Whole Genome Analysis of Gene Expression Reveals Coordinated Activation of Signaling and Metabolic Pathways during Pollen-Pistil Interactions in Arabidopsis[1][C][W]

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Plant reproduction depends on the concerted activation of many genes to ensure correct communication between pollen and pistil. Here, we queried the whole transcriptome of Arabidopsis (*Arabidopsis thaliana*) in order to identify genes with specific reproductive functions. We used the Affymetrix ATH1 whole genome array to profile wild-type unpollinated pistils and unfertilized ovules. By comparing the expression profile of pistils at 0.5, 3.5, and 8.0 h after pollination and applying a number of statistical and bioinformatics criteria, we found 1,373 genes differentially regulated during pollen-pistil interactions. Robust clustering analysis grouped these genes in 16 time-course clusters representing distinct patterns of regulation. Coregulation within each cluster suggests the presence of distinct genetic pathways, which might be under the control of specific transcriptional regulators. A total of 78% of the regulated genes were expressed initially in unpollinated pistil and/or ovules, 15% were initially detected in the pollen data sets as enriched or preferentially expressed, and 7% were induced upon pollination. Among those, we found a particular enrichment for unknown transcripts predicted to encode secreted proteins or representing signaling and cell wall-related proteins, which may function by remodeling the extracellular matrix or as extracellular signaling molecules. A strict regulatory control in various metabolic pathways suggests that fine-tuning of the biochemical and physiological cellular environment is crucial for reproductive success. Our study provides a unique and detailed temporal and spatial gene expression profile of in vivo pollen-pistil interactions, providing a framework to better understand the basis of the molecular mechanisms operating during the reproductive process in higher plants.

Seeds develop as a result of sexual reproduction in plants, a process relying on a series of complex signaling interactions collectively known as the progamic phase. Despite apparent anatomical simplicity, the pistil plays a central role in the success of this process. Its structure is precisely adapted to bear the female gametophyte and to act in a way that influences pollen tube growth (Boavida et al., 2005a, 2005b; Feijó, 2010). The first pollen-stigma recognition event involves molecules present on both partner surfaces to promote pollen adhesion and subsequent hydration (Hiscock and Allen, 2008). Upon germination, the pollen tube grows from the stigma through the style and ovary tissues, where localized cellular interactions potentially define unique phases or checkpoints for pollen tube guidance. In flowering plants, compatible signaling molecules and chemical gradients produced by the female diploid tissues were identified as playing major roles supporting pollen tube growth and guidance along their way within the pistil (Cheung et al., 1995; Wu et al., 1995; Palanivelu et al., 2003; Dong et al., 2005), while more precise signals emitted by the female gametophyte should act as short-range attractants guiding pollen tubes into their final target, the embryo sac (Shimizu and Okada, 2000; Higashiyama et al., 2006). Specific signal interactions between pollen tubes and the embryo sac determine the final guidance steps toward sperm cell release into the embryo sac (Huck et al., 2003; Rotman et al., 2003; Higashiyama et al., 2006), a process that seems to be at least partially controlled by the female gametophyte (Escobar-Restrepo et al., 2007).

Knowledge about signaling pathways that operate during pollen tube growth following self-incompatible and compatible pollinations has increased considerably in recent years (Hiscock and Allen, 2008), but the

molecular basis underlying the production of female signals, and how pollen tubes perceive and decode them to correctly target the embryo sac, remain largely unknown. The transcriptional profiles of pollen (Becker et al., 2003; Honys and Twell, 2003, 2004; Pina et al., 2005; for review, see Becker and Feijó, 2007), embryo sac (Yu et al., 2005; Johnston et al., 2007; Jones-Rhoades et al., 2007; Steffen et al., 2007), pistil (Scutt et al., 2003; Tung et al., 2005), and embryo or seed developmental stages (Chen et al., 2001; Hennig et al., 2004; Yoshida et al., 2005, Le et al., 2010) were recently analyzed. More notably, recent progress in the isolation of Arabidopsis (*Arabidopsis thaliana*) gametes allowed genome-wide expression profiling of purified sperm cells (Borges et al., 2008) as well as of isolated egg and central cells (Wuest et al., 2010). In addition, the transcriptomes of in vitro pollen tube growth (Wang et al., 2008) and of pollen tubes growing in a semi-in vitro system (Qin et al., 2009) unfolded a new level of complexity of gene regulation occurring during the reproductive process. However, the analysis of individual partners or particular cellular processes cannot provide a complete outline of the molecular events acting during plant reproduction, in that they do not fully represent the way that individual elements relate with each other in vivo in a precise timing and developmental context. To address this question, we used the Affymetrix ATH1 whole genome array to profile gene expression in unpollinated pistils (UP) and dissected unfertilized ovules, based on comparative transcriptomics with different tissues. In addition, the same technology was used to analyze the kinetics of gene regulation along several time points that could represent the most significant developmental events from pollination to fertilization. By combining these approaches with thorough bioinformatics analysis, we could identify 1,373 genes whose behavior would be compatible with crucial roles needed to ensure fertilization. This study represents, to our knowledge, the first microarray-based approach on pollen-pistil interactions using a complete in vivo system, thus providing a more comprehensive view into the complex signaling and genetic networks underlying sexual plant reproduction.

RESULTS AND DISCUSSION

Prediction of Stage-Specific and Reproductive Organ-Enriched Transcripts

The reproductive process involves the regulation of cellular interactions that occur between the male gametophyte (pollen/male gametes), diploid female sporophytic tissues, and the female gametophyte (embryo sac/female gametes). Our first approach aimed to identify stage-specific and/or stage-enriched transcription in UP and unfertilized ovules, which are the more relevant organs in terms of cellular interactions occurring after pollination. The UP expression profile should include transcripts that are abundantly expressed in stigmatic and transmitting tissue (TT) cells that may function to support and modulate pollen tube growth. Similarly, the expression profiles of ovules should show enrichment for transcripts expressed in the female gametophyte but also in other sporophytic transcripts that may be equally important for pollen tube growth modulation or guidance. A primary transcriptomic comparison of UP, ovules, and siliques enriched the UP and ovule data sets for stage-specific transcripts by excluding common sporophyte-expressed genes potentially involved in carpel and ovule determination, such as *SEEDSTICK* (*STK*; Rounsley et al., 1995) and *SHATTERPROOF1* (*SHP1*; Flanagan et al., 1996), a phosphogluconolactonase (*At5g24420*) that is expressed in ovule integuments and in the ovary wall (Scutt et al., 2003), as well as genes involved in later stages of seed development, such as *MEDEA* (*MEA*; Vielle-Calzada et al., 1999), *FERTILIZATION INDE-PENDENT SEED2* (Luo et al., 1999), and *FLOWERING WAGENINGEN* (Kinoshita et al., 2004). Using the parameters outlined in "Materials and Methods" and further comparison with publicly available data sets (Zimmermann et al., 2004; Tung et al., 2005; Yu et al., 2005; Steffen et al., 2007; Borges et al., 2008; Wang et al., 2008; Qin et al., 2009; Wuest et al., 2010), a total of 42 genes were considered enriched or preferentially expressed in ovules (Supplemental File S1). As anticipated, 27 out of 42 transcripts preferentially expressed in ovules and 96 out of 1,275 ovule-enriched transcripts were previously reported to be expressed specifically in the embryo sac or enriched in female gametophytic cells (Supplemental File S1; Yu et al., 2005; Johnston et al., 2007; Punwani et al., 2007; Steffen et al., 2007; Wuest et al., 2010). Ovule-expressed transcripts likely include plausible candidates for short-range pollen tube attractants expressed in the embryo sac (Higashiyama et al., 2001, 2003; Chen et al., 2007; Okuda et al., 2009) but also of other modulators expressed in the surrounding sporophytic tissues that could function as long-range pollen tube attractants. UP express the lowest number of enriched transcripts (340), due to the exclusion of overlapping transcript expression with mature siliques. Among these, we confirmed the detection of 17 UP-enriched transcripts previously reported to be expressed in receptive stigma or TT (Supplemental File S1; Scutt et al., 2003; Wellmer et al., 2004; Tung et al., 2005).

