UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Study of Downstream Targets and Other Characteristics of *NTT*

A Thesis submitted in partial satisfaction of the requirements

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in

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by

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The thesis of Michelle W. Fung is approved and is acceptable in quality and form for publication on microfilm and electronically:

Chair

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DEDICATION

I would like to dedicate this thesis to my family, especially my parents, for all their support. Without them, I never would have gotten five valuable years of college education. Without their motivation, I never would have gotten into a lab, much less a Masters degree. From the bottom of my heart, thank you! And even though I never say it, “I love you!”

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ABSTRACT OF THE THESIS

The Study of Downstream Targets and Other Characteristics of *NTT*

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Professor Martin Yanofsky, Chair

An important part of the female reproductive tract, enclosed within the gynoecium of angiosperms such as *Arabidopsis thaliana*, is the transmitting tract. It is a major component of the reproduction process, as the majority of the pollen tube growth occurs in the transmitting tract tissues. The transmitting tract region fails to develop in *no transmitting tract (ntt)* mutants, indicating that the *NTT* gene is necessary for specifying the transmitting tract. *NTT* encodes a putative transcription factor that is specifically expressed in the transmitting tract of the developing gynoecium. Here we generated and characterized promoter-GUS fusions of *NTT* and a number of putative downstream targets of NTT, including *HALF FILLED* (*HAF*), At1g30795 and At1g72290. Recently, the *HAF* gene was also shown to encode a transcription factor that plays an important role in formation of the transmitting tract. We therefore also characterized the expression pattern of a gene that is closely related to *HAF* called *At1g18400*, because it likely has

functions that are redundant with *HAF*. We found that all of these promoter-GUS fusions were expressed in the transmitting tract. These studies provide new insights into the possible functions of several previously uncharacterized genes and lay the foundation for future efforts aimed at dissecting the cascade of gene activity that underlies transmitting tract formation.

INTRODUCTION

The process of plant reproduction has evolved dramatically since land plants arose, with the female egg cell being progressively more enclosed and protected. In ancient plants such as ferns, the egg cells are on an open surface where the sperm can freely fertilize it (Figure 1A). Fertilization in gymnosperms occurs when the sperm, transported by the pollen tube, are released to the egg cells that are in the exposed ovules (Figure 1B). In angiosperms, such as *Arabidopsis thaliana*, the egg cells develop inside the ovules that are enclosed within the ovary. The ovary is inside the gynoecium, the female reproductive organ of angiosperms, which functions as a barrier to protect and nourish the developing ovule and subsequent seed. The gynoecium includes a reproductive tract, consisting of the stigma, style, transmitting tract, and funiculus, that facilitates the fertilization the egg cell (Crawford et al., 2008) (Figure 2). The stigma contains papillary cells that form the stigmatic papillae, to which the pollen grain adheres. The stigma-pollen interaction is absolutely necessary to condition the pollen tube for fertilization (Palanivelu et al., 2006). From the stigma, the pollen germinates and the pollen tube grows through the transmitting tract within the style, and continues traveling down the ovary transmitting tract. The transmitting tract produces extracellular matrix (ECM) that contains a combination of polysaccharides, glycoproteins, and glycolipids that are suggested to assist pollen tube movement and nourishment (Lord et al., 2002). As the pollen tube travels down the transmitting tract, it exits onto the septum epidermis, then grows along the funiculus until it reaches the ovules. It then enters the

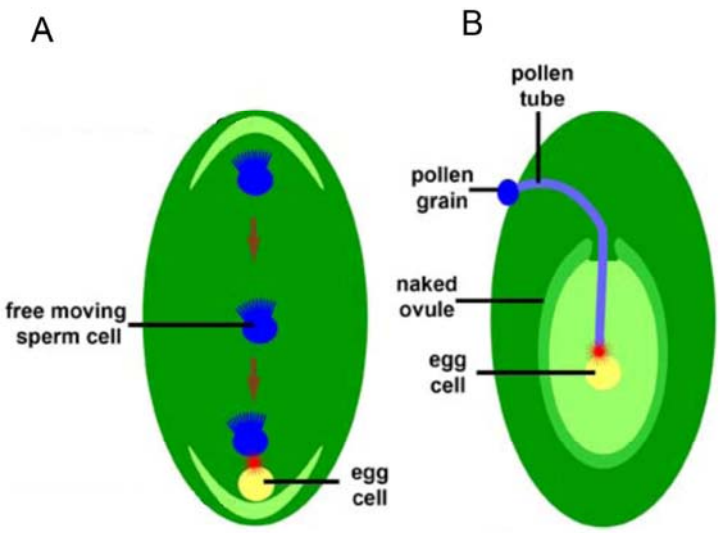

Figure 1. Reproduction process of ferns and gymnosperms

Model diagram of the reproduction process that occurs in (A) ferns and (B) gymnosperms.

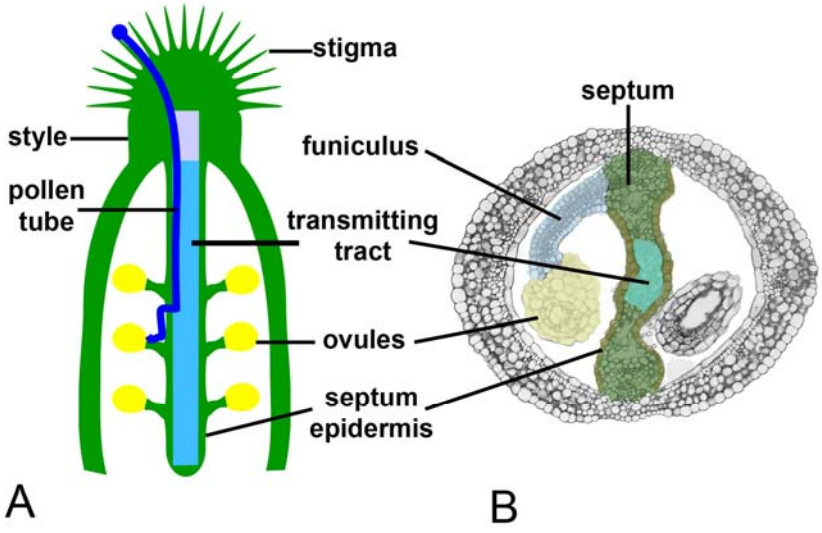

Figure 2. Structure of the Arabidopsis gynoecium

(A) Longitudinal and (B) transverse cross sections of the gynoecium are shown. Different parts of the reproductive tract are labeled.

Crawford et al., 2008

micropyle, an opening in the ovule integuments, in order to reach the embryo sac. The sperm cells are released to one of the two synergid cells. One sperm fertilizes the egg, forming a zygote that develops into the embryo; the second sperm fuses with the central cell, resulting in an endosperm that provides nourishment for the embryo (Yadegari et al., 2004).

The gynoecium, the ovule-producing part of the flower, may consist of one or more carpels. In *A. thaliana*, two carpels are fused to form the gynoecium. The gynoecium begins to form at stage 6 of flower development, and grows as a hollow tube at stage 8. At stage 9, the septum is formed. The stigmatic papillae begins to form at stage 10, and fully covers the stigma at stage 11. At stage 12, the gynoecium is ready for fertilization, in which the different parts of the gynoecium are morphologically distinct. At stages 13 and 14, when the flower self-pollinates and fertilization occurs, the programmed cell death of the transmitting tract also takes place. The purpose of the cell death is to assist the movement of the pollen tube. At stages 15 and 16, the gynoecium continues to elongate until stage 17, when the fruit has completely developed. At this stage, the ovule integuments have undergone changes to develop into the seed coat. The lignified layer of the valve margin becomes lignified, causing the fruit to open to release the seeds (Roeder et al., 2003).

It has been proposed that several genes are involved in the regulation of the development of these female-derived tissues. *SPATULA* and the *HECATE* genes (*HEC1, HEC2, HEC3*) are found to be necessary for correct stigma, septum, and transmitting tract development. The *spt* mutant shows a defect in the septum, the loss of the

transmitting tract, and a decreased development of the stigma. The *hec1 hec3* double mutant has defects in the stigma, style, and interior gynoecium, and *hec1,2,3* RNAi plants are completely infertile with severe defects in stigma and septum development (Gremski et al. 2007). Two closely related genes, *AUXIN RESPONSE FACTOR* (*ARF), ARF6* and *ARF8*, are expressed in the style, transmitting tract, and funiculus, and redundantly regulate the development of the female reproductive tract. The *arf6 arf8* double mutants are female sterile and do not have fully developed stigmatic papillae. The genes are also necessary for anther development, where the *arf6 arf8* double mutants have anthers are defective in releasing pollen (Wu, M.F. et al., 2006). These genes are all involved in a part of the female reproductive tract, therefore a mutation in any of the genes leads to a decrease in fertility.

Another gene that is especially important for the development of the transmitting tract is *NO TRANSMITTING TRACT* (*NTT*). *NTT* is part of the WIP gene family and encodes a transcription factor that contains the C2H2 zinc finger domain. The *ntt* mutant shows a lack of cell death in the transmitting tract, and is also defective in the production of ECM, shown by a lack of Alcian Blue staining of acidic polysaccharides, a major component of the ECM (Crawford et al., 2007). As a result, the pollen tube cannot penetrate to the basal region of the *ntt* mutant gynoecium, and the ovules on the bottom are not fertilized. Therefore the bottom part of the *ntt* mutant fruit does not have seeds.

Cell death and the production of ECM appear to be coordinated, shown with an increase in Alcian staining. Arabinogalactans (AGPs), a type of acidic glycoprotein, is a major component of the ECM and has been suggested to be involved in programmed cell

death in plants (Gao et al., 1999). Therefore, the production of AGPs may be involved in promoting programmed cell death in the transmitting tract.

In addition to being expressed in the transmitting tract, *NTT* is also expressed the developing ovule, but its function was never discovered in this region (Crawford et al., 2007). The ovule has cell layers that eventually undergo cellular changes to form the seed coat (Figure 3). The cell layers of the ovules consist of two integuments, outer and inner. There are two layers of the outer integuments and three layers of the inner integuments, and they undergo differentiation in response to fertilization. The innermost layer, the endothelium, produces condensed tannins that are responsible for the brown color of the eventual seed coat (Dixon et al., 2005). The outermost layer, the epidermal layer, contains cells that produce and secrete mucilage, a pectinaceous carbohydrate, into the apoplast underneath the outer wall of the eventual seed coat. The outer two layers of the inner integuments undergo cell death first, at an early stage of seed development. By seed maturity, the cells of all the layers undergo plant programmed cell death, and all the layers, except the epidermal layer, are crushed together, forming the seed coat (Haughn, et al., 2005).

It has been reported that caspase activity is important in programmed cell death, as it was conserved in plants and animals. The enzyme, δVPE, has been found to have caspase-like activity in plants and is expressed in outer two layers of the inner ovule integument, the layers that undergo cell death the earliest. The δVPE-deficient mutant has delayed cell death of those two layers of the seed coat, with thick cell layers all

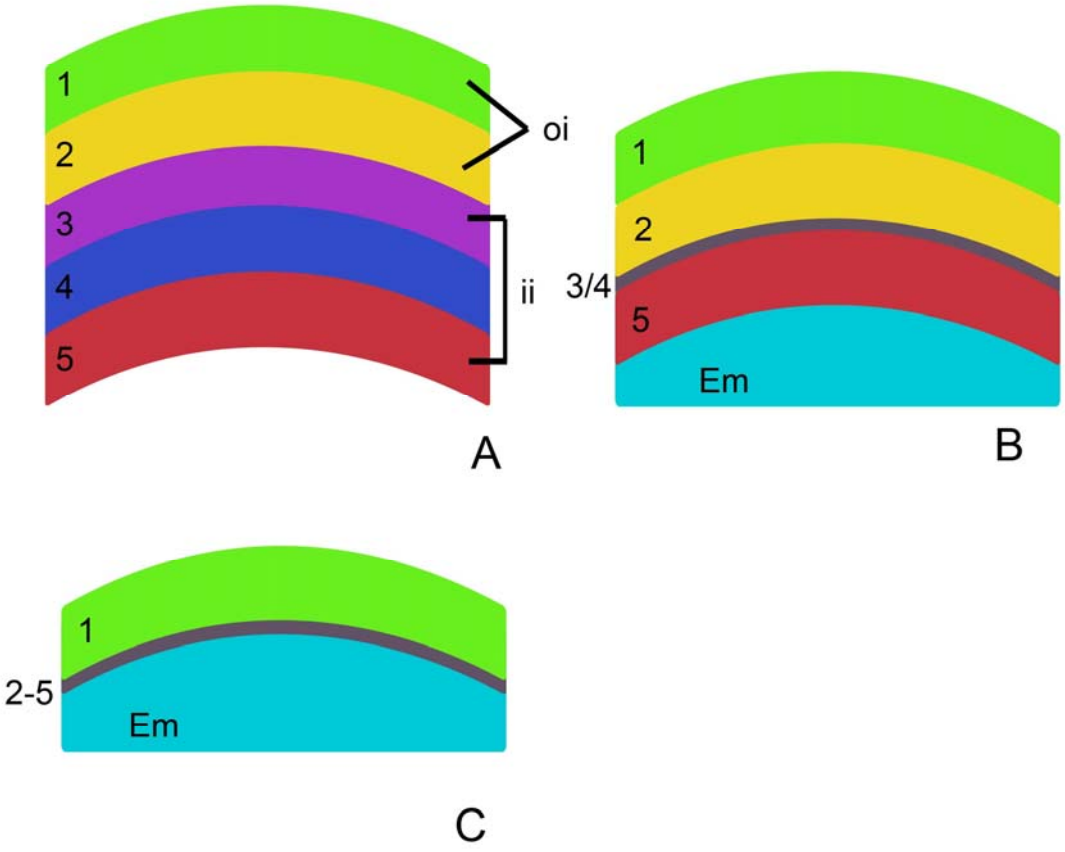

Figure 3. Seed coat development

(A-C) The process of seed coat development from ovule integuments shown. Cell layers 1 and 2 are the outer integuments, and layers 3-5 are the inner integuments.
oi = outer integument ii = inner integument

through development. However, at stage 17, the layers have diminished and the thin seed coat has formed (Nakaune et al, 2005).

