscope (Zeiss Axioplan 2, Carl Zeiss MicroImaging) microscope using the UV set (UV excitation max 347 nm, blue emission max 435 nm).

2.5. Germination analysis

Mutant and control seeds were collected from plants grown together under identical environmental conditions, and stored for two days, two weeks, or three weeks at room temperature in paper bags (Midco Enterprises, Inc.). The experiments were completed twice using two sets of plants grown at different times. For the dark treatment conditions, plates were wrapped in two layers of aluminum foil immediately after sowing the seeds and placed in sealed cardboard boxes. Germination was scored as the onset of cotyledons.

2.5.1. Germination under four different light/temperature conditions in Gamborg's B-5 basal medium

Fifty dry seeds were sown on Gamborg's B-5 basal medium plates and germination was scored under the following four combinations of light/temperature conditions: (1) Cold/Light: 3 days at 4°C in the dark followed by 3 days at 22°C in 16 hours light per 8 hours dark, (2) Cold/Dark: 3 days at 4°C followed by 3 days at 22°C all the time in the dark, (3) No Cold/Light: 6 days at 22°C all the time in 16 hours light per 8 hours dark, and (4) No Cold/Dark: 6 days at 22°C all the time in the dark. Germination was scored at day six.

2.5.2. Germination timeline in water

Seeds were tested two weeks after harvesting. Two 70 mm filter papers (Whatman #1) were placed in the lid of a 100 mm plastic petri dish and wetted with 2 mL of distilled water before sowing 50 seeds. The plates were incubated three days at 4°C in the dark, followed by six days at 22°C in 16 hours light/8 hours dark cycle. Germination was scored daily for six days and at day eight to ensure 100% germination in all lines.

2.6. Seed coat permeability

2.6.1. Germination in water reduced conditions

Seeds were tested two weeks after harvesting. Two 70 mm filter papers (Whatman #1) were placed in the lid of a 100 mm plastic petri dish and wetted with 1.5 mL of distilled water (control) or 1.5 mL of 12%, 15%, 18%, 21%, and 25% polyethylene glycol (PEG) 6000 solution (Sigma, Aldrich). The plates were incubated for 3 days at 4°C

in the dark followed by 5 days at 22°C in 16 hours light per 8 hours dark. Germination was scored as the appearance of green cotyledons at day five.

2.6.2. Tetrazolium Red assay

Fifty seeds were placed in a 1.5 mL Eppendorf tube containing 1.0 mL of 1% (w/v) 2,3,5-triphenyltetrazolium chloride aqueous solution and incubated for two days in the dark at 30°C. The seeds were placed in a white porcelain depression plate and observed on a dissection microscope (Leica Zoom 2000 Stereozoom) to calculate the percentage of seeds that changed colour from brown to red.

2.7. Analysis of root growth and development

Four groups of seeds were sown on square, gridded petri dishes (120 mm x 120 mm x 17 mm) containing 40 mL of Gamborg's B-5 basal medium, as follows: a row of six control seeds followed by six mutant seeds on top of the plate, and a row of six mutant seeds followed by six control seeds in the middle of the plate. The plates were placed in a horizontal position for three days at 4°C in the dark, followed by 14 days in a vertical orientation at 22°C in 16 hours light per 8 hours dark. Observations of root length, gravity, root hairs, and secondary roots development were made daily starting on the fifth day of the assay. Root length was measured for the primary root only starting at the base of the stem and to the root tip.

2.8. Positional mapping of the *MEN2* locus

2.8.1. Genomic DNA extraction

Leaves of about 0.5 cm in length were placed in 1.5 mL Eppendorf tubes and stored at -80°C for 10 minutes, after which 750 µL of EDM buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 5 mM EDTA, 0.5% SDS) were added and incubated at 95°C for five minutes. After incubation overnight at room temperature, 750 µl of isopropyl alcohol was added. The tubes were centrifuged at 14000 rpm for three minutes at room temperature and the supernatant was discarded. The pellet was washed three times with 1 mL 70% ethanol inverting several times during each wash. After a one minute centrifugation at 14000 rpm at room temperature, the pellets were dried at 37°C for one hour by placing the tubes open in a tube incubator. 250 µl of TE buffer (10 mM Tris-HCl, 1mM EDTA PH 8.0) was added and the tubes were placed in a shaker (100 rpm) for 10 minutes.

2.8.2. Primers for amplification of polymorphic locus

Design of Col and L*er* primers for amplification of polymorphic locus was facilitated by information available from the TAIR website (http://www.arabidopsis.org/). The polymorphisms chosen were InDels[4] that showed a difference of about 40 bp between the Col and L*er* sequences. The flanking sequences were used to BLAST and compare against the Col-0 sequence using the TAIR BLAST and TAIR mapviewer tools. The sequences obtained were about 200 bp, which were copied into the primer 3 software at http://frodo.wi.mit.edu/ adjusting the parameters to Min: 20, Opt: 25, Max: 27 before

[4] InDel refers to the mutation class that includes both insertions, deletions, and the combination thereof.

selecting the primers. Selected primers were analyzed using the oligo analyzer tool at http://www.idtdna.com/analyzer/Applications/OligoAnalyzer/Default.aspx to obtain annealing temperature (desirable around 55°C) and GC content (desirable around 50%). To confirm that there were size differences between the predicted Col-0 and the *Ler* products to be amplified, pairwise BLAST searches were performed using the TAIR BLAST tool.

Table 2. Primers used in amplification of polymorphic locus for positional mapping of the Men2-1 *locus*.

Marker Name	Chr	Position (Mbp)	Forward primer	Reverse primer	Col size (bp)	L*er* size (bp)
F21M12	1	3.2	GGCTTTCTCG AAATCTGTCC	TTACTTTTTG CCTCTTGTCA TTG	200	160
nga63	1	3.6	ACCCAAGTGA TCGCCACC	AACCAAGGC ACAGAAGCG	111	89
Cer452156	1	5.5	GATTATTTCAT TATAATGAA	TGTTTAACGT ATTTTTTGTT	207	174
Cer453258	1	7.8	ACACACACAC ACACACACAC	GCACAAAAG TGAGAGAGT	190	180
Cer464611	1	8.0	GTTATTAGTTT GGTCTCTTT	TTTCTTCTCTT CTTTCTCTA	197	185
Zfpg	1	8.7	TGGGTCAATTC ACATGTAGAG	TTGCGTTTCC ACATTTGTTT	143	139
Ciw12	1	9.6	AGGTTTTATTG CTTTTCACA	CTTTCAAAAG CACATCACA	128	115
nga392	1	9.8	GGTGTTAAATG CGGTGTTC	TTGAATAATT TGTAGCCATG	170	162
Cer450309	1	10.0	TAACATCTGTT TTAGTCACC	ATTAATCTTAT TATGGTGAT	199	161
AC17118	1	11.5	GTTGGATTGA GTTTGGTCAC	AATTGGCTAT GTAGAGTTGG	224	203
T15K4-1	1	12.4	AAAGTCCACG TAGACCGAAG	CGCAAATCAA ACACGAACA	165	183
Ciw1	1	18.4	ACATTTTCTCA ATCCTTACTC	GAGAGCTTCT TTATTTGTGAT	159	135
nga280	1	20.8	GGCTCCATAA AAAGTGCACC	CTGATCTCAC GGACAATAGT GC	105	185
nga128	1	20.6	ATCTTGAAAC CTTTAGGGAG G	GGTCTGTTGA TGTCGTAAGT CG	180	190
nga111	1	27.4	TGTTTTTTAGG ACAAATGGCG	CTCCAGTTGG AAGCTAAAG GG	128	162
ciw2	2	1.2	CCCAAAAGTT AATTATACTGT	CCGGGTTAAT AATAAATGT	105	90

Marker Name	Chr	Position (Mbp)	Forward primer	Reverse primer	Col size (bp)	L*er* size (bp)
ciw3	2	6.4	GAAACTCAAT GAAATCCACT	TGAACTTGTT GTGAGCTTTG	230	200
nga1126	2	12.6	GCACAGTCCA AGTCACAACC	CGCTACGCTT TTCGGTAAAG	191	199
nga168	2	16.3	GAGGACATGT ATAGGAGCCT CG	TCGTCTACTG CACTGCCG	151	135
nga162	3	4.6	CTCTGTCACTC TTTTCCTCTGG	CATGCAATTT GCATCTGAGG	107	89
ciw11	3	9.8	CCCCGAGTTG AGGTATT	GAAGAAATTC CTAAAGCATT	179	230
ciw4	3	18.9	GTTCATTAAAC TTGCGTGTGT	TACGGTCAGA TTGAGTGATT	190	215
nga6	3	23.0	ATGGAGAAGC TTACACTGATC	TGGATTTCTT CCTCTCTTCA	143	123
ciw5	4	0.8	GGTTAAAAATT AGGGTTACGA	AGATTTACGT GGAAGCAAT	164	144
ciw6	4	7.9	CTCGTAGTGC ACTTTCATCA	CACATGGTTA GGGAAACAA TA	162	148
ciw7	4	11.5	AATTTGGAGAT TAGCTGGAAT	CCATGTTGAT GATAAGCACA	130	123
nga1107	4	18.1	CGACGAATCG ACAGAATTAG	GCGAAAAAA CAAAAAAATC	150	140
ciw8	5	7.5	TAGTGAAACC TTTCTCAGAT	TTATGTTTTCT TCAATCAGTT	100	135
Phyc	5	14.0	CTCAGAGAAT TCCCAGAAAA	AAACTCGAG AGTTTTGTCT	207	222
ciw9	5	17.0	CAGACGTATC AAATGACAAA TG	GACTACTGCT CAAACTATTC GG	165	145
Cer462550	5	17	AAACATTTGG GCAGGGGTGG	GTGGCACTCA CTGGTGTGAT	165	145
ciw10	5	24.5	CCACATTTTCC TTCTTTCATA	CAACATTTAG CAAATCAACT	140	130

2.8.3. Polymerase chain reaction (PCR)

Amplifications by PCR were conducted on a thermocycler (533114515, Eppendorf) executing the following program: 1 minute at 94°C (denaturation step), followed by 40 cycles of 30 seconds at 94°C, 30 seconds at the optimized annealing temperature for each set of primers usually between 55°C and 60°C (annealing step), and 30 seconds at 72°C (extension step), and then one incubation of 10 minutes at 72°C. The reactions were kept at 4°C. The PCR reaction contained 50 mM KCl, 50 mM $(NH_4)_2SO_4$, 100 mM Tris-HCl pH 8.0, 1% Triton X-100, 1 mM dNTPs, 1 µM of each forward and reverse primers, 0.2 µl of Taq DNA polymerase, and 1.5 µl of DNA template.

2.8.4. Agarose gel electrophoresis

Gel electrophoresis of the PCR amplified DNA products were performed using 4% (w/v) agarose gels. To make the gels, 5 g of high melting point agarose (AGA001.500, Bioshop Canada Inc.) were diluted in 125 mL of TAE buffer (40 mM Tris acetate, 1 mM EDTA (pH 8.0)), and 5 g of low melting point agarose (AGA103.25 Bioshop Canada Inc.) were slowly added. After swirling the solution for about 3 minutes, another 125 mL of TAE buffer were added and the flask was covered with aluminum foil and kept at 4°C overnight (to allow small agarose clumps to hydrate). On the next day, the solution was heated at low temperature in the microwave oven for about 1.5 minutes until the agarose was completely dissolved and 3 µL of 0.05% Ethidium bromide were added. 3 µL of 10X orange dye (20 g sucrose, 100 mL orange G) were added to each 20 µL sample before loading them into the gel for electrophoresis at 60 V for about three hours.