Analysis of the major functional classes represented in UP-enriched transcripts reveals as predominant classes cell wall biosynthesis and regulation (4.7%), stress- and defense-related transcripts (8.8%), transcription (13.2%), and development (6.3%; Table I). Although much of the constitutive expression in pistils is represented by stress and defense-related genes (Scutt et al., 2003; Lan et al., 2004), these transcripts likely encode proteins that, in addition, may perform functions in the modulation of pollen tube growth. On the other hand, transcription-, cell cycle-, and DNA processing-related transcripts are major

Table I. *Functional classification of UP, ovules, and pollen-expressed transcripts*

Functional categories are based on criteria of the Munich Information Center for Protein Sequences Arabidopsis database. Data refer to frequency (%) of annotated genes assigned to each functional category. Overrepresented categories are highlighted in boldface, and the total number of genes is indicated in parentheses. Asterisks indicate significant categories at $P < 0.05$.

Functional Category	Enriched or Preferentially Expressed			Enriched		
	ATH1 (22,325)	Ovules (46)	Pollen (420)	UP (317)	Ovules (1,245)	Pollen (964)
Metabolism	20.6	15.2	22.9	19.6	16.9	**27.0***
Cell wall	1.7	**8.7***	**4.8***	**4.7***	1.4	3.0
Cytoskeleton	0.6	0.0	**1.4***	0	1.1	**2.5***
Energy pathways	1.9	0.0	0.7	0.7	1.0	1.1
Stress and defense	5.7	0.0	4.3	**8.8***	2.9	5.2
Signal transduction	5.2	0	**6.9***	5.4	5.6	**9.1***
Cellular transport	10.0	0.0	**12.9***	9.1	7.9	12.3
Interaction with cellular environment	6.1	0.0	3.3	**11.4***	5.9	6.6
Cell cycle and DNA processing	5.9	6.5	2.6	6.0	**10.0***	3.8
Transcription	9.7	4.3	5.2	**13.2***	**16.1***	5.3
Protein fate	12.7	10.9	**15.0***	12.3	13.7	**17.8***
Development	3.4	**10.9***	4.0	**6.3***	4.6	3.8
Cell fate	1.6	**4.3***	1.0	2.2	1.8	2.0
Protein regulation	2.5	4.3	3.80	2.2	2.4	**5.6***
Protein synthesis	2.7	0.0	0.0	0	3.9	0.9
Unclassified	25.6	**50.0***	25.5	20.8	23.4	23.3

functional classes in the ovule-enriched data set. Overrepresented functional categories in transcripts preferentially expressed in ovules include cell wall biosynthesis (8.7%), development (10.9%), and cell fate (4.3%) as well as a significant number of unclassified transcripts (50%; Table I), while components of signal transduction and cellular transport were surprisingly poorly represented in these data sets. Yet, apparently lacking tissue or cell specificity at the expression level, many of these signal transduction components may still perform crucial functions during pollen-pistil interactions. As an example, the *FERONIA* gene, which encodes a receptor-like kinase required for pollen tube reception (Escobar-Restrepo et al., 2007), is under gametophytic selection, albeit being also expressed in siliques, seedlings, and leaves.

Gene Regulation Underlying the Progamic Phase

In order to identify genes involved in cell-cell communication, we generated expression profiles for three distinct time points after pollination assumed to represent important time frames for biological switches on the nature of cell-to-cell interactions along the pollen tube pathway. In Arabidopsis, within the first half-hour after pollination (0.5HAP), most pollen grains have hydrated, germinated, and invaded the stigmatic papilla cells. At 3.5HAP, pollen tubes are growing through the style TT cells, and at 8.0HAP, few pollen tubes might be in their final guidance stages to or interacting with the embryo sac, but most ovules are fertilized.

In order to analyze global expression patterns, we used principal component analysis (PCA), projected onto the first three principal components (Fig. 1A). The first principal component captures the highest vari-

ance among samples (42.9%) and underlines the unique expression profiles of UP, 0.5/3.5HAP and 8.0HAP, undoubtedly revealing major transcriptional changes occurring in response to pollination. The data sets of 0.5HAP and 3.5HAP are more similar to each other than to any other, suggesting that this particular stage, corresponding to pollen tube growth in the TT of the style, has a distinct transcriptional profile compared with other stages. The second and third principal components explain the remaining variance between samples, accentuating the differences between all time points. A similar separation of the samples could be obtained with clustering analysis (Fig. 1B). The time-course expression profiles were then compared with each other using UP as baseline and a more stringent cutoff (lower confidence bound of fold change [LCB] $\geq$ 1.5; $P < 0.01$) to increase biological significance. We found 1,298 genes regulated at least in one of the time points after pollination. Regulated genes include those that showed a significant change in expression between two consecutive time points as well as genes in which the kinetics of regulation was slower. Therefore, a significant change in gene expression was only detected in nonconsecutive time points. We hypothesized that some of the enriched pollen transcripts could be highly diluted in female sporophytic tissues during in vivo growth. Using a lower cutoff (LCB $\geq$ 1.2), we identified 75 additional transcripts identified initially as enriched or preferentially expressed in the pollen data sets.

