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HALF FILLED promotes reproductive tract development and fertilization efficiency in *Arabidopsis thaliana*

Brian C. W. Crawford and Martin F. Yanofsky*

SUMMARY

Successful fertilization in angiosperms requires the growth of pollen tubes through the female reproductive tract as they seek out unfertilized ovules. In *Arabidopsis*, the reproductive tract begins with the stigma, where pollen grains initially adhere, and extends through the transmitting tract of the style and ovary. In wild-type plants, cells within the transmitting tract produce a rich extracellular matrix and undergo programmed cell death to facilitate pollen movement. Here, we show that the *HAF*, *BEE1* and *BEE3* genes encode closely related bHLH transcription factors that act redundantly to specify reproductive tract tissues. These three genes are expressed in distinct but overlapping patterns within the reproductive tract, and in *haf bee1 bee3* triple mutants extracellular matrix formation and cell death fail to occur within the transmitting tract. We used a minimal pollination assay to show that *HAF* is necessary and sufficient to promote fertilization efficiency. Our studies further show that *HAF* expression depends on the *NTT* gene and on an auxin signaling pathway mediated by the *ARF6*, *ARF8* and *HEC* genes.

KEY WORDS: *Arabidopsis*, Reproductive tract, Fertility

INTRODUCTION

Throughout plant evolution the egg cell has become progressively less exposed to fertilization by the male gametophyte. Indeed, the defining feature of angiosperms is the enclosure of the ovule within a carpel. This presents a problem of accessibility for pollen tubes (Crawford and Yanofsky, 2008; Williams, 2009). Not only are ovules distant from the point of pollen germination, in some angiosperms, such as *Arabidopsis*, the ovule is buried within the layers of tissue of the carpel. Additionally, the pollination and fertilization process occurs more rapidly in angiosperms compared with gymnosperms (Williams, 2008; Williams, 2009). To overcome these difficulties, angiosperms have developed a unique set of tissues to assist pollen tubes in reaching the ovules. These tissues are collectively known as the reproductive tract and consist of the stigma, style, transmitting tract and funiculus (Fig. 1A,B).

Pollen grains initially contact and germinate on elongated papillary cells of the stigma. Once germinated, they develop into pollen tubes that grow into the style, the connecting tissue between the stigma and ovary chamber (Fig. 1A,B). In *Arabidopsis thaliana*, pollen tubes must grow through the short, enclosed style to reach the ovary (Lennon et al., 1998). In other angiosperms, however, such as lily, pollen tubes can grow on the surface of an open style to reach the ovary (Kim et al., 2003). The closed style of *Arabidopsis* contains the start of the transmitting tract, a pathway for pollen tube growth that undergoes programmed cell death (Crawford et al., 2007). Transmitting tract tissue develops in the center of the septum between the two fused carpels (Fig. 1A) and connects the style to the bottom of the ovary chamber (Fig. 1B). Pollen tubes grow basally through the transmitting tract and exit laterally onto the septum epidermis, whereupon they grow towards and upon funiculi to reach ovules. The funiculus develops at the boundary of the septum and carpel walls and connects the ovules to the carpel (Fig. 1A). At the end of the funiculus, pollen tubes enter the micropyle to fertilize the egg cell.

The reproductive tract is essential for successful fertilization of ovules by pollen, and seed set is reduced in mutants that interfere with reproductive tract development (Alvarez and Smyth, 2002; Gremski et al., 2007; Heisler et al., 2001). Pollen tubes also target ovules more efficiently in vitro if they have first grown through stigma and style (Palanivelu and Preuss, 2006).

The *HECATE* (*HEC1*, *HEC2*, *HEC3*) and *SPATULA* (*SPT*) genes encode putative basic helix-loop-helix (bHLH) transcription factors that have key roles in reproductive tract development as they control overall growth of the stigma, style and transmitting tract. Mutations in these genes lead to varying degrees of reduced fertility, and whereas *HEC2*-RNAi *hec1 hec3* mutants are completely infertile, *spt* mutants show moderate infertility (Alvarez and Smyth, 2002; Gremski et al., 2007; Heisler et al., 2001). HEC and SPT proteins have been shown to interact, suggesting that they act together to control development. Although the entire reproductive tract is affected in these mutants, mutations in other genes show more specific defects in reproductive tract differentiation. The *NO TRANSMITTING TRACT* (*NTT*) gene, for example, is required for normal differentiation of the ovary transmitting tract. Transmitting tract cells normally produce an extracellular matrix (ECM) containing a mixture of glycoproteins, glycolipids and polysaccharides (Lennon et al., 1998). The specific contribution of ECM to pollen tube growth is unknown, but growth studies have demonstrated that pollen tubes grow faster in vivo than in vitro, and this has been speculated to be due to interactions with ECM (Palanivelu and Preuss, 2006). Transmitting tract cells also undergo a process of programmed cell death essential for efficient pollen tube growth. In the *ntt* mutant, both ECM production and cell death are absent from the ovary transmitting tract. As a result, pollen tubes progress normally through the style but have great difficulty entering the ovary and the basal ovules remain unfertilized (Crawford et al., 2007).

Here, we report the identification of the *HALF FILLED* (*HAF*) gene, a bHLH-encoding transcription factor required for reproductive tract development. We show that *HAF* acts

Section of Cell and Developmental Biology, University of California San Diego, La Jolla, CA 92093, USA.

*Author for correspondence (marty@ucsd.edu)

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redundantly with two closely related genes, *BRASSINOSTEROID ENHANCED EXPRESSION1* (*BEE1*) and *BEE3*. All three genes are expressed in an overlapping pattern during reproductive tract development and contribute jointly to ECM production and programmed cell death. A *haf bee1 bee3* triple mutant drastically reduces the efficiency of pollen tube growth throughout the reproductive tract. We also investigated the genetic interactions of *HAF*, *BEE1* and *BEE3* with other transcription factors involved in reproductive tract development. Finally, we used loss-of-function and misexpression studies to explore the role of *HAF* in regulating reproductive tract development and growth.

MATERIALS AND METHODS

Plants

All wild-type plants used in this study were in the Columbia background. For all phenotypic analyses and in situ hybridization experiments, *Arabidopsis thaliana* seeds were stratified for 3-5 days at 4°C after sowing. Plants were grown at 22-24°C under long-day (LD) conditions. The *haf* mutant CSHL_ET2536 has an insertion into the third exon and is an RNA-null allele. It was obtained from the Cold Spring Harbor collection in the Ler background and was backcrossed into the Columbia background. The *bee1*, *bee3*, *ntt-1*, *arf6-2*, *arf8-3*, *hec1* and *hec3* mutants were previously published (Crawford et al., 2007; Friedrichsen et al., 2002; Gremski et al., 2007; Ichihashi et al., 2010; Nagpal et al., 2005). The *hec2* allele SM.17339 was crossed into the *hec1* and *hec3* mutants. Seed numbers for different genotypes were determined by counting the number of seeds in each silique after the fifth silique produced. Infertile siliques and siliques containing fewer than ten seeds were excluded. The statistical significance of the difference in seed number was analyzed using Student's *t*-test.

Genotyping and overexpression lines

All primers used in this study are listed in Table S1 in the supplementary material. To genotype *haf* we used HAF7x and HAF8x. HAF8x was used along with the Ds3-2 primer to detect the insertion. *HAF* corresponds to AT1G25330. The *HAF* cDNA was subcloned behind an operator array in BJ36 plasmid to generate responder lines (Moore et al., 1998) using HAFPOP1+ and HAFPOP1– and transformed into *Arabidopsis* using the pGREEN0029 binary plasmid.

In situ RNA hybridization, microscopy and histology

In situ hybridization was carried out as described previously with the following modifications (Dinneny et al., 2006). Tissue samples were fixed in formalin-acetic-acid-alcohol (FAA) for 2.5 hours at room temperature and slides were not treated with RNase. Substrate solution for alkaline phosphatase color reaction was prepared using 2% of a nitro-blue tetrazolium and 5-bromo-4-chloro-3′-indolyphosphate (NBT/BCIP) stock solution (Roche Diagnostic, Germany) in 100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$.