3. Results

3.1. Phenotypic characterization of eight *mum4-1* enhancer lines

3.1.1. Mucilage production and release

The seed coat of *Arabidopsis* produces gelatinous mucilage that swells upon hydration and is released after breaking the primary cell wall, forming a thick layer that surrounds the seed. The released mucilage can be observed by staining with the pectin-specific dye ruthenium red (Figure 5). *Mum4-1* mutants produce significantly less mucilage than wild-type seeds and this mucilage is not released upon hydration (Figure 5B). However, its release can be induced by shaking the seeds in a solution of chelator such as EDTA, which withdraws Ca^{2+} ions from the cell wall-associated pectins. This weakens the cell wall and allows increased release of the stored mucilage (Arsovski *et al.* 2009*a*). Seeds mutant for *ttg1-1*, which is epistatic to *mum4-1* in the pathway for mucilage production (Western *et al.*, 2004), generate very little mucilage and this is not released from the seed coat even after EDTA treatment (Figure 5C). Seeds of *ttg1-1* were used as a control to illustrate the appearance of seeds that produce very little mucilage.

To compare the amount of mucilage produced and released by *mum4-1* enhancer lines to that of wild type Col-2 and *mum4-1,* the seeds were treated in EDTA with shaking and stained with ruthenium red. A thick layer of stained mucilage was present around the Col-2 wild type seeds, whereas this layer was reduced in the *mum4-1* seeds, and totally absent in the *mum4-1* enhancer lines (Figures 6D and 7). There were no apparent differences in the amount of mucilage released between the *mum4-1* enhancer lines (Figure 7, A-J).

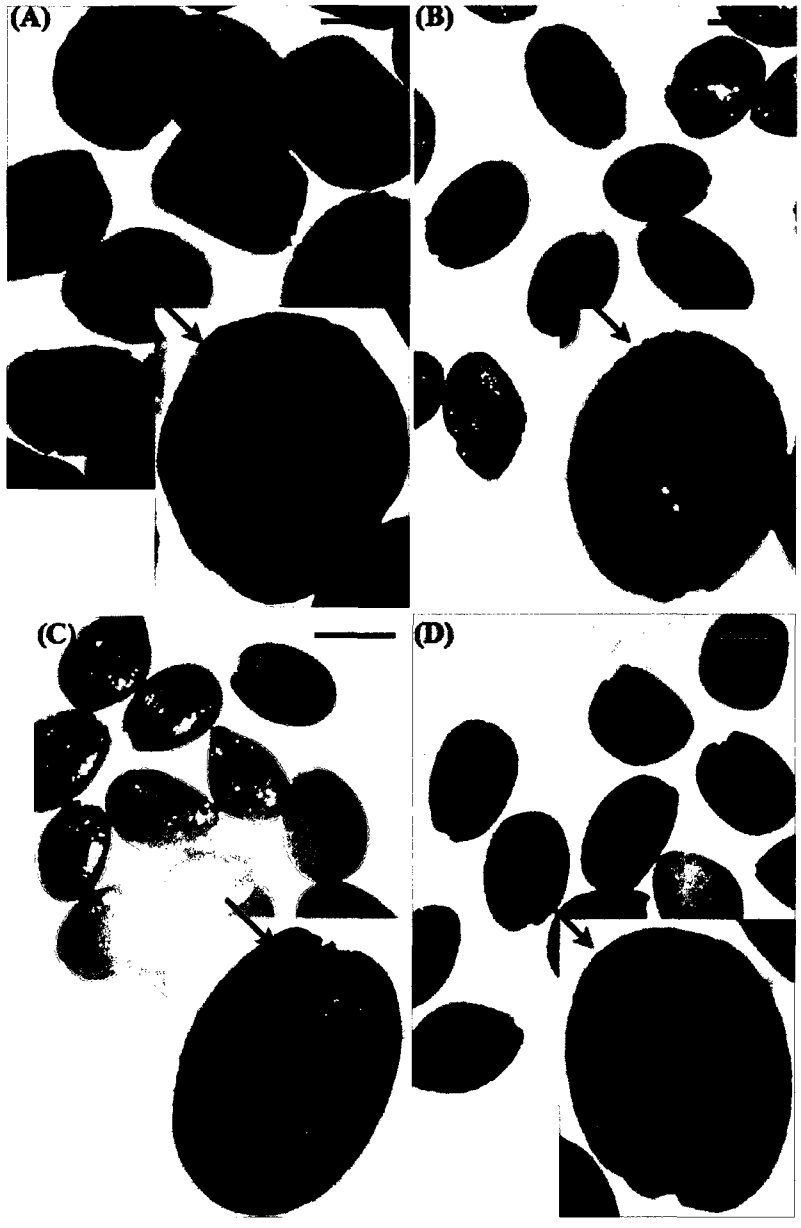


Figure 6. Mucilage release of (**A**) Col-2, (**B**) *mum4-1*, (**C**) *ttg1-1*, and (**D**) *men2-1 mum4-1* seeds. The mucilage appeared as a thick red halo surrounding the wild-type Col-2 seeds (arrow), which was significantly reduced around *mum4-1* seeds, and completely absent from the seeds of *ttg-1* mutants and *men2-1 mum4-1* double mutants. Scale bars: A: 100 μm, B, C and D: 500 μm.

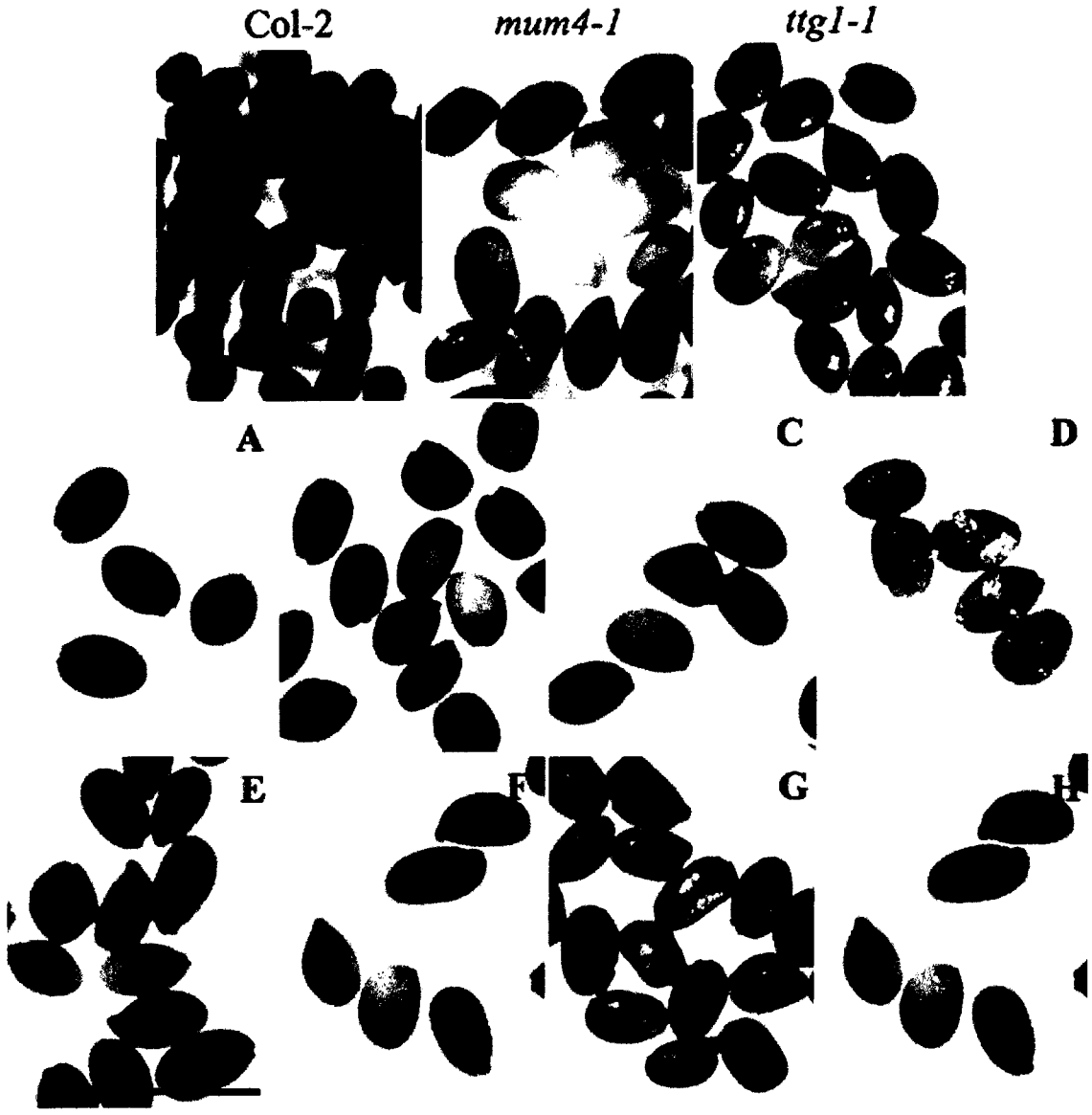


Figure 7. Mucilage release of *mum4-1* enhancer lines and controls. The mucilage appeared as a thick red halo surrounding the wild-type Col-2 seeds, which was significantly reduced around *mum4-1* seeds, and completely absent from seeds of *ttg1-1* and *mum4-1* enhancer lines. (**A**) *men1-1 mum4-1*, (**B**) *men2-1 mum4-1*, (**C**) *men3-1 mum4-1*, (**D**) *men4-1 mum4-1*, (**E**) *men5-1 mum4-1*, (**F**) *men6-1 mum4-1*, (**G**) *mum2-13 mum4-1*, (**H**) *myb61-6 mum4-1*. Scale bar: 500 μm.

3.1.2. Seed coat permeability

The uptake of tetrazolium salts by the embryo has been used to assess the permeability of the seed coat in *Arabidopsis*, as it is difficult to evaluate water uptake due to the hygroscopic properties of the mucilage in the seed coat (Debeaujon *et al.*, 2000). Increased seed coat permeability allows the penetration of tetrazolium into the embryo, where it is metabolized in the endoplasmic reticulum by NADH-dependent reductases to form red coloured formazans (Debeaujon *et al.*, 2000). The seed coat becomes impermeable to tetrazolium salts during the last stage of seed development, when the brown pigments accumulate prior to seed desiccation. In this way, wild type *Arabidopsis* Col-2 seeds with brown seed coats remain unstained by tetrazolium salts, while pigmentation or structural seed coat mutants, such as *ttg1* and *ap2*, respectively, exhibit different shades of red upon exposure to tetrazolium salts solution (Debeaujon *et al.*, 2000).

Staining with tetrazolium salts staining was used to determine the permeability of the seed coats of *mum4-1* enhancer lines (Figure 8). *Ttg1-1* seeds were included in the assay as a positive control because they produce little or no mucilage and their seed coats are highly permeable to tetrazolium salts (Debeaujon *et al.*, 2000). From all the enhancer lines tested, only the seeds of *men3-1 mum4-1* turned red upon treatment with tetrazolium salts indicating increased seed coat permeability (Figure 8C). However, these seeds were slightly paler than wild-type seeds, and later studies demonstrated that this seed colour phenotype segregates away from the mucilage phenotype. Therefore it was most likely a consequence of a background *transparent testa* mutation or related type of mutation, and not related to variations in mucilage release (Arsovski *et al.*, 2009*a*).