Interestingly, *transparent testa 1* (*TT1*), the first gene to be characterized in the six-member WIP gene family, is expressed in all five cell layers, but most strongly in the endothelium. *TT1*, or *WIP1*, is suggested to be involved in the pigmentation of the development in seeds, as the *tt1* mutant in L*er* has seeds that appear yellow rather than brown. This is a result of the lack of condensed tannins in endothelium of the seed coat. The role of *TT1* has not been defined in the transparent testa pathway, as its role in tannin biosynthesis and endothelial cell shape establishment is still uncertain (Debeaujon et al., 2003). Therefore it is proposed that *TT1* may be involved in the differentiation of the endothelium.

The gene, *HAF*, may also play a role in regulating transmitting tract development, as the *haf* mutant in L*er* background has a similar fruit phenotype to the *ntt* mutant (Crawford unpublished data). *HAF*, which encodes for basic helix-loop-helix (bHLH) transcription factor, is also known to be expressed in the transmitting tract, which is consistent with its phenotype. *HAF* has two closely related genes, *Brassinosteroids Enhanced Expression* (*BEE*) *1* (At1g18400) and *BEE3* (At1g73830). The double mutant, *bee1 bee3*, also did not show any major phenotypic differences from Col (Friedrichsen et al., 2002). Brassinosteroids (BR) are polyhydroxylated steroids that are important in the regulation of plant growth and development such as cell elongation in floral organs. They are also found to be involved in enhancing seed germination. *BEE1* and *BEE3* are found to be up-regulated in response to BL, the most active BR. They are early response

genes that are necessary for full BR response. Also, *BEE1* and *BEE3* encode for basic helix-loop-helix (bHLH) transcription factors, which are often associated with developmental processes (Friedrichsen et al., 2002). It is possible that *BEE1* and *BEE3* may function redundantly with *HAF*. Although there has been an analysis of the *BEE1* and *BEE3* genes, no expression data was generated.

Not many genes have been identified that have downstream roles in the development of the transmitting tract. Genes that were up-regulated in the *pi-1* inflorescences, composed of carpel and sepal organs (Sablowski et al., 1998), were identified. These up-regulated genes in this mutant, termed *PUP* (*pistillata-up-regulated*), are anticipated to be up-regulated in the wild-type gynoecium. Two genes identified using this method, *PUP5* and *PUP3*, were of particular interest. *PUP5* (At1g72290), a trypsin and protease inhibitor family protein, was identified by in situ as being expressed in the transmitting tract within the style and septum (Scutt et al., 2003). According to its expression pattern, *PUP5* is a possible downstream target of *NTT* and *HAF*.

PUP3 encodes a hydroxyproline-rich glycoprotein and in situ hybridization reveals expression of this gene in the epidermis of the septum (Scutt et al., 2003), which is where the pollen tube grows to fertilize the ovules. Glycoproteins have been found to be involved in transmitting tract movement, as shown with *TTS*, a gene found in the tobacco that also encodes a glycoprotein. It was found to be a downstream gene of transmitting tract function. The TTS glycoprotein was found in the transmitting tract to attract pollen tube and enhance its growth by providing a guidance of the pollen tube. It

was proposed that the gradient of the glycosylation of the sugars attached to TTS may be the mechanism for the guidance (Wu H.M. et al., 2005). In addition to *PUP3*, another gene in the *Arabidopsis* genome that encodes a glycoprotein is At1g30795, which is a homolog of *PUP3*. Therefore, it is important to investigate the expression pattern of At1g30795, as it may have redundant functions with *PUP3* in the role of pollen tube growth.

In this work, we created a GUS expression construct for *NTT*, to further investigate its role in the formation of the transmitting tract and ovules. Different stages of the fruit were checked to observe exactly when the promoter of the gene is active. The function of *NTT* in the ovule was examined by making sections of *ntt* mutant ovules. We also investigated the possibility of redundancy of *ntt* its homolog, *TT1*, by observing the cell layers of the ovule in the double mutant. The GUS constructs were made of the *HAF* homologs, *BEE1* and *BEE3*, to check their expression profile in relation to *HAF*. We also made GUS constructs for the potential downstream targets of *HAF* and *NTT*, At1g72290 and At1g30795, to investigate their role in the development of the transmitting tract.

MATERIALS AND METHODS

Strategy for Cloning

To create transcriptional beta-glucuronidase (GUS) reporters, the promoter of the genes At1g30795, At1g72290 (*PUP5*), At1g18400 (*BEE1*), and At1g73830 (*BEE3*) were cloned into the vector pDW294, which contains a minimal CaMV 35S promoter used to drive transcription of the GUS gene (Hong et al., 2003). The promoter fragment of each gene was amplified and isolated using phusion PCR, with restriction sites added to the ends of both the 5' and 3' primers (Table 1 for sequence of oligonucleotides). The fragments were then subcloned into the pCR2.1 TOPO vector, cut out with the relevant restriction enzymes, and ligated into the final vector, pDW294 (Table 1 for restriction enzymes used and the sizes of the promoter fragments).

The *NTT::GUS* construct is a translational GUS reporter line created using the vector pJJGUS (Ripoll et al., 2006). It includes a 6.0 kb fragment, which includes 3.8 kb of the promoter, both exons, and the intron of the *NTT* gene, At3g57670. The construct was made by first amplifying the fragments ntt 9-/13+ and ntt 27-/26+ of the gene and separately cloning them into pTOPO. Then the two fragments were excised with the restriction enzymes SwaI and NotI and ligated together, forming the final 6.0 kb fragment. This fragment is cut out of pTOPO by the restriction enzyme ApaI and inserted into the GUS vector, pJJGUS. See Table 2 for the size of the fragments and the sequence of the oligonucleotides used. Refer to Appendix A for the constructs we tested that were no successful.

Refer to Appendix A for a summary of the all GUS contructs.

GUS Staining

The tissues were prefixed in acetone for 20 minutes before being infiltrated in staining solution (0.2% Triton-X-100, 50 mM sodium phosphate buffer pH 7.2, 10 mM potassium ferrocyanide, 10 mM potassium ferricyanide, 1 mM X-gluc) on ice for 15 minutes (Session et al., 1999). The whole-mounts were taken with a standard dissecting scope. For cross sections, the samples were then fixed in FAA for 2.5 hours, taken through an ethanol and Histoclear series, embedded in Paraplast Plus, and sectioned at 8 μm. Slides were prepared and viewed as described (Roeder et al., 2003).

JB-4 Embedding and Alcian Blue, Nuclear Fast Red, Neutral Red Staining

The tissues were fixed and embedded either as described (Crawford et al., 2007), or with the fixative of 4F1G (McDowell, 1976). The 3 μm plastic sections of tissue samples were then stained using one of two methods. The first method stains with Nuclear Fast Red (0.1 g Nuclear Fast Red, 2.5 g Aluminum Sulfate, 100 ml H_2O) for 30 minutes then with Alcian Blue (0.5 g Alcian Blue 8GX, 0.5 ml Glacial Acetic Acid, 100 ml H_2O) for 30 minutes (Crawford et al., 2007). The other method is to stain with Alcian Blue for 60 minutes then with Neutral Red (0.1 g Neutral Red, 100 ml H_2O) for 15 seconds (Alvarez et al., 2002). Slides were prepared and viewed as described (Crawford et al., 2007).

Ruthenium Red Staining

Seeds were placed on a petri dish with water for imbibing, then were stained by adding 0.2% w/v aqueous ruthenium red (Sigma) solution (Arsovski et al., 2009).

Phloroglucinol Staining

Wax sections of tissue samples were taken through a histoclear and ethanol series, stained with phloroglucinol staining solution (2% in 95% ethanol) for 2 minutes, and soaked in 50% HCl. Slides were prepared and viewed as described (Liljegren et al., 2004).

Plant Transformation

To generate the transgenic lines, the floral dip method was performed with Columbia (Col) plants (Weigel and Glazebrook, 2002). The transgenics were selected on either kanamycin for the vector, pDW294, or hygromycin for the vector, pJJGUS.

Table 1. List of size, restriction enzymes, and oligonucleotides used to create GUS reporter constructs

The 5' and 3' oligonucleotides listed were used to generate 4 lines of GUS reporter constructs. For each construct, the promoter fragment was excised with restriction enzymes and cloned into pDW294 containing the GUS reporter gene. The size of the fragments are also listed.

Gene	5' primer	3' primer	Size (kb)	Restriction Enzyme
At1g30795	ttggatgtagaatccttgctc	ggttcatgtgttgtaggatc	1.8	HindIII + BamHI
At1g72290	atttgttctcgtagtaattaac	ttgtgattgttatgtgtg	1.1	HindIII + BamHI
At1g18400	acctagagagggaccgtaac	aatataatgaattgagatatg	1.4	HindIII + BamHI
At1g73830	ccaatcaacatcacaacagagacg	ttctgagtttcaatttttatttttttttgaaaattgg	2.6	PstI + BamHI

Table 2. List of size and oligonucleotides used to create *NTT::GUS* reporter line

The 5' and 3' oligonucleotides listed were used to generate the two fragments of the *NTT* gene that were combined to make the ntt GUS reporter line.

Fragment of *NTT*	5' primer	3' primer	Size (kb)
9-/13+	ccaatataggtcgaactcgaatagc	TTAGACTGCATTGACTCATGATCATTGTC	3.5
27-/26+	agcctcttgtataaggccgaag	gtaaggcttaatagcacaggaac	2.7

RESULTS

Seed Size Phenotype

The *ntt* mutant has seeds that are larger than wild-type Col seeds (Figure 4). One potential explanation is that *NTT* has a role in seed development. Alternatively, the seed size difference could be a result of *ntt* mutants having fewer seeds per silique. To test this hypothesis, minimal and maximal pollination were performed on wild-type and *ntt* mutants to examine the effect on seed size. For wild-type plant, it was found that the seeds from minimal pollination were bigger compared to seeds from maximal pollination (Figure 5A, 5B). In addition, when minimal pollination was performed for Col and *ntt* plants, and the resulting seeds have similar size (Figure 5C, 5D). These seeds also have a larger size than the wild-type seeds from maximal pollination.

Indehiscence of *ntt* mutant

The fruits of the *ntt* mutant appear to have an indehiscent phenotype, as they do not seem to break open to release their seeds when compared to wild-type fruit (Figure 6). During wild-type fruit development, dehiscence is caused by the development and subsequent lignification of valve margin cells. Lignification can be observed by phloroglucinol staining of wax sections of the fruit. To determine if lignification was altered in *ntt* mutants, stage 17 fruits of Col and the *ntt* mutant were compared to observe any differences. At first, it was observed that a difference at the proximal region of the fruit between wild-type and *ntt* mutant was seen in the lignification of the valve margin

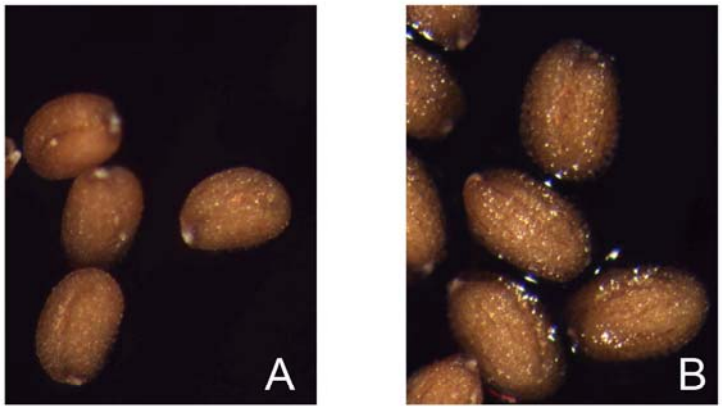

Figure 4. Seed size of wild-type and *ntt* mutant

Typical seed size of (A) wild-type and (B) *ntt* mutant.

Figure 5. Seed size phenotype of *ntt* mutant

(A) Maximum and (B) minimum pollination of wild-type seeds, showing seed with minimum pollination is larger. Minimum pollination of (C) wild-type and (D) *ntt* mutant seeds, showing the seeds having similar size.

