The HECATE genes regulate female reproductive tract development in Arabidopsis thaliana

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Successful fertilization in plants requires the properly coordinated development of female reproductive tissues, including stigma, style, septum and transmitting tract. We have identified three closely related genes, HECATE1 (HEC1), HECATE2 (HEC2) and HECATE3 (HEC3), the expression domains of which encompass these regions of the Arabidopsis gynoecium. The HEC genes encode putative basic helix-loop-helix (bHLH) transcription factors with overlapping functionality. Depending on the amount of HEC function missing, plants exhibit varying degrees of infertility, defects in septum, transmitting tract and stigma development and impaired pollen tube growth. The observed phenotypes are similar to those reported for mutations in the SPATULA (SPT) gene, which also encodes a bHLH transcription factor required for development of the same female tissues. We show that the HEC proteins can dimerize with SPT in a yeast two-hybrid system, indicating that the HEC genes work in concert with SPT to coordinately regulate development of the female reproductive tract. Furthermore, when the HEC genes are ectopically expressed from the CaMV 35S promoter, some of the resulting transgenic plants show pin-shaped inflorescences, suggesting that the HEC genes are probably involved in auxin-mediated control of gynoecium patterning.

KEY WORDS: Arabidopsis, Development, Stigma, Transmitting tract, Pollen tube growth

INTRODUCTION
The Arabidopsis gynoecium is a complex organ specialized for seed production and dispersal. It arises from two congenitally fused carpels and at maturity consists of an apical stigma for pollen capture, a short intervening style and a large central ovary where ovule and seed development occurs (Fig. 1A,B). The ovary is divided into two compartments by the septum. A specialized tissue called the transmitting tract, crucial for pollen tube growth, develops in the center of both the style and the septum.

During fertilization, pollen grains germinate on the stigma and grow downward through the stigma and transmitting tract before diverging laterally toward the ovules. Transmitting tract cells facilitate pollen tube growth by secreting a complex extracellular matrix (ECM) rich in acidic polysaccharides, and by undergoing a program of developmentally controlled cell death (Lennon et al., 1998; Wang et al., 1996; Crawford et al., 2007). A number of mechanisms involving chemical gradients and/or signal molecules have been proposed, whereby pollen tubes are guided from the stigma into the transmitting tract and out of the transmitting tract towards the awaiting ovules (Johnson and Preuss, 2002; Palanivelu et al., 2003; Palanivelu and Preuss, 2006). In the absence of proper transmitting tract differentiation, pollen tube growth is limited and fertility is reduced (Crawford et al., 2007).

Since proper development of female reproductive tissue is essential to the reproductive success of the plant, this process is likely to be highly regulated. A number of genes have been identified as important for patterning the stigma, style, septum and transmitting tract. These include SPATULA (SPT), STYLISH1 (STY1), STYLISH2 (STY2) and ETTIN (ETT). SPT encodes a basic helix-loop-helix (bHLH) transcription factor expressed early in septum and stigma development. Loss of SPT function leads to defects in septum and apical carpel fusion, loss of transmitting tract and a decrease in stigmatic tissue development (Alvarez and Smyth, 2002; Heisler et al., 2001). STY1 and STY2 encode RING-finger proteins that function in the development of the style (Kuusk et al., 2002; Sohlberg et al., 2006). spt mutants are epistatic to sty1 mutants, suggesting that SPT and STY act in the same pathway (Sohlberg et al., 2006). ETT, which encodes an auxin-response factor (ARF), has been shown to restrict the expression domain of SPT. In ett mutants, transmitting tract tissue develops on the outside of the gynoecium, and removing SPT function from ett mutants rescues this defect (Heisler et al., 2001).

Previous studies indicate that the hormone auxin plays an important role in controlling development of the gynoecium. High levels of auxin have been postulated to accumulate in the style and form a gradient downward through the gynoecium (Nemhauser et al., 2000). Treatment of wild-type gynoecia with the auxin transport inhibitor NPA produces enlarged stigmas and styles reminiscent of weak ett phenotypes. Furthermore, application of NPA to spt gynoecia partially restores gynoecium development, indicating that auxin is especially important in patterning the development of the female reproductive tract (Nemhauser et al., 2000).