In total, we detected significant changes in the expression of 1,373 genes from pollination to fertilization. A comparison between tissue and time-course expression allowed us to determine that 78% (1,066) of the regulated transcripts were first expressed before pollination in UP or unfertilized ovules, 7% (96) were
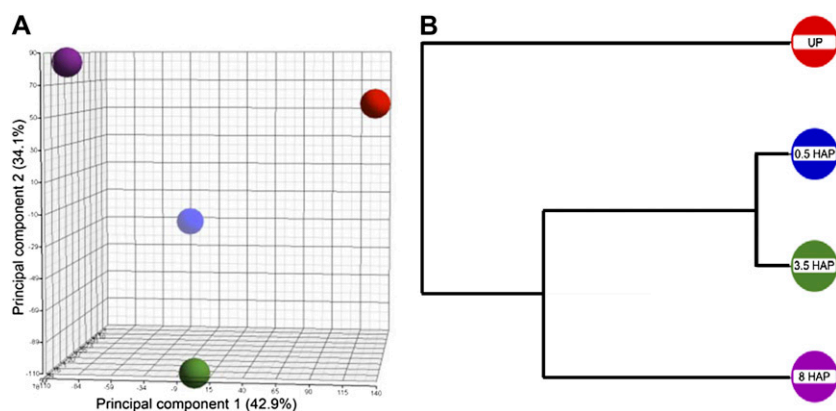
Figure 1. PCA and hierarchical clustering of time-course transcriptome data. A, PCA is an exploratory technique used to describe the structure of high-dimensional data (e.g. derived from microarrays) by reducing its dimensionality. Here, expression values for approximately 22,400 genes during the four time points are projected onto the first three principal components. B, Hierarchical clustering was used to group similar objects into "clusters," producing a tree (called a dendrogram) that shows the hierarchy of the clusters. [See online article for color version of this figure.]

exclusively induced by pollination (absent in any other tissue directly involved in the reproductive process), 12% (170) represent transcripts identified initially as preferentially expressed in our pollen data set (absent from UP or ovules), and 3% (36) were enriched (Supplemental File S2). Despite the predominant expression of genes in the pistil at receptivity, only a few proteins expressed in the sporophytic female tissues were identified with functions in pollen-pistil interactions (Wu et al., 2000; de Graaf et al., 2003; Palanivelu et al., 2003; Park and Lord, 2003), and only one gene was identified to be strictly induced by pollination (Bui and O'Neill, 1998).

The comparison of our time-course data set with recently reported transcriptomes of in vitro (Wang et al., 2008) and semi-in vitro pollen tube growth (Qin et al., 2009) confirmed an expected overlap of multiple genes "de novo" transcribed during pollen tube growth and possibly induced due to interactions with the female tissues. This overlap includes 13 genes classified in our data sets as "induced by pollination," 129 as "expressed before pollination," and 103 already identified as preferentially expressed and/or enriched in the pollen data sets. Yet, 119 regulated genes classified as pollen enriched and/or preferentially expressed in the initial data sets were not represented in the in vitro or semi-in vitro transcriptomes. These include genes predominantly up- or down-regulated upon interactions with the TT of the style (0.5HAP versus 3.5HAP), genes in which significant changes in gene expression were detected in nonconsecutive time points (i.e. there was a gradual change in expression from 0.5HAP up to 8.0HAP), and genes in which transcriptional changes occur in later stages of pollen tube growth in the ovary (3.5HAP versus 8.0HAP). These may represent either pollen-expressed genes modulated in later stages (Supplemental File S2) or genes elicited in female tissues upon pollen tube interaction with the ovary TT, ovules, or embryo sac (3.5–8.0 HAP), conditions that were not considered in any of the previous studies. These transcripts may thereby function in both pollen and pistil tissues to ensure a proper fertilization.

Using cluster analysis, the 1,373 transcripts were grouped in 16 distinct time-course clusters according to their expression patterns (Fig. 2; Supplemental File S3). Even though it was initially thought that the success of the reproductive process would depend predominantly on down-regulation of pistil-expressed transcripts (Chen et al., 2001; Lan et al., 2004), recent genome-wide analyses in rice (*Oryza sativa*) and Arabidopsis contributed to change this perspective (Endo et al., 2004; Hennig et al., 2004). Our time-course analysis further reveals that pronounced transcriptional changes take place in response to pollination, and these involve the concerted activity of multiple genes that ultimately follow distinct patterns of regulation. In fact, 46% of the transcripts are regulated at least in one time point after pollination, 38% are consistently up-regulated (clusters 6, 8, and 9), while only 16% (clusters 13 and 14) are continuously down-regulated after pollination (Fig. 2). As an example, clusters 1 and 2 show a biphasic regulation: these transcripts are induced as fast as 0.5HAP, down-regulated during pollen tube growth in TT, and induced again at 8.0HAP. On the other hand, in clusters 7, 10, 11, and 12, transcripts are preferentially induced at 3.5HAP; in clusters 3 and 4, transcripts are slowly down-regulated during pollen tube growth in the stigma and TT but are specifically up-regulated at 8.0HAP; while clusters 15 and 16 seem to include transcripts predominantly down-regulated with a slight induction in later stages of fertilization (Fig. 2). Therefore, each cluster contains a group of dynamically coregulated transcripts, likely performing specific functions in particular cellular interactions between pollen tubes and female tissues (i.e. stigma, style, or ovary). While most clusters consist of genes that are involved in varied biological functions, other clusters seem to contain transcripts involved in more specific functions (Supplemental Table S1). Interestingly, cluster 2 contains a significant representation of metabolism and energy-related transcripts as well as a unique enrichment of unclassified proteins, while cluster 10 is almost exclusively represented by transcripts related to cell wall and cell fate. Clusters 15 and

Figure 2. Clustering analysis of the time-course profile of pollen-pistil interactions. The expression values of 1,373 genes were normalized to the baseline (UP; time 0), and the ratios were $\log_2$ transformed to reveal expression kinetics. Genes were clustered using the SOTA array clustering method (GEPAS) and organized in a hierarchical tree (SOTATREE). Total number of genes is given in gray circles, ordered from top to bottom. Graphs show expression patterns for all genes within each cluster along the time course; the thick line indicates the average expression trend line for each cluster. Clusters are numbered from left to right and top to bottom.
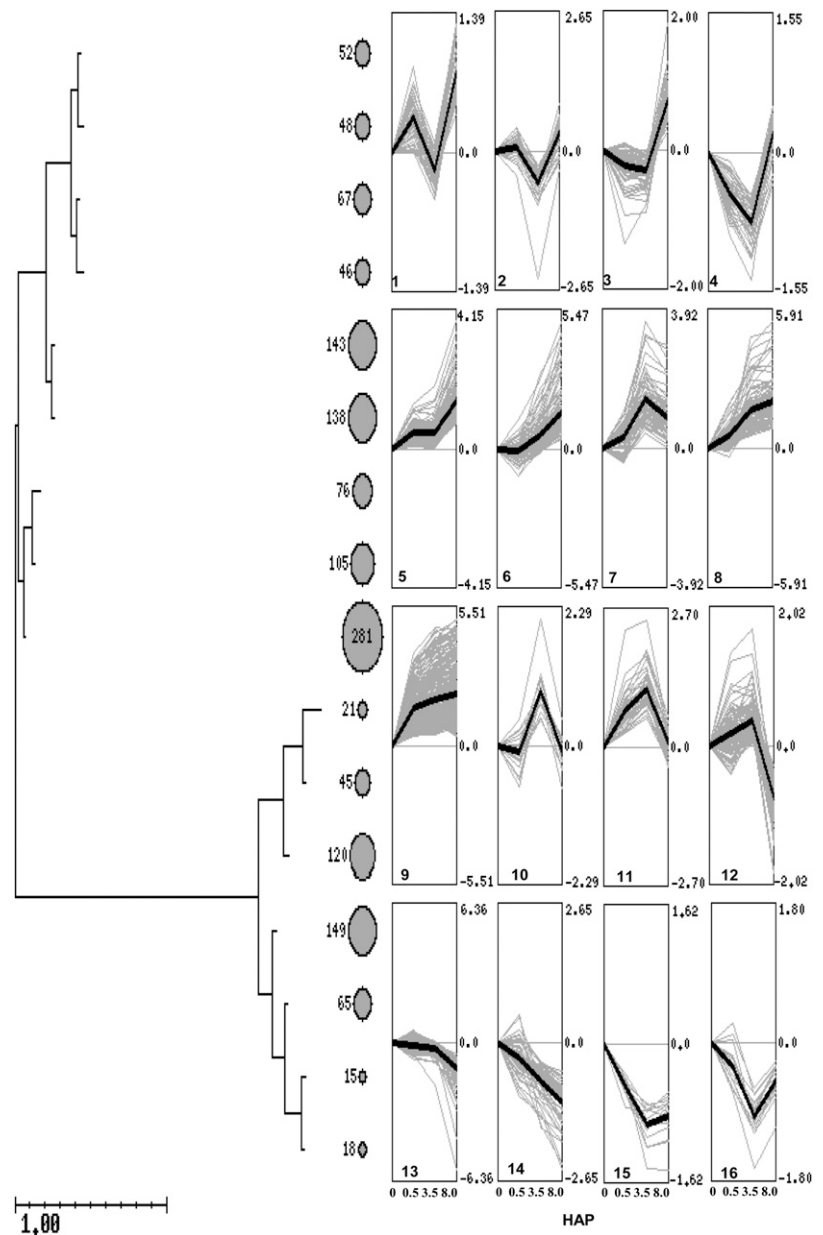

