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MUM ENHANCERS are important for seed coat mucilage production and mucilage secretory cell differentiation in *Arabidopsis thaliana*

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Abstract

Pollination triggers not only embryo development but also the differentiation of the ovule integuments to form a specialized seed coat. The mucilage secretory cells of the *Arabidopsis thaliana* seed coat undergo a complex differentiation process in which cell growth is followed by the synthesis and secretion of pectinaceous mucilage. A number of genes have been identified affecting mucilage secretory cell differentiation, including *MUCILAGE-MODIFIED4 (MUM4). mum4* mutants produce a reduced amount of mucilage and cloning of *MUM4* revealed that it encodes a UDP-L-rhamnose synthase that is developmentally up-regulated to provide rhamnose for mucilage pectin synthesis. To identify additional genes acting in mucilage synthesis and secretion, a screen for enhancers of the *mum4* phenotype was performed. Eight *mum enhancers (men)* have been identified, two of which result from defects in known mucilage secretory cell genes (*MUM2* and *MYB61*). Our results show that, in a *mum4* background, mutations in *MEN1, MEN4,* and *MEN5* lead to further reductions in mucilage compared to *mum4* single mutants, suggesting that they are involved in mucilage synthesis or secretion. Conversely, mutations in *MEN2* and *MEN6* appear to affect mucilage release rather than quantity. With the exception of *men4*, whose single mutant exhibits reduced mucilage, none of these genes have a single mutant phenotype, suggesting that they would not have been identified outside the compromised *mum4* background.

Key words: *Arabidopsis thaliana*, cell wall, germination, *MEN*, mucilage, *MUM4*, pectin, *RHM2*, rhamnogalacturonan I, seed coat.

Introduction

Pollination in flowering plants leads not only to the initiation of embryogenesis and endosperm development, but also to differentiation of the ovule integuments to form the seed coat. The seed coat layers are derived from maternal tissue and can undergo a number of specializations that aid embryo nutrition, seed dispersal, germination, and seed longevity (Esau, 1977; Fahn, 1982; Boesewinkel and Bouman, 1984). One such specialization is the production of a hydrophilic polysaccharide slime, known as mucilage, in the seed coat epidermis. This trait, known as myxospermy, is found in a number of species,

including the Brassicaceae, Solanaceae, Linaceae, and Plantaginaceae. Seed coat mucilage has been suggested to play a number of roles, including the promotion of seed hydration and germination, the prevention of gas exchange, and attachment to soil substrates and animal vectors (Esau, 1977; Fahn, 1982; Grubert, 1981).

The seed coat mucilage secretory cells of the model genetic plant, *Arabidopsis thaliana* (*Arabidopsis*), undergo a complex differentiation process, including separable stages of pectinaceous and cellulosic cell wall production, making them an excellent model in which to study the regulation of

cell wall biosynthesis in a developmental context (Haughn and Chaudhury, 2005; Western, 2006). Epidermal cells of the seed coat first grow by vacuolar expansion. This growth phase is followed by the biosynthesis of a large quantity of pectinaceous mucilage, which is secreted to the apical tangential regions of the cell, forming a doughnut shaped mucilage pocket between the plasma membrane and primary cell wall (Beeckman *et al.*, 2000; Western *et al.*, 2000; Windsor *et al.*, 2000). Mucilage production is accompanied by an increase in the number of Golgi stacks, consistent with the synthesis of pectins in the Golgi apparatus (Western *et al.*, 2000; Young *et al.*, 2008). Concurrent with mucilage synthesis and accumulation, the vacuole contracts towards the bottom of the cell and the cytoplasm is constricted to a volcano shape in the centre of the cell. Pectin biosynthesis and secretion is succeeded by the production of a cellulosic secondary cell wall that fills in the remaining cytoplasm to form a volcano-shaped columella in the centre of the cell (Beeckman *et al.*, 2000; Western *et al.*, 2000; Windsor *et al.*, 2000). Programmed cell death is followed by seed desiccation and the shrinking of the mucilage around the columella to reveal hexagonal-shaped cells with thickened radial cell walls surrounding the columella. Seed wetting leads to the almost instantaneous hydration of the hydrophilic mucilage, followed by rupture of the primary cell wall and the release of mucilage to form a gel capsule surrounding the seed (Western *et al.*, 2000; Windsor *et al.*, 2000). *Arabidopsis* seed mucilage is primarily composed of an unbranched form of the pectin rhamnogalacturonan I (RG I), with smaller quantities of pectic side chains (arabinans, galactans), homogalacturonan, hemicellulose, and cellulose (Penfield *et al.*, 2001; Willats *et al.*, 2001*a*; Western *et al.*, 2004; Macquet *et al.*, 2007*a*).

Mutations in several genes have been identified to have pleiotropic effects on *Arabidopsis* mucilage secretory cell differentiation. These include the developmental regulator, *APETALA2 (AP2)*, the epidermal cell differentiation factors *TRANSPARENT TESTA GLABRA1 (TTG1)*, *TTG2*, *GLABRA2 (GL2)*, *TRANSPARENT TESTA2 (TT2)*, *TT8*, *ENHANCER OF GLABRA3 (EGL3)*, *MYB5*, and the transcription factor *MYB61* (Koornneef, 1981; Bowman and Koornneef, 1993; Jofuku *et al.*, 1994; Rerie *et al.*, 1994; Penfield *et al.*, 2001; Johnson *et al.*, 2002; Zhang *et al.*, 2003; Gonzalez *et al.*, 2009; Li *et al.*, 2009). Loss of function mutants of each of these regulators result in a reduced amount of mucilage and flattened columellae. The most severe are *ap2* mutants, which completely lack mucilage and columellae. Reduced mucilage has also been observed in mutants for the KANADI family transcription factor *ABERRANT TESTA SHAPE (ATS)*, the putative glucosidase II, *RADIAL SWELLING3*, *MICROTUBULE ORGANIZATION1*, the abscisic acid biosynthetic gene *ABSCISIC ACID1*, and the gibberellin biosynthetic gene *GIBBERELLIN-3 OXIDASE4* (Karssen *et al.*, 1983; Léon-Kloosterziel *et al.*, 1994; Burn *et al.*, 2002; Kim *et al.*, 2005; Messmer McAbee *et al.*, 2006; McFarlane *et al.*, 2008). A screen for mucilage-specific genes led to the identification of *MUCILAGE-MODIFIED1–5 (MUM1–5)* (Western *et al.*,

