Current Biology 17, 1101–1108, July 3, 2007 ©2007 Elsevier Ltd All rights reserved DOI 10.1016/j.cub.2007.05.079

Article

The *NTT* Gene Is Required for Transmitting-Tract Development in Carpels of *Arabidopsis thaliana*

Brian C.W. Crawford,[1] Gary Ditta,[1] and Martin F. Yanofsky[1,*]
[1] Section of Cell and Developmental Biology
University of California, San Diego
La Jolla, California 92093

Summary

Background: The majority of pollen-tube growth in *Arabidopsis* occurs in specialized tissue called the transmitting tract. Little is currently known about how the transmitting tract functions because of a lack of mutants affecting its development. We have identified such a mutant and we used it to investigate aspects of pollen-tube growth.
Results: Reverse genetics was used to identify mutations in a gene, *NO TRANSMITTING TRACT (NTT)*, encoding a C2H2/C2HC zinc finger transcription factor specifically expressed in the transmitting tract. The *ntt* mutants have a negative effect on transmitting-tract development. Stage-specific analysis of transmitting-tract development was carried out and was correlated with investigations of pollen-tube behavior. In *ntt* mutants, pollen tubes grow more slowly and/or terminate prematurely, and lateral divergence is accentuated over apical-to-basal movement. Normal transmitting-tract development is shown to involve a process of programmed cell death (PCD) that is facilitated by, but does not depend upon, pollination.
Conclusions: This is the first report of a gene that is specifically required for transmitting-tract development in *Arabidopsis*. Mutations in *NTT* cause reduced fertility by severely inhibiting pollen-tube movement. The data support the idea that the function of the transmitting tract is to increase fertilization efficiency, particularly in the lower half of the ovary. This occurs by facilitating pollen-tube growth through differentiation and then death of transmitting-tract cells.

Introduction

In *Arabidopsis*, self-fertilization occurs when pollen grains germinate on the stigmatic surface of the carpel and form pollen tubes to carry sperm cells to ovules in the ovary chamber [1–4]. The journey through the female reproductive tract, which includes the stigma, style, transmitting tract, septum epidermis, funiculus, and micropyle, terminates at synergids contained within each ovule (Figures 1A and 1B). Each of these tissues represents a potentially unique phase of pollen-tube growth [1]. Incomplete formation of portions of the reproductive tract, as occurs, for example, in *spatula* (*spt*) mutants, results in very low seed yield [5].

*Correspondence: marty@ucsd.edu

Most pollen-tube growth in the carpel occurs within the transmitting tract. Two types of transmitting-tract tissue are generally recognized, represented by either the open style of lily (*Lilium longiflorum*), where pollen tubes grow within a channel filled with secretions, or the closed style of *Arabidopsis,* where pollen tubes must grow through various cell layers rather than on epidermal surfaces [6, 7]. The precise mechanism by which pollen tubes translocate within a closed transmitting tract remains unclear. It has been suggested that there is facilitated movement within intercellular spaces and that cell death might contribute to this process [7, 8]. It has also been suggested that movement of the pollen tube itself might promote breakdown of transmitting-tract tissue [8]. The recent identification of a pollen-specific gene, *VANGUARD1* (*VGD1*), encoding a pectin methylesterase, is consistent with this idea [9]. The contribution of the transmitting-tract tissue to pollen-tube movement, however, is largely undefined although there have been many speculations involving support, nutrition, and guidance. Importantly, a pollen tube must at some point exit the internal environment of the transmitting tract and locate the micropylar opening of an ovule. Targeting of ovules is presumed to involve one or more guidance mechanisms, possibly involving chemical gradients and signal molecules [10, 11].

Here we report the identification and characterization of a gene critical for proper development of transmitting-tract tissue and show that perturbations in transmitting-tract function profoundly affect pollen-tube growth. The *NTT* (*NO TRANSMITTING TRACT*) gene encodes a putative transcription factor expressed in both the transmitting tract and the funiculus of ovules. In *ntt* mutants, which are female specific, pollen tubes germinate and travel through the style efficiently but experience considerable difficulty moving within the carpel. Fertilization of basal ovules is impaired, leading to partial infertility. We also examined the behavior of limiting numbers of pollen tubes and found that in wild-type, ovules in the central to basal portion of the ovary are preferentially targeted for fertilization.

Results

Fruit Morphology and Fertility in *ntt*

Both fruit length and seed set are reduced in *no transmitting tract (ntt)* mutants. Mature fruit are approximately 30% shorter than wild-type (Figures 1E and 1F), and the number of fertilized seeds is reduced by 60% (Col = 51.4 ± 5.6 seeds per silique, n = 16; *ntt* = 20.4 ± 2.4 seeds per silique, n = 28). Seeds develop only in the apical part of the fruit (Figures 1C–1F) and are larger than wild-type. Only female reproductive tissue is affected in *ntt* mutants, as shown by the fact that *ntt* mutant pollen produces a normal seed set when applied to wild-type flowers (data not shown). Considerably more siliques are produced in *ntt* mutants, probably because of the reduced fertility of individual flowers [12].

