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Expression profiling of *Arabidopsis* stigma tissue identifies stigma-specific genes

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Abstract Plants discriminate among pollen grains that land on the stigma surface, providing compatible pollen with the nutrients and signals required to proceed in pollination, and in many species, recognizing and inhibiting foreign pollen adhesion, hydration, germination and invasion. Much of the stigma machinery involved in these processes remains unknown. It is likely that the expression of a stigma-specific gene program confers specialized structural and functional properties. Here we used microarray technology and cDNA subtraction to build a profile of candidate stigma genes that facilitate early pollination events. Of over 24,000 *Arabidopsis* genes probed, we identified 11,403 genes expressed in stigma tissue; 317 of these were not expressed in control tissues. Analysis of the stigma transcriptome demonstrated a unique transcriptional profile. Functional specialization of the stigma for extracellular interactions is reflected by an increased number of stigma-specific and stigma-expressed genes involved in sculpturing the cuticle and cell wall, lipid metabolism, as well as genes potentially involved in pollen–stigma interactions.

Keywords *Arabidopsis* · Expression profiling · Microarray analysis · Stigma-specific genes

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Introduction

Successful pollination in flowering plants requires communication and coordination between the sperm-containing pollen grain and the ovule-containing pistil. This process is initiated when the stigma, a specialized portion of the pistil, captures a desiccated, metabolically inert pollen grain. On dry stigmas, the pollen grain is hydrated, and germinates to produce a pollen tube that invades the stigma cell wall on its path to the ovary (Elleman 1992). The extending pollen tube uses metabolites provided by the stigma, and later by the transmitting tract, to traverse the enormous distances required to reach the ovary (Sanchez et al. 2004). In order to locate unfertilized ovules, pollen tubes perceive guidance signals provided by the pistil. In many species, the stigma also discriminates between compatible pollen grains that lead to successful fertilization and foreign or incompatible pollen grains, promoting compatible pollen growth while preventing incompatible pollen grain adhesion, hydration, penetration, and growth (for recent reviews, see Kachroo et al. 2002; Swanson et al. 2004). These processes require communication between the pollen grain and stigma cells, yet much of the stigmatic cellular machinery that mediates these functions is unknown.

It is likely that the specialized structural and functional properties of the stigma are based on the expression of a stigma-specific gene program. Characterizing this program should lead to a better understanding of the molecular basis of stigma–pollen interactions. In an effort to build a profile of candidate stigma genes that facilitate early pollination events, we carried out microarray and cDNA subtraction experiments, comparing the suite of genes expressed in the stigma, ovaries

and 10-day old seedlings. In each case, multiple replicates were performed and statistically collapsed to provide consensus profiles that were then compared, identifying stigma-specific and stigma-enhanced transcripts. Also, to compare and complement the microarray data, a cDNA subtraction screen was performed, yielding a library of stigma-specific cDNAs that were subsequently sequenced. Analysis of individual gene categories as well as complete metabolic pathways revealed that lipid and cell wall metabolism genes are preferentially expressed in the stigma compared to other tissue types, consistent with the role of the stigma surface in interaction with pollen grains. These data build a stigma-specific expression profile useful for studying pollen–stigma communication and guidance.

Materials and methods

RNA Preparation

Stigma and ovary tissues were isolated from pistils after emasculating stage 8 buds (Bowman 1994) of Landsberg *erecta* (Ler) flowers. After 1 day of growth, pistils were harvested and frozen in liquid nitrogen. Stigmas were separated from pistils using superfine tip scissors (World Precision Instruments, Inc., Sarasota, FL, USA). Stigmas and the remaining ovaries were placed in separate Eppendorf tubes on dry ice until daily collections were complete. Tissue was stored at −80°C until RNA isolation. Seedling tissue was collected by growing Ler seedlings on Murashige and Skoog media plates for 10 days, and whole seedlings were harvested for RNA preparations. Roughly 1,500 stigmas, 400 ovaries or 60 seedlings were used for each RNA preparation. Total RNA was isolated using the RNeasy plant mini kit according to manufacturer's instructions (Qiagen, Valencia, CA, USA). RNA concentration and quality were determined by spectrophotometric measurements and agarose gels, respectively.

cDNA subtraction library construction and analysis

cDNA was prepared using the SMART PCR cDNA synthesis kit (Clontech, Palo Alto, CA, USA), starting with 1 μg of total RNA for each tissue. The subtraction was performed using amplified cDNAs from stigma and ovary tissue according to the PCR-Select cDNA subtraction kit (Clontech, Palo Alto, CA, USA). Subtracted cDNAs were size fractionated on agarose gels and cloned into pGEM-T Easy vector (Promega, Madison, WI, USA). cDNA clones obtained from the library were sequenced by the DNA Sequencing Core, The University of Chicago, using M13 forward or reverse primers. Nucleotide sequences were identified by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/), using default parameters.