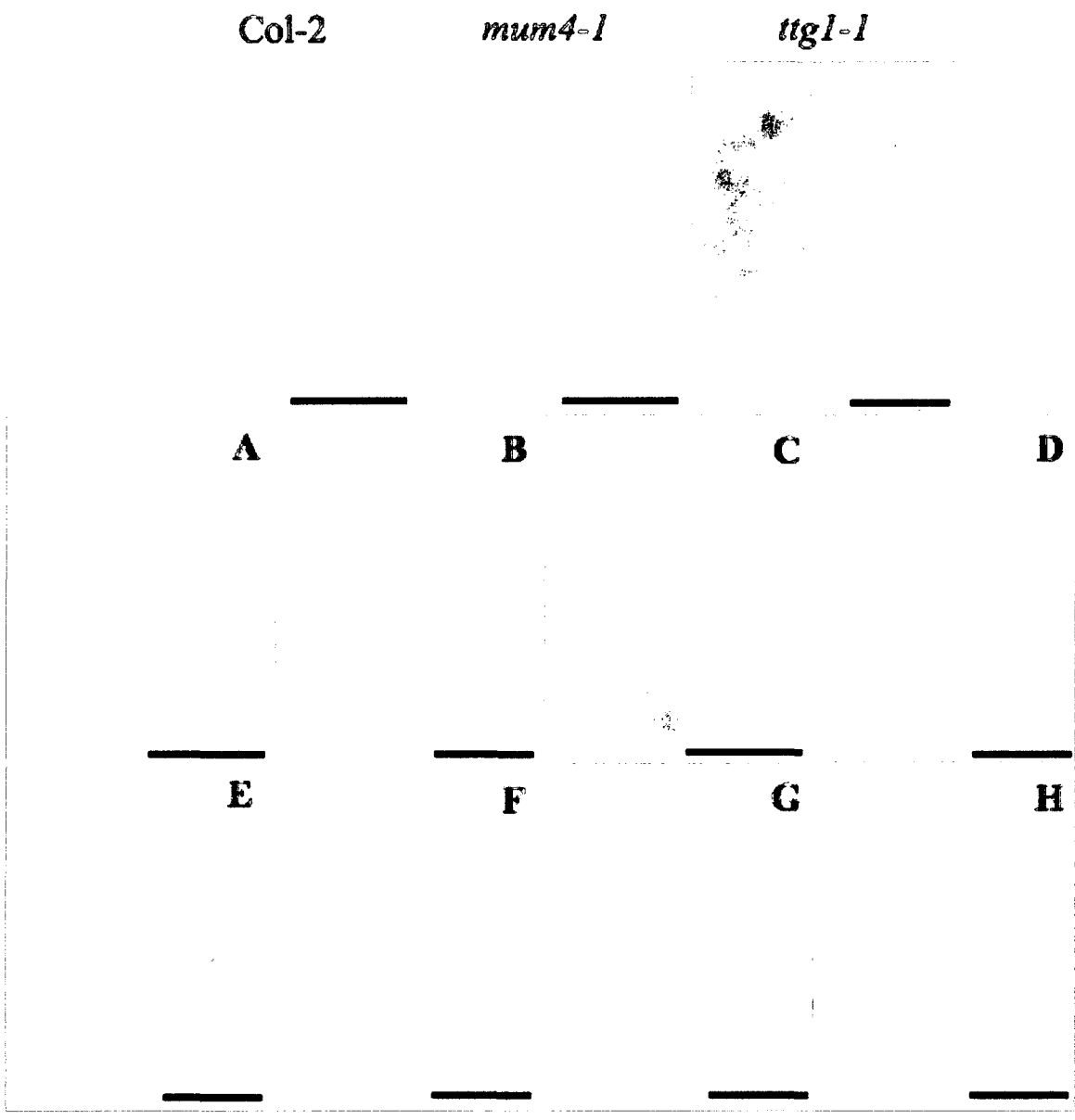


Figure 8. Variations of seed coat permeability of *mum4-1* enhancer lines. Permeability to tetrazolium salts causes a change in the seed colouration to different shades of red. (**A**) *men1-1 mum4-1*, (**B**) *men2-1 mum4-1*, (**C**) *men3-1 mum4-1*, (**D**) *men4-1 mum4-1*, (**E**) *men5-1 mum4-1*, (**F**) *men6-1 mum4-1*, (**G**) *mum2-13 mum4-1*, (**H**) *myb61-6 mum4-1*. Scale bar: 500 μm.

3.1.3. Germination conditions

The onset of germination is marked by three key events: (i) uptake of water by the dry seed, (ii) embryonic axis elongation, and (iii) radicle tip protrusion. Seed dormancy can be imposed by the embryo, the seed coat, the endosperm or a combination of various factors, depending on the plant species. The seed coat imposes dormancy on *Arabidopsis* seeds by limiting water and oxygen exchange with the environment, and also by mechanically restricting radicle protrusion. Mature *Arabidopsis* seeds remain dormant (unable to germinate even under appropriate environmental conditions) for about one month after being released or harvested from the mother plant (Debeaujon *et al.* 2000); However, germination can be induced by dormancy-breaking agents such as stratification or the addition of gibberellins (Koornneef and Karssen, 1994). Germination tests were performed on the seeds of *mum4-1* enhancer lines, as reduced mucilage production has been associated with reduced germination rates in *Arabidopsis* (Debeaujon *et al.*, 2000; Penfield *et al.*, 2001) and mutations affecting seed coat structure and/or pigmentation have been associated with altered dormancy patterns (Debeaujon *et al.* 2000). Seeds of the pigmentation and seed coat structure mutant *ttg1-1* were used as a control because of their extreme non-dormancy phenotype (100% germination after only two days of harvest). The degree of dormancy in wild-type and mutants was assessed by determining the percent germination of seed lots at three different times after seed harvest (2 days, 2 weeks, and 3 weeks), which indicates the after-ripening time requirement to reach 100% germination. Incubation in the dark at 4°C for three days, followed by three days of incubation in the light at 22°C produced 80% germination in all controls and *mum4-1*

enhancer lines when seeds were tested 2 days and 3 weeks after harvest (Figure 9A). The germination rate for all lines increased to 90% three weeks after harvest.

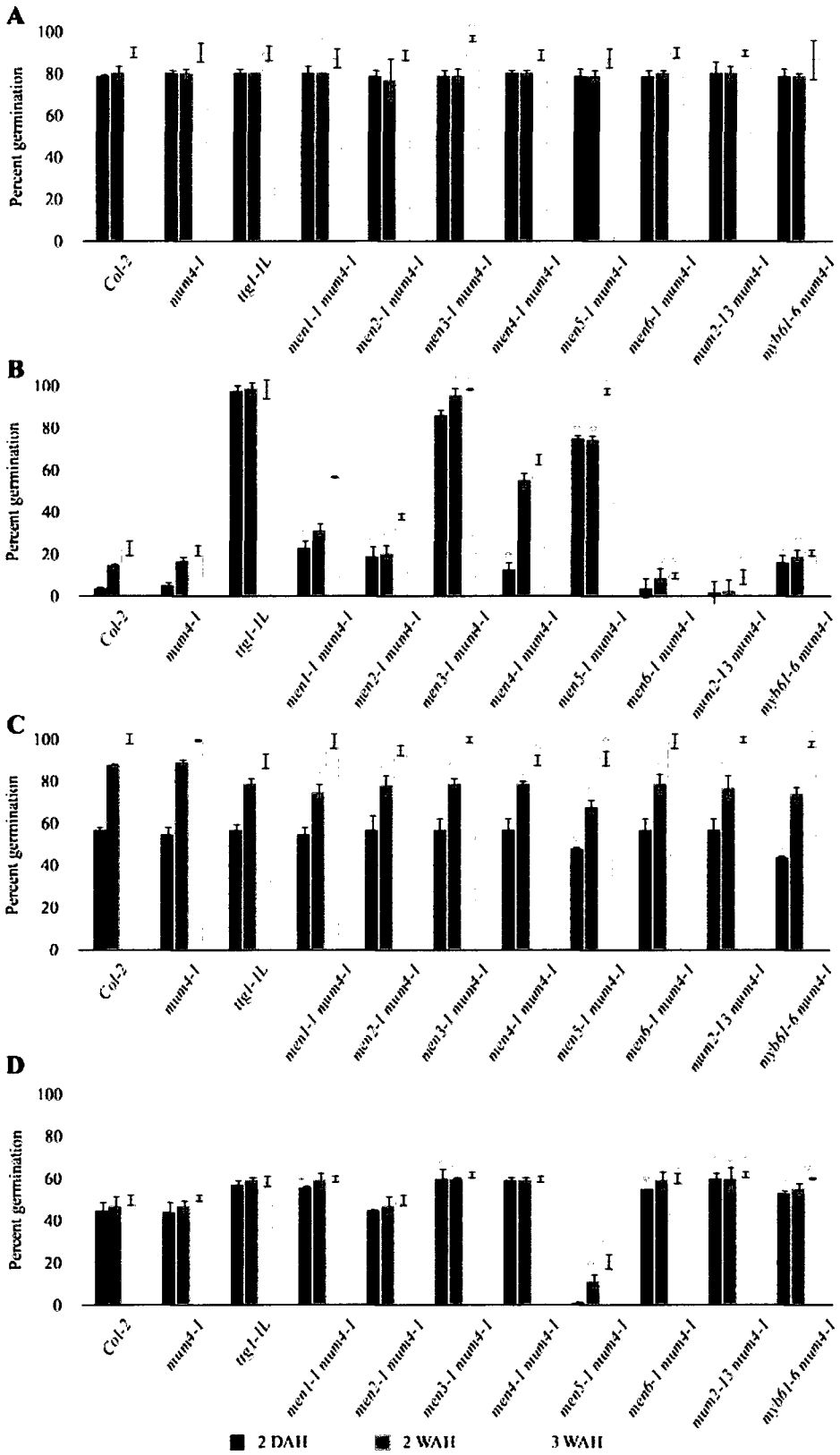
Incubation of seeds in darkness at 22°C revealed a significantly lower germination rate for Col-2 and *mum4-1* seeds, which achieved only 20% germination three weeks after harvest, indicative of the requirement of cold and light to break the dormancy in these seeds. In contrast, *men3-1 mum4-1,* and *men5-1 mum4-1* showed a more rapid release from dormancy, even at two days after harvesting, achieving 83%, and 79% germination respectively (Figure 9B). These results are similar to those seen for reduced dormancy in structural and pigmentation mutants *ap2* and *ttg1-1* (Debeaujon *et al.*, 2000) leading to a closer examination of these two mutants for further seed coat defects. Unlike *ap2* mutants, we did not observe any changes in their seed shape. Only *men3-1 mum4-1* seeds showed staining with tetrazolium red, indicative of increased permeability of the seed coat to solutes as in *ttg1-1* seeds (Figure 8C).

There were no differences in the rate of germination between wild-type, *mum4-1*, and the enhancer mutant lines when incubated for six days in the light at 22°C, at any of the tested times after harvest. When comparing these results to those obtained for three days of dark incubation at 4°C followed by three days of light treatment at 22°C (Figures 9A vs. 9C), we can conclude that stratification helped release dormancy of the *Arabidopsis* seeds, as the addition of the cold treatment resulted in 80% germination two days after harvest (Figure 9A), compared to only 60% germination two days after harvest in the absence of the cold treatment (Figure 9C).

