# The GAP in Fruit Development: Determining the Role of RanGAP in Arabidopsis Fruit Development

Honors Research Thesis

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By

Bailey Tilford

The Ohio State University

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Project Advisor: Professor Iris Meier, Department of Molecular Genetics

**Abstract:**

RanGAP is the activating protein for the small GTPase Ran, and is known to be involved in nucleocytoplasmic transport and mitotic cell division across kingdoms. Arabidopsis has two RanGAP proteins, RanGAP1 and RanGAP2, which share 63% amino acid homology. Both proteins contain two functional domains: a localization domain known as the WPP domain, which contains a conserved tryptophan-proline-proline motif necessary for interaction with localization binding partners, and a GTPase activation (GAP) domain, which is responsible for Ran binding and activation. Double null mutants in the genes encoding RanGAP1 and RanGAP2 are lethal at an early stage of development. However, a homozygous mutant combining the *RanGAP1* null allele and *RanGAP2* knockdown allele, called short silique knockdown (SILK), exhibits a reduced fruit (silique) length phenotype. What role RanGAP plays in fruit development, however, is unknown. In order to determine its function, constructs containing wildtype *RanGAP1* or mutant *RanGAP1* with one or both functional domains mutated were inserted into SILK mutant plants. Their phenotypes were quantified by measuring fruit length and seed number to determine if the *RanGAP1* transgene had rescued the short-fruit phenotype. *RanGAP1* transgenes with mutations in the GAP domain did not rescue the SILK phenotype whereas mutants that maintained this function did, regardless of localization. These results indicate that it is the GAP function of RanGAP that is important in fruit development. Seed count, however, varied greatly among individual lines with the same transgene, indicating that seed development may depend on the location of the *RanGAP1* insertion into the genome. Further analysis of silique cell types indicate that the length phenotype may be due to defects in the differentiation of the cells of the fruit itself. This work highlights the intersection between cell biological processes and developmental events, and shows the relevance of intracellular events to the understanding of whole-plant processes.

## Introduction:

### RanGAP

RanGAP (Ran GTP-ase Activating Protein) is the activating protein of the small GTPase Ran: a small Ras superfamily GTP-ase primarily involved in the transport of

RNA and protein between the nucleoplasm and the cytoplasm, with homologues in many different species[1]. This transport role is dependent on a gradient of Ran across the nuclear membrane, with GTP-bound and GDP-bound forms dominant in the nucleoplasm and the cytoplasm, respectively[2]. The transition between these forms is dependent on the interaction of Ran with its activating protein, RanGAP, and its nucleotide exchange factor, RanGEF[2–4]. The ability of RanGAP to help Ran to hydrolyze GTP is referred to as its GTPase-activation (GAP) activity [5–7]. This interaction occurs via the leucine-rich repeat (LRR) domain of the protein[8]. All known RanGAP proteins contain an LRR domain, as well as a c-terminal acidic tail with no known function[8,9]. Yeast RanGAP, which is a cytoplasmic protein, contains no other known functional domains[10]. Animal RanGAP, on the other hand, is tethered to the nuclear envelope via interactions with nuclear pore protein NUP358 through a SUMOylated c-terminal domain[10]. Arabidopsis RanGAP contains an N-terminal domain known as the WPP domain which contains a conserved tryptophan-proline-proline motif and is necessary and sufficient for localization of RanGAP to the nuclear periphery[10,11]. This localization is accomplished through protein-protein interactions with two families of outer nuclear envelope proteins: the WIPs (WPP Domain Interacting Proteins) and WITs (WPP Interacting Tail-Anchored Proteins)[12,13]. The WPP domain also targets RanGAP to specific mitotic sites, including the preprophase band, the cortical division site, the cell plate, kinetochores, the spindle midzone, and the outward-growing rim of the phragmoplast[7,10,11,14]. *Arabidopsis thaliana* contains two paralogous copies of RanGAP: AtRanGAP1 and AtRanGAP2 which share around 60% amino acid identity with each other and around 20% identity with *Saccharomyces cerevisiae* Rna1p and *Homo sapiens* RanGAP[10]. Although single null mutants of either *AtRanGAP1* (AT3G63130) or *AtRanGAP2* (AT2G34150) create no observable phenotype, a double null mutant was reported to be female gametophyte lethal and to exhibit arrested nuclear division, indicating a redundant and essential role for the proteins in mitotic progression in plant female gametophytes[15].

