A Spatial Dissection Of
The Arabidopsis Floral Transcriptome
By MPSS

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A SPATIAL DISSECTION OF THE ARABIDOPSIS FLORAL TRANSCRIPTOME BY MPSS

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“From our acquaintance with this abnormal metamorphosis, we are enabled to unveil the secrets that normal metamorphosis conceals from us, and to see distinctly what, from the regular course of development, we can only infer. And it is by this procedure that we hope to achieve most surely the end which we have in view.”

~ Johann Wolfgang Goethe
Die Metamorphose Der Pflanzen (1789)

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Dedication

This thesis is dedicated to Bill Serfass, whom helped further my interests in plant science through my employment at “PrydenJoy” Orchard and ultimately led me to focus on the discipline as a career. In addition, I would like to dedicate this thesis to Dr. Blake Meyers, a professor who has continued to bolster my interests and self-confidence in plant biotechnology by providing me with the opportunities and guidance necessary to prepare for doctoral study.

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Abstract

We have further elucidated the transcriptional complexities of floral organogenesis and organ-localized expression in the inflorescence of *Arabidopsis thaliana* by comparative analysis of massively parallel signature sequencing (MPSS) data. Six libraries of sequence tags corresponding to mRNA extracted from immature inflorescence tissues were constructed and matched to their respective loci in the annotated *Arabidopsis* genome. These signature libraries survey the floral transcriptome of wild type tissue as well as that of the floral homeotic mutants *apetala-1-10, apetala-3-6, agamous*, *superman/apetala 1-10* and ovules dissected from the gynoecia of wild type inflorescences. With the aid of a binary system, comparative *in silico* analysis of these expression libraries permitted a genome-wide dissection of organ-specific expression as measured by MPSS. Transcripts expressed specifically in the petals, stamen, stigma/style, gynoecium, and those putatively specific to the sepal/perianth, petal/stamen, or gynoecium/stamen were identified and quantified.

Implementing a binary system of determinants across the MPSS floral expression libraries, a total of 2,537 genes were categorized within spatial subgroups of the inflorescence. The quantitative nature of MPSS technology permitted these genes to be sorted by their expression level relative to a baseline of expression undetected by the technology. This sorting based on expression level enabled a relative ranking of confidence in the computational assessment. The bulk of characterized organ-specific transcript diversity was

noted in the gynoecium and stamen, whereas fewer genes possessed perianth localized expression. This exemplifies the molecular intricacies and organ specificity associated with reproductive organ development as opposed to vegetative floral tissue. Validation of the computational analysis across MPSS floral libraries was performed by comparison of previous expression data, *in situ* hybridization, promoter-reporter fusions, and RT-PCR.

This extensive analysis has illustrated the accuracy of MPSS at assigning spatial floral gene expression. A number of well-characterized genes with known expression patterns were accurately demarcated within our system of transcript filtration. Moreover, biological validations of these *in silico* predictions are in accordance with MPSS predictions for numerous genes with previously uncharacterized expression patterns. This bolsters support for the application of MPSS expression library analysis, LIBAN, toward preliminary genome-wide functional analysis; and will be advantageous in the elucidation of more comprehensive genetic regulatory networks which govern floral development.

Chapter 1

BACKGROUND

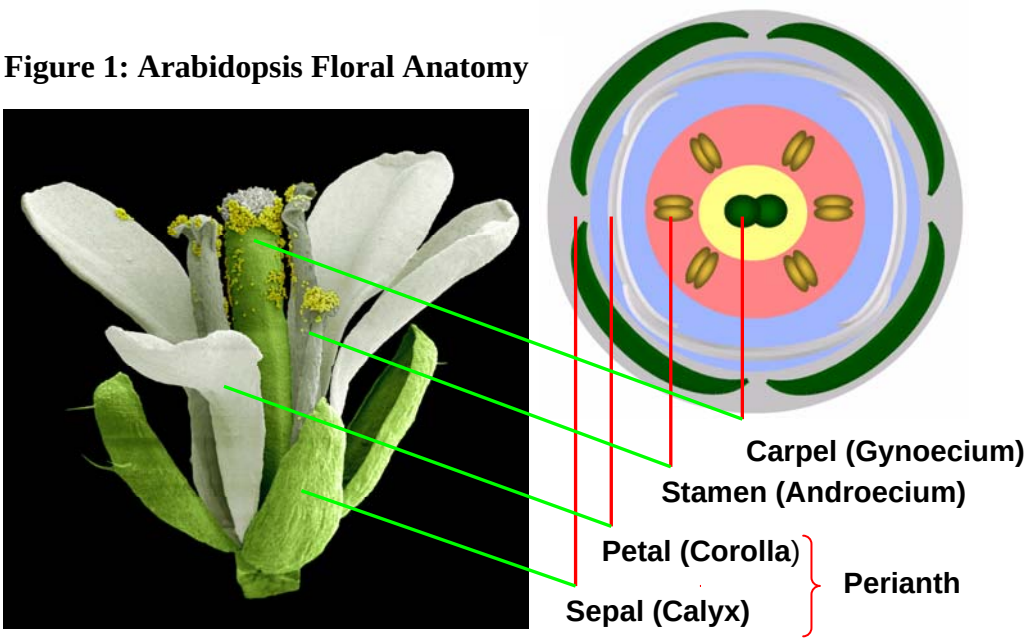
1.1 Floral Anatomy and Homeotic Morphology

The central role held by flowers in plant reproduction, as well as their economic significance in agriculture and horticulture has led to intensive study of floral development. Like many other angiosperms, the floral architecture of *Arabidopsis* consists of four organs. Each of these organs is confined to one of the four concentric whorls which compose the flower. In *Arabidopsis*, the outermost whorl consists of four sepals which define the calyx. In conjunction with these sepals, the four petals of the corolla constitute the vegetative perianth. Housed inward of the perianth, are the reproductive whorls of the stamen-bearing androecium and central gynoecium. The gynoecium of the fourth whorl is a compound pistil composed of two fused carpel which may be further dissected into the stigma, style and ovary. Despite much previous characterization, further investigation into the molecular causality for such anatomical complexity remains a fundamental goal of plant biology.

Presently, the majority of genes implicated in floral development have been identified through characterization of mutants displaying severe phenotypic deviations from wild type development. An interesting subset of these mutants is the homeotic floral phenotypes. In these mutants, the

organs of a single whorl of the inflorescence are duplicated within another distinct whorl at the expense of the organs typically present. Many of these phenotypes were first reported by Johann Wolfgang von Goethe in "The Metamorphosis of Plants" in 1789 [3]. However, the molecular basis of Goethe's prescience was not elucidated until 1991 with the formulation of the "ABC model" of floral development for *Arabidopsis* and *Antirrhinium* [4, 5].

Figure 1: Arabidopsis Floral Anatomy



1.2 Molecular Causality of Morphological Prescience

1.2.1 ABC Model of Genetic Association

Based on the cloning of homeotic mutants, the ABC model identifies three genetic interactions among MADS-box transcription factors which govern organ patterning within the inflorescence. In *Arabidopsis*, the A function responsible for sepal and petal development was found to necessitate the presence of *APETALA 1* within the first and second whorls. A loss of *apetala 1* function, results in a loss of whorl identity, and induces the formation of apetalous secondary inflorescences (Figure 2B) [6]. Epistatic to the A

function genes, the C function gene *AGAMOUS* directs the establishment of the carpel within the central whorl. Absence of C function genes results in a complete loss of reproductive organs. The stamens are homeotically replaced by petals, and carpels by sepals (Figure 2D). As in the case of *apetala1, agamous* results in a loss of whorl determinacy, resulting in an alternating pattern of sepals and petals [7]. The B function gene, *APETALA 3,* regulates proper development of the petal and androecium in the second and third whorls respectively. The absence of *APETALA 3* results in the replacement of petal and stamen with sepal and carpelloid organs (Figure 2C). Nonetheless, for proper development, B function genes cannot determine petal formation without the A function genes [4]. Similarly, proper development of the androecium cannot occur without the C function *AGAMOUS* [7].

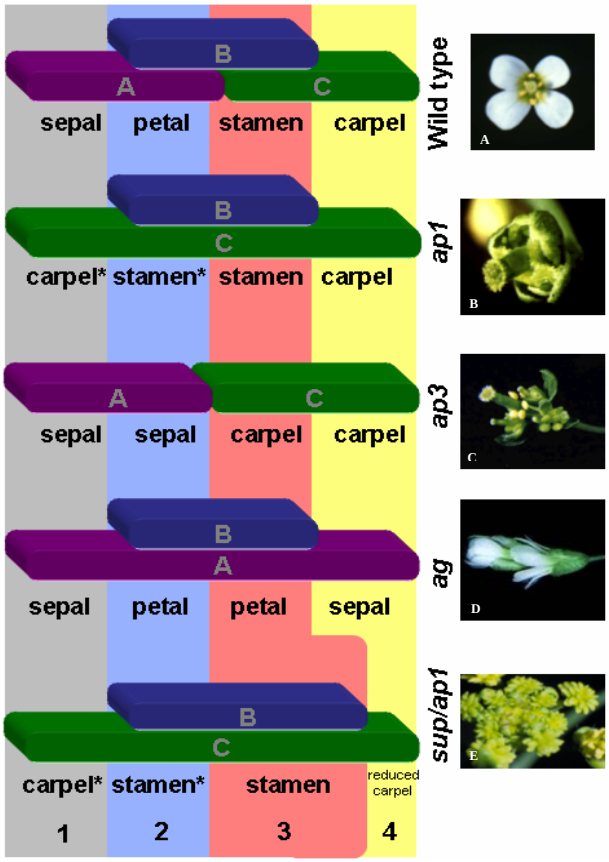
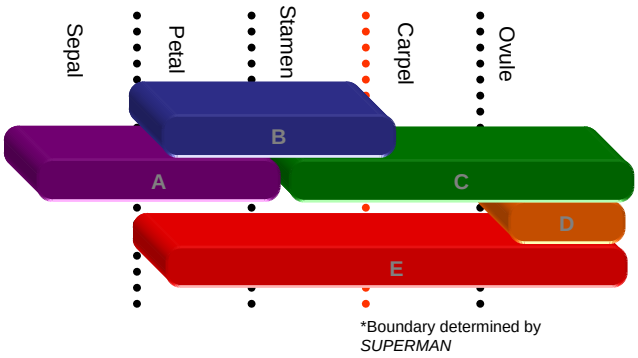


Figure 2. ABC Model and Homeotic Floral Mutants

Idealized illustration of ABC models for each homeotic floral mutant employed within the study. **ap1* and *ap1/superman* do not follow the parameters of the model precisely, secondary apetalous inflorescence develop in the first two whorls. *superman* is not a homeotic mutation; however the third whorl expands at the expense of the fourth. Photographs obtained from *Plant Cell Vol.16, 1314-1326, May 2004.*

More recently, the ABC model has been expanded to include the D function genes *SHATTERPROOF 1, 2,* and *SEEDSTICK*. These genes are expressed within the ovules, and induce proper formation of ovule tissue in the presence of *AG*. This reveals the ovule as a homeotically replaceable organ despite its presence within the gynoecia. Mutations within *Shatterproof 1,2* and *SEEDSTICK* result in the homeotic conversion of ovules to carpelloid tissue [8]. *SEPALATA* 1,2,3,4 genes have been referred to as E function genes and were found to act in conjunction with A-D function genes to further govern the development of petals, stamen, and gynoecia [9].