No differences were observed between the germination rate of *mum4-1*, Col-2, and any of the *mum4-1* enhancer lines when the seeds were exposed to cold in the dark for six days (Figure 9D), with the exception of *men5-1 mum4-1* seeds that had a much lower germination rate, achieving only 20% germination after six days. These results appeared to be independent of the time of seed storage, as it was consistent for the seeds tested 2 days, 2 weeks, and 3 weeks after harvest.

Overall, these experiments indicate that the dark and cold treatment (dark at 4°C for three days/light at 22°C for three days) helps release dormancy in *Arabidopsis* seeds as 80% germination was achieved only two days after harvest (Figure 9A). This same level of germination was achieved 2 weeks after harvest in the absence of the cold treatment (Figure 9C) and was not achieved even three weeks after harvest when seeds were germinated in the dark without exposure to light (Figure 9D). Although most of the *men* enhancers display the same behaviour as *mum4-1* and Col-2 seeds in all treatments, *men3 mum4* and *men5 mum4* seeds appear to have a reduced requirement for stratification to release dormancy of the seeds as they achieved a higher percent germination than *mum4* and Col-2 control seeds (Figure 9B).

Figure 9. Effect of temperature and light on germination of *mum4-1* enhancer lines 2 days (blue bars), 2 weeks (green bars), and 3 weeks (yellow bars) after harvest (2 DAH, 2 WAH, and 3 WAH). Seeds were incubated in four different environmental conditions: (**A**) 3 days at 4°C in dark/3 days at 22°C in light, (**B**), 6 days at 22°C in dark, (**C**) 6 days at 22°C in light, (**D**) 6 days at 4°C in dark. *men3-1 mum4-1, men4-1 mum4-1,* and *men5-1 mum4-1* presented reduced dormancy compared to Col-2 and *mum4-1* parent lines, when incubated in the dark at room temperature. Error bars: standard deviation (based on 3 plates per line and 50 seeds per plate). Significance assessed by Student *t*-test (*, $P<0.05$). Germination values of *mum4-1* enhancer lines were compared to those of *mum4-1* single mutant lines.


A
B
C
D
Percent germination
100
80
60
40
20
0
Col-2
mum4-1
ttg1-1L
men1-1 mum4-1
men2-1 mum4-1
men3-1 mum4-1
men4-1 mum4-1
men5-1 mum4-1
men6-1 mum4-1
mum2-13 mum4-1
myb61-6 mum4-1
2 DAH
2 WAH
3 WAH

3.1.4. 'Gantlet' analysis of general plant growth and developmental characteristics of *mum4-1* enhancer lines

The *Arabidopsis* Gantlet Project (http://thale.biol.wwu.edu/) was designed to determine the function of 25,000 genes in *Arabidopsis thaliana* by the year 2010. A collection of protocols describing a broad range of environmental and developmental assays is available as part of this project, aiming to reveal phenotypic differences between wild-type and mutants of genes with unknown function.

The *mum4-1* enhancer lines were subjected to the *Arabidopsis* Gantlet protocol and assessed for changes in whole plant developmental phenotypes and compared to both the parental lines. The phenotypes that were assessed included germination time; cotyledon size and shape; time of bolting and emergence of true leaves; bolt length and gravity; length of petiole, root and stem; size and shape of leaves; phenotypes of roots including length and branching. Overall conclusion from these results was that with the exception of *men2-1 mum4-1*and *men5-1 mum4-1,* no significant differences were observed in the other six *men* mutants. Both *men2-1 mum4-1* and *men5-1 mum4-1* displayed a two day germination delay, and a shorter main root with increased branching. The following sections describe detailed characterization of *men2-1 mum4-1*. Characterization of *men5-1 mum4-1* is being performed in the laboratory of Tamara Western at McGill University and therefore will not be further discussed here.

3.2. Phenotypic characterization of *men2-1 mum4-1*

3.2.1. Seed coat structure

The dyes toluidine blue and calcofluor white were used to study the structure of the seed coat of *men2-1 mum4-1*. Toluidine blue reveals the shape of the columella and the amount of mucilage stored in the seed coat and/or released, as it binds to acidic polysaccharides such as pectins staining them pink-purple (Figure 10A). Calcofluor white reveals the outline of the seed coat cells as well as the columella, as it binds to β-glucans in the cell walls and mucilage (Figure 10B). The seeds used for these assays were collected 13 days post anthesis (DPA), the time at which mucilage production is completed and the columella are fully formed (Western *et al.*, 2000).

The wild-type Col-2 seeds had volcano-shaped columella and a very thick layer of pink mucilage that was released by the epidermal cells upon exposure to the aqueous toluidine blue dye. Conversely, *mum4-1* seeds had a flattened columella and a thin layer of mucilage that remained inside the seed coat cells above the large vacuole. The columella of *men2-1 mum4-1* resembled that of *mum4-1*, but the amount of mucilage stored inside the seed coat cells appeared to be significantly reduced (Figure 10A).

The alteration in cell shape caused by the reduction in mucilage production and storage in the seed coat was revealed by the calcofluor white staining. The hexagonal shape shown by the epidermal cells of the seed coat of wild type Col-2 seeds appeared distorted in the epidermal seed coat cells of the *mum4-1* mutant. This seed coat epidermal cell shape alteration was even more pronounced in the *men2-1 mum4-1* mutant which is displayed by a very faint staining of the cell walls of the seed coat epidermal cells (Figure

10B). These results suggest that the further reduction in mucilage produced and stored in the seed coat epidermal cells might result in extensive alterations of cell shape. It is also possible that given the faint staining in the *men2-1* seeds, mutation could affect the production or structure of β-glucans in the cell walls of the epidermal seed coat cells.

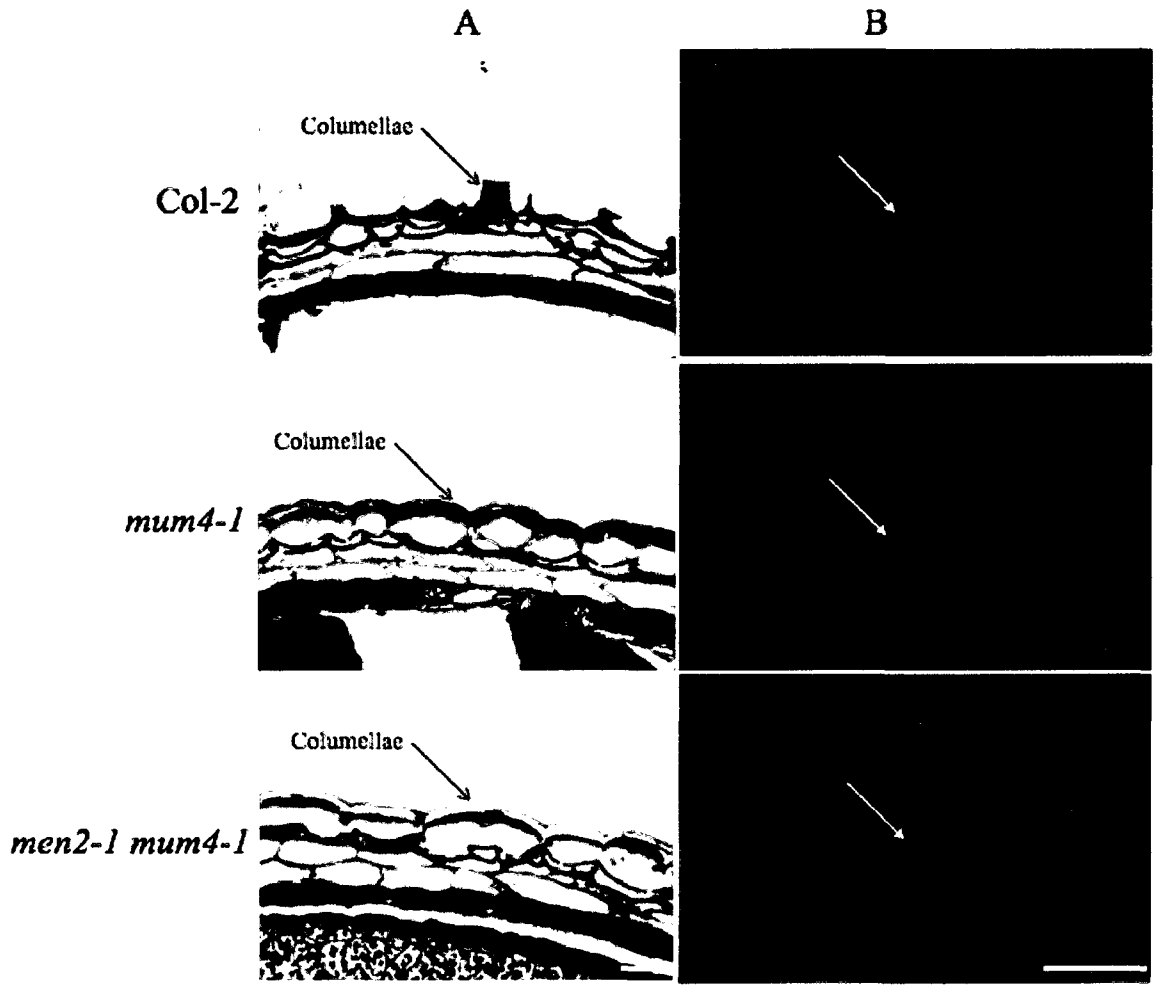


Figure 10. Seed coat structure of *men2-1 mum4-1*. (**A**) Toluidine blue staining of resin-embedded seed sections, and (**B**) Calcofluor white staining of whole seeds. The volcano-shaped columella present in Col-2 seeds is flattened in seeds of *mum4-1*, as well as in seeds of the double mutant *mum2-1 mum4-1*. Scale bars: (**A**) 10 μm, (**B**) 50 μm. Arrows indicate differences in epidermal cells appearance.

3.2.2. Germination analysis of *men2-1 mum4-1*

3.2.2.1. Germination timeline

A germination timeline assay was performed to compare the germination times of *men2-1 mum4-1* seeds to those of Col-2 wild type seeds and of *mum4-1* seeds (parent line). Seeds of the *ttg1-1* mutant were included in the assay as an additional control, because these seeds produce a very limited amount of mucilage that is not released upon hydration. It has been demonstrated that defects in mucilage production and release can affect germination (Debeaujon *et al.*, 2000; Penfield *et al.*, 2001; Rautengarten *et al.*, 2008; Arsovski et al., 2009*b*).

Control (*Col-2*, *mum4-1*, and *ttg1-1*) and *men2-1 mum4-1* seeds were incubated three days at 4°C in the dark, followed by six days at 22°C in long day conditions (16 hours light/8 hours dark cycle). The wild type Col-2 seeds reached 65% germination at day three of the assay, while only 25% and 10% germination was achieved by *mum4-1* and *men2-1 mum4-1* seeds, respectively, during that period (Figure 11). There was no discernible difference between two parental lines at day five and in fact, by day six the germination of *mum4-1* seeds and Col-2 wild type reached 100%. The *men-2 mum4-1* germination on the other hand, even at day six, lagged behind that of both Col-2 and *mum4-1* seeds. The delay was more pronounced at day four (65% germination compared to 79% and 98% from *mum4-1* and Col-2, respectively) and closely resembled the time line observed with the *ttg-1* mutant. These results indicated that the delay in the onset of germination was related to the reduction in mucilage production in the seed coat cells, as *ttg1-1* seeds, which produce the least amount of mucilage from the lines tested had the greatest delay, followed closely by *men2-1* seeds that produce less mucilage than *mum4-1* seeds.