2001). *mum4* mutants have reduced mucilage and flattened columellae, while *mum3* and *mum5* mutants have mucilage of altered composition. By contrast, *mum1*, *mum2*, and the recently identified *subtilase1.7 (Atsbt1-7)* mutants have defects in mucilage release (Western *et al.*, 2001; Rautengarten *et al.*, 2008). Both *MUM2* and *MUM4* have been cloned. *MUM2* encodes a β-galactosidase, which, along with a putative pectin methylesterase target of *SBT1.7*, appears to be required to modify pectin structure in the mucilage and/or primary cell wall to facilitate mucilage release (Dean *et al.*, 2007; Macquet *et al.*, 2007*b*; Rautengarten *et al.*, 2008). Conversely, *MUM4* encodes a UDP-L-rhamnose synthase (also known as RHM2) required for the production of the primary mucilage pectin RG I (Usadel *et al.*, 2004*a*; Western *et al.*, 2004; Oka *et al.*, 2007). Expression of *MUM4* is specifically up-regulated at the time of mucilage synthesis. AP2 and a TTG1–EGL3–TT8–MYB5–TT2 transcription factor complex activate GL2, which, in turn, regulates *MUM4* gene expression (Western *et al.*, 2004; Gonzalez *et al.*, 2009; Li *et al.*, 2009). Alternate pathways of mucilage production appear to be regulated by TTG2, also downstream of AP2 and the TTG1–EGL3–TT8–MYB5–TT2 complex, and MYB61, which may be acting indirectly on mucilage production through a role in sugar allocation (Johnson *et al.*, 2002; Zhang *et al.*, 2003; Newman *et al.*, 2004; Western *et al.*, 2004). Thus, while many regulatory genes, and even some cell wall modification genes, have been identified for roles in mucilage secretory cell differentiation, only one biosynthetic gene has yet been identified.

An enhancer mutant screen of the reduced mucilage mutant *mum4* was performed to identify additional downstream genes in the mucilage production pathway. In addition to isolating new alleles of *mum2* and *myb61*, six new mucilage secretory cell differentiation genes, *MUM ENHANCER1–6 (MEN1–6)* were identified and characterized. *men1–6* mutants demonstrate varying degrees of enhancement of the *mum4* phenotype, with three having significant loss of mucilage, suggesting direct roles in mucilage biosynthesis or secretion.

Materials and methods

Plant lines, mutagenesis, and growth conditions

Lines of *Arabidopsis thaliana* used were *mum4-1* (Col-2 ecotype) (Western *et al.*, 2004) and *ttg1-1* (L*er* ecotype; Arabidopsis Biological Resource Centre, Columbus, OH). Seeds were planted on AT minimal medium plates (Haughn and Somerville, 1986) or directly on soil (Sunshine Mix No. 5, SunGro Horticulture), stratified for 3–4 d at 4 °C and then transferred to growth chambers at 22 °C under continuous light (90–120 μE m^{-2} s^{-1} photosynthetically active radiation), unless otherwise specified. Flower staging for days post anthesis (DPA) was performed as in Western *et al.* (2001).

For mutagenesis, 0.33 g *mum4-1* seeds (~15 000) were treated for 12 h with 0.25% (v/v) ethyl methanesulphonate.

After rinsing, mutagenized seeds were planted in 80 batches of ~150 plants (M1) and bulk-harvested. For screening, seeds from individual M2 plants were isolated and stained with ruthenium red after pretreatment with EDTA as described below. The nine mutants described were isolated from screening approximately 5000 M3 lines from 10 batches (~1500 parental lines). *men2 mum4* and *men3 mum4* were isolated from a common parental batch, as were *men6-2 mum4* and *myb61-6 mum4*; the rest were single isolates from separate parental batches. Prior to study, all double mutant lines were backcrossed at least twice to *mum4-1*.

In accordance with journal policy on the distribution of novel materials, the *men* mutants will be made available by the authors upon request.

Sequencing of mum2-13 and myb61-6

The coding regions of *MUM2* and *MYB61* were PCR amplified from the *mum2-13 mum4-1* and *myb61-6 mum4-1* double mutants, respectively, using the overlapping primer sets presented in Supplementary Table S1 at *JXB* online. Sequencing was performed at the McGill-Genome Quebec Innovation Centre sequencing facility and alignments were performed against the wild-type sequences using DNAMAN (Lynnon Corporation).

Microscopy

For ruthenium red staining, seeds were either placed directly in 0.01% (w/v) ruthenium red without shaking, shaken directly in ruthenium red for 90 min, or pre-hydrated with shaking in 0.05 M EDTA for 90 min followed by ruthenium red stain, as indicated. For the seeds stained with shaking, samples were rinsed in dH_2O prior to visualization. Seeds were observed on a Leica MZ-16F stereomicroscope and imaged with a Micropublisher 3.3 camera (Qimaging) operated via Openlab 5 (Perkin Elmer).

Developing seeds were prepared for brightfield microscopy, sectioned, and stained with toluidine blue O as described in Western *et al.* (2001). Samples were examined using a Leica DM 6000B compound microscope and images captured with a Qimaging Retiga CCD camera operated through Openlab. Scanning electron microscopy of dry seeds was performed as described in Western *et al.* (2001).

To test for mucilage release after extraction with ammonium oxalate, intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30 °C. Seeds were then either shaken in 0.01% (w/v) ruthenium red for 90 min, mounted on a depression slide and observed with a Leica DM 6000B compound microscope, or air-dried before mounting on stubs and observed by scanning electron microscopy.

Seed coat permeability was determined using tetrazolium salts as described by Debeaujon *et al.* (2000). In short, seeds were incubated in 1% (w/v) tetrazolium red for 2 d in the dark at 37 °C and the percentage of red seeds calculated as a measure of permeability.

Chemical analysis

To quantify neutral sugars in crude mucilage extracts, 50 mg of intact seeds were incubated in 0.2% (w/v) ammonium oxalate with vigorous shaking for 2 h at 30 °C. 1 μmol of myo-inositol was added to the supernatant and samples were precipitated with 5 vols ethanol, directly hydrolysed with 2 M trifluoroacetic acid, and derivatized to alditol acetates. Derivatization to alditol acetates and gas chromatography were performed as in Gibeault and Carpita (1991), but with an HP-23 glass capillary column (30 m×0.25 mm i.d.; Agilent Technologies). Seeds used for chemical analyses were collected from mutant and control plants cultivated together.

Germination time-course

Two 70 mm diameter Whatman No. 1 filter papers (Whatman) were placed in the lid of a 100 mm plastic Petri dish. To these were added 2 ml of water and 40–80 seeds of each mutant line. The plates were sealed with parafilm and stratified in the dark at 4 °C for 72 h. Seeds were incubated at 22 °C under 16/8 h light/dark, following which they were counted every day for 6 d and germination was scored by the presence of open green cotyledons. The plates were counted again after 9 d to confirm that all lines had reached approximately 100% germination. Seeds used for germination analyses were collected from mutant and control plants cultivated together and stored as distinct seed sets for 6 or 8 months, depending on the set. Seeds were stored in microfuge tubes with holes in the lids at room temperature under ambient humidity and light conditions. Each time-course was done in triplicate and the whole was performed twice using two independent sets of seeds with similar results.