Preparation of fluorescent probes and microarray conditions

Three separate RNA preparations of stigma tissue, four of ovary tissue and four of seedling tissue were subjected to microarray analysis. Fluorescent probe preparations and microarray experiments were performed by the Keck Foundation Biotechnology Resource Laboratory (Yale University, New Haven, CT, USA). Briefly, double stranded cDNA was synthesized from 6 μg of total RNA using the Superscript Choice System (InVitrogen, Carlsbad, CA, USA), with an HPLC-purified oligo $(dT)_{25}$ primer containing a T7 RNA polymerase promoter sequence at its 5′ end (Genset Corp., La Jolla, CA, USA). After precipitation, biotinylated cRNA was generated by in vitro transcription using the Bioarray high yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY, USA). cRNA fragmentation was performed at 94°C for 35 min in fragmentation buffer (40 mM Tris–acetate, pH 8.1, 100 mM potassium acetate, 30 mM magnesium acetate). Integrity of cDNA, cRNA and fragmented cRNA was assessed on a bioanalyzer. Sample quality was assessed using the Test-3 Gene Array, as described online (http://ingo.med.yale.edu/wmkeck/affymetrix/). Quality controls and initial analysis were performed with Affymetrix Microarray 5.0 (MAS) software (Affymetrix, Santa Clara, CA,USA). Once quality control parameters were met, the samples were hybridized to the ATH1 *Arabidopsis* gene array (Affymetrix, Santa Clara, CA, USA).

Statistical analysis of microarray data

MAS was used to make an absolute call for each transcript (present, absent or marginal) with a corresponding statistical confidence value. For comparisons between two tissue types, we included only transcripts that were consistently present in all replicates in at least one of the two tissue types. If this criterion was not met for either tissue, the transcript was excluded from further analysis. The probability (*P*-value) of differential expression of a transcript between two compared tissues was determined using the Wilcoxon–Mann–Whitney test, a non-parametric, two-sample method. A weighted average (Tukey's biweight) was then used to determine the fold difference between tissues.

Genome ontology assignment and metabolic pathway analysis

The genes on the ATH1 chip were classified using the gene annotation present in the BINS of the MAPMAN data-mining program (Thimm et al. 2004). Gene assignments were based on information from TAIR, the Gene Ontology Consortium, the Kyoto Encyclopedia of Genes and Genomes, and TIGR, followed by manual

correction. Existing BINS were manually collapsed to create the more general categories used to construct Fig. 1d. These categories were modeled after the functional categories initially used by the *Arabidopsis* genome initiative (AGI 2000). Metabolic pathway analysis was performed using the AraCyc *Arabidopsis thaliana* biochemical pathways (Mueller et al. 2003) available publicly (http://www.arabidopsis.org/tools/aracyc/).