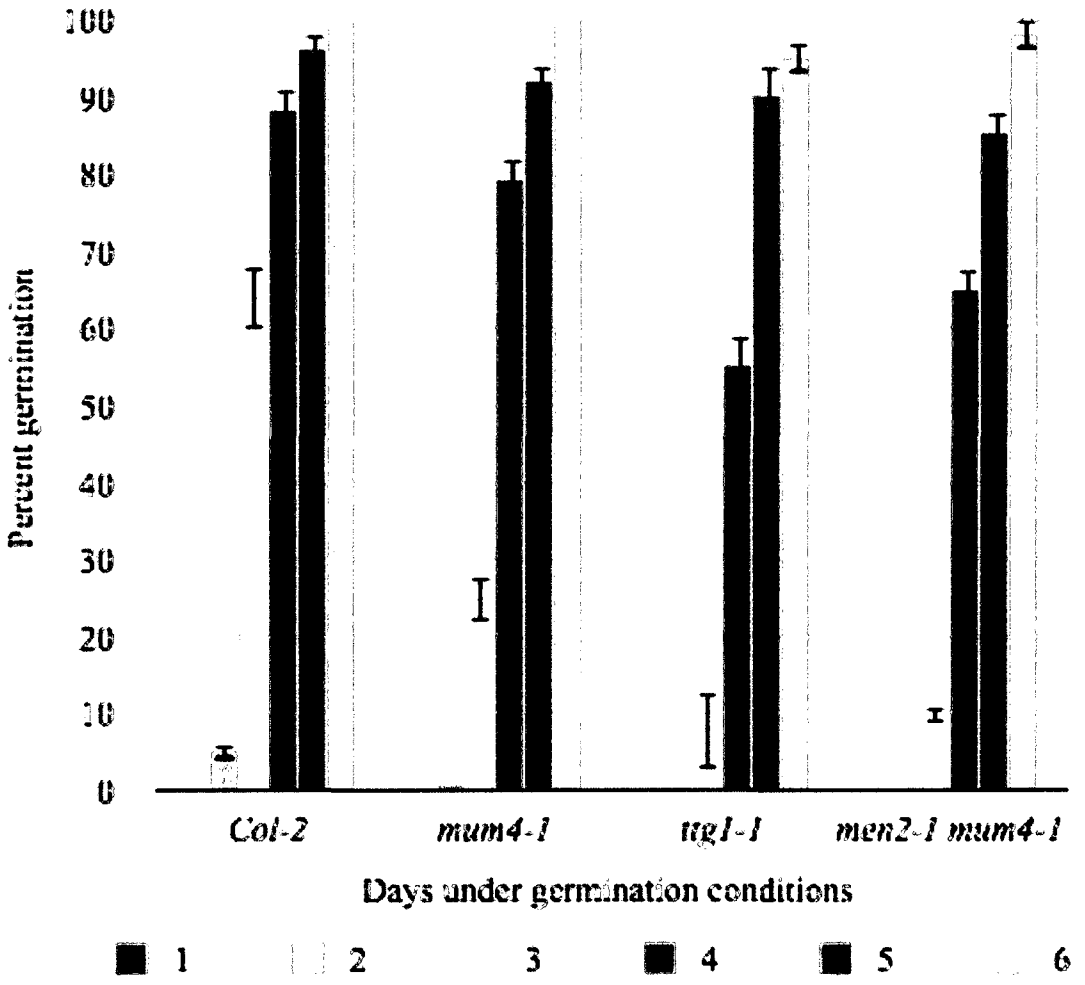


Figure 11. Timeline of germination of *men2-1 mum4-1*. Seeds were stratified at 4°C followed by germination at 22°C under a 16 hours of light per 8 hours of dark photoperiod regime. At day six, under germination conditions, Col-2 and *mum4-1* reached 100% germination while *men2-1 mum4-1 and ttg1-1* took two days for complete germination. Error bars: standard error (based on 3 plates per line and 50 seeds per plate).

3.2.2.2. Germination in water reduced conditions

Mucilage in the seed coat of *Arabidopsis* is thought to aid germination as it absorbs water, swells, breaks through the cell walls, and is released as a gelatinous capsule around the seed that facilitates hydration (Western *et al.*, 2000; Rautengarten *et al.*, 2008). To further investigate *men2-1 mum4-1* germination defects, seeds of *men2-1 mum4-1* and Col-2, *mum4-1*, and *ttg1-1* controls were set to germinate in aqueous solutions with increasing amounts of polyethylene glycol (PEG) 6000 to mimic drought conditions. Seeds were incubated for three days at 4°C in the dark followed by five days at 22°C in long day conditions. At 15% PEG concentration, 70% of the wild type Col-2 seeds germinated, however, only 50% of the seeds of *men2-1 mum4-1* and *mum4-1* germinated under these conditions (Figure 12). The germination efficiency of *men2-1 mum4-1* at all PEG concentrations resembled that of the *mum4-1* parent line and both of these lines had reduced germination efficiency under water limiting conditions compared to Col-2 wild type seeds. Seeds of *ttg1-1* had dramatically reduced germination efficiency under water limiting conditions with only 10% germination in 15% PEG. All of the tested lines were unable to germinate at 21% concentration of PEG. These results indicated that reduced mucilage production in the seed coat cells negatively impacted germination in drought conditions, most likely due to inadequate amount of mucilage, such as that in Col-2 wild-type seeds, which limited water absorption and retention at the time of germination.

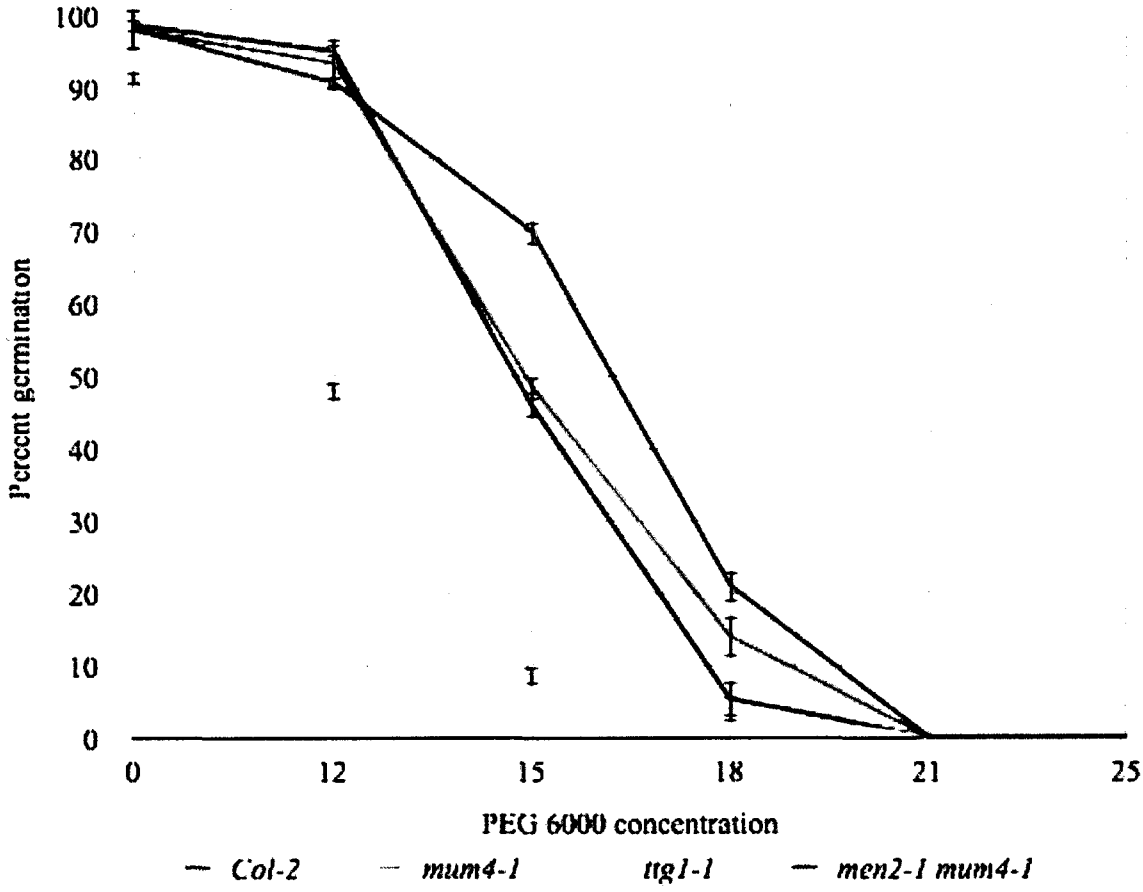


Figure 12. Germination of *men2-1 mum4-1* in water limiting conditions. Seeds were stratified at 4°C and transferred to a growth chamber at 22°C under a 16 hours of light per 8 hours of dark photoperiod regime. The germination efficiency of *men2-1 mum4-1* resembled that of *mum4-1* under water reduced conditions. Error bars: standard error (based 50 seeds per plate and three plates per line).

3.2.3. Study of root growth and development

The results of the Gantlet assay indicated that there were differences in the length and branching of the root of the *men2-1 mum4-1* mutant compared to wild-type Col-2 and *mum4-1* parent line plants. To further investigate these differences, the root growth and development of the mutant and controls were studied over a 14 day period. All seeds were stratified for three days at 4°C in the dark, before placing the plates vertically in a growth chamber at 22°C in long day conditions. The differences between the Col-2 and the mutants was minimal (about one millimeter) during the first three days, but became more pronounced as time progressed, resulting in a difference of about 10 millimeters after 14 days for *ttg1-1* and *men2-1 mum4-1* plants (Figure 13). Although the root length of the *mum4-1* parent line plants lagged behind that of the wild type Col-2 plants (about 5 mm), the delay in root growth of the *men2-1 mum4-1* double mutant resembled more that of the *ttg1-1* plants. Study of root gravitropism showed no differences between *men2-1 mum4-1* and Col-2 or *mum4-1*, as the roots of all mutant and control plants elongated in the direction of the gravitational pull when the plates were placed in vertical orientation. Microscopical observation of root hairs development at the end of the 14 day experimental period showed no difference in the amount and distribution of root hairs between *men2-1 mum4-1* mutant plants and Col-2 or *mum4-1* controls. Timing of secondary roots development as well as number of secondary roots at the end of the 14 day experimental period and indicated no differences between *men2-1 mum4-1* and Col-2 or *mum4-1* plants. These results indicated that mutations that negatively impact mucilage production in the seed coat epidermal cells such as *mum4-1*, *ttg-1*, and *men2-1* could also

negatively impact root growth. The fact that the delay in root elongation was more severe in *men2-1 mum4-1* than in *mum4-1* plants indicated that the *men2-1* mutation enhances the delayed root growth phenotype in addition to enhancing the reduced seed coat mucilage production phenotype of *mum4-1* mutant plants.