The *HAF* probe was transcribed using T7 RNA polymerase (Promega) from a PCR product produced from a PCR 3.1 vector using M13 forward and reverse primers. A *HAF* cDNA clone was obtained by PCR amplification of cDNA obtained from stage 12 carpels using the primers HAFCDNA+ and HAFCDNA– and cloned into the PCR 3.1 vector.

Embedding of plant material in JB-4 media, sectioning and Alcian Blue staining of thin sections was performed as previously described (Crawford et al., 2007). Paraplast sections were embedded as previously described (Roeder et al., 2003). Samples were sectioned at 8 μm with a disposable steel blade on a Jung Biocut microtome and mounted on slides. Slides were dewaxed in three changes for 10 minutes each of Histoclear and mounted with permount.

Minimal pollinations, Aniline Blue staining and carpel measurements

Aniline Blue staining of pollen tubes in pistils was performed as described by Jiang et al. (Jiang et al., 2005). The flowers were emasculated at stage 12 and left for 24 hours. For the time series, we added maximal pollen with a paintbrush. The pollinated pistils were collected after 2, 6 and 24 hours. For the *qrt* pollinations we used a fine hair to pick up a single pollen cluster from a slide under a compound microscope. The pollen cluster was then transferred to the stigma under a dissecting microscope. We then waited 14 days for the seeds to set and used ImageJ (NIH) to analyze the lengths of carpels (Abramoff et al., 2004).

GUS staining

We used pollen containing the *ACA9::GUS* construct (Schiott et al., 2004). We created the *HAF::GUS*, *BEE1::GUS* and *BEE1::GUS* construct using the PD137 vector with the primers in Table S1 in the supplementary material to amplify 3191 bp, 1384 bp and 2635 bp regions of the *HAF*, *BEE1* and *BEE3* promoters, respectively (Blazquez et al., 1997). The flowers were emasculated at stage 12 and pollen was added after 24 hours. GUS staining of tissue sections was as previously described (Dinneny et al., 2006).

RESULTS

Phenotype of *half filled* (*haf*)

In the Landsberg *erecta* ecotype, fruit of *half filled* (*haf*) mutants were smaller than those of wild type and contained few seeds that were preferentially localized to the upper portion of the fruit (see Fig. S1A,B in the supplementary material). To determine whether these defects are female specific, we pollinated wild-type carpels with *haf*-mutant pollen and found that a normal seed set resulted. Conversely, when we pollinated *haf*-mutant carpels with wild-type pollen, the *haf* mutant phenotype was observed, indicating that the *haf* phenotype is female-specific. Surprisingly, when the *haf* allele was introgressed into the Columbia (Col) ecotype, siliques had restored fertility. This suggests that one or more genes act redundantly with *HAF* in the Col ecotype and that these genes are either not present or not functional in the Ler ecotype.

HAF is predicted to encode a 223 amino acid protein with similarities to bHLH transcription factors. It is grouped within a subfamily of bHLH genes containing 16 members (Friedrichsen et al., 2002), and is structurally most similar to two that have previously been characterized, *BEE1* and *BEE3* (Friedrichsen et al., 2002). These two genes, along with a third less closely related member, *BEE2*, were initially identified as responding to exogenous application of brassinosteroid (BR). However, unlike *BEE1*, *BEE2* and *BEE3*, *HAF* was not activated by application of BR in seedlings (Friedrichsen et al., 2002). Within the bHLH protein subdomain, HAF (bHLH number 75) has 93% similarity to BEE1 (bHLH number 44) and 91% similarity to BEE3 (bHLH number 50), and the three genes have identical intron-exon structures. The *bee1 bee3* double mutant was reported to have normal levels of fertility (Friedrichsen et al., 2002). To test for genetic redundancy with *HAF*, we generated different mutant combinations of *haf*, *bee1* and *bee3*. Whereas the *haf* single mutant produced the same number of seeds as wild type, the *haf bee1* double mutant produced an average of only 27.3 seeds per silique (n=46), compared with 51.4 seeds per silique in wild type (n=16). The *haf bee1 bee3* triple mutant (labeled *hbb* in figures) had the most severe effect on fertility, yielding an average of only 22.3 seeds per silique (n=60; compared with *haf bee1* P<0.001). In both *haf* combinations, seeds were produced predominantly in the apical part of the silique (Fig. 1). As our previous work involving *NTT* was done in the Columbia ecotype, and because the *haf bee1 bee3* mutant had the most severe fertility defect, we used this genotype for the remainder of our analyses.

The *haf bee1 bee3* mutant phenotype is very similar to that of *ntt* (Crawford et al., 2007). In both cases, seed production is reduced relative to wild type, the seeds are largely localized to the upper portion of the fruit, and the phenotype is female specific. Genetic studies have shown *NTT* to be required for proper formation of the

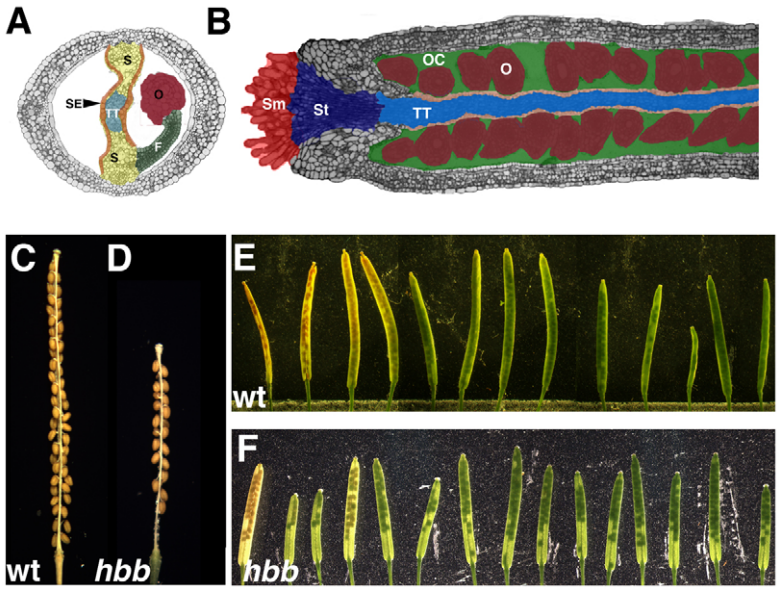

Fig. 1. Reproductive tract structure and *haf bee1 bee3* (*hbb*) mutant phenotype. (**A**) Cross section of carpel with structures colored. Transmitting tract (TT) is blue, septum (S) yellow, septum epidermis (SE) orange, funiculus (F) green and ovule (O) maroon. (**B**) Longitudinal section of carpel with structures colored. In addition to the structures labeled in A, the ovary chamber (OC) is green, the style (St) dark blue and stigma (Sm) red. (**C**) Wild-type seed set showing equivalent apical and basal seed distribution. (**D**) *haf bee1 bee3* mutant seed set showing a loss of basal seeds. (**E**) Wild-type siliques showing seeds uniformly distributed throughout fruit. (**F**) *haf bee1 bee3* mutant siliques showing consistent loss of basal seeds.

transmitting tract. To understand better the relationship between *ntt*, *haf*, *bee1* and *bee3*, we generated multiple mutants. Whereas the *ntt* single mutant produced an average of 20.4 seeds per silique (n=57), the *ntt haf* double mutant produced an average of only 14.9 seeds (n=56). The most severe effect on seed number was found in the *ntt haf bee1 bee3* quadruple mutant, which produced an average of only 13.5 seeds per silique (n=39; compared with *ntt haf* P=0.017).

HAF, *BEE1* and *BEE3* expression

To investigate how *HAF*, *BEE1* and *BEE3* function in reproductive tract development, we analyzed their expression patterns using both RNA in situ hybridization and β-glucuronidase (GUS) reporter gene constructs. *HAF* was expressed throughout the reproductive tract during stages 8 to 15 of flower development, whereas *BEE1* and *BEE3* showed more restricted patterns of expression (Fig. 2).