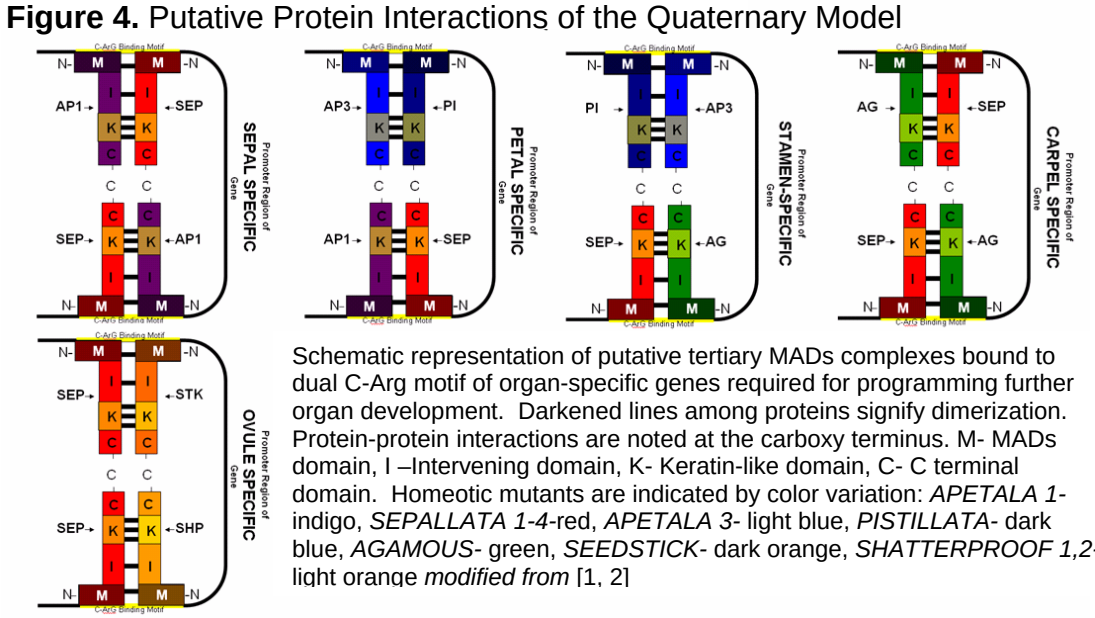
Figure 3. Revised ABC Model
ABC model inclusive of recent addition of ovule as a homeotically replaceable organ



1.2.2 Transcription Factors and Quartet Complexes

Analysis of the genetic associations within the classical ABC model has led to the "quartet model" of protein interaction [2]. In this model, MADS-box proteins interact to form five distinct whorl-specific tetrameric complexes capable of binding DNA and activating downstream genes responsible for organ development through *cis*-regulation at dual C-ArG boxes [2]. *In vitro* analysis has revealed heterodimeric interactions among B and C functions gene products, as well as A and E function proteins [10]. *In vivo* interactions of homologous petunia MADS-box proteins FBP2, FBP11, and FBP24 involved in a putative ovule-defining quaternary complex were also observed [11].

Figure 4. Putative Protein Interactions of the Quaternary Model



Schematic representation of putative tertiary MADs complexes bound to dual C-Arg motif of organ-specific genes required for programming further organ development. Darkened lines among proteins signify dimerization. Protein-protein interactions are noted at the carboxy terminus. M- MADs domain, I –Intervening domain, K- Keratin-like domain, C- C terminal domain. Homeotic mutants are indicated by color variation: *APETALA 1*-indigo, *SEPALLATA 1-4*-red, *APETALA 3*- light blue, *PISTILLATA*- dark blue, *AGAMOUS*- green, *SEEDSTICK*- dark orange, *SHATTERPROOF 1,2*- light orange *modified from* [1, 2]

Despite structural support for the quartet model of protein interaction and its role in floral organ specification, many regulatory aspects of this model have yet to be identified. A number of genes activated by hormonal and abiotic factors have been determined to regulate meristem-identity genes earlier in the developmental pathway. However, few meristem-identity genes have been linked to the regulation of the organ identity genes encoding these quaternary complexes. Similarly, few downstream organ-specific genes directly activated by these complexes have been identified [12]. Moreover, genes characterized as downstream targets of the homeotic mutant gene products such as *FRUITFUL, SPOROCYTELESS/NOZZLE* and *NO APICAL MERISTEM* do not obey the single whorl premise of the quartet model. [13] [14] [15]. Characterization of organ-specific genes downstream of the putative quaternary complexes is necessary to validate the functionality of the complex and characterize the nature of its targets.

1.3 Gene Expression Profiling Technologies and Previous Analyses

Genomic approaches have become an invaluable tool in elucidating the genetic networks of floral development at a global level. Genome-wide spatial analyses of transcript enrichment among Arabidopsis organs has been performed with the aid of hybridization based approaches such as cDNA and oligonucleotide microarrays [16-26] and represent a strong first step in spatial characterization of the floral transcriptome. However, microarray analyses and other hybridization-based approaches are subject to a number of innate limitations, including sensitivity to RNA quantity, non-specific probe hybridization, and substantial background levels capable of masking transcripts with low expression rates [27]. Furthermore, quantitative analysis across multiple microarrays requires standardization and calibration of chips to ensure equivalent hybridization patterns. Previous microarray analysis of organ-specific genes have revealed an enrichment of MADS box family and NAC-like proteins. This warranted further analysis of MADS box and putative AGAMOUS binding motifs. However, no significant enrichment of binding sites for these factors was noted within the putative promoter regions of the previous organ-specific datasets. In addition, it was noted that some overexpressed binding motifs existed; however, these were insignificant [16]. This suggests floral organogenesis is not regulated by a select few *cis*-factors, and instead relies on a number of transcription factors and molecular interactions to orchestrate development and govern the expression of structural genes responsible for organ maturity and maintenance after organ initiation as determined by the quaternary complexes. Additional levels of

regulation have been identified through the characterization of microRNA such as the repression of *Apetala2* by miR172 [28]. MPSS analysis has predicted the expression of over 90 distinct miRNA regulating gene translation within the inflorescence of *Arabidopsis* [29].

1.4 Execution and Validation of Floral Organ-Specific Spatial Expression

In this study, we have implemented the sequence-based approach of massively parallel signature sequencing (MPSS) as an alternative means of spatially dissecting the entire floral transcriptome into those genes expressed specifically within the petal, stamen, gynoecium, stigma/style, and those localized in the sepal/perianth, petal/stamen, or stamen/gynoecium. Using the web-based MPSS expression library analysis interface LIBAN (http://mpss.udel.edu/at/) [30], wildtype inflorescences, homeotic mutants (*apetala 1-10, apetala 3-6, agamous)*, as well as the double mutant *superman/ apetala1-10* and dissected ovules were cross-analyzed for the respective presence/enrichment, or absence/ diminishment of specific floral organs within their inflorescence to develop an organ occurrence profile (OOP) for each organ or group of organs under observation (Figure 5).

Figure 5. Organ Occurrence Profiles (OOPs) of MPSS Expression Libraries

MPSS Libraries					Putative Organ(s) of Expression
wt	*ap1*	*ap3*	*ag*	*sup/ap1*	
1	0	0	1	0	Petal
1	1	0	0	1	Stamen
1	1	1	0	0	Carpel
1	1	1	0	1	Stamen/Carpel
1	1	0	1	1	Petal/Stamen
1	0	1	1	0	Sepal/Petal, Sepal
1	1	1	1	0	Sepal/Carpel, Sepal/Petal/Carpel, Petal/Carpel
1	1	1	1	1	Sepal/Stamen, Petal/Stamen/Carpel, Sepal/Stamen/Carpel, Sepal/Petal/Stamen, All floral organs

Gene expression profiles of these inflorescences were then obtained through MPSS and matched to their respective OOP to demarcate genes with putative organ-specific expression. Root and leaf MPSS expression libraries were also compared to reveal plant-wide specificity. Unlike hybridization-based expression analysis, MPSS relies on the synchronous sequencing of 17bp cDNA fragments and is less constrained by initial RNA quantity thus enabling a lower background level when compared to hybridization based technologies. This permits detection of transcripts with lower expression levels, such as transcription factors [31]. Analysis has revealed a greater number of transcripts are present within the reproductive organs than the vegetative perianth. More specifically, a greater diversity of organ-specific expression was noted in the gynoecium than the androecium. In addition, MPSS determined transcript diversity within the gynoecium was irrespective of the transcripts presence within the ovule.

In situ hybridization, promoter: GUS fusions and RT-PCR have been implemented and further indicate the accuracy of MPSS at assessing spatial expression within the inflorescence. The results of this study and previous microarray analyses represent a significant step toward further detailing the pathways of floral development. In addition, they provide a resource for floral organ-specific reporters, which may be integrated into techniques such as fluorescence-activated cell sorting [32] to enable more detailed profiling of gene expression in future floral studies .

Chapter 2

MATERIALS AND METHODS

2.1 Plant Materials, Tissue Collection, and Nucleic Acid Isolation

All plant materials procured for MPSS analysis as well as that obtained for RT-PCR were from *Arabidopsis thaliana* ecotype *Columbia-0*. Floral inflorescences were harvested from plants grown in pro-mix soil in a growth chamber with 16h of light for 5 weeks at 22^{o}C with 60% humidity. These floral tissues included inflorescence meristems as well as floral buds corresponding to the first 13 stages of development [33]. Leaf and root tissues were obtained from the same plants grown in 16h of light for 21d under sterile conditions in vermiculite and perlite. Ovules were dissected using micro-aspiration as previously described (CITATION). Stigma and petal tissue utilized in RT-PCR analysis were hand-dissected from *agamous* inflorescences. All tissue samples were harvested less than 2h after dark and frozen at -80^{o}C prior to nucleic acid extractions. *A. thaliana* ecotype *Columbia-0* plants utilized for transformation of promoter: GUS fusion plasmids were grown in 16h of light for 5 weeks under the same conditions prior to floral inoculation with *Agrobacterium tumefacierens*.

Floral tissues utilized within the *in situ* hybridization validation were derived from *Arabidopsis thaliana* of the *Landsberg Erecta* ecotype. This was primarily due to the increased size of floral inflorescences as compared to the

Columbia-0 relative. Plants were grown in a growth chamber with light, temperature and humidity conditions similar to those implemented to grow the plants for the creation of the aforementioned MPSS expression libraries.

RNA used to create the cDNA implemented in massively parallel signature sequencing, *in situ* hybridization, and RT-PCR validation was isolated using TRIzol (Invitrogen) reagent as per instructed within the manufacturer's protocol. Genomic DNA isolated for the amplification of promoter sequences within the promoter: GUS biological validation was obtained using the DNeasy Minispin Column Extraction Kit (Qiagen) as per the manufacturer's protocol.

2.2 Signature Sequencing and Genomic Correspondence

MPSS was performed as previously described [31, 34]. Signatures for each floral library were produced in multiple sequencing runs and in two distinct types of sequencing reactions[31, 35]; these sequencing runs and reactions were joined to compute a single normalized abundance for each signature observed in each of the floral, root, and leaf MPSS libraries[35]. All raw and normalized signature data have been made available at http://mpss.udel.edu/at. These signatures were matched to their respective loci within the *A. thaliana* genomic sequence. Briefly, potential MPSS signatures were computationally derived from all possible *DpnII* restriction site (GATC) and 13 adjacent bases within the genome. Potential MPSS signatures located on the sense-strand corresponding to an exon, intron, exon-intron splice boundary, or present within 500bp of the 3' end of an annotated ORF were fitted with the empirically derived MPSS sequences

within each of the floral, leaf, and root sequencing reactions to delineate the expression level of the respective gene or pseudogene.

2.3 MPSS Library Filtration, Cross-Analysis, and Sorting

All MPSS libraries implemented within this study were filtered with a "reliability" filter in order to remove potentially erroneous signatures and distinguish a subset of valid expression levels. This filter eliminates all signatures identified within only a single sequencing run across all current expression libraries. Each tissue utilized within MPSS library corresponds to a minimum of four distinct sequencing runs. Therefore, signatures not identified within any other runs are likely resultant from random MPSS sequencing errors, which have been estimated to occur at a rate of ~ 0.25% per base [35].

Once reliable MPSS expression data was accrued, libraries corresponding to floral tissue were evaluated on the premise of organ presence "1" or absence "0" to demarcate an organ occurrence profile (OOP) among the homeotic floral mutants (refer to fig. 4). By maintaining the order of the four homeotic mutants under study the OOP acts as a bar code to identify organ-specific expression. In order to isolate the subset of genes expressed specifically within a given organ, the normalized signature data corresponding to each gene's expression was matched to each OOP through the use of our publicly available library analysis (LIBAN) interface. The expression of floral organ specific genes within a mutant lacking that specific floral organ(s) logically possesses a normalized transcription level of 0 transcripts per million assayed (TPM). In contrast, those homeotic mutants

that possess the specific floral organ consequently express the transcript at a normalized rate of 1 or greater TPM.