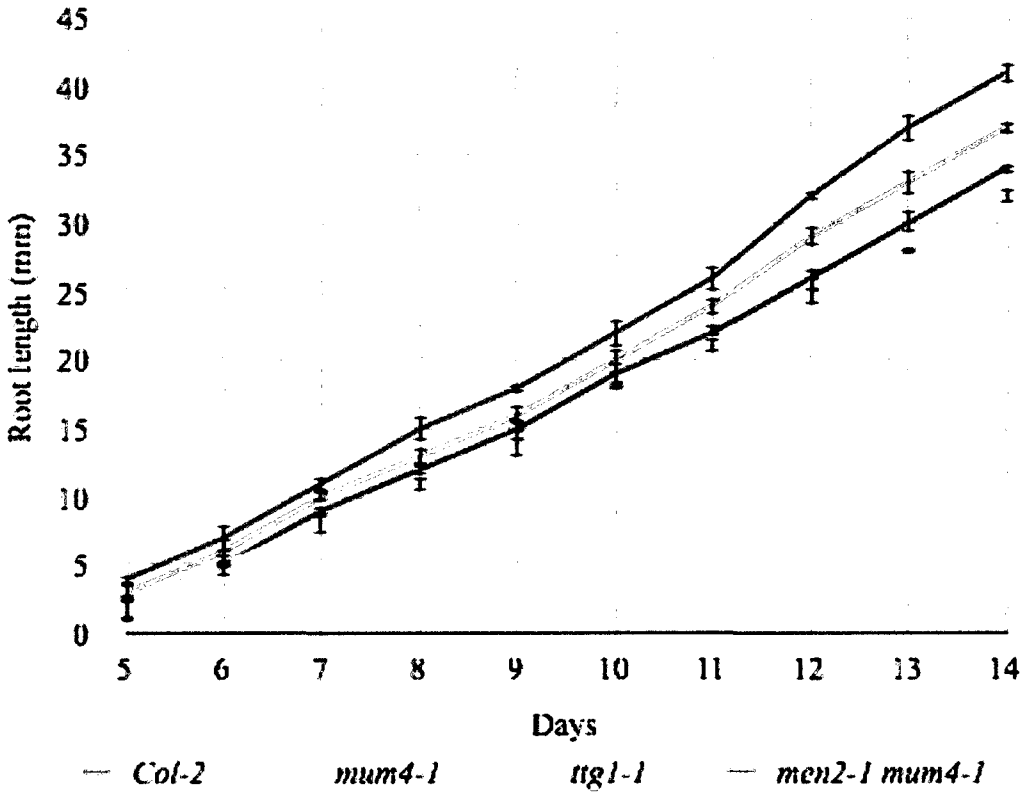


Figure 13. Root growth of *men2-1 mum4-1*. Seeds were stratified for 3 days at 4°C and transferred to a growth chamber at 22°C under 16 hours of light by 8 hours of dark. The root growth of *men2-1 mum4-1* lagged behind that of the *mum4-1* parent line, resembling the root growth shown by the *ttg1-1* mutants. Error bars: standard error (based on the average root length of 12 plants in 3 plates per line).

3.3. Positional mapping of *MEN2* locus

3.3.1. *men2-1 mum4-1* enhancer line

men2-1 mum4-1 was identified from a *mum4-1* (Col-2 ecotype) EMS mutagenized population (performed by Prof. Tamara Western, McGill University), by screening the seeds (M3) of over 5000 M2 lines for reduced levels of mucilage compared to *mum4-1*, as shown by ruthenium red staining after EDTA pretreatment.

The Chi square test results of the backcross of *men2-1 mum4-1* to Col-2 revealed that the null hypothesis of a 12:3:1 segregation ratio in the F2 generation cannot be rejected, indicating a two gene independent assortment pattern, and therefore that *men2-1* is genetically unlinked to *mum4-1* (wild type to *mum4-1* to *men2-1 mum4-1* phenotypic ratio of 98:21:8; n= 127; $0.5 > p > 0.1$ (Prof. T. Western, McGill University, unpublished results). Two backcrosses of *men2-1 mum4-1* to *mum4-1* were performed showing that the seed phenotype of reduced mucilage production was the result of a recessive mutation at a single locus (*mum4-1* to no mucilage ratio of 60:17; Chi square 0.3506, $P>0.5$. Arsovski *et al.*, 2009*a*).

Complementation tests were performed between *men2-1 mum4-1* and the other eight enhancer lines, as well as between *men2-1* and *mum1*, *mum2*, *mum5*, *fly*, and *myb61*, which are genes known to be involved in mucilage production (Performed by Prof. T. Western, McGill University). Complementation was observed in every case, indicating that *men2-1* is a mutant locus not yet characterized (Arsovski *et al.*, 2009*a*). *men2-1 mum4-1* was backcrossed to wild-type Col-2 plants to determine if there was a single mutant phenotype (in the absence of *mum4-1*). Staining of seeds after EDTA pretreatment revealed that the *men2*-dependent reduction in seed mucilage production

phenotype is only visible in the presence of the *mum4-1* mutation. Therefore, mucilage phenotype associated with *men2-1* is dependent on the presence of the *mum4-1* mutation.

3.3.2. Mapping population for positional mapping of *MEN2* locus

To create the *men2-1 mum4-1* mapping population given the absence of a *men2-1* single phenotype, the Col-2 *mum4-1* allele was introgressed into Landsberg (L*er*) through five backcrosses, and the *mum4-1* phenotype was selected in each generation (Prof. T. Western, McGill University). This procedure assured that the chromosomal region at the *MUM4* locus is Col-2 ecotype but with the rest of the genome mostly of L*er* ecotype. However, as only five backcrosses were performed, some stretches on either side of this locus, and in the other chromosomes, are also expected to be Col-2 ecotype (3.125% of the entire genome). PCR amplification of molecular markers along chromosome I revealed that the *mum4-1* L*er* line used to create the *men2-1 mum4-1* mapping population was of Col-2 ecotype around the *mum4-1* locus, and that this Col-2 region extended 2 Mbp above the centromere up to the location of molecular marker F15C21-1 (Table 3 and Figure 14). The six molecular markers on the top arm of chromosome I, north of F15C21-1 (13.6 Mbp) were L*er* homozygous in the *mum4-1* L*er* introgressed line.

The *men-2 mum4-1* mapping population was created by crossing *men2-1 mum4-1* (Col-2 ecotype) to *mum4-1* (L*er* ecotype introgression) to produce the F1 seeds. The F1 seeds were planted and self-pollinated to produce F2 seeds. Planting of the F2 seeds resulted in a mapping population of 1,150 F2 plants that were individually harvested (F3 seeds) and screened for the *mum4-1* enhancer phenotype by ruthenium red staining after EDTA pretreatment (homozygous for the *men2-1* recessive mutation).

Table 3. Molecular markers amplified in the *mum4-1* L*er* introgressed parental DNA to verify the ecotype of chromosome I of the plant line used to create the *men2-1* mapping population. **Col-2**, Columbia-2; **L*er***, Landsberg.

Marker	Location (Mbp)	Resulting Ecotype
ZFPG	8.7	L*er*
ciw12	9.6	L*er*
AC17118	11.5	L*er*
Cer451062	12.1	L*er*
T15K41	12.4	L*er*
F12A41	13.1	L*er*
F15C21-1	13.6	Col-2
F28J9-1	13.7	Col-2
T32E20-1	14.0	Col-2
Centromere 15.5		
ciw1	18.3	Col-2
MUM4	**19.9**	**Col-2**
Nga128	20.6	Col-2
Nga280	20.8	Col-2
Nga111	27.3	L*er*

3.3.3. Screening of double mutant phenotype in F3 seeds

A total of 500 of the 1,150 plants representing the *men2-1 mum4-1* mapping population were screened for the enhanced mutant phenotype by Ruthenium red staining (Figure 6). *Mum4-1* seeds release a very thin layer of mucilage upon EDTA treatment with shaking, in contrast to the double mutant seeds which do not release mucilage at all under the same treatment. Given the difficulty in observing the very small difference in the mucilage released in *mum4-1* versus the double mutant seeds, all samples were treated and stained in triplicate and only those that displayed the double mutant phenotype for all three samples were selected. The double mutant phenotype was observed in 80 of the 500 screened plants representing 16% of the mapping population. This is lower than expected (25%) most likely due to the subtle differences in phenotypes between the *mum4-1* and *men2-1 mum4-1* double mutant and the stringent criteria used for selection.

3.3.4. First-pass mapping of the *MEN2* locus

First pass mapping of the *MEN2* locus was performed by testing 37 double mutant samples with a collection of 16 polymorphic markers that spanned the five *Arabidopsis* chromosomes (Table 4). Linkage to chromosome I and chromosome V was indicated by the less than 50% recombination frequency shown by three markers located on the top arm of chromosome I (RF=0 for two markers) and by three markers located on the bottom arm of chromosome V (RF≈35% for the three markers). In addition, the results of the Chi-square test (Table 4 and Appendix 1), showed that the null hypothesis of independent segregation of these markers from the *MEN2* locus (no linkage between the *MEN2* locus and the molecular markers) had to be rejected.

Linkage to chromosome I was confirmed by amplifying the four polymorphic markers located in chromosome I on DNA using an additional 43 double mutant homozygous samples (80 double mutant homozygous samples in total), again obtaining recombination frequencies of less than 50% in this extended population. The low recombination frequency found in chromosome V in the 10Mbp region located between polymorphic markers PHY (RF=40%) and ciw10 (RF=43%) indicated that the *men2-1* locus could be linked to chromosome V. However, the recombination frequencies of the 13 markers tested on chromosome I strongly support linkage to the top arm of this chromosome in the region between molecular markers Cer464611 (8.0Mbp) and T15K4-1 (12.4Mbp).

Eight additional polymorphic markers on chromosome I (Table 5) were selected to test the 80 double mutant homozygous samples, resulting in the narrowing of the position of the *Men2-1* locus to a 4.4 Mbp region (Figure 14) located between polymorphic markers Cer464611 (8.0Mbp) with a recombination frequency[5] of 1.9% and T15K4-1 (12.4 Mbp) with a recombination frequency of 9.4% (Table 5). The order of the mutation with respect to the two molecular markers Cer464611 and T15K4-1 was determined by a three point cross (Table 6). From the three possible orders tested: (A) Cer464611-*men2-1*-T15K4-1, (B) *men2-1*-Cer464611-T15K4-1, and (C) T15K4-1-Cer46461-*men2-1*, order (A) presented the least number of meiotic recombination events (18 vs. 20 and 33 from orders (B) and (C) respectively), and therefore it was most likely that the *MEN2* locus is located in the region of chromosome I flanked by molecular markers Cer464611 and T15K4-1.

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[5] RF = $[n_{Het} + (2 \times n_{Ler})] / 2n$. Where n=total number of samples; n_{Het}=number of heterozygous samples; and n_{Ler}=number of Landsberg samples.

Table 4. Linkage analysis of *men2-1* to 16 polymorphic molecular markers spanning the five chromosomes of *Arabidopsis* for the first pass-mapping experiments.

