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 funiculus 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...
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 genotypic 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, ntr-1, arf6-2, arf8-3, hcf1 and hcf3 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 hcf2 allele SM.17339 was crossed into the hcf1 and hcf3 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 from 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 the PCR 3.1 vector. HAF::GUS and ACA9::GUS constructs were used to transform Arabidopsis (Atac, Inc.). HAFPOP1− was used for the Landsberg erecta ecotype, and because the haf bee1 bee3 mutant phenotype is very similar to that of hbb (Crawford et al., 2007). In both cases, seed production is reduced 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 (Schött et al., 2004). We created the HAF::GUS, BEE1::GUS and BEE3::GUS constructs 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 (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
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 siliques (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 siliques (n=39; compared with ntt haf bee1 bee3 mutant 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-Glucuronidase (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 only grown half the length of the carpels in the triple mutant (Fig. 3E, arrowhead). Moreover, pollen tubes were restricted to a smaller and smaller region of growth within the transmitting tract (compare Fig. 3I and 3J). 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). 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 Alcian 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 siliqua 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.
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. Emasculated 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 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 massively pollinated with wild-type pollen and the length of the resultant fruit was measured after 7 days. AtML1>>HAF silique measuring 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 septon 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.

The hcc1 hcc3 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 hcc1 hcc3 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 the activity of all three hcc genes caused complete loss of reproductive tract tissue (Gremski et al., 2007). This phenotype was obtained using a combination of hcc1 hcc3 insertional mutants along with RNAi to target HEC2. We obtained an insertion mutation in hcc2 and created a hcc1 hcc2 hcc3 triple mutant that confirmed the complete loss of reproductive tract tissue phenotype (Fig. 6G). Consistent with previous work, the hcc1 hcc2 hcc3 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 hcc1 hcc2 hcc3 mutant carpels (compare Fig. 6G and 6H). As a result of this stigmatic tissue it is possible to pollinate the AtML1>>HAF hcc1 hcc2 hcc3 carpels.
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 ARF6 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; 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 double mutant, showing only faint expression in the style and transmitting tract (Farage-Barhom et al., 2008)). Expression from a *BFN1::GUS* reporter was noticeable rescue of the *arf6 arf8* double mutant (Farage-Barhom et al., 2008)). Expression from a *BFN1::GUS* reporter was

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 *AtMLI>>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 NUCLEASE 1* (*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-emasculaion (dpe).
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.

**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 stalar 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 stalar-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-emasculating 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 haf triple mutant carpels. The most closely 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 mt, 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 stylis 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.

Acknowledgements
We thank M. Fung, G. Goelmlski and J. Wang for help with plant stocks and general laboratory assistance. We thank G. Ditta and J. J. Ripoll-Samper for careful reviewing of this manuscript and for helpful discussions. This work is funded by a National Science Foundation grant to M.F.Y.

Competing interests statement
The authors declare no competing financial interests.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.067793/-/DC1

References

Controlling the efficiency of fertilization


<table>
<thead>
<tr>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
<th>Primer name</th>
<th>Primer sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>HAF-X7</td>
<td>HAF – X8</td>
<td>CATCAAGCATCACCTGCCATT</td>
</tr>
<tr>
<td>MUTANT</td>
<td>DS3-2</td>
<td>HAF – X8</td>
<td>CATCAAGCATCACCTGCCATT</td>
</tr>
<tr>
<td>WT</td>
<td>BEE1+</td>
<td>BEE1–</td>
<td>CTGCATGGGACTCACTGATCT</td>
</tr>
<tr>
<td>MUTANT</td>
<td>JMLB2</td>
<td>BEE1+</td>
<td>GGTCTTTGAGAAGAGGTAGGTT</td>
</tr>
<tr>
<td>WT</td>
<td>BEE5+</td>
<td>BEE6–</td>
<td>TCAAGCATAGGACCATCCCA</td>
</tr>
<tr>
<td>MUTANT</td>
<td>JMLB2</td>
<td>BEE5+</td>
<td>CTCCCTCTCTCTCTCTCTGATTC</td>
</tr>
<tr>
<td></td>
<td>25330PROM1+</td>
<td>25330PROMO3–</td>
<td>GATCC TAGAGACACTCAGAAATTTAATGAA</td>
</tr>
<tr>
<td>WT</td>
<td>HAFCDNA</td>
<td>HAFCDNA–</td>
<td>TCAAAGGGGTAATGTTGAACTGAA</td>
</tr>
<tr>
<td>MUTANT</td>
<td>BEE1PROMO1+</td>
<td>BEE1PROMO2–</td>
<td>GATCCGAGACACTCAGAAATTTAATGAA</td>
</tr>
<tr>
<td>MUTANT</td>
<td>BEE1PROMO11+</td>
<td>BEE1PROMO2–</td>
<td>GATCCGAGACACTCAGAAATTTAATGAA</td>
</tr>
<tr>
<td>WT</td>
<td>HEC2LP</td>
<td>HEC2RP</td>
<td>TGCATCAAGAGGACTCAGACAG</td>
</tr>
<tr>
<td>MUTANT</td>
<td>HEC2RP</td>
<td>DSPM1</td>
<td>CTATTTGGAAGAGGTGAGGTTGAGG</td>
</tr>
</tbody>
</table>

WT, wild type.