Results

Identification of mum enhancers

When *Arabidopsis* seeds are hydrated, the seed coat mucilage swells rapidly, leading to the bursting of the primary cell wall and the release of mucilage to surround the seed in a gel-like capsule (Fig. 1A) (Western *et al.*, 2000; Windsor *et al.*, 2000). *mum4* mutants make a significantly reduced amount of mucilage (Western *et al.*, 2001, 2004) that remains within the cells when seeds are hydrated. Addition of a heavy metal chelator such as EDTA or EGTA, however, leads to the release of *mum4* mucilage. This is probably due to the withdrawl of Ca^{2+} ions from the cell wall pectins, leading to weakening of the cell wall and/or permitting increased swelling of the mucilage present. *mum4* seeds shaken in EDTA prior to ruthenium red staining reveal a thin layer of stained mucilage around the seeds, consistent with their reduced mucilage production (Fig. 1A) (Western *et al.*, 2004). By contrast, mutants for *TTG1*, which acts upstream of both the *GL2* and *TTG2* pathways of mucilage production, make very little mucilage and show no obvious mucilage release when EDTA-treated
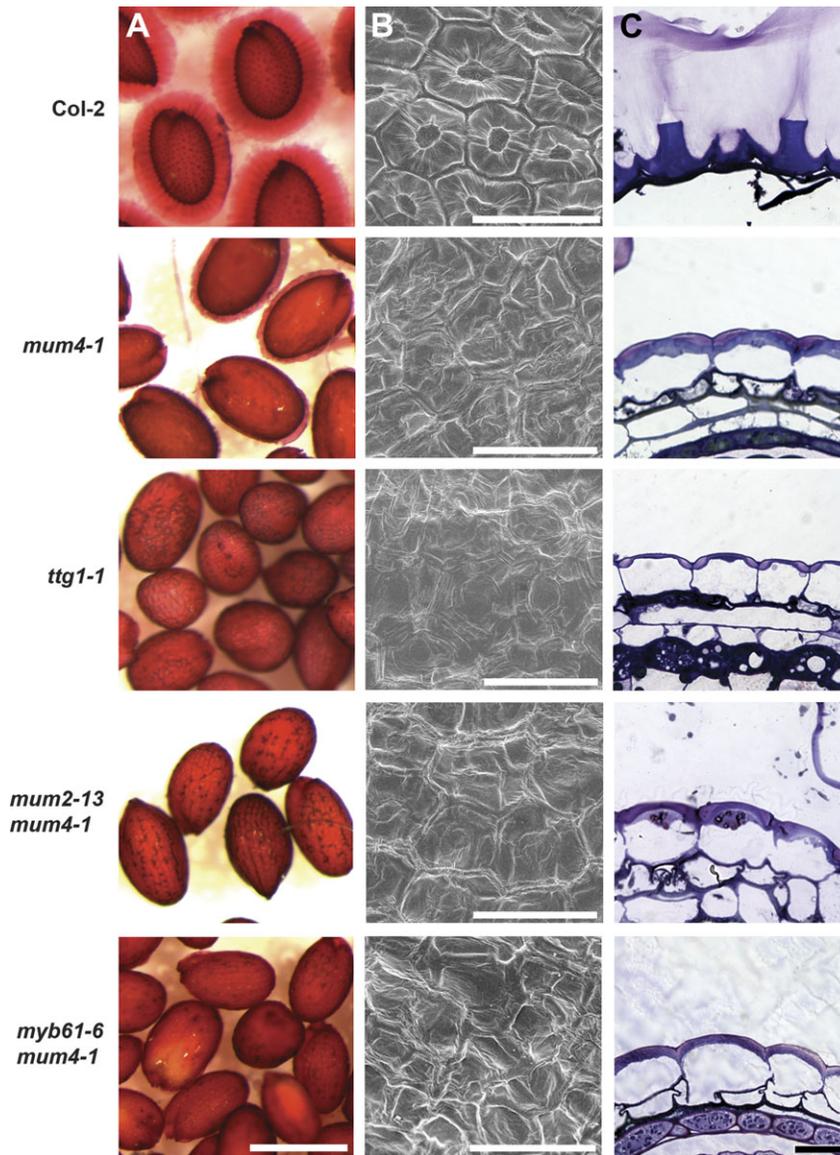
Fig. 1. Mucilage release and seed coat structure of controls and *mum4* enhancer lines resulting from mutations in *MUM2* and *MYB61*. (A) Seeds pretreated in EDTA with shaking and stained with ruthenium red. Note the thick layer of mucilage surrounding wild-type Col-2 seeds and the very thin layer around *mum4-1* seeds. (B) Scanning electron microscopy of dry seeds. Note the prominent hexagonal cell walls and central volcano-shaped columella in the centre of Col-2 cells. (C) Toluidine blue-stained resin sections of 13 DPA seeds. Wild-type mucilage cells have burst open in the aqueous fixative, leaving tall, blue-stained, volcano-shaped columellae with some cell wall material attached to the centre of the columella. *mum4-1* cells do not burst, and contain small pockets of purple-stained mucilage in the upper apical corners, subtended by a blue dome of secondary cell wall. *ttg1-1* cells, similar to *mum4-1*, do not burst, however, the mucilage pockets are smaller and the secondary cell wall is thinner in appearance. Scale bars: (A) 500 μm, (B) 50 μm, (C) 10 μm.

(Fig. 1A). The moderate level of mucilage release found for *mum4* mutants, as well as the ability to differentiate *mum4* mutants from mutants with further reduced mucilage, allowed us to perform a genetic screen for phenotypic enhancers of *mum4*. *mum4-1* seeds were mutagenized with ethyl methanesulphonate and seeds from individual M2 plants (M3 lines) were collected and screened for reduced levels of mucilage compared with *mum4-1* as observed with ruthenium red staining after EDTA pretreatment. Over 5000 M3 lines derived from ten parental M1 batches (1000–1500 M1 parents) were screened, leading to the identifica-tion of nine *mum4-1* enhancers [named *mum enhancers (men)*] that have no visible mucilage release when treated with EDTA (Figs 1A, 2A).