Expression of *HAF* was first observed by RNA in situ hybridization during stage 8 of gynoecium development, in the medial ridges of the septum, which at this time have grown together and fused (Fig. 2A, arrowhead) (Bowman, 1993). By stage 10, *HAF* expression was observed in the center of the septum where the transmitting tract arises (Fig. 2B, arrowhead). Expression had expanded to include both the septum transmitting tract and funiculus (Fig. 2C, arrow) by pre-fertilization stage 12. Expression in the funiculus was predominantly in the epidermis (Fig. 2C, arrow and see Fig. S2 in the supplementary material).

To compare *HAF*, *BEE1* and *BEE3* expression patterns, we made GUS constructs driven by their respective promoters. Fertilization of the carpel occurs during stage 13 and the *HAF* promoter was seen to be active before and after this stage in the stigma, style, transmitting tract and funiculus (Fig. 2D). The *BEE1* promoter was active in the stigma and the top of the style, whereas the *BEE3* promoter was active in the transmitting tract and style, albeit at a lower level compared with *HAF* (Fig. 2E,F). The expression of *BEE1* within the carpel was confirmed by in situ hybridization (see Fig. S2 in the supplementary material). *BEE1* and *BEE3*, therefore, have restricted, overlapping expression patterns with *HAF* within the reproductive tract.

In later stages of fruit development after fertilization, *HAF* expression is maintained in the style, transmitting tract and funiculus and only diminishes during late maturation at stage 17 (Fig. 2G-I). *HAF* is expressed exclusively in the developing carpel according to our results and publicly available microarrays (Atgenexpress, http://www.arabidopsis.org/portals/expression/microarray/ATGenExpress.jsp), but *BEE1* and *BEE3* are expressed more widely. The promoter-GUS constructs for *BEE1* and *BEE3* showed expression in other parts of the plant, including vegetative tissues (see Fig. S2 in the supplementary material). However, no obvious aberrant phenotype was observed in any of these tissues in the *haf bee1 bee3* triple mutant.

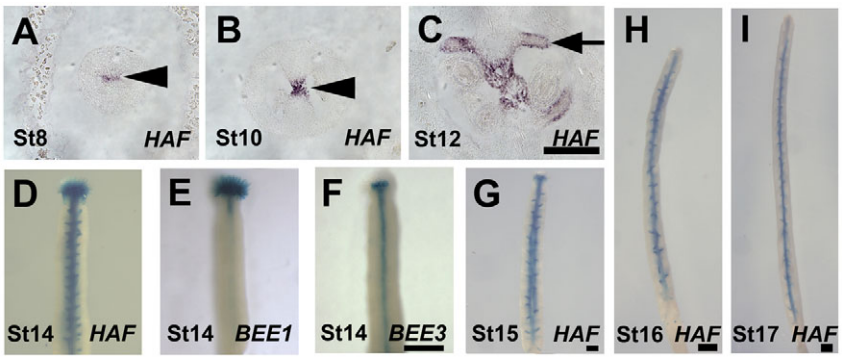

Fig. 2. *HAF*, *BEE1* and *BEE3* expression in developing wild-type carpels. (**A-C**) Transverse sections of stage eight (A), ten (B) and 12 (C) wild-type carpels probed with *HAF* by in situ hybridization. Arrowheads indicate position of *HAF* expression. Arrow indicates epidermal expression in funiculus. (**D-I**) β-Glucoronidase (GUS) staining on whole-mount carpels and fruits with *HAF::GUS* at stage 14 (D), 15 (G), 16 (H) and 17 (I). Stage 14 *BEE1::GUS* (E) and *BEE3::GUS* staining (F). Scale bars: 50 μm.

Pollen tube growth in the *haf bee1 bee3* triple mutant

Pollen tubes grow basally from the stigma through the different tissues of the reproductive tract to reach ovules. As *HAF*, *BEE1* and *BEE3* are expressed in reproductive tract tissues, it seemed likely that at least part of the phenotype of *haf bee1 bee3* might be due to impaired pollen tube growth. We therefore used Aniline Blue staining to reveal callose, a component of the pollen tube cell wall, to visualize pollen tubes in the carpel. We analyzed the extent of tube growth in both Col and the triple mutant at 2, 6 and 24 hours post-fertilization. The first unopened flower was emasculated and grown for 24 hours to allow full development of the reproductive tract before being maximally pollinated with wild-type pollen and then fixed at the indicated times.

At 2 hours, pollen germination and/or stylar passage were already seen to be impaired in the triple mutant. The width of the stylar pollen tube pathway (Fig. 3A,B, arrowheads) was 88.3±9.7 μm (n=11) for Col, and in the *haf bee1 bee3* triple mutant it was only 55.2±8.8 μm (n=13; P<0.001). Although fewer pollen tubes entered the upper ovary chamber, their extent of ingress was roughly similar to that of wild type. The farthest extent of apical-basal pollen tube growth into the carpel at this time point was 0.66±0.07 mm (n=11) in wild type compared with 0.58±0.11 mm (n=13) in *haf bee1 bee3* (Fig. 3B).

The effect on apical-basal pollen tube growth was much more pronounced at 6 hours (compare Fig. 3D and 3E). Pollen tubes were found throughout the transmitting tract in wild type, but had significantly less apical-basal and lateral growth in *haf bee1 bee3*, where they were funneled into a narrow path down the center of the transmitting tract (Fig. 3E, arrowhead).

At 24 hours, pollen tubes had reached the base of carpels in wild type, but had only grown half the length of the carpels in the mutant (Fig. 3G,H). Moreover, pollen tubes were restricted to a smaller and smaller region of growth within the transmitting tract (compare Fig. 3I and 3J).

Whole-mount analysis of carpels displaying pollen-specific GUS expression 24 hours after pollination was consistent with results from Aniline Blue staining (see Fig. S3A,B in the supplementary material). Cross-sections at the midpoint of the carpels confirmed that, in contrast to the widespread distribution of pollen tubes in wild type, pollen tubes in the triple mutant were constrained to a very small region within the transmitting tract (compare Fig. 3K and 3L, arrows).

Transmitting tract structure in *haf bee1 bee3* mutants

We undertook a detailed morphological analysis of the transmitting tract in *haf bee1 bee3* triple mutant plants. To visualize transmitting tract tissue in the triple mutant, carpels were thin-sectioned and stained with Alcian Blue to reveal acidic polysaccharides, major components of the ECM of the transmitting tract. Neutral Red was used as a counterstain to highlight cell walls. Sections were obtained from stages 12 (pre-anthesis), 14 (post-anthesis) and 17 (mature fruit) of wild type and *haf bee1 bee3*.

At stage 12, the wild-type transmitting tract region stained strongly with Alcian Blue, indicating the presence of ECM (Fig. 4A). In *haf bee1 bee3* mutants, the region of septum corresponding to the transmitting tract septum appeared cytologically normal, but stained only slightly (Fig. 4D), indicating severely reduced ECM production. Immediately after fertilization at stage 14, wild-type cells within the transmitting tract undergo increased cell death and stained intensely for Alician Blue (Fig. 4B, arrowhead) (Crawford et al., 2007). At this stage in the triple mutant, there was only a minimal increase in the very low level of Alcian Blue staining seen at stage 12, and there was still no indication of cell death (Fig. 4E, arrow). In the mature wild-type silique at stage 17, all cells between the septum epidermal layers were absent, leaving a two cell-layered structure (Fig. 4C) (Crawford et al., 2007). In the triple mutant, residual cells could still be seen between the epidermal cell layers (Fig. 4F, arrow)

We also examined transmitting tract structure in the style of *haf bee1 bee3*. In wild type, the stylar transmitting tract consisted of tightly packed smaller cells, the majority of which stained for ECM (Fig. 4G), whereas in the triple mutant, the width of tightly packed cells in the style was significantly reduced and relatively few showed evidence of Alcian Blue ECM staining (compare Fig. 4G and 4H, chevrons).