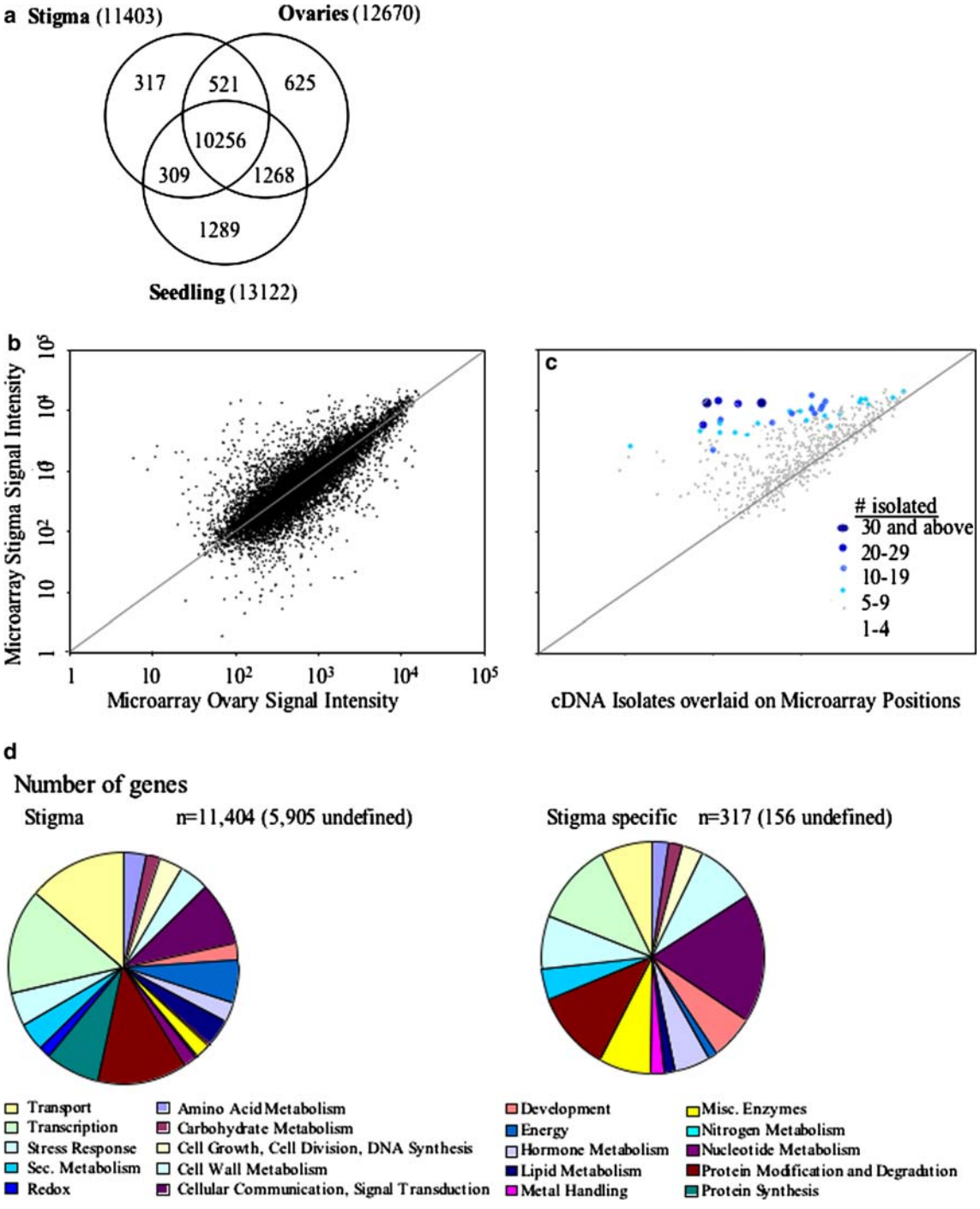

Fig. 1 **a** Venn diagram showing relationships between the three transcriptome data sets (stigma, ovaries and seedling), including the number of genes in each overlapping category. Numbers in parentheses indicate total number of expressed genes in each tissue type. **b** Scatter plot of ratio of signal intensities in stigma (*Y*-axis) and ovaries (*X*-axis) for each expressed gene on the microarray. **c** Stigma isolates from cDNA subtraction experiment plotted onto their corresponding positions from Fig. 1b. Points are size graded based on the number of times isolated in the cDNA subtraction screen, with largest dots corresponding to genes isolated as many as 53 times, while the smallest dots corresponding to genes isolated as few as 1–4 times. **d** Pie chart of distribution of total number of genes expressed in the stigma as well as stigma-specific genes

Table 1 Genes up-regulated in stigma relative to ovary (cDNA subtraction, 32 top genes)