Backcrosses to *mum4-1* plants revealed, in each case, that the seed phenotype was the result of a recessive mutation to a single locus (Table 1). Complementation tests were also performed between the nine *men mum4-1* lines. Only one pair of mutants did not complement each other, revealing the identification of eight mutant loci. To determine if any of the *men mum4-1* lines represented known mucilage mutant loci beyond *MUM4*, several assays were performed.
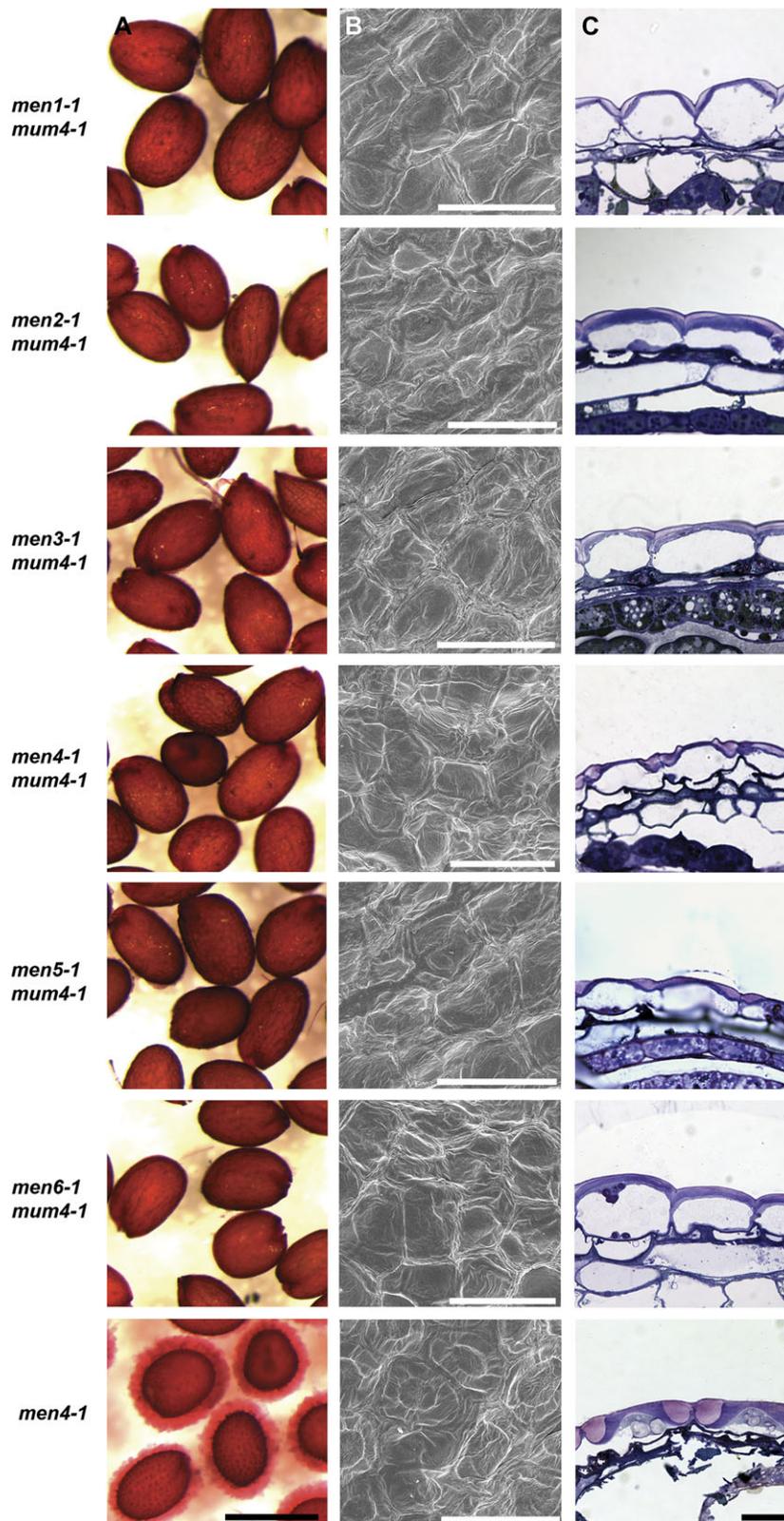
Fig. 2. Mucilage release and seed coat structure of *mum4* enhancer lines. (A) Seeds pretreated in EDTA with shaking and stained with ruthenium red. (B) Scanning electron microscopy of dry seeds. (C) Toluidine blue-stained resin sections of 13 DPA seeds. Scale bars: (A) 500 μm, (B) 50 μm, (C) 10 μm.

First, no changes in seed shape were observed as in *ap2* and *ats* mutants (Léon-Kloosterziel *et al.*, 1994). Second, their identity as new alleles of *GL2*, *TTG1*, and *TTG2* was tested through an examination of seed coat colour and trichome presence (Koornneef, 1981; Rerie *et al.*, 1994; Johnson *et al.*, 2002). All *men mum4-1* lines had trichomes, and none

Table 1. Segregation analysis and Chi-square test results for *mum4* enhancer lines backcrossed to *mum4-1*

Mutant line	*mum4*:no mucilage[a]	Chi square[b]
men1-1 mum4-1	68:17	1.1333, *P* >0.1
men2-1 mum4-1	60:17	0.3506, *P* >0.5
men3-1 mum4-1	85:27	0.0476, *P* >0.5
men4-1 mum4-1	57:16	0.3699, *P* >0.5
men5-1 mum4-1	60:21	0.0370, *P* >0.5
men6-1 mum4-1	80:19	1.7811, *P* >0.1

[a] F$_3$ seed phenotype (seed of F$_2$ plants), shaken for 90 min in EDTA followed by staining in 0.01% ruthenium red.
[b] Null hypothesis of 3:1 *mum4*:no mucilage; degrees of freedom=1; cutoff at *P*=0.05.