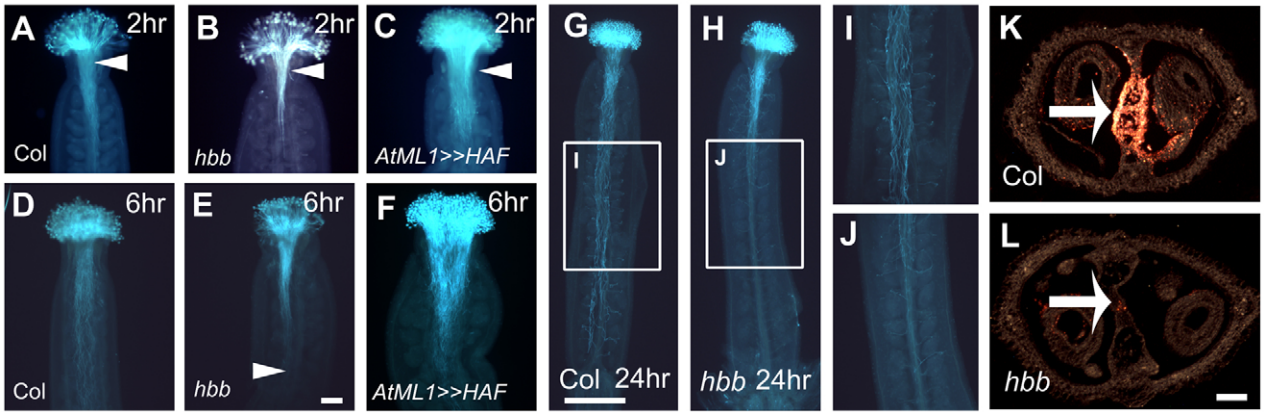

Fig. 3. Pollen tube growth in wild type, *haf bee1 bee3* (*hbb*) mutant and *AtML1>>HAF*. (**A-J**) Pollen tube growth stained with Aniline Blue of wild type (A,D,G,I), *haf bee1 bee3* mutant (B,E,H,J) and *AtML1>>HAF* (C,F) 2 (A-C), 6 (D-F) and 24 hours post-pollination (G-J). Boxed areas in G and H are enlarged in I and J, respectively. (**K**,**L**) Distribution of pollen tubes visualized with GUS-labeled pollen (Schiott et al., 2004). Transverse sections were taken 24 hours after pollination and processed after an additional 24 hours in wild type (K) and *haf bee1 bee3* mutant (L). The cross sections were taken from the middle of the carpel. Arrows indicate GUS staining. Scale bars: 50 μm.

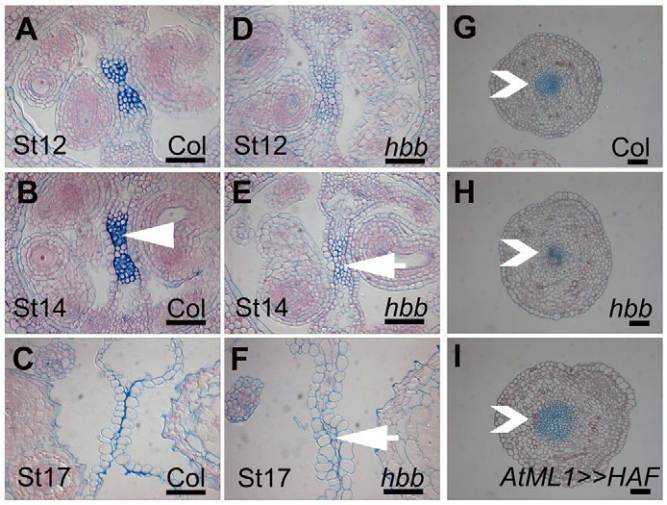

Fig. 4. Transverse sections of different stages of transmitting tract development. (**A-I**) Sections stained with Alcian Blue and Neutral Red were taken from wild type (A-C,G), *haf bee1 bee3* (*hbb*) mutant (D-F,H) and *AtML1>>HAF* (I) at stage 12 (A,D), 14 (B,E,G-I) and 17 (C,F) style region. Arrowhead in B indicates cell breakdown in wild type. Arrows in E and F indicate cells intact in *haf bee1 bee3* mutant. Chevrons in G-I indicate position of style transmitting tract. Scale bars: 100 μm.

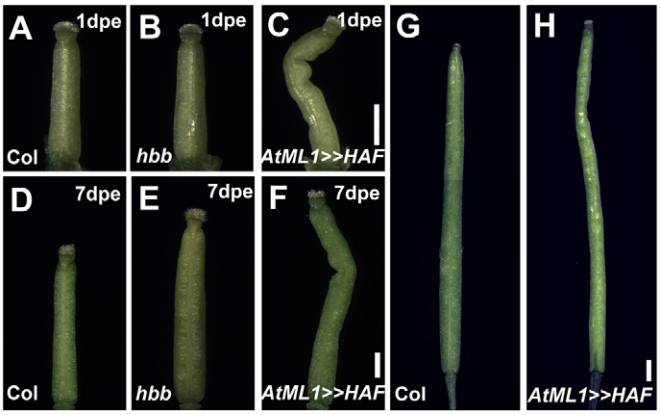

Fig. 5. Growth in emasculated carpels and fruit of *haf bee1 bee3* (*hbb*) mutant and *AtML1>>HAF*. (**A-F**) Emasculated carpels of wild type (A,D), *haf bee1 bee3* mutant (B,E) and *AtML1>>HAF* (C,F) left for one day (A-C) and seven days (D-F). (**G**,**H**) Examples of wild type (G) and *AtML1>>HAF* (H) fruits. dpe, days post-emasculation. Scale bars: 500 μm.

Ectopic expression of *HAF* promotes both pollen and carpel growth

Loss-of-function studies have demonstrated that *HAF* is required for efficient fertilization and plays an important role in development of the reproductive tract. In order to determine whether *HAF* is sufficient to produce phenotypes when ectopically expressed, we initially tried to express *HAF* from the 'constitutive' CaMV35S promoter. However, we were unable to recover transgenic plants, as tested by in situ hybridization, that misexpressed *HAF*, suggesting that constitutive misexpression might be lethal. To circumvent this problem, we expressed *HAF* from the epidermal-specific *AtML1* promoter (*AtML1>>HAF*) (see Fig. S4 in the supplementary material) (Sessions et al., 1999; Sessions et al., 2000). A number of transcription factors have been misexpressed from the *AtML1* promoter, often leading to non-autonomous effects in internal tissues (Savaldi-Goldstein et al., 2007; Sessions et al., 2000).

We first investigated the effect of *AtML1>>HAF* on growth of unfertilized carpels and fertilized fruit. The most striking growth phenotype of ectopic *AtML1>>HAF* expression was an increased size and bent morphology for *AtML1>>HAF* carpels. Similar phenotypes were also observed when *HAF* was expressed under the control of the *AGAMOUS* promoter (data not shown). To quantify the effect of *HAF* on carpel growth, we measured carpel length one day (1 dpe) and seven days (7 dpe) after flower emasculation in wild type, *haf bee1 bee3* and *AtML1>>HAF*. Emasculation was used to prevent the dramatic increase in carpel size resulting from fertilization. At 1 dpe, there was no difference between wild type 1.65±0.15 mm (n=10) and *haf bee1 bee3* triple mutant carpels 1.65±0.17 mm (n=10) (Fig. 5A,B). By contrast, the *AtML1>>HAF* carpels were significantly larger, 1.82±0.21 mm (n=17) with a characteristic bent morphology (compared with wild type P=0.037) (Fig. 5C). By 7 dpe, wild-type carpels had grown slightly even though they had not been pollinated, reaching 3.25±0.22 mm (n=12). In the *haf bee1 bee3* triple mutant, carpels were noticeably bigger than wild type, averaging 3.71±0.26 mm (n=10; compared with wild type P<0.001) (Fig. 5D,E). The largest carpels were produced by the *AtML1>>HAF* genotype, 4.49±0.46 mm (n=17; compared with wild type P<0.001), and these again had a bent morphology (Fig. 5F). These data show that misexpression of *HAF* is sufficient to promote substantial overgrowth in unfertilized carpels, but that such growth is uncoordinated and leads to distortions in carpel shape.

