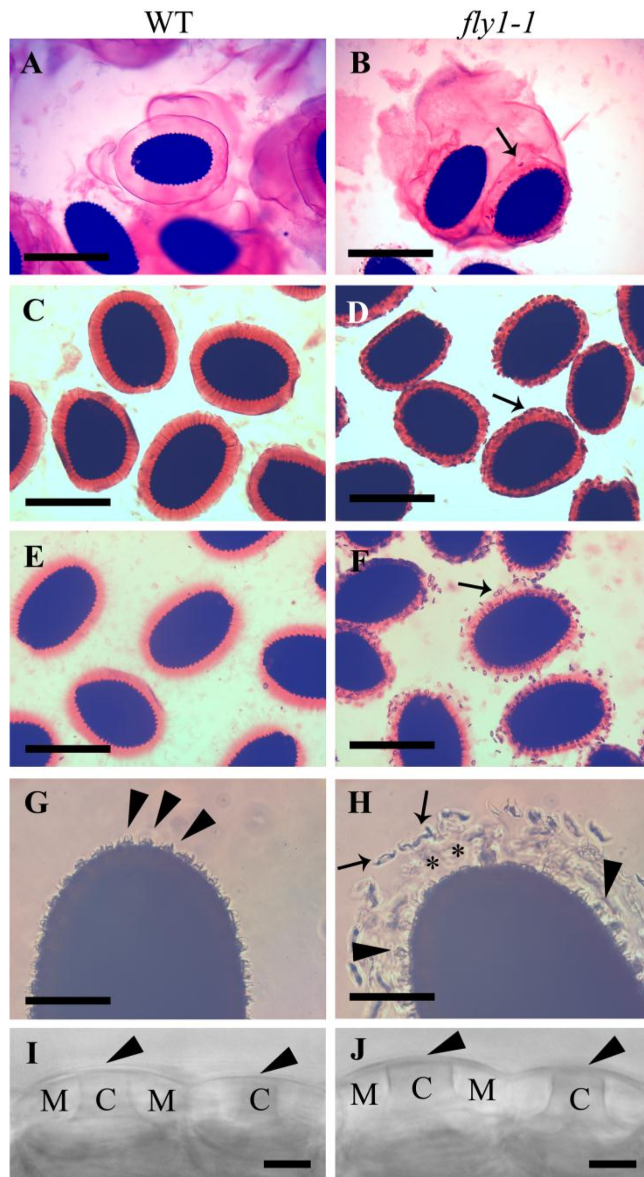
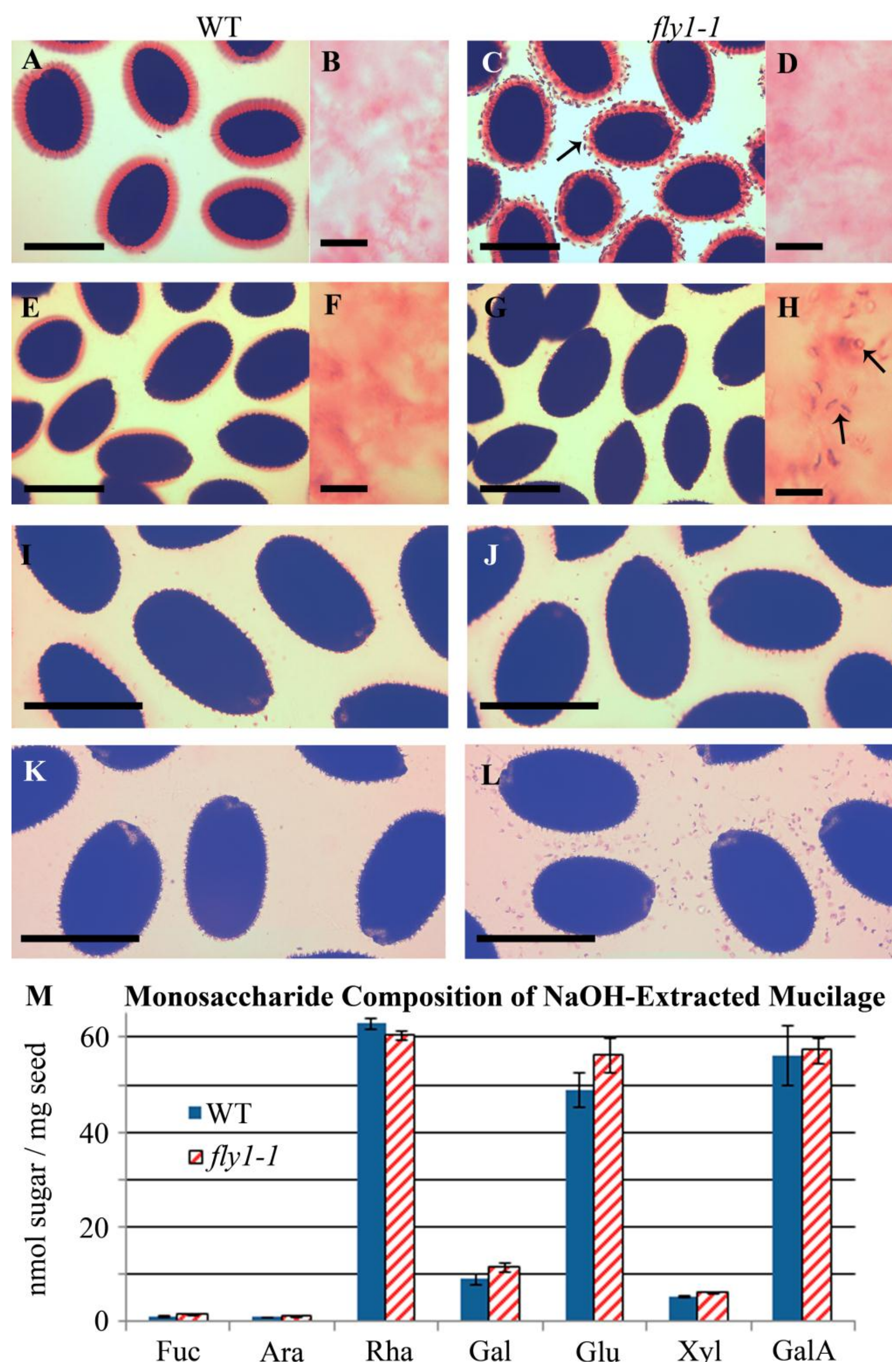