SILK Mutant

A mutant called short silique knockdown (SILK) was generated by crossing to obtain a homozygous null of *RanGAP1* (*rg1-1* allele) and a homozygous knockdown of *RanGAP2* (*rg2-2* allele) *(rg1-1/rg1-1 rg2-2/rg2-2*). This line was seen to have a slight

developmental delay and shortened siliques. We utilized the silique length phenotype to investigate the role of Arabidopsis RanGAP in a specific part of the plant's development.

Fruit development in Arabidopsis

Arabidopsis fruits, called siliques, develop from two fused carpels that form the flower's gynoecium[16,17]. Mature fruits are elongated structures with three distinct regions: The replum, which extends longitudinally through the center of the silique and to which the seeds are tethered via their funiculi; the valve, which forms a wall around the seeds; and the valve margins, which separate the valve from the replum at two locations[16–20]. Although much is known about flower morphogenesis in Arabidopsis, relatively little is known about the further development of flowers into siliques. It is known that elongation of Arabidopsis siliques is initiated after fertilization and occurs in order to make room for the expanding seeds [21,22]. This timing is regulated by proteins that actively suppress fruit development until fertilization has occurred[21,22]. Mutants which initiate silique elongation without fertilization, including *fis1*, *fis2*, and *fwf*, demonstrate the complex regulations that occur to ensure that fruit development and seed development coincide[2122]. Other genes involved in the development of siliques determine cell fate in the context of the regions outside the seeds once fertilization has released fruit elongation from its inhibition. After fertilization, cells in the valve region divide primarily anticlinaly and differentiate into valve cell types, which include fully developed stomata[17]. The MADS-Box gene *FRUITFULL* has been found to be necessary for establishing valve identity, and *fruitfull* mutants develop short, compact fruits in which the valve region has failed to elongate and differentiate, but which contain a full set of developed seeds[16,17]. FRUITFULL negatively regulates the valve margin identity gene *INDEHICENT* which works together with the *SHATTERPROOF* genes and *ALCATRAZ* to develop the valve margin region, which is composed of small, thin, lignified cells and which is necessary for the separation of the valves from the replum during seed dispersal[18,20]. The replum region requires the activity of REPLUMLESS, which negatively regulates both *SHATTERPROOF* and *FRUITFULL* in the replum region to prevent valve or valve margin fates from being adopted in the replum, although it is not required for replum fate[19]. Because so many genes are involved in fruit development, determining which

processes are disrupted in the SILK mutant requires a number of phenotypic analyses, the beginning of which will be presented in this thesis.

**Results:**

Assays

In order to determine whether the GTPase activation function and/or localization of RanGAP are necessary for its role in silique development, we used previously created constructs that contain point mutations in one or both of RanGAP's functional domains. The first mutant, AAP, includes two point mutations converting tryptophan 18 and proline 19 of RanGAP1 to alanines. This prevents RanGAP's interaction with its nuclear envelope binding partners WIP and WIT, causing delocalization to the cytoplasm[10–13]. The two mutants that nullify RanGAP's GAP activity, termed the "no GAP" mutants, are D330A and N219A. D330A and N219A carry point mutations in aspartate 330 and asparagine 219, respectively, which convert these conserved residues in loop regions of the LRR domain to alanines. These mutations allow the protein to localize properly, but not to interact with Ran[8]. An additional mutant combines the AAP mutation with the N219A mutation, to create AAP+N219A mutants. SILK plants were transformed with these constructs and the progeny resulting from two generations of selection (T2) were grown alongside wildtype and SILK plants, and their silique lengths were quantified. To determine which part of the developmental process was being affected, a seed count assay was performed as well as sectioning of siliques to observe non-seed cell types. These data were compared and used for analysis of the role of RanGAP in silique development.

Silique Length

The siliques of SILK plants compared to wildtype were shorter (Figure 2A), with a median at approximately 0.7 centimeters compared to approximately 1.1 centimeters for wildtype. The insertion of wildtype RanGAP1 or AAP into the SILK background completely complemented the short silique phenotype to wildtype levels, while insertion of D330A yielded no complementation, and insertion of N219A or AAP + N219A yielded partial complementation, with siliques measuring at levels about halfway between SILK and wildtype (Figure 2B).