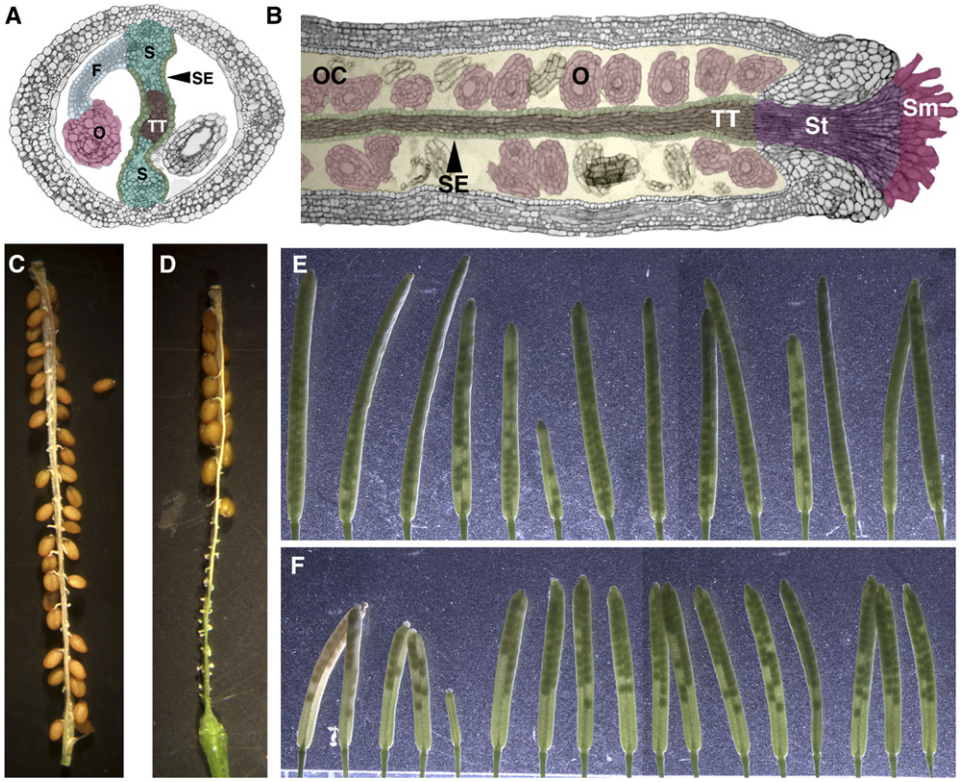

Figure 1. Carpel Structure and Seed Set in Wild-Type and *ntt* Mutant

(A and B) Cross-section and longitudinal section of stage 12 carpel.

(A) Cross-section of carpel with structures colored and labeled. Transmitting tract (TT) is brown, septum (S) is green, septum epidermis (SE) is olive green, funiculus (F) is light blue, and ovule (O) is pink.

(B) Longitudinal section of carpel with structures colored and labeled. In addition to the structures labeled in (A), the ovary chamber (OC) is yellow, the style (St) is purple, and the stigma (Sm) is maroon.

(C and D) Siliques with valves removed to show seed set.

(C) Wild-type seed set with both apical and basal seed distribution.

(D) *ntt* mutant seed set lacking basal seeds.

(E and F) Series of siliques removed from single plant illuminated from below.

(E) Wild-type siliques showing seeds throughout fruit.

(F) *ntt* mutant siliques showing consistency of loss of basal seeds.

Gene Structure and In Situ Hybridization

NTT is predicted to encode a 383 amino acid protein with C2H2/C2HC zinc finger motifs in the C-terminal region of the protein [13] and a proline-rich region near the N terminus (Figure S1A in the Supplemental Data available online). *NTT* is a member of the A1 zinc finger family in *Arabidopsis* that contains 24 members with conserved arrangements of the zinc finger domains [14]. Within this family, it is most closely related to group A1d that has six members recently described as the WIP genes [13]. Two phenotypically indistinguishable alleles were identified from the Salk collection, *ntt-1* and *ntt-2* (Figures S1A and S1B).

In situ hybridization analysis was performed on transverse sections from four stages of fruit development. *NTT* expression was first detected and was most intense at stage 9 at the point of fusion where the septum and transmitting tract will later develop (Figure 2A, sideways V). During stage 11, when septum development has been completed, expression was found within the central region of the septum where the transmitting tract will form (Figure 2B). Expression was also seen in the funiculus, particularly the epidermis (Figure 2B, arrow). There also appeared to be a small region of expression in developing ovules (Figure 2B, arrowhead). Stage 12 in *Arabidopsis* immediately precedes fertilization, and at this stage *NTT* expression could still be observed throughout the transmitting tract (Figure 2C). The epidermal cells of the funiculus continued to display strong expression (Figure 2C, arrow). At stage 13, during fertilization, *NTT* expression was reduced in both the transmitting tract and epidermal cells of the funiculus (Figure 2D). A longitudinal section probed with *NTT* is shown in Figure S1C. At stage 12, *NTT* expression was observed to be strongest within the transmitting tract and weak or absent in the style or the stigma.

Maximal Pollination Experiments

To characterize pollen-tube movement, we used whole-mount staining of carpels with aniline blue, allowing the tubes to be visualized by fluorescent microscopy. We also examined the apical-to-basal progress of pollen tubes in cross-sections of carpels displaying pollen-specific GUS expression (by using the ACA9-promoter::GUS construct [15]).

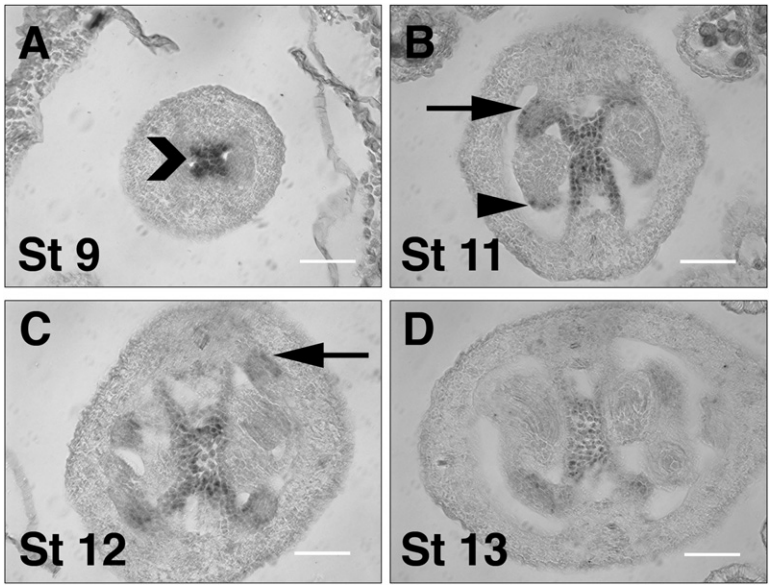

Figure 2. *NTT* Expression in Developing Wild-Type Carpels

Transverse sections of wild-type carpels probed with *NTT* by in situ hybridization.
(A) Stage 9 carpel with sideways V indicating expression at the point of carpel fusion.
(B) Stage 11 carpel with arrowhead indicating expression in the ovule and arrow indicating expression in the funiculus epidermis.
(C) Stage 12 carpel with arrow indicating expression in the funiculus epidermis.
(D) Stage 13 carpel showing a decrease in expression levels.
Scale bars represent 50 μm.