We next tested how ectopic *HAF* expression influenced the final size of fertilized fruit. Wild type and *AtML1>>HAF* flowers were emasculated and carpels maximally pollinated with wild-type pollen and the length of the resultant fruit was measured after 7 days. *AtML1>>HAF* siliques measured on average 14.3±2.9 mm (n=44), and were larger than wild-type siliques, 12.0±1.6 mm (n=55; P<0.001) (Fig. 5G,H). The increased silique size was due to an increased number of cells in the silique, as the average valve cell length in wild type, 0.243±0.045 mm (n=49), was comparable to that in *AtML1>>HAF*, 0.239±0.046 mm (n=43). The overall number of seeds remained the same but when the internal septum of *AtML1>>HAF* was compared with wild type, there was more space between seeds along the septum. The increased septum size could be one cause of the bent morphology observed in the *AtML1>>HAF* carpels. Interestingly, the fertilized *AtML1>>HAF* fruit (H) were much straighter than those unfertilized carpels (C). We excluded *haf bee1 bee3* triple mutants from these studies as the reduction in seed number of this genotype leads to smaller fruit regardless of any effect on growth.

Because the most striking phenotype of the *haf bee1 bee3* triple mutant is a lack of proper transmitting tract development and an impairment of pollen tube growth, we examined these features in *AtML1>>HAF* carpels. Aniline Blue staining was used to monitor pollen tube growth at both 2 and 6 hours after pollinating emasculated flowers. There was a significant increase in the number of pollen tubes in *AtML1>>HAF* carpels compared with wild type at both time points (Fig. 3A,C,D,F, arrowheads). At 2 hours (Fig. 3A,C), the average width of the pollen tube pathway in the style was 88.3±9.7 μm (n=11) in wild type, and was 111.7±22.7 μm (n=15) in *AtML1>>HAF* (P<0.01). The farthest extent of

apical-basal pollen tube growth into the carpel was 0.66±0.07 mm (n=11) in wild type, and 0.75±0.1 mm (n=15) in *AtML1>>HAF* (P=0.013). At 6 hours, pollen tubes appeared more widespread in the style in *AtML1>>HAF* compared with wild type whereas the extent of the pollen tubes within the ovary transmitting tract was similar (compare Fig. 3D and 3F). Pollen tube growth is thus significantly enhanced in the *AtML1>>HAF* reproductive tract.

HAF controls efficiency of fertilization

Whereas impaired pollen tube growth in *haf bee1 bee3* carpels implies that basal ovules are less likely to be fertilized, it was unclear how the enhanced pollen tube growth seen in *AtML1>>HAF* carpels might influence fertility. To quantify how the fertilization efficiency is affected in the different genotypes, we performed minimal pollinations on carpels from emasculated carpels using *quartet* (*qrt*) mutant pollen (Preuss et al., 1994). In the *qrt* mutant, pollen grains failed to separate in the final stage of pollen development, leaving a characteristic four pollen grain bundle. During fertilization, the pollen grains separated and acted independently. This allowed us to add an exact number of pollen grains and measure how many fertilized siliques were produced by each pollination event.

We placed one of these *qrt* bundles on the stigma of wild type (n=97) or *haf bee1 bee3* (n=63) and *AtML1>>HAF* (n=85). In wild type, 33% of carpels were fertilized, compared with only 8% of carpels in the *haf bee1 bee3* triple mutant and 49% of *AtML1>>HAF* carpels. An average of 0.54 (s.e.m.=0.09) seeds were produced per carpel in wild type compared with 0.91 (s.e.m.=0.12) seeds in *AtML1>>HAF* carpels (P=0.015). This indicates that the ability of ovules to be fertilized is reduced in the triple mutant and significantly increased in the *AtML1>>HAF* genotype.

HAF acts downstream of the *NTT* and *HEC* genes

Previous studies have shown that the transmitting tract does not develop correctly in the *ntt* mutant, causing very inefficient pollen tube growth (Crawford et al., 2007). The fertility defects of *haf bee1 bee3* triple mutants are very similar to those of *ntt*, and *HAF* expression in the septum of stage 9 to 12 carpels overlapped with that of *NTT* (Fig. 2A-C) (Crawford et al., 2007). This suggests that *HAF* and *NTT* could act in the same pathway to promote reproductive tract development. To check this, we analyzed *HAF* expression in *ntt* carpels using the *HAF::GUS* construct. *HAF::GUS* expression was greatly reduced at both stage 12 and stage 14 in *ntt* mutant carpels (compare Fig. 6A,B and 6C,D). By contrast, no change was seen for *NTT* expression in *haf* mutant carpels, as determined by in situ hybridization (see Fig. S5 in the supplementary material). These data suggest that *NTT* acts upstream to promote *HAF* expression in the transmitting tract.

As expression of *HAF* is partially dependent on *NTT*, we wanted to determine whether misexpression of *HAF* could rescue the *ntt* mutant phenotypes. To test this, we introduced the *AtML1>>HAF* transgene into *ntt* and examined pollen growth by Aniline Blue staining. Both *ntt* and *ntt AtML1>>HAF* flowers were emasculated and the carpels maximally hand-pollinated with wild-type pollen before staining 6 hours later with Aniline Blue. In *ntt*, pollen tubes passed normally through the style but were severely inhibited upon reaching the ovary transmitting tract (compare Fig. 6K and 6L), as previously reported (Crawford et al., 2007). In *ntt AtML1>>HAF*, pollen tubes still had great difficulty entering the ovary transmitting tract, but the extent of basal pollen tube growth was modestly increased compared with *ntt* (Fig. 6L, arrowhead). Expression of *HAF* from *AtML1>>HAF*, therefore, does partially rescue *ntt* pollen tube growth. However, as the number of seeds produced by *ntt AtML1>>HAF* carpels was the same as for *ntt* (data not shown), this improvement in pollen tube growth was not sufficient to rescue fertility.

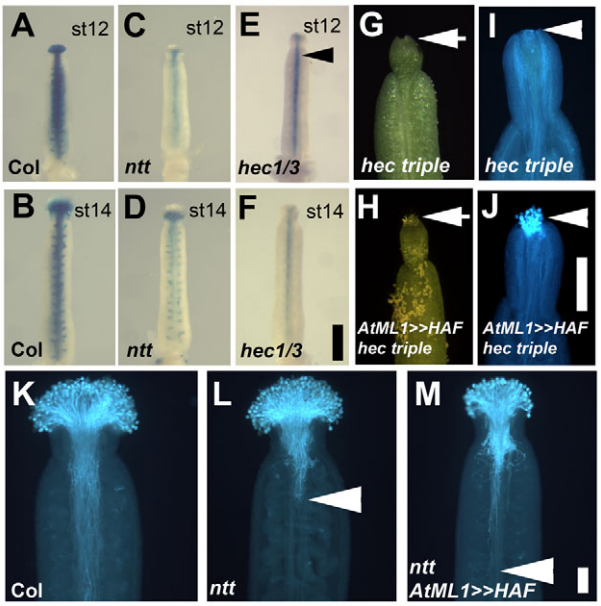

Fig. 6. Genetic relationship between *HAF* and *NTT* and *HEC* genes. (**A-F**) *HAF::GUS* staining in wild type (A,B), *ntt* (C,D) and *hec1 hec3* (E,F) carpels at stages 12 (A,C,E) and 14 (B,D,F). Arrowhead indicates low level staining (E). (**G**,**H**) Stigma and style of *hec1 hec2 hec3* triple mutant (G) and *hec1 hec2 hec3* triple mutant with *AtML1>>HAF* (H). Arrow indicates stigmatic tissue or lack of stigmatic tissue. (**I**,**J**) Aniline Blue staining of pollen tube growth blue fixed at 24 hours post-pollination in carpels in *hec1 hec2 hec3* triple mutant (I) and *hec1 hec2 hec3* triple mutant with *AtML1>>HAF* (J). Arrowhead indicates pollination or lack of pollination. (**K-M**) Pollen tube growth stained with Aniline Blue fixed at 24 hours post-pollination in carpels of wild type (K), *ntt* (L) and *ntt AtML1>>HAF* (M). Arrowheads indicate extent of pollen tube growth. Scale bars: 500 μm for A-J; 100 μm for K-M.