Gene	Function or Comment	cDNAs Isolated	Microarray Fold Change (Stigma relative to ovary)
At4g11290	peroxidase, putative	53	35
At2g33850	expressed protein	39	144
At5g19880	peroxidase, putative	28	122
At2g37170	aquaporin (plasma membrane intrinsic protein 2C)	26	8[1]
At3g03670	peroxidase, putative	25	71
At3g12000	S-locus related protein SLR1 homolog (AtS1)	25	64
At3g01530	myb family transcription factor	16	10
At1g65450	expressed protein	13	12
At2g20340	putative tyrosine decarboxylase	13	5
At5g20230	blue copper binding protein	13	13
At5g59810	subtilisin-like serine protease	13	56
At1g55020	lipoxygenase, putative	12	6
At1g22380	UDP-glucose glucosyltransferase, putative	11	21
At4g23690	disease resistance response protein family/ dirigent protein family	11	7
At2g34810	FAD-linked oxidoreductase family	10	5
At2g39350	ABC transporter family protein	10	7
At1g67090	ribulose-bisphosphate carboxylase small unit, putative	9	1
At2g37180	aquaporin (plasma membrane intrinsic protein 2C)	9	8[1]
At1g04250	putative auxin-induced protein, IAA17/AXR3-1	8	2
At1g06520	expressed protein	8	9
At2g45580	cytochrome p450 family	8	17
At3g23410	expressed protein	8	5
At5g67340	putative protein	8	49
At2g38540	putative nonspecific lipid-transfer protein	7	1
At2g45960	aquaporin (plasma membrane intrinsic protein 1B)	7	2
At3g45970	putative protein	7	12
At4g30190	H+-transporting ATPase type 2, Plasma membrane	7	3
At4g38770	extensin-like protein	7	2
At5g11400	putative protein	7	220
At5g20820	putative protein	7	35
At5g44020	vegetative storage-like protein	7	23
At5g50040	pectin methylesterase inhibitor-related	7	–[2]

[1]Affymetrix probe set designed for both At2g37180 and At2g37170[2] not included in Affymetrix ATH1 array

Results

Microarray comparisons of *Arabidopsis* stigma, ovary, and seedling mRNA

To identify genes that confer unique stigma functions, we performed microarray analysis, harvesting 1,500 stigmas from mature, wild-type pistils (stage 14, Bowman 1994) for each array. Because *Arabidopsis* pistils are quite small, it is not possible to completely remove style tissue from the stigma fractions. Thus, we also probed microarrays with mRNA collected from 400 ovaries and from sixty 10-day old seedlings, enabling us to distinguish messages that are stigma specific from messages that contribute to common cellular activities or style functions. We hybridized cRNA from each sample to the ATH1 GeneChip, which contains 22,500 probe sets corresponding to 24,000 genes—more than 95% of the *Arabidopsis* genome. Our complete microarray dataset is available online (http://www.ncbi.nlm.nih.gov/geo/).

A total of 11,403 genes (51% of the probe sets) were consistently detected in the stigma samples; similar numbers were expressed in the ovary (12,670, 56%) and seedlings (13,122, 58%). Of these genes, 10,256 were common to all three tissues, while 1,463 were primarily in floral, but not seedling samples. A substantial number of genes showed expression that was highly tissue-specific; 317 in the stigma, 625 in the ovary, and 1,289 in the seedlings, accounting for 2.1, 4.2, and 8.8% of the expressed genes, respectively (Figure 1a). We also measured the genes that were preferentially expressed in stigma and ovary tissue: 2,595 were more abundant in the stigma ($P < 0.05$) and 3,783 in the ovary ($P < 0.05$); 6,869 genes did not show statistically meaningful differences between these two tissues ($P > 0.05$). The relative levels of the entire set of genes expressed in stigma and ovary are plotted in Fig. 1b.

A stigma-specific cDNA library confirms genes identified in microarray hybridizations

To further define the set of stigma-enriched genes, we used stigma and ovary mRNA preparations to prepare a stigma-enriched cDNA library, using subtractive hybridization to remove messages shared by ovaries (see Materials and methods). Sequencing 1,292 of the resulting cDNA clones identified 688 distinct genes, 153 of which were isolated multiple times (See Supplementary Table S1). A majority of the genes identified in the cDNA subtraction experiment also displayed preferential stigma expression when assayed on a microarray; none showed higher ovary expression ($P < 0.05$) (Fig. 1c). Four hundred and forty-two (34%) of the most frequently recovered cDNAs corresponded to only

Table 2 Genes up-regulated in stigma relative to ovary (microarray, top 30 genes)