Supplemental Figure 1. Analysis of *fly1-1* Mucilage Extrusion and Primary Wall Attachment.

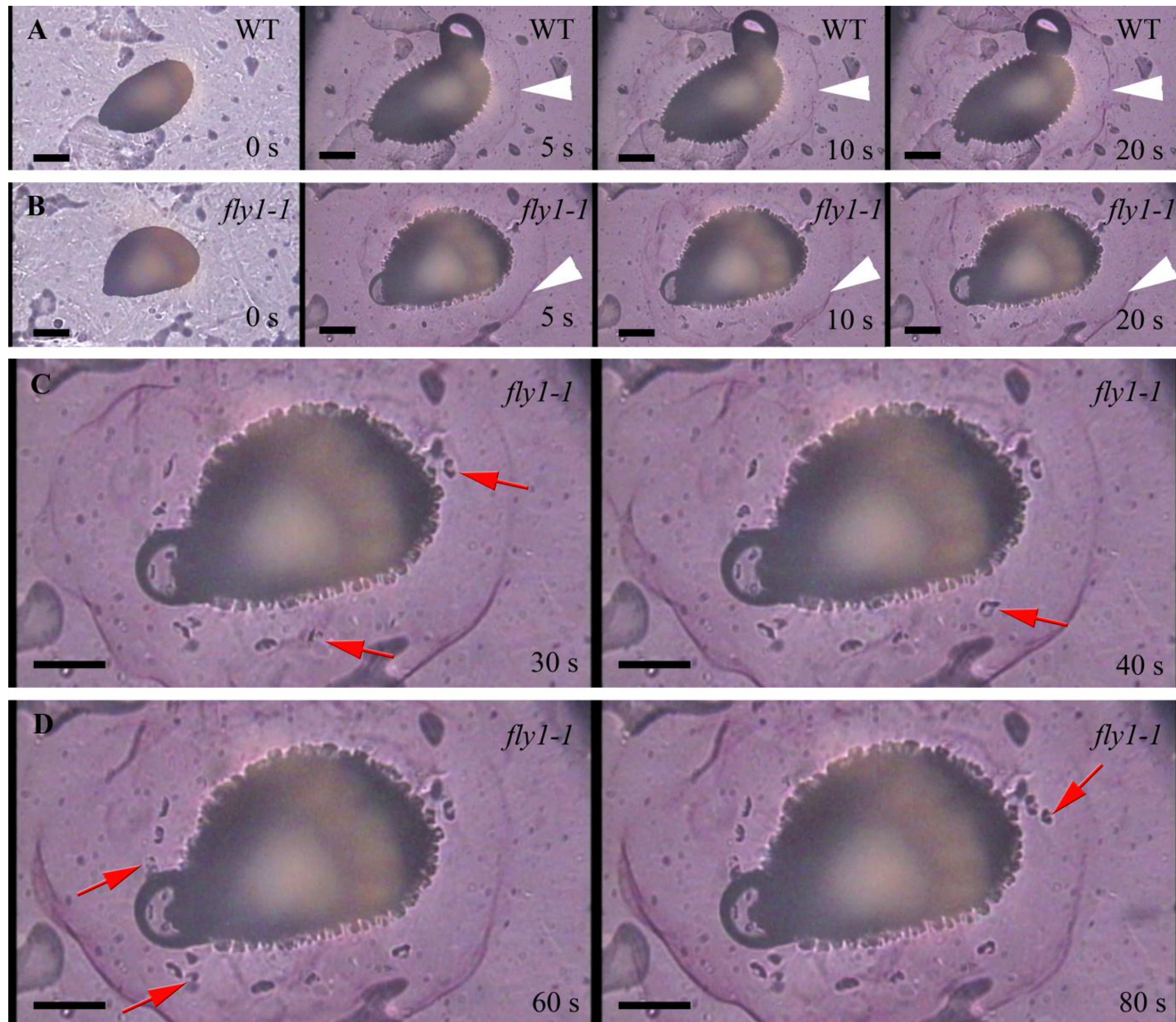
(A) and **(B)** Mature seeds hydrated in RR, without mechanical agitation. Both wild type (WT) and *fly1-1* release large amounts of non-adherent mucilage.
(C) and **(D)** Mature seeds shaken directly in RR. The *fly1-1* seeds display discs and a smaller mucilage capsule than WT.
(E) and **(F)** Mature seeds shaken in water for 24 h and then stained with RR. The inner, adherent mucilage layer and the *fly1-1* discs remain attached to seeds.
(G) and **(H)** Phase contrast micrographs of seeds shaken in water. Unlike *fly1-1*, all WT seed coat epidermal cells have primary cell walls attached to their columellae (arrowheads). The *fly1-1* seeds display discs (arrows) and loss of primary wall attachment (asterisks).
(I) and **(J)** Transmitted light images of live seed coat epidermal cells at 12 DPA. Both WT and *fly1-1* display primary cell walls (arrowheads) in close association with the columellae (C) during development. M, mucilage pockets.