In this work, we report three new genes that play an important role in the complex program of gynoecium development. HEC1, HEC2 and HEC3 encode closely related bHLH transcription factors with overlapping functionality. Loss of HEC function leads to defects in the development of the transmitting tract, septum and stigma and to a decrease in fertility. Conversely, overexpression of HEC genes causes both the production of ectopic stigmatic tissue and gain-of-function phenotypes implicating them as components of the auxin-signaling pathway. HEC proteins heterodimerize with SPT in a yeast two-hybrid system, suggesting that these proteins are likely to cooperatively interact in controlling development.

MATERIALS AND METHODS
Mutant plants
The hec1 allele corresponds to the GABI-KAT line 297B10 (Rosso et al., 2003). It was genotyped using the gene-specific primers oKG156 (5'-ACCACAACACTTACCTTTC-3') and oKG157 (5'-GTTCCTAC
CACCCTTTCAACT-3') to amplify a wild-type fragment and the T-DNA-specific primer (5'-CCCTATTTTGACCTGTAATGAC-3'), sequence provided by GABI-KAT in combination with oKG156.

The hec3 allele corresponds to the SALK_005294 line (Alonso et al., 2003). It was genotyped using primers C-X1 (5'-GTGCAATATTGTGAGAAGACCAAGA-3') and C-X4 (5'-TCTAACAACACCTTTGTGATTCCA-3') to amplify a wild-type fragment and C-X4 in combination with the T-DNA-specific primer JML21 (5'-TGGGTTGATGTTGACCG-3').

The etr-7 allele was kindly provided by Patricia Zambryski (University of California, Berkeley, CA). The spr-2 allele was kindly provided by David Smyth (Heisler et al., 2001).

**Generation of transgenic plants**

**HEC2**-RNAi lines were generated by amplifying a 180 bp fragment, using primers oKG93 (5'-GGGATCCCTTGTCTAGATTAATTCC-3') and oKG110 (5'-TATCAGATACCCGGGTTGGTGACCGG-3') for the 5' orientation, and primers oKG94 (5'-CTTCGAGTCTAATTGAA-3') and oKG91 (5'-GGTACCCCGGGTTGGTGGATTGACCGG-3') for the 3' orientation. Both fragments were cloned into the pAHNIBAL vector (Wesley et al., 2001) and the entire cassette was subsequently cloned into pART27 (Gleave, 1992) to generate KG154-1. Phenotypes were analyzed in the T1 and T2 generations. In situ analysis was using primers oKG119 (5'-AATCAC-3') and oKG187 (5'-GGTCGACATCTTTCTCTATGGATTCTGAC-3') to amplify a wild-type fragment and the T-DNA-specific primer JML21 (5'-TGGGTTGATGTTGACCG-3').

**RT-PCR**

RNA was isolated using the Qiagen RNeasy Plant Mini Kit. The reverse transcriptase reaction was performed using the Promega Reverse Transcription System. **HEC1** was amplified with primers oKG83 and oKG84. **HEC2** was amplified with primers oKG89 and oKG90. **HEC3** was amplified with primers oKG95 and oKG96. A B-TUBULIN control amplification was performed using primers (5'-GGACAACTCGGATCCAGG-3') and N-1137 (5'-CGTCTCCACTCTTGCAGACC-3'). The annealing temperature was 58°C. The PCR amplification in Fig. S2A,B (see Fig. S2A,B in the supplementary material) was carried out using 2 μl of reverse transcriptase reaction, the HEC amplification was performed with 35 cycles, and TUBULIN was amplified using 25 cycles. In the PCR amplification shown in Fig. S2C (see Fig. S2C in the supplementary material), **HEC2** was amplified with 30 cycles using 1 μl of reverse transcriptase reaction, **SP1** was amplified with 28 cycles using 1 μl of reverse transcriptase reaction, and **TUB** was amplified with 25 cycles using 0.5 μl of reverse transcriptase reaction.

**In situ hybridization**

In situ hybridization was performed as described by Dinney et al. (Dinney et al., 2004). An antisense **HEC1** probe was transcribed with T7 polymerase (Promega) using a full-length coding sequence in pCR2.1 (KG62-1) that had been linearized with HindIII. An antisense **HEC2** probe was transcribed with T7 polymerase using a full-length coding sequence in pBluescript (KG100-1) linearized with SpeI. An antisense **HEC3** probe was transcribed with T7 polymerase using a full-length coding sequence in pCR2.1 (KG76-2) linearized with SpeI.