The *hec1 hec3* double mutant shows a significant loss of reproductive tract tissue and a corresponding reduction in fertility (Gremski et al., 2007). It is therefore not surprising that we found *HAF::GUS* expression to be dramatically reduced in the *hec1 hec3* double mutant (compare Fig. 6A,B with 6E,F). These data suggest that *HEC* acts upstream of *HAF* to promote reproductive tract development.

Previous work had determined that removing the activity of all three *hec* genes caused complete loss of reproductive tract tissue (Gremski et al., 2007). This phenotype was obtained using a combination of *hec1 hec3* insertional mutants along with RNAi to target *HEC2*. We obtained an insertion mutation in *hec2* and created a *hec1 hec2 hec3* triple mutant that confirmed the complete loss of reproductive tract tissue phenotype (Fig. 6G). Consistent with previous work, the *hec1 hec2 hec3* mutant cannot be pollinated as no stigmatic tissue is produced. Interestingly, misexpression of *HAF* through *AtML1>>HAF* was sufficient to restore some stigmatic tissue to the *hec1 hec2 hec3* mutant carpels (compare Fig. 6G and 6H). As a result of this stigmatic tissue it is possible to pollinate the *AtML1>>HAF hec1 hec2 hec3* carpels

(Fig. 6I,J). A limited number of seeds can be produced from *AtML1>>HAF hec1 hec2 hec3* plants compared with the complete infertility of *hec1 hec2 hec3* triple mutants.

ARF6 and *ARF8* control reproductive tract development and are positive regulators of *HAF* expression

ARF8 is a member of a transcription factor family that activates or represses gene expression in response to auxin. The first mutant allele of *ARF8* to be characterized was called *fruit without fertilization* (renamed *arf8-4*) (Vivian-Smith et al., 2001). The *arf8-4* mutant allele has a fertility phenotype similar to both *ntt* single mutants and *haf bee1 bee3* triple mutants (Goetz et al., 2007; Goetz et al., 2006; Vivian-Smith et al., 2001). Subsequent analysis of *ARF8* revealed that it acts redundantly with *AUXIN RESPONSE FACTOR 6* (*ARF6*). These two closely related transcription factors control maturation and development of the carpel (Nagpal et al., 2005; Wu et al., 2006). Because *ARF6* and *ARF8* are both expressed in a pattern that resembles *HAF* expression (Wu et al., 2006), we analyzed *HAF* expression in *arf6* single and *arf6 arf8* double mutants to determine whether these *ARF* genes have a role in regulating *HAF*.

HAF::GUS expression was severely reduced in the *arf6 arf8* double mutant, showing only faint expression in the style and transmitting tract (compare Fig. 7A and 7C, arrowhead indicates expression). We also compared *HAF* expression in stage 14 wild-type carpels with a carpel from an equivalent position from the *arf6 arf8* double mutant. *HAF::GUS* expression was almost completely absent in the *arf6 arf8* mutant at this stage (compare Fig. 7B and 7D). Although *arf6 arf8* mutants showed some loss of transmitting tract tissue that will reduce *HAF* expression for trivial reasons, most of the effect observed in Fig. 7 appears to be due to impaired regulation. Indeed, in contrast to the loss of *HAF* expression, *HEC2* expression was unaffected in *arf6 arf8* double mutants as shown by the *HEC2::GUS* construct (Fig. 7H,I) (Gremski et al., 2007). To determine whether *ARF6*/*ARF8* expression is independent of *HAF*, we analyzed *ARF8* expression in *haf bee1 bee3* using the *gARF8::GUS* construct (Wu et al., 2006) and found no reduction in *gARF8::GUS* expression (data not shown). Taken together, these data suggest that *ARF6* and *ARF8* act upstream of *HAF*, but not the *HEC* genes, in control of reproductive tract development.

Carpels of the *arf6 arf8* double mutant arrest development at stage 12 and do not undergo anthesis, preventing self fertilization. After hand pollination, pollen tubes grow aberrantly within *arf6 arf8* double mutant carpels, suggesting a defect in reproductive tract development (Wu et al., 2006). To investigate this further, we performed fertility and morphological studies with *arf6 arf8* mutants.

Fertility was examined in the *arf6 arf8* double mutant by applying wild-type pollen to *arf6 arf8* carpels and examining seed distribution after 2 weeks. An average of only 7.8 seeds per fruit (n=11) was produced from mutant plants, compared with 50 seeds for wild type. The fertility of *arf6 arf8* mutant carpels was quite variable, with one carpel of the 11 pollinated producing 25 seeds. The seeds were smaller than those of wild type and the resulting fruit lacked structures characteristic of a wild-type silique, such as a valve margin. Seeds were always found apically within the carpel, similar to *haf bee1 bee3* mutants (Fig. 7E, chevron).

We created a *haf bee1 bee3 arf6 arf8* quintuple mutant to determine whether *haf bee1 bee3* could enhance the fertility defect of *arf6 arf8*. Carpels of the quintuple mutant could still be pollinated to produce an average of 6.1 seeds per fruit (n=10) and the maximum number of seeds obtained from an individual carpel was 12. As the fertility of *haf bee1 bee3 arf6 arf8* was equivalent to that of *arf6 arf8*, these data are consistent with the idea that *ARF6* and *ARF8* act upstream of *HAF*, *BEE1* and *BEE3*.

Transmitting tract structure was examined in *arf6 arf8* carpels using thin cross sections of stage 12 carpels stained with Alcian Blue. We chose to perform the comparison at stage 12 because *arf6 arf8* carpels stop development at this stage. ECM production was found to be substantially reduced in the septum of *arf6 arf8* (compare Fig. 7F and 7G, arrows). The reduction in ECM staining supports the idea that *ARF6* and *ARF8* act in the same pathway as *HAF*, *BEE1* and *BEE3* to promote reproductive tract development. An additional growth-related phenotype in the *arf6 arf8* reproductive tract was a failure of the septum to fuse completely in the basal region of the carpel (see Fig. S6 in the supplementary material), indicating that *ARF6* and *ARF8* are also involved in promoting generalized growth of the septum. There was no noticeable rescue of the *arf6 arf8* double mutant by the *AtML1>>HAF* construct. This is not surprising considering the wide roles that *ARF6* and *ARF8* have in overall flower and fruit development.

HAF controls cell death within the reproductive tract

Cell death in the ovary transmitting tract is necessary for efficient pollen tube growth (Crawford et al., 2007). Disintegrative cell death is initiated prior to fertilization and is potentiated by the arrival of pollen. In contrast to wild type, cells in the transmitting tract of developing *haf bee1 bee3* triple mutants remain intact before and after fertilization, suggesting that *HAF*, *BEE1* and *BEE3* function in promoting cell death. To examine this further, we used the *BIFUNCTIONAL NUCLEASE1* (*BFN1*) (Farage-Barhom et al., 2008) gene as a marker because it is normally expressed in tissues undergoing cell death, including the transmitting tract (Farage-Barhom et al., 2008). Expression from a *BFN1::GUS* reporter was examined in unfertilized *haf bee1 bee3* carpels at 1, 3 and 5 days post-emasculation (dpe).