Seed Count

Seed positions (all possible locations where a seed could develop) in each silique were given one of three values: normal seed, aborted ovule, or aborted seed (Figure 3A). SILK plants had a high level of abnormal seed positions (aborted seeds or aborted ovules) with a higher aborted ovule number than aborted seed number. Wildtype siliques were consistently normal with few aborted seeds or aborted ovules and large seed sets. The seed count for the transgenic SILK lines varied greatly among each independent insertion line (Fig 3B) with no correlation to the length of siliques. Within lines, however, seed count was generally consistent.

Cell Type Analysis

A preliminary assay of silique cells was performed on the valve and valve margin layers of wildtype, SILK, and single-construct plant siliques. Siliques were sectioned by hand, and stained with Toluidine blue, which dyes lignified cell walls turquoise and cellulosic cell walls purple. Siliques from all plants had fully developed valve tissues with stomata visible in the epidermal layer, and fully developed xylem. SILK plants appeared to have a reduced valve margin identity, with lignified cells concentrated in a central position and not extending to the outer layers, but all constructs analyzed appeared to rescue that phenotype (Figure 4). Cell size and number in the valve, and quality of replum cells have not been analyzed.

**Discussion:**

Arabidopsis RanGAPs are responsible for activation of the small GTPase Ran and subsequent import and export of proteins and RNA between the nucleoplasm and the cytoplasm. Reduction of this function by eliminating the RanGAP1 protein and reducing the amount of the RanGAP2 protein in Arabidopsis leads to defects in fruit (silique) development. The quantification of these defects as well as an investigation into its exact causes was performed via a complementation experiment and subsequent analysis of various aspects of the siliques themselves.

The SILK plants expressing RanGAP1 wildtype or RanGAP1AAP had their silique lengths rescued completely to wildtype length, indicating that the reintroduction of RanGAP1 into the SILK background could complement the phenotype, and that a

specific RanGAP1 subcellular localization is not necessary for proper silique elongation. RanGAP1D330A was unable to rescue the silique phenotype, indicating that the GAP function of RanGAP is necessary for proper silique elongation. RanGAP1N219A and RanGAP1AAP+N219A were able to partially rescue the short silique phenotype. One possible explanation for this partial rescue is the retention of some GAP function in the N219A point mutant that its counterpart, D330A, does not retain. Further analyses of these data are necessary to fully understand this result.

Seed count data did not match with silique length rescue, but SILK seeds showed defects in development while wildtype seeds were consistently well-developed, indicating that although RanGAP plays a role in seed development, that role is independent of its role in silique elongation. The high variation in seed position identity among independent insertion lines could be based on different levels of protein in the individual transgenic lines; an idea that can be tested by performing Western blots for the RanGAP1 variants in each line. The existence of this variation points to a dependence on the location of the *RanGAP1* gene itself in the plant's genome, making it an interesting topic for further study of the importance of protein import and export and gene regulation during the development of seeds.

Because seed count did not correlate with silique length, it is likely that the length phenotype is due entirely to problems in the development of the tissues of the silique itself. Preliminary assays have established that the defects are not in the loss of valve cell identity, and are not entirely due to a loss of valve margin identity, although SILK plants do display a decrease in the spread of valve margin identity, or perhaps the spread of another tissue identity which suppresses valve margin identity. The relationships between identity genes in silique development are complex and not completely understood, but further analysis of cell types in these mutant siliques may lead to some understanding of which identities or developmental events are being repressed, and which are being activated. Because RanGAP canonically participates in nucleocytoplasmic transport, it is possible that these defects are due to a regulatory protein failing to be imported into the nucleus. Discovery of which proteins in silique development may fail to be imported without RanGAP may lead to a better understanding of the genetic interactions which pattern siliques.