16 denote a slight up-regulation at 8.0HAP, which is associated with the enrichment of cell wall- and cytoskeleton-related transcripts, cell cycle, DNA processing, and transcription, as well as a large number of unclassified proteins possibly associated with the termination of pollen tube growth, fertilization, or the initiation of early zygote/embryo development.

Microarray Data Validation

For validation of our microarray analyses, we followed distinct approaches: (1) overlapping our microarray data sets with the literature, both in terms of transcriptomics and single gene profile studies; (2) reverse transcription (RT)-PCR analysis of candidate genes to confirm tissue expression or regulatory patterns within different gene clusters (Fig. 3); and (3) characterization of gene and enhancer trap insertion lines (Fig. 4). Among 1,373 genes regulated during the reproductive process, we selected 36 genes with relatively high expression levels and for which the change in expression in consecutive or nonconsecutive time points could potentially be detected by RT-PCR (greater than 1.3-fold change).

Comparison of our data sets with the literature confirmed that the expression patterns of several of the regulated genes were consistent with the described functions in pollen tube growth or guidance. Such examples include the Arabidopsis plantacyanin (*At2g02850*; Dong et al., 2005) and the well-known *POP2* gene (*At3g22200*) encoding a transaminase involved in the degradation of γ-aminobutyrate (Palanivelu et al.,
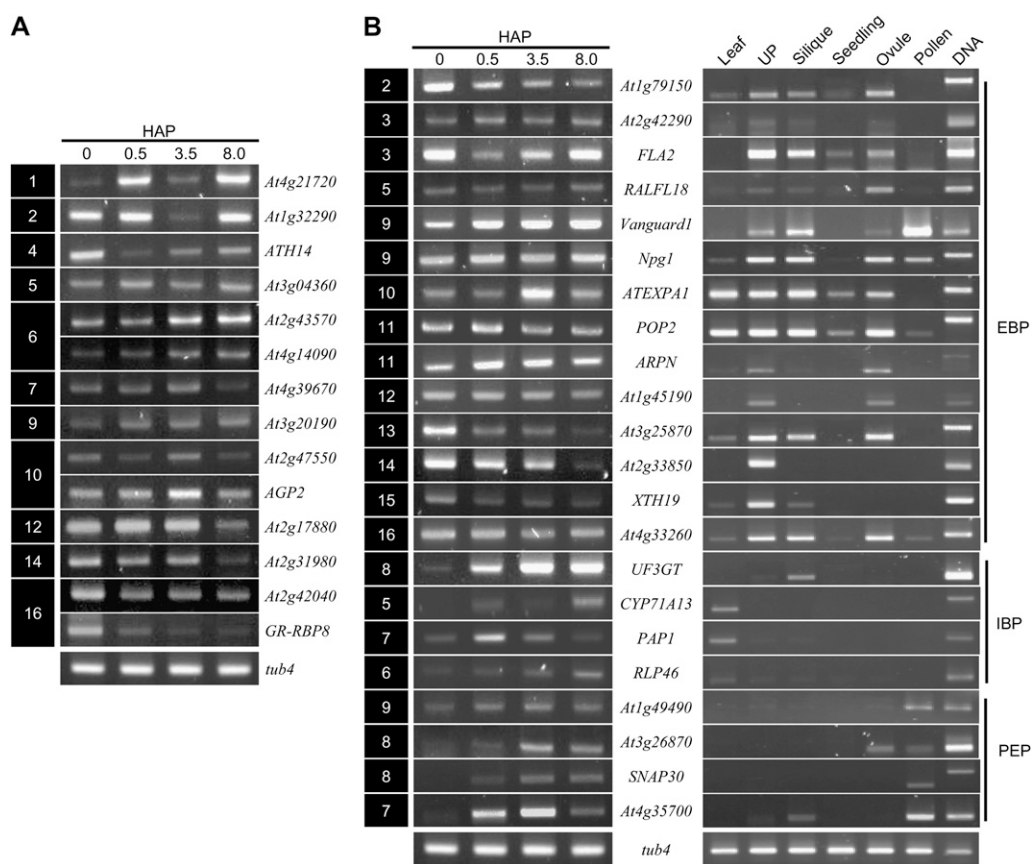
Figure 3. RT-PCR analysis of genes regulated during pollen-pistil interactions. A, Confirmation of changes in expression for several regulated genes during the time course. B, Expression of several time-course-regulated genes classified as EBP (expressed before pollination), IBP (induced by pollination), and PEP (pollen enriched or preferentially expressed) and corresponding expression reproductive and vegetative tissues. *TUB4* (*At5g04180*) was used as a positive control. The number of the hierarchical cluster is indicated for each gene.

