Transcriptomic characterization of a synergistic genetic interaction during carpel margin meristem development in *Arabidopsis thaliana*

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Transcriptomic Characterization of a Synergistic Genetic Interaction during Carpel Margin Meristem Development in Arabidopsis thaliana

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Abstract

In flowering plants the gynoecium is the female reproductive structure. In Arabidopsis thaliana ovules initiate within the developing gynoecium from meristematic tissue located along the margins of the floral carpels. When fertilized the ovules will develop into seeds. SEUSS (SEU) and ANTENQUERA (ANT) encode transcriptional regulators that are critical for the proper formation of ovules from the carpel margin meristem (CMM). The synergistic loss of ovule initiation observed in the seu ant double mutant suggests that SEU and ANT share overlapping functions during CMM development. However the molecular mechanism underlying this synergistic interaction is unknown. Using the ATH1 transcriptomics platform we identified transcripts that were differentially expressed in seu ant double mutant relative to wild type and single mutant gynoecia. In particular we sought to identify transcripts whose expression was dependent on the coordinated activities of the SEU and ANT gene products. Our analysis identifies a diverse set of transcripts that display altered expression in the seu ant double mutant tissues. The analysis of overrepresented Gene Ontology classifications suggests a preponderance of transcriptional regulators including multiple members of the REPRODUCTIVE MERISTEMS (REM) and GROWTH-REGULATING FACTOR (GRF) families are mis-regulated in the seu ant gynoecia. Our in situ hybridization analyses indicate that many of these genes are preferentially expressed within the developing CMM. This study is the first step toward a detailed description of the transcriptional regulatory hierarchies that control the development of the CMM and ovule initiation. Understanding the regulatory hierarchy controlled by SEU and ANT will clarify the molecular mechanism of the functional redundancy of these two genes and illuminate the developmental and molecular events required for CMM development and ovule initiation.

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Introduction

In both gymnosperms and angiosperms, ovules are critical for reproductive competence. Ovules contain the female gametophyte and thus the egg cell. Additionally, upon fertilization the ovules develop into the seeds that nurture and protect the developing embryos. In Arabidopsis thaliana, two rows of ovules develop from a ridge of meristematic tissue on the inner surface of the seed pod or gynoecium. Within the developing ovule primordia, much is known about molecular patterning events along the proximal to distal axis and the mechanisms of integument development [1,2,3]. Also dramatic progress has been made with respect to understanding the subsequent development of the female gametophyte within the maturing ovule [4,5,6]. However, considerably less is known about the earliest steps in ovule development: the mechanisms of ovule initiation, and in the establishment and maintenance of the meristematic tissues of the carpel margin meristem (CMM) that generate the ovule primordia.

Gynoecial development in Arabidopsis initiates at stage 6 of floral development (floral stages according to Smyth; [7]). The gynoecial primordium is first morphologically recognizable as a dome or mound of cells, oval in cross section, that forms from the cells of the central most portion of floral meristem (i.e. floral whorl 4). During stage 6 the different spatial domains of the gynoecial tube are already discernable based on the differential expression of genes within the medial portion of the gynoecium versus the lateral domains, as well as along the inner to outer (adaxial to abaxial) axis [8,9,10] (Fig. 1A). During floral stages 6 and 7 the proliferation of cells along the perimeter of the gynoecial dome leads to the formation of a tube-shaped structure (Fig. 1B).

The single gynoecial primordium likely represents a composite of two congenitally-fused carpel organs in a phylogenetic sense (Fig. 1A) [8,11]. In this scenario, the medial portions of the gynoecium represent the fused margins of the two component carpels. The adaxial portions of the medial/marginal domain maintain meristematic potential throughout the elongation of the gynoecial tube and these regions have been termed carpel margin meristems (CMMs) [12,13,14]. Each Arabidopsis gynoecium contains two CMMs that are positioned within the adaxial portions of the medial domain of the gynoecium. During floral stages 7 and 8 the CMM takes the shape of a ridge of tissue (the apical ridge) that extends along the apical basal extent of the
Adaxial fate specification is compromised in the **seu** **ant** double mutant

A variety of experimental data suggest that the disruption of CMM development observed in the **seu** **ant** mutant is not conditioned simply by a de-repression of **AG**, but rather that **SEU** and **ANT** function to maintain or specify adaxial fate in the gynoecium and that this fate specification is critical for proper CMM development [12,16,19]. These studies demonstrated that expression levels of **PHABULOSA** (**PHB**) and **REVOLUTA** (**REV**) are reduced in the adaxial core of the stage 6 gynoecium in **seu** **ant** mutant plants. **PHB** and **REV** encode transcriptional regulators of the Homeodomain Leucine Zipper Class III type (**HDZip-III**) that are known to play a key role in the specification of adaxial identity in lateral organs [33,34,35,36,37,38,39,40]. These genetic studies, however, were not able to determine if the effect of the loss of **SEU** and **ANT** activity on HDZip-III expression was due to a direct or indirect regulation of their expression or accumulation. Additionally the defects in ovule and CMM development observed in the **seu** **ant** double mutant were not rescued when **PHB** activity was replaced, suggesting that either that **PHB** could not substitute for the other **HDZip-III** family members or that gene functions in addition to **HDZip-III** are required downstream of **SEU** and **ANT** for CMM development [12]. Synergistic disruptions of gynoecial and CMM development observed in the **ant rev** double mutant, but not in **ant phb** double mutant support the idea of a functional differentiation between the **PHB** and **REV** activities within the CMM [15]. The analysis of higher order mutants of the **HDZip-III** family members also suggests a diversification of functional roles within this gene family [40]. However these data do not exclude the possibility that there are a large number of additional gene regulation events critical for CMM development downstream of **SEU** and **ANT** that remain to be elucidated.