Once a subset of genes bearing organ(s) specific expression was identified they were further sorted by their level of expression within the mutants which most overexpresses the specific organ. Although biasing data for genes of higher expression, this permitted the establishment of a relative degree of confidence in the MPSS prediction of organ-specific expression. For example, putative stamen-specific transcripts were sorted first based on their level of expression within the *superman/ap1* mutant, then by their level of expression within *ap1* (which also overexpresses stamen) and finally by wild type expression level. In addition a “significance” filter may be applied on our online user interface LIBAN to negate those transcripts expressed at less than 4 TPM.

In order to identify the genome-wide correlation of expression across the floral tissues assayed within this study, the Pearson product-moment correlation coefficients were determined. MPSS expression data accrued for each mutant and wild type tissue were plotted in a multivariate correlation plot. In order to demarcate differences in the expression of organ-specific genes in mutant and wildtype MPSS libraries a best linear was identified for each organ-specific subset in a bivariate comparison. The slope of this line was indicative of the relative ratio of expression within the organ-specific genes of both floral tissues.

2.5 GUS:Promoter Fusion and Histochemical Assay

A ~1.5kb promoter fragment upstream of each respective start codon was amplified from genomic DNA of *A. thaliana- Col-0*. Gateway Cloning Technology (Invitrogen) was implemented to insert the amplicon into pDONR221, and ultimately into the binary expression vector pK2GWFS7 wherein the promoter was used to drive the expression of the reporter gene Beta-Glucuronidase. Binary vectors were transformed into *Agrobacterium tumefacieriens* strain *gc101*. A previously described spray method [36] was utilized to develop transgenic *Arabidopsis thaliana*. First generation transformed plants were subjected to histochemical analysis for GUS activity. Transgenic and wild type floral tissues were infiltrated using two vacuum pulses at 7 min each in GUS assay buffer (H_20, 0.1M NaH_2PO_4, 10mM Na_2EDTA,. 0.5M $K_3Fe(CN)_6$, 0.1% Triton X-100 and 0.3% 5-bromo-4chloro-3-indoly$_l$ b-D-glucuronide (X-Gluc) and incubated at 37^{o}C for 12 h. Chlorophyll de-staining was performed with consecutive ethanol washes at 22^{o}C. GUS staining regions were identified and fixed. Photographs were captured using an AxioVision digital camera (Zeiss) and compiled with the GNU Image

Chapter 3

RESULTS

3.1 Elucidation of Expression Data in the Floral Transcriptome

Massively parallel signature sequencing has proven to be a capable technology in the realm of transcriptomics and presents an alternative to overcome a number of innate limitations associated with microarrays and other conventional methods of wide scale gene expression analysis [27, 35]. Briefly, MPSS entails the parallel selective coupling of cDNA restriction fragment amplicons to over a million beads. These beads are then arrayed in a flow cell. With the aid of digestion-hybridization based sequencing, it is possible to identify “signature” tags of 17 to 20 nucleotides in length from each bead. It is then possible to align these tags to their respective loci within the genome, which in most instances results in a single match [37] and in others allows identification of previously uncharacterized transcribed regions of the genome (in progress). The length of MPSS signatures permit highly specific quantification of transcription, with a background level set to remove only those transcripts found in a single sequencing run across all libraries. Having likely developed from sequencing errors, these signatures are unreliable; however, this minute background level is superior to the level of transcript detection permitted by other technologies [27]. Also, the linear normalized

nature of MPSS data acquisition eliminates the necessity of signal standardization between those cDNA libraries under study.

We have excised inflorescence meristems representative of the first 13 stages of development [33]. RNA was extracted from the wild type as well as four floral homeotic mutants *agamous, apetala 1-10, apetala 3-6,* the double mutant *superman/apetala 1-10*, and vacuum-aspirator dissected ovules of *Arabidopsis thaliana* all in a *Col-0* ecotype. Individual MPSS libraries were constructed for all samples and sequenced at Lynx Therapeutics, Inc. (Hayward, CA). We matched resulting signatures to their corresponding sequence in the genome as annotated by TAIR Version 6 and have developed the data into a publicly available web interface (http://mpss.udel.edu/at/) [38] . Analysis of signature locality and the annotated genome enabled the quantification of floral gene expression from individual signatures. In our analysis, MPSS analysis of wild type inflorescence samples identified signature tags representing the expression of a total of 15,769 genes at the instant of RNA extraction. Subtractive analysis of previously determined *Arabidopsis* root, and leaf derived MPSS expression libraries permitted the dissection of 1,763 distinct genes putatively expressed exclusively within wild type inflorescences during the first 13 stages of floral development [33] (see table 1). In accordance with the view of floral organs as modified leaves [39], the correlational coefficient of the wildtype inflorescence gene expression profiles was significantly higher for leaf (r = .73) than root tissue (r = .27).

Despite the diversity of organs present within the wild type flowers as compared to the floral homeotic mutants, the number of distinct transcripts detected within wild type tissue was not as great as that possessed by the A and B function mutants both of which are enriched for reproductive organs relative to the wild type. MPSS analysis of the A function mutant *apetala1-10* revealed a total of 16,463 actively expressed genes. Of these genes, 1,943 were determined to be specifically expressed within the inflorescence at the developmental stages under investigation. This represents the most diverse floral transcriptome surveyed by MPSS. Analysis of the *ap1* gene expression pattern with that of leaf and root expression patterns revealed an even greater similarity with leaf than wildtype inflorescences (r = .79) despite the increase in reproductive organs and loss of vegetative tissue. In addition greater similarity was noted between the root and *ap1* inflorescence than wildtype (r = .31).

The B function mutant *apetala 3-6* was found to express a detectable total of 15,840 distinct genes with a total of 1,454 genes putatively expressed exclusively within the inflorescence. However, despite less diversity in expression when compared to the A function mutant; *ap3*, which overexpresses carpels and sepals, was found to have the greatest correlation with leaf tissue gene expression of any homeotic mutant under study (r = .88). The similarity between gene expression patterns within the root were found to be comparable to *ap1* (r = .30)

In contrast to the A and B function mutants, the C function mutant *agamous* lacks reproductive organs and was found to possess the least diverse whole-

inflorescence transcriptome. Furthermore, dissected ovule tissue revealed more transcriptional diversity than that noted within *agamous*. The expression of 13,644 genes was detected in *agamous*. After subtractive analysis of root and leaf MPSS expression libraries, only 966 genes were found expressed exclusively within the inflorescence of this mutant. Despite an increase in the amount of vegetative tissue at the expense of reproductive organs, *agamous* inflorescence were found to contain the least correlation in gene expression with leaf tissue (r = .66). Root tissue gene expression correlation with *ap2* was equivalent to the other homeotic mutants (r = .31).

Analysis of MPSS signatures corresponding to the double mutant *superman/apetala1* which possesses only stamen and highly reduced gynoecia, has revealed the expression of 14,078 genes. 1,425 genes were determined to be expressed specifically within the inflorescence during the analyzed developmental stages. Despite the lack of the vegetative sepals and petals, a higher correlation of gene expression with leaf tissue (r = .72) than that noted within the leaf and *agamous* correlation, root tissue was similar to other inflorescence transcriptomes under analysis (r = .26).

In addition to the whole-inflorescence MPSS libraries, the expression of 11,651 distinct genes was detected in dissected ovules. Of these transcripts, a total of 983 genes were not detected in leaf or root tissue. The correlational coefficient of gene expression in dissected ovule and leaf tissue (r = .23) was found to be lower than that of root tissue (r = .45). The transcripts detected within the dissected ovules were compared to the *ap1* and *ap3* inflorescences which are enriched for gynoecia. 92% (10,745 genes) and 91% (10,608

genes) of the ovule transcriptome diversity was captured within the *ap1* and *ap3* mutants, respectively. This is greater than the 90% (10,459 genes) captured within the wildtype inflorescence which contain only a single whorl of gynoecia.

Table1. MPSS detected genes across homeotic mutants

Floral Strain or Tissue	Number of Gene with MPSS detected Expression	Genes with inflorescence specific Expression	Average Expression Level (TPM)	Correlation of Gene Expression Profile with Leaves	Correlation of Gene Expression Profile with Roots
ap1	16,463	1,943	48.8	.79	.31
ap3	15,840	1,454	51.5	.88	.30
Wildtype	15,769	1,763	53.0	.73	.27
ap1/sup	14,078	1,425	53.4	.72	.26
ag	13,644	966	53.7	.66	.31
ovule	11,651	983	56.2	.23	.45

TPM- Transcripts per million; Correlations are determined r - values in the Pearson Product-Momentum Correlation

Correspondence of floral phenotype and MPSS gene expression profile was distinguished by intersecting distinct transcripts detected among the floral homeotic mutants and wild type tissue (table 2). The *superman/ap1* mutant possesses the same mutation as *apetala1*, with the additional non-homeotic *superman* mutation. To determine the effect of the *superman* mutation on *apetala1* the transcriptomes of both *superman/ap1* and *apetala1* were compared and found to contain the second highest coefficient of correlation (*r* =.88). The mutant with gene expression patterns most closely resembling *apetala1* was found to be *apetala3* (*r* = .91). This correlation was the highest of any two floral inflorescences under analysis. The greatest number of conserved distinct transcripts between any two mutants was also found in these mutants (14,431 distinct transcripts). Both *ap1* and *ap3* express reproductive organs (albeit different reproductive organs) in multiple whorls

and are devoid of petals. Homeotic mutants showing the least number of conserved distinct transcripts were among the stamen-enriched *sup/ap1* and vegetative *agamous* (11,778 distinct genes). However, the least correlation of gene expression was noted between *ap1* which is enriched for both gynoecia and stamen at the expense of the perianth, and *agamous* which possesses no reproductive organs (r = .75).

Figure 6. Transcriptional Diversity Across Homeotic Mutants

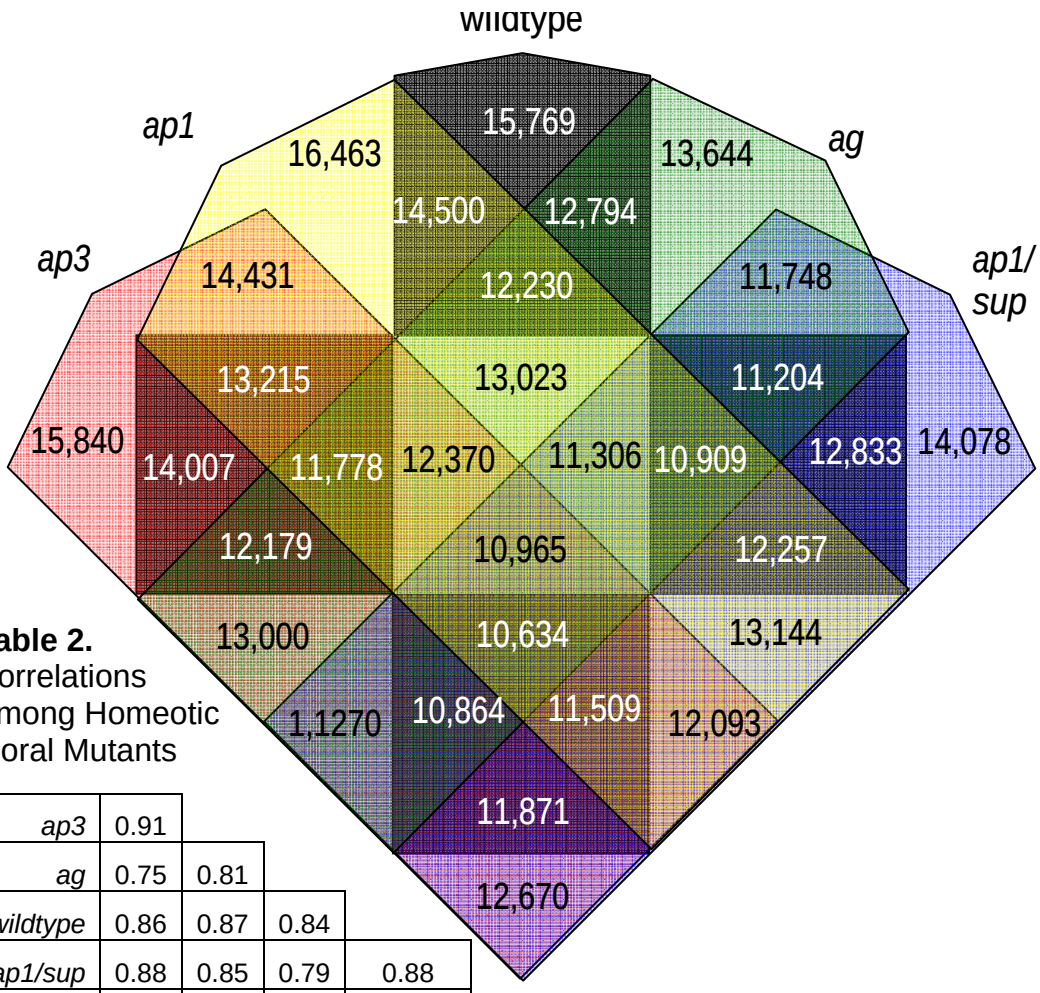


Table 2. Correlations Among Homeotic Floral Mutants

ap3	0.91			
ag	0.75	0.81		
wildtype	0.86	0.87	0.84	
ap1/sup	0.88	0.85	0.79	0.88
	ap1	*ap3*	*ag*	*wildtype*

Figure 6 represent those transcripts shared across surveyed floral mutants revealing similarities in phenotype and gene expression profile. Table 2 reveals the r-value determined by the Pearson Product Momentum Correlation by comparing the expression levels across the homeotic mutants and wildtype tissue.