**Microscopy and histology**

Staining for β-glucuronidase expression was as described (Blázquez et al., 1997) with minor modifications. Wild-type (Columbia ecotype) and transgenic fruit and flowers were fixed and analyzed by scanning electron microscopy as previously described (Liljegren et al., 2000). Aniline Blue staining for pollen tubes was performed after emasculating flowers just prior to pollination (late stage 12), growing them for another 18-24 hours to allow transmitting tract and ovule development to be completed, and then hand-pollinating them maximally. After allowing another 24 hours for pollen growth, they were fixed, cleared, stained with Aniline Blue (Jiang et al., 2005) and examined under a fluorescence microscope.

Staining with Alcian Blue 8GX was used to visualize the transmitting tract. Alcian Blue stains the acidic mucopolysaccharide component of the transmitting tract ECM (Scott and Dorling, 1965). Papalast-embedded flowers and inflorescences were sectioned at 4 μm and fixed to slides. Slides were then de-waxed with Histoclear (National Diagnostics), rehydrated through a gradual ethanol series, counterstained for 5 minutes with 0.1% Nuclear Fast Red, rinsed, stained for 5 minutes with Alcian Blue pH 3.1, rinsed again, dried briefly at 37°C, then mounted directly in Permount (Fischer Scientific).

**Yeast two-hybrid system**

Directed yeast two-hybrid interactions were conducted as described (Pelaz et al., 2001). Full-length **HEC1** was cloned into both the bait vector pBl-880 to make SP7-2 and into the prey vector pBI-771 to make SP18. Full-length **HEC2** was cloned into both the bait vector pBl-880 to make SP8-4 and into the prey vector pBI-771 to make SP19. Full-length **HEC3** was cloned into the prey vector pBI-771 to make SP20. The full-length **HEC3** in the bait vector was able to activate the reporters on its own. Hence, a partial **HEC3** fragment, which included amino acids 92-224, was cloned into pBluescript (KG100-1) linearized with SpeI. A partial **HEC3** fragment, which included amino acids 92-224, was cloned into pBluescript (KG100-1) linearized with SpeI.

**RESULTS**

**Identification of the HECATE (HEC) genes**

Previous work identified the bHLH transcription factor INDEHISCENT (IND) as a key regulator of valve margin development in the Arabidopsis fruit (Liljegren et al., 2004).
Reasoning that bHLH proteins related to IND might also function in plant development, we performed a BLAST search to find those genes most closely related to *IND*. *HEC1* (At5g67060; BHLH088), *HEC2* (At3g50330; BHLH037) and *HEC3* (At5g09750; BHLH043) were thus identified. All three *HEC* genes were subsequently found to function in gynoecium development, but to have roles distinct from that of *IND*.

Like *IND*, each of the *HECs* is composed of a single small exon (Fig. 1C), *HEC1*, *HEC2* and *HEC3* encode proteins of 242, 232 and 225 amino acids, respectively. The *IND* and *HEC* gene products share extensive protein sequence similarity in the bHLH domain, as well as in a 30 amino acid N-terminal extension of this region (Fig. 1D). Similarity in this 30 amino acid region leads to both IND and HECs being grouped as a subfamily of bHLH proteins (Heim et al., 2003). *HEC1* and *HEC2* are the most closely related and share 61% amino acid identity across the length of the proteins and 100% identity within the bHLH domain. Previous phylogenetic analysis of the *Arabidopsis* bHLH transcription factor family placed *HEC1* and *HEC2* in regions that arose from a putative interchromosomal duplication event (Toledo-Ortiz et al., 2003). Protein alignment of the HECs with IND and more-distantly related bHLH factors reveals that the HECs, like IND, lack a conserved glutamate at position 13 of the basic domain (Fig. 1D, asterisk). This glutamate has been shown to be important for DNA binding (Ellenberger et al., 1994; Ma et al., 1994). Thus, if the HECs regulate gene transcription through DNA binding, they are likely to do so through the use of other residues.

The **HEC** genes are expressed in the developing septum, transmitting tract and stigma

To investigate whether the *HEC* genes function in gynoecium development, we analyzed their expression patterns using both RNA in situ hybridization and β-glucuronidase (GUS) reporter gene constructs. In contrast to the valve margin expression pattern described for *IND* (Liljegren et al., 2004), all three *HEC* genes were found to be expressed in the stigma and septum during stages 8 to 12 of flower development.