**Conclusion:**

Fruit development is a vital process in the plant life cycle. The GAP domain of RanGAP plays a role in this process in Arabidopsis and is necessary for proper silique elongation. The proper anchoring of RanGAP to the nuclear envelope is not necessary for elongation. Although seed development is also affected by the loss of RanGAP activity in Arabidopsis it is independent of elongation, as constructs which rescued the short length phenotype of the RanGAP mutant SILK showed variability in seed viability which did not correlate with silique length. The loss of RanGAP does seem to have an effect on the tissues of the silique itself, specifically in the valve margin, but further analysis of silique cell types is necessary to draw conclusions about how RanGAP contributes to cell growth and identity in siliques. As such, it is as of yet unknown how RanGAP affects silique development and what role the Ran gradient plays in fruit elongation independent of seed development, but a basis has been set on which further research can progress.

**Materials and Methods:**

Plant Materials and Growth Conditions

*Arabidopsis thaliana* (ecotype Columbia) plants were grown at 22$^{o}$C under long-day conditions (16 hours of light followed by 8 hours of darkness) on plates with Murashige and Skoog (MS) medium and later moved to soil under the same light and temperature conditions. SILK plants were produced via crosses of T-DNA insertion mutants *rg1-1* (SALK_058630) described previously[14,15], and *rg2-2* (SALK_006398) acquired from the Arabidopsis Biological Resource Center (ABRC). Both insertions are in the Columbia background. Successive generations yielded plants that were homozygous for *rg1-1* and *rg2-2* (SILK).

Construct design and transformation into SILK background

All RanGAP1 variant constructs are driven by the RanGAP1 native promoter and C-terminally fused with GFP. The RanGAP1 construct contains full-length RanGAP1. The AAP construct contains a copy of RanGAP1 which has the tryptophan and first proline of the WPP motif mutated into alanines. The D330A construct has a copy of RanGAP1 with its aspartic acid 330, a conserved residue located in the LRR domain,

mutated to alanine. The N219A construct has a copy of RanGAP1 with its Asparagine 219, another conserved residue in the LRR domain, mutated to alanine. These constructs were transformed into *Agrobacterium tumefaciens.* SILK plants were transformed via agrobacterium-mediated flower dip, and transgenic offspring were selected for on MS plates containing carbenicillin and hygromycin. Those selected were imaged for RanGAP1-GFP localization under an Eclipse C90i (Nikon, Tokyo, Japan) confocal microscope using a 388nm laser. Phenotypes were analyzed in the T2 generation (the progeny of the plants obtained after the first round of selection).

Silique Collection and Measurement Analysis

Ten siliques were collected from each plant, with about six plants grown for each independent insertion line and for wildtype and SILK. Siliques were collected from the middle section of the plant, and only siliques that had begun to yellow were collected to ensure that only fully developed siliques were measured. Siliques were scanned using a Canon Canoscan LIDE 200 Scanner and measured using ImageJ software (Figure 2A).

Seed Analysis

Five siliques were collected from each independent insertion line as well as wildtype and SILK, with siliques taken randomly from among the plants in that line. Only siliques that were fully developed and beginning to yellow were taken. Siliques were opened under a Zeiss Stemi 2000-C dissecting scope and the total numbers of normal seeds, aborted ovules, and aborted seeds were counted and recorded for each silique.

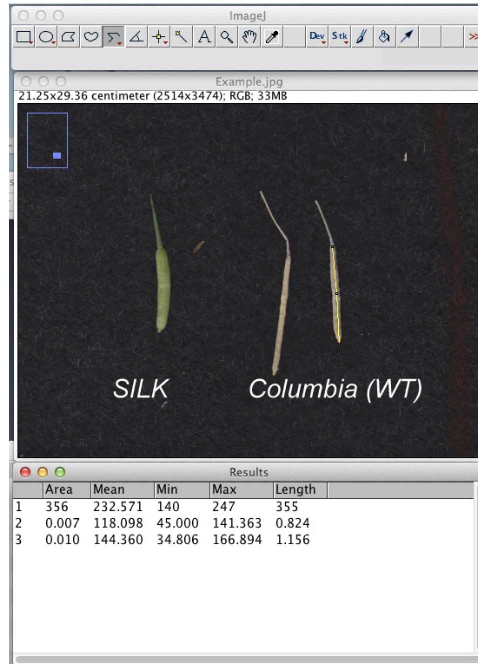
Tissue Analysis

Three siliques from each single construct type (RanGAP1, D330A, N219A, AAP) as well as SILK and wildtype were sectioned periclinally. Sections were mounted on glass slides and stained with Toluidine Blue stain (TBO), then photographed using a Nikon E100 light microscope. Thicker sections were rotated to view valve epidermal tissues.