**Genetic analyses reveal a complex and highly redundant mechanism supporting CMM development**

Although no single mutant has been identified that eliminates CMM development or ovule initiation, a number of double mutant or higher order mutant combinations condition a severe...
disruption of the CMM and CMM-derived tissues (e.g. ovules) [12,16,18,19,20,27]. These data suggest that one or more redundant genetic programs support the development of the CMM. A portion of this resiliency is likely supported by the action of multiple members of structurally related genes families. Both SEU and ANT are members of gene families whose members have been shown to share redundant function [16,27]. With respect to the CMM, the SEUSS-LIKE genes, SLK1, and SLK2 genetically enhance the ant mutant phenotype with respect to ovule initiation defects [16]. Similarly the ANT-LIKE family member, ALL6, shares a critical redundant function with ANT as the ant all6 double mutants flowers display reduced medial domain development and initiate very few ovule primordia [27]. Other mutant combinations indicate instances of molecularly dissimilar molecules sharing overlapping functions during floral and CMM development. A redundant function shared between ANT and the IABBY family members YAB1 and YAB3 is suggested by the synergistic disruption of ovule initiation observed in the ant yab1 and ant yab1yab3 mutants [18]. Analysis of ant shatterproof1 (shp1) shatterproof2 (shp2) crabs claw (cc) mutants implicates the SHP MADS domain transcription factors in CMM development. These studies together highlight an important role for ANT function during CMM development and ovule initiation as well as reveal a high degree of functional redundancy within this tissue.

A high degree of redundancy hinders genetic approaches to the study of the CMM

A number of key regulators of CMM development may be difficult to recover with standard forward genetic approaches due to a high degree of redundancy. Identifying genes that have specific patterns of spatial and temporal expression in the CMM would generate a set of candidate genes that could then be analyzed by reverse genetic approaches. In this paper we employ a transcriptomic profiling approach to identify sets of genes that are differentially expressed in the developing carpels of the seu ant double mutant. In particular we sought to identify transcripts whose expression was dependent on the coordinated activities of SEU and ANT gene products. We hoped to both identify novel regulators of CMM development and to examine the molecular mechanism of the functional redundancy of SEU and ANT during CMM development. Our analysis identified a diverse set of transcripts that display altered expression in the seu ant double mutant tissues. Our in situ hybridization analyses indicate that many of these genes are preferentially expressed within the developing CMM. The analysis of overrepresented Gene Ontology classifications suggests a preponderance of transcriptional regulators including multiple members of the REPRODUCTIVE MERISTEMS (REM) and GROWTH-REGULATING FACTOR (GRF) families of transcriptional regulators are mis-regulated in the seu ant gynoecia. This study is the first step toward a detailed description of the transcriptional regulatory hierarchies that control the development of the CMM and ovule initiation.

Results

Transcriptomic analysis reveals putative targets of SEU and ANT regulation important for CMM development

In an effort to identify novel regulators of CMM development and ovule initiation we identified genes that are preferentially expressed within the CMM within the context of the gynoecium. Additionally we endeavored to prioritize genes whose expression is synergistically disrupted in the seu ant double mutant relative to either single mutant. We isolated RNA from staged (floral stages 8 through 10) and hand-dissected gynoecia to limit the developmental window of the sample to the period just before and then during ovule primordia initiation, the earliest steps of ovule development. This differentiates our work from that of the Gasser and Colombo groups that have focused on later ovule developmental stages when identifying ovule-specific transcripts [2,41].

We utilized the Arabidopsis ATH1 Gene Chip (Affymetrix) to compare transcript levels between four different genotypes (Col-0, seu-3, ant-1, and seu-3 ant-1 double mutant). We first analyzed mRNA accumulation in each single mutant relative to the Col-0 wild type gynoecial samples. To identify transcripts whose steady-state levels were altered in the single mutants relative to wild type, we utilized a 1-way ANOVA and identified probe sets (transcripts) that displayed a statistically significant difference in accumulation by the genotype term. This analysis identified 120 under-expressed and 200 over-expressed transcripts in the seu single mutant and 219 under-expressed and 241 over-expressed transcripts in the ant single mutant (Tables S1, S2, S3, S4) Throughout this manuscript we refer to transcripts that display a differential steady state level of accumulation in a given sample as differentially “expressed” with the caveat that we are measuring steady state levels and cannot differentiate transcriptional from post-transcriptional effects on RNA accumulation with these approaches.

Over-represented GO categories for the genes displaying reduced expression in seu are reported in Table S5 and include “sequence specific DNA-binding transcription factor activity” (GO:0003700) and “leaf development” (GO:0048366). For genes that are over-expressed in the seu single mutant the over represented GO categories are reported in Table S6. Over represented GO categories for the genes under-expressed in ant single are reported in Table S7 and include “sequence specific DNA-binding transcription factor activity” (GO:0003700) and “flower development” (GO:0009908). Over represented GO categories for the genes over-expressed in ant single are reported in Table S8. As the seu ant double mutant fails to initiate ovule primordia, we reasoned that genes critical for the earliest steps of ovule initiation would display reduced expression in the seu ant double mutant gynoecia, relative to either single mutant or the wild-type tissues. To identify transcripts that are differentially expressed in the seu ant double mutant relative to the other genotypes, we utilized two statistical approaches. For Approach I we used a 1-way ANOVA to identify probe sets for which the mean expression level was significantly different in the double mutant relative to the overall expression mean: 210 transcripts displayed reduced accumulation (Table S9) and 128 displayed elevated accumulation in the double mutant using this analysis approach (Table S10). In the set of genes with reduced accumulation in the seu ant double mutant statistically enriched GO categories included “transcription factor activity”, “ad/abaxial polarity specification”, “flower development”, and “transmembrane receptor protein kinase activity” (Table S11). The GO terms that were significantly enriched in the gene set with elevated accumulation are presented in Table S12.

We focused our attention on the genes that displayed reduced expression within the seu ant double mutant because: 1) over-represented GO terms suggest a role for this gene set in transcriptional regulation and relevant developmental processes, and 2) the reduced accumulation of these transcripts in the seu ant double mutant suggests that they may be preferentially expressed in the CMM in the wild-type gynoecium and, thus, are candidates for novel regulators of CMM development.