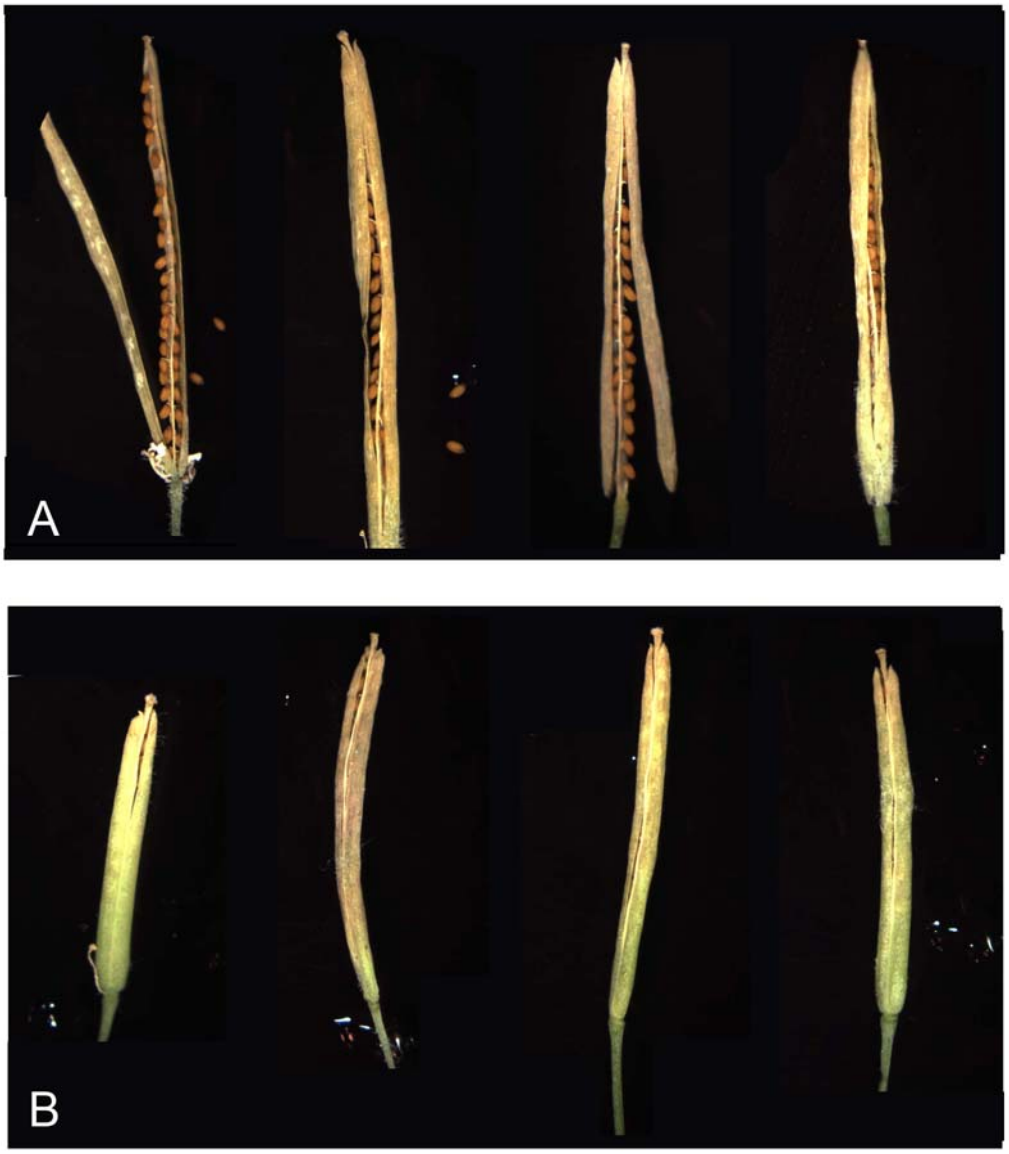

Figure 6. Fruit series of wild-type and *ntt* mutant

Examples of stage 17 fruits of (A) wild-type and (B) *ntt* mutant.

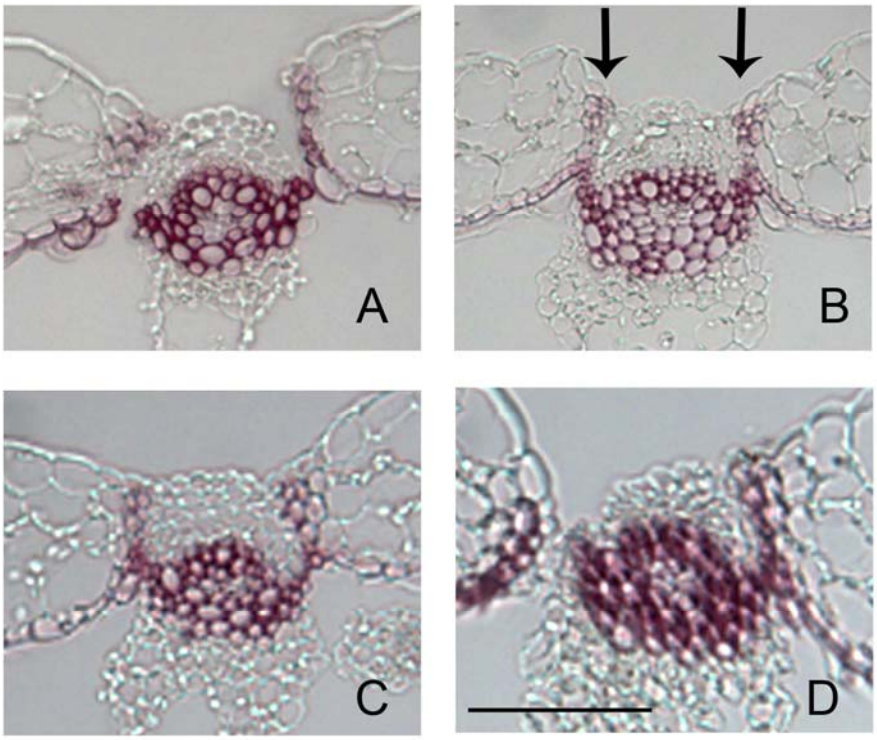

Figure 7. Indehiscence of *ntt* mutant

Cross sections of fruits with phloroglucinol staining of stage 17 fruit of (A) wild-type and (B) *ntt* mutant, showing a difference in the lignification in the valve margin cells. But (C) *ntt* stage 17 proximal, and (D) *ntt* stage 17 distal, when compared with (A), did not show a difference in the lignification of the valve margin cells. Arrows indicate valve margin. Scale bar represents 50 μm.

cells (arrows in Figure 7A, 7B). However, when examining more closely by looking at the distal region as well as the proximal region of stage 17 *ntt* fruits, little or no difference in the lignification of valve margin cells was seen when compared with stage 17 wild-type fruit (Figure 7A, 7C, 7D).

Reporter GUS Activity by Promoter of Downstream Targets of *ntt* and *haf* mutants

The GUS reporter line, *72290::GUS*, is a transcriptional GUS construct that contains 1.1 kb fragment of the promoter region of the gene At1g72290. At1g72290 encodes a protease inhibitor protein and is highly down-regulated in both the *ntt* and *haf* mutant. Understanding the expression pattern of this may indicate a potential role for At1g72290 in a pathway for transmitting tract development. In the wild-type background, GUS expression was observed exclusively in the whole transmitting tract that traveled through the style and the septum in both the pre-fertilized and post-fertilized gynoecium (sideways V and arrow in Figure 8A, 8B). When the cross sections of the inflorescence were examined, showing different stages of the gynoecium, GUS expression throughout the whole transmitting tract was seen in as early as stage 12 and continued on through stage 14 (Figure 9A-D). However, the staining was not seen in the epidermal layers of the septum (Figure 9). Also, in agreement with previous results, GUS activity was seen in the transmitting tract in the style of a pre-fertilized gynoecium as well (Figure 9A).

In the *bee3* mutant, At1g72290 GUS expression was noticeably reduced compared with wild-type in the transmitting tract in the pre-fertilized and post-fertilized

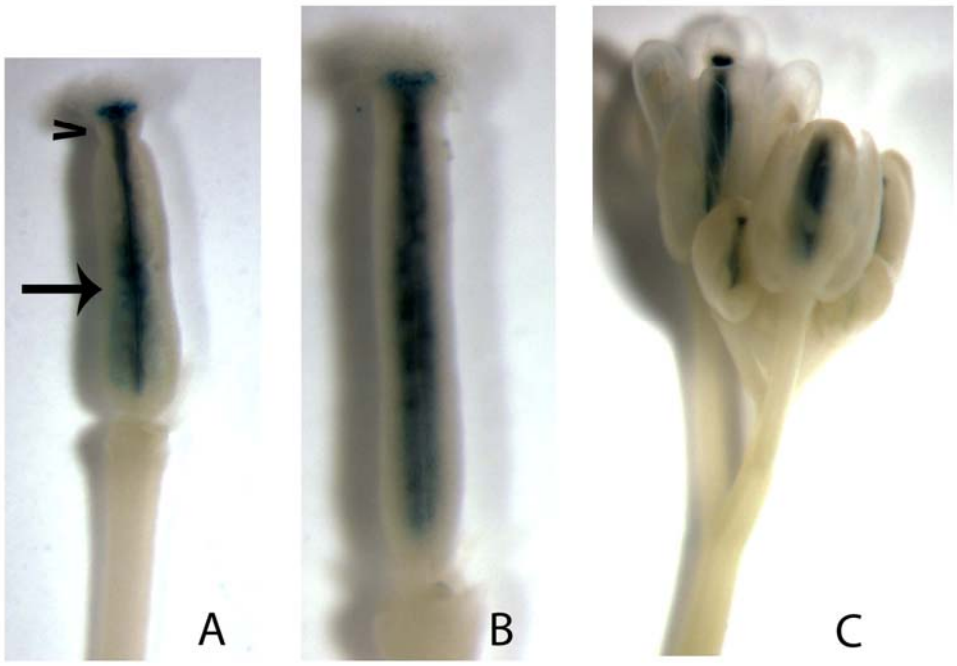

Figure 8. ***72290::GUS*** **expression pattern in wild-type gynoecium (whole-mount)**

(A-C) GUS expression is shown in the transmitting tract of wild-type gynoecium at (A) pre-fertilization, (B) post-fertilization, and (C) inflorescence. Arrow indicates transmitting tract within the septum, sideways V indicates transmitting tract within the style.

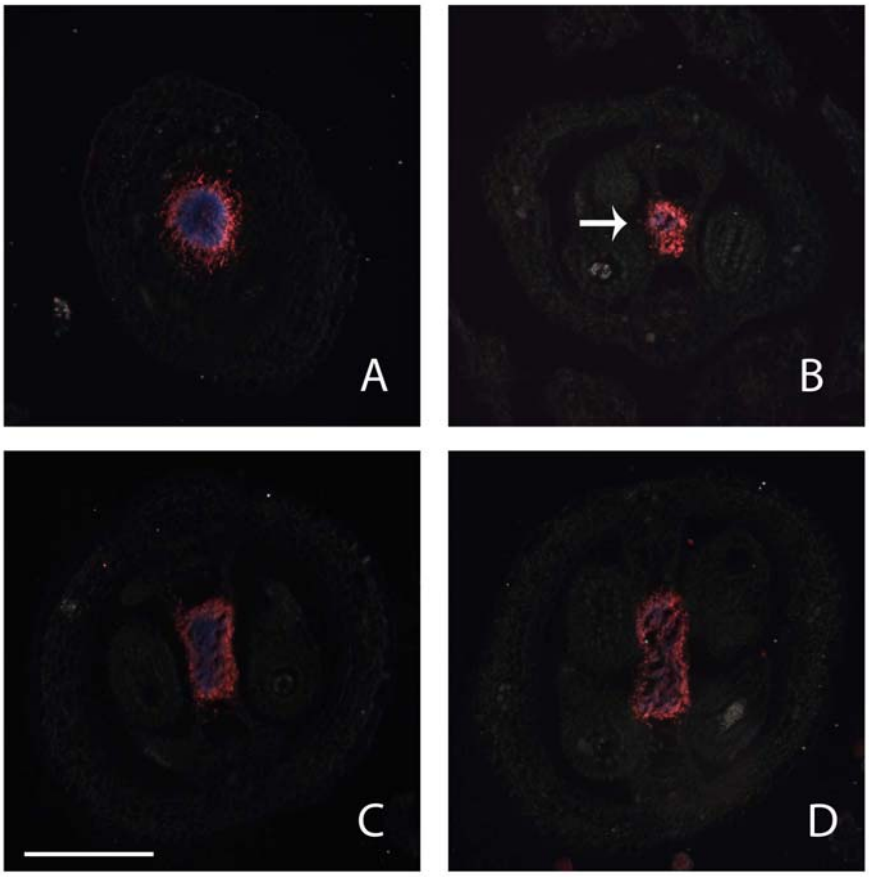

Figure 9. *72290::GUS* expression pattern in wild-type gynoecium (cross section)

(A-D) Cross sections of the infloresence, showing (A) the transmitting tract within the style at stage 12, and (B-D) different stages of wild-type gynoecium. Developmental stages (B)12, (C) 13, and (D) 14 were used. Arrow indicates transmitting tract. Scale bar represents 50 μm.