Fig. 3. Mucilage release of *men4-1* versus wild-type seeds in ruthenium red with and without shaking. (A, B) Col-2 wild-type seeds. (A) Seeds put directly into ruthenium red without shaking are surrounded by an outer, diffuse layer of mucilage and an inner, dense layer of mucilage, while those shaken in dye lose the soluble outer layer (B). (C, D) *men4-1* seeds lack mucilage release when treated directly with ruthenium red, with or without agitation. Scale bar: 500 µm.

had obviously yellow seeds, suggesting that they are different genes. Third, to eliminate *tt* mutants that were not obviously yellow, tetrazolium red staining was used to detect the increased permeability to solutes found for most *tt* mutants, including *ttg1*, *tt2*, and *tt8* that are known to affect mucilage production (Debeaujon *et al.*, 2000). One line that appeared to be wild-type seed colour (named *men3-1 mum4-1*) showed significant staining with tetrazolium red (data not shown). However, closer examination of *men3-1 mum4-1* seeds revealed that they were slightly paler than wild-type seeds. The seed colour phenotype was found to segregate away from the mucilage phenotype, suggesting a background mutation in a *tt* or related gene that is unlikely to significantly affect mucilage release (data not shown). Fourth, complementation tests were performed with *myb61*, another reduced mucilage mutant (Penfield *et al.*, 2001). One line was found not to complement *myb61-1* and sequencing confirmed that it is a new allele of *MYB61*, which has been named *myb61-6* (G to A transition leading to the conversion of Trp at position 252 to a stop codon). Fifth, the remaining *men mum4-1* lines were back-crossed to wild-type Columbia-2 (Col-2) plants to determine if there was a mutant phenotype in the absence of *mum4-1*, as all mucilage mutants identified to date other than *egl3*, *tt2*, and *tt8* have detectable mucilage release phenotypes in a wild-type background. Only two mutant lines had detect-able single mutant phenotypes where no mucilage was released when shaken in ruthenium red stain without EDTA pretreatment (Fig. 3; data not shown). Since they were already shown not to be allelic to known reduced mucilage mutants, both of these lines were backcrossed to the mucilage release mutants *mum1-1*, *mum2-1*, and *patchy* (Western *et al.*, 2001; AA Arsovski, TL Western, unpub-lished results). One line was found to complement all three mutants, while the other only complemented *mum1-1* and *patchy*. While sequencing did not reveal an obvious mutation in the coding sequence of *MUM2* for the non-complementing line, regulatory region or intron-related mutations cannot be ruled out. This line did, however, map to the *MUM2* region, which, in combination with multiple complementation tests using both the double and isolated single mutants, and similar results between the single

mutant and *mum2* in all further assays (data not shown), suggest that this is, indeed, a new allele of *MUM2* that has been named *mum2-13*. The other line represents a different gene that has been named *MEN4*. EDTA pretreatment of *men4* single mutants revealed the release of a reduced amount of mucilage compared to wild-type seeds (compare Fig. 1A with Fig. 2A).

Phenotypic characterization of men seed coats

Wild-type epidermal seed coat cells, when observed by scanning electron microscopy (SEM), are shown to be roughly hexagonal in shape with thickened radial cell walls and a narrow, volcano-shaped columella in the centre of each cell (Fig. 1B) (Beeckman *et al.*, 2000; Western *et al.*, 2000; Windsor *et al.*, 2000). A characteristic of reduced mucilage synthesis mutants, such as *mum4* and *ttg1*, is a flattened columella that is subtly visible or missing when observed with SEM (Fig. 1B) (Koornneef, 1981; Western *et al.*, 2001, 2004). To determine if an exacerbated phenotype was apparent in the *mum4* enhancer lines, dry seeds of each double mutant plus the *men4-1* single mutant were subjected to SEM. In each case for the double mutants, no significant enhancement of the *mum4* pheno-type was obvious (Figs 1B, 2B). *men4-1* epidermal seed coat cells, however, have apparent columellae, but they are much broader and less prominent than those of wild-type seeds (Fig. 2B).

A more detailed view of the presence of mucilage and the shape of the columella can be gained through the use of sectioning and staining of seed coats with toluidine blue. Toluidine blue is a polychromatic dye that stains various cell components different colours. For example, acidic

polysaccharides such as pectins stain pink-purple, and cell walls purple-blue (O'Brien *et al.*, 1964). The timing of mucilage and columella production has been extensively studied, demonstrating that both are generally complete by 13 d post anthesis (DPA) (Western *et al.*, 2000). Wild-type mucilage secretory cells at 13 DPA tend to release their mucilage upon wetting in aqueous fixative, leaving only the tall, volcano-shaped columellae and empty spaces where the mucilage accumulated prior to hydration (Fig. 1C) (Western *et al.*, 2000). *mum4-1* mucilage secretory cells, by contrast, remain intact, with a small amount of pink-purple staining mucilage found in the apical cell corners above a dome-shaped secondary cell wall (columella), all found above a large vacuole (Fig. 1C) (Western *et al.*, 2004). *ttg1-1* mucilage cells have a more severe phenotype than that of *mum4-1*, with less mucilage and a very thin secondary cell wall (Fig. 1C) (Penfield *et al.*, 2001; Western *et al.*, 2001). All of the *mum4* enhancer lines resemble *mum4-1* or *ttg1-1* to varying degrees (Figs 1C, 2C). *mum2-13 mum4-1* and, *men2-1 mum4-1* resemble *mum4-1*, while *men1-1 mum4-1*, *men4-1 mum4-1*, and *men5-1 mum4-1* are similar to *ttg1-1*. *myb61-6 mum4-1*, *men3-1 mum4-1* (with background *tt* mutation), and *men6-1 mum4-1* all appear to have an intermediate phenotype.

Quantitative analysis of men mucilage

To quantify the amount of mucilage produced by the different *mum4* enhancers, ammonium oxalate soluble mucilage was extracted from intact seed samples, hydrolysed, derivatized to alditol acetates, and subjected to gas chromatography. Alditol acetate derivatization allows for the production of a complete neutral sugar profile of cell wall material (fucose, rhamnose, arabinose, xylose, mannose, galactose, and glucose) (Chaplin, 1986). However, the soluble cell wall material from these mutants could be derived from the cell wall and/or the mucilage. *Arabidopsis* mucilage is primarily comprised of unbranched RG I, a pectin whose backbone is composed of alternating residues of rhamnose and galacturonic acid (Penfield *et al.*, 2001; Western *et al.*, 2004). Comparison between ground wild-type seeds and those of an *ap2* mutant, which makes little or no mucilage, suggests that approximately 80% of the rhamnose of *Arabidopsis* seeds is found in the mucilage (Western *et al.*, 2001). Thus, to focus more specifically on changes to mucilage levels, only rhamnose was considered for comparison between mutants (Fig. 4). Wild-type Col-2 and L*er* extracted mucilage contained 339.6±9.7 and 332.9±6.3 μg of rhamnose per 50 mg seed, respectively (SE, n=3). By contrast, *mum4-1* seeds have approximately one-tenth that amount (38.90±3.5 μg per 50 mg seed) and *ttg1-1* seeds have approximately half as much rhamnose as *mum4-1* mutants (17.5±2.6 μg per 50 mg seed) (Fig. 4).