3.2 Mutant Profile Based Predictions of Organ Localized Expression

In an attempt to characterize the localization of those transcripts exclusively expressed within specific floral organs within the inflorescence a binary system was implemented. First, all possible patterns of organ expression within the four floral whorls were identified among the gynoecia, stamen, petal, and sepals. For example, the occurrence of exclusively gynoecia provides a potential expression pattern, as does the development of only stamen; however, categorizing the presence of both gynoecias and stamen or gynoecia-stamen-petal allows the formation of 16 patterns of possible organ expression. Next, the occurrence of a specific organ or group of organs within a single floral strain was characterized as a “1” if present and a “0” if absent. By maintaining the order of the floral mutants under observation, a four digit binary code was developed to represent the organ occurrence profile (OOP) among the four homeotic libraries under study (Figure 5). For instance, surveying the occurrence of stamen across all floral MPSS libraries would follow an OOP of “11001”, wherein stamen are expressed in wildtype as well as the mutant *ap1* (1), absent in both *ap3* (0), and *ag* (0), and expressed in the whorls of *sup/ap1*(1). However, the ability to demarcate all 16 possible patterns of organ expression is limited by the types of homeotic mutant libraries employed within the study. Therefore, the presence of a specific organ or multiple organs can only be recognized if their respective OOPs are unique. For example, sepal specific transcripts cannot be isolated from sepal and petal specific transcripts; because they both brandish the same OOP, “10110”. Consequently, with the MPSS libraries

currently available, the presence of organ(s) specific transcripts within the inflorescence may only be fully dissected on seven occasions: sepal-perianth, petal, stamen, petal-stamen, stamen-gynoecium and gynoecium both inclusive and exclusive of the ovule.

Although unable to detect sepal specific transcripts due to the nature of the ABC model and those homeotic mutant employed within this study, genes whose expression was localized in the perianth or strictly within the sepal were determined to have an OOP of “10110.” Cross analysis of the mutant libraries revealed a total of 177 genes expressed within these criteria. A total of 79% (139 genes) were also found present in leaf and root libraries. Corresponding to the enrichment of floral organs within the homeotic mutants, an average expression fold increase in sepal and perianth localized genes was noted in *ap3* (x1.3 fold) and *agamous* (x1.1 fold) when compared to wildtype inflorescence. Unlike previous microarray analysis, the nature of MPSS data acquisition is non-relative and linear in nature based on a threshold of MPSS detectable transcription. Using the promoter motif analysis program developed by the University of Leeds, a significant overexpression ($p = .021$) of the SV40 core promoter motif was noted among sepal and perianth expressed genes. Three distinct SV40 promoter elements containing the SV40 core motif have been previously ascribed to cell-specific expression within mammalian cell lines [40].

Housed inward of the sepal, petal specific expression was demarcated with an OOP of “10010”. Expressed in only the C function mutant *ag* and absent from all other MPSS homeotic libraries, a total of 93 genes were identified as

petal specific within the inflorescence. In agreement with the increase in petals in *agamous* relative to wildtype, an average 2 fold level of enrichment was found in predicted petal specific transcripts in *agamous* relative to wildtype. 51% (47 genes) of petal specific genes were found expressed in root and leaf tissue. Concurring with previous studies, no significant enrichment of any binding motifs was noted within the petal specific dataset.

Those transcripts found in both the stamen and petals wield an OOP of "11011". Signatures were only absent from one mutant, *ap3*, and are therefore less statistically reliable. The number of genes identified pertaining to reproductive organ localized expression was found to be 271 genes, of which 79% (213 genes) are also predicted to be expressed in the leaf and root tissue. A 1.6 and 1.4 fold increase in average gene expression level among stamen/petal specific transcripts in *sup/ap1* and *ap1* was noted when compared to wild type. However, a four fold decrease in average expression in stamen and petal localized transcripts was noted in stamen-less *agamous* despite the increase in petalloid organs. Analysis of the promoter regions of those genes putatively expressed within the stamen and petals revealed an over-expression of the MYB3 binding site motif (p= .0043). MYB3 has been implicated in various disease responses, formation of secondary metabolites, cell shape and its expression has been correlated with auxin, ethylene and many other growth regulators [41].

Putative stamen-specific transcripts were determined to follow an OOP of "11001". Whereby, MPSS signatures were absent from B and C function mutants *ap3* and *ag*, and present within the A function *ap1* and *sup/ap1*.

Signatures which met these criteria were found to illustrate the expression of 475 genes within the inflorescence libraries. Putative stamen specific transcripts were expressed an average of 1.8 fold higher in *superman/* in relation to wildtype inflorescence. The average expression level of stamen specific transcripts within *ap1* was found to be 1.5 fold that identified in wildtype inflorescences. With the added filtering of leaf and root detected transcripts 46% (219 genes) were revealed to be expressed within other regions of the plant. A significant overexpression of MYB2 binding site motif (p=.0036) was noted among the stamen specific transcripts. Expression of the MYB2 transcription factor has been largely implicated in abscisic acid signalling [42].

Those transcripts found throughout both reproductive organs but absent from the perianth wield an OOP of “11101”. Due the absence of signatures from only one mutant, *ag,* the results are less statistically reliable. The number of genes identified pertaining to reproductive organ localized expression was found to be 1,009 genes, of which 88% (886 genes) are also predicted to be expressed in the leaf and root tissue. Although, *ap1* is enriched for both reproductive floral organs, the largest increase in average reproductive organ specific gene expression within the mutants under study was found within *ap3* (1.9 fold higher than wildtype). The homeotic mutant *ap1* revealed an expression fold increase of only 1.3 fold higher than wildtype. No significant overexpression of transcripts was denoted within *superman/apetala1* despite an overall increase in the number of stamen within this mutant.

In order to dissect those genes expressed exclusively within the gynoecia an OOP of “11100” was identified (Figure 5). Therefore, identification of gynoecia specific transcripts was based on absence in the sterile C function homeotic floral mutant *ag* as well as the *sup/ap*1 double mutant which only weakly expresses gynoecious tissue in a diminished central whorl. By intersecting the signatures present within all the inflorescence MPSS libraries based on the OOP criteria of expected expression a total of 506 genes were identified with patterns suggestive of gynoecium specificity within the inflorescence. In order to further distinguish the data set, an additional MPSS library corresponding to dissected ovules was analyzed. The average expression level of ovule specific expression was increased two fold in dissected ovules when compared whole wildtype inflorescences, whereas expression was only 1.5 fold higher in dissected ovules than *ap3* which possesses multiple whorls of gynoecia-like tissue. Those gynoecium-specific transcripts expressed within the ovule represented 45% (230 genes) with the remaining genes expressed solely within the stigma and style. Ovule-specific expression could not be fully dissected with the available MPSS libraries. 80% (220 genes) of the genes thought to be expressed exclusively within the gynoecium of the inflorescence are putatively expressed within the roots and leaves of the plant as well and only organ-specific within the inflorescence. The relative level of MPSS predicted gynoecium specific gene expression within dissected ovules was found to be two fold higher than that noted within the wildtype inflorescences.

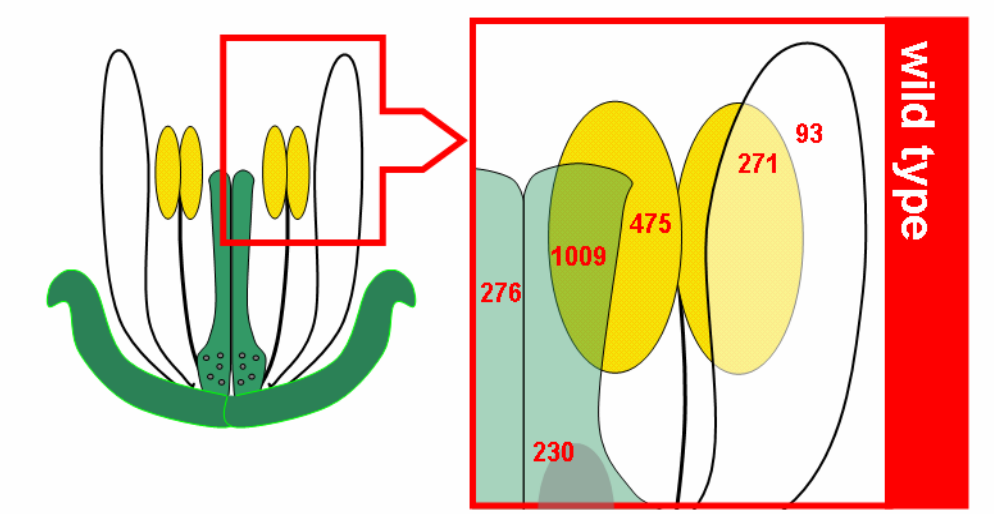


Figure 7. Putative Organ-Specific Gene Expression
The number of distinct genes putatively expressed within specific floral organs as determined by MPSS filtration of homeotic mutants. Figure does not display genes of the sepal/perianth number present within the sepal and the number present throughout the perianth could not be fully dissected. Similarly Ovule specific expression could not be dissected from gynoecia expression; however, those transcripts expressed in the stigma/style but not within the ovule could be demarcated.

To further elucidate floral organ specific expression, the spatial data accrued from this study was superimposed with temporal data identified in GENEVESTIGATORS online microarray database [26]. The majority of genes with MPSS determined floral organ-specific expression were detected in later stages of floral development after 10% of the flower buds have opened (>36 days after planting). Further analysis of previous microarray temporal assays [24] were overlaid on MPSS spatial analysis; however, little commonality was notable among the data sets.

3.3 Validation of Organs expressed transcripts

3.3.1 MPSS Acquiescence with Previous Expression Analyses

MPSS data sets have revealed significant overlap with genes acknowledged in previous genome-wide analyses as well as expression with known genes, especially in the identification of stamen-specific transcripts.

A total of 215 genes were identified as stamen specific within this study as well as a previous study which utilized a whole genome microarray to cross-analyze floral homeotic mutants in a similar manner [23]. Furthermore, a later study implemented additional whole genome microarray analysis on RNA extracted from isolated floral organs [21]. Merging both previously published microarray datasets with MPSS data has revealed a total of 74 putative stamen specific genes with expression profiles concurring in all three data sets. Of the 74 putative stamen specific genes, 19 have unknown functions. Further cross comparison of a previous microarray analysis from pollen [43] reveals a total of 17 genes expressed specifically within the pollen which are also in accordance with MPSS and aforementioned microarray studies.