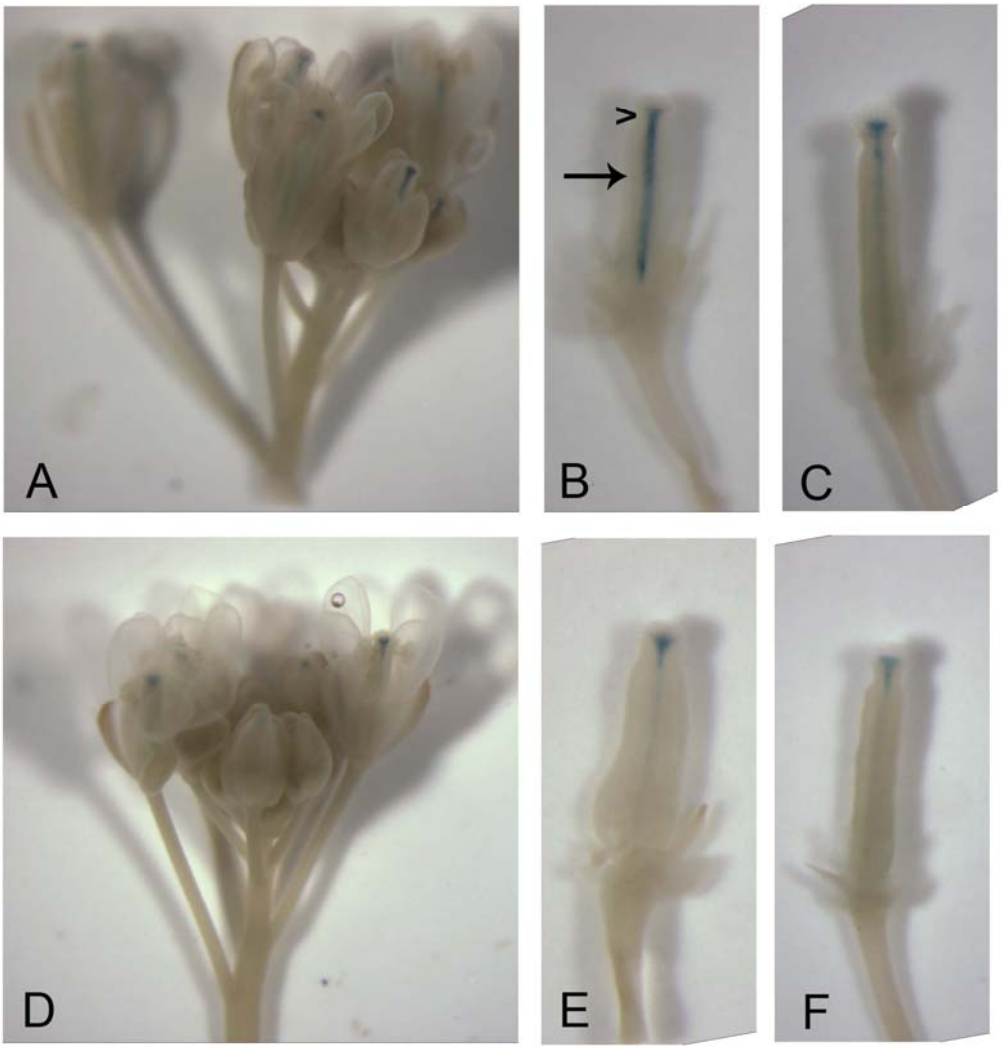

Figure 10. ***72290::GUS*** **expression pattern in** ***bee3*** **mutant and** ***haf bee1*** **double mutant**

GUS expression in (A-C) *bee3* mutant and (D-F) *haf-/- bee1 -/- bee3 +/-* double-heterozygous mutant background shown in gynoeciums at (B and E) pre-fertilization and (C and F) post-fertilization. Arrow indicates transmitting tract within the septum, sideways V indicates transmitting tract within the style.

gynoecium (Figure 8A-C, 10A-C). In *haf bee1 bee3* +/- double-heterozygous mutant, the expression in the transmitting tract was further reduced with residual GUS activity still observed within the style and almost completely absent within the septum (Figure 8A-C, 10D-F).

The GUS reporter line, *30795::GUS*, contained a 1.8 kb promoter fragment of the gene, At1g30975. This gene encodes a hydroxyproline-rich glycoprotein that is highly down-regulated in the *haf* mutant but only slightly in the *ntt* mutant. The GUS activity slightly differed in the pre-fertilized and post-fertilized of the wild-type gynoecium. GUS activity was observed in region to become the transmitting tract in the pre-fertilized gynoecium (arrow in Figure 11B), while in addition to the transmitting tract within the septum, the post-fertilized gynoecium also showed some but not strong GUS expression in the stylar transmitting tract and the funiculus (sideways V and asterisk in Figure 11C). The cross sections of the wild-type inflorescence showed that at the pre-fertilization stage of 11, there was GUS expression in the septum where the transmitting tract will form (arrow in Figure 12A), and at the post-fertilization stage of 14, GUS expression was mainly observed in the epidermis of the septum, transmitting tract, and funiculus (sideways V in Figure 12B).

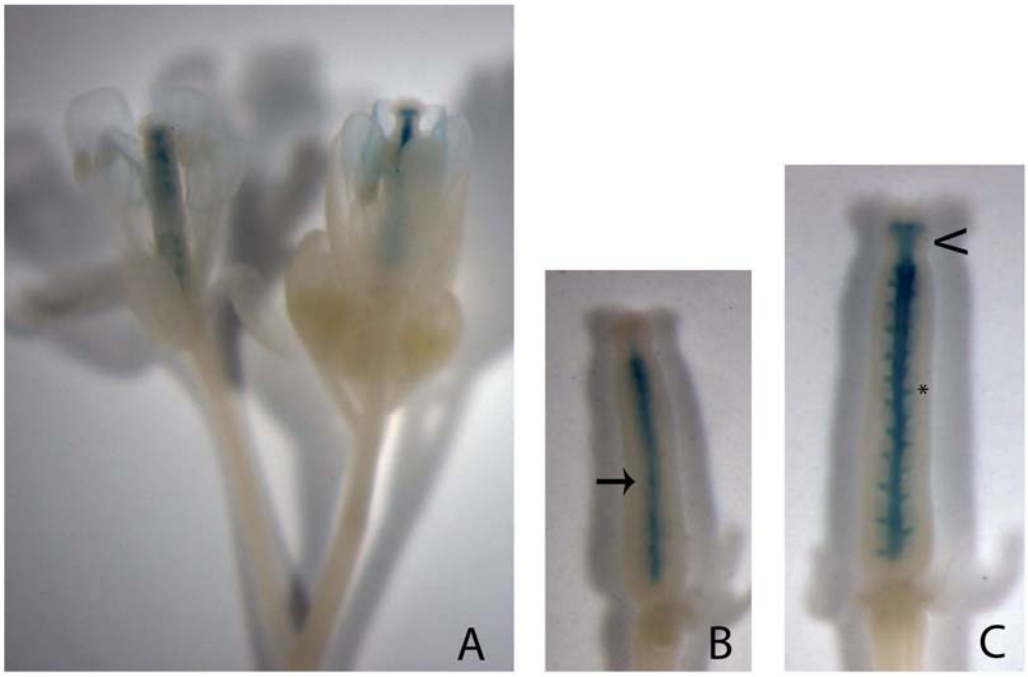

Figure 11. ***30795::GUS*** **expression in wild-type gynoecium (whole-mount)**

GUS expression shown in, (A) inflorescence, (B) transmitting tract of the pre-fertilized gynoecium, and (C) transmitting tract and funiculus of the post-fertilized gynoecium. Arrow indicates transmitting tract within the septum, sideways V indicates transmitting tract within the style, asterisk indicates funiculus.

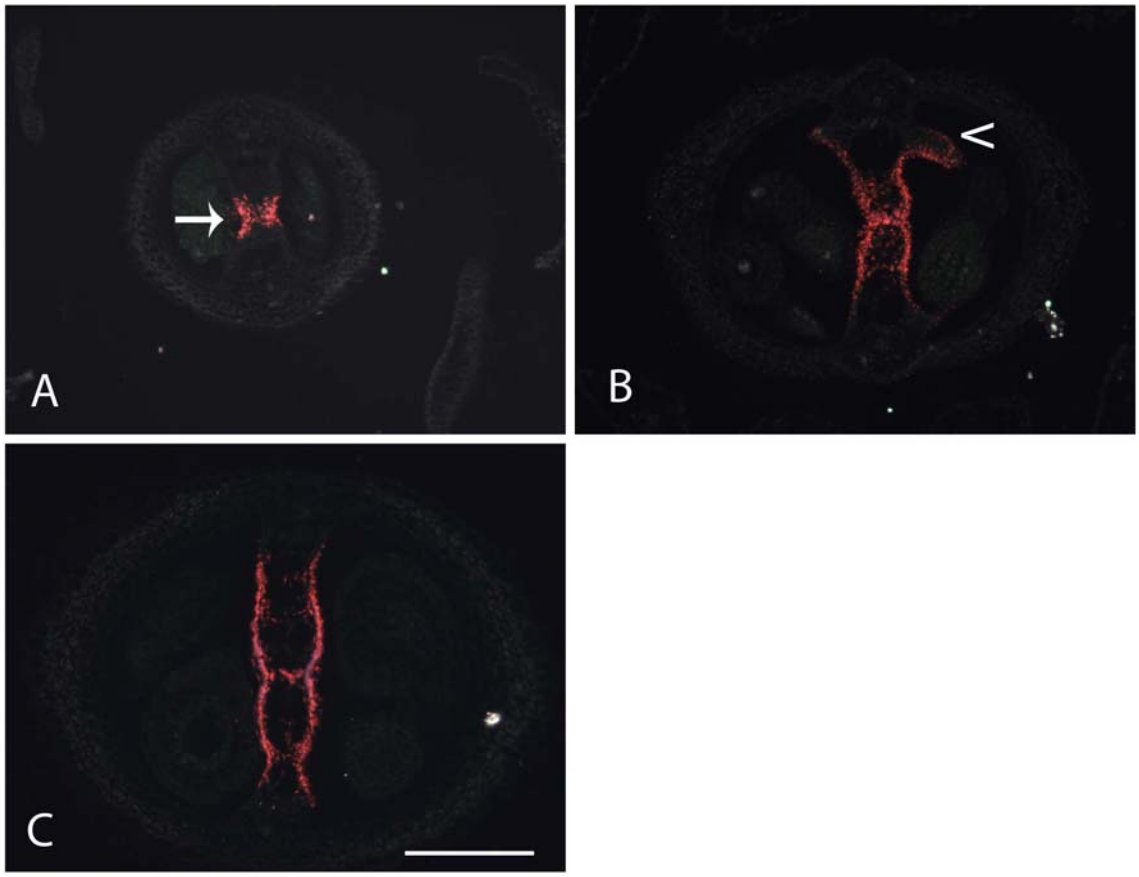

Figure 12. ***30795::GUS*** **expression in wild-type gynoecium (cross section)**

(A-C) Cross sections of the infloresence showing different stages of wild-type gynoecium. Developmental stages (A) 11, (B) 14, and (C) 15 were used. (A) Arrow indicates septum. (B) Sideways V indicates funiculus. Scale bar represents 50 μm.

Reporter GUS Activity by Promoter of At1g18400 (*BEE1*), Homolog of *HAF*

The GUS reporter line, *18400::GUS*, is also a transcriptional GUS construct, and it contains 1.4 kb of the promoter region of the gene At1g18400. This gene is homologous to the *HAF* gene, and because the *haf* mutant does not have a phenotype in Col, GUS expression pattern of *BEE1* and *HAF* were compared to see whether these genes act redundantly. When examining the 24 transformants for this reporter line, GUS expression was observed in the stigma and transmitting tract for all samples, but the expression pattern in the stamens varied. The majority of the samples showed strong GUS expression in the stigma, so the expression in the stigma was used as a reference to calculate the percentage of gynoecia with expression in the stamens (Table 3). In the wild-type background, strong GUS expression was observed in the stigma, including the stigmatic papillae, and the stylar transmitting tract, and less strongly in the transmitting tract in the septum of the pre-fertilized gynoecium (arrows in Figure 13A). Similar expression was seen in the post-fertilized gynoecium, but with expression also in the stamens (arrow in Figure 13B). Cross sections of the wild-type gynoecium showed that GUS expression in the transmitting tract was seen in stages as early as 10 (Figure 14A) and on through 15, where the expression is only faintly present (arrow in Figure 14C).

In the GUS reporter line, *HAF::GUS* (Crawford unpublished data), strong expression was observed in the transmitting tract in the septum and funiculus, with lower expression in the stigma and transmitting tract in the style in the pre-fertilized and post-fertilized gynoecium (Figure 15A-C). Expression in the stigmatic papillae was also

Table 3. Varied GUS expression pattern of *18400::GUS*
List shows the percentages of transformants with GUS expression in certain parts of the wild-type carpel.

Expression level	Locale	Percentage (%)
Strong	Stigma	71
Strong	Stigma + stamens	21
Normal	Stigma	100
Normal	Stigma + stamens	62.5

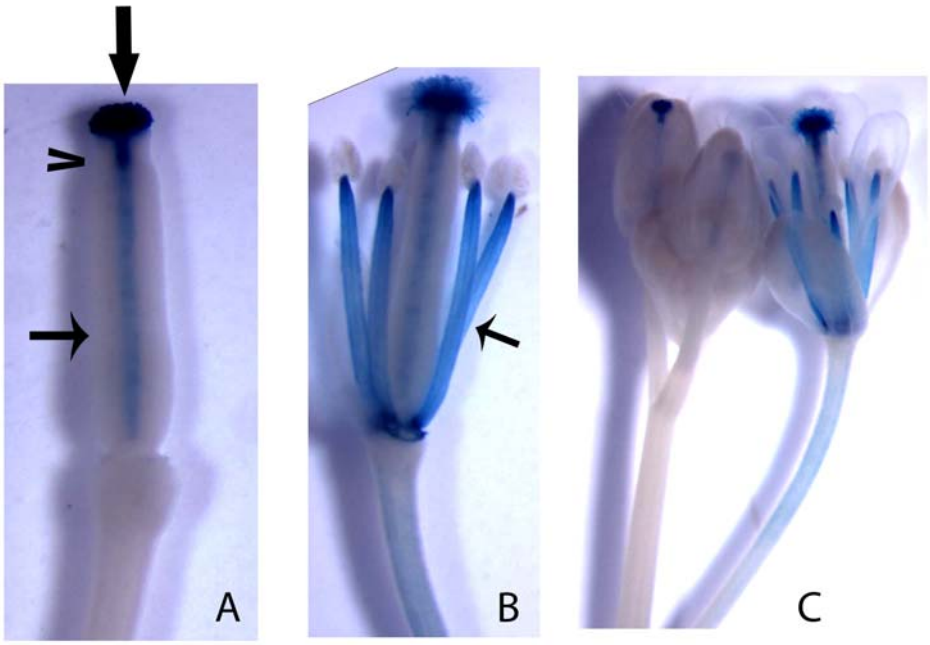

Figure 13. *18400::GUS* expression pattern in wild-type gynoecium (whole-mount)

(A-C) GUS expression is shown in the stigma, transmitting tract, and stamens of wild-type gynoecium at (A) pre-fertilization, (B) post-fertilization, and (C) inflorescence. (A) Thick arrow at the top indicates stigma, sideways V indicates transmitting tract within the style, and thin arrow at the bottom indicates transmitting tract within the septum. (B) Arrow indicates stamens.