Analytical Approach II yields 31 high-priority putative CMM regulators

To further identify genes exhibiting reduced expression in the seu ant double mutant we used a second analytical approach
Appropriately comprising two steps, we first selected probe sets for which the mean expression was significantly lower in the *sea* or *ant* single mutant relative to wild type (The union of the genes sets reported in Tables S3 and S4). As the *sea* and *ant* single mutants display very minor morphological disruptions in stage 8 and 9 gynoecia, we reasoned that transcripts displaying reduced accumulation in the single mutants would not simply reflect a morphological loss of CMM tissue in these samples, but might be more likely to reflect a reduction in the level of transcription of a given gene in the mutant. We then applied a second selection criterion such that we additionally required that the transcript abundance in *sea* and *ant* double mutant gynoecia be lower than an expected value that was estimated via an additive model using the data from each single mutant. This was done with the JMP Genomics estimate builder with a significance cutoff of alpha <0.05. By using these selection criteria, we hoped to enrich for genes that were synergistically reduced in expression in the *sea* and *ant* double mutant and that might uncover the molecular basis of the synergistic phenotypic enhancement in the *sea* and *ant* double mutant.

Approach II yielded just 31 candidate genes (Table 1). Hereafter referred to collectively as “Approach II candidate genes”. The majority (55%) of the Approach II candidate genes encode transcriptional regulators. Several observations suggest that many of these candidates are preferentially expressed in the developing carpel margin and are likely important regulators of CMM development that are downstream of *SEU* and *ANT* regulation. Firstly, twenty-eight of the thirty-one Approach II candidate genes were also found within the set of 210 genes showing significantly reduced accumulation in the double mutant as identified by Approach I. Secondly, 11 of the 31 genes have been previously shown to be expressed preferentially in the CMM or in CMM-derived tissues (e.g. ovules). These include *AT1G02080* (ATCE2), *AT2G55550* (AP2), *AT2G57720* (REM15), *AT2G46870* (NGA1), *AT1G66840* (PAN), *AT2G34710* (PHB), *AT4G37750* (ANT1), *AT1G07560* (TA1), *AT3G18000* (VDD), *AT3G17010* (REM22), and *AT4G31610* (REM4 - previously AtREM1). [2,12,15,22,23,40,41,42,43,44,45,46,47,48]. The expression levels of two of these (PHB and TA1) has been previously shown to be reduced in *sea* and *ant* double mutant gynoecia [12,15,16]. Interestingly even though *SEU* and *ANT* have been implicated in the repression of *AG* in perianth organs, the levels of *AG* expression were not significantly different from wild type in the *sea* or *ant* single mutant gynoecial RNA samples (Table 3).

qRT verification of candidates

From this set of 31 genes that displayed reduced expression in the *sea* and *ant* double mutant we have confirmed by qRT-PCR nine out of ten genes tested (Table 2). We also confirmed an additional 7 of 7 genes that displayed increased expression in the *sea* and *ant* double mutant (Table 3).

**REM family and AtGRF family transcriptional regulators are significantly over-represented in Approach II candidates**

Surprisingly, seven out of the 31 Approach II candidate genes were members of the B3 superfamily of transcription factors (Table 1) [49,50]. This enrichment for genes encoding B3 transcription factors within our sample is highly unlikely to have occurred by chance alone ($p = 2.5 \times 10^{-10}$ by hypergeometric probability test). The Arabidopsis B3 superfamily consists of 118 genes all of which encode proteins containing one or more B3-type DNA binding domains. The B3 superfamily is comprised of four sub-families: REM (REPRODUCTIVE MERistem); LAV (LEAFY COTYLEDON2 [LEC2]; ABCISIC ACID INSENSITIVE [ABI]); VAL; ARF ( Auxin RESPONSE FACTOR); and RAV (RELATED TO A Be; and VP1). Six of the seven B3 regulators that were identified in our transcriptomics approach are from the REM subfamily for which there is little functional data. Four of these genes, *AT4G31610* (REM34/AIREM1), *AT3G18000* (VDD), *At5G57720* (REM15) and *AT3G17010* (REM22), have been previously reported to display CMM-enriched expression [41,42,43,47,51].

The GRF family of genes is also over-represented in the list of Approach II candidates ($p = 6.9 \times 10^{-5}$ by hypergeometric probability test). Members of the GRF family in Arabidopsis have been shown to regulate growth and development of leaves, cotyledons and floral organs [52,53,54]. Over expression of members of this gene family result in larger and wider leaves while *gfi2* single mutants and *gfi1,2,3* triple mutants display narrower leaves, suggesting a role in the regulation of cell proliferation within the medial to lateral axis of the leaf.

**Expression of several REM family genes marks the gynoecial medial domain**

We used *in situ* hybridization to further characterize the temporal and spatial expression patterns of a number of the Approach II candidates during early gynoecial development. Although for several of these genes expression data from *in situ* hybridization experiments were previously published, these data did not examine the expression pattern of these genes in detail during gynoecial development. We specifically focused on the developing gynoecium and examined cross sections to determine the expression patterns within the medial versus lateral gynoecial domains. In some cases we also examined the expression in *sea*, *ant*, and *sea ant* double mutant tissues. Our results indicate that all six REM family members identified with Approach II analysis are expressed preferentially within the medial gynoecial domain with varying developmental profiles.

*At4G353310* (REM16) was previously reported in stamen primordia at stage 4 and carpel primordia at stage 6 [43]. We detected expression of *At4G353310* (REM16) weakly in the stage 1–4 floral primordia, chiefly in L1 layer and in peripheral portions of the floral meristem that will give rise to the sepal primordia that will give rise to the sepal primordia (Fig. 2A). Expression in the gynoecium is difficult to detect before early stage 7 when expression is observed in the abaxial portions of the medial gynoecial domain (Fig. 2B). Expression during stage 8 is seen in the medial domains and begins to be detected in both adaxial and abaxial portions. However expression is not observed in the L1 layer (Fig. 2C and G). The stage 8 medial domain expression appeared reduced in the *sea* single and the *sea ant* double mutant relative to wild type (Figs. 2D, E, F). In wild type tissue expression continues to be detected in the ovule primordia throughout stages 9 through 11 and is confined to subepidermal cell layers (Fig. 2I). During stage 7 and 8 expression in the stamens is detected mostly in the subepidermal cells from which archesporial and tapetal cells are derived (Fig. 2 C, H). During stage 9 expression is most strongly detected in the tapetal cells (Fig. 2 H). Expression within the stamen primordia also appeared to be reduced in the *sea* mutant tissues (Fig. 2D, K). Hybridizations with sense strand probes gave very little background staining (Fig. 2L).