**Figures**

**Figure 1**: Illustration of point mutations used to create RanGAP1 constructs. The localization domain (WPP domain) is in green, and the GAP domain (LRR domain) is in orange. The Acidic Tail domain has no known function in plants. The AAP+ D330A construct has not yet been inserted into the SILK background. (Figure modified from Anna Newman)

A.



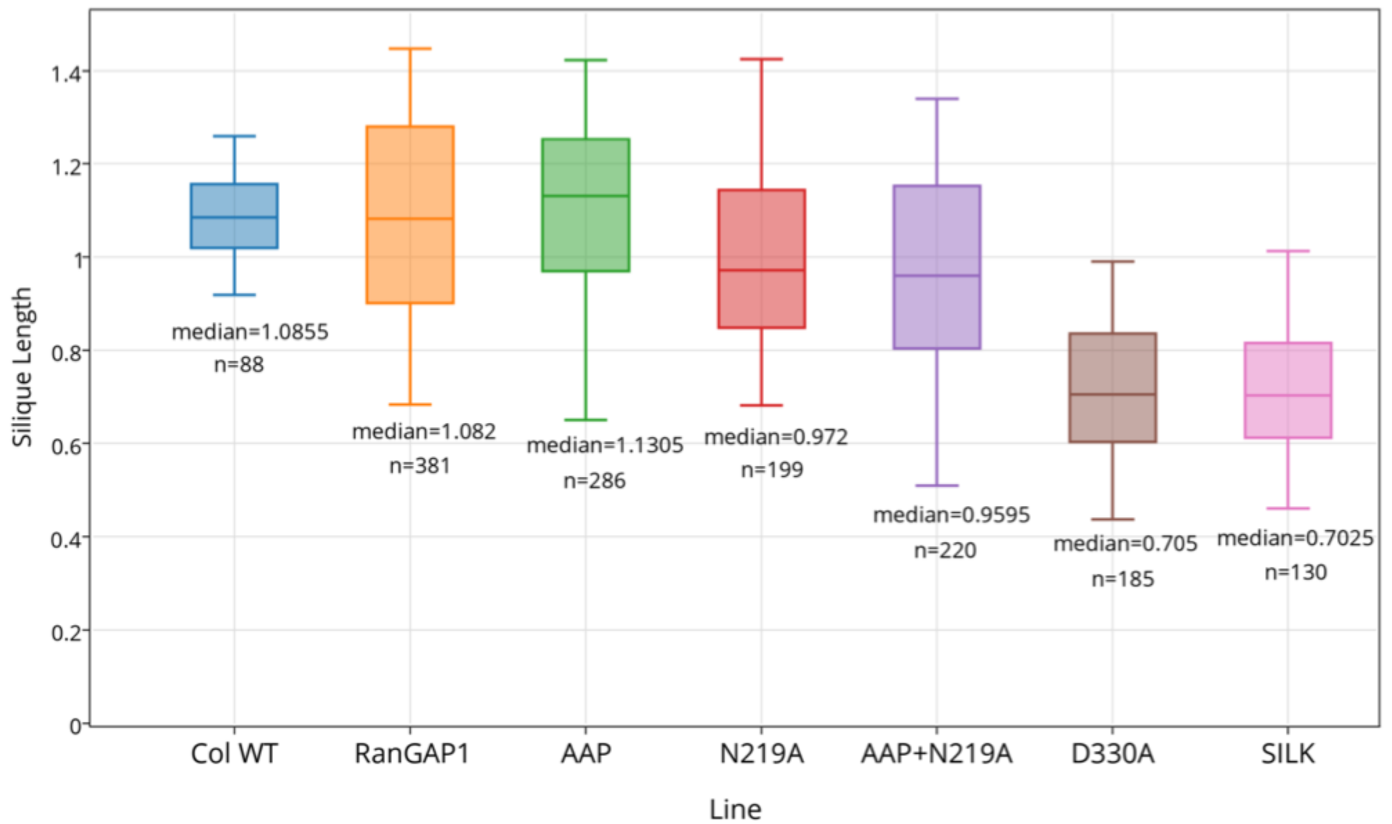



**Figure 2**: (A) ImageJ software interface and comparison of SILK and Columbia (wildtype) siliques. SILK siliques are noticeably shorter. (B) Quantification of silique length measurements. There is a large difference between the lengths of SILK and wildtype siliques, with a median of around 1.1 for Columbia