2 hr after hand-pollinating emasculated carpels, the *ntt* mutant and wild-type showed no significant difference in the amount or pattern of pollen-tube growth (Figures 3A and 3B). This indicates that pollen-tube growth in *ntt* mutants is essentially unaffected prior to entry into the ovary chamber.

The first difference between the wild-type and *ntt* mutant was observed 4 hr after pollination. Pollen tubes in wild-type had traveled a substantial distance into the transmitting tract, but in *ntt* mutants, tubes had only slightly penetrated the ovary chamber (Figures 3C and 3D). In wild-type carpels, the first pollen tubes reach the base of the ovary after 10 hr, whereas in *ntt* carpels, pollen tubes made little additional progress (Figures 3E and 3F). Even after 48 hr, pollen tubes in the *ntt* mutant had penetrated only approximately a third of the way into the ovary chamber (Figures 3G and 3H).

An analysis of cross-sections of carpels displaying pollen-specific GUS expression 24 hr after pollination was consistent with results from aniline blue staining. There was no difference in stylar expression between mutant and wild-type (Figures 3J and 3K). Expression at the apex of the ovary was also roughly comparable between mutant and wild-type, although the pattern in the mutant was more dispersed (Figures 3L and 3M). More basal sections revealed a significant reduction in the number of pollen tubes for the mutant compared with wild-type (Figures 3N–3Q).

In *ntt* mutants, a number of pollen tubes at the top of the ovary grew toward the sides of the ovary chamber in a manner not seen for wild-type (Figures 3D and 3I, arrowheads). This behavior can also be seen in the cross-sectional analysis shown in Figure 3 where GUS staining is more widely distributed at the top of the *ntt* mutant ovary than it is in wild-type (Figures 3L and 3M).

Overall, the results indicate that pollen tubes in *ntt* mutants have considerable difficulty growing within the transmitting tract, causing them to terminate travel prematurely and to frequently leave the transmitting tract. This is in contrast to wild-type, where apical-to-basal tube growth is rapid and lateral growth is seen only rarely, presumably reflecting movement toward ovules for fertilization.

Minimal Pollination Experiments

It has been reported that pollen tubes within the transmitting tract grow straighter than tubes on the septum epidermis [16]. To observe the growth morphology and growth pattern of individual pollen tubes, we pollinated wild-type and *ntt* mutant carpels with minimal amounts of pollen. Two pollen-tube morphologies were observed for wild-type. One type (Figure 4A) was thick, intensely staining, and rather crooked compared to the other type, which was straighter and thinner (Figure 4B). Straight, thin tubes were found associated only with linear apical-to-basal movement, whereas crooked tubes often pursued wandering sideways paths that terminated in the vicinity of ovules. For *ntt* mutants, only the intensely staining crooked type of pollen-tube growth was ever seen (Figure 4C).

Increased callose deposits in pollen tubes are associated with a decreased speed of travel and more difficult movement [17]. Our observations regarding pollen-tube growth suggest that pollen tubes in wild-type grow relatively easily within the environment of the transmitting tract (straight/thin morphology) but grow less efficiently after leaving the transmitting tract to target ovules (crooked/thick morphology). Pollen tubes in the *ntt* mutant experience an environment where movement is difficult whether inside or outside the transmitting tract, consistent with their crooked/thick morphology.

Previous studies with limited pollination described a preference for pollen tubes to emerge from the transmitting at the most apical ovule and to travel preferentially to nearby ovules [18]. We used minimal pollination studies (1–5 grains per carpel) to examine the behavior of pollen tubes in both wild-type and the *ntt* mutant. First, the spatial distribution of seed set within each silique was recorded at maturity (Figures 4F and 4G). Second, individual tubes were examined by staining with aniline blue after 24 hr. The approximate location of the end point of each pollen tube was noted (Figures 4D and 4E).