Bars = 500 µm in **(A)** to **(F)**, 150 µm in **(G)** and **(H)**, and 10 µm in **(I)** and **(J)**.



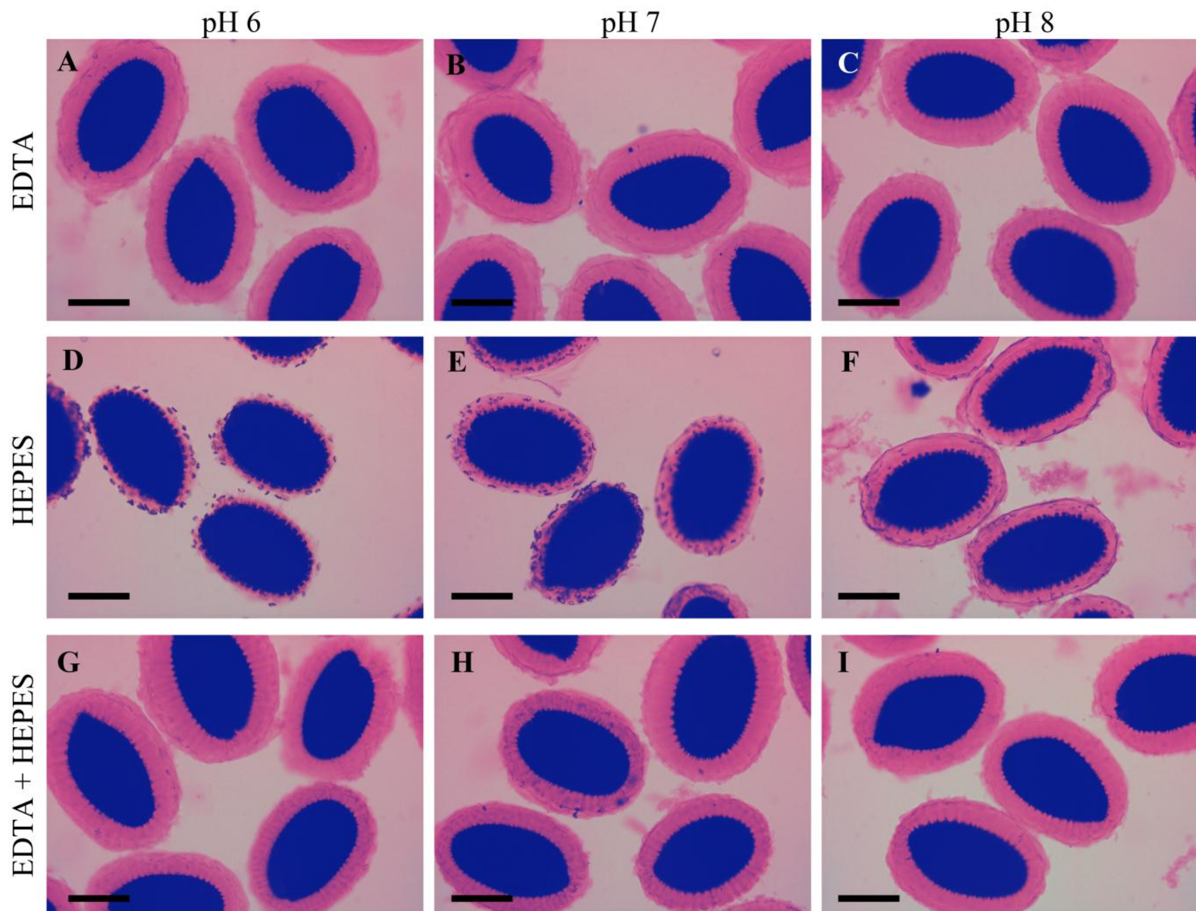
Supplemental Figure 2. Mucilage Extractions and Total Monosaccharide Composition.