and 0.7 for SILK. D330A silique length is similar to SILK length while RanGAP1 and AAP siliques were close to wildtype length, with N219A and N219A + AAP in between.

A.

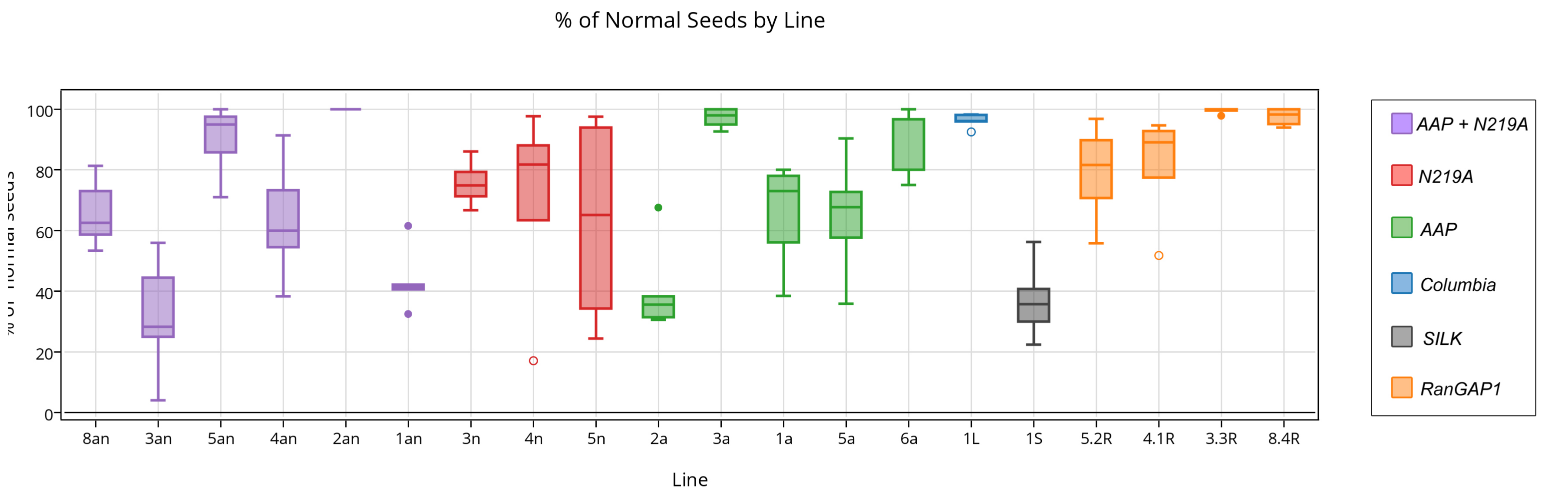



**Figure 3:** (A) Illustration of seed assay. Positions considered "Normal seed", "Aborted Ovule" and "Aborted Seed" positions are labeled. Image is of a SILK silique. (B) Quantification of seed assay data. Seed counts were highly variable between different

lines with the same construct, and did not correlate with length rescue. Each column represents five siliques selected randomly among plants in a single line. No D330A plants were available at the time of this assay, so the data is not included.