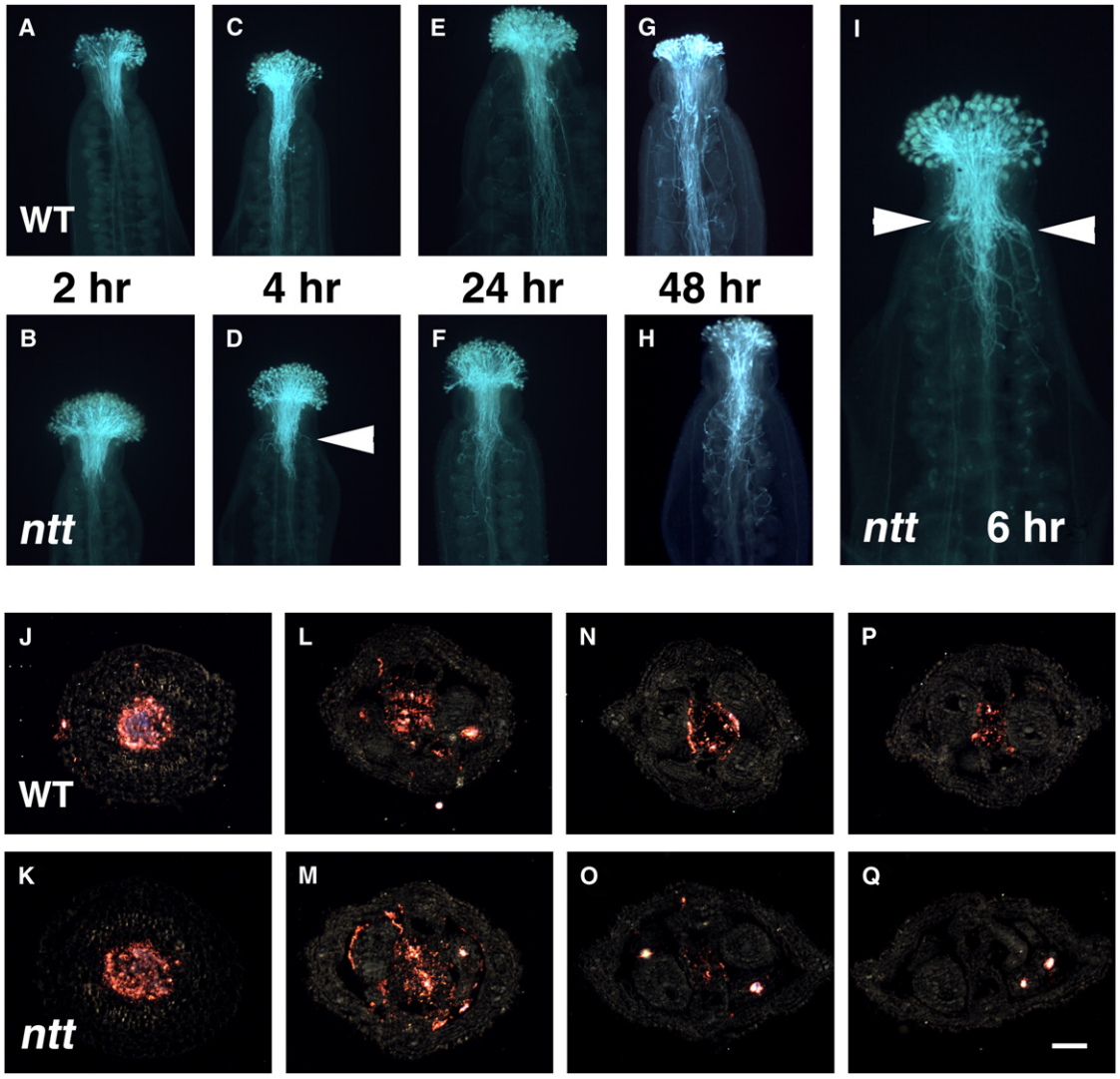

Figure 3. Pollen-Tube Movement in Wild-Type and *ntt* Mutant

(A–I) Time series of pollen-tube movement stained with aniline blue. Carpels were emasculated, left for 24 hr, and then pollinated. Carpels of wild-type (A, C, E, and G) and *ntt* mutant (B, D, F, and H) were fixed at 2, 4, 24, and 48 hr after pollination. Arrowheads indicate sideways pollen-tube movement in carpels of *ntt* 6 hr after pollination (D and I).

(J–Q) Distribution of pollen tubes visualized with GUS-labeled pollen in transverse sections 24 hr after pollination. Carpels were emasculated, left for 24 hr, pollinated with pollen expressing the ACA9-promoter::GUS construct [15], and processed after an additional 24 hr. A carpel of wild-type (J, L, N, and P) and *ntt* mutant (K, M, O, and Q) was sectioned. The cross-sections were taken from the style immediately beneath the stigma (J, K), the top of the ovary chamber at the first ovule (L, M), and at around 100 μm (N, O) and 200 μm (P, Q) from the top of ovary chamber.

Scale bar represents 50 μm.

Figure 5 shows seed distribution within siliques of wild-type (n = 96) and *ntt* (n = 54) after minimal pollination. The distribution is divided into tenth percentiles starting from the top (0–10 percentile) of the ovary chamber (indicated between two arrows in Figure 4G). In wild-type, 62% of the seeds were found in the bottom half of the silique (50–100 percentile) and 5% were found in the apical region (0–20 percentile). On average, seeds formed toward the center of the silique (50–60 percentile). No preference was observed for fertilization of the most apical ovules. In the *ntt* mutant, most seed formed in the upper half of the carpel (90%), consistent with its general phenotype. Despite this fact, there was again no preference for fertilization of the most apical ovules (0–20 percentile).

The number of data points for the microscopic analysis (wild-type, n = 34; *ntt* mutant, n = 19) was more limited than that for seed set, but again there was no indication of preferential targeting of the most apical ovules in either wild-type or mutant. In wild-type, the average pollen tube reached the bottom half of the carpel (60–70 percentile) whereas for *ntt* mutants they terminated in the top half (20–30 percentile).

Transmitting-Tract Structure in *ntt* Mutants and Wild-Type

Plastic cross-sections were obtained from five different stages of wild-type and *ntt* mutant carpels (Figures 6A–6J). The sections were stained with neutral red to highlight cell walls and with alcian blue to stain acidic

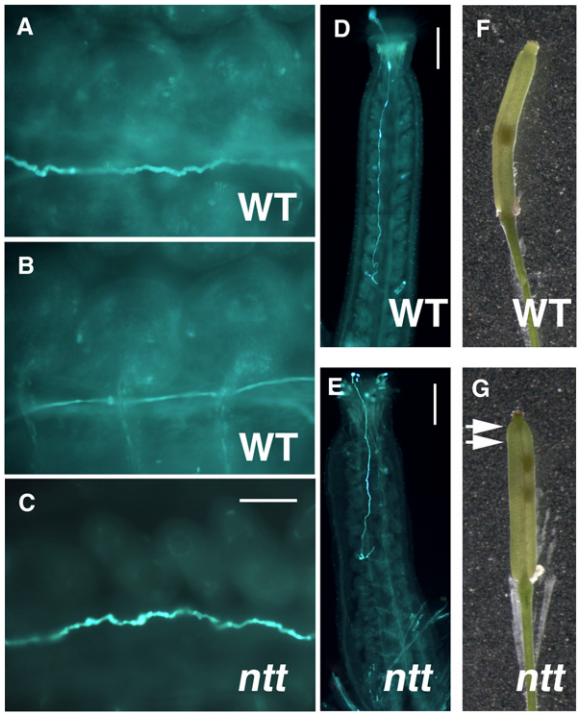

Figure 4. Minimal Pollination Studies in Wild-Type and *ntt* Mutant

(A–C) Close examination of pollen-tube growth morphology within the transmitting tract of wild-type (A, B) and *ntt* mutant (C). Scale bar represents 25 μm.

(D and E) Examples of pollen tubes arising from minimal pollination events after staining with aniline blue in wild-type (D) and *ntt* mutant (E). Scale bars represent 100 μm.

(F and G) Examples of seed set resulting from minimal pollination events in wild-type (F) and *ntt* mutant (G). Arrows indicate distance of 10% in fruit.

polysaccharides, a major component of the extracellular matrix (ECM) of the transmitting tract [7, 19].