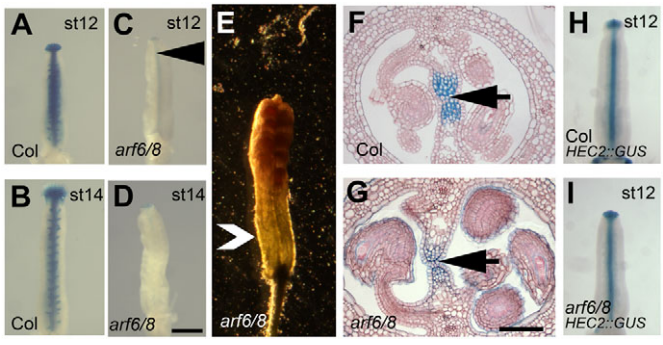

Fig. 7. Relationship between *HAF*, *ARF6*, *ARF8* and *HEC2*. (**A-D**) *HAF::GUS* staining in wild type (A,B) and *arf6 arf8* (C,D) at stages 12 (A,C) and 14 (B,D). Arrowhead indicates low level staining (C). (**E**) Hand-pollinated and fertilized fruit of *arf6 arf8* mutant; chevron indicates the loss of basal seed. (**F**,**G**) Transverse sections stained with Alcian Blue and Neutral Red of wild type (F) and *arf6 arf8* mutant (G) stage 12 carpels. Arrow indicates transmitting tract. (**H**,**I**) *HEC2::GUS* staining in stage 12 of wild type (H) and *arf6 arf8* (I). Scale bars: 500 μm for A-D,H,I; 50 μm for F,G.

In wild type, *BFN1::GUS* was strongly expressed in the ovary transmitting tract at both 1 and 3 dpe (Fig. 8A,B, arrowhead). Faint expression was also seen in the stigma at 3 dpe (Fig. 8B, arrow), suggesting that stigmatic papillae also undergo cell death in the absence of pollination, similar to cells in the ovary transmitting tract. *BFN1::GUS* was expressed strongly in ovules 5 dpe, showing that ovules abort around this time (Fig. 8C). In contrast to wild type, no GUS expression was detected in the *haf bee1 bee3* mutant in the ovary transmitting tract at either 1 or 3 dpe (Fig. 8D,E), and expression was also absent from the stigma at 3 dpe (Fig. 8E). However, ovule expression was the same as in wild type and was clearly observed 5 dpe (Fig. 8F).

It is interesting that *haf bee1 bee3* stigmata never showed any evidence of *BFN1::GUS* expression. We examined this in more detail by examining the structural integrity of wild-type and mutant stigma for both fertilized and unfertilized carpels up to 7 days after emasculation. Consistent with a lack of *BFN1::GUS* expression, mutant stigma (Fig. 8H,J) remained intact longer than did wild-type stigma (Fig. 8G,I) under both conditions.

It seemed possible that delayed cell death in mutant stigmata might prolong pollen receptivity (Carbonell-Bejerano et al., 2010). Wild-type carpels have been reported to be receptive to pollination and fertilization for up to 4 days after the flower has opened at anthesis (Vivian-Smith et al., 2001). To test this, we emasculated wild-type and *haf bee1 bee3* flowers and maximally pollinated carpels over a period of 7 days. We found that both wild-type and mutant carpels could only be fertilized up to 4 dpe. Furthermore, Aniline Blue staining showed no pollen grain germination in either wild type or mutant beyond 4 dpe. It therefore appears that stigmatic cell death does not determine how long carpels remain receptive for fertilization.

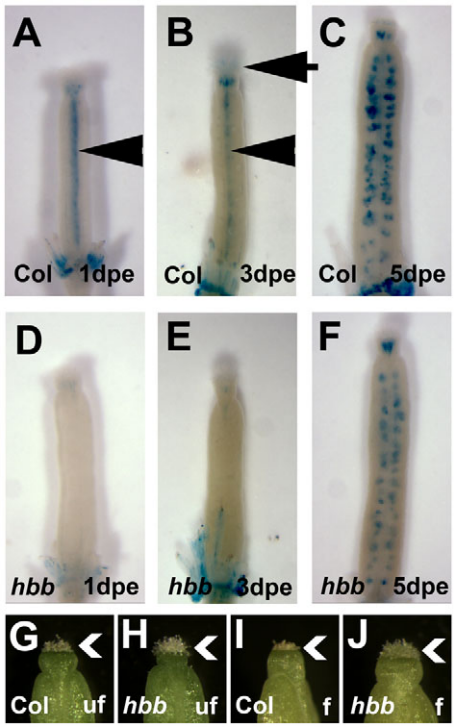

Fig. 8. *BFN1::GUS* staining and stigma cell death phenotype in wild type and *haf bee1 bee3* (*hbb*) mutant. (**A-F**) *BFN1::GUS* staining of emasculated carpels of wild type (A-C) and *haf bee1 bee3* mutant (D-F) at one (A,D), three (B,E) and five (C,F) days post-emasculation. Arrowheads indicate staining in the transmitting tract; arrow indicates staining in the stigma. (**G-J**) Stigma structure in unfertilized (G,H) and fertilized (I,J) in carpels seven days after emasculation of wild type (G,I) and *haf bee1 bee3* mutant (H,J). Chevron indicates stigma. dpe, days post-emasculation, uf, unfertilized; f, fertilized.

DISCUSSION

Genetic redundancy of *HAF*, *BEE1* and *BEE3*

In the Columbia ecotype, *haf* single mutants resemble wild-type plants. However, when mutations in *HAF* are combined with mutations in *BEE1* and *BEE3*, the resulting triple mutants display dramatic defects in reproductive tract formation. HAF, BEE1 and BEE3, which share more than 90% sequence similarity within the bHLH domain and identical intron and exon structures, are the three most closely related members of a 16-member subfamily of bHLH transcription factors. Not only are *HAF*, *BEE1* and *BEE3* closely related, but they also share overlapping expression patterns in the female reproductive tract. The previous observation that *BEE1* and *BEE3* have redundant roles in brassinosteroid signaling (Friedrichsen et al., 2002) raises the intriguing possibility that brassinosteroids could have a role in reproductive tract formation. Interestingly, *haf* single mutants display a dramatic fruit phenotype in the Ler background, indicating that the *BEE1* and *BEE3* genes are unable to substitute for *HAF* in this ecotype. To circumvent the complexities of the ecotype and functional redundancy, we carried out most of our studies in the Columbia background using the *haf bee1 bee3* triple mutant.

The expression patterns of *HAF*, *BEE1* and *BEE3* within the reproductive tract suggest that each gene has a subtly different function. Whereas *HAF* is expressed in all the tissues of the reproductive tract, *BEE1* is expressed only in the stigma and stylar transmitting tract, and *BEE3* is expressed only in the ovary transmitting tract. The more widespread expression of *HAF* could explain why both the *haf bee1* and *haf bee3* double mutants both show reduced fertility, whereas the *bee1 bee3* double mutant does not.

Previous work demonstrated that *BEE1* and *BEE3* share functional redundancy in the brassinosteroid signaling pathway with *BEE2*, a more distantly related member of the bHLH transcription factor family (Friedrichsen et al., 2002). Although no expression studies were previously reported for *BEE1* and *BEE3*, our analyses show that these genes are expressed in vegetative tissues. It will be interesting to determine the expression patterns of all members of this sub-family of bHLH genes to identify additional candidates for functional redundancy. Our observation that ectopic expression of *HAF* leads to a variety of effects on the overall architecture of the plant suggests that other members of this subfamily of genes probably have significant roles during plant development.