(A) to **(L)** RR-staining of mucilage extractions for biochemical analysis. For sequential water extractions, seeds were gently shaken for 1 h **(A)** and **(C)**, or vortexed for an additional 2 h **(E)** and **(G)**. RR-stained extracted mucilage is shown in **(B)**, **(D)**, **(F)** and **(H)**. Discs (arrows) are removed from *fly1-1* seeds only after vigorous shaking.
(I) and **(J)** For DM analysis, seeds vortexed in 50 mM EDTA pH 8 for 1 h.
(K) and **(L)** Seeds vortexed in 0.2 NaOH for 1 h to remove all mucilage.
(M) Monosaccharide levels in wild type (WT) and *fly1-1* mucilage extracted from **(K)** and **(L)** respectively.. Values are the mean ± SE of four samples.Results were verified using two additional biological replicates.
Bars = 100 μm in **(B)**, **(D)**, **(F)** and **(H)**, and 500 μm in the other panels.



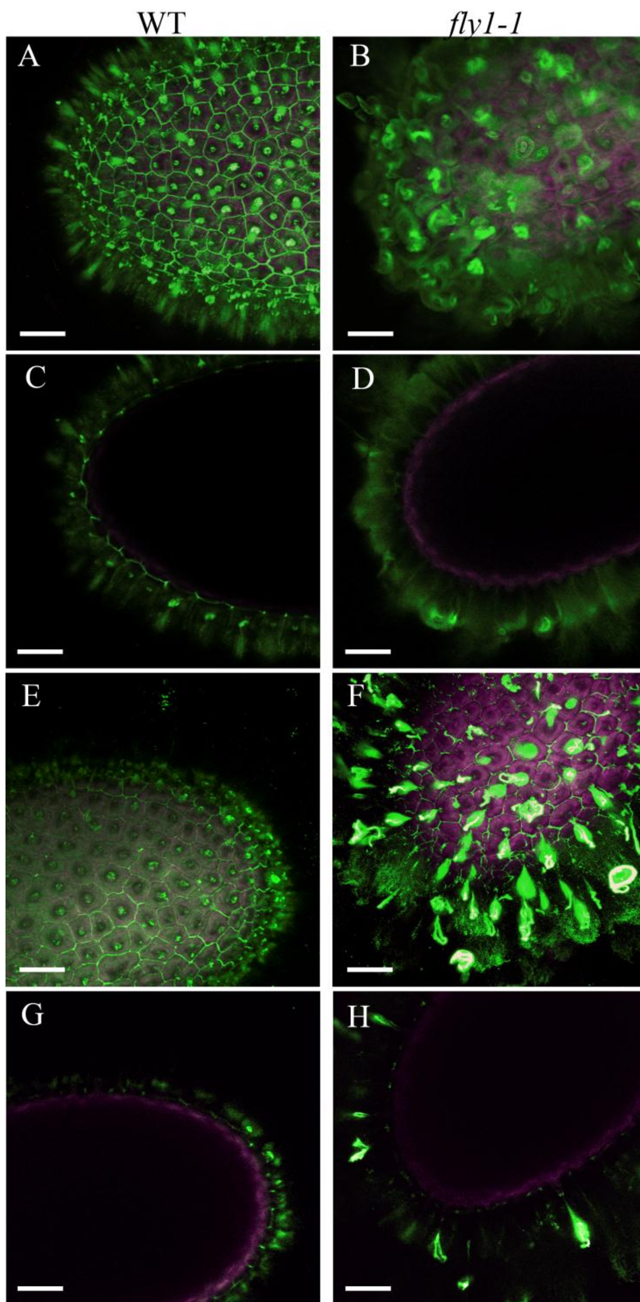
Supplemental Figure 3. Time Course Analysis of Mucilage Release From Seeds Upon Hydration.

(A) Wild type (WT) seed releases a large mucilage halo (arrowheads) within 10 s of hydration in RR. **(B)** to **(D)** *fly1-1* seed releases an equally large mucilage halo (arrowheads) within 10 s, but discs continue to be released after more than 60 s (arrows). Bars = 150 µm.