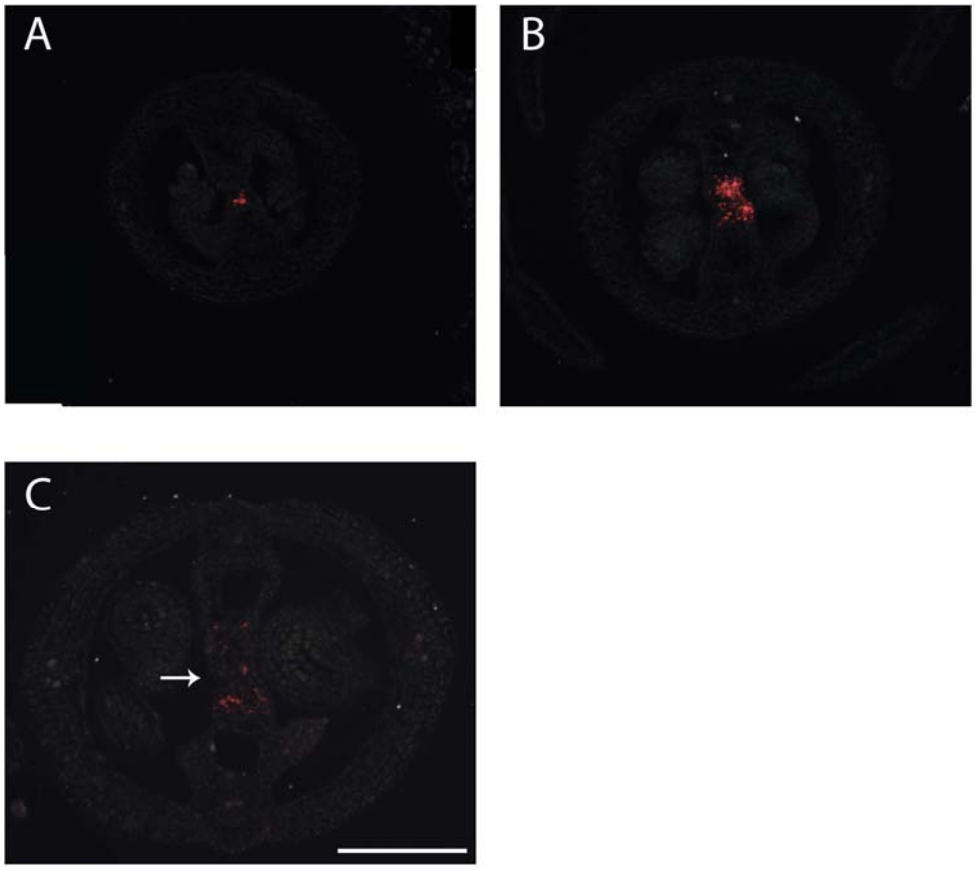

Figure 14. ***18400::GUS*** **expression pattern in wild-type gynoecium (cross section)**

(A-C) Cross sections of the inflorescence, showing different stages of wild-type gynoecium. Developmental stages (A) 10, (B) 14, and (C) 15 were used. Arrow indicates transmitting tract. Scale bar represents 50 μm.

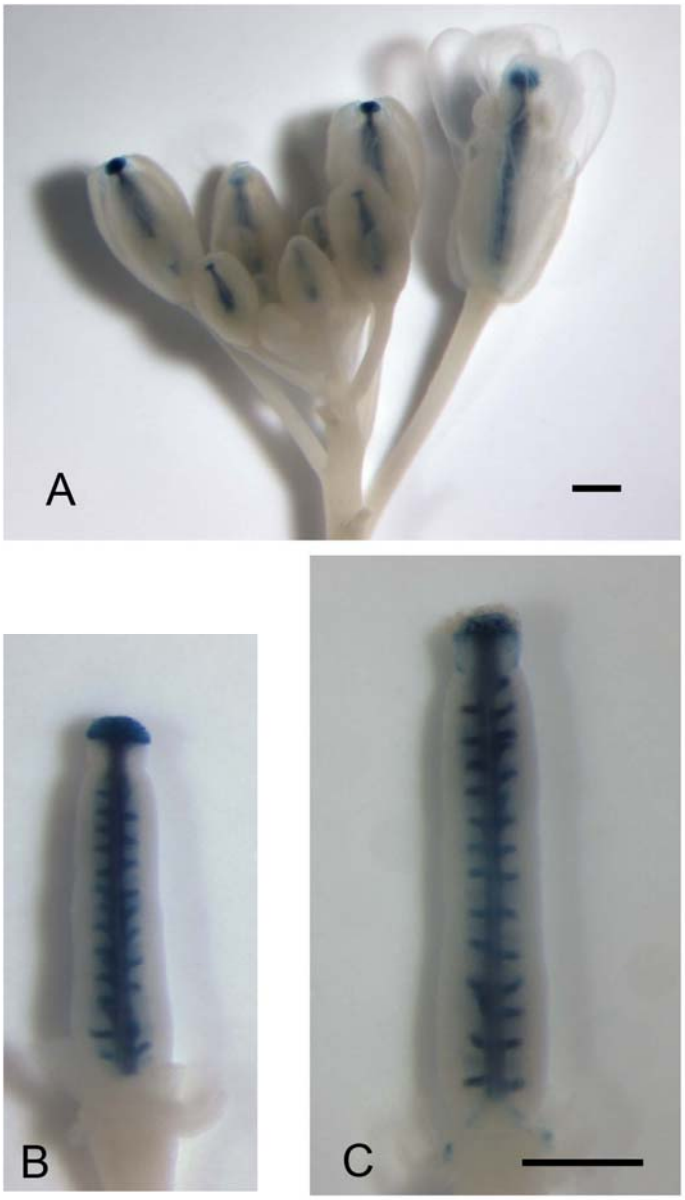

Figure 15. *HAF::GUS* expression pattern in wild-type gynoecium

(A-C) GUS expression is shown in the stigma, style, transmitting tract, and funiculus in (A) inflorescence, (B) pre-fertilized gynoecium, and (C) post-fertilized gynoecium.

absent. When comparing the pattern of *HAF::GUS* and *18400::GUS*, an overlap and complementation of GUS expression was observed.

Another gene that is a homolog of *HAF* is At1g73830 (*BEE3)*, and a GUS reporter line containing the 2.6 kb promoter region of the gene was also made but not yet available for staining.

Reporter GUS Activity of *NTT* gene (At3g57670)

The GUS reporter line, *NTT::GUS*, is a translational GUS construct that contains the two exons, the intron, and 3.8 kb upstream of the ATG translation initiation site of the *NTT* gene. The GUS expression was not observed until the samples were incubated for 2-3 days, suggesting that the expression was very weak.

From whole-mounts, GUS expression was observed in the transmitting tract in the pre-fertilized gynoecium (Figure 16B), but absent in post-fertilized gynoecium (Figure 16C). Similarly shown in cross sections, GUS expression was seen in the transmitting tract beginning in stage 9 and continued through stage 12 (Figure 17A-E). At stage 14, after fertilization, the expression was no longer present (arrow Figure 17F). Expression in the funiculus was not observed at any stage (Figure 16, 17, and 18).

The GUS expression in ovules was not seen in pre-fertilized gynoecium, but was strongly observed in post-fertilized ovules in the whole-mounts (Figure 16B, 16C). However, at the pre-fertilization stage of late stage 12 gynoecium, there was GUS activity observed the early ovules. It was only present in the most proximal region in

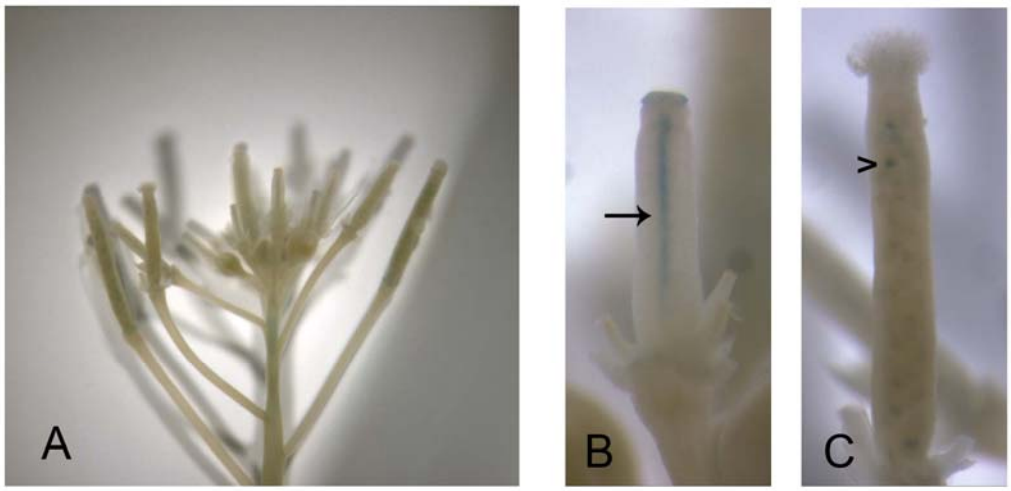

Figure 16. ***NTT::GUS*** **expression pattern of wild-type gynoecium**

GUS expression shown in, (A) inflorescence, (B) transmitting tract of the pre-fertilized gynoeciums, and (C) ovules of the post-fertilized gynoeciums. Arrow indicates transmitting tract, sideways V indicates ovule.

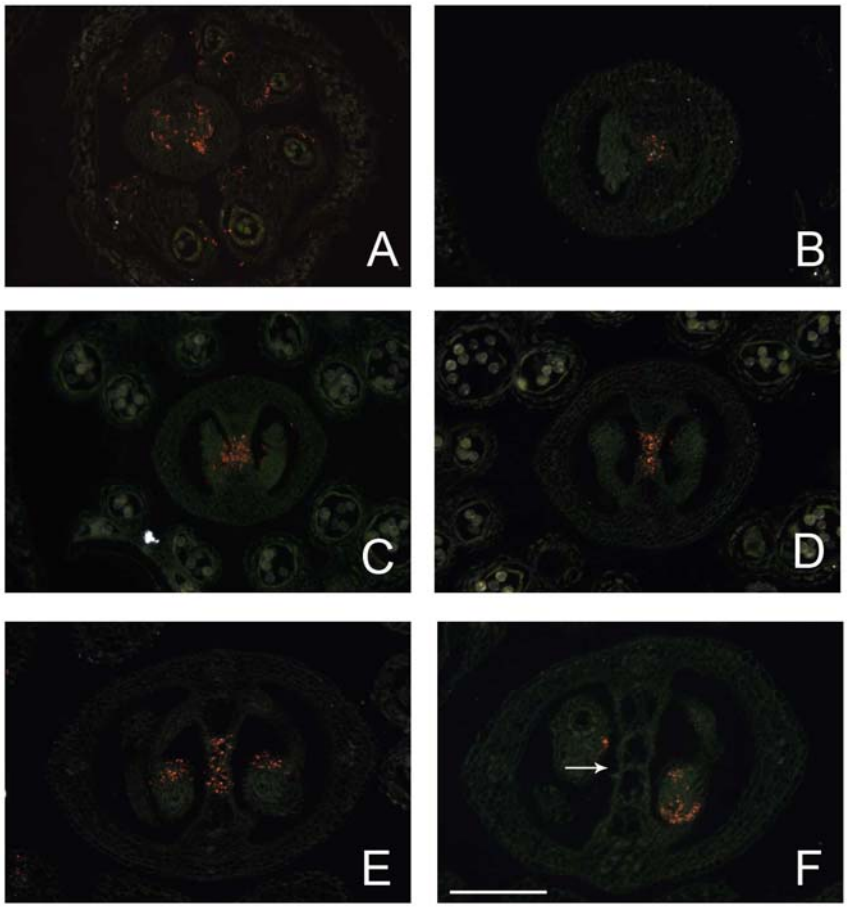

Figure 17. *NTT::GUS* expression pattern in of wild-type transmitting tract

(A-F) Cross sections of different stages of wild-type gynoecium. Developmental stages (A) 9, (B) 10, (C) 11, (D) early 12, (E) late 12, and (F) 14 were used. Arrow indicates transmitting tract. Arrow indicates transmitting tract. Scale bar represents 50 μm.