Expression of *At4G353160* (REM34/AIREM1) in the inflorescence meristem and in floral stages 2-5 has been previously reported [42]. Franco-Zorrilla et al. also report expression of *AT4G31610* (REM34/AIREM1) is confined to gynoecial primordium from stage 6 onward and later expressed in the medial ridge, sepal, style and stigma [42]. Our analysis of gynoecial expression patterns reveals that *At4G353160* (REM34/AIREM1) is expressed in...
the adaxial core of the stage 6 and stage 7 gynoecial primordia (Fig. 3C and D). During stage 7 and 8 expression is strongest at the apex of the gynoecium in the medial domain (Fig. 3E, G). During late stage 8 expression is detected in the ovule anlagen (Fig. 3H).

Table 1. Approach II Candidate Genes.

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<th>AGI</th>
<th>Gene Title</th>
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<th>B3 Family member</th>
<th>wt log2 Lsmean</th>
<th>ant log2 Lsmean</th>
<th>seu log2 Lsmean</th>
<th>seu ant log2 Lsmean</th>
<th>−log(10) P-value*</th>
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<td>8.22</td>
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Expression of AT5G18000 (VDD) was previously reported in inflorescence and floral meristems and in ovules, as well as within the developing female gametophyte [41]. Within the gynoecium we first observe expression of VDD at stage 7 when it is detected weakly throughout the primordium (Fig. 4A). Expression was stronger at the medial portions of the apex of the stage 7 and 8 gynoecial primordia relative to more basal positions (Fig. 4B, C). Expression continues in the apical medial domain into stage 10 (Fig. 4G). Expression is also detected in ovule primordia as they arise at stage 8 and continues throughout ovule development (Fig. 4E and data not shown) [41]. Expression in ovule primordia at stage 11 was strongest in the chalazal portions of the ovule. Expression in the ant single mutant tissue at this stage appeared reduced, suggesting that ANT may regulate the expression of VDD in the chalazal portions of the ovule. Strong expression was also detected in tapetal cells of the anther at stage 9 (Fig. 4H).

Expression of AT5G17010 (REM22) in stamen and carpel primordia has been previously reported [47,51]. We first detected expression of AT5G17010 starting at late stage 4 or early stage 5 in the stamen primordia as they arise (Fig. 5A). Within the gynoecium expression can be detected preferentially in the medial domain as early as stage 6 (Fig. 5B, C). AT3G17010 continues to be preferentially expressed subepidermally within the medial domain through stage 8 (Fig. 5E, F) and is strongly detected in the medial

Table 1. Approach II Candidate Genes.
### Table 2. qRT PCR verification of candidates under-expressed in the seu ant double mutant.

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
<th>wild type expression mean*</th>
<th>wild type standard error mean</th>
<th>seu mutants expression mean*</th>
<th>seu mutants standard error mean</th>
<th>ant mutants expression mean*</th>
<th>ant mutants standard error mean</th>
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<th>Tukey HSD level</th>
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*mean normalized expression [normalized to ADENOSINE PHOSPHORIBOSYL TRANSFERASE1 (At1g27450)].
1Tukey honest statistical difference test level.
n.d. not detected.
doi:10.1371/journal.pone.0026231.t002

### Table 3. qRT PCR verification of candidates over-expressed in the seu ant double mutant

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Gene Name</th>
<th>wild type expression mean*</th>
<th>wild type standard error mean</th>
<th>seu mutants expression mean*</th>
<th>seu mutants standard error mean</th>
<th>ant mutants expression mean*</th>
<th>ant mutants standard error mean</th>
<th>seu ant double mutants expression mean*</th>
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*mean normalized expression [normalized to ADENOSINE PHOSPHORIBOSYL TRANSFERASE1 (At1g27450)].
1Tukey honest statistical difference test level.
# Expression level of AG was not statistically different across the four genotypes in data from the ATH1 arrays, nor as estimated with qRT PCR.
doi:10.1371/journal.pone.0026231.t003
domain at apical positions of the stage 8 gynoecium (Fig 5G). Expression is detected in the ovule primordia during stage 9 and 10 in subepidermal layers (Fig. 5H and data not shown).

The $AT3G19184$ ($REM1$) transcript is detected throughout the inflorescence meristem and throughout stage 1–4 floral meristems (Fig. 6A). During stage 5 expression of $AT3G19184$ ($REM1$) is strongest in stamen and petal primordia as they arise (data not shown). Expression is detected throughout stage 6 and 7 gynoecia (Fig. 6B). It is strongly detected at the apical regions of stage 7 gynoecia, particularly in medial positions (Fig. 6C). Expression in stage 8 gynoecia is strongest in ovule primordia as they arise. Expression in stage 7 stamen primordia is detected strongly in the precursors of the archesporial and tapetal cells (Fig. 6B, C) and is later expressed in microspores and tapetal cells during stage 9 (data not shown).

Expression of $AT3G57720$ ($REM15$) in stamen and carpel primordia was previously reported [43]. We first detect expression of $AT3G57720$ during early stage 4 as a ring of expression that appears to mark whorl three positions just interior or adaxial to the sepal primordia (Fig. 7A). During stage 6 $AT3G57720$ ($REM15$) is detected in the gynoecium in the medial domain, most strongly in abaxial positions (Fig. 7B). During stage 7 $AT3G57720$ ($REM15$) is detected throughout the medial domain of the gynoecium (Fig. 7C) and continues to be detected in adaxial portions of the medial domain.
domain during stage 8 (Fig. 7 D and E). *AT3G57720 (REM15)* is detected in ovule primordia as they arise during stage 8 (Fig. 7F) and continues to be expressed in the megaspore mother cell and in nucellar portions of the ovule through stage 12 (Fig. 7G and H). *AT3G57720 (REM15)* is also detected strongly in stamen tapetal cells during stage 9 (data not shown).