Table 3. Putative Stamen-Specific Transcripts

Accession Number	Description	*agamous*	*ap1*	*ap3*	*wild type*	*ap1/sup*
At4g35010	glycosyl hydrolase family 35 protein	0	147	0	122	93
At1g55570	multi-copper oxidase type I family protein	0	146	0	273	59
At4g02250	invertase/pectin methylesterase inhibitor family	0	36	0	33	52
At1g55560	multi-copper oxidase type I family protein	0	34	0	71	41
At2g45800	LIM domain-containing protein	0	8	0	1	26
At2g46860	Pyrophosphatase (soluble) / PPase, putative	0	1	0	11	24
At4g25780	pathogenesis-related protein, putative	0	2	0	8	18
At2g19770	profilin 4 (PRO4) (PFN4)	0	41	0	39	17
At2g07040	leucine-rich repeat transmembrane kinase,	0	7	0	10	14
At4g33970	leucine-rich repeat family protein / extensin	0	6	0	8	10
At2g18470	protein kinase family protein	0	2	0	4	8
At2g13570	CCAAT-box binding transcription factor,	0	2	0	8	7
At4g13240	hypothetical protein	0	1	0	3	3
At2g02140	plant defensin-fusion protein, putative (PDF2.6)	0	7	0	3	2
At4g07960	glycosyl transferase family 2 protein	0	19	0	23	2
At1g15350	expressed protein	0	5	0	10	1
At4g39670	expressed protein	0	1	0	7	1

Transcripts were identified as stamen-specific within MPSS analyses, and microarray analyses[16, 22], as well as pollen-specific in an additional microarray analysis[43]. Numbers indicative of Transcripts Per Million.

The union of these distinct approaches to identifying organ specific genes overcomes technical biases associated with both microarray analysis and MPSS, and permits the removal of non-specific transcripts. In addition to those genes previously characterized through microarray analysis, we have also confirmed the expression of several known stamen expressed genes including *CALLOSE SYNTHASE5, PROFILIN5*, *ABORTED MICROSPORE, MALE STERILITY 2, ARABIDOPSIS TAPETUM 1, 27* and numerous members of the glycine-rich protein family which is known to possess high expression within the androecium [43].

SPOROCYTELESS/NOZZLE transcripts were identified as stamen-specific although their expression has been characterized in both the stamen and ovules. Previous analyses reveal a much greater level of expression in the anthers than ovule. Initial *in situ* hybridizations performed in previous analyses did not enable visualization of expression within the ovule. Ovule expression was only identified through an *SPL* promoter: GUS fusion and was not found in the integuments of funiculus [44]. Similarly, the homeodomain transcription factor WUSCHEL was identified as stamen specific despite well characterized expression within the ovule. This concurs with previous microarray expression data and *in situ* hybridization [23] which revealed a large expression domain within the locules of the anthers that was ascribed to the masking of the ovule localized transcripts in previous microarray analyses [23].

Analysis of MPSS determined gynoecium specific expression also corroborated with previous studies. 19 of the non-ovule contained gynoecium

specific genes were suggested to have gynoecia or pistil specific expression in two previous whole genome microarray screens [21, 23]. The expression of three EMBRYO DEFFECTIVE genes, a gene encoding, *RIE1;* a ring H2 zinc finger protein essential for seed development [45], and the kinase partner protein-like ATROPGEF6 responsible for pollen tube development [46] have been identified within our predicted non-ovule gynoecium specific dataset. The kinase *TOUSLED* has been shown in previous studies to be required in leaf and floral development and is localized to the style by stage 13 of development [33]. As expected, expression of the respective gene was identified within the gynoecia exclusive of the ovule. Additional gynoecium analyses inclusive of the ovule tissue are confirmed for 15 distinct transcripts in aforementioned microarray studies. Moreover, the well-characterized transcription factor *SHATTERPROOF 2,* which is carpel specific after floral buds have opened [47], as well as several additional *EMBRYO DEFECTIVE* genes, were identified in the gynoecium specific data set inclusive of the ovule.

Among the set of sepal/perianth localized transcripts, 8 sepal specific specific genes were noted previously in a microarray analysis of RNA isolated from specific organs [22]. Furthermore, expression of Arabidopsis Receptor Kinase, *ARK2*, has been noted within vegetative above-ground tissues, specifically cotyledons, leaves, and sepals and correlated with maturation of these structures [48]. This gene was correctly assigned in the sepal/sepal-perianth specific MPSS dataset. Furthermore, expression of the gene was identified in the MPSS leaf but not root libraries as expected. The alternative

oxidase isoform AOX1A, although expressed in multiple organs of the inflorescence is most strongly expressed in the sepals [49]. For this reason, our analysis has placed this gene in the sepal/sepal-perianth dataset; thus suggesting MPSS analysis is still subject to a threshold of transcription quantity and libraries may possess genes enriched rather than localized within a floral organ.

Analysis of the petal-specific MPSS derived dataset reveals concurrence of 7 genes within the microarray analysis of RNA isolated from petals in a previous study [22]. Expression of genes encoding the circadian rhythm dependant protein kinase WNK4 [50] was identified in this study and previous microarray analysis [22] to possess petal specific expression. Genevestigators [26], and AtGenExpress [18] revealed strong expression of WNK4 within the perianth as opposed to reproductive tissue as well.

Genes containing MPSS derived petal and stamen expression were also noted within previous microarray analyses. However, MPSS analysis suggests a number of genes with previously determined petal or stamen specific expression are expressed within both petals and stamen. Interestingly, genes such as At4g37800, a putative xyloglucosyl transferase, have dissenting predictions regarding microarray expression. Previous studies have suggested their presence within the petals whereas others have suggested presence within the stamen [16, 22]. Similarly, those genes predicted by MPSS to be located within both the stamen and the gynoecia have been placed into organ-specific groups within previous studies as well [16, 22].

3.3.2 *In situ* Hybridization

In addition to validating MPSS methodology with previously assessed genes and microarray analyses, the previously uncharacterized expression of putative organ-specific genes was biologically validated through *in situ hybridization*. We have selected genes whose MPSS expression profiles matched the expected profile for gynoecia/stamen and petal/stamen localization as well as stamen specificity, and gynoecia specificity (Figure 8). Expression of the galactosyltransferase family protein, At1g33430, was correctly assigned by MPSS with expression in the stamen and carpel primordia and strong expression specifically within the tapetum and microspores [Figure 8A-C (anti-sense) D, E (sense)]. Our filtering system of MPSS data predicted At1g72290, a gene encoding a trypsin and protease inhibitor family protein, to be expressed in both the gynoecia and stamen. However, *in situ* hybridization has only revealed expression within the carpel [Figure 8M (anti-sense)] and no expression was noted within the stamen. Nonetheless, it must be noted that despite conformation of expression profile to our binary filtering procedure, the double mutant *sup/ap1* which is highly enriched for stamen tissue has less than half the number of At1g72290 transcripts as wild type tissue. In contrast, the number of transcript within *ap3* which is enriched for carpel tissue is over nine fold higher than wild type tissue. Similarly, a gene predicted by MPSS to be localized within the petal-stamen is At2g19070 and encodes an anthranilate N-hydroxycinnamoyl/ benzoyltransferase protein. *In situ* hybridization patterns revealed presence within only the tapetum.

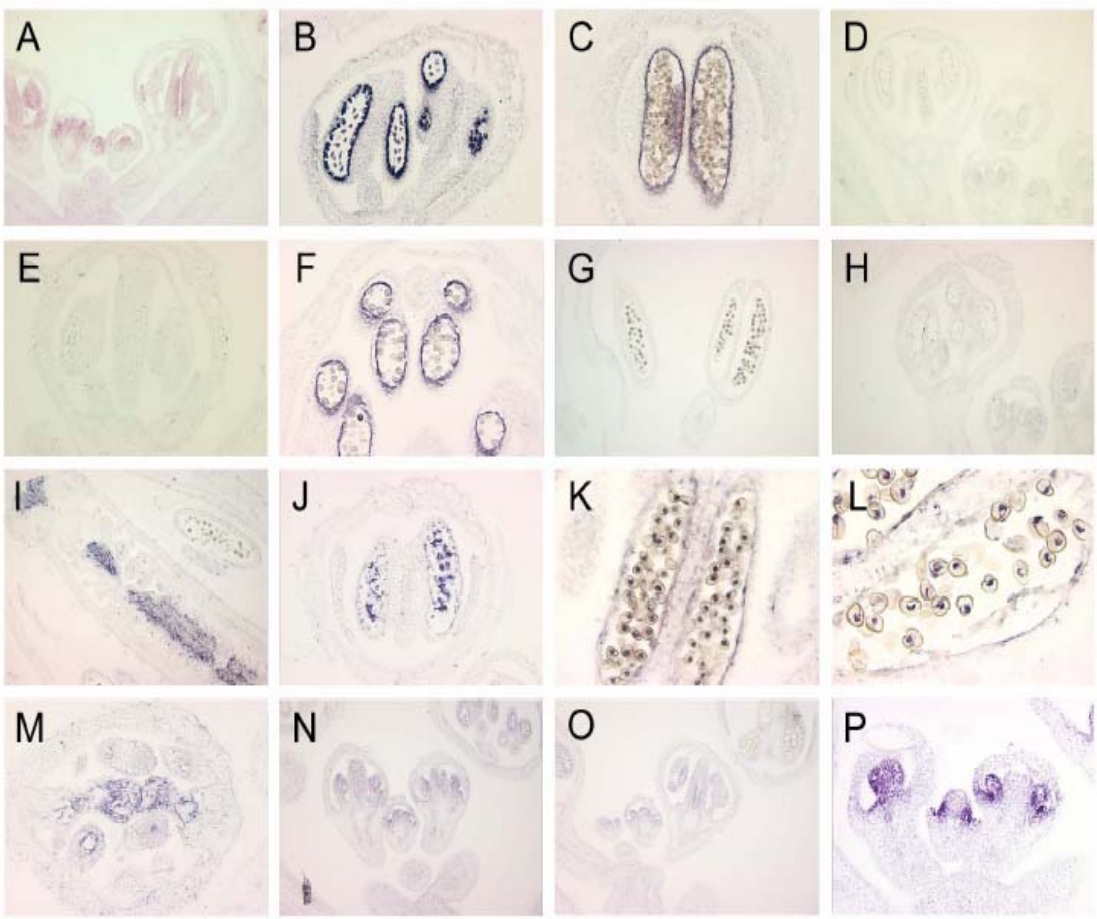


Figure 8. Biological Validation via *in situ* hybridization

A-C At1g33430 (anti-sense probe), D-E (sense control probe) noted signal on stamen and carpel primordial with strong expression detected in the tapetum and microspore as predicted by MPSS.

F-G At2g19070 (anti-sense probe), H (sense control probe) signal identified on the tapetum. Stamen and petal localized expression predicted by MPSS

I-J At1g54860 (anti-sense probe) signal on stigmatic papillae as well as septum and developing microspore. Predicted to be stamen specific by MPSS analysis.

K-L At5g59810 (anti-sense probe) transient signal noted on a specific microspore of the tetrad. Carpel specificity predicted by MPSS.

M At1g72290 (anti-sense probe) signal identified on the septum. Predicted to be localized within the stamen and the carpel by MPSS

N At2g42940 (anti-sense probe) O (sense control probe) weak signal on stamen and carpel primordial. Identified as a putative stamen specific transcript by MPSS.

P AP3 (anti-sense control probe)

However, additional analysis revealed the expression of only 1 transcript within the *agamous* mutant. This sole transcript led our filtering procedure to predict petal and stamen localized expression. This suggests that expression in the petal may be below the level required for an observable signal in *in situ* hybridization [Figure 8F-G (anti-sense) H (sense)]. The expression of an unknown expressed protein, At1g54860 was characterized as stamen specific. *In situ* hybridization revealed expression within the microspore as well as the stigmatic papillae and septum [Figure 8I-J]. Another putative stamen specific gene, At2g42940, encoding a DNA binding family protein was found expressed in both carpel and stamen primordial [Figure 8N (anti-sense) O (sense)]. An additional discrepancy between MPSS and *in situ* data was noted in the putative carpel specific At5g59810. This gene encodes a subtilase family protein and was identified strictly within the stamen in a single microspore of the tetrad by *in situ* hybridization [Figure 8K-L]. Nonetheless, previous publications have revealed stigma-specific expression of At5g59810 via microarray, RNA -gel blot, and *in situ* analysis [51].