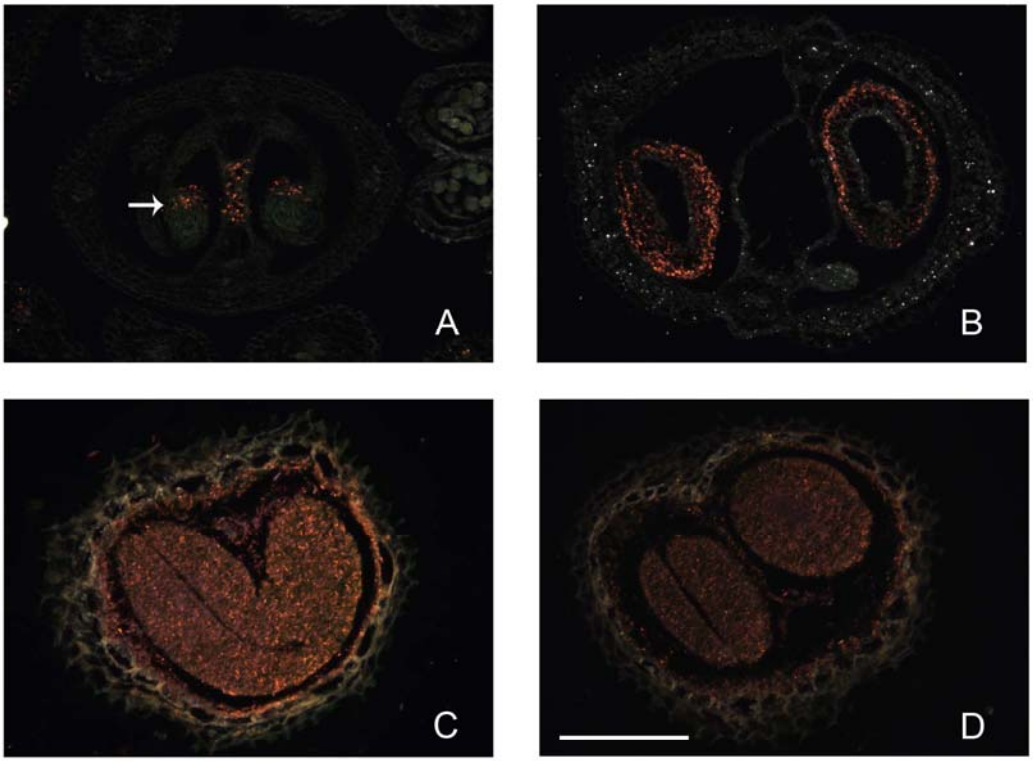

Figure 18. *NTT::GUS* expression pattern in of wild-type ovules/embryos

(A-D) Cross sections of different stages of wild-type gynoecium. Developmental stages (A) late 12, (B) 15, (C) early 17, and (D) late 17 were used. Arrow indicates ovule. Scale bar represents 50 μm.

relation to the funiculus (arrow in Figure 18A). After fertilization at stage 15, the expression was strong in the outermost layer of the integuments, with lower expression in the inner cell layers (Figure 18B). At early stage 17, expression was had expanded to the whole embryo and was observed in the inner layer of the integuments (Figure 18C), but was reduced by late stage 17 (Figure 18D).

The GUS expression was not observed until the samples were incubated for 3 days, suggesting that the expression was very weak.

Role of *NTT* in ovules and seed development

The expression of *NTT* was observed in the cell layers of the developing ovule and seed. One known role for *NTT* in the transmitting tract is to promote cell death. Cell death is a known phenomenon in the development of the five cell layered integuments to the two cell layered seed coat. We therefore examined the cell layers in seed development to investigate whether cell death of the cell layers in seeds was affected in *ntt* mutants. Cross sections of fruit samples of different stages were taken from wild-type and *ntt* plants and stained with Alcian Blue to compare and analyze the cell death that occurs. For choosing different stages, fruits were taken 5, 10, and 20 down from the first opened flower. Therefore when comparing wild-type and *ntt* mutant, the stages were more similar and less ambiguous. The wild-type and *ntt* fruit taken 5 fruits down from the first opened flower still clearly had 5 cell layers (Figure 19A, 19C). In the later stage of 10 fruits down, the cell layers of the wild-type fruit showed strong Alcian Blue staining, while in the *ntt* fruit, there were still 5 distinct cell layers and they did not have

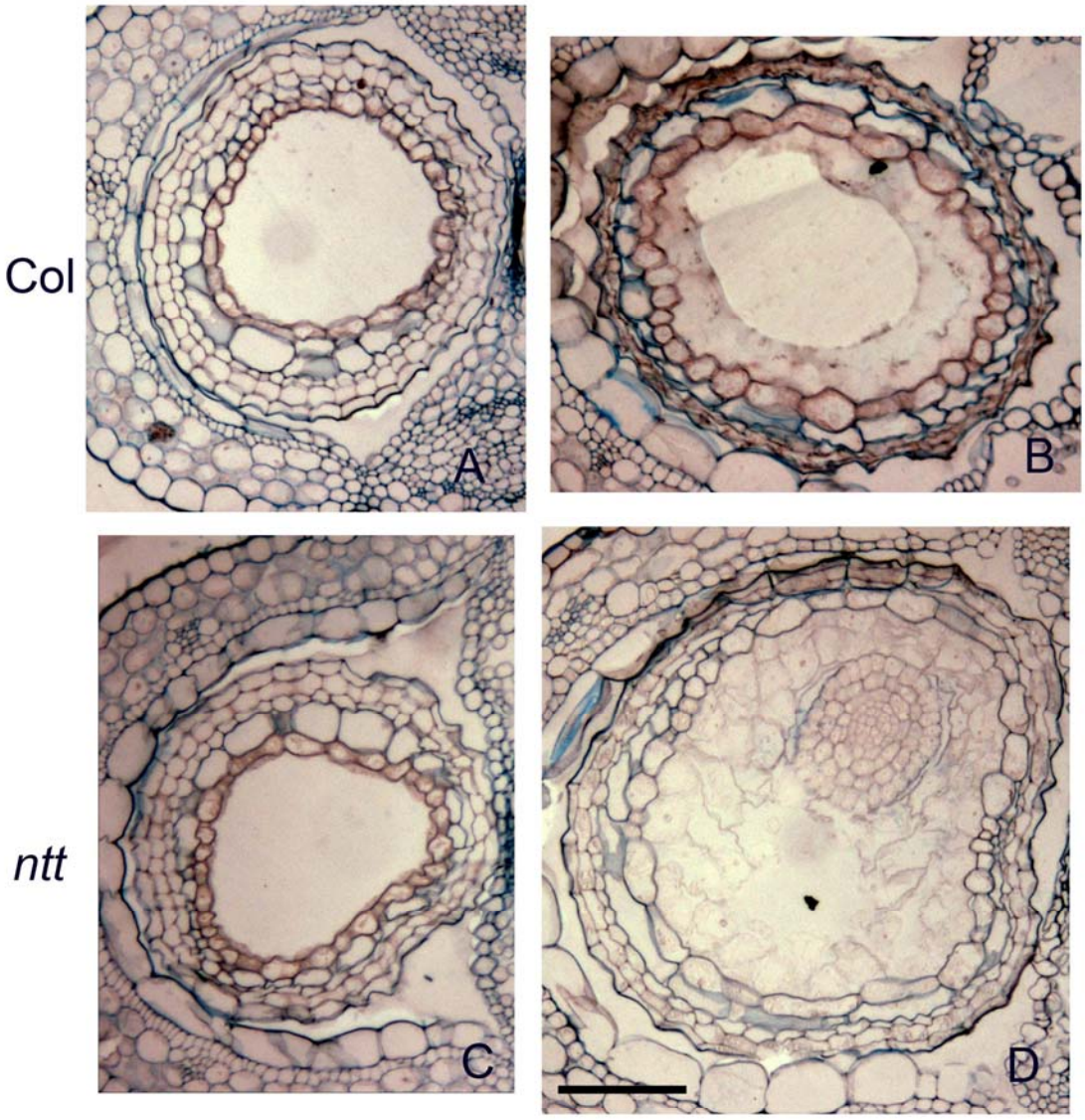

Figure 19. Cell layers in different stages of wild-type and *ntt* fruits

Plastic sections (3 μm) stained with Alcian Blue and Nuclear Fast Red were taken from wild-type (A-B) and *ntt* mutant (C-D). Samples were taken 5 (A and C) and 10 (B and D) fruits down from first opened flower. Scale bar represents 50 μm.

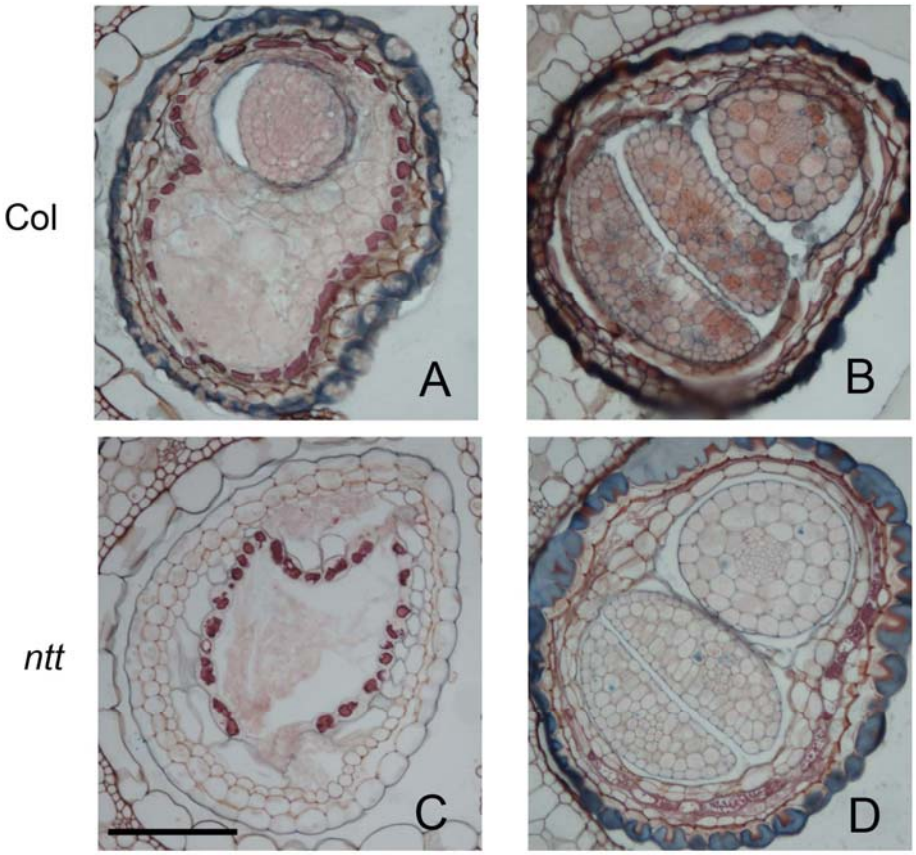

Figure 20. Cell layers in different stages of wild-type and *ntt* fruits

Plastic sections (3 μm) stained with Alcian Blue and Neutral Red were taken from wild-type (A-B) and *ntt* mutant (C-D). Samples were taken 10 (A and C) and 20 (B and D) fruits down from first opened flower. Scale bar represents 50 μm.

strong Alcian Blue staining (Figure 19B, 19D). With a slightly different staining procedure, the experiment was repeated using 10 and 20 fruits down from the first opened flower. Once again, there was strong Alcian Blue staining in the wild-type fruit 10 fruits down, but not in the *ntt* mutant (Figure 20A, 20C). The wild-type and *ntt* fruits taken 20 down both showed Alcian Blue staining, but the wild-type had stronger staining (Figure 20B, 20D). Also, in both stages, the cell layers were more distinct in *ntt* than wild-type.

Role of *TT1* in ovule and seed development

TT1 gene is a homolog of *NTT* gene and is suggested to have a function in seed pigmentation, expressing in the endothelium, or the innermost layer of the seed coat, where the condensed tannins are accumulated to produce the brown color of seeds. Because the Col mutant of *TT1* was not available, the *tt1 ntt* double mutant was made by crossing the *tt1* mutant in L*er* background and *ntt* mutant in Col background. To investigate whether *TT1* is involved in ovule and seed development, the cell layers of *tt1* mutant and *tt1 ntt* double mutant were examined using the same technique of taking 5, 10, and 20 fruits down from the first opened flower. When comparing the cell layers of the two mutant fruits, the Alcian Blue staining seemed to show at an earlier stage in the *tt1* mutant, where staining was already present at 5 fruits down (Figure 21A), but not clearly visible in the *tt1 ntt* mutant (Figure 21B). At 10 fruits down, the staining was present in both mutants, but stronger in the *tt1* mutant (Figure 21C, 21D). Finally, at 20 fruits down, the staining is stronger in the *tt1 ntt* double mutant (Figure 21E, 21F).

Seed Mucilage

Because there was a difference in the cell layers of the wild-type and *ntt* mutant seeds, and of *tt1* mutant and *tt1 ntt* double mutant, it was important to examine aspects of seed development. A simple test of correct development of the seed coat is by observing the production of mucilage by seeds. The mucilage of seeds can readily be stained by Ruthenium red. The procedure was performed with wild-type Col and *ntt* mutant seeds, and with L*er*, *tt1*, and *tt1 ntt*, and it was shown that all showed similar production of mucilage (Figure 22).