Developmental stages 10, 11, 12, 14, and 17 were analyzed [20]. Stage 10 shows no detectable ECM staining in either mutant or wild-type, and the overall cellular structure of the septum in *ntt* mutants is comparable to wild-type (Figures 6A and 6F). Stage 11 in wild-type shows the first alcian blue staining occurring in the internal cell layers of the septum (Figure 6B). Such staining is absent in *ntt* mutants (Figure 6G) and suggests that ECM is either not being produced or is different in overall composition. Another feature of the wild-type septum at stage 11 is the presence of intercellular spaces in the region of the septum adjacent to the transmitting tract (Figure 6B, arrowheads). These spaces represent the beginning of cellular breakdown and are not present in *ntt* mutants. At stage 12, the transmitting tract stains intensely in the wild-type but does not stain in *ntt* mutants (Figures 6C and 6H). Stage 14 is after fertilization and shows an almost complete cell breakdown within the septum epidermis of the transmitting tract of wild-type (Figure 6D). Cells in the flanking region appear loosely packed though still intact. In contrast, at stage 14 in *ntt* mutants (Figure 6I), cells in the transmitting-tract region remain intact with no alcian blue staining (Figure 6I). Some intercellular spaces have appeared in the area adjacent to the replum (Figure 6I, arrow). In mature wild-type fruit, stage 17, breakdown of cells within the transmitting tract has been completed and the mature two-cell layered septum has formed (Figure 6E). In *ntt* mutants, cellular breakdown has not occurred, leaving several layers of cells between the two epidermal walls (Figure 6J).

Cell Death in Emasculated Carpels

As described above, the cells of the transmitting tract in wild-type undergo breakdown when pollination (stage 12) and fertilization (stage 14) occur. To determine whether cell death was autonomous or required pollination, wild-type and *ntt* mutant carpels were emasculated at stage 12 and allowed to develop without pollination. Cross-sections were examined at 1, 3, 5, and 7 days after emasculation.

1 day after emasculation, the wild-type transmitting tract had intense alcian blue staining and large amounts of intercellular space (Figure 7A, arrow), indicative of transmitting-tract breakdown. In *ntt* mutants, there was no alcian blue staining and no cell breakdown in the region corresponding to the wild-type transmitting tract (Figure 7E). After 3 and 5 days, staining was still present in the transmitting tract of wild-type but intact cells were mostly absent (Figure 7B, arrow; Figure 7C). In contrast, cells in the transmitting-tract region of *ntt* remained intact and unstained (Figures 7F and 7G). At 7 days, the two cell layers of the septum in wild-type had fused and all traces of the transmitting tract were gone (Figure 7D). In *ntt* mutants, the two cell layers of the septum had also joined by this time, but the center of the septum still contained many cells from the transmitting tract (Figure 7H).

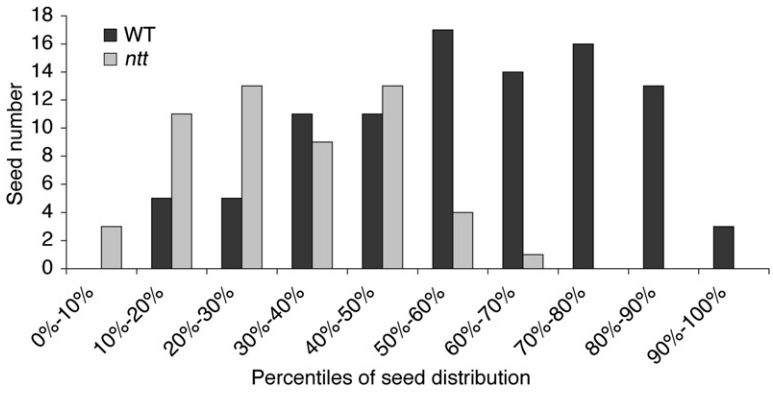

Figure 5. Results of Minimal Pollination Studies in Wild-Type and *ntt* Mutant

Seed-set distribution in wild-type and *ntt*. Bar charts represent the number of seeds present in each ten percentile of fruit length from the most apical (1%–10%) to the most basal (90%–100%) region of the fruit.

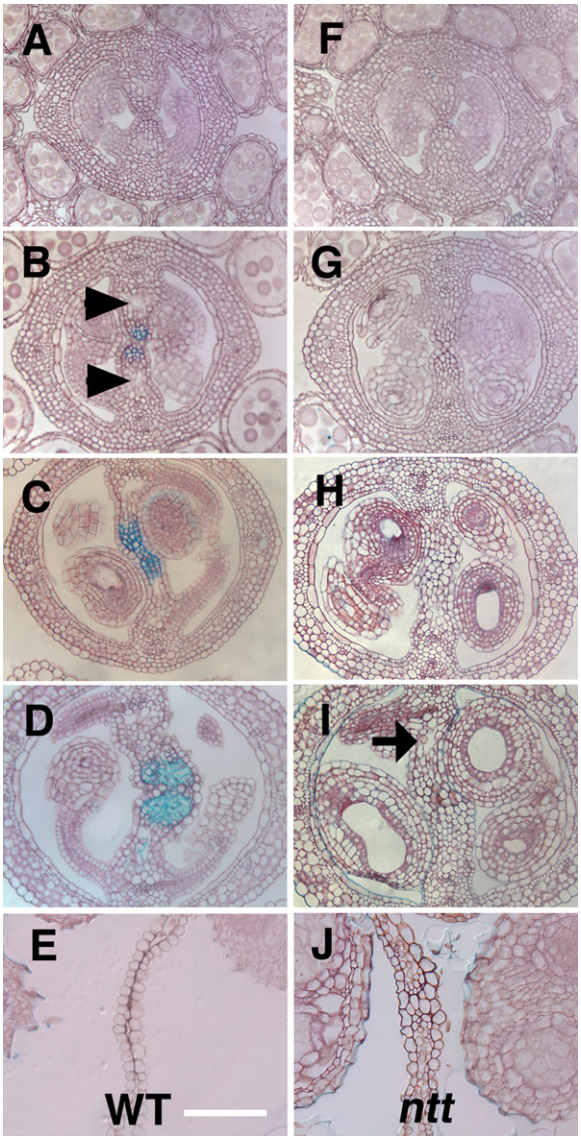

Figure 6. Transverse Sections of Different Stages of Transmitting-Tract Development

Thin sections (3 μm) stained with alcian blue and neutral red were taken from wild-type (A–E) and *ntt* mutant (F–J). Developmental stages 10 (A and F), 11 (B and G), 12 (C and H), 14 (D and I), and 17 (E and J) were used. Arrowheads in (B) and arrow in (I) indicate the appearance of intercellular spaces between the replum and transmitting tract. Scale bar represents 50 μm.