Chromosome (size Mbp)	Marker	Location (Mbp)	Recombination Frequency (%)[a]	Chi square[b]
I (29.2)	F21M12	3.2	36.7	59.72, $P<0.005$
	ciw12	9.6	0	240.00, $P<0.005$
	ciw1	18.3	0	240.00, P<0.005
	nga111	27.4	39.6	1.59, $P>0.1$
II (17.5)	ciw2	1.2	50.0	0.13, $P>0.9$
	ciw3	6.4	48.0	0.07, $P>0.95$
	nga168	16.3	48.3	0.07, $P>0.95$
III (23.6)	ciw11	9.8	48.3	0.07, $P>0.95$
	nga6	23.0	60.0	5.73, $P>0.05$
IV (22.1)	ciw5	0.7	55.0	1.13, $P>0.1$
	ciw6	7.9	56.7	2.27, $P>0.1$
	nga1107	18.1	45.0	1.13, $P>0.1$
V (26.2)	CTR1	0.9	56.7	1.20, $P>0.1$
	ciw8	7.5	48.3	2.20, $P>0.1$
	PHYC	14.0	40.0	3.60, $P>0.1$
	ciw9	17.0	31.7	12.87, $P<0.005$
	Cer462550	17.1	35.1	17.49, $P<0.005$
	ciw10	24.0	43.3	1.20, $P>0.1$

[a] Based on 80 samples for chromosome I and 37 samples for chromosomes II to V. Data for markers in chromosomes I to V provided by Natalie Martin (McGill University) except for marker Cer462550 in chromosome V, which was performed myself.
[b] Null hypothesis of 1:1:2 Col:Ler:Het genotype; degrees of freedom=2; $P=0.05$ (See Appendix 1:Table of Chi-Square critical values).

Table 5. Linkage analysis of *men2-1* to 9 molecular markers located on *Arabidopsis* chromosome I. Recombination frequency and Chi-square test results indicate that the *men2-1* locus is located between polymorphic markers Cer464611 and T15K4-1.

Marker	Location chr. I (Mbp)	R.F (%)[a]
nga63	3.6	27.6
Cer452156	5.5	30.0
Cer453258	7.8	32.9
Cer464611	8.0	1.9
Zfpg	8.7	0
nga392	9.8	0
Cer450309	10.0	0
AC17118	11.5	0
T15K4-1	12.4	9.4

[a] Based on 80 samples.

Table 6. Three point test cross to determine the location of the *MEN2* locus with respect to two molecular markers on chromosome I. The genotypes of the homozygous double mutant samples with recombination events are shown. Crossovers are indicated with **X** and double crossovers with **XX**. **Col**, Homozygous Columbia-2; **L*er***, Homozygous Landsberg *erecta*; **Het**, Heterozygous allele; **M 1**, Marker Cer464611; **M 2**, Marker T15K4-1; **Mut**, *men2-1*. F2 lines with zero recombinants are not shown (66 lines).

F2 line #	**M 1**		**Mut**		**M 2**	**Mut**		**M 1**		**M 2**	**Mut**		**M 2**		**M 1**
10	Het	X	Col		Col	Col	X	Het		Col	Col		Col	X	Het
26	Col		Col	XX	L*er*	Col		Col	XX	L*er*	Col	XX	L*er*	XX	Col
29	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
31	Het	X	Col		Col	Col	X	Het	X	Col	Col		Col	X	Het
32	Het	X	Col		Col	Col	X	Het	X	Col	Col		Col	X	Het
41	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
42	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
43	Col		Col	XX	L*er*	Col		Col	XX	L*er*	Col	XX	L*er*	XX	Col
57	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
59	Col		Col	XX	L*er*	Col		Col	XX	L*er*	Col	XX	L*er*	XX	Col
60	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
67	Col		Col	XX	L*er*	Col		Col	XX	L*er*	Col	XX	L*er*	XX	Col
74	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
80	Col		Col	X	Het	Col		Col	X	Het	Col	X	Het	X	Col
	Order A 18 crossovers					**Order B 20 crossovers**					**Order C 33 crossovers**				

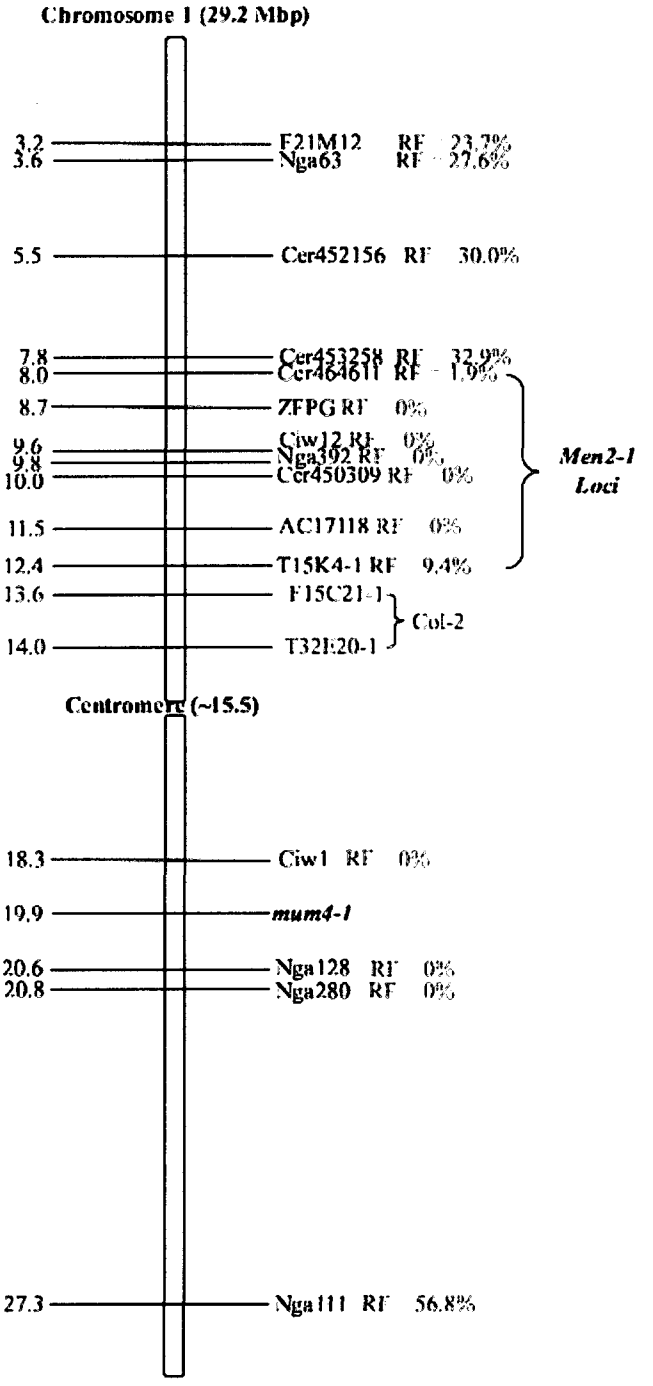

Figure 14. Map of the chromosome I of *Arabidopsis* indicating location of *MEN2* locus relative to polymorphic molecular markers. The *men2-1* locus is located in a 4.4 Mbp region flanked by the Cer464611 (8.0Mbp) and T15K4-1(12.4Mbp) molecular markers.

4. Discussion

The development of the seed coat from the ovule integuments starts immediately after fertilization. One specialization found in various plant families such as the *Brassicaceae*, *Solanaceae*, *Linaceae*, and *Plantaginaceae*, is the production of pectinaceous mucilage in the epidermal cells of the seed coat (myxospermy). This mucilage is released upon imbibition forming a gel capsule that is thought to aid seed hydration and germination (Western *et al.*, 2001). Although various genes involved in the differentiation of mucilage secretory cells have been identified, only the *MUM4* gene is known to be directly involved in mucilage synthesis (Arsovski *et al.*, 2009*a*) and *AtBXL1*, *DCR*, and *AtSBT1.7* have been recently linked to mucilage release (Arsovski *et al.*, 2009*b*; Panikashvili, *et al.*,2009; Rautengarten *et al.*, 2008). The enhancer/suppressor screen and the results described in this thesis allowed the isolation and phenotypic characterization of eight *mum4-1* enhancers (*men* mutants), two of which were identified as new alleles of known mucilage secretory cell genes *MUM2* and *MYB61* (Arsovski *et al.*, 2009*a*). Here, the *MEN2* locus was mapped to a 4.4 Mbp region on chromosome I, located between polymorphic markers Cer464611 (8.0Mbp) and T15K4-1 (12.4 Mbp).

4.1. *MEN* genes affect mucilage production and release

The biosynthesis and secretion of mucilage in the seed coat proceeds through at least three predicted pathways, two of them dependent on APETALA2, while another involves MYB61. A key enzyme in one of these pathways is MUM4, which is a UDP-L-rhamnose synthase required for mucilage biosynthesis. *MUM4* encodes a protein of 667 amino acids containing two domains: (i) N-terminal domain similar to bacterial dTDP-d-

Glucose-4,6-dehydratases, and (ii) C-terminal domain similar to bacterial 4-reductases (Western *et al.*, 2006). The mucilage of the *Arabidopsis* seeds is composed of alternating residues of rhamnose and galacturonic acid that form the unbranched pectin rhamnogalacturonan I (RG I) (Penfield *et al.*, 2001; Western *et al.*, 2004). The secretion of mucilage to the apoplast causes the contraction of the central vacuole forming a cytoplasmic column in the centre of the cell, called the columella. *mum4* mutants have reduced mucilage accumulation in the extracellular space and consequent altered columella appearance (Western *et al.*, 2006).

The *men mum4* double mutants have reduced amounts of mucilage compared to *mum4* as determined by staining with ruthenium red after EDTA treatment (Figures 5 and 6). These results were further analyzed by quantification of the mucilage produced by each mutant using ammonium oxalate extraction and gas chromatography (Arsovski *et al.*, 2009*a*), and it was demonstrated that while *mum4* seeds produce approximately one-tenth of the rhamnose produced by Col-2 and L*er* seeds, *men1-1 mum4-1*, *men4-1 mum4-1*, and *men5-1 mum4-1* make an even smaller amount of mucilage according to their levels of soluble rhamnose. By contrast, the same experiments showed that *men2-1 mum4-1* and *men6-1 mum4-1* produce almost the same amount of rhamnose as *mum4-1* mutants. However, seeds of *men2-1 mum4-1* and *men6-1 mum4-1* release only a very limited amount of mucilage upon ammonium oxalate treatment (Arsovski *et al.*, 2009*a*).

4.2. The *men5 mum4-1* double mutant has reduced dormancy

The seed is a protective structure that encases the developing plant embryo protecting it from maturation through seed dispersal and until seedling establishment.

Harvested wild-type *Arabidopsis* seeds are dormant until about one month after-ripening, which means that the intact and viable seeds can not germinate even under favorable conditions (Debeaujon *et al.*, 2000; Koornneef *et al.*, 2002). Although very little is known about the mechanisms of dormancy and germination, they are controlled by environmental factors such as light and temperature, as well as by the duration of seed storage, the growth of the embryo, and the restraint imposed over the embryo by the tissues surrounding it (Koornneef *et al.*, 2002). In addition, while the presence of ABA has been implicated in the maintenance of seed dormancy, the presence of GA and the role of ethylene in the inhibition of ABA, have been associated with the onset of germination (Bewley, 1997). The crucial role of the seed coat in the dormancy of *Arabidopsis* seeds was demonstrated by the induction of germination in GA-deficient mutants as well as in dormant wild-type embryos upon seed coat removal (Koornneef *et al.*, 2002; Debeaujon *et al.*, 2000). Therefore it was tested whether mutants affected in mucilage deposition affected dormancy.