Additional Approach II candidate genes are preferentially expressed within the medial gynoecial domain

*PERIANTHIA (PAN)* expression patterns have been previously published [44,55]. *PAN* is detected strongly in the stage 6 gynoecial primordium within the medial domain (Fig. 8B). *PAN* expression continues to be expressed at declining levels within the medial domain throughout stage 7 and 8 (Fig. 8C, and E). Expression is again strongly detected at late stage 8 or early stage 9 in the early ovule primordia (Fig 8F). Expression of *PAN* in the *sea ant* double mutant appeared reduced within the stage 7 medial domain and later (Fig 8D). Additionally *PAN* was not detected in the *sea ant* stage 9 gynoecia (Fig 8G).

Expression of *AtGRF5 (AT3G13960)* has been previously reported as strongly expressed in actively growing tissues but only weakly detected in mature tissues suggesting a role in regulation of cellular proliferation [53]. Analysis of a *GRF5:GUS* reporter line revealed expression within the proximal half of the young leaf primordia, a domain with a high proportion of actively dividing cells, however a detailed description of the expression within the flower was not reported [54]. We detected expression of *AtGRF5 (AT3G13960)* in stage 1 floral primordia, and in a line that marks the boundary between later stage floral primordia and the inflorescence meristem (Fig. 9A). Expression was not detected in floral stage 2 meristems, nor in the inflorescence meristem. Expression was again detected in stage 3 floral meristems in the sepal primordia and then in stamen and petal primordia as they arise during stage 5 (data not shown). Within the gynoecium expression is detected during stage 6 and 7 in the marginal portion of the gynoecium, most strongly detected in abaxial portions of the margin (Fig. 9C and D). During stage 8 *AtGRF5* is detected in a somewhat punctate pattern throughout the gynoecial primordia,
but with highest expression within the medial portions (Fig. 9E). 
GRF5 is also detected in the ovule primordia are they arise (Fig. 9G) and in subepidermal layers through at least stage 11 (Fig. 9H). Expression of AtGRF5 in the seu ant double mutant tissue appeared to be slightly reduced in stage 1 and stage 3 floral meristems (Fig. 9B) and then strongly reduced within the later stage gynoecia (Fig. 9F).

The expression of EXCESS MICROSPOROCYTES (EMS) (also named EXTRA SPOROGENOUS CELLS) has been previously reported during stamen development [56,57]. During stage 6 EMS is expressed weakly throughout whorls 3 and 4 (data not shown). During stage 7 and early stage 8 EMS is expressed throughout the gynoecium, but expression levels are slightly higher in medial domain particularly in the apical region (Fig. 10B, C, D). During stage 8 and 9, expression is evident in ovule anlagen and primordia as they form (Fig. 10E). Expression continues in the ovule primordia in the nucellar and chalazal domains during megaspore mother cell stage and as integuments arise (Fig. 10F).

We detected strong LEAFY (LFY) expression in the stage 1–3 floral primordia as previously reported (Fig. 11A) [58]. In the seu ant double mutant tissue expression of LFY in the floral stages 1–3 appeared reduced relative to wild type levels (Fig. 11B). During floral stages 4 and 5 LFY is expressed strongly in the petal and stamen primordia as they arise, but only weakly detected in the central floral dome (data not shown). During floral stages 6 and 7 LFY expression is strongly detected in the adaxial core of the gynoecium and within the medial domain at the apex (Fig. 11C and D). Expression of LFY in the stage 6 seu ant gynoecia was very reduced relative to wild type levels (Fig. 11E). In early stage 8 wild type tissue LFY expression is detectable in the early ovule primordia (Fig. 11 F).

Other Approach II candidate genes are expressed outside the medial gynoecial domain

Several of the Approach II candidate genes that we assayed by in situ hybridization displayed preferential expression within the gynoecial valve domains or expression in both the medial and valve/lateral domains. These data suggest that the effects of the loss of SEU and ANT on gynoecial development are not specific for the medial domain, but rather alterations of gene regulation occur in both the medial and lateral domains in the seu ant double mutants. 

**YABBY3 (YAB3)** (AT1G75430) expression has been previously reported as expressed within the abaxial portions of all lateral organs derived from both the apical and floral meristems [59]. Expression of YAB3 is seen in the abaxial valve domains within the gynoecium during stages 6 through 9 (Fig. 12). Expression is fairly weak in the stage 6 gynoecia and becomes stronger in stages 7 and 8 (Fig. 12A–C). YAB3 expression in not detected within the medial portions of the gynoecium. Expression in the stage 8 seu ant double mutant gynoecium is very reduced or undetectable (Fig. 12D).

**BELL-LIKE HOMEODOMAIN 11 (BLH11)** (AT1G75430) expression was detected weakly in the inflorescence meristem and stage 1 and 2 floral meristems (data not shown). Expression is more strongly detected in sepal primordia during stages 3 and 4 and in stamen and petal primordia during stage 5 (data not shown). Within the gynoecium expression is detected at stage 6 throughout the primordium, but at higher levels in the valve domains. Expression during stage 7 and 8 is predominantly within the valve domains, but is detected within both valve and medial domains at the gynoecial apex (Fig. 13B, C, and D). Expression is detected in young ovule primordia during stage 8 and 9 (Fig. 13E), and continues to be detected in nucellar portions of the ovule through stage 11 (data not shown).
**UGT84A2 (At3G21560)** is expressed within the inflorescence meristem and stage 1 and 2 floral meristems, chiefly in the L1 cell layer (Fig. 14H). In stage 3 floral meristems UGT84A2 is strongly expressed in the L1 epidermal cells of the sepals (Fig. 14H). Within the stage 7 gynoecium UGT84A2 expression is detected most strongly in the abaxial portions of the valve domains (Fig. 14A and B). However, at the apex of the gynoecium expression is detected in the L1 layer in both medial and lateral/valve domains (Fig. 14 C). During stage 8 expression was detected in the L1 epidermis of the valve domains in both abaxial and adaxial positions (Fig. 14G). Expression in the **seu ant** double mutant tissue was slightly reduced in the inflorescence meristem and young floral buds and strongly reduced in the stage 7 and 8 gynoecia (Fig. 14D, E, F, I).