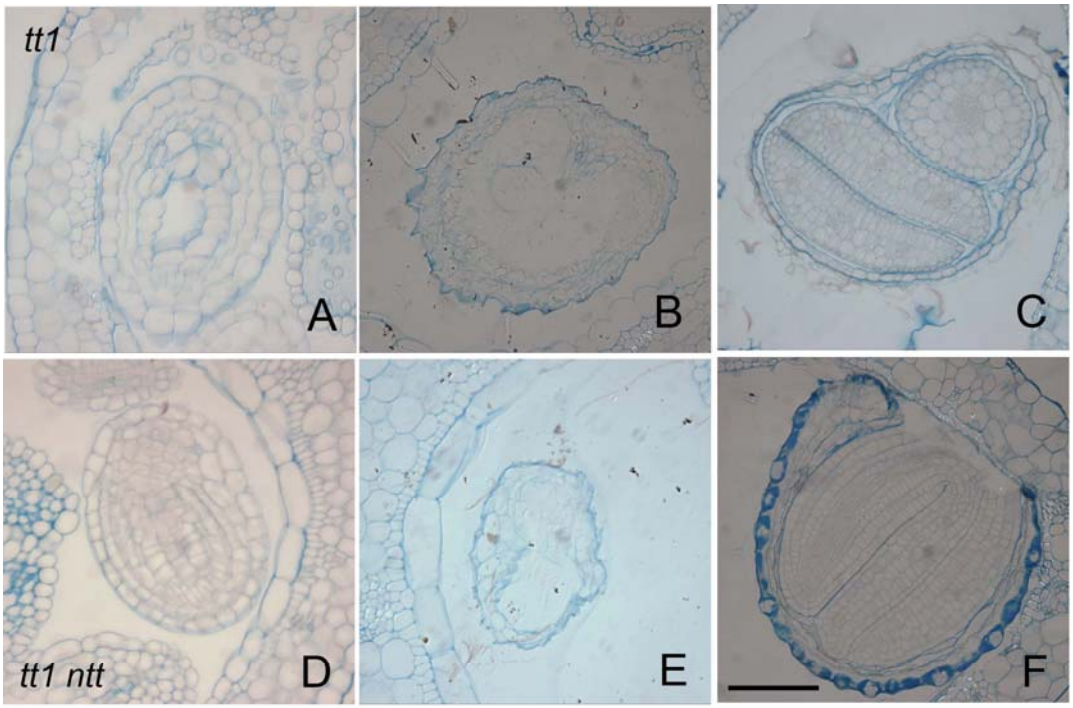

Figure 21. Cell layers in different stages of *tt1* mutant and *tt1 ntt* double mutant

Plastic sections (3 μm) stained with Alcian Blue and Nuclear Fast Red were taken from *tt1* mutant (A-C) and *tt1 ntt* double mutant (D-F). Samples were taken 5 (A and D), 10 (B and E), and 20 (C and F) fruits down from first opened flower. Scale bar represents 50 μm.

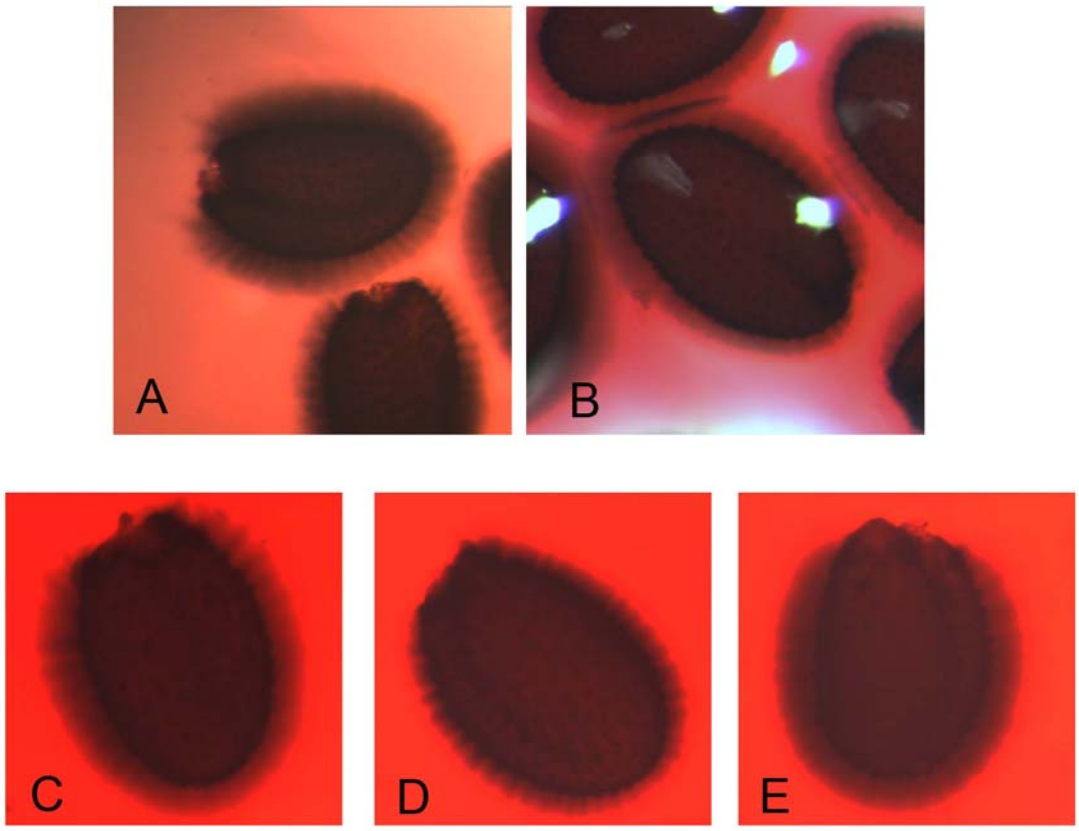

Figure 22. Seed mucilage of Col, L*er*, and various mutants

(A) Wild-type, (B) *ntt* mutant, (C) L*er*, (D) *tt1* mutant, and (E) *tt1 ntt* double mutant seeds were stained with Ruthenium Red to show seed mucilage.

DISCUSSION

Some genes suggested to be involved in transmitting tract development were examined, and GUS reporter lines were made with these genes. By examining the GUS activity, we can further investigate the genes' role in the transmitting tract and possibly other parts of the reproductive tract, and their effect on reproduction.

NTT::GUS Expression Pattern in Transmitting Tract and Ovule/Embryo

The GUS staining of the reporter line, *NTT::GUS*, was observed in the transmitting tract and a part of the ovule, and later throughout the embryo. The expression in the transmitting tract was very similar to the results of the in situ hybridization of wild-type gynoecia probed with *NTT* (Crawford et al. 2007). GUS staining in the gynoecium was first observed at stage 9 when fusion to form the septum has just occurred. Consistent with the *ntt* mutant phenotype, staining occurs in the region that will eventually form the transmitting tract. The staining continues to be observed in this region throughout stages 9 to 12, before decreasing expression levels at stage 13 in the in situ, and almost completely absent at stage 14 in the GUS. Thus the expression was observed at pre-fertilization, but absent after fertilization. This suggests that the *NTT* gene is not required in the transmitting tract after fertilization. As *NTT* is observed at the earliest point of septum fusion, *NTT* is likely important in setting up the pattern for the differentiation of the cells in the transmitting tract. This is consistent with the *ntt* mutant phenotype, where no Alcian Blue staining was observed within the developing transmitting tract (Crawford et al. 2007). To determine the precise role of *NTT* in

forming the transmitting tract, it will be now possible to use this *NTT::GUS* to cross to other known mutants affecting the transmitting tract such as *spt, arf6,8,* and *hec1,3*. *HEC1*, *HEC2*, and *HEC3* are expressed in the septum beginning at stage 8, when the medial ridges of the septum, which later forms the transmitting tract, have fused (Gremski et al., 2007), and this expression was observed up to stage 12. This pattern is very similar to the GUS expression of *NTT*. The *SPT* gene is expressed in the gynoecium earlier than the *HEC* genes (Gremski et al., 2007). *ARF6* and *ARF8* are both expressed in the transmitting tract, and in the double mutant, the plants arrest at pre-fertilization stage 12 (Wu M.F. et al., 2006). By crossing the *NTT::GUS* to these mutants and observing the change in the GUS expression level, we can determine whether *NTT* acts upstream or downstream of these genes.

One difference in suggested expression between *NTT::GUS* and *NTT* in situ was seen in the funiculus. In the in situ, funiculus expression was observed at stage 12 while GUS staining in the *NTT::GUS* line was not observed. One possible reason is that because the GUS expression of this line was very weak, showing only after two to three days of incubation. It is possible that the expression in the funiculus was too weak to be seen. Another possible explanation may be that a further amount of the promoter is needed for the expression in the funiculus. To test the effectiveness and validity of the *NTT* promoter fragment, we have crossed the *NTT::GUS* construct to the *ntt* mutant. Unfortunately, at this time, the F2 population of this line is not yet available. However, if the reporter line rescues the *ntt* mutant phenotype, it is indicative that the promoter fragment is sufficient for *NTT* function. However, this experiment relies on the funiculus

expression being important to full seed set.

At pre-fertilization stage 12, the *NTT::GUS* staining was observed in the developing ovules in the region closest to the funiculus. In post-fertilization stage 15, staining was present strongly in the outer cell layer of the ovule integuments and less strongly in the inner layers. This staining pattern was similar to the GUS staining of the *TT1* promoter construct. The *TT1::GUS* staining was observed in the cell layers, with the strongest expression in the endothelium (Sagasser, et al., 2002), leading to the theory that *NTT* and *TT1* are redundant genes, see below.

When the embryo developed, the expression was strongly observed throughout the entire embryo at early stage 17, as the levels reduced at late stage 17. This pattern was found in the majority but not all embryos, and a reason may be that the transformants were hemizygous for the transgene. Therefore, 1:3 of the embryos of this future T2 population did not inherit a copy of the transgene. We will be able to test this in future generations after creating a stable homozygous line. Once a homozygous line has been generated, future work will include a comprehensive analysis of GUS expression in all tissues of the plant.

GUS Expression of Downstream Targets of *NTT* and *HAF*

Two genes, At1g72290 (*PUP5*) and *PUP3*, were identified as being up-regulated in the inflorescence of the mutant *pi-1*, suggesting that they are expressed in the gynoecium. In situ hybridization also shows that *PUP3* is expressed in the epidermis of

the septum (Scutt et al., 2003). At1g30795 is a homologous gene to *PUP3*, and the genes At1g72290 and At1g30795 encode proteins that are potential downstream targets of *NTT* and *HAF*. This suggests they may play a role in the function of the transmitting tract.

According to the microarray, At1g72290 was the most down-regulated gene of both *NTT* compared with Col and *HAF* compared with L*er* (Crawford unpublished results). At1g72290 encodes for a trypsin and protease inhibitor family protein. Consistent with the microarrays, *72290::GUS* was observed in the transmitting tract in both pre-fertilized and post-fertilized gynoecia. This GUS expression partially overlapped the expression pattern of *NTT::GUS* and *HAF::GUS*, showing that the promoter of the gene is expressed in the same tissues as *NTT* and *HAF*. Although staining was not observed in the funiculus for *72290::GUS*, a more detailed analysis was performed using wax the cross sections of the gynoecium. Staining was observed in the inter-epidermal regions of the transmitting tract and absent in the epidermal layers of the septum. This part of the transmitting tract where the GUS stains is the region where cell death occurs. This could suggest that the role of the At1g72290 is related to the cell death that occurs in the transmitting tract.

At1g72290 in the background of *bee3* mutant and *haf bee1 bee3* +/- double-heterozygous mutant was examined. GUS staining in the *bee3* mutant background was observed in the transmitting tract in the style and septum. The expression was reduced in comparison to the wild-type background, showing that *BEE3* partially controls the expression of At1g72290. In the background of the *haf bee1 bee3* +/- double-heterozygous mutant, the GUS expression was still observed in the transmitting tract

within the style and septum. The expression was much weaker compared to wild-type, especially the region within the septum, which was almost absent. This confirms the microarray results of the *HAF*/L*er* array in Col with redundancy of *BEE1*, *BEE3*, and *HAF*, and may suggest this gene as a target of these genes. One method to test whether it is a direct target of *HAF* is to mis-express *HAF* in different tissues and check whether that is sufficient to drive At1g72290 expression.

The gene At1g30795 encodes a hydroxyproline-rich glycoprotein that functions in molecular pathways and is down-regulated highly in *haf* mutants compared to L*er*. The GUS expression for the reporter line, *30795::GUS*, was observed in the transmitting tract within the septum in the pre-fertilized gynoecium. In addition to the ovary transmitting tract, the post-fertilized gynoecia also showed staining in the stylar transmitting tract and the funiculus. Staining was also observed in the epidermis of the septum. The gene *PUP3*, which also codes for a glycoprotein, is found to be homologous to At1g30795, and is also down-regulated in the *haf* mutant compared with L*er*. The two genes also have similar expression pattern, with At1g30795 showing GUS expression and *PUP3* showing in situ expression in the epidermal cells of the septum after fertilization (Scutt et al., 2003). The region where these genes are expressed is tissues where the pollen tube grows within the gynoecium. The difference between the expression patterns is that At1g30795 showed GUS expression in the funiculus while *PUP3* was not expressed in the funiculus. The knockout of the gene At1g30795 alone was not enough to affect the growth of pollen tubes as shown with aniline staining (Crawford unpublished data). A possible reason is that At1g30795 and *PUP3* are redundant and both genes have to be

knocked out to affect pollen tube growth as in the down-regulation in the *haf* mutant. Currently, we are knocking out both At1g30795 and *PUP3* to see whether there is an effect of pollen tube growth.

A gene called *TTS* encodes for a glycoprotein found in the stylar transmitting tissue in tobacco. It has been identified to enhance pollen tube growth from the stigma to the ovaries. The TTS protein has an adhesive property and it binds to the pollen tube tip, and the sugar moieties of the glycoprotein are glycosylated by the pollen tubes. An increasing gradient of glycosylation was found from the stigmatic to the ovarian transmitting tract tissues, suggesting that the gradient may help with the directionality of the pollen tube growth (Wu H.M. et al. 1995). *TTS* and At1g30795 both show expression in the transmitting tract within the style and both encode glycoproteins. It is possible that At1g30795 and *PUP3* may be the equivalent genes to *TTS* in *Arabidopsis*. A way to further investigate this matter is to examine the level of glycosylation of the glycoprotein encoded by At1g30795 along the transmitting tract, and whether there is a gradient similar to what is observed in the tobacco transmitting tract.