RNA in situ analysis showed that for all three *HECs*, expression was first observed during stage 8 of gynoecium development, in the medial ridges of the septum, which have grown together and fused at this time (Fig. 2A-C, large arrowheads), and in the apical tips (Fig. 2G, lower arrow), where the stigma will arise. By stage 10 to early stage 12, hybridization signal was localized to the transmitting tract (Fig. 2D-H, large arrowheads) and developing stigmas (Fig. 2G, H, arrows). Patchy signal was also apparent in the ovules for *HEC1* and *HEC2* (Fig. 2D-F, small double arrowheads) and in the ovule funiculus for *HEC3* (Fig. 2F, arrow). By late stage 12, just prior to fertilization, *HEC3* expression was still evident in the transmitting tract (Fig. 2I, large arrowheads) and was strong in the ovule funiculus (small arrowheads), but was no longer visible in the stigma (arrow). *HEC1* and *HEC2* expression could no longer be detected by late stage 12 (data not shown).

GUS reporter results confirmed the septum and stigma expression of *HEC1* and *HEC2* (see Fig. S1A-D in the supplementary material) and also indicated that *HEC3* funiculus expression continued even after pollination (see Fig. S1E in the supplementary material, arrowheads). Some *HEC2p::GUS* lines also expressed GUS in pollen and the nectaries (data not shown), and a number of the *HEC3p::GUS* lines showed expression in vasculature (see Fig. S1E in the supplementary material, arrow). GUS analysis did not confirm *HEC3* transmitting tract and stigma expression, *HEC1* and *HEC2* ovule expression, or *HEC3* anther expression, presumably because the GUS constructs lack essential DNA regulatory elements necessary for them to represent the entire pattern of expression indicated by RNA in situ hybridization.

The close sequence similarity of the *HEC* genes and their overlapping expression patterns suggest that they might have partially redundant functions in stigma and septum development.

**hec1** and **hec3** mutations reduce fertility

To determine the functions of the *HEC* genes, we identified T-DNA insertion lines in *HEC1* and *HEC3* from available mutant collections (Fig. 1C) and confirmed these lines as RNA-nulls by RT-PCR (see Fig. S2A, B in the supplementary material). An absence of *HEC3*...
RNA in the *hec3* mutant was further demonstrated by in situ hybridization (data not shown). No satisfactory mutants in *HEC2* were available at the time of this work.

*hec1* mutant plants showed no alteration in fruit phenotype (Fig. 3A,B; Table 1). *hec3* mutant plants had smaller fruit and a modest reduction in fertility compared with wild type (59% wild-type seed set) (Fig. 3A,C; Table 1). Reciprocal crosses revealed the fertility defect to be female-specific (data not shown). The *hec1 hec3* double mutant had a sizable reduction in overall fertility (17% wild-type seed set), along with significant variations in individual fruit size and seed yield (Fig. 3A,D; Table 1). Seed distribution was biased toward the apical half of the carpel, but substantial fertilization also occurred in the basal half (Fig. 3D). The fact that the double mutant had a more severe phenotype than either single mutant demonstrates that both genes are required for wild-type levels of fertility.

We were able to rescue the fertility defect of *hec1 hec3* plants to approximately wild-type levels by transforming them with a *HEC3* rescue construct composed of 3 kb of the *HEC3* promoter driving the *HEC3* coding region (Fig. 3E). This result would be anticipated as the *hec1* mutation alone produces no obvious change in the fruit, and it confirms that the observed mutant phenotypes are due to the *hec1* and *hec3* mutations.

**Defects in pollen tube growth in the *hec* mutants**

Stigma and transmitting tract provide the apical-to-basal tissue path for pollen tube growth in *Arabidopsis*. Since the *HEC* genes are expressed in these tissues, it seemed likely that the loss of fertility in *hec* mutants would correlate with aberrant or reduced pollen tube growth. To visualize pollen tubes within the ovary, we used an...
Aniline Blue staining technique (Jiang et al., 2005) 24 hours after hand-pollinating emasculated carpels (Materials and methods). Aniline Blue stains callose, a component of pollen tubes, allowing them to be visualized by fluorescence microscopy.