Genes controlling transmitting tract development

The transmitting tract begins at the boundary between the stigma and style and extends through the style and to the base of the ovary. Within the style, the *HAF*, *BEE1* and *BEE3* genes have important roles as evidenced by the restricted pollen tube growth and dramatically reduced ECM production in *haf bee1 bee3* mutants. Moreover, ectopic *HAF* expression increases transmitting tract tissues within the style, leading to a corresponding increase in the number of pollen tubes passing through this region. By contrast,

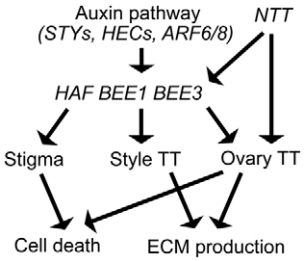

Fig. 9. Genetic network controlling *HAF* expression and reproductive tract development. The *STY*, *HEC* and *ARF* genes mediate different aspects of the auxin signaling pathway to positively regulate *HAF* expression in combination with *NTT*. *HAF*, *BEE1* and *BEE3* promote ECM production in the style and ovary transmitting tract along with cell death in the stigma and ovary transmitting tract. *NTT* also controls cell death and ECM production in the ovary transmitting tract.

the *NTT* gene does not appear to play a role within the stylar transmitting tract and there is no restriction on pollen tube movement through this region of *ntt* mutants.

HAF, *BEE1* and *BEE3* are also important for normal development of the ovary transmitting tract, and in *haf bee1 bee3* mutants, ECM production in this region is severely reduced. Moreover, the cell breakdown leading to cell death that normally occurs in this region, which has been linked to components of the ECM (Crawford et al., 2007; Crawford and Yanofsky, 2008; Gao and Showalter, 1999; Wu and Cheung, 2000), fails to occur normally in *haf bee1 bee3* mutants. As with the style, there are clear differences between the functions of *NTT* and the *HAF*, *BEE1* and *BEE3* genes within the ovary. For example, all ECM production appears to be absent from *ntt* mutants, but some residual staining is still present in *haf bee1 bee3* mutants. Moreover, some pollen tubes penetrate the transmitting tract within the ovary in *haf bee1 bee3* mutants, whereas this never occurs in *ntt* mutants. It is possible that the decreased fertility in the *ntt haf bee1 bee3* quadruple mutant compared with either the *ntt* mutant or *haf bee1 bee3* triple mutant is caused by the severe ovary transmitting tract defect of the *ntt* mutant combined with the stylar-transmitting tract defect of the *haf bee1 bee3* triple mutant (Fig. 9).

We used a minimal-pollination assay to assess the efficiency of fertilization. Using this assay, we found that *haf bee1 bee3* carpels were less efficient at producing seeds than wild-type plants are. Conversely, when *HAF* was misexpressed, fertilization efficiency was increased. To our knowledge, *HAF* is the first gene shown to be capable of increasing the potential of the carpel to be fertilized. It is worth noting that we were somewhat surprised to find, both here and in the earlier work involving *ntt* (Crawford et al., 2007), that wild-type carpels are relatively inefficient at producing seed when minimally pollinated. One explanation for these results is that there is normally an abundance of pollen, thus minimizing the requirement for all pollen tubes to complete their growth toward ovules (Williams, 2009). Indeed, it is possible that the necessity to grow through the reproductive tract allows the maternal parent to select for the most robust pollen tubes that reach the ovules (Mulcahy, 1979).

Exactly how ectopic expression of *HAF* can lead to increased fertilization efficiency is unclear, although regulation of ECM production is certainly an important aspect. Recent studies have shown that the interaction between pollen tubes and reproductive tract dramatically changes the pollen transcriptome as well as the efficiency by which pollen tubes target ovules (Qin et al., 2009). Given the expression of *HAF*, *BEE1* and *BEE3* throughout the reproductive tract and the reduced fertilization efficiency of the triple mutant, it is possible that these genes function in the interaction of the reproductive tract with pollen tubes.

We found that the *BFN1::GUS* cell death marker (Farage-Barhom et al., 2008) is expressed in the transmitting tract of wild-type carpels and that its expression is absent in the *haf bee1 bee3* triple mutant. Previous studies involving a cytological analysis of the transmitting tract indicated that cell death begins around the time of fertilization and does not depend on the presence of pollen (Crawford et al., 2007). Confirming this result, we found that the *BFN1::GUS* marker is initially expressed just prior to fertilization (stage 13) and does not depend on pollination.

In addition to their importance in controlling cell death in the transmitting tract, *HAF*, *BEE1* and *BEE3* also influence cell death in the stigma (Carbonell-Bejerano et al., 2010). Stigmatic cells degenerate soon after pollination in wild type, but remain intact in *haf bee1 bee3* mutants even 7 days post-anthesis. However, we did not find that the lack of stigmatic cell death in *haf bee1 bee3* resulted in an increase in the post-emasculation period in which stigmas were receptive to pollen.

Genes regulating *HAF* expression

We have identified a number of genes that function as upstream regulators of *HAF* expression (Fig. 9). The *NTT* gene is required for ovary transmitting tract formation, and *HAF* expression is dramatically reduced in *ntt* mutants. Moreover, *AtML1>>HAF* can partially rescue pollen tube growth in the *ntt* mutant. Although these data show that *NTT* acts upstream to promote *HAF* expression, *HAF* is still expressed at low levels in *ntt* mutants and the phenotype of *ntt* mutants is further enhanced by mutations in *haf*, *bee1* and *bee3*, suggesting that other factors also contribute to *HAF* expression.

We also show that wild-type *HAF* expression is dependent on the auxin pathway within the reproductive tract (Sundberg and Ostergaard, 2009). The *HEC* genes, which encode bHLH proteins capable of interacting as heterodimers, are crucial for formation of the reproductive tract (Gremski et al., 2007). *HAF* expression is dramatically decreased in *hec* mutants, indicating that *HEC* genes act upstream to promote *HAF* expression. *HAF* misexpression is also sufficient to partially restore the stigma and allow pollination in *hec* triple mutant carpels. The most closest related gene to the *HEC* genes in *Arabidopsis* is *INDEHISCENT* (*IND*), and both the HECs and IND have been implicated in regulation of auxin signaling (Gremski et al., 2007; Liljegren et al., 2004; Sorefan et al., 2009). *HAF* expression is, therefore, dependent on the auxin signaling pathway.

The transcriptional response to auxin signaling is mediated by the ARF transcription factor family. The partially redundant *ARF6* and *ARF8* genes, which provide a link between auxin signaling and fruit development, are good candidates for ARF regulators of *HAF* expression (Nagpal et al., 2005). The fruit of *arf6 arf8* mutants fail to stain for ECM, develop seeds primarily at their apical ends and show a dramatic reduction in *HAF* expression. Moreover, the fertility defects of *arf6 arf8* double mutants are similar to those seen in *arf6 arf8 haf bee1 bee3* quintuple mutants, suggesting that all of these genes act in the same pathway. Taken together, the data suggest that *ARF6* and *ARF8* are essential for promoting *HAF* expression and for reproductive tract development.

We have shown that the normal expression pattern of *HAF* is dependent on several genes and that misexpression of *HAF* can partially rescue *ntt* and *hec1 hec2 hec3* mutants. Although these data strongly suggest that *HAF* acts downstream of these genes, we cannot exclude the possibility that *HAF* acts at least partially in a parallel pathway. For example, the reduction in *HAF* expression in these mutants could be caused in part by the corresponding reduction or loss of reproductive tract tissues. The *STYLISH* genes, which have been implicated in controlling the expression of auxin biosynthesis genes, provide additional evidence for the important role of auxin in the development of the reproductive tract as these tissues are dramatically reduced in *stylish* mutants (Eklund et al., 2009). Taken together, these data suggest that auxin controls *HAF* expression through the combined action of the *STY*, *HEC* and *ARF6/8* genes.

In addition to auxin-mediated transcriptional control through ARF transcription factors, *BEE1* and *BEE3* have also been implicated in post-translational control by atypical bHLH proteins regulating brassinosteroid signaling (Wang et al., 2009). *HAF* corresponds to the recently reported gene *CRESTA*, whose protein product was shown to be regulated post-translationally by BIN2, a kinase associated with brassinosteroid signaling (Poppenberger et al., 2011). Thus, although auxin probably controls transcription of *HAF*, *BEE1* and *BEE3* through *ARF6* and *ARF8*, post-translational control could involve brassinosteroids. Given their roles in controlling the expression of these bHLH genes, it will be interesting to examine further the role of auxin and brassinosteroids in production of a fully functioning reproductive tract.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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