The gene **At1G68780** is annotated as a member of the RNase inhibitor-like superfamily containing multiple leucine rich repeat InterPro domains (InterPro:IPR001611) [60]. Expression of **At1G68780** was detected weakly throughout the inflorescence meristem and floral stages 1–2 (Fig. 15A). During floral stage 3 expression was strongly detected within the sepal primordia. During floral stages 6 through 8, **At1G68780** is most strongly detected in apical portions of the gynoecium throughout both medial and lateral domains (Figs. 15B through 15D). Gynoecial expression was significantly reduced in the stage 7 **seu ant** double mutant gynoecia. Expression was detected in wild type flowers throughout petal development during floral stages 5 through 12 (Figs. 15B, C, G and data not shown). Expression within the petals was reduced in the **ant** single mutant relative to wild type at stage 11 (Fig. 15H).

**Discussion**

Here we report the transcriptomic signature of the **seu ant** double mutant gynoecium relative to wild type and single mutant gynoecia in an effort to characterize both the set of genes important for CMM development and those that are synergistically regulated by the coordinated activities of the **SEU** and **ANT** transcriptional regulators. We have identified a diverse set of transcripts displaying altered expression levels in the **seu ant** double mutant tissues. The analysis of the set of genes displaying reduced accumulation in the **seu ant** double mutant tissue indicates a preponderance of transcriptional regulators including multiple members of the **REPRODUCTIVE MERISTEMS (REM)** and **GROWTH-REGULATING FACTOR (AtGRF)** families. Our in situ hybridization analyses indicate that many of these genes are preferentially expressed within the medial domain of the wild type gynoecia further suggesting a role for these genes during CMM development.

**GROWTH-REGULATING FACTOR (AtGRF) family**

Members of the **AtGRF** gene family encode proteins with a conserved QLQ domain that functions as a protein/protein interaction domain and a conserved WRC domain that functions as a nuclear localization signal and contains a putative DNA
binding C_{3}H motif. *AtGRF1, AtGRF2, AtGRF3* and *AtGRF5* have been shown to regulate growth and development of leaves, cotyledons and floral organs [52,53,54]. Over-expression of members of this gene family result in wider leaves and petals while *AtGRF3* single mutants and *AtGRF1,2,3* triple mutants display narrower leaves and petals. The phenotypic effects of the loss of function of *AtGRF1,2,3* and *AtGRF5* are enhanced by mutations in *GRF-INTERACTING FACTOR1* (*AtGIF1*) [52,54]. *AtGIF1* encodes a transcriptional co-regulator that physically interacts with members of the *AtGRF* family. The *AtGIF* and *AtGRF* family members are thus likely to support cell proliferation required for the lateral (laminal) expansion of the leaf blade. Additionally, mutations in *AtGIF1* reduce female fertility and this effect was enhanced as the dosage of wild type *GRF* family members was reduced in the *gif1* mutant background [52]. Recently, it has been observed that an *AtGIF* triple mutant, *gif1 gif2 gif3*, develops unfused gynoecia, that lack replum and septal tissues, and contain fewer ovules ([61]; personal communication J. H. Kim) These results support a role for *AtGIF1* and *AtGRF* family members in female reproductive development.

**Figure 10. Results of in situ hybridization with EMS antisense probe.** Numbers indicate floral stages. All panels show transverse (cross sectional) tissue orientation. ov - ovule; Scale bars in all panels are 100 microns. All panels are Col-0 wild type tissue. doi:10.1371/journal.pone.0026231.g010

**Figure 11. Results of in situ hybridization with LFY antisense probe.** Numbers indicate floral stages. All panels show transverse (cross sectional) tissue orientation. Arrowheads indicate medial domain expression. ov - ovule; Scale bars in all panels are 100 microns. All panels are Col-0 wild type tissue except as marked. doi:10.1371/journal.pone.0026231.g011

**Figure 12. Results of in situ hybridization with YAB3 (At4g00180) antisense probe.** Numbers indicate floral stages. All panels show transverse (cross sectional) tissue orientation. Scale bar in D represents 100 microns for all panels. Arrows indicate abaxial valve domain expression. All panels are Col-0 wild type tissue unless otherwise marked. doi:10.1371/journal.pone.0026231.g012

**Figure 13. Results of in situ hybridization with BLH11 (AT1G75430) antisense probe.** Numbers indicate floral stages. All panels show transverse (cross sectional) tissue orientation. Arrowheads indicate medial domain expression. Arrows indicate valve domain expression. ov - ovule; sp - microsporogenic cells. Scale bars in all panels are 100 microns except for panel A where scale bar is 50 microns. All panels are Col-0 wild type tissue. doi:10.1371/journal.pone.0026231.g013
REPRODUCTIVE MERISTEMS (REM) family

A function has been determined for two of the REM family transcriptional regulators, VERNALIZATION1 (VRN1) and VERNALIZATION1 (VRN1). Although VRN1 is expressed within the medial gynoecial domain and was identified in our transcriptomics analysis as a potential regulator of CMM development, VRN1-silencing constructs do not disrupt CMM development [41]. Mutations in At4G31610 (REM34 - previously AtREM1) and At3G17010 (REM22) do not condition obvious developmental defects [42]. Our unpublished analysis of loss-of-function alleles of At4G31610 (REM34) and At3G17010 (REM22) also failed to detect developmental defects. Given the strong expression of several members of the REM family during early CMM development, it is possible that REM family members share a redundant function that may be revealed in the analysis of higher order mutant combinations.