Wild-type and hec1 gynoecia showed an abundance of pollen tubes throughout the length of the transmitting tract (Fig. 3G,H). Fertilization events, evident as lateral deviations in tube growth, likewise occurred throughout the entire length of the ovary. hec3 carpels showed significantly fewer pollen tubes and fertilization events, particularly in the basal half of the gynoecium (Fig. 3I). This difference was even more pronounced for the hec1 hec3 double mutant (Fig. 3J). A similar pattern of reduced pollen tube growth was found when carpels were examined only 6 to 7 hours after pollination, when wild-type pollen tubes had not yet reached the bottom of the ovary (data not shown).

**HEC1 and HEC3 are necessary for stigma and transmitting tract development**

Both stigma and style showed obvious developmental abnormalities in hec mutants. Compared with wild type (Fig. 4K), stigmas were smaller and more variable in size in hec1 hec3 mutants (Fig. 4L). Although not evident in Fig. 4, there was also a slight tendency for the style to be somewhat longer in the double mutant. To visualize the transmitting tract in hec mutants, post-fertilization flowers were thin-sectioned and stained with Alician Blue, a dye that detects acidic polysaccharides characteristic of the transmitting tract ECM. Wild type showed a characteristically large, intensely staining transmitting tract (Fig. 4A,H, arrowheads). The transmitting tract of hec1 was indistinguishable from that of wild type in size, staining intensity and cytology (Fig. 4B). The hec3 transmitting tract was smaller in size than wild type in both the septum and the style (Fig. 4C,I), but had the same general appearance as wild type. The hec1 hec3 double mutant, however, had dramatically reduced Alician Blue staining in both the style and the septum compared with wild type (Fig. 4D,E,J). In analyzing pre-fertilization stages of development, we found that the transmitting tract of the hec1 hec3 double mutant had no delay in onset of ECM production, but produced less ECM than wild type (see Fig. S3 in the supplementary material). Taken together, these data demonstrate that HEC1 and HEC3 are redundantly required for transmitting tract differentiation.

**Reducing HEC2 RNA levels in the hec1 hec3 double mutant intensifies defects in stigma and septum development**

Given the sequence similarity and overlapping expression domains among the HEC genes, and considering the synergistic nature of hec1 and hec3 single mutations, it seemed likely that all three HEC genes would share functionally related roles in Arabidopsis. To confirm this hypothesis and to substantiate a role for HEC2 in gynoecium development, we used RNAi to create the equivalent of a hec1 hec2 hec3 triple mutant. To make the RNAi construct we used 180 bp from the 5′ coding region of HEC2 (Fig. 1C). This region contains some areas of close sequence similarity to HEC1, but little similarity to HEC3. The construct was transformed into both the wild type and the hec1 hec3 double mutant.

RT-PCR analysis of several independent lines of HEC2-RNAi in the wild type revealed a strong to moderate reduction in the level of both HEC1 and HEC2 RNA and only a slight effect on HEC3 RNA (see Fig. S2C,D in the supplementary material). These HEC2-RNAi lines, equivalent to hec1 hec2 double mutants, showed little or no effect on fertility (data not shown). However, when the HEC2-RNAi construct was transformed into the hec1 hec3 double mutant, 8 of 32 lines exhibited complete sterility (Fig. 3F). Such lines had dramatic defects in apical gynoecium development, with a complete absence of stigmatic tissue and, in many cases, an incomplete fusion of the style to be somewhat longer in the double mutant. To visualize the transmitting tract (Fig. 4A,H, arrowheads). The transmitting tract of the wild type revealed a strong to moderate reduction in the level of HEC2 RNA and only a slight effect on HEC3 RNA (see Fig. S2C,D in the supplementary material). These HEC2-RNAi lines, equivalent to hec1 hec2 double mutants, showed little or no effect on fertility (data not shown). However, when the HEC2-RNAi construct was transformed into the hec1 hec3 double mutant, 8 of 32 lines exhibited complete sterility (Fig. 3F). Such lines had dramatic defects in apical gynoecium development, with a complete absence of stigmatic tissue and, in many cases, an incomplete fusion of the

<table>
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<th>Seeds/fruit (n)</th>
<th>% seed set</th>
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<td>Wild type</td>
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<tr>
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<tr>
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*The average number of seeds per fruit plus or minus the standard deviation (n=the number of fruits sampled). Fruits were collected from the primary shoot of five plants per genotype, starting at fruit number five, and going up to fruit number twenty.