Gene	Function or Comment	Stigma Signal	OvarySignal	Fold Change	cDNAs isolated
At5g05260	cytochrome P450	1568	5	264	
At5g11400	putative protein	2510	11	220	7
At3g55870	anthranilate synthase alpha-1 chain	1659	10	153	4
At2g33850	unknown protein	12734	88	144	39
At5g19880	peroxidase, putative	14312	117	122	28
At1g47280	expressed protein	1015	8	116	1
At4g00910	expressed protein	1963	19	102	
At3g54940	cysteine protease	1999	23	86	2
At3g03670	peroxidase, putative	5722	80	71	25
At3g12000	S-locus related protein SLR1 homolog (AtS1)	12970	200	64	25
At1g61560	mlo protein	4507	72	62	6
At1g05450	lipid-transfer protein	6549	115	56	3
At5g59810	subtilisin-like serine protease	7025	124	56	13
At3g57950	putative protein	1468	27	54	1
At5g67340	putative protein	5897	120	49	8
At5g53710	expressed protein	2855	71	40	1
At2g02010	glutamate decarboxylase	2811	73	38	1
At4g11290	peroxidase, putative	13101	365	35	53
At5g20820	putative protein	4302	120	35	7
At5g10800	RRM-containing protein	1527	44	34	
At2g17000	hypothetical protein	3134	91	34	1
At3g02940	myb family transcription factor	760	22	33	
At3g14060	expressed protein	5870	181	32	
At3g56060	mandelonitrile lyase-like protein	3106	110	28	3
At1g22990	copper chaperone related protein	2627	95	27	1
At5g38900	frnE protein-like protein	2190	82	26	2
At3g13790	glycosyl hydrolase family 32	2468	98	25	
At5g40630	putative protein	1523	61	24	
At5g44020	vegetative storage-like protein	4256	184	23	7
At1g06120	delta 9 desaturase	1592	74	21	

32 genes (Table 1). Three of these genes were represented in the microarray:stigma:ovary ratios at >100:1, and 16 at >10:1. In addition, of the 30 stigma messages that were most enriched relative to ovary messages in the microarray experiment, 22 were recovered in the subtracted stigma cDNA library (Table 2). Taken together, these data indicate that cDNA subtraction screening provides a powerful approach that can independently confirm large datasets generated in a microarray expression profile.

Interestingly, the cDNA subtractions and microarray hybridizations showed some important differences. Eight genes that were implicated on the microarrays as being expressed at 20 to 300-fold higher levels in stigmas compared to ovaries were not represented by the sequences obtained from the cDNA library. These genes are likely represented at a reduced level in the cDNA library due to their lower absolute levels of expression (Table 2). In addition, not all messages recovered in the cDNA library were preferentially expressed in the stigma, presumably due to incomplete subtraction. For example, 35% of the recovered cDNAs corresponded to messages detected at approximately equal levels in both tissues ($P<0.05$). In addition, although the subtractive process should have eliminated common messages, including those derived from the style, the cDNA library nonetheless contained four clones corresponding to rDNA and one that represented a telomere repeat.

Functional comparisons of genes identified using stigma and ovary microarrays

We used the quantitative nature of the microarray data to examine the distribution of genes comprising the stigma, ovary and seedling transcriptomes. Sorting the total set of expressed genes into 20 functional categories (see Materials and methods) showed no striking differences between the three tissues, with a similar distribution of expressed gene functions and comparable expression levels in each category (See Supplementary Figs. S2, S3). Consistent with other functional comparisons, the largest category of genes was undefined (Tung et al. 2005). Of the defined genes, most stigma genes functioned in (i) protein modification and degradation, (ii) transport, and (iii) transcription, making up 13, 14, and 15% of the total defined genes, respectively. Analysis of the ovary and seedling transcriptomes showed similar profiles, again with most defined expressed genes also falling into (i) protein modification and degradation, (ii) transport, and (iii) transcription categories (ovary:12, 14, 16% seedling:12, 14, 15%, respectively). These data suggest that, at this broad level of classification, neither stigmas nor ovaries activate processes that are highly distinct.

We did find interesting distinctions, however, when we examined the 317 messages that were specifically elevated in the stigma. In contrast to the distributions of

total stigma messages, three functional categories were expanded, and six were under-represented (Fig. 1d). Among those over-represented were (i) cellular communication and signal transduction (9 versus 18%), (ii) cell wall metabolism (4 versus 9%), and (iii) miscellaneous enzymes (2 versus 7%). We observed no induction of stigma-specific genes that play a role in nitrogen metabolism, nucleotide metabolism, redox, or protein synthesis, and only a few stigma-specific genes that function in transport and energy, indicating that stigmas rely on messages common to ovaries or seedlings for these processes.