Characterization of the transcriptional hierarchies required for CMM development

Although in situ hybridization is only a semi-quantitative technique, in most cases the reductions in gene expression in the single and double mutants that were detected in the ATH1 microarray and qRT PCR analyses were confirmed in our in situ hybridization experiments. The in situ hybridization technique is advantageous in that it allows a finer spatial and temporal characterization of the expression differences between the genotypes. In many cases, our in situ hybridization experiments revealed a reduction in gene expression of a candidate gene before an alteration in gynoecial or ovulate morphology was apparent in the mutant tissue. In these cases it is unlikely that the reduction of transcript level is simply due to a loss of the tissue in the mutant. However, the data we present here cannot distinguish between direct and indirect transcriptional targets of SEU and ANT regulation and thus our ability to define the transcriptional hierarchy of CMM development is limited at this time. Future analyses aimed to identify direct transcriptional targets of SEU and ANT regulation through chromatin-immunoprecipitation or glucocorticoid-inducible activities will help to identify the subset of candidates listed here that are directly regulated by SEU and ANT. These analyses will help to better delineate the levels of the transcriptional hierarchy required for CMM development and may illuminate the mechanistic basis for the synergistic genetic interaction between seu and ant mutants during CMM development. Synergistic genetic interactions are commonly observed in animal, plant and fungal systems and yet the mechanistic basis for the synergistic effect typically is poorly understood.

SEUSS may mediate the action of MADS domain-containing protein complexes required for medial domain development

Interestingly, ten of the 31 Approach II candidate genes (including five of the seven B3 candidate genes) have been previously identified by Gomez-Mena and colleagues as induced in response to the MADS domain-containing transcription factor AG [47]. Based on our ATH1 data and follow-up qRT-PCR (Table 3), the levels of AG transcript accumulation are not statistically different between the wild-type, seu, ant, or seu ant double mutant in the gynoecial samples. These data suggest that
SEU and ANT do not alter the levels of AG accumulation in the CMM, but rather they may work in parallel to AG and/or might alter the ability of the AG protein to function. The SEU transcriptional adaptor is known to physically interact with dimers of MADS domain DNA-binding proteins (including AP1, SEP3, SVP, and AG124) during the specification of floral organ identity [21,29,30,31,64]. We speculate that SEU may function in the developing gynoecium by mediating the action of MADS domain proteins (AG and others) and thus support the expression of a subset of the identified Approach II candidate genes. It is notable that both FDD and REM16 (AT3G55310) are direct targets of the MADS protein SEEDSTICK (STK) [41].

The medial apex of the gynoecium as a developmental domain

Our in situ analyses together with the work of other groups indicates that at least 16 of the 31 Approach II candidate genes are expressed preferentially within the medial gynoecial domain with respect to their gynoecial expression. The exact timing and position of expression within the medial domain varies between the candidates. Yet many of these genes similarly display strong expression within the apical-most portion of the medial gynoecial domain. These expression patterns suggest that the medial apex might be functionally distinct from other portions of the gynoecium as early as stage 6. The common expression pattern of many of these candidates suggests that gene regulation events within the apical medial domain of the gynoecium may be critical for the subsequent initiation of ovule primordia from the medial ridge tissues. In this scenario the maintenance of a particular transcriptional or cellular state within the medial apex would be required to maintain the meristic potential of the medial domain during elongation of the gynoecial tube.

The medial apex of the stage 6 gynoecium is also marked by the expression of TRYPHTHON AMINOTRANSFERASE OF ARABIDOPSIS (TAA1) [15,45]. TAA1 encodes a tryptophan amino-transferase required for the synthesis of auxin via the indole-3-pyruvic acid (IPA) branch of the auxin biosynthesis pathway [45,65]. We previously demonstrated an enhanced sensitivity of the medial domain to the action of auxin transport inhibitors and suggested a model in which patterning along the medial-lateral axis of the gynoecium requires an auxin dependant signal [15]. Among the list of Approach II candidates, TAA1 as well as INDOLE-3-ACETIC ACID INDUCIBLE 1 (IAA18), PINFORMED 1 (PEN1) and LIKE AUXIN RESISTANT2 (LAX2) all are known to encode auxin synthesis, transport or response functions [66]. Additional experiments will be required to test the role of these genes during medial domain development.

Non-cell autonomous functions during CMM development

Several of the Approach II candidate genes displaying reduced expression in the seu ant double mutant were not expressed specifically in the medial domain in wild type gynoecia, but rather displayed strong expression in valve domains (e.g. LAB3, BLH11 and UGT804A2). YABBY family members are expressed in abaxial portions of aerial lateral organs and support laminal expansion in response to the juxtaposition of abaxial and adaxial fates during organ growth [67]. The loss of CMM development in the ant yab1 yab3 triple mutant indicates a role for YABBY genes during CMM development [18]. The YABBY genes likely exert a non-cell-autonomous effect on CMM development suggesting that interactions between the developing valve and medial domains may be important during early gynoecium development. Our transcriptomics data indicates that expression of YABBY1/FILAMENTOUS FLOWER (AT2G45310) is also significantly reduced (to 45% of wild type levels) in the seu ant double mutant (Table S9). However, YABBY1/FILAMENTOUS FLOWER did not make our list of Approach II candidates because it did not display a reduction in either of the single mutants.

The alterations of gene expression in the lateral domain of the seu ant double mutant point to a role for SEU and ANT function within the lateral domain. Although the most dramatic gynoecial defects in the seu ant double mutant are observed within the CMM and its derived structures, the size of the carpel valve, the overall floral size and plant height are also reduced in the seu ant double mutant indicating that the loss of SEU and ANT activity alters more than just medial domain development [12]. The enhanced effect of the seu ant double mutant on CMM development may reflect an enhanced sensitivity of the medial domain to the loss of SEU and ANT activities.

Methods

Transcriptomics data analysis

Whole inflorescences were fixed in ice cold 100% ethanol overnight and then stored for up to one week in 100% ethanol before hand-dissection of gynoecia from floral stages 8–10 under a dissecting scope. RNA was isolated from staged gynoecia using the RNeasy Plant Mini Kit from Qiagen. Linear amplification, labeling, and fragmenting of the cDNA was carried out according to GeneChip 3′ IVT Express Kit instructions from Affymetrix. The initial 25 ng of total RNA was amplified to approximately 11 to 15 micrograms of fragmented and labeled aRNA. Affymetrix ATH1 microarrays were hybridized by Expression Analysis (Durham, NC). Probe density data was imported into JMP Genomics 4.1 (SAS, Cary, NC). The CEL or intensity files for each array were compared with a distribution analysis for similarity of the arrays. After visual inspection, none of our arrays was excluded. The data for the arrays was normalized using the Loess Model of Normalization. The probe set values were then summarized by calculation of the mean for each probe set.