### Table 1. Loss of HEC function leads to a reduction in fertility

- **A-G** Transverse sections of stage 14 Arabidopsis ovaries stained with Alician Blue to reveal the transmitting tract (arrowheads) and with Fast Red as a counterstain. In the wild-type (Col-0) gynoecium (A), the ECM of the transmitting tract stains bright blue in the center of the septum. The hec1 transmitting tract (B) is essentially equivalent to wild type, but the hec3 transmitting tract (C) is noticeably smaller. Typical examples of hec1 hec3 transmitting tracts (D,E) are severely reduced in size within narrowed septa. Typical examples of HEC2-RNAi hec1 hec3 gynoecia (F,G) exhibit no blue staining at the transmitting tract, and have either only a few cells at the septum fusion point (F, arrow) or an unfused septum (G, arrow). (**H-J**) Transverse sections of stage 14 styles stained with Alician Blue and Fast Red. The transmitting tract (arrowhead) is reduced for hec3 in the stilar region (J) in comparison with wild type (H). hec1 hec3 has very little transmitting tract in the style (J). (**K-O**) Scanning electron micrographs of stigma and style regions of stage 14 gynoecia. The stigma of hec1 hec3 gynoecia (L) are significantly less well developed than those of wild-type Col-0 (K). HEC2-RNAi hec1 hec3 gynoecia (M,N) lack any stigmatic development and have longer styles than wild type. Some fruit displayed a defect in apical fusion (N, arrow) similar to that of spt-2 (O, arrow; Ler background). Scale bars: 50 μm in A-J; 100 μm in K-O.
redundant and play a fundamental role in stigma and transmitting tract development and in the post-genital fusion of the septum and the apical gynoecium. Owing to the requirement of the three HEC genes for female fertility, we named them after the Greek goddess of fertility Hecate, often portrayed as three women.

**Interactions between SPATULA and the HECs**

The well-studied developmental regulator *SPATULA (SPT)* encodes a bHLH protein that is considerably larger than any of the HEC proteins (373 amino acids versus approximately 230 amino acids) and is poorly conserved with the HEC proteins (Fig. 1D). Nevertheless, SPT is expressed in both septum and stigma during stages 6 to 11 (Heisler et al., 2001), and genetic studies have shown it to be required for septum, transmitting tract and stigma formation (Alvarez and Smyth, 2002). We therefore investigated possible interactions between *SPT* and the HECs.

Since *SPT* expression is detectable at earlier stages of gynoecium development than is *HEC* expression (Heisler et al., 2001), the possibility existed that *SPT* might be a transcriptional regulator of the HECs. We examined this by analyzing the expression of *HEC1*, *HEC2* and *HEC3* in early carpels of plants carrying the strong *spt-2* allele (Fig. 5A-C). All three HECs continued to be expressed in this mutant background, indicating that a functional *SPT* protein is not required for *HEC* gene expression.

A more likely possibility was that the SPT and HEC proteins might interact cooperatively to regulate development. bHLH proteins are known to both homodimerize and heterodimerize, and dimer formation is essential for transcriptional regulation (Murre et al., 1994; Massari and Murre, 2000). We therefore used a yeast two-hybrid system to investigate protein-protein interactions among the *HEC1*, *HEC2*, *HEC3* and *SPT* gene products. The HEC proteins do not form either homodimers or heterodimers in yeast, but each is capable of heterodimerizing with SPT (Fig. 5G,H). If the HEC proteins function as transcriptional regulators, the data strongly suggest that SPT is likely to be a required partner.

**ETTIN is a negative regulator of *HEC* gene expression**

Since both *SPT* and the *HEC* genes are required for aspects of interior carpel development, specifically septum and transmitting tract development, it is relevant that mutants in the ARF factor *ETT* display a dramatic phenotype in which transmitting tract tissue develops on the outside of the gynoecium. This externalization of transmitting tract derivatives at least in part from the unrestricted expression of *SPT* in carpel valves (Heisler et al., 2001). We wanted to determine whether the *HEC* genes might also be under *ETT* control and play a similar role in the formation of external ectopic transmitting tract in *ett* mutants. *HEC* expression was examined in *ett*-7 gynoecia and found to be equivalent to that seen for *SPT*. The HECs were ectopically expressed on the outside of *ett* gynoecia (Fig. 5D-F). *ETT* therefore negatively regulates *HEC* expression in the abaxial gynoecium in a similar manner as it does *SPT*.