men2-1 mum4-1 and *men6-1 mum4-1* were found to have approximately the same amount of rhamnose as *mum4-1* single mutants (Fig. 4). While this correlates with the
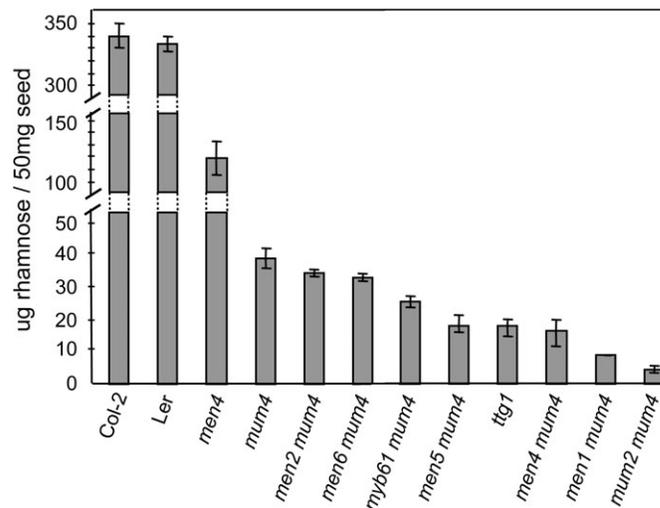
Fig. 4. Rhamnose levels of soluble mucilage extracted from *mum4* enhancers and controls. Ammonium oxalate extracts of concurrently-grown seed batches were hydrolysed with trifluoroacetic acid and derivatized to alditol acetates, followed by gas chromatography. Extractions were done in triplicate, error bars=SE.

similar appearance of mucilage and columella between these two double mutants and *mum4-1* observed with toluidine blue-stained sections (Figs 1C, 2C), it is intriguing that the double mutant seeds pretreated with EDTA do not appear to release mucilage (Fig. 2A). In order to determine if this resulted from the differential release of mucilage with ammonium oxalate at 30 °C versus EDTA at room temperature, ammonium oxalate-treated seeds were stained with ruthenium red. Similar to the EDTA results, *mum4-1* seeds showed substantial mucilage release with ammonium oxalate treatment, while *men2-1 mum4-1* and *men6-1 mum4-1* seeds had only slight puffing of the cell walls (Fig. 5). No mucilage release or cell wall puffing was seen for *mum2-1* mutants that cannot release mucilage due to mucilage hydration defects (Fig. 5A; Dean *et al.*, 2007; Macquet *et al.*, 2007b).

myb61-6 mum4-1 has an approximately 30% drop compared with *mum4-1*, confirming the enhanced phenotype seen with toluidine blue-stained sections. This was also the case for *men4-1 mum4-1* and *men5-1 mum4-1*, which are similar to *ttg1-1*, both in terms of a 50% reduction in rhamnose compared with *mum4-1* and in their phenotype in their cross-sections. *men1-1 mum4-1*, which also appears similar to *ttg1-1* in toluidine blue-stained sections, has a further 40–50% drop in soluble mucilage compared to *ttg1-1*. The lowest amount of rhamnose observed was for *mum2-13 mum4-1*, reflecting the lack of mucilage release observed in *mum2* single mutants (Fig. 5; Dean *et al.*, 2007; Macquet *et al.*, 2007b). The rhamnose level was also determined for the *men4-1* single mutant and found to be approximately 35% of wild-type rhamnose levels in its extracted mucilage, consistent with the ruthenium red and toluidine blue section results.

Germination of men *lines*

Altered seed germination responses have been correlated with changes in seed coat structure, including mucilage quantity and release (Léon-Kloosterziel *et al.*, 1994; Debeaujon *et al.*, 2000; Penfield *et al.*, 2001; Rautengarten *et al.*, 2008). To determine the effect of reduced mucilage
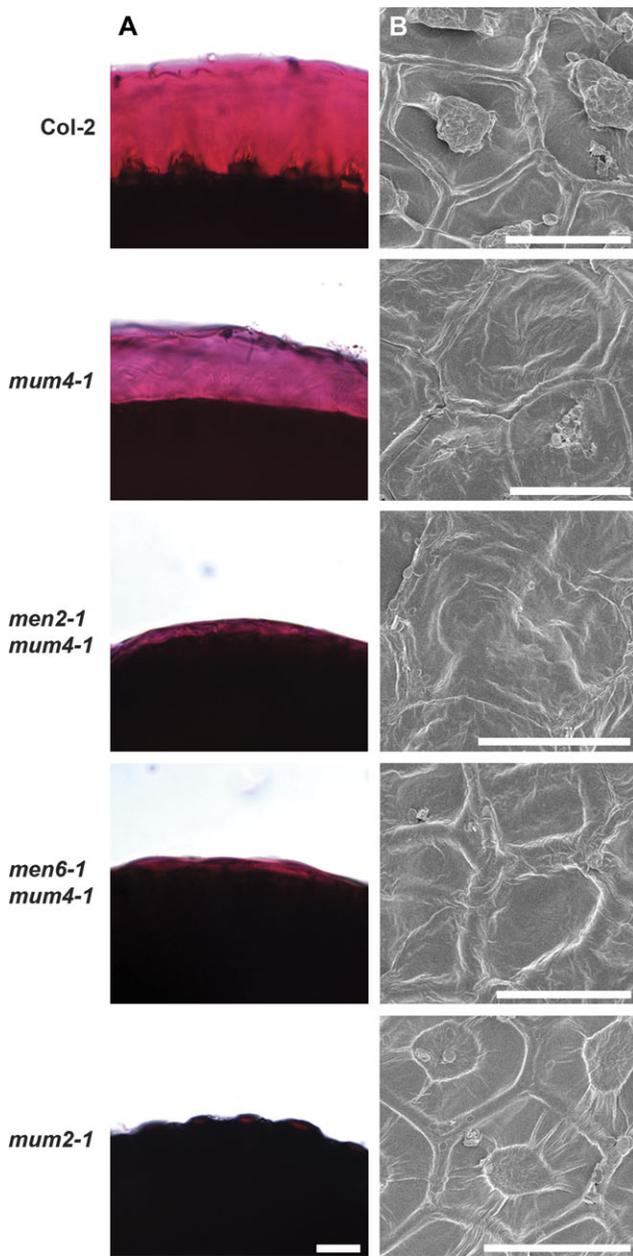
Fig. 5. Seed coat phenotype of *men2 mum4*, *men6 mum4*, and control seeds following extraction with ammonium oxalate. (A) Seeds extracted with ammonium oxalate with shaking at 30 °C, then stained with ruthenium red. Note substantial mucilage release for Col-2 seeds. *mum4-1* seeds release less mucilage, with the outer wall appearing to remain largely intact. (B) Scanning electron microscopy of air-dried ammonium oxalate extracted seeds. Only Col-2 seeds show obvious rupture of outer primary cell wall. Scale bars: (A) 10 µm, (B) 25 µm.