We expanded this analysis from the stigma-specific genes to the broader set that was enriched in the stigma, relative to the ovary and seedling ($P < 0.05$). Two categories were over-represented: lipid synthesis and cell wall synthesis. Of the 232 expressed *Arabidopsis* genes annotated as playing a role in lipid functions, 56 showed preferential expression in the stigma, compared to 33 in the ovary and 51 in seedlings; for genes implicated in cell wall functions, 69 / 290 showed elevated expression in the stigma, compared to 63 in the ovary and 52 in the seedling (See Supplementary Fig. 2c).

Stigma-specific expression of genes encoding metabolic pathways

We also examined stigma-specific metabolic processes that were up-regulated, using the annotations deposited in the AraCyc database—a collection that assigns over 1,100 enzymatic reactions to 185 metabolic pathways (Mueller et al. 2003). We gauged pathway activity by summing gene expression levels of the pathway genes. Sixty-three pathways showed comparable expression in all the three tissues, varying by $\leq$ 10%, whereas 33 pathways were up-regulated in stigmas, 26 in ovaries and 63 in seedlings (See Supplementary Fig. S3). In stigmas, pathways associated with lipid and cell wall metabolism were induced. Long-chain lipid synthesis pathways and those required to produce a waxy cuticle were elevated 30–40 and 100%, respectively. Lipoxygenases, which contribute to the production of unsaturated fatty acid hydroperoxides were also up-regulated (30–60% increase), as were jasmonic acid production pathways (50% increase) suggesting important signaling roles. The implications of this distinct repertoire of stigma functions is intriguing and provides further support for the importance of lipids in pollen–stigma communication.

Discussion

To identify genes involved in stigma functions, we constructed a stigma transcriptional profile using microarrays and cDNA subtraction. In previous studies, isolated RNA from stigma-ablated plants was compared to RNA from wild-type plants; known stigma-expressed genes were identified, including SLR1, a protein putatively involved in pollen adhesion, SLG, a protein involved in *Brassica* incompatibility response, and Pis63–1, a gene of unknown function, as well as four genes not previously known to be expressed in the stigma (Kang and Nasrallah 2001). Recently, this same strategy was used with microarray analysis to define genes expressed in the pistil along the path of pollen tube growth (Tung et al. 2005). That study and this report of 11,403 stigma-expressed genes and 317 stigma-specific genes represent a dramatic increase in our knowledge of the genes involved in stigma function. To better exploit this information, we employed two separate bioinformatics techniques; functional categories and metabolic pathway analysis.

Functional categories

Our initial analysis of the stigma transcriptome was performed utilizing functional categories that organize and display data in the context of pre-existing biological and homology-based information. The categories were chosen to reflect those initially used to describe the *Arabidopsis* genome (AGI 2000). Although no gross differences were noted in the number of genes in each functional category in stigma, ovary and seedling, when the genes expressed specifically in the stigma were analyzed, several functional categories were non-proportionally expanded. The cellular communication and signal transduction category displayed the most pronounced increase, with 16 of the 29 stigma-specific genes encoding kinases. Six of these kinases are putative LRR (Leucine-Rich Repeat) transmembrane receptor kinases, a protein category previously implicated in a variety of cell signaling processes, including pollen–pistil interactions and pollen tube growth (Muschietti et al. 1998; Tang et al. 2002). Transmembrane receptor kinases have also been implicated in pollen recognition by the stigma in the *Brassica* self-incompatibility system (Kachroo et al. 2002). The stigma-specific *Arabidopsis* transmembrane kinases implicated by this study may play central roles in coordinating pollen arrival and stigma response.