**Overexpression of the HEC genes produces ectopic stigmatic tissue, ett-like and pin-like phenotypes**

To further examine the effects of ectopic *HEC* activity, we generated overexpression lines in which *HEC* gene expression was driven from the constitutive 35S promoter. Overexpression of each HEC gene resulted in flowers that produced ectopic carpelloid tissue, most often stigmatic tissue (Fig. 6B,C,D, arrowheads; Fig. 6B,C, insets). The data indicate that the HEC genes are able to activate carpel identity factors when ectopically expressed.
The overexpression of HEC1 and HEC3 also occasionally led to the production of gynoecia with defects in apical-basal polarity reminiscent of a weak ett phenotype. Carpels had enlarged stigmas, reduced ovaries and elongated gynophores (Fig. 6G-I). The HEC genes thus could be involved in the ETT-mediated auxin-signaling pathway needed for apical-basal development. This possibility is further supported by even more extreme phenotypes seen among overexpressing lines, such as those shown in Fig. 6K,L. Here, pin-shaped inflorescences or carpelloid stalks were observed, resembling those that result from loss of the auxin efflux carrier PIN-FORMED1 (PIN1) or from treatment with the chemical NPA, an auxin transport inhibitor (Okada et al., 1991). These dramatic phenotypes suggest that in these 35S::HEC1 and 35S::HEC3 lines there was an alteration of auxin levels, auxin transport and/or auxin perception.

**DISCUSSION**

**The role of the HECATE genes in the development of the transmitting tract and stigma**

Pollen tubes must travel through several distinct tissues before reaching ovules, including the stigma, the stylar transmitting tract and the septum transmitting tract. Coordinated development of these tissues is crucial for successful fertilization. We report here the identification of three related bHLH transcription factors, HEC1, HEC2 and HEC3, that are required for this process. All three genes share some degree of functional equivalency as shown by the synergistic effect of deficiencies in each. Whereas no abnormal phenotype was observed with an RNA-null mutation in HEC1, an RNA-null mutant of HEC3 displayed a moderate loss of fertility and decreased transmitting tract tissue in both septum and style. A hec1 hec3 double mutant displayed substantial defects in both fertility and transmitting tract development. The development of stigmatic tissue was reduced and there was a slight increase in the size of the style.

To examine a possible contribution of HEC2 toward the development of female reproductive tissues, we created a HEC2RNAi construct and introduced it into the hec1 hec3 background, establishing the equivalent of a hec1 hec2 hec3 triple mutant. Transgenic lines with the most-extreme phenotypes displayed a complete loss of fertility and severe defects in stigma and septum development. HEC2-RNAi hec1 hec2 hec3 plants lacked any stigmatic or transmitting tract cells and frequently lacked both style and septum fusion. These results are consistent with the idea that all three HEC genes share some measure of functional redundancy. Overexpression studies support this contention. Overexpression of any of the individual HECs led to the ectopic production of stigmatic tissue, consistent with their requirement for stigma development.

**The relationship between the HEC/IND subfamily and the SPT subfamily of bHLH transcription factors**

The HEC proteins and the previously characterized valve margin specification factor IND (Liljegren et al., 2004) belong to an atypical group of bHLH transcription factors. Most Arabidopsis bHLH proteins are thought to have evolved from an ancestral group of bHLHs common to both plants and animals (group B) and contain a conserved glutamate in the basic region (Heim et al., 2003; Toledo-Ortiz et al., 2003). This glutamate contacts DNA at the bHLH recognition sequence, the E-box (Ellenberger et al., 1994). SPT contains this crucial glutamate, but the HECs and IND have an alanine substitution (Fig. 1D). Animal group-C bHLH proteins, which also lack the conserved glutamate, have been shown to bind DNA in combination with group-B bHLHs using a different recognition site (Basci et al., 1995; Swanson et al., 1995).