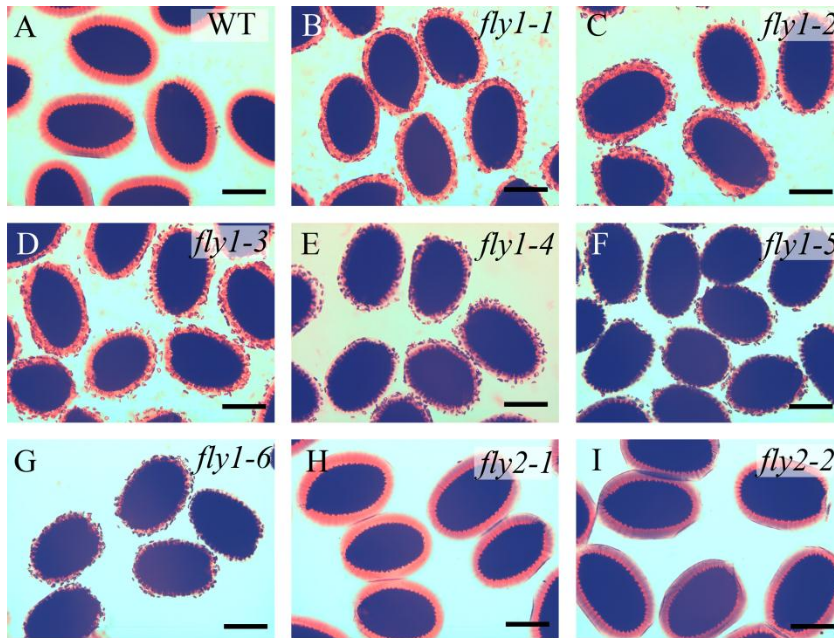
Supplemental Figure 4. Analysis of the Effect of Buffer pH on *fly1-1* Mucilage Defects.

(A) to **(I)** *fly1-1* seeds were shaken in 50 mM EDTA, 50 mM HEPES or both for 1 h before being rinsed and stained with RR.Unlike EDTA, HEPES does not chelate Ca^{2+} ions. EDTA pH 6 and 7 treatments **(A)** and **(B)** resemble the EDTA pH 8 treatment, which fully rescues the *fly1-1* mucilage defects **(C)**. HEPES partially rescues the defects at pH 8 **(F)** but not at pH 6 **(D)**. HEPES pH 7 shows an intermediate phenotype **(E)**. The EDTA + HEPES treatments **(G)** to **(I)** phenocopy the EDTA treatments **(A)** to **(C)**. Bars = 150 µm.



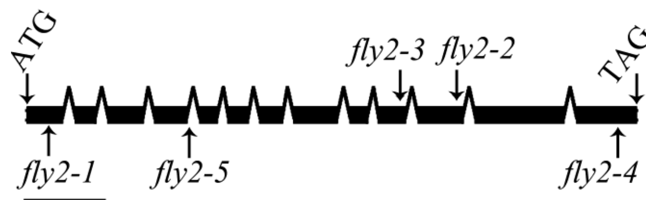
Supplemental Figure 5. Immunolabeling of Partially Methylesterified HG in Seeds.

(A) to **(D)** JIM5 and **(E)** to **(H)** JIM7 antibody signals are shown in green, and intrinsic seed fluorescence in magenta. **(A)**, **(B)**, **(E)** and **(F)** Maximum intensity signals from multiple optical slices. **(C)**, **(D)**, **(G)** and **(H)** represent single optical sections through the middle of seeds. JIM5 binds to low DM (up to 40%) HG (VandenBosch et al., 1989), and JIM7 labels high DM (35 to 81%) HG (Knox et al., 1990). The mucilage of *fly1-1* displays increased JIM5 and JIM7 immunofluorescence compared to wild type (WT). Bars = 50 µm.