Two *men* lines (*men3 mum4* and *men5 mum4*) demonstrated reduced dormancy when tested for germination in the absence of light and chilling, showing a 75% increase in germination (2 days of storage after harvest) compared to Col-2 and *mum4* seeds (Figure 8B). Considering that increased permeability to endogenous inhibitors such as ABA or to exogenous stimulants such as water or oxygen can cause reduced dormancy, and given that pigmentation and structural mutants such as *ttg1* and *ap2* have altered germination patterns (Debeaujon *et al.*, 2000), the permeability of the seed coat was assessed by the tetrazolium salts assay (Figure 7). The results demonstrated that *men3*

mum4 seeds had increased seed coat permeability, but the seeds were paler than wild-type seeds. It was concluded that the reduced dormancy of the *men3 mum4* was most likely a consequence of a background *transparent testa* mutation and not an effect of the *men* mutation. Considering that *men5-1 mum4-1* double mutant seeds were not paler than wild-type seeds and that it does not have seed coat increased permeability, as per tetrazolium assay results, it is possible that there is a germination phenotype in this line.

4.3. The *men2-1 mum4-1* double mutant has altered seed coat cellular structure, germination patterns, and root growth

Epidermal wild-type seed coat cells are hexagonal in shape, with a volcano-shaped columella in the centre and thick secondary cell walls (Arsovski *et al.*, 2009*a*). Staining of the seed coat with the polychromatic dye toluidine blue, which stains the pectins in mucilage pink-purple, and the cell walls purple-blue, shows the complete absence or flattening of the columella in mucilage production observed in mutants like *mum4* and *ttg1*. While wild-type seed coat cells release their mucilage upon imbibition in the aqueous dye displaying their volcano-shaped columella (Figure 9), *mum4-1* seed coat cells remain intact, with a small amount of pink-purple mucilage above the dome-shaped columella. The columella of *men2-1 mum4-1* resembled that of *mum4-1*, but the amount of mucilage stored inside the seed coat cells appeared to be significantly reduced, and there was absolutely no mucilage released upon wetting (Figure 9).

The production of hygroscopic mucilage in the *Arabidopsis* seeds is thought to be an adaptation that enhances seed hydration, and therefore it can aid germination and seedling establishment when water is a limiting factor (Penfield *et al.*, 2001). It has been

demonstrated that while the efficiency of germination of wild-type seeds under water reduced conditions (imposed by increasing concentrations of polyethylene glycol) was only slightly affected by PEG concentrations greater than 15%, germination of seed mucilage mutants *ttg1-1, myb61, gl2-1, and atsbt1.7* were almost completely inhibited at 10% PEG concentration (Penfield *et al.*, 2001; Rautengarten, 2008). The germination efficiency of the *men2-1 mum4-1* double mutant resembled that of the *mum4-1* parent line (50% vs. 70% of the Col-2 wild-type seeds) at 15% PEG (Figure 11). However, the germination of the double mutant was much lower than that of the parent line at PEG concentrations above 15%. While in *mum4-1* seed hydration is negatively affected by its reduced quantity of mucilage (Arsovski *et al.*, 2009*a*), the further reduction in seed germination displayed by the double mutant at high PEG concentrations could be due to its defects in mucilage release, as this line produces about the same quantity of mucilage than the *mum4-1* parent line (Arsovski *et al.*, 2009*a*). These results and those from a germination timeline (Figure 11), which revealed that *mum4-1* has delayed germination compared to Col-2 wild-type seeds and that this delay is even greater in the *men-2 mum4-1* double mutant, emphasize that both mucilage quantity and release are important for efficient seed hydration and germination.

The epidermal cells of *Arabidopsis* roots produce mucilage composed of homogalacturonan (HG) and rhamnogalacturonan I (RG-I), in contrast to the seed mucilage which is mainly composed of RG-I (Willats *et al.*, 2001). Root mucilage may have a wide variety of roles such as the adhesion to soil, accumulation of heavy metals, control of pH at the root surface, and access to water and nutrients (Willats *et al.*, 2001).

A root growth assay (Figure 12) demonstrated that the root length of the *mum4-1* parent line lagged behind that of the wild-type Col-2 plants (12% shorter after 14 days) and that this delay was even more pronounced in the *men2-1 mum4-1* (24% shorter after 14 days). Given that seed coat mucilage-related genes such as *TTG1* and *GL2* are involved in the differentiation of root epidermal cells (trichoblasts vs. atrichoblasts) and trichome formation, respectively, a role for the mucilage related gene *MEN2* in root cell differentiation, growth, and development should be considered and studied in more detail in the future.

4.4. *MEN2* maps to chromosome I

Men2-1 was selected from an enhancer/suppressor screen of the *mum4-1* phenotype, and identified as a mutant locus not yet characterized, according to the results of the following genetic tests: (i) backcross to Col-2 indicated that it is genetically unlinked to *mum4-1*, (ii) two backcrosses to *mum4-1* showed that its phenotype was the result of a recessive mutation to a single locus, and (iii) complementation tests eliminated known mucilage mutants and the other eight enhancer lines (Arsovski *et al.*, 2009*a*).

Preliminary mapping of this locus was initiated with 80 homozygous double mutant samples selected from 500 screened F3 plants. *Mum4-1* seeds release a very thin layer of mucilage upon EDTA treatment with shaking, while *men2-1 mum4-1* double mutant seeds do not release mucilage, but the differences between the two phenotypes is subtle. As a consequence, a very conservative approach was taken to select the homozygous double mutant samples from the 500 screened plants resulting on the identification of only 16% samples instead of the 25% expected by Mendelian

segregation. The position of the *MEN2* locus was narrowed down to a 4.4 Mbp region in chromosome I (Figure 13) between polymorphic markers Cer464611 (8.0Mbp) T15K4-1 (12.4 Mbp). In order to further narrow down the location of the *MEN2* locus, the other 750 plants need to be screened to increase the number of recombinants for fine-scale mapping and additional polymorphic molecular markers need to be designed inside the area of interest.

The mapping results also revealed that *men2-1* could be linked to chromosome V between polymorphic markers PHY and ciw10. However, the recombination frequencies of the 13 markers tested on chromosome I strongly support linkage to this chromosome specifically to the top arm of chromosome I in the region between molecular markers Cer464611 (8.0Mbp) and T15K4-1 (12.4Mbp). The low recombination frequency found in chromosome V could be attributed to several reasons, (i) selection of false positives for mapping (double mutant homozygous samples) given that the phenotype is difficult to differentiate from that of *mum4-1*, (ii) a Col-2 locus on chromosome V that enhances the *mum4-1* phenotype and therefore was preferentially selected when screening for *men2 mum4* homozygous double mutants, considering that after five backcrosses 3.125% of the genome is expected to be Col-2 ecotype, and (iii) another mutation in chromosome V, which is different from *men2-1* influences mucilage deposition. Although chromosome I is the most likely location of *MEN2*, future extension of the set of homozygous double mutant samples used for mapping should confirm the location of the locus.

Men2-1 mum4-1 double mutants did not release mucilage after pretreatment with EDTA, but appear to make a similar amount of mucilage to *mum4-1* as assessed by

rhamnose levels in soluble mucilage (Arsovski *et al.*, 2009*a*). The lack of mucilage release by *men2-1 mum4-1* could be the result of (i) a further decrease in mucilage production but not large enough to be detected by observation of secretory cell structure or rhamnose levels (Arsovski *et al.*, 2009*a*), (ii) a defect in the cell wall that physically impedes release, or (iii) a defect in mucilage structure that prevents the completion of the release process (Arsovski *et al.*, 2009*a*).

Although it is very early in the mapping process to identify possible genes for the *MEN2* locus, some candidates include proteins involved in the production of the pectins that form the mucilage such as glycosyl transferases, nucleotide sugar interconversion factors, pectin methyl estereases, and nucleotide sugar transporters, or pectin methyl transferases, which modify the pectins before being transported to the extracellular space via exocytosis, or a gene required for secretion of mucilage to the apoplast, such as small G-proteins, their effectors or activators (Arsovski *et al.*, 2009*a*).

5. Conclusion

The enhancer/suppressor screen of the reduced mucilage mutant *mum4-1* allowed the identification of eight genes involved in the mucilage production and/or release pathways. While two of these genes were identified as new alleles of *MUM2* and *MYB61* genes (Arsovski *et al.*, 2009*a*), the other six are considered new mucilage secretory cell differentiation genes (*MEN1-6*). Characterization of these mutants revealed that apart from the reduction of mucilage production beyond the levels found in *mum4-1*, *men5-1 mum4-1* had reduced dormancy, and *men2-1 mum4-1* had altered seed coat cells structure, germination patterns, and root growth. The *MEN2* locus was mapped to the top arm of chromosome I in a 4.4 Mbp region flanked by molecular markers Cer464611 and T15K4-1.

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Appendix 1: Table of critical values of the Chi-Square distribution[6]

df \ *p*	**0.005**	**0.01**	**0.025**	**0.05**	**0.10**	**0.90**	**0.95**	**0.975**	**0.99**	**0.995**
1	4E-05	0.0002	0.001	0.0039	0.0158	2.71	3.84	5.02	6.63	7.88
2	0.0100	0.0201	0.0506	0.1026	0.2107	4.61	5.99	7.38	9.21	10.60
3	0.0717	0.115	0.216	0.352	0.584	6.25	7.81	9.35	11.34	12.84
4	0.207	0.297	0.484	0.711	1.064	7.78	9.49	11.14	13.28	14.86
5	0.412	0.554	0.831	1.15	1.61	9.24	11.07	12.83	15.09	16.75
6	0.676	0.872	1.24	1.64	2.20	10.64	12.59	14.45	16.81	18.55
7	0.989	1.24	1.69	2.17	2.83	12.02	14.07	16.01	18.48	20.28
8	1.34	1.65	2.18	2.73	3.49	13.36	15.51	17.53	20.09	21.96
9	1.73	2.09	2.70	3.33	4.17	14.68	16.92	19.02	21.67	23.59
10	2.16	2.56	3.25	3.94	4.87	15.99	18.31	20.48	23.21	25.19
11	2.60	3.05	3.82	4.57	5.58	17.28	19.68	21.92	24.73	26.76
12	3.07	3.57	4.40	5.23	6.30	18.55	21.03	23.34	26.22	28.30
13	3.57	4.11	5.01	5.89	7.04	19.81	22.36	24.74	27.69	29.82
14	4.07	4.66	5.63	6.57	7.79	21.06	23.68	26.12	29.14	31.32
15	4.6	5.23	6.26	7.26	8.55	22.31	25	27.49	30.58	32.80
16	5.14	5.81	6.91	7.96	9.31	23.54	26.30	28.85	32.00	34.27
18	6.26	7.01	8.23	9.39	10.86	25.99	28.87	31.53	34.81	37.16
20	7.43	8.26	9.59	10.85	12.44	28.41	31.41	34.17	37.57	40.00
24	9.89	10.86	12.40	13.85	15.66	33.20	36.42	39.36	42.98	45.56
30	13.79	14.95	16.79	18.49	20.60	40.26	43.77	46.98	50.89	53.67
40	20.71	22.16	24.43	26.51	29.05	51.81	55.76	59.34	63.69	66.77
60	35.53	37.48	40.48	43.19	46.46	74.40	79.08	83.30	88.38	91.95
120	83.85	86.92	91.58	95.70	100.62	140.23	146.57	152.21	158.95	163.64

[6] Modified from Griffiths *et al.*, 2005. Introduction to Genetic Analysis. 8th Ed. Freeman & Company, NY.