Stigma-specific genes were also enriched in the Cell Wall Metabolism category. The plant cell wall, largely composed of cellulose, hemicelluloses and pectins, is involved in cell growth and development, intercellular communication and interaction with the environment (McCann et al. 2001). The cell wall is sculpted by a number of enzymatic activities. Interestingly, of the 14 stigma-specific Cell Wall Metabolism genes, eight are involved in pectin metabolism. Of these genes, six are putative pectinases and the remaining two are pectin methylesterase inhibitors. Pectin has long been implicated in pollen tube growth; in lily, a large pectic polysaccharide is involved in a two component-adhesive interaction between the pollen grain and the extracellular matrix of the stigma and style (Mollet et al. 2000). Several pectinases have also been identified that are

secreted from pollen; it is hypothesized that they are involved in pollen tube penetration into the stigma (Kim et al. 1996; Wu et al. 1996; Kalinowski et al. 2002). It is likely that stigma enzymes are also involved in pollen penetration. Degradation of stigma surface proteins results in an inability of pollen to penetrate the stigma; one enzymatic activity consistently seen on the stigma surface and used as a marker of receptivity is an esterase activity (Heslop-Harrison and Heslop-Harrison 1975; Heslop-Harrison 1977; Heslop-Harrison and Shivanna 1977). Indeed, multiple esterase activities have been identified in the stigma and their inhibition results in an inability of pollen to penetrate the stigma cell wall (Hiscock et al. 2002).

Metabolic pathway analysis

We also used the publicly available AraCyc database of biochemical pathways to analyze stigma function (Mueller et al. 2003). There are 186 metabolic pathways containing 1161 reactions in the database; approximately 53% of the reactions include annotated genes. Because it is estimated that approximately 4,000 enzymes are involved in metabolism in *Arabidopsis*, AraCyc may represent up to 25% of the metabolism of the plant (Mueller et al. 2003). By overlaying and comparing the expression data of stigma, ovary and seedling onto the metabolic pathways of AraCyc, we were able to identify which discrete metabolic pathways may be up-regulated in each tissue type.

Of the ten most up-regulated metabolic pathways in the stigma, four are specifically involved in lipid metabolism. In the stigma, the initial steps in fatty acid biosynthesis, unsaturated and saturated fatty acid elongation show 30–40% up-regulation in transcriptional throughput compared to that of ovary and seedling, while cutin biosynthesis shows a $>100\%$ transcriptional induction. Previous work suggests that a specialized lipid-rich, cutin-containing cuticle overlying the stigma is crucial for pollen interaction. Mutants have been identified that modify the permeability of the plant cuticle to small molecules (Lolle et al. 1992, 1998). These mutants allow pollen germination and pollen tube penetration onto non-physiological plant surfaces, such as leaf tissue. Long-chain lipids of pollen are required for establishing functional interaction between the pollen grain and the stigma. Screens for male sterility have consistently isolated mutants that display the bright, glossy appearance of waxless *eceriferum* (*cer*) mutants (Preuss et al. 1993; Hülskamp et al. 1995). Finally, a fourth pathway, glycerol metabolism, is up-regulated by more than 40% in the stigma compared to ovary and seedling. Although involved in a wide range of functions in the cell, glycerol is essential for the synthesis of lipids, raising the possibility that it is related to an increase in stigma lipid metabolism.

Homogalacturonan (HGA) biosynthesis also shows $>$ 40% increase in transcriptional throughput in the stigma. HGA is a linear chain of 1, 4-linked d-galacturonic acid residues that contain some methyl esterified carboxyl groups. HGA accounts for roughly 60% of the pectin found in the cell wall of the plant. As noted above in the functional category assignments, the Cell Wall Metabolism category showed an expansion in stigma-specific defined genes, with the majority involved in pectin metabolism.

The jasmonic biosynthesis pathway shows $>50\%$ increase in transcriptional throughput in the stigma compared to ovary and seedling. Jasmonic acid is a linolenic acid-derived cyclopentanone that acts as a signaling molecule in a variety of different processes, including fruit ripening, tendril coiling and defense. In *Arabidopsis*, jasmonic acid is required for pollen development and anther dehiscence. In several instances, such as the triple mutant (*fad3-2*|*fad7-2*|*fad8*) that lacks linolenic acid, and in *delayed dehiscence1* that eliminates 12-oxophytodienoate reductase, male sterility and defective anther dehiscence timing can be rescued by exogenous application of jasmonic acid. Given that genes involved with jasmonic acid biosynthesis are developmentally regulated during pollination and that jasmonic acid is a volatile signal, the up-regulation of this pathway in the stigma may contribute to the proper timing of anther dehiscence to coordinate pollination (Sanders et al. 2000). The lipoxygenase pathway is also up-regulated; lipoxygenase transcriptional throughput shows an increase in stigma by 30–60% over ovary and seedling. Lipoxygenases are iron-containing dioxygenases that catalyze the addition of oxygen to polyunsaturated fatty acids to produce an unsaturated fatty acid hydroperoxide. Although lipoxygenases produce products involved in diverse pathways, one of the major products of lipoxygenases is jasmonic acid. Thus, it is formally possible that the up-regulation of lipoxygenases in the stigma is tied to the increased production of jasmonic acid.