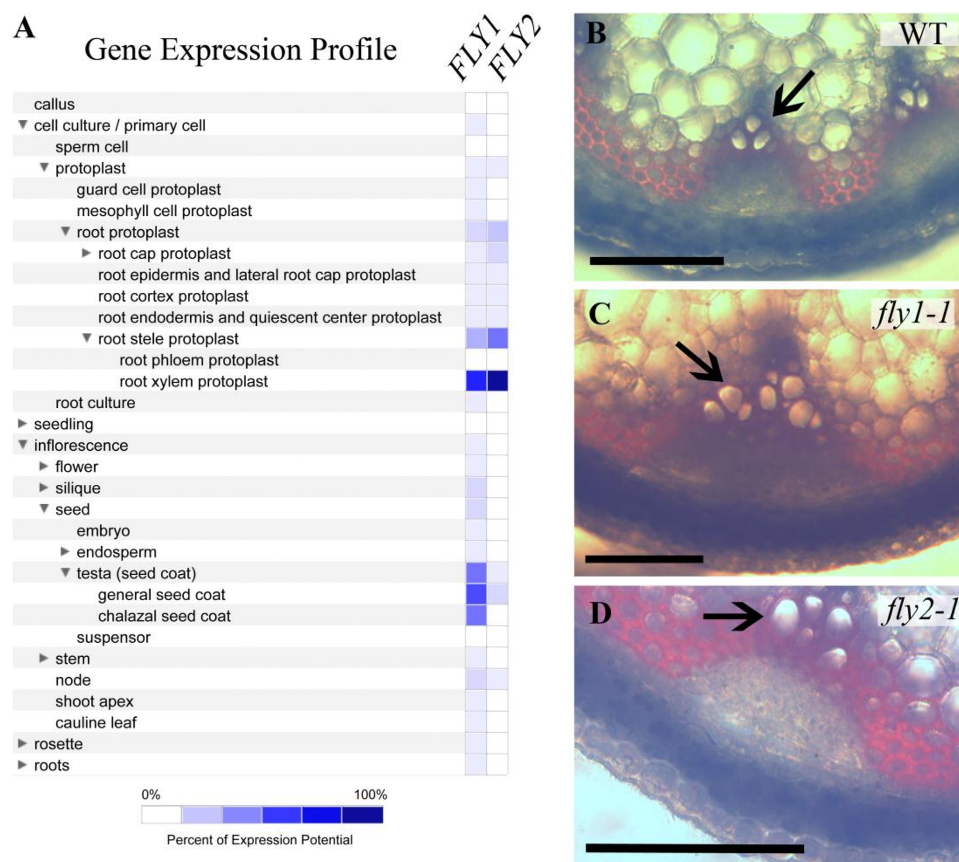
Supplemental Figure 6. Analysis of *fly1* and *fly2* Mucilage Extrusion.

(A) to **(I)** Seeds were shaken in water for 2 h and stained with RR. All *fly1* mutants have discs at the edge of the extruded mucilage. Wild type (WT) and *fly2* are indistinguishable. Bars = 300 µm.



Supplemental Figure 7. *FLY2* Gene Structure and Mutations.

T-DNA insertions (*fly2-1* to *fly2-5*) are indicated with arrows. Boxes and connecting lines represent exons and introns. Bar = 300 amino acids.



Supplemental Figure 8. *FLY1* and *FLY2* May Be Involved in Xylem Development.