levels in the *mum4* single mutant, as well as in the *men* lines, a time-course of germination was performed (Fig. 6). *mum4-1* germination lagged significantly behind that of wild-type seeds at 3 d (23% for *mum4-1* versus 67% for Col-2), but reached approximately wild-type levels by 4 d (Fig. 6). Similar, or even more severe delays, at 3 d were detected for the set of *men mum4-1* lines plus *myb61-6 mum4-1* and *mum2-1 mum4-1* double mutants, all of which continued to stay significantly below wild-type germination levels at day four, with the exception of *men4 mum4* (Fig. 6). All lines reached 95–100% germination within 9 d (data not shown). Together, these results suggest not only that the reduction of mucilage in *mum4-1* has an effect on the speed of germination, but also that this delay may be enhanced by further defects in both mucilage release and quantity. Conversely, *men4-1*, which has approximately three times more mucilage than *mum4-1* (Fig. 4), shows no delay in germination. Interestingly, both *men4-1 mum4-1* and *men5-1 mum4-1* seeds demonstrate precocious germination relative to *mum4-1* at 2 d. This is reflected in *men4-1*, which germinates faster than the wild type at 2 d. This 'early germination' may explain the similar if not faster germination exhibited by *men4-1* versus wild type, and *men4-1 mum4-1* and *men5-1 mum4-1* versus *mum4-1* exhibited at 3 d. It is possible that, in these lines, there is a germination phenotype beyond that resulting from reduced mucilage levels.

The *mum4* enhancer lines were also tested for gross changes in whole plant developmental phenotypes by following the Arabidopsis Gantlet Project protocol (http://thale.biol.wwu.edu/). In short, seeds were plated with wild-type and *mum4-1* control seeds side-by-side, grown vertically on plates for 14 d, and then transplanted to soil, with regular observation across all stages (daily while on plates
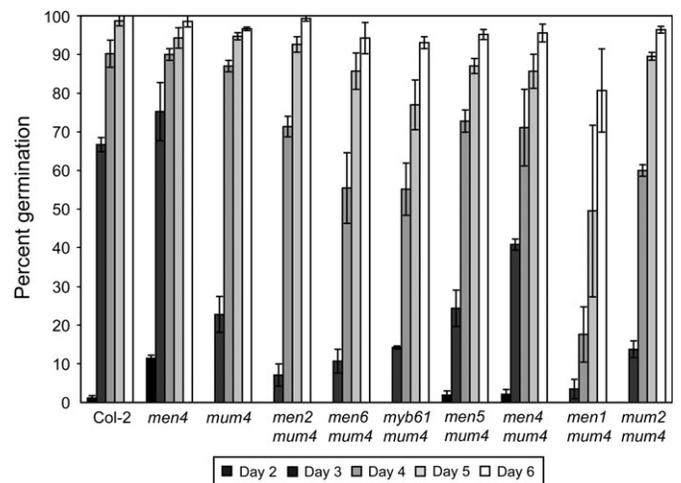
Fig. 6. Time-course of germination of *mum4* enhancers and controls. Genotypes are organized in the same order as Fig. 4 for comparison. Seeds were stratified for 3 d at 4 °C, followed by germination at 22 °C under 16/8 h light/dark. 40–80 seed of each genotype were sowed on filter paper with water, error bars=SE. Similar results were obtained in a separate experiment using seed from an independent set of plants.

and weekly once in soil). No gross developmental phenotypes were observed for any of the lines.

Discussion

The mucilage secretory cells of the *Arabidopsis* seed coat are a useful model for the identification and study of genes involved in cell wall production and metabolism. In particular, they have started to allow the dissection of genes involved in the regulation of pectin synthesis and in pectin modification (Penfield *et al.*, 2001; Johnson *et al.*, 2002; Zhang *et al.*, 2003; Western *et al.*, 2004; Dean *et al.*, 2007; Macquet *et al.*, 2007b; Rautengarten *et al.*, 2008; Gonzalez *et al.*, 2009; Li *et al.*, 2009). However, to date, only one gene involved directly in mucilage synthesis has been identified, and no genes have been directly implicated in polar secretion of mucilage (Usadel *et al.*, 2004a; Western *et al.*, 2004). Here, six new genes involved in mucilage production have been identified as enhancers of the *mum4* reduced mucilage mutant. Three of these genes appear to have further reductions in mucilage production compared with *mum4*, making them promising candidates for roles in mucilage synthesis and/or secretion.

MEN *genes affect mucilage production*

Mutations in six new genes (*MEN1–6*) affecting mucilage secretory cell differentiation were identified, along with new alleles of two known genes: *MUM2* and *MYB61*. The finding of new alleles of these two genes validated the screen in its ability to find genes acting in parallel with *MUM4* for mucilage production (*MYB61*) (Penfield *et al.*, 2001), as well as genes acting in pectic mucilage modifications that are required for mucilage swelling and release (*MUM2*) (Dean *et al.*, 2007; Macquet *et al.*, 2007b). Further, this screen revealed the utility of such a sensitized screen, as only one of the new genes identified (*men4*) had an obvious single mutant phenotype. Characterization of the *mum4* enhancers revealed two phenotypic categories: reduced mucilage production and lack of mucilage release.

Three of the *men mum4* double mutants identified in this screen (*men1 mum4*, *men4 mum4*, and *men5 mum4*) appear to make reduced amounts of mucilage compared to *mum4* as determined by both their cell structure and their soluble rhamnose levels (Figs 1, 2, 4). *men1 mum4* double mutants have the most significant reduction of mucilage, while *men4 mum4* and *men5 mum4* have slightly more mucilage. The interpretation of these mutants as being affected in mucilage production is supported by their shared phenotypes with *myb61 mum4* and *ttg1*. TTG1 regulates both the GL2 and TTG2 pathways of mucilage production (Johnson *et al.*, 2002; Zhang *et al.*, 2003; Western *et al.*, 2004), thus, *ttg1*-like mutants may be expected to be affected in both pathways downstream of TTG1. *myb61* mutants have reduced mucilage resulting from disruption of a TTG1-independent pathway (Penfield *et al.*, 2001; Western *et al.*, 2004), so the *myb61 mum4* double mutant serves as a control for the disruption of the two independent pathways of

mucilage production, as *MUM4* acts downstream of TTG1. In addition, the single mutant for *men4* has significantly reduced mucilage compared to wild-type seeds (Figs 1, 3, 4), confirming a role in mucilage production for one member of this class of *mum4* enhancers.