In the current study, we investigated possible interactions between SPT and the HECs. Because SPT is expressed at earlier stages of gynoecium development than the HEC genes (Heisler et al., 2001), the possibility existed that it might function as an upstream regulator of HEC expression. We therefore examined HEC expression in...
plants carrying the strong spr-2 allele and found that the HEC genes were still expressed, implying that SPT is not required for activation. We then considered the possibility that SPT and HEC gene products might interact with each other. This was shown to be the case. HEC proteins can form heterodimers with SPT in a yeast two-hybrid system, but cannot heterodimerize or homodimerize with each other. SPT can also heterodimerize with IND, the closest relative of the HECs (data not shown). Since SPT is expressed more widely during development than either the HECs or IND, but nevertheless encompasses the expression domains of both (Heisler et al., 2001), it seems likely that SPT and the HECs work in concert to carry out certain developmental programs and that SPT, because of its broader expression domain, interacts with yet other bHLH proteins to carry out additional developmental programs. It is relevant to note here that constitutive overexpression of SPT does not produce mutant phenotypes (M. Groszmann, PhD thesis, Monash University, 2005), as does overexpression of the HECs. This observation is consistent with the possibility that the HECs are able to dimerize with broadly expressed proteins, whereas SPT requires partners with more limited expression domains.

Do the HEC genes play a role in the auxin-signaling pathway in the gynoecium?

A fundamental role has been suggested for the hormone auxin in patterning the Arabidopsis gynoecium, with high levels of auxin conferring apical tissue identity (stigma/style) and low levels of auxin leading to basal (gynophore) development (Nemhauser et al., 2000). The ETT gene is an important mediator of auxin effects. Mutations in ETT cause severe defects in gynoecium development, including an enlarged stigma, an elongated gynophore and a reduced ovary that develops transmitting tract tissue on the outside (Sessions and Zambryski, 1995). SPT is also likely to be involved in auxin patterning, both as a target of auxin regulation and as a mediator of auxin effects. SPT is ectopically expressed in the ett gynoecium, most notably in the inverted transmitting tract tissue on the outside of carpels (Heisler et al., 2001). Mutations in SPT can suppress mutations in ETT (Heisler et al., 2001). spt-2 mutants can also be partially rescued by the auxin transport inhibitor NPA, and spt-2 gynoecia are less sensitive than wild-type gynoecia to NPA effects on apical-basal patterning (Nemhauser et al., 2000).

If the HECs operate coordinately with SPT as protein partners, it is likely that both proteins are targets of auxin regulation and would be similarly affected by mutations in ETT. We found that all three HEC genes were, like SPT, ectopically expressed in external transmitting tract tissue in the ett-7 mutant. The HECs, like SPT, are therefore implicated as possible targets of auxin regulation.

The overexpression phenotype of the HECs further suggests involvement in auxin patterning. Some of the phenotypes of HEC-overexpressing lines were similar to those of auxin-related mutants. 35S::HEC1, 35S::HEC3 and, to a lesser degree, 35S::HEC2 lines occasionally produced gynoecia with defects in apical-basal patterning resembling those of a weak ett mutant (Fig. 6G-J; data not shown). Several independent 35S::HEC1 lines produced pin-shaped, flowerless inflorescences. We also observed stalk-like floral structures capped by stigmatic tissue for both 35S::HEC1 and 35S::HEC3 (Fig. 6f,l). Both of the latter phenotypes are very similar to what has been reported for the pin1 mutant (Okada et al., 1991). PIN1 belongs to the PIN family of auxin efflux carriers, which play an important role in setting up auxin gradients or patterns of flow that pattern the plant (Benkova et al., 2003; Friml, 2003; Friml et al., 2003). The overproliferation of stigmatic tissue in 35S::HEC lines suggests the pooling of auxin or an increased auxin response at these sites. This interesting link between the HECs and auxin should be investigated in future studies.

In summary, the HEC genes function redundantly in patterning tissues crucial for reproductive success in the Arabidopsis gynoecium. Elucidating additional details about how the HECs interact with other carpel-patterning genes will help to provide insights into various aspects of gynoecium function, including carpel development, pollen tube growth and fertilization.

We thank Yat Long Poon and Nancy Lee for excellent technical assistance in the laboratory; Evelyn York for technical help with SEM work carried out at the Scripps Institution of Oceanography; Adrienne Roeder for many helpful discussions and support throughout the project, and Brian Crawford, Juan Jose Ripoll and Sangho Jeong for carefully reviewing this manuscript. This work is funded by a National Science Foundation grant to M.F.Y.

Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/20/3593/DC1

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