(A) *FLY1* and *FLY2* are expressed highest in xylem cells. Relative transcript levels across Arabidopsis tissues and cell types are shown using a GENEVESTIGATOR heat map (Hruz et al., 2008). Values are normalized to the maximum expression level (darkest blue color) recorded for each gene. *FLY1*, unlike *FLY2*, also shows strong expression in the seed coat.
(B) to **(D)** Wild type (WT), *fly1-1*, and *fly2-1* display similar xylem cell morphology. Hand sections from the base of stems were stained with phloroglucinol-HCl. Arrows indicate xylem cells. Bars = 100 µm.

```
FLY1 504 CVICMTAIDL-------RQHTS--DCMVTPCEHFFHSGCLQRWMDIKMECPTCRRSLPP 561
FLY2 501 CVICMTTIDL-------RHRIN--DCMVTPCEHIFHSGCLQRWMDIKMECPTCRRPLPP 558
TUL1 698 CAICMSDVPIYIEEIPETHKVDQHSYMVTPCNHVFHTSCLENWMNYKLQCPVCRSPLPP 757
         *.***: : :        ::  .   . *****:*.**:.**:.**: *::**.** .***
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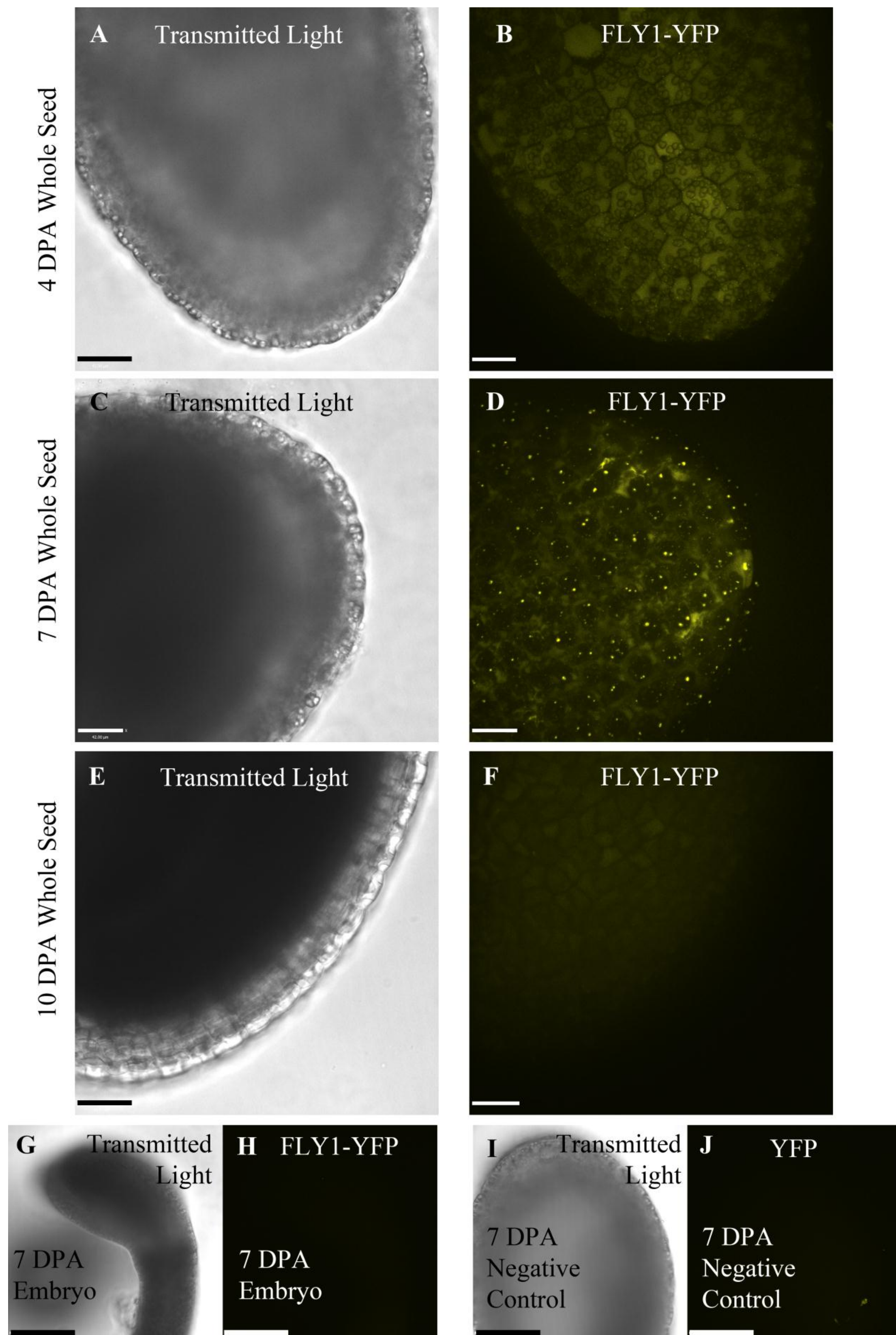
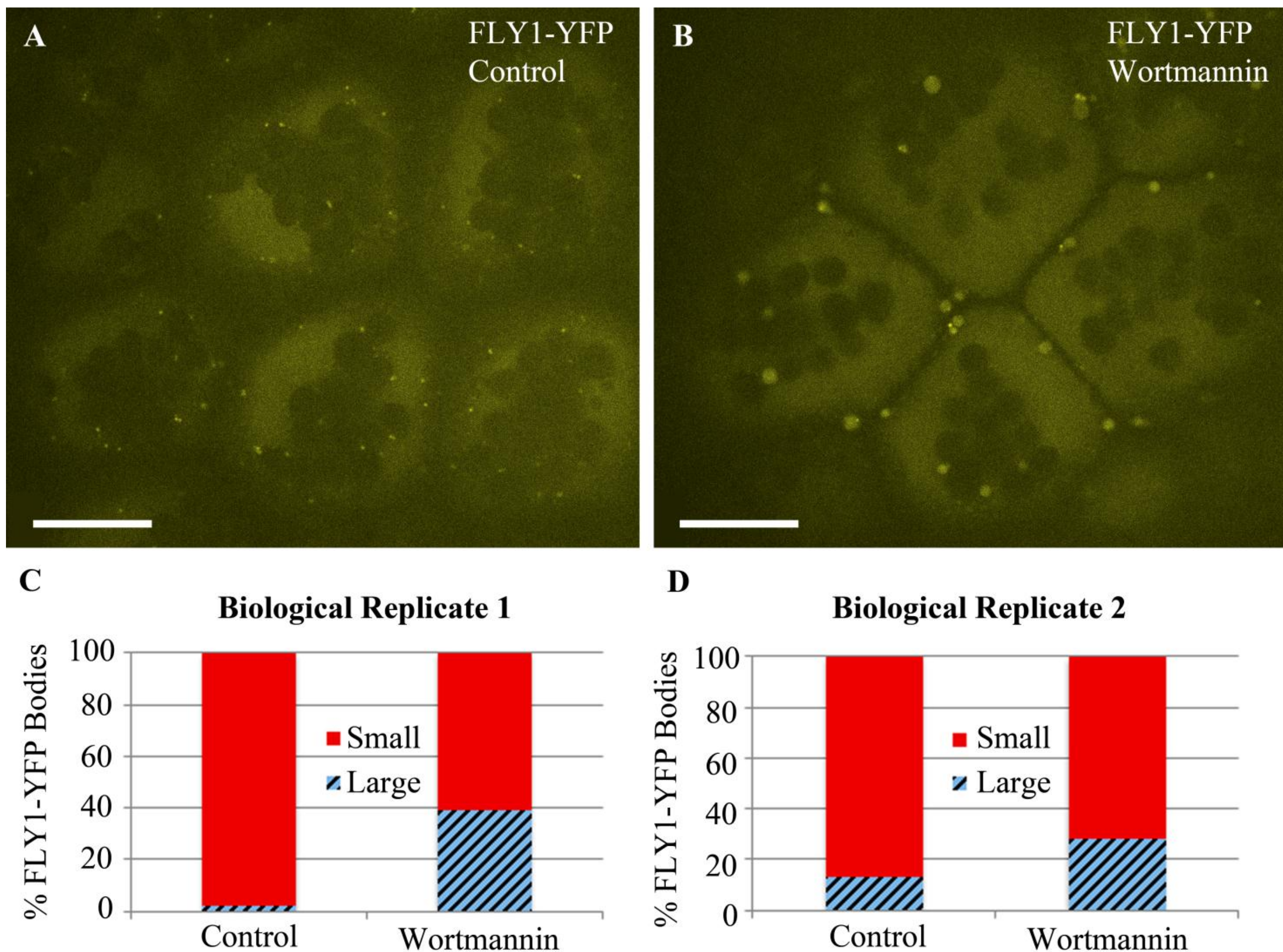
Supplemental Figure 9. Alignment of the RING-H2 domains of FLY1, FLY2 and TUL1.

Shading indicates the eight conserved Cys (C) and His (H) amino acids that define the RING-H2 domain (Stone et al., 2005). Fully conserved residues are indicated by an asterisk. A colon denotes conservation between groups with strongly similar properties, while a period indicates groups with weakly similar properties.



Supplemental Figure 10. Analysis of $FLY1_{pro}$:*FLY1-YFP* expression in developing seeds.

(A) to **(J)** Light micrographs of developing seed tissues, left of the corresponding YFP signals (maximum intensity signals from multiple stacks).