3.3.3 GUS histochemical assay

Another means of assessing the accuracy of MPSS predicted floral organ localization was the analysis of transgenic GUS reporter lines. A 1.5 kilo base pair region flanking the 5' end of organ-specific genes was used to drive the expression of the GUS reporter gene. Constructs were then stained with a phosphate buffer to determine spatial expression of the MPSS predicted gene. The majority of organ-specific expression predicted by MPSS was mirrored within the GUS staining activity of the 14 genes under

observation. Putative stamen-specific expression was evaluated by promoter fusion in three genes. At1g20130, a putative family II extracellular lipase [52], At2g42940 encoding a DNA binding protein, and At3g27025, encoding a protein of unknown function were all found to be expressed within the stamen as predicted. However, in the case of At2g42940, *in situ* analysis revealed additional expression in the carpel primordia. Two genes with putative gynoecia specific expression were assayed as well. After staining, the promoter: GUS fusions for At1g07370, a proliferating cellular nuclear antigen, and At1g27900, a putative RNA helicase were both found to stain within the gynoecia as predicted. In addition to whole gynoecium promoter:GUS fusions, ovule expression was identified in five genes with MPSS predicted ovule localization. INSERT JEAN PHILLIPE DATA WHEN ACCESSIONS IDENTIFIED At1g33430, a gene encoding a galactosyltransferase family protein was predicted to be expressed throughout the reproductive organs. This result was further substantiated through the histochemical assay, with staining occurring in both the stamen and gynoecia. Two additional gene with MPSS predicted stamen and petal localized expression were also assessed. Confirming previous results of *in situ* hybridization, At2g19070, was found to be expressed only in the stamen. In contrast, expression of At5g07550, a gene encoding a glycine-rich oleosin protein (GRP19) found to regulate the size and character of lipid droplets within the pollen coat [53] was demonstrated within both the stamen and the petals as predicted. Existence within the petal has not previously been documented in the literature and may indicate activity within the petal. At1g26270, a gene encoding a

phosphatidylinositol 3- and 4-kinase family protein which was present in all floral MPSS libraries was implemented as an additional validation of MPSS data. In these promoter:GUS fusions staining was prevalent throughout all organs of the inflorescence. Similarly, the putative RNA helicase, At2g35340, was assayed and confirmed MPSS prediction of no expression within the inflorescence tissues as expected by MPSS analysis despite previous microarray characterizations within floral tissues [26].

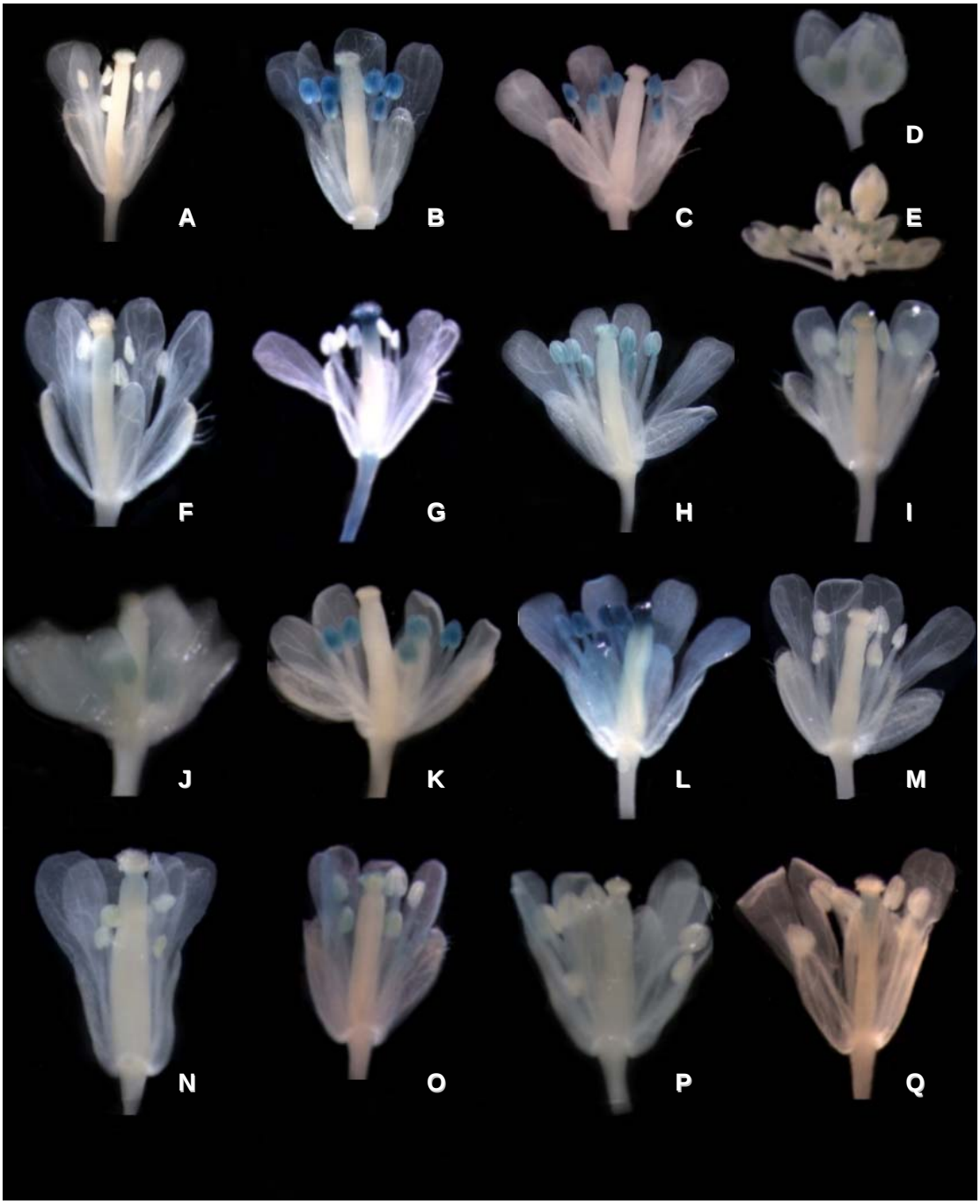


Figure 9. Biological Validation by Promoter: GUS fusions

(A) Wild type Control
(B) to **(E)** MPSS-Predicted Stamen-Specific Promoter:*GUS.*
At1g20130 **[B]**, At3g27025 **[C]**, At2g42940 (**[D]** and **[E]**)
(F) to **(G)** MPSS-Predicted Carpel-Specific Promoter:*GUS.*
At1g07370 **[F]**, At1g27900 **[G]**
(H) MPSS-Predicted Stamen/Carpel-Specific Promoter:*GUS*.
At1g33430 **[H]**
(I) to **(J)** MPSS-Predicted Stamen/Petal-Specific Promoter:*GUS*.
At5g07550 **[I]**

3.3.4 RT-PCR

Validation of MPSS organ specific filters was performed in five putative gynoecia expressed genes by real time PCR (figure 11). Expression patterns were found to correlate highly with those predicted by MPSS. At1g04620, a gene encoding ATG8b, a microtubule associated protein involved in autophagy was found to wield expression within the ovule as predicted by MPSS, without any expression present within sepal or petal organs. This isoform of ATG8 has been previously characterized by microarray studies [54] which reveal the highest level of floral expression within petal tissue, followed by stamen, with lesser amounts in petal and style tissue; however, MPSS analysis reveals gynoecia specific expression inclusive of the ovule. MPSS predictions of gynoecia specificity have been confirmed by RT-PCR. No expression was deduced in petal or sepal tissues. Ovule expression was also noted in At4g27860, a gene encoding an integral membrane family protein of unknown function and At1g19240, a gene of unknown function. At4g14420, a gene with homology to a hypersensitive response lesion inducer in *Nicotiana tabacum* was also found to possess ovule expression by RT-

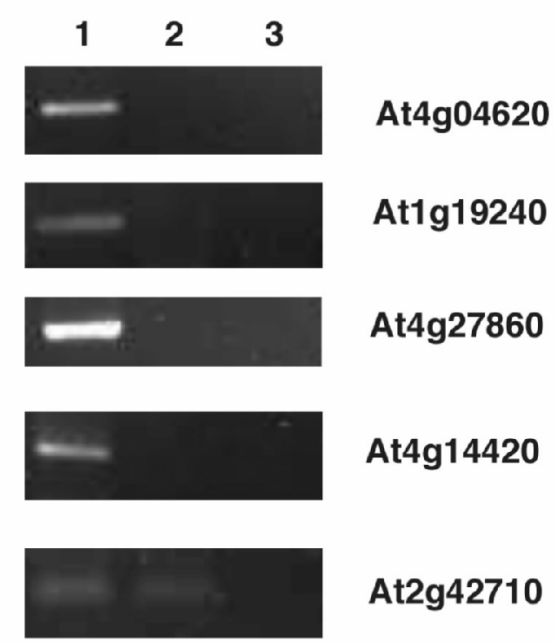


Figure 11. RT-PCR
Validation of Gynoecium Specific Expression inclusive of the ovule. RNA extracted from (1) Mature Ovule; (2) Petal; (3) Sepal

PCR. No sepal or petal expressed transcripts were noted within these RT-PCR analyses. In contrast to MPSS predictions, RT-PCR revealed the expression of At2g42710, a putative structural constituent of the large ribosomal subunit in both ovule and petal tissues. This suggests the presence of some false positives within the dataset. However, comparison with additional technologies will enable further filtration of these transcripts and provide even more robust data.

Chapter 4

DISCUSSION

4.1 Organ-Specificity Across Multiple Profiling Platforms

Previous genome-wide studies have predicted the enrichment of transcripts within a specific floral organ based upon their expression level in microarray analyses. We have implemented another method to complement prior investigations. Numerous genes with known expression patterns have been accurately assigned through our transcript filtering procedure. In addition, genes characterized in previous microarray analyses have been further validated through MPSS. The magnitude of data assessed within these analyses prohibits confirmation via independent means. Nonetheless, integration of multiple whole-genome techniques such as MPSS, oligonucleotide microarrays and cDNA microarrays alleviates the technical biases associated with each platform of transcriptome analysis and permits the removal of non-organ specific transcripts which were erroneously predicted by any single filtering process. The results of this study provide a list of candidate genes which may be incorporated into future studies in functional genomics such as overexpression and knockout studies as well as identification of candidate genes responsible for quantitative trait loci. Expression information accrued through this and previous studies will continue to increase the ease and effectiveness of reverse genetics and

further the viability of selecting candidate genes from forward genetic approaches, ultimately enabling the elucidation of more complex networks of floral development and maintenance. Furthermore, the validation of GUS: promoter fusions have revealed promoter regions 1.5 kb upstream of most surveyed genes were capable of governing floral organ-specific expression.

Microarray analysis and other hybridization-based techniques of surveying genome-wide expression are subject to a number of inherent limitations, including non-specific probe hybridization, sensitivity to initial RNA quantity, and substantial background levels [27]. In addition, quantitative analysis across multiple microarrays requires standardization and calibration of chips to ensure equivalent hybridization patterns. Nonetheless, the widespread use of this technology as well as its replicability further justifies its application in gene expression profiling.

MPSS contains its own innate limitations. Previously described “bad words” in sequencing reactions, [26] as well as the absence of the necessary restriction sites within cDNA, and cost prohibitive replicability partially limit this technology. MPSS is not as limited by initial RNA quantity and is able to detect expression with a much smaller background level than microarrays; however, analysis across the homeotic mutants has suggested MPSS is still only a sampling of the RNA present within a specific tissue.

4.2 Assessment of Floral Organ-Specific Expression Subsets

The greatest number of distinct floral organ specific transcripts were identified as expressed throughout the reproductive organs (1,009 genes). This was nearly twice as many as that noted as specific to the gynoecia alone

(511 genes). The myriad of genes expressed within the reproductive organs may be less statistically significant due to their absence in only a single floral mutant *agamous.* This initially implies the magnitude of reproductive specific expression is artifactual due to a decrease in filtering stringency. However, a similar situation arose in the identification of petal-stamen expressed transcripts which are putatively absent from only *apetala3*. A total of only 271 transcripts were identified within these screening criteria of petal-stamen localized expression. In contrast a greater number of distinct stamen specific transcripts (475 genes) were identified with strictly stamen specific expression, despite the increased filtering criteria provided by stamen absent in both *agamous* and *apetala3*. This paradigm repudiates views of excessive bias by transcript filtering stringency, and further substantiates the scale of genes expressed throughout the reproductive organs and not within the perianth.