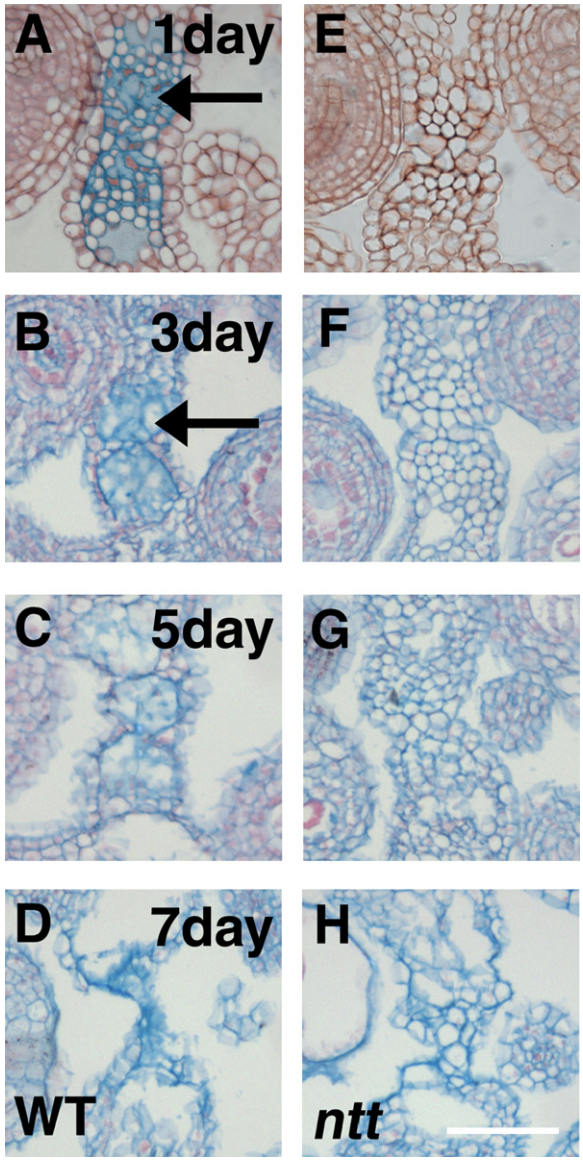

Figure 7. Development of Transmitting Tract in Unpollinated Carpels

(A and E) Thin (3 μm) plastic sections of carpels.

(B–D and F–H) 4 μm wax sections of carpels.

Wild-type (A–D) and *ntt* mutant (E–H) carpel sections taken at 1 day (A and E), 3 days (B and F), 5 days (C and G), and 7 days (D and H) after emasculation. Arrows indicate intercellular spaces that are arising. Scale bar represents 50 μm.

Discussion

Transmitting-Tract Function and Pollen-Tube Growth

The transmitting tract in *Arabidopsis* facilitates the transport of pollen tubes to the ovules for fertilization. In *ntt* mutants, where transmitting-tract development, ECM production, and PCD are defective, pollen-tube growth is severely impaired. Despite this, *ntt* mutant plants still display significant fertility. One explanation could be that the remaining transmitting-tract cells still retain some functionality in terms of assisting pollen-tube growth. Another possibility is that the transmitting tract in *Arabidopsis* is not strictly required for fertilization and simply increases fertilization efficiency. As previously noted, the transmitting tract may operate as a "highway" for pollen-tube movement, enabling pollen tubes to travel longer distances [1]. It is interesting to speculate that a mutation such as *ntt* might render the carpel considerably more infertile in species such as tobacco, where pollen tubes must travel a much greater physical distance to reach the ovules through tissue that also produces ECM and displays PCD [8].

Differentiation of the Transmitting Tract

Transmitting-tract cells secrete a complex extracellular matrix composed of carbohydrates, glycoproteins, and lipids whose purpose is to assist and perhaps guide

pollen-tube movement [10, 11]. Acidic glycoproteins such as arabinogalactans (AGPs) are major components of the ECM. Their exact function is unknown, but it has been suggested that they provide nutrition and adhesion for pollen-tube growth and movement [7]. Alcian blue is a stain for acidic polyanions and is used to represent transmitting-tract development and localization in plant studies [7]. We have shown in this work that the expression of *NTT* precedes and accompanies the accumulation of alcian blue staining in the transmitting tract. The *ntt* mutant completely lacks staining and displays severe perturbations in transmitting-tract differentiation, breakdown, and pollen-tube growth. The ECM has other components besides acidic polysaccharides, and we have no information as to the effect of *ntt* upon these molecules. Additional mutations eliminating other ECM components might be additive with *ntt* mutants in terms of their effect upon pollen-tube growth.

A possible involvement of AGPs in programmed cell death in plants has been reported [21, 22]. These studies are intriguing because they suggest that the production and/or secretion of these large molecules somehow triggers an apoptosis-like event in plant cells.

Programmed Cell Death in Pollen-Tube Growth

Cell death occurs in the transmitting tract around the time of pollination. We have documented the structural progression of this process in detail for *Arabidopsis*. Intact cells enclosed by the septum epidermis prior to pollination are absent by complete fruit maturation. Pollen-specific production of a pectin methylesterase was shown to enable pollen-tube growth [9] and was hypothesized to facilitate penetration of the pollen tube into the ovary chamber. Our studies show, however, that initiation and progression of PCD in the transmitting tract does not specifically require pollination. PCD occurs in unpollinated carpels and is absent in *ntt* mutants despite the presence of pollen tubes. The fact that PCD occurs in the absence of pollination indicates a mechanism of developmental control. Although pollen tubes are apparently not required to initiate PCD, our results suggest that pollen tubes assist in promoting PCD, probably by some sort of synergy with the normal mechanism(s) of transmitting-tract development. Lastly, PCD most closely correlates with the rise in alcian blue staining and suggest the involvement of AGPs or other AGP-like molecules in promoting cell breakdown.