2003), which are up-regulated after pollination with a decrease in expression in later stages of pollen tube growth (Fig. 5). Consistently up-regulated are general genes with known functions in pollen germination and tube growth, such as *Vanguard1* (*At2g47040*; Jiang et al., 2005) and *No Pollen Germination1* (*At2g43040*; Golovkin and Reddy, 2003; Fig. 5). Other examples include the RopGEFs known to interact with leucine-rich repeat (LRR) kinases during pollen tube growth in the pistil (Kaothien et al., 2005). In our data sets, we identified two pollen-specific RopGEFs (Zhang and McCormick, 2007), RopGEF12 (At1g79860) and Rop-GEF11 (At1g52240), both of which are up-regulated during pollen tube growth (cluster 9). Interestingly, two out of six up-regulated LRR kinases are preferentially expressed in pollen and follow a similar regulatory pattern (cluster 9). One is the ortholog of LePRK2 (for pollen receptor-like kinase), AtPRK2a (At2g07040), recently found to interact with RopGEF12 (Zhang and McCormick, 2007). The second pollen LRR (At1g49490; Fig. 3) may represent the ortholog of LePRK1. However, several crucial molecular players in this signaling pathway are still missing (Wengier et al., 2010), namely

those expressed in the female partner tissues. It is plausible that this particular gene cluster (cluster 9) may contain other partner components of this complex signaling transduction pathway, namely the most wanted pistil ligand.

The expected expression patterns of several potential candidates encoding unknown, cell wall-, and signaling-related components were confirmed by RT-PCR (Fig. 3). We validated the expected gene expression in 94% of the 144 independent contrast points tested (36 genes in four time points) and confirmed the expected regulatory trend for all four time points in 27 out of the 36 genes. Examples of this validation include transcripts induced in response to pollination, such as a MYB transcription factor (At1g56650, PAP1), which is elicited at 0.5HAP and then down-regulated during pollen tube growth in the TT and ovary, or of a glycosyltransferase (At5g54060, UF3GT) strongly induced by pollination (Fig. 3). We obtained a similar confirmation (94%) for tissue expression. From 132 independent contrasts tested (22 genes in six different tissues), only nine showed discrepancies in expression (approximately 6%), seven of which were in seedlings.
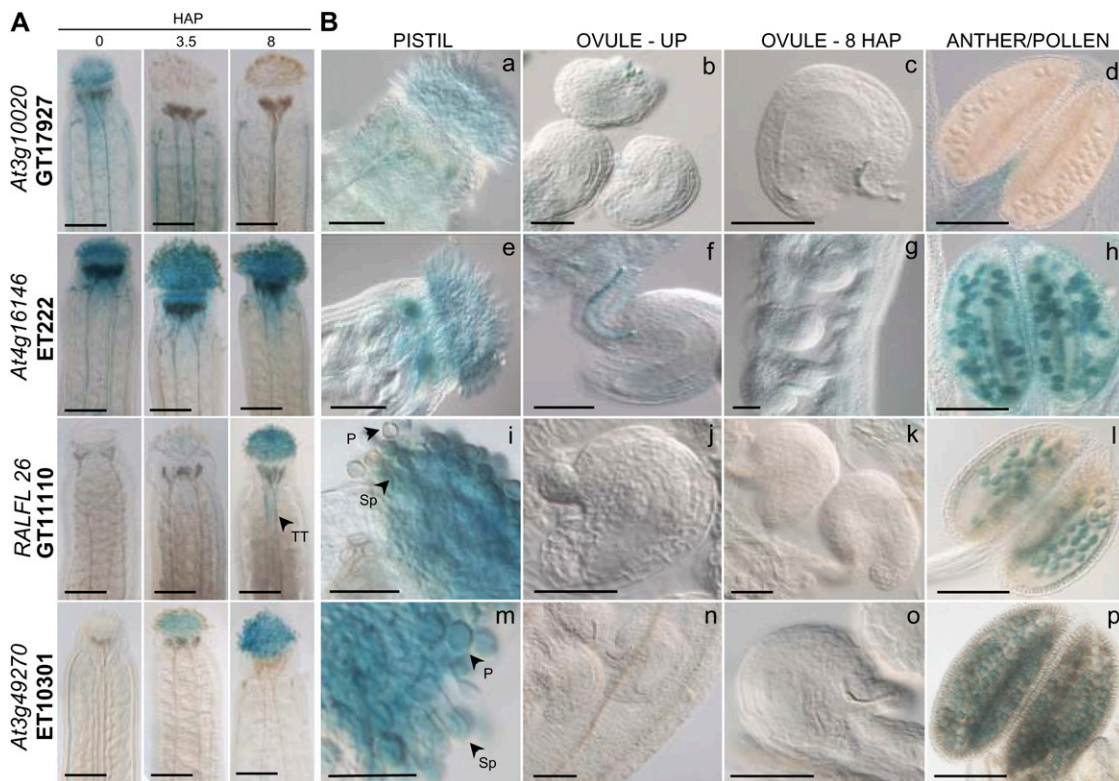
Figure 4. Histochemical detection of GUS enzymatic activity in gene trap (*DsG*) and enhancer trap (*DsE*) lines. A, Young pistils were emasculated and pollinated 24 h later. GUS activity was assayed at specific time points after pollination (0, 3.5, and 8.0 HAP) for each insertion line. B, GUS activity was assayed in different tissues: UP (a and e), 8HAP pistils (i and m), unfertilized ovules (b, f, j, and n), 8HAP fertilized ovules (c, g, k, and o), and dehiscent anthers/mature pollen (d, h, l, and p). Arrowheads show pollen (P), stigma papillae (Sp), and transmitting tract (TT). Bars = 400 μm for A and for B as follows: a, e, m, and i, 100 μm; b, c, f, g, j, k, n, and o, 50 μm; d, h, l, and p, 400 μm. [See online article for color version of this figure.]

We think that the discrepancies observed in seedling expression may reflect biological variation and may be due to different developmental stages used in the microarray data sets and in the RT-PCR. In a few cases, we were able to detect a faint band by RT-PCR in saturation conditions upon 35 cycles of amplification for some transcripts called absent by the MAS5 algorithm, suggesting limitations in the sensitivity of microarrays to detect very low expressed genes. In addition to the RT-PCR, we selected 12 transposon lines from a collection of gene (*DsG*) and enhancer trap (*DsE*) lines (Sundaresan et al., 1995; Supplemental Table S2). These lines were selected based on availability, position, and orientation of the GUS construct within the genes as well as differential expression along the time course. No phenotypical defects in plant fertility or deviation in kanamycin resistant/kanamycin sensitive (Kan^R/Kan^S) segregation ratios (3:1) were associated with any of the insertion lines. GUS activity was only detected in inflorescence tissue of four lines, GT17927, GT11110, ET222, and ET10301, with insertions in *At3g10020*, *At3g25170* (*RAFL26*), *At4g16146*, and *At3g49270*, respectively (Supplemental Table S2). The changes in gene expression during the

time course were confirmed in pollinated pistils and monitored by GUS staining (Fig. 4).