Two analyses were run on our data. The first was a simple 1-way ANOVA by genotype. This method identified genes with expression levels that were statically different from that of the mean of the expression values. Class variables were specified as the genotype and the genotype was modeled as a fixed effect. The data was not compared to any baseline, but the LSMeans were run for simple differences of genotype using pFDR for the multiple testing method with an alpha of 0.05. Additionally we required that log2 of the magnitude of the expression level difference between the compared genotype means was greater than 0.33. The fixation method for the data points with large residuals was set as the False Positive Rate and the LSMeans standardization rate was set for Standard Deviation. The second analysis method (Approach II) was directed at detecting genes whose expression was synergistically affected in the seu ant double. The null hypothesis tested here was that the value for the seu ant double was equal to that of the addition of the seu value with the ant value. We then accepted all values for the seu ant double that were statistically different from the additive estimate. This analysis was done in JMP Genomics using the estimate builder feature. Results of the ANOVA and the calculated statistical significance of non-additivity estimates from the estimate builder feature for all 22,810 probe sets are reported in Table S14. We then applied a second criterion to this list by requiring that the mean expression in the seu or ant single mutant was significantly lower than the wild type mean expression level (by ANOVA). This reduced the list of Approach II candidates to 31 genes (See Table 1). Gene lists were moved to virtual plant [68] to convert Affymetrix probe set IDs to AT gene
identifiers and to generate intersection and union sets. GO TERM enrichment analysis was carried out using ChipEnrich [69]. Chip Enrich selects for \( p < 0.001 \) hypergeometric probability without correcting for multiple testing. The ChipEnrich program also returned statistically overrepresented DNA binding motifs in a 1 kilobase region 5' to the annotated ATG of the genes in the set and overrepresented transcription factor gene families. ATH1 data sets have been submitted to the Gene Expression Omnibus (GEO) database [70] (series record GSE30492) and the Array Express database [http://www.ebi.ac.uk/arrayexpress/] with experiment number E-MEXP-3293.

Quantitative Real time RT PCR and in situ hybridization analysis of candidate gene expression

For analysis of transcript abundances, RNA from stage 8–10 gynoecia isolated for microarray analysis (pre-amplification) was used. cDNA synthesis and qRT-PCR were performed as previously described [12], except we used the SuperScript III First-Strand Synthesis System (Invitrogen) to generate cDNA and the cDNA was diluted 1:4 for qRT-PCR analysis. A single qRT-PCR experiment assayed four biological replicates each of wild type, \( \text{seu} \), \( \text{ant} \), and \( \text{seu ant} \) genotypes. Each biological replicate was assayed in triplicate. Results in Tables 2 and 3 are the mean expression of the indicated gene normalized to the expression level of \( \text{ADENOSINE PHOSPHORIBOSYL TRANSFERASE} \) (\( \text{APT1} \), At1g27450). Results shown are the average expression normalized to \( \text{APT1} \) and the standard error of the mean for four biological replicates. \( \text{APT1} \) was shown to be unaffected by genotype in our gynoecial RNA samples by comparison of \( \text{APT1} \) expression levels with two other standards (\( \text{TUB6} \), \( \text{AT5G12250} \) and \( \text{G6PD5} \), \( \text{AT1G62420} \)) across the four genotypes. Statistical analysis of one way ANOVA was conducted in JMP8 (SAS Institute Incorporated, Cary NC) using a Tukey-Kramer HSD test and a \( p \) value cutoff of 0.05. Sequences of the oligonucleotides used for qRT-PCR analysis are described in Table S13. The \( \text{in situ} \) hybridizations were carried out as reported previously [21] with the following modifications: acetic anhydride and RNase treatment steps were omitted. A detailed protocol is available at http://www4.ncsu.edu/~rfgfranks/research/protocols.html.

Supporting Information

Table S1  Genes under-expressed (reduced accumulation) in the \( \text{seu} \) single mutant via one way ANOVA. (XLS)

Table S2  Genes over-expressed (increased accumulation) in the \( \text{seu} \) single mutant via one way ANOVA. (XLS)

Table S3  Genes under-expressed (reduced accumulation) in the \( \text{ant} \) single mutant via one way ANOVA. (XLS)

Table S4  Genes over-expressed (increased accumulation) in the \( \text{ant} \) single mutant via one way ANOVA. (XLS)

Table S5  Over-represented GO categories for gene set displaying reduced expressed in the \( \text{seu} \) single mutant. (XLS)

Table S6  Over-represented GO categories for gene set over expressed in the \( \text{seu} \) single mutant. (XLS)

Table S7  Over-represented GO categories for gene set displayed reduced expressed in the \( \text{ant} \) single mutant. (XLS)

Table S8  Over-represented GO categories for gene set over expressed in the \( \text{ant} \) single mutant. (XLS)

Table S9  Genes under-expressed (reduced accumulation) in the \( \text{seu ant} \) double mutant via one way ANOVA. (XLS)

Table S10  Genes over-expressed in the \( \text{seu ant} \) double mutant via one way ANOVA. (XLS)

Table S11  Over-represented GO categories for gene set displaying reduced expressed in the \( \text{seu ant} \) double mutant. (XLS)

Table S12  Over-represented GO categories for gene set over expressed in the \( \text{seu ant} \) double mutant. (XLS)

Table S13  Sequences of oligonucleotides used for qRT PCR analysis of candidate gene expression. (XLS)

Table S14  Output from JMP Genomics (SAS) ANOVA analysis of candidate gene expression. (XLS)

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Author Contributions

Conceived and designed the experiments: ANW EER RGF. Performed the experiments: ANW EER RGF. Analyzed the data: ANW EER RGF. Wrote the paper: ANW RGF.

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