Regulation of Pollen-Tube Exit from the Transmitting Tract

Pollen-tube growth involves attraction and guidance mechanisms acting along the apical-basal axis and along lateral axes toward ovules. The simplest model for apical-basal movement is mechanical. Studies by Sanders and Lord [23] showed that appropriately sized nylon beads could be efficiently drawn down transmitting tracts in several types of plants. This suggests that a role for chemical attraction along the apical-to-basal axis might be secondary. One possibility for chemical involvement is that the ECM might, through nutritional support, act to draw pollen tubes downward through the ovary because the concentration of nutrient would always be highest in advance of the tubes. This idea derives from studies on a unique class of AGPs in tobacco (TTS proteins) [11]. PCD could play a role in generating the materials necessary for pollen-tube growth.

The work of Hulskamp et al. [18] strongly suggests that ovule development influences pollen-tube movement within carpels. This in turn implies some sort of signaling by ovules to pollen tubes. However, the pattern of pollen-tube movement described by these authors predicts a wave of fertilization peaking apically and proceeding basally at continually diminishing levels. We found this not to be the case. In our experiments, the probability of fertilization peaked in the central-basal region of the carpel. Even the *ntt* mutant, which displayed fertilization mainly in the apical half of the carpel, showed no preference for the most apical ovule. These results are not easily interpreted but argue against a strictly stochastic model of tube divergence. It is clear that lateral deviations in pollen-tube movement must in some way reflect the presence and availability of awaiting ovules. Recent in vitro experiments purport to show the existence of diffusible ovule-specific signals directing pollen-tube growth [24]. This type of molecule clearly offers promise as part of a guidance mechanism.

The ratio of forces acting to promote apical-basal and lateral growth in the pollen tube will determine the probability for exit from the transmitting tract. These forces undoubtedly change temporally and spatially as the transmitting tract undergoes PCD and possible nutrient consumption by pollen tubes, and ovules are constantly fertilized and removed as potential signal sources. Initial pollen tubes (represented by our limiting pollination experiments) might consequently experience a considerably different environment than later-arriving pollen tubes. Added to the complexity of these various possibilities is the consideration that pollen tubes might compete with each other to fertilize ovules [25]. An excess of microgametes competing to reach the macrogamete is a well-known feature of sexual reproduction.

In summary, the observed pattern of pollen-tube movement is probably the sum of multiple influences, and additional work will be necessary to dissect the individual contributions. However, it is clear that pollen-tube movement within the carpel is nonrandom and that the overall path to fertilization includes influences acting to promote both basal travel through the transmitting tract and lateral travel over epidermal surfaces toward ovules. Mutants such as *ntt* should prove useful in dissecting these different influences.

Experimental Procedures

Plants

Wild-type was Col-O. *ntt-1* and *ntt-2* were obtained from the SALK T-DNA collection [26]: *ntt-1* (SALK_049220) is 934 base pairs and *ntt*-2 (SALK_007406) is 1909 base pairs from the start codon, respectively. We focused on *NTT* (At3g57670) as a gene of interest after screening 36 candidate genes by in situ hybridization for their pattern of carpel-specific tissue expression. Candidates were initially identified by a survey of publicly available microarray data (Supplemental Data).

Genotyping

To genotype *ntt-1*, we used SALK_049220+LP (5′-TCTCAGATTCTC ATTGGCCCT-3′) and SALK_049220+RP (5′-TCTAACCAGCAGACG CAACTT-3′). To genotype *ntt-2*, we used HAD1-RP (5′-TGGTCCATA GATTTAGGTTTCTT-3′) and HAD1-LP (5′-TTGTTAACCTAAGCATG

CATTGAC-3′) to detect wild-type. SALK_049220+LP and HAD1-RP were used with the JMLB2 primer to detect the insertion.

In Situ RNA Hybridization, Microscopy, and Histology

In situ RNA hybridization was carried out as described previously [27]. The *NTT* probe was transcribed with T7 RNA polymerase (Promega) from a PCR product produced from a pCR2.1 vector with M13 forward and reverse primers. An *NTT* cDNA clone was obtained from stage 12 carpel cDNA with the primers 5′-ATGACTGATCCTTATTCCA ATTTCTTCAC-3′ and 5′-CTATTTAGACTGCATTGACTCATGATC-3′.

Embedding plant material in JB-4 media and sectioning was performed as previously described [28]. Alcian blue staining of thin sections was performed as previously described [19]. Paraplast sections were embedded as previously described [28]. Samples were sectioned to 4 μm with a disposable steel blade on a Jung Biocut microtome and mounted on slides. Slides were dewaxed in three changes of Histoclear 10 min each and rehydrated in an ethanol series. The slides were counterstained with 0.1% Nuclear Fast Red for 5 min and then stained with 0.5% alcian blue (pH 3.1). The slides were then rinsed in water, air dried, mounted in Permount, and viewed under DIC optics.

Minimal Pollinations and Aniline Blue Staining

Aniline blue staining of pollen tubes in pistils was performed as described [9]. The flowers were emasculated at stage 12 and left for 24 hr. For the time series, we added maximal pollen with a paintbrush. The pollinated pistils were collected after 2, 4, 6, 24, and 48 hr. For the minimal pollinations, we used a fine hair to pick up 1–5 pollen grains from a slide under a compound microscope. The pollen grains were then transferred to the stigma under a dissecting microscope. The pistils were fixed and stained as described [9]. We used the software program ImageJ (http://rsb.info.nih.gov/ij) to analyze the lengths of the pollen tubes within the carpel and to determine the position of the seeds within the fruit [29].

GUS Pollen

Cross-sections of pollen tubes could be visualized after pollinating with pollen containing the ACA9-promoter::β-glucuronidase (GUS) construct [15]. Flowers were emasculated at stage 12 and pollen was added after 24 hr. Sections of the GUS-staining tissue were prepared as previously described [27].

Supplemental Data

One figure and Experimental Procedures are available at http://www.current-biology.com/cgi/content/full/17/13/1101/DC1/.

Acknowledgments

We thank R. Willner and J. Young for help with plant stocks and general laboratory assistance. We thank S. Jeong and J.J. Ripoll-Samper for careful reviewing of this manuscript and S. Frietsch for helpful discussions and materials. This work is funded by a National Science Foundation grant to M.F.Y.