Aside from those transcripts denoted throughout both reproductive structures, the greatest single organ-specific expression was observed within the gynoecia (511 genes). This contrasts previous microarray analyses which report over four fold less organ-specific diversity within the gynoecia as compared to the stamen[16]. The number of stamen specific transcripts within this study represents the second most diverse single organ (475 genes). However, the relative increase in distinct gynoecia specific transcripts within this study relative to microarray may be ascribed to the difference in filtering. Previous microarray analyses were initially based on significant downregulation of transcripts within a single mutant *agamous*. This led to an

exceedingly large number of transcripts which were further filtered on the assumption of large expression ratios between *agamous* and gynoecium deficient mutants [16]. Implementation of these secondary filtering criteria was unnecessary within our analyses due to the incorporation of another gynoecia deficient mutant *superman/apetala1* as well as further validation through dissected ovule tissue. Moreover, this analysis enabled the dissection of those transcripts found expressed within the ovule (230 genes) and gynoecium and those within the stigma and style but absent from the ovule (281 genes). Interestingly, a greater number of transcripts were found within the stigma and style than included within the ovule. Nonetheless, the relative lack of developmental stages assessed within the dissected ovule transcriptome may lend to bias data towards increasing stigma/ style specific expression.

Over two fold less stamen specific transcripts were identified within this study when compared to previous microarray homeotic mutant derived subsets [16]. This may also be ascribed to an increase in specificity due to the analysis of additional mutants for comparison. The number perianth expressed transcripts increased in this study relative to homeotic microarray analyses; nonetheless, this number is still greatly reduced in comparison to those identified within the reproductive organs of either study, thus emphasizing the relative molecular complexity of reproductive tissue. More transcripts were identified as expressed within the sepal and/or throughout the perianth (177 genes) than those detected as petal specific (94 genes).

Interestingly, distinct genes expressed within the perianth in this and previous homeotic mutant microarray analyses are much less than those identified in microarray analysis integrating micro-dissected floral tissues [22]. In these analyses the greatest number of distinct transcripts within assayed floral organs was identified within the petals with a total of 827 genes with petal specific expression. This number exceeds those identified within the study as stamen enriched (805 genes). Those genes identified within the sepal are nearer to our analyses equalling a total of 141 genes with sepal-enriched expression. The primary difference in these analyses may be attributed to the stringency with which significant expression differences are noted during microarray data acquisition.

A cross analysis of the organ expression of genes within previous oligonucleotide, cDNA microarrays and gene trap lines revealed varying levels of overlap with the greatest consistency present in stamen-specific transcripts. Despite some low levels of overlap, it must be noted not all analyses were on a genome-wide basis, not all demarcated specific expression and instead revealed expression within the organ. For example, numerous microarrays of previous cDNA analyses suggested ~200 genes were dependant upon the activation of *AP3/PI* and therefore would be expressed specifically within the petals, stamen, or both based on the number of genes on a cDNA and a fold increase to account for the entire genome. Oligonucleotide analyses have suggested this level is much higher identifying nearly 6 fold more genes expressed specifically within the stamen or petal[16]. Our analysis confirms

previous oligonucleotide microarray analysis to a lesser degree with the identification of 840 genes with expression in the petals, stamen, or both.

4.3 Gynoecia Enriches Detectable Transcript Diversity

In addition to possessing the greatest number of distinct organ-specific transcripts within the gynoecia, a positive correlation was noted between the level of reproductive biomass, especially gynoecia tissue, and the level of MPSS detectable transcript diversity. The homeotic mutants *ap1* and *ap3* both actively expressed a more diverse set of genes than wildtype despite a lack of organ diversity. The increase in gynoecia in both these mutants may be ascribed to the increase in detectable transcript diversity. Furthermore, the most diverse transcriptome, *ap1*, overexpresses both gynoecia and stamen reproductive organs relative to wildtype and further increases floral organs relative to vegetative peduncle tissue due to the development of secondary inflorescences. In contrast, *superman/ap1* transcribes from a less diverse transcriptome than wildtype or *ap1*. This insinuates the degradation of gynoecia within this mutant greatly lessens the transcript diversity of the mutant despite increases in stamen tissue; further suggesting the gynoecia are responsible for the most transcript diversity.

Despite the correlation between detectable diversity and gynoecia tissue, the MPSS expression library acquired from micro-aspirator dissected ovules was the least diverse noted within this study. Similarly, cross-analysis of MPSS libraries reveals a greater number of distinct transcripts specifically expressed within the stigma and style than those ubiquitously expressed exclusively within the gynoecia. Nonetheless, this relative lack of transcript

diversity may be attributed to a limitation in assayed developmental stages. Homeotic and wildtype inflorescences represent floral buds throughout the first 13 stages of development, whereas ovules were dissected from only those flower buds which possessed emergent petals.

In order to further investigate the correlation among gynoecia biomass and MPSS detectable transcriptome diversity, as well as the putative dilution of gynoecia transcript diversity by high gene expression from the less diverse vegetative tissues, the average gene expression level within the floral tissues was determined. The average wildtype expression was found to be less than the homeotic mutants which overexpress gynoecia. Therefore, wildtype inflorescences were found to possess less transcript diversity with a higher quantity of those transcripts identified. This is in accordance with notions of dilution of MPSS expression predictions by high expression from a less diverse transcript set.

4.4 Gynoecia Correlations with Gene Expression Profiles of the Leaf

Analysis across all the homeotic mutants has revealed increases in gynoecia within the inflorescence were found to increase correlation with the leaf transcriptome. If one accepts the view of floral organs as modified leaves it is possible this correlation insinuates a greater loss or disruption of gene expression profiles within sepal and petals occurs when compared to gynoecia and suggests a greater degree of leaf modification in the perianth than the reproductive organs. The diversity of genes expressed within leaf tissue was found to be less than wildtype and all homeotic mutants possessing an enrichment of carpel tissue; however, leaf expression libraries

reveal a significantly greater number of distinct genes than those expressed in libraries possessing diminished or absent gynoecia. Countering the transcriptional diversity of petals previously identified in micro-dissection analyses [22], this suggests leaf tissue transcribes from a larger subset of genes than the petal or sepals, and significant downregulation of genes was necessary during leaf modification into the perianth.

4.5 Phenotypic Resemblances Concur with Gene Expression Profiles

Correlations among homeotic mutants revealed a correspondence of gene expression patterns with phenotypic similarity further supporting the ability of utilitizing floral homeotic mutants to demarcate organ specific expression. Those homeotic mutants overexpressing similar floral organs were found to correlate more closely with other inflorescences containing the same organ. The homeotic mutants *ap1* and *ap3* which overexpress gynoecia and are lacking petals were found to contain the most similar expression profile. In addition, *ap1* and *superman/ap1* were found to possess the second most similar expression profile. These mutants express the same floral organs; however, the double mutant *superman/ap1* increases expression of stamen at the expense of gynoecia tissue. In contrast to these levels of correlation, the A function mutant *apetala1* which is enriched for reproductive organs at the expense of vegetative tissue possessed the least similar expression pattern when compared to the C function mutant *agamous* which is enriched for the vegetative tissue and posses no reproductive organs.

4.6 Differential Expression in Wildtype and Homeotic Flowers

MPSS predicted gene expression within all four homeotic floral mutants was compared to the genes expressed in the wild type. It was found that a total of 331 genes ubiquitously expressed in homeotic mutant inflorescences are unaccounted for in the set of genes detected within the wild type inflorescence. Conversely, a total of 417 genes were identified within the wild type inflorescence and were not detected by MPSS in any of the floral mutants under observation. These transcripts illustrate a threshold of detection in MPSS. The average level of expression of the genes expressed within all homeotics and absent from the wildtype as well as those expressed within wildtype but absent from all homeotic mutants was equivalent to less than 5% the average level of gene expression within the floral libraries. This suggests transcripts with less than 5% the average level of transcription or a normalized transcription rate of 4 times per million may not be significant within our analyses. For this reason, we have sorted the organ-specific transcripts identified within our filtering system by their level of expression within the homeotic mutant which most overexpresses the respective organ (see methods). Therefore those transcripts which are at the top of each organ specific subgroup are the most dependable genes to possess organ-specific expression. In addition, we have placed a “significance” filter in our online user interface LIBAN to remove transcripts expressed at less than 4 times per million.

4.7 Limitation to Spatial Dissection

4.7.1 Cross-Analysis of Floral Homeotics Limits Analysis

This study was limited to floral organs and has not dissected the expression patterns of individual cell types. In addition, comparison of the expression profiles of transcripts across the homeotic floral mutants does not permit the identification of expression exclusivity of transcripts within all floral organs, due to the inadvertent inclusion of non-specific transcripts. For instance, although a transcripts may be present or upregulated in the homeotic mutants containing multiple whorls of sepals; *ag*, *ap3*, and absent from the A function mutants; *ap1, ap2,* and *sup/ap1*. This does not suggest specificity of the transcript to the sepals because a transcript found both in the sepals as well as the petals may follow the same expression pattern. A sepal or sepal-petal expressed transcript would be present and upregulated in *ag* because the number of both vegetative organs is increased to more than 70 organs per inflorescence [55]. Similarly, both expression patterns would be absent or greatly downregulated in the A function mutants *ap1* and the double mutant *sup/ap1*. However, the expression become less well defined in cases such as *ap3* wherein the number of sepals is increased but petals are absent. In these instances, the expression level of sepal-petal expressed transcripts would be increased to an unknown degree due to the enrichment of sepals. However, the quantity of these transcripts would also be abated owing to an absence of petals. Therefore, it is impossible to dissect those transcripts expressed only within the sepals from sepal/petal expressed transcripts using homeotic floral mutants. It is likely for this reason, the A function gene

apetala1 which is epistatic to *ag* and confined to the sepal and petals was identified as one of the sepal specific transcripts detected with the microarray screening criteria [16]. A similar issue arises in dissection of gynoecia expressed transcripts from those expressed within both the gynoecium and the androecium.

The fundamental issue underlying these incapacities can be attributed to the second and third whorl specificity exhibited by the B function of the classical ABC model in floral development (Figure 2). By only utilizing mutants lacking one of the three “classical” functions in the ABC model, the first and fourth whorl organs are left relatively unaffected by a B function mutant. Consequently, it is improbable to develop an A, B, or C function mutant which expresses petals without the presence of sepals. Petal specific transcripts may be dissected from the sepal/petal expressed transcripts by noting the presence of a C function mutant’s transcripts to its absence in A and B mutants which when combined allow the removal of transcripts that abide by all other possible organ specific expression patterns. However, sepal specific transcripts cannot be isolated from those expressed in the sepal and petal; because sepals are still present in B function mutants therefore eliminating the ability to negate transcripts embodying non-organ specific expression. Similarly, classical ABC function mutant do not permit the occurrence of stamen without the presence of the gynoecia. Stamen specific transcripts may be dissected from the Stamen-Gynoecia expressed transcripts by comparing the occurrence of transcripts within the A function mutant’s transcripts to its absence in the B and C function mutants which represent all

other possible expression profiles. Nonetheless, gynoecia specific transcripts cannot be isolated from those expressed in the sepal and petal; because gynoecia are still present in B function mutants and absence from the C function mutant alone do not permit removal of all other possible expression profiles. Even in the case of double function homeotic mutants A/B (ubiquitous carpel inflorescence), B/C (ubiquitous sepal inflorescence) or A/C (sepals/petals/leaf-like organs), or the triple mutant (leaf-like inflorescence), petals are expressed in tandem with sepals and gynoecia are expressed in the presence of stamen (Figure 2).