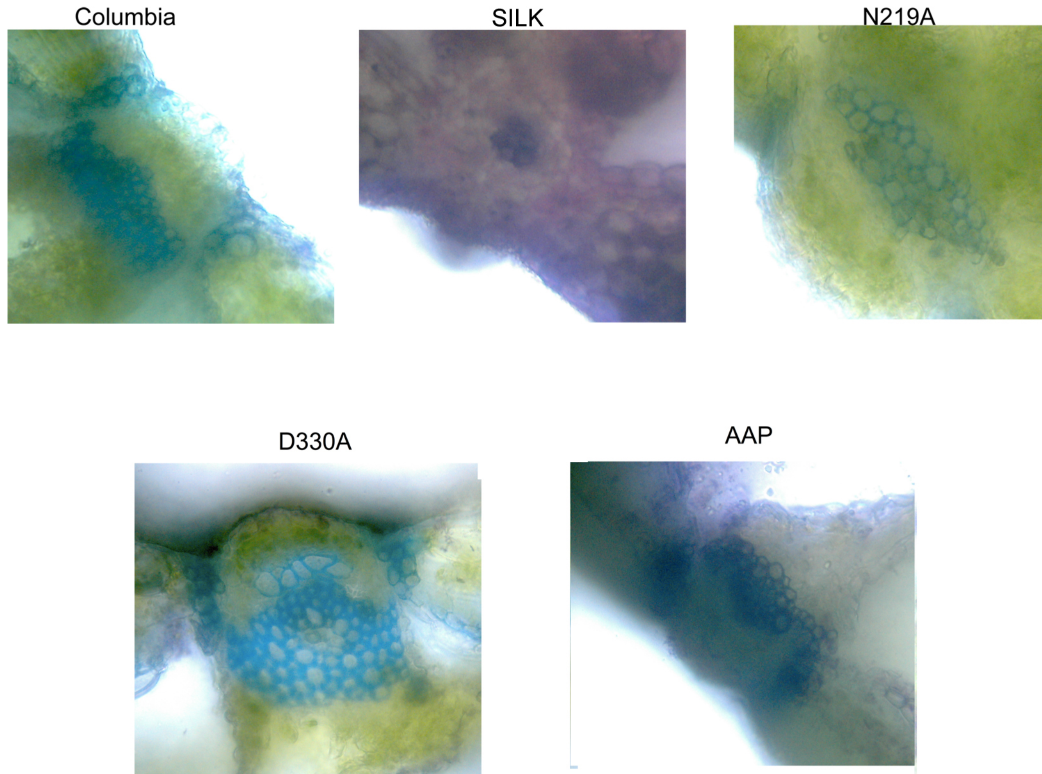



**Figure 4:** Comparison of cross-sections of siliques. Notable is the reduction of the lignified layer in SILK plants, but this reduction is not present in D330A, which maintained the short silique phenotype.

## Supplementary Figures

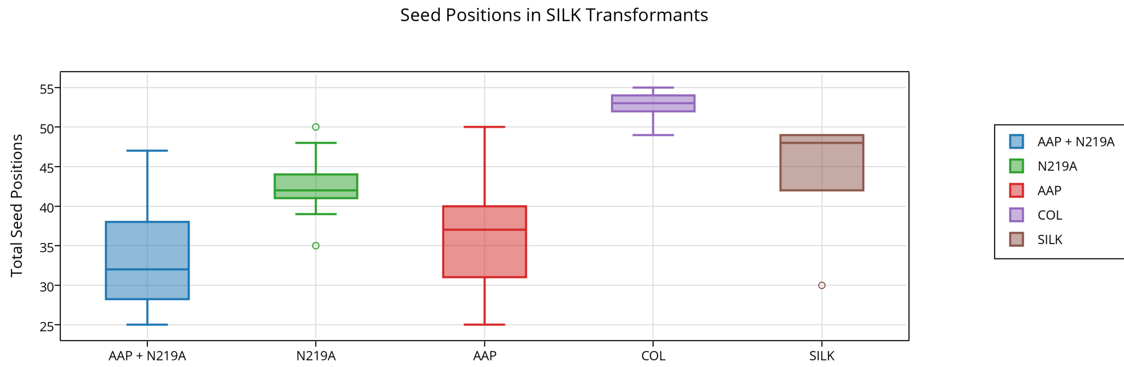



**Figure S1:** Quantification of seed positions in Columbia, SILK, and SILK transformants. Total number of seed positions was variable for all plants, but tended to be higher and less variable in Columbia, and lower in plants with constructs. . The number of siliques used to form averages varied based on the number or lines available. The number of lines for each plant type was as follows: Columbia: 1; RanGAP1: 4; AAP: 5; N219A: 3; AAP + N219A: 6; SILK: 1.

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