Received: May 2, 2007
Revised: May 24, 2007
Accepted: May 25, 2007
Published online: June 28, 2007

References

1. Johnson, M.A., and Preuss, D. (2002). Plotting a course: multiple signals guide pollen tubes to their targets. Dev. Cell *2*, 273–281.
2. Ray, S.M., Park, S.S., and Ray, A. (1997). Pollen tube guidance by the female gametophyte. Development *124*, 2489–2498.
3. Lord, E. (2000). Adhesion and cell movement during pollination: cherchez la femme. Trends Plant Sci. *5*, 368–373.
4. Cheung, A.Y., and Wu, H.M. (2001). Plant biology. Pollen tube guidance—right on target. Science *293*, 1441–1442.
5. Alvarez, J., and Smyth, D.R. (1999). *CRABS CLAW* and *SPATULA*, two *Arabidopsis* genes that control carpel development in parallel with *AGAMOUS*. Development *126*, 2377–2386.
6. Erbar, C. (2003). Pollen tube transmitting tissue: place of competition of male gametophytes. Int. J. Plant Sci. *164*, S265–S277.
7. Lennon, K., Roy, S., Hepler, P., and Lord, E. (1998). The structure of the transmitting tissue of *Arabidopsis thaliana (L.)* and the path of pollen tube growth. Sex. Plant Reprod. *11*, 49–59.
8. Wang, H., Wu, H.M., and Cheung, A.Y. (1996). Pollination induces mRNA poly(A) tail-shortening and cell deterioration in flower transmitting tissue. Plant J. *9*, 715–727.
9. Jiang, L., Yang, S.L., Xie, L.F., Puah, C.S., Zhang, X.Q., Yang, W.C., Sundaresan, V., and Ye, D. (2005). *VANGUARD1* encodes a pectin methylesterase that enhances pollen tube growth in the *Arabidopsis* style and transmitting tract. Plant Cell *17*, 584–596.
10. Palanivelu, R., Brass, L., Edlund, A.F., and Preuss, D. (2003). Pollen tube growth and guidance is regulated by *POP2*, an *Arabidopsis* gene that controls GABA levels. Cell *114*, 47–59.
11. Cheung, A.Y., Wang, H., and Wu, H.M. (1995). A floral transmitting tissue-specific glycoprotein attracts pollen tubes and stimulates their growth. Cell *82*, 383–393.
12. Hensel, L., Nelson, M., Richmond, T., and Bleecker, A. (1994). The fate of inflorescence meristems is controlled by developing fruits in *Arabidopsis*. Plant Physiol. *106*, 863–876.
13. Sagasser, M., Lu, G.H., Hahlbrock, K., and Weisshaar, B. (2002). *A. thaliana TRANSPARENT TESTA 1* is involved in seed coat development and defines the WIP subfamily of plant zinc finger proteins. Genes Dev. *16*, 138–149.
14. Englbrecht, C.C., Schoof, H., and Bohm, S. (2004). Conservation, diversification and expansion of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. BMC Genomics *5*, 39.
15. Schiott, M., Romanowsky, S.M., Baekgaard, L., Jakobsen, M.K., Palmgren, M.G., and Harper, J.F. (2004). A plant plasma membrane Ca2+ pump is required for normal pollen tube growth and fertilization. Proc. Natl. Acad. Sci. USA *101*, 9502–9507.
16. Pruitt, R., and Hülskamp, M. (1994). From Pollination to Fertilization in Arabidopsis (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).
17. Bassiri, A., Ahmad, F., and Slinkard, A. (1987). Pollen grain germination and pollen tube growth following in vivo and in vitro self and interspecific pollinations in annual Cicer species. Euphytica *36*, 667–675.
18. Hulskamp, M., Schneitz, K., and Pruitt, R.E. (1995). Genetic evidence for a long-range activity that directs pollen tube guidance in *Arabidopsis*. Plant Cell *7*, 57–64.
19. Sessions, R.A., and Zambryski, P.C. (1995). *Arabidopsis* gynoecium structure in the wild type and in *ettin* mutants. Development *121*, 1519–1532.
20. Smyth, D.R., Bowman, J.L., and Meyerowitz, E.M. (1990). Early flower development in *Arabidopsis*. Plant Cell *2*, 755–767.
21. Gao, M., and Showalter, A.M. (1999). Yariv reagent treatment induces programmed cell death in *Arabidopsis* cell cultures and implicates arabinogalactan protein involvement. Plant J. *19*, 321–331.
22. Guan, Y., and Nothnagel, E.A. (2004). Binding of arabinogalactan proteins by Yariv phenylglycoside triggers wound-like responses in *Arabidopsis* cell cultures. Plant Physiol. *135*, 1346–1366.
23. Sanders, L.C., and Lord, E.M. (1989). Directed movement of latex particles in the gynoecia of three species of flowering plants. Science *243*, 1606–1608.
24. Palanivelu, R., and Preuss, D. (2006). Distinct short-range ovule signals attract or repel *Arabidopsis thaliana* pollen tubes in vitro. BMC Plant Biol. *6*, 7.
25. Mulcahy, D. (1979). The rise of the angiosperms: a genecological factor. Science *206*, 20–23.
26. Alonso, J.M., Stepanova, A.N., Leisse, T.J., Kim, C.J., Chen, H., Shinn, P., Stevenson, D.K., Zimmerman, J., Barajas, P., Cheuk, R., et al. (2003). Genome-wide insertional mutagenesis of *Arabidopsis thaliana*. Science *301*, 653–657.
27. Dinneny, J.R., Weigel, D., and Yanofsky, M.F. (2006). *NUBBIN* and *JAGGED* define stamen and carpel shape in *Arabidopsis*. Development *133*, 1645–1655.
28. Roeder, A.H., Ferrandiz, C., and Yanofsky, M.F. (2003). The role of the *REPLUMLESS* homeodomain protein in patterning the *Arabidopsis* fruit. Curr. Biol. *13*, 1630–1635.
29. Abramoff, M., Magelhaes, P., and Ram, S. (2004). Image processing with ImageJ. Biophotonics Int. *11*, 36–42.