GT17927 corresponds to an insertion in the first exon of *At3g10020*, an unknown gene that, according to our microarray data, was highly expressed in UP but down-regulated after pollination. GUS expression was detected in stigma and style TT as well as in ovary and weakly in the sporophytic tissues of ovules, consistent with the pathway taken by the pollen tubes. The GUS expression clearly decreases after pollination, being almost undetectable at 8.0HAP (Fig. 4A), suggesting a possible role during pollen tube growth or guidance. A reduction of the expression level expected for *At4g16146* was less evident in ET222, where GUS expression was predominant in pollen and stigma throughout the time course analyzed. The transposon was confirmed to be inserted in an intron but in an opposite orientation to the *At4g16146* coding region; thus, GUS localization for this line might not be as comprehensible. However, it is possible that the GUS expression could be induced by a proximal enhancer sequence derived, for example, from an antisense transcript. In the GT11110 and ET10301 insertion lines, the transposons are located in the exons of
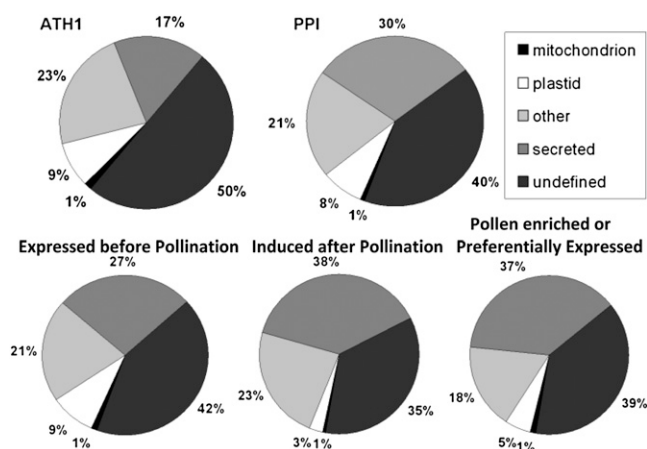
Figure 5. Predictions of subcellular localization for proteins regulated during pollen-pistil interactions. Wedges indicate the percentage of proteins assigned to each subcellular localization according to The Arabidopsis Information Resource 9.0 prediction. Whole charts represent proportions in the ATH1 array, PPI (genes regulated during pollen-pistil interactions), and in categories of genes expressed before pollination, induced after pollination, and in the subset of genes pollen enriched or preferentially expressed and regulated during the time course.

RALFL26 (for rapid alkalinization factor-like family-like 26; *At3G25170*) and an unknown protein (*At3g49270*), respectively. In both lines, GUS staining was not detected in UP but was present in mature pollen (Fig. 4B, l and p). A slight GUS activity was detected in the stigma at 3HAP, while at 8HAP, when most pollen tubes have discharged their cytoplasmic contents in the embryo sac, GUS expression significantly increased in stigma papilla (GT11110 and ET10301; Fig. 4, A and B) and in the upper part of the TT (GT11110; Fig. 4A), confirming up-regulation of these genes in female tissues, as expected from the microarray data. The regulation of GUS activity in these insertion lines is compatible with a function of the targeted genes in the support or modulation of pollen tube growth. In the GT11110 and ET10301 insertion lines, the gene function might be associated with postpollination responses such as cell wall modifications or degradation of proteins related to pistil receptivity.

Based on these results, we conclude that our microarray data reflect with confidence the dynamics and strict regulatory control occurring during pollen-pistil interactions and can be used to predict potential molecular interactions associated with specific cellular events during the reproductive process and in terms of temporal clustering patterns (Fig. 2).

## Swinging Partners: Regulation of Signal Transduction and Cell Wall

Cell-to-cell communication between pollen tubes and their partner cells in the pistil involves signaling events that take place across cell walls. The kinetics of

regulation observed in the time-course experiment supports the hypothesis that many of the regulated transcripts (transcripts in which a change in abundance was detected across the different time points; greater than 1.2-fold change) may be locally produced to mediate specific interactions between the pollen tube and particular cell types of the pistil. During pollen tube growth, the pistil extracellular matrix plays a variety of roles in addition to its structural function. It can act as a conductor of signals, as a source of signals, and also as an extracellular domain for plasma membrane receptors (Brownlee, 2002). On the ATH1 genome array, about 17% of the probe sets represent transcripts encoding proteins predicted to contain an N-terminal signal peptide, and according to our data set, 30% of the regulated transcripts encode proteins predicted to follow the secretory pathway (Fig. 5). These are similarly represented in transcripts "expressed before pollination" (27%), "induced after pollination" (38%), or in the subset of transcripts identified in the pollen data sets as "enriched and preferentially expressed" in pollen but that can also have been induced in pistil tissues during pollen tube growth (37%). Many of these genes encode for small proteins that are likely to function as ligands or extracellular signal molecules. Such examples include transcripts belonging to the clavata family (*CLE21*), the phytosulfokine 1 precursor (*PSK1*), a low-molecular-weight Cys-rich protein (*LCR59*), the COBRA-like family (*COBL5*, *COBL6*, *COBL10*), and members of the *RALFL* family (*At4g14020*, *RALFL4*, *RALFL9*, *RALFL18*, *RALFL26*), some of which are preferentially enriched in the female gametic cells and are induced during later stages of pollen tube growth in the ovary (Supplemental File S4; Jones-Rhoades et al., 2007; Wuest et al., 2010). Interestingly, a 15-amino acid secreted peptide (C-terminally encoded peptide 1), which was shown to cause growth arrest when applied externally to roots (Ohyama et al., 2008), seems to be induced at 8.0HAP.

Based on the classification of the Cell Wall Genomics (http://cellwall.genomics.purdue.edu/) database, 131 (9.5%) of the regulated genes represent cell wall-related components. Overrepresented families include members of the polygalacturonase family, glucorosyltransferases, glycosyl hydrolases, expansins, extensins, arabinogalacturans (AGPs) and fasciclins, COBRAs, pectate lyases, and pectinesterases (Fig. 6A). These proteins, in particular glycosylphosphatidylinositol-anchored proteins, are thought to play important roles in cell wall remodeling, cell expansion, or mediating cell-to-cell signaling interactions (Borner et al., 2003). Among these, we identified seven predicted AGPs, four pistil expressed (*AGP2*, *AGP6*, *AGP7*, *AGP23*) and three preferentially expressed or enriched in pollen (*AGP11*, *AGP22*, *AGP40*). Additionally, we identified five fasciclins, four expressed in the pistil (*FLA1*, *FLA2*, *FLA7*, *FLA15*) and one pollen specific (*FLA3*). Fasciclins were implicated in growth cone guidance in *Drosophila* (Grenningloh et al., 1991) as well as signal-