4.7.2 Justification of Floral Mutants Employed within this Study

In an attempt to overcome the inherent restrictions associated with the second and third whorl specificity of the B function and the sole usage of homeotic mutants. We have performed MPSS analysis on the double mutant *superman/apetala1* in addition to the *A, B,* and *C* function mutants to try to express stamen in the near absence of gynoecia. *SUPERMAN* encodes a C2H2-type zinc finger protein which regulates the third and fourth whorl boundary and is not a homeotic mutant [56]. The *sup* mutant exhibits development of additional stamen at the expense of carpel tissue in the central whorl by increasing proliferation of third whorl cells and greatly reducing cellular divisions within the fourth whorl. Furthermore, the double mutant sup*/ap1* acts in an additive manner whereby the first and second whorls contain axial apetalous inflorescences and the fourth whorl is greatly diminished accommodating an increase of stamen. The combined effect of the *sup* and *ap1* increased the number of stamen to as many as 14 in

observed inflorescences, and greatly reduced the first and fourth whorl containing carpelloid structures. Furthermore, the double mutant inflorescences chosen for MPSS analysis were preferentially selected for the greatest degree of stamen enrichment and carpel deficiency. This permitted the isolation of the stamen from gynoecia; thereby availing the dissection of gynoecia specific transcripts. Previous microarray analyses predicting organ specificity across the homeotic mutants relied only upon significant downregulation of putative gynoecia specific transcripts within a single homeotic mutant *agamous*. Integration of the carpel deficient *superman/ap1* mutant as well as micro-aspirator dissected ovules has permitted a more reliable dissection of gynoecia specific transcripts within our analyses.

The expansion of the third whorl by *SUPERMAN* stretched the domain of the B function and permitted dissection of the gynoecia and stamen. Although, a similar situation would be advantageous in the case of isolating the petal from the sepal, no developmental mutants have been identified which act on the boundary between the first and second whorls of *Arabidopsis* in an analogous manner to the phenotype exhibited in the inner whorls by *superman*. Furthermore, ectopic expression of the B function *AP3*, bearing other necessary known factors such as presence of *PISTILLATA* would theoretically expand the B function to all whorls of the inflorescence. However, 35S:AP3 mutants exhibit a phenotype similar to *superman*, with no accumulation of AP3 within the first whorl; thus suggesting an as of yet undetermined form of post transcriptional regulation [57] possibly a miRNA acting in a manner analogous to the relationship noted between AP2 and

miR172 [58]. Therefore, the degradation of the first whorl as well as ubiquitous expression of the B function, AP3 are not currently viable alternatives to isolation of petals from sepals to enable the *in silico* dissection of sepal specific transcripts from those expressed throughout the perianth.

4.8 Characterization of Floral Organ Specific cis-regulation

A number of genes involved in the regulation of flowering time and meristem identity have been identified and associated with the homeotic genes which compose the quaternary complex responsible for organogenesis. Nonetheless, little characterization of those downstream elements responsible for the maintenance and development of the distinct floral organs have been identified. This study confirms previous assessments regarding a lack of enrichment of those CArG boxes targeted by the quaternary complex. However, an enrichment of MYB2 binding domains was noted within the subset of genes possessing stamen specific expression. Genes containing these binding domains have been largely involved in abscisic acid (ABA) signalling [42]. Low temperature regulation of male sterility has been shown to be regulated by a reduction in the level of ABA within the inflorescence of tomato (*Lycopersicon esculentum*) [59]. Furthermore, this interaction occurs after initiation of stamen development has ensued. An addition MYB binding motif MYB3 was overexpressed within the petal and stamen localized dataset. MYB3 has also been implicated in various hormone signalling pathways and is likely responsible for developmental regulation as a result of fluctuations within these signalling hormones [41]. The SV40 binding motif of Arabidopsis is a nuclear localization signal. However, in *Arabidopsis* little information has

been published regarding its activation or downstream effects of SV40 antigen expression. SV40 promoter elements have been identified as bearing cell-specific expression in mammalian cell lines [40] which suggests cell specificity of those genes regulated by the transcription factor.

Chapter 5

CONCLUSION

Analysis of MPSS floral expression data has produced a robust floral-organ specific dataset to further corroborate and supplement previous expression data accrued through microarray technologies. Numerous well-characterized genes with known expression patterns were accurately dissected as organ-specific within our system of transcript filtration. Furthermore, biological validation of MPSS expression library analysis predictions acquiesce with expected expression patterns for several previously uncharacterized genes. In concurrence with previous analyses, reproductive structures possess the most diverse transcriptome when compared to vegetative tissue. Analysis of putative promoter regions within the putative organ-specific datasets concurs with previous microarray analysis. No significant enrichment of c-arg boxes was noted upstream of putative organ-specific genes, this suggests floral organogenesis and maintenance requires a multitude of signalling cascades as opposed to extensive direct cis-regulation by the ternary complex. A slight enrichment of MYB2, MYB3, and SV40 motifs were noted within the stamen, stamen/petal and sepal/perianth specific genes, respectively; warranting further analyses of these collectively regulated genes.

The culmination of both microarray hybridization and MPSS-sequence survey based approaches overcomes the inherent shortcoming which may bias the data when utilizing the technologies independently. Limitations in initial RNA quantity as well as issues with probe hybridization, and high background in microarray analyses are mitigated with sequence survey based analyses of MPSS. However, our floral analysis has revealed the sequencing depth of MPSS is still limited by a threshold of transcript detection as evident by increases detectable transcript diversity with increases in reproductive organ biomass in several homeotic mutants. Nonetheless, demarcating petal, stamen, stigma/style, gynoecia, sepal/perianth, petal/stamen, and stamen/gynoecia localized gene expression within *A. thaliana* via MPSS has further elucidated patterns of spatial regulation of transcription and will provide a valuable reference for preliminary reverse genetic characterizations as well as a source for floral organ-specific promoters to drive transgene expression. In addition, these promoters may be integrated into fluorescence-activated cell sorting, [32] subsequent RNA extraction and employment of Sequencing By Synthesis (Illumina) [60]; a sequence survey platform with ten fold higher sensitivity than MPSS. This will enable even more detailed spatial profiling of gene expression in future floral studies.

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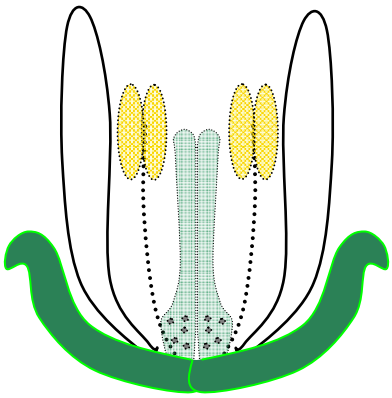
Appendix



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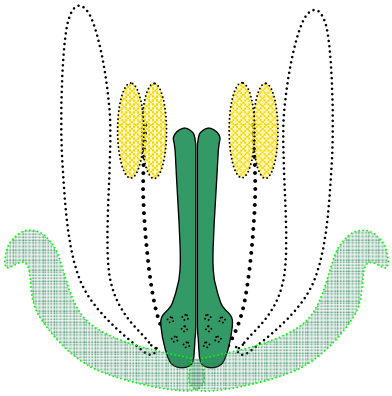

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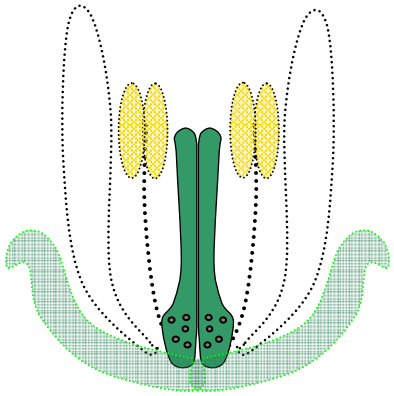

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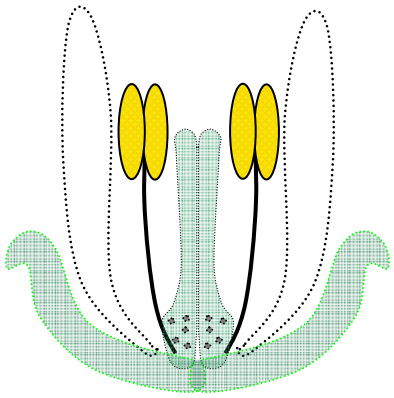

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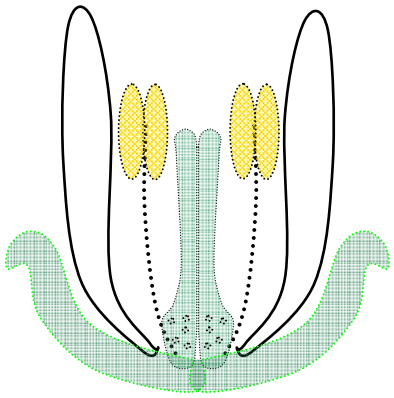

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At4g27570	At5g48770	At1g56120	At3g28100	At1g26530	At2g32340	At4g30510	
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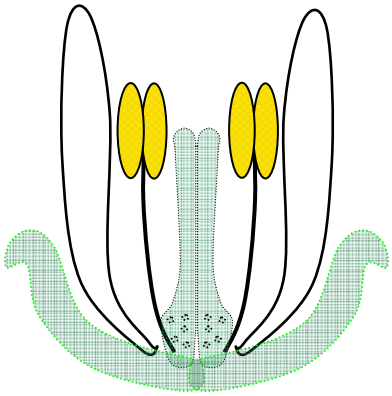

ACCESSIONS WITH MPSS DETERMINED PETAL-STAMEN LOCALIZED EXPRESSION							
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At2g18550	At3g28580	At1g19910	At4g12310	At2g18380	At5g49130	At1g34020	At1g71160
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At2g45230	At5g18770	At2g26400	At5g10610	AtCg00580	At3g09560	At1g75030	At3g06940
At1g16705	At1g02380	At3g14590	At5g36220	At2g07560	At5g63090	At4g39390	At5g49840
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At5g11530	At5g23130	At1g03120	At5g66800	At5g10930	At1g69940	At3g21720	At3g09530
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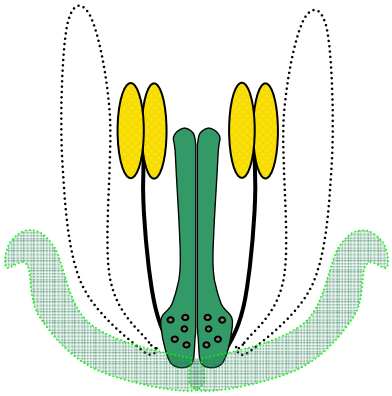

ACCESSIONS WITH MPSS DETERMINED STAMEN-GYNOECIA LOCALIZED EXPRESSION							
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At5g24420	At1g60790	At3g11480	At3g27770	At1g68030	At5g50740	At2g43430	At4g18250
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At1g33430	At3g09890	At3g16150	At3g52200	At1g71210	At5g56450	At3g05650	At4g23430
At5g21150	At4g01700	At3g19020	At3g53370	At1g71330	At5g61060	At3g06620	At4g27630
At2g27600	At5g09240	At3g19980	At3g55080	At1g77310	At5g64880	At3g07960	At4g36260
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At2g46260	At5g15880	At5g24790	At5g50350	At3g15354	At1g31010	At3g54130	At5g13820
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ACCESSIONS WITH MPSS DETERMINED STAMEN-GYNOECIA LOCALIZED EXPRESSION CONT"D							
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ACCESSIONS WITH MPSS DETERMINED STAMEN-GYNOECIA LOCALIZED EXPRESSION CONT'D							
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At2g44410	At4g12340	At1g75230	At5g67540	At5g07910	At1g68580	At3g53510	At4g26390
At3g63440	At4g19870	At1g76740	At1g02010	At5g15070	At1g69545	At3g54140	At4g32360
At4g06634	At5g13700	At1g80270	At1g08490	At5g15600	At1g74055	At3g55060	At4g38480
At5g01700	At5g16820	At2g01620	At1g09870	At5g18240	At2g01650	At3g59470	At5g13920
At1g30835	At5g21090	At2g11140	At1g13030	At5g18310	At2g02790	At4g00170	At5g20580
At1g71270	At5g24530	At2g23610	At1g16540	At5g22830	At2g06000	At4g01670	At5g43750
At1g72320	At5g43790	At2g26710	At1g19220	At5g23550	At2g06845	At4g03110	At5g46390
At4g16444	At5g51970	At2g27030	At1g26260	At5g27380	At2g12490	At4g04180	At5g51810
At4g19180	At5g54670	At2g30920	At1g33560	At5g27830	At2g16400	At4g09920	At5g57735
At5g02540	AtCg00450	At2g33850	At1g44740	At5g41740	At2g16570	At4g10110	At5g61540
At5g05350	At1g08710	At2g36145	At1g50910	At5g42400	At2g27330	At4g12710	At5g62020
At5g20710	At1g26310	At2g40690	At1g56180	At5g45350	At2g30330	At4g12760	At5g67240