GUS Expression of *BEE1* and *HAF*

HAF plays a role in transmitting tract development, and two very closely related genes to *HAF* that exist in the genome are *BEE1* (At1g18400) and *BEE3* (At1g73830). We therefore set out to check the expression of these genes in the reproductive tract.

The GUS expression for the reporter line, *18400::GUS*, was observed strongly in

the stylar transmitting tract and the stigma, including the stigmatic papillae. Expression was present but weak in the transmitting tract in the septum. In the GUS reporter line, *HAF::GUS*, expression was strongly observed in the transmitting tract in the septum and funiculus. Weak expression was observed in the transmitting tract in the style and stigma, but was not seen in the stigmatic papillae. When comparing the two expression patterns, there is an obvious overlap of expression in the transmitting tract. The *haf* mutant in the L*er* background has a phenotype with no seeds on the basal region of the fruit, while it does not have a phenotype in the Col. The overlapping expression suggests that *HAF* and *BEE1* may be redundant in the transmitting tract in the Col background.

HAF::GUS expression was strong in the ovary transmitting tract, while *18400::GUS* expression was strong in the style region. The difference in expression levels suggests that the transmitting tract is different in the style and ovary, with different genes, such as *HAF* and *BEE1*, expressed in those regions to carry out different functions.

In addition to the transmitting tract, both genes have unique area of expression, with the two GUS constructs also revealing expression in other regions of the gynoecium. In *HAF::GUS*, staining was observed in the funiculus and stigma, but not stigmatic papillae, while *18400::GUS* had expression in the stigma including the stigmatic papillae. The expression of *BEE1* in the stigmatic papillae may reveal that it is responsible for specific functions of these cells in interaction with the pollen tube. *HAF*, expressed in the funiculus, may control a similar process of pollen tube interaction with the funiculus. Also, *HAF* may be more important in transmitting tract development, while *BEE1* may be involved in stigma growth. The differences of expression also suggest that *HAF* and

BEE1 may have diverged through evolution to control different tissues. These genes may have evolved from one gene in an ancestral plant that controlled development of tissues that interact with pollen tubes. This ancestral gene has diverged to regulate different tissues, in the more complex gynoecium of *Arabidopsis*, with *HAF* specifically regulating the funiculus and *BEE1* regulating the stigmatic papillae. An interesting project would be to obtain the *HAF* homolog in basal angiosperms that have a relatively simple gynoecium.

Also, the majority of the transformants of the *18400::GUS* line showed expression in the stamens as well. This suggests that *BEE1* may play a role in this structure. *BEE1* is also known to be expressed in vegetative tissue, and future work will fully examine the expression of this gene in all tissues.

Another gene that is in the family is At1g73830 (*BEE3*), the construct has been made but the GUS expression of the reporter line needs to be analyzed.

ntt Phenotype

Seed Size

The typical size of a *ntt* mutant seed is larger than that of a wild-type seed. This may be a result of the *NTT* gene affecting the seed maturation process, or due to the simple fact that there are less seeds in each silique in *ntt* mutants. To test the latter hypothesis, maximal and minimal pollination were first performed on wild-type gynoecium. As expected, the gynoecium pollinated with only a few grains of pollen

produced less seeds in comparison to the gynoecium pollinated with many grains of pollen, and results showed that seeds from minimal pollination were larger than seeds from maximal pollination. Then, minimal pollination was performed on both wild-type and *ntt* mutant gynoecium, and the resulting seeds had similar size. These results strongly indicate that the larger seed size of *ntt* mutants is a consequence of having less seeds per fruit. This may be a result of more room for seeds to grow, as in the *ful* mutant, where the ovary chamber is smaller than wild-type, the seeds were reduced in size (Ferrándiz et al., 2000).

Indehiscence

The *ntt* mutant has fruits that seem to be indehiscent, as they do not break open easily when they have matured enough to release the seeds. In wild-type fruits, the dehiscence is caused by the lignification of valve margin cells, which can be observed by phloroglucinol staining. When examining the proximal and distal regions of stage 17 fruits of wild-type and *ntt* mutant, there was no difference in the lignification of the valve margin cells, indicating that the indehiscence of *ntt* fruits is not due to the lack of lignification of those cells. A possible explanation for the indehiscence is that there is a lack of cell death in the separation layer of the valve margin in the fruits *ntt* mutants. However, no apparent difference was observed in plastic sections of stage 17, and the expression of the *NTT::GUS* suggests that *NTT* is not expressed in the valve margin. However, funiculus expression was not observed in *NTT::GUS* although it was shown by the in situ. It is still possible that *NTT* could be expressed in the valve margin. Another

plausible hypothesis for the indehiscent phenotype is *ntt* mutant's effect on fertility. The lack of seeds on the bottom of the fruit somehow inhibits the opening of the fruit, maybe as a result of the lack of localized ripening signal.

Cell layers of *ntt* and Col

Another focus of the *ntt* mutant is the development of the seed coat. The normal development of the seed coat involves programmed cell death of the ovule integuments. The enzyme δVPE, expressed in the outer two layers of the inner integument, was proposed to be involved in programmed cell death (Nakaune et al., 2005). It is also shown to be down-regulated in the *ntt* mutant array. To see whether the cell death of cell layers is affected the *ntt* mutant ovules, different stages of the *ntt* mutant fruit were examined.

There are five cell layers of the ovule, two of the outer integument and three of the inner integument. All the layers except the outermost layer undergo programmed cell death and are crushed together to develop into the seed coat (Haughn et al., 2005). To examine different stages of seed coat development, 5, 10, and 20 fruits were taken from the first opened flower. When examining the cross section of the wild-type and *ntt* fruits taken 5 fruits down from the first opened flower, the five layers of the ovule integuments were still clearly visible. Beginning at 10 fruits down, and even more apparently seen at 20 fruits down, the cell layers of the wild-type seemed to be undergoing cell death, while the five cell layers in *ntt* mutant were still distinct. These results indicate that there is

indeed a lack of cell death in the ovule integuments that eventually develop into the seed coat. In the δVPE-deficient mutant, the two outer layers of the inner integument had delayed cell death, as the layers eventually undergo necrosis as a result of the lack of nutrients (Nakaune et al., 2005). In the end, the seeds have wild-type phenotype. The development of the seed coat of the *ntt* mutant may be similar to that of the δVPE-deficient mutant.

There was also weaker Alcian Blue staining in the cell layers of the *ntt* tissues, showing that there are less acidic polysaccharides being produced in the cell layers. These acidic polysaccharides, a component of the ECM, are also made during the development of the transmitting tract. It has been suggested that that the production of the ECM, shown by strong Alcian Blue staining, may be correlated with programmed cell death (Crawford et al., 2007). This is consistent with the lack of Alcian Blue staining in the transmitting tract of the *ntt* mutant, which lacks programmed cell death. AGPs, also a component of the ECM, are acidic glycoproteins that have been suggested to be involved in programmed cell death in plants (Gao et al., 1999). Therefore, the lack of production of AGPs may attribute to the lack of programmed cell death in the ovule integuments.

Cell Layers of *tt1* and *tt1 ntt*

TT1 and *NTT* are homologous genes that belong in the WIP gene family. This six-member family is characterized by the conserved C2H2 zinc finger domain (Sagasser et al., 2002). In the L*er* background, the *tt1* mutant shows a phenotype of yellow seeds,

caused by the lack of condensed tannins in the endothelium of the seed coat, suggesting that *TT1* may be involved in the differentiation of this cell layer. From the GUS staining results, it was found that there was overlapping *TT1* and *NTT* expression in the ovule integuments. However, the *TT1* GUS construct showed stronger staining in the endothelium, while the *NTT::GUS* showed stronger staining in the outer layers. It is possible that these two closely related genes function redundantly in assisting the development and differentiation of the cell layers in the Col background. To test this, the cross sections of *tt1* and *tt1 ntt* fruits of different stages were analyzed. There is not much to conclude from these results because the *tt1* mutant is of L*er* background, while the ntt mutant is in Col. Therefore, the *tt1 ntt* double mutant is of L*er*/Col hybrid background, and it wasn't possible to compare directly with a wild-type. Differences were observed between the cell layers between the *tt1* mutant and *tt1 ntt* mutant, in which at 5 and 10 fruits down, the double mutant did not seem to have five distinct cell layers as seen in the *tt1* mutant. But for a further and more accurate analysis, a backcross into L*er* or Col is needed. There also appeared to be a difference in Alcian Blue staining, where it was present at an earlier stage in *tt1* mutant than in the *tt1 ntt* double mutant, but stronger in the *tt1 ntt* double mutant at a later stage, suggesting that the occurrence of cell death may be delayed in the double mutant. For the future, the *tt1 ntt* double mutant should be backcrossed several times to L*er* or Col before being used for comparison.

Seed Mucilage

A difference was observed in the cell layers of wild-type and *ntt* ovules, and of *tt1* and *tt1 ntt* mutant ovules. These cell layers ultimately develop into the seed coat, therefore it was of interest to investigate whether the eventual seed coat was developed correctly. A method to check this is to examine the production of seed mucilage. In normal seed coat development, all cell layers undergo cell death except the outermost layer, the epidermal layer, where the cells of this layer produce and secrete a large amount of mucilage (Haughn et al. 2005). When comparing wild-type and *ntt* mutant, there was no apparent difference in the production of seed mucilage. Therefore, although *NTT* affects the development of the cell layers of the ovule and seed, apparently this does not affect the eventual production of mucilage. Similar results were observed in L*er*, *tt1*, and *tt1 ntt*, where there was also no noticeable difference in the production of seed mucilage. It may suggest that the seed coat was eventually developed. A possible explanation for this is that the inner cell layers eventually undergo necrosis as a result of the lack of nutrients, similar to what occurs in δVPE-deficient mutants.

In summary, we have examined expression of genes that are expressed in the female reproductive tract, especially the transmitting tract. This is to hopefully help determine the development of these tissues, the most evolutionarily and intricate tissues produced in plants.

APPENDIX A

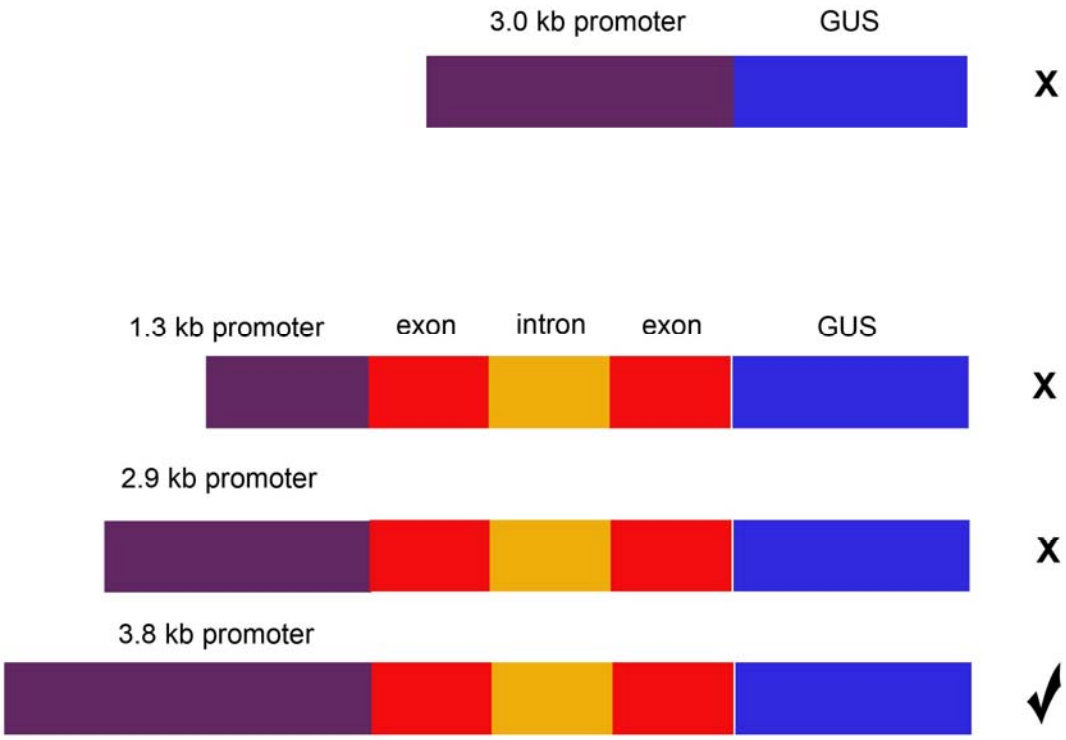

Figure 23. Different *NTT::GUS* constructs

Above are the different *NTT::GUS* constructs we tried making, with the last fragment being the only one that was successful.

Table A-1. Summary of GUS expression of GUS reporter lines at pre-fertilization

Below is a chart showing the areas of expression in the gynoecium at pre-fertilization for the five GUS reporter lines. Shaded boxes represent positive expression.

	Stigma	**Stylar TT**	**Ovary TT**	**Septum Epidermis**	**Funiculus**	**Ovule**
72290::GUS						
30795::GUS						
18400::GUS						
HAF::GUS						
NTT::GUS						

Table A-2. Summary of GUS expression of GUS reporter lines at post-fertilization

Below is a chart showing the areas of expression in the gynoecium at post-fertilization for the five GUS reporter lines. Shaded boxes represent positive expression

	Stigma	**Stylar TT**	**Ovary TT**	**Septum Epidermis**	**Funiculus**	**Ovule**
72290::GUS						
30795::GUS						
18400::GUS						
HAF::GUS						
NTT::GUS						

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