Figure 6. Signal transduction and cell wall families regulated during pollen-pistil interactions. A, Cell wall families. B, Signal transduction families. Values represent percentages of genes assigned to each family on the ATH1 array and regulated during pollen-pistil interactions, as indicated. [See online article for color version of this figure.]
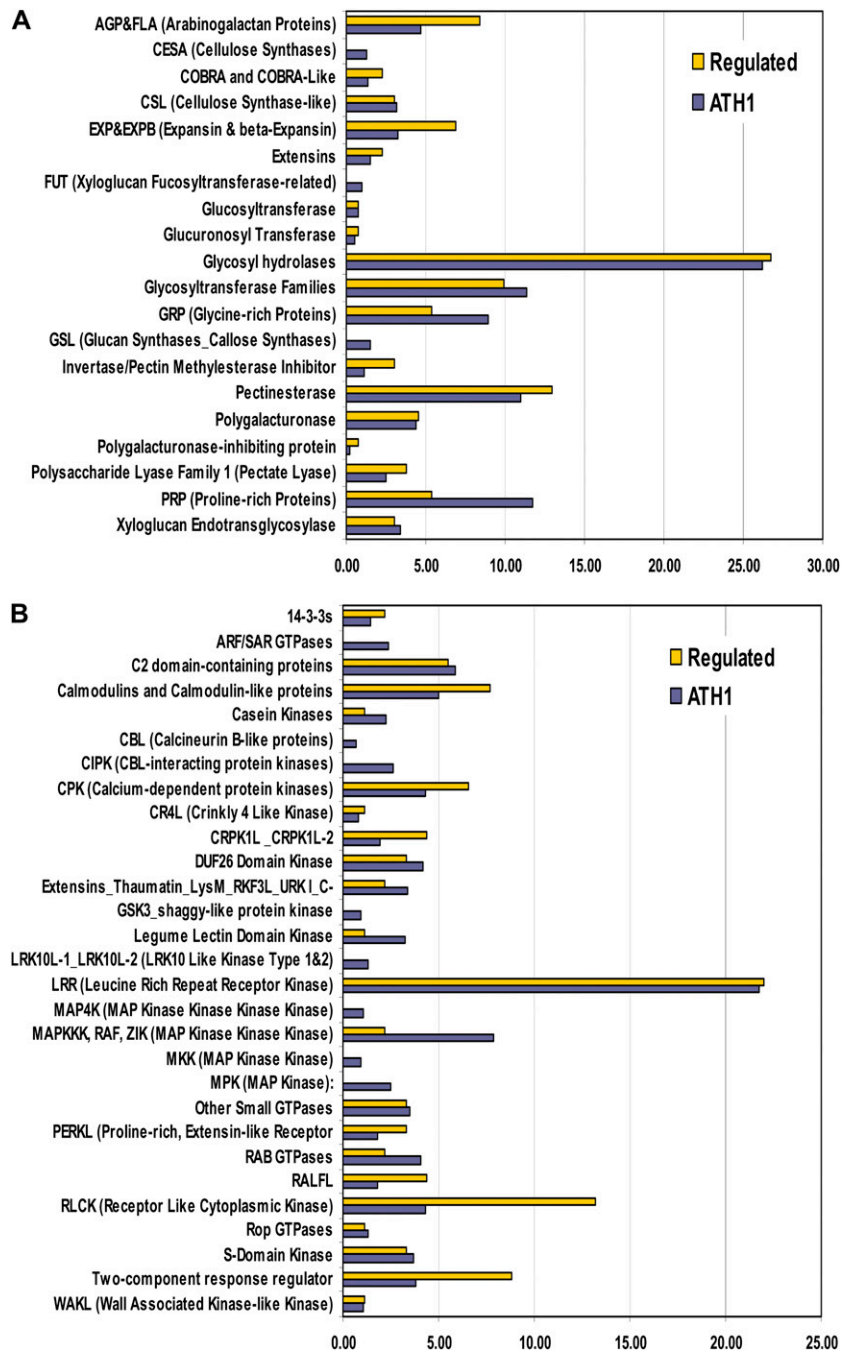
ing via Tyr kinases (Elkins et al., 1990) and represent good candidates to function as receptors or cell adhesion molecules. We also found that 89 (6.5%) of the regulated transcripts encode signal transduction components. Overrepresented families include the 14-3-3 proteins, calcium- and calmodulin-related proteins, CRPK1L, LRRs, Pro-rich and extensin-like kinases, RALFLs, receptor-like cytoplasmic kinases, and members of the two-component signaling transduction system (Fig. 6B). Receptor kinases located at the plasma membrane are particularly interesting, since they trans-

duce external signals via activated signaling pathways (Morris and Walker, 2003). Such examples include the PRKs belonging to the LRR family involved in the regulation of pollen tube growth in tomato (*Solanum lycopersicum*) and petunia (*Petunia hybrida*; Muschietti et al., 1998; Tang et al., 2004), but so far, no receptor kinases were identified in the female tissues. Here, we identify several kinases expressed in female tissues. This induction (or regulation) in different time points and/or expression in a particular tissue is compatible with a possible function in a specific cellular stage

during the reproductive process (Fig. 5; Supplemental Table S4).

These localized cellular interactions are known to define unique checkpoints along the pollen tube pathway in self-compatible crosses. However, these interactions are also often the reason for the failure of a successful fertilization upon self-incompatible or interspecific and intergeneric crosses (Higashiyama et al., 2006; Escobar-Restrepo et al., 2007). Understanding the molecular basis of postpollination events in compatible crosses will undoubtedly provide new insights in how self-incompatibility and interspecific responses are regulated, as evidence emerges supporting an intrinsic link between the cellular pathways operating in both systems (Samuel et al., 2009).

Signal Integration and Cross Talk: The Key for Reproductive Success?

Several metabolic pathways were differentially regulated during the progamic phase. Components of PSI and PSII light-harvesting complex systems and chlorophyll-associated genes were predominantly down-regulated, indicating a general trend to shut down photosynthesis during pollen tube growth (clusters 13 and 14). Other components of metabolic pathways, such as the glycolytic and starch degradation pathways, general carbohydrate and polysaccharide metabolism, phosphate, amino acid, secondary metabolism (phenylpropanoid, stilbenes, and flavonoids), and hormone metabolism, were positively regulated (clusters 5–9).

Based on the Arabidopsis Transcription Factors Database (http://arabidopsis.med.ohio-state.edu/), 84 transcription factors are regulated during the reproductive process. Several transcription factor families were significantly represented in the data set, such as bHLH, MYB, ARR, GRAS, GRF, SBP, TVP trihelix, and ABI3/VP1, while several other members, such as YABBY and MADS box transcription factor families, which were previously reported to be important during the reproductive process (Hennig et al., 2004), were poorly represented or absent. It is possible that the function of these transcription factors may be related to carpel and ovule development, since the previous study compared immature pistils, fertilized pistils, and siliques, while our analysis was performed on a shorter temporal developmental window (time course of 8 h). Some of these transcription factors are coexpressed in different clusters (Supplemental File S5), suggesting that they might be involved in the regulatory control of particular genetic pathways, alone or through combinatory interactions (Singh, 1998). As an example, TCP21, REM16, and two MYB transcription factors (MYB70 and MYB123) may represent the major regulators of clusters 2 and 3 (Supplemental File S5).