We found strong correlations between the functional categories and metabolic pathway analyses. Lipid and cell wall metabolism genes showed significant enrichment in the stigma, and these metabolic pathways were also identified in AraCyc analysis. These metabolic pathways could represent stigma-specific rate limiting steps in lipid and cell wall metabolism that can be used to further analyze the roles of lipids and pectins in pollen–stigma interaction.

Recently, microarray analysis has been employed to identify expressed genes in the stigma and transmitting tract, specifically along the path of the pollen tube (Tung et al. 2005). Although the analysis methods differ between that study and this one, the corresponding data sets generated similar numbers of stigma-expressed genes and functional conclusions. After analyzing genes for the presence of signal peptides, (Tung et al. 2005) noted that signal peptide containing stigma-specific genes fell into functional categories including cuticle biogenesis, extra cellular matrix modification and cell–cell communication and signal transduction; similar

functional categories are highlighted in this work. These two separate characterizations of the gynoecial transcriptome, which employed differing methodologies, provide both confirmatory and complementary data that represent an excellent resource for the further investigation of stigma biology.

cDNA subtraction screen

To confirm that our microarray results were accurate, we performed replicate experiments to assess variability, and also used a high-throughput subtractive cDNA screen to verify genes identified with the arrays. Often, northern blot analysis, ribonuclease protection assays, in situ hybridization, immunohistochemistry, RT-PCR, or real-time RT-PCR are used to confirm the transcriptional levels of specific genes. These methods are most useful when only one or a few genes identified in the microarray are of interest, but become less useful when validating the large number of genes that make up the bulk of the microarray profile. Because we are interested in the entire transcriptome of the *Arabidopsis* stigma, we constructed an independent stigma expression profile by performing cDNA subtraction, comparing and validating the microarray-based stigma expression profile. These techniques complement each other; microarrays provide reproducible measures of gene expression that can be compared from different tissues or similar treatments of single tissues. Construction of the arrays, however, depends on the accuracy of the existing genome annotation; if a gene is mis-annotated or overlooked, that gene's expression cannot be measured using designed, pre-fabricated microarray chips. The cDNA expression profile is not limited by knowledge of existing annotation, but is biased in a number of other ways, including by restriction site presence in gene sequences as well as by the relative expression level of individual genes in the cell.

The overall correlation between the two methods was quite good. Interestingly, the cDNA subtraction screen revealed information that was not apparent from microarray analysis alone. Part of this difference is due to the fact that the ATH1 chip utilizes the same probe sets for multiple genes; for example, the 265444_s_at probe recognizes two separate aquaporins, At2g37180 and At2g37170. According to the microarray analysis, these genes together are expressed eightfold more in stigma tissue relative to ovary. The cDNA subtraction analysis isolated At2g37170 almost three times more often than At2g37180, indicating that one aquaporin is more likely prevalent in the stigma compared to the other. Also, some genes are mis-annotated on the ATH1 chip, and some genes are not present at all. For example, the pectin methylesterase inhibitor, At5g50040 is not present on the ATH1 chip, but was identified as a stigma enriched transcript via the cDNA subtraction screen. These data demonstrate that the cDNA subtraction screen can both validate gene profiles for a large number of genes, as well as provide information unattainable by microarray analysis alone.

Conclusions

Analysis of the stigma transcriptome demonstrates unique features of the stigma transcriptional profile. Functional specialization of the stigma for extracellular interactions is reflected by an increased number of stigma-specific and stigma-expressed genes involved in sculpting the cell wall, cuticle, as well as manufacturing signaling molecules such as kinases and jasmonic acid. This data set provides important insight into fertilization biology. The pollen grain, which produces the pollen tube that penetrates the stigma, has recently been analyzed by microarray analysis, identifying a prevalence of genes involved in cell wall metabolism, the cytoskeleton and cell signaling (Honys and Twell 2003; Becker et al. 2003). This study analyzed genes expressed in the tissue that facilitates pollen tube elongation within the stigma and ovary, making it possible to design experiments that differentially address both sides of the fertilization process.

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