As mucilage primarily comprises the pectin RG I, genes involved in its manufacture and transport, or the regulation of these processes, would be the most obvious candidates for the *men* genes affected in mucilage quantity. While our phenotypic and complementation analyses have ruled out most mucilage regulatory mutants, it is possible that one of the *men* genes could encode a new allele of *EGL3* or a weak allele of *MYB5* without an obvious single mutant phenotype (Zhang *et al.*, 2003; Gonzalez *et al.*, 2009; Li *et al.*, 2009). Our preliminary mapping data for *MEN1*, *MEN4*, and *MEN5*, however, suggest that this is not the case (AA Arsovski, M Wang, N Martin, J Schafhauser, TL Western, unpublished results). *MUM4* is a member of a small gene family that encodes three full-length, trifunctional UDP-L-rhamnose synthase proteins (RHM1, MUM4/RHM2, RHM3), and a protein that catalyses only the latter part of the conversion of UDP-D-glucose to UDP-L-rhamnose (UER) (Reiter and Vanzin, 2001; Usadel *et al.*, 2004a; Watt *et al.*, 2004; Western *et al.*, 2004; Oka *et al.*, 2007). All members are expressed throughout the plant, allowing for genetic redundancy in rhamnose synthesis and the production of some mucilage in *mum4* seeds. A mutation in *RHM1*, *RHM3* or *UER* could result in a further reduction in mucilage production. By contrast, one of the *men* genes could encode a member of the *UDP-D-GLUCOURONATE 4-EPIMERASE (GAE)* family, which are required for the synthesis of UDP-D-galacturonic acid, the other sugar comprising the backbone of RG I, as well as the backbone of homogalacturonan (Willats *et al.*, 2001b; Usadel *et al.*, 2004b). Pectins are synthesized in the Golgi apparatus through the activity of glycosyltransferases (GT) that use nucleotide sugars as substrates. Members of GT family 8 have been implicated in pectin synthesis through both mutant studies and enzyme isolation (Scheller *et al.*, 1999; Sterling *et al.*, 2001, 2006; Willats *et al.*, 2001b; Bouton *et al.*, 2002; Lao *et al.*, 2003; Shao *et al.*, 2004; Mohnen, 2008), so it is possible that one of the *men* genes could encode a GT8 protein required either for RG I backbone synthesis or for the synthesis of one of the other pectins found in *Arabidopsis* mucilage. Indeed, a gene encoding a GT8 family protein that has a mild mucilage production phenotype has been identified as being up-regulated in seed coats at the time of mucilage synthesis, similar to *MUM4* (J Schafhauser, A Abdeen, TL Western, unpublished results). Alternately, a *men* mutant could be affected in a gene required for secretion of mucilage from the Golgi apparatus to the apoplast. These could include vesicle trafficking factors such as small G-proteins, their effectors or activators, or the multi-subunit exocytosis complex known as the exocyst (Cole and Fowler, 2006; Hála *et al.*, 2008; Nielsen *et al.*, 2008; Rojo and Denecke, 2008; Yalovsky *et al.*, 2008).

Both *men2 mum4* and *men6 mum4* double mutants did not release mucilage after pretreatment with EDTA, but

appear to make a similar amount of mucilage to *mum4* as assessed by both cellular appearance and rhamnose levels in soluble mucilage (Figs 2, 4). Specific mucilage release in ammonium oxalate used for mucilage extraction was tested by staining seeds after shaking in ammonium oxalate and revealed only slight puffing of the cell wall for *men2 mum4* and *men6 mum4* (Fig. 5). It is possible that some primary cell wall breakage occurred in parallel with this puffing, allowing mucilage extraction through fissures in the wall. Conversely, chelator treatment may have extracted pectins from the primary cell wall, allowing further extraction of highly soluble mucilage through the weakened wall. Extraction of only primary cell wall pectins in the case of *men2 mum4* and *men6 mum4* is unlikely since both have higher levels of soluble rhamnose than other non-releasing mutants that have little or no mucilage (e.g. *ttg1*) or that have been shown to affect mucilage hydration (*mum2 mum4*) (Figs 2, 4, 5) (Western *et al.*, 2000; Dean *et al.*, 2007; Macquet *et al.*, 2007b).

The *mum4*-like phenotypes make it difficult to assess the cause of the lack of mucilage release in *men2 mum4* and *men6 mum4*. One possibility is that there is a decrease in mucilage production that is sufficient to prevent release, but not large enough to be detected though observation of secretory cell structure or rhamnose levels. Alternately, there could be a defect in the cell wall or mucilage structure that prevents mucilage release. While the extremely low soluble rhamnose levels of *mum2 mum4* double mutants would seem to argue against this latter hypothesis, *mum2* mutants are characterized by a lack of mucilage swelling that probably results from reduced hydration capacity, explaining the *mum2 mum4* double mutant phenotype (Fig. 5; Dean *et al.*, 2007; Macquet *et al.*, 2007b). It is possible for the *men2 mum4* and *men6 mum4* double mutants that the mucilage and/or outer cell wall structural changes are such that mucilage solubility is less significantly affected than for mutations in *MUM2*. Candidate genes for *men2 mum4* and *men6 men2*, therefore, may be new cell wall modification factors. While preliminary mapping places *MEN2* away from known mucilage release genes (MM Villota, TL Western, O Rowland, R Subramaniam, unpublished data), it cannot be ruled out that the two *men6* alleles are weak alleles of *MUM1*, *MUM2* or *SBT1.7* that lack obvious phenotypes in the absence of *mum4*.

Effect of mucilage changes on seed germination

A time-course of germination revealed that *mum4-1* has delayed germination compared with wild-type seeds (Fig. 6). This is consistent with previous studies that demonstrated that reduced mucilage mutant seeds (*myb61-1*, *ttg1-1*, *gl2-1*) had a decreased ability to germinate under conditions of limited water supply (as exerted by increasing concentrations of polyethylene glycol) compared to wild-type seeds (Penfield *et al.*, 2001). A similar reduction in germination in the presence of polyethylene glycol was seen for *Atsbt1.7* mutants, which are defective in mucilage release (Rautengarten *et al.*, 2008). Due to its significant hydrophi-

licity, *Arabidopsis* mucilage has been proposed to promote seed hydration, and thus germination, through the attraction and retention of water surrounding the seed (Penfield *et al.*, 2001). In *mum4-1*, seed hydration, and thus imbibition, could be slowed by either the reduced quantity of mucilage or the lack of release of mucilage to form a hydrated gel around the seed. While pleiotropic effects cannot be ruled out, the enhanced delay seen in both *myb61 mum4* and *mum2 mum4* double mutants, as well as *men mum4* double mutants (Fig. 6), strongly suggests that both mucilage quantity and release are important for efficient seed hydration and germination, even under moist conditions.

Supplementary data

Supplementary data can be found at *JXB* online.

Supplementary Table 1. Primers used for the sequencing of *mum2-13* and *myb61-6*.

Acknowledgements

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