(A) to **(H)** *fly1-1* seeds rescued with the $FLY1_{pro}$:*FLY1-YFP* transgene show the highest FLY1-YFP expression in the seed coat epidermis at 7 DPA **(D)**, but not in the embryo **(H)**.
(J) 7 DPA *fly1-1* seeds without the $FLY1_{pro}$:*FLY1-YFP* transgene show no YFP signal. Bars = 50 µm in **(A)** to **(F)**, 25 µm in **(G)** to **(J)**.



Supplemental Figure 11. Wortmannin Induces Fusions of FLY1-YFP punctae.

(A) and **(B)** $FLY1_{pro}$:*FLY1-YFP* seeds excised from the same 7 DPA silique. **(A)** Control seed displays many small punctae. **(B)** Seed treated with 20 µM wortmannin for 2 h displays many large bodies. Bars = 20 µm.
(C) and **(D)** Wortmannin induced the fusion of small FLY1-YFP punctae (0.1 μm^2 to 1.5 μm^2), thereby increasing the ratio of large bodies (2.5 μm^2 to 8 μm^2) to punctae. Approximately 200 punctae/bodies were counted in each of the two biological replicates.

Supplemental Table 1. Gene-Specific Primers Used for T-DNA Genotyping and RT-PCR Analysis.

Locus	**Left Primer (5' to 3')**	**Right Primer (5' to 3')**
fly1-2	CGCAAGTTCAGATGCTAATGC	AAAAAGGAACCGACAAACCTG
fly1-3	AGGCACAAATAAGCATCCATG	ATGAACAAAATGTGGGTGGTG
fly1-4	TCTGCTAATGGCTTGTTTGATG	ACGGGTTGCTTTCCATATAGC
fly1-5	TTTTCACTAGAAGCCACACGG	CTTGCAGTGGCTCTTTGGTAG
fly1-6	GCACTCAAGATTCAGTGCAGG	ATGACGGAGATTGTTTTTCCC
fly2-1	AACTGCACCCTGTTCACATTC	ACTCCGACATTCCAAGTTTCC
fly2-2	CGATTCCTAAGGAACCAAAGG	TTCTTGTATACAAGGGTGCCG
FLY1	TGTAGAGCCCAACAAGGTTTG	GATCAATAGCGGTCATGCAG
FLY2	CAAAAAAGAAAAAGGTGGAGCAG	AATTTTTGTTGGACTTGTCAC
GAPC	GATTCGGAAGAATTGGTCGTTT	CTTCAAGTGAGCTGCAGCCTT