Among the regulated transcripts, we found a significant representation of transcriptional regulators, receptors, response factors, and transporters related to hormonal regulation (Supplemental Fig. S1, A–C). Hormones usually do not function in discrete pathways but rather exhibit extensive cross talk and signal integration with each other and with environmental and developmental signaling pathways (Lorenzo et al., 2003; Nemhauser et al., 2004). Therefore, their dynamic regulation may indicate that a precise control of the local concentrations for defined hormonal metabolites might be essential to regulate downstream signaling events along the pollen tube pathway. Several studies suggested that certain hormones such as ethylene, auxin, brassinosteroids, and methyl jasmonate could act as primary signals in the pollination response (O'Neill, 1997). Supporting an important role of hormones in the success of the reproductive process (Aloni et al., 2006; Chen and Zhao, 2008), we identified several biosynthetic and signaling-related components of the auxin (Supplemental Fig. S1B) and ethylene (Supplemental Fig. S1C) pathways within the classes expressed before pollination, which seem to be differentially regulated or induced in specific stages of pollen tube growth. A good example of auxin/ethylene cross talk is the induction during pollen tube growth in the style of a 1-aminocyclopropane-1-carboxylate synthase (At4g37770), a main component of ethylene biosynthesis and known to be auxin responsive (Supplemental Fig. S1C; Supplemental File S2). Cross talk of cytokinin and auxin in the regulation of cell division is another well-known process, namely in specific cell-cycle transitions (Hartig and Beck, 2006). This cross talk seems to be important for the regulation of expression and activity of CdkA at the G2/M transition (Sorrell et al., 2001) and to induce the expression of the D3 cyclins at the G1/S transition (Riou-Khamlichi et al., 1999). Our data show that the regulatory kinetics of cytokinin-related genes (Supplemental Fig. S1A) resembles mostly that found in cluster 1, where there is also a significant enrichment for transcripts related to cell cycle and DNA processing. According to the classification of Shultz et al. (2007), 16 out of 48 ATH1-represented transcripts encode proteins associated with DNA replication regulated during the reproductive process (Table II). Interestingly, with the recent description of Arabidopsis male and female gamete transcriptomes, we verified that, in fact, many of these transcripts are enriched in sperm cells (Borges et al., 2008) and expressed in the egg and central cell (Wuest et al., 2010; Table II). This is exemplified by a cytokinin-induced *CYCD3;1* (*At4g34160*), known as a key regulatory element for the G1/S transition of the cell cycle (Menges et al., 2006), and a member of CdkA, *CYCA2;4* (*At1g80370*), known as a major regulator of the G1/S checkpoint. Both are down-regulated during early stages of pollen tube growth in the pistil but up-regulated at 8.0HAP. Given this association, an involvement of these transcripts, either in synchronization of female and male gametes in the cell cycle prior to fertilization and karyogamy (Friedman, 1999) or upon successful fertilization during early zygote and endosperm patterning, is plausible.

Table II. *Time-course regulation of genes assigned to cell cycle and DNA processing*

Genes were classified according to molecular function in the DNA core replication machinery. The number of the hierarchical cluster for the time-course analysis is indicated for each gene, and genes were classified according to reproductive tissue expression, enrichment, and selectivity. Male and female gamete expression is according to Borges et al. (2008) and Wuest et al. (2010), respectively. Signal intensities are indicated for each time point.

Core DNA Replication Machinery	Arabidopsis Genome Initiative Identifier	Cluster	Ovules Expressed	Ovules Enriched	Female Gamete Expressed	Pollen Expressed	Pollen Enriched	Preferentially Expressed in Pollen	Sperm Expressed	Signal			
										0HAP	0.5HAP	3.5HAP	8.0HAP
Origin recognition complex (ORC)													
ORC2	At2g37560	6	x	x	x	x			x	151.3	146.0	150.9	251.4
ORC6	At1g26840	5					x		x	97.4	172.4	171.0	348.7
NOC3	At1g79150	2	x		x				x	663.4	649.8	379.7	1020.4
Replicative helicase complex (MCM)													
MCM2	At1g44900	3		x	x				x	245.9	235.0	194.9	403.2
MCM3	At5g46280	1		x	x				x	573.0	585.7	491.9	868.4
MCM4	At2g16440	3		x	x				x	618.3	617.9	562.1	959.0
MCM5	At2g07690	4	x						x	336.4	247.5	185.5	419.4
MCM7 (PROLIFERA, PRL)	At4g02060	2		x	x				x	802.1	757.7	536.7	1,042.6
MCM helicase loading (licensing) factor													
CDT1A	At2g31270	5	x		x				x	295.0	320.4	303.2	554.2
DNA replication initiation factor													
CDC45	At3g25100	1	x		x				x	287.4	385.8	278.5	500.4
DNA synthesis:DNA polymerase α-complex													
POLA1	At5g67100	2		x					x	312.9	354.2	221.3	428.9
POLA3	At1g67320	4		x	x				x	513.0	445.2	366.7	640.3
POLA4	At5g41880	1		x	x				x	500.6	590.5	455.9	908.6
Single-stranded DNA binding													
RPA1B	At5g08020	5	x							196.9	266.3	217.7	498.7
RPA1D	At5g61000	5	x		x				x	344.8	401.0	385.1	685.4
RPA2A	At2g24490	3		x	x				x	465.6	481.9	439.2	921.5
Other													
CYCD3;1	At4g34160	4	x							599.9	377.1	273.5	490.0
CYCA2;4	At1g80370	4	x		x				x	333.0	293.8	221.1	386.9
¹H-3	At2g18050	13		x						171.5	143.5	152.8	59.7
NAP1;2	At2g19480	2	x		x				x	2,047.7	2,185.4	1,536.1	2,494.4
Nucleolar protein, putative	At4g26600	4								253.6	184.9	124.8	249.7
HTA10	At1g51060	5			x				x	1,817.0	2,346.0	1,959.3	3,010.3
PHR1	At1g12370	5			x		x		x	292.5	501.3	373.2	939.2
RH18	At5g05450	1	x		x					237.5	292.0	202.6	351.3
NAP57	At3g57150	1	x		x				x	1,734.5	1,874.1	1,302.5	2,978.2

CONCLUSION

A spatial and temporal transcriptional study was performed in order to follow variations in gene expression and de novo transcription through different cellular events occurring from pollination to fertilization. This way, we could complement earlier work that focused on individual transcriptomes of isolated gametophytes or on specific aspects of pollen tube growth with that of a complete in vivo time-series profiling that analyzes the regulation of genes of all biological players in specific cellular events. Consequently, we were able to identify 1,373 transcripts differentially regulated up to 8 h after pollination. Namely, we identified several signaling and cell wall-related transcripts whose regulation over time and preferential expression in a particular reproductive tissue may be correlated with activation in a specific cellular event during the reproductive process. Our results also suggest that pathways responsible for the production of certain metabolites and hormones require a precise molecular control and balanced distribution, ensuring the correct physiological and metabolic cellular environment for the success of the reproductive process. We believe that our results can provide a roadmap to detailed genetic dissection of the progamic phase.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Seeds of Arabidopsis (*Arabidopsis thaliana* ecotype Columbia [Nottingham Arabidopsis Stock Centre]) were sown on soil and kept for 3 d at 4°C in the dark to promote seed stratification. Seedlings were grown in short-day conditions (8 h of light/16 h of dark at 22°C–24°C) for 2 weeks and then transferred to long-day conditions (16 h of light/8 h of dark at 22°C–24°C) to induce flowering. Flowers at developmental stage 12c (Smyth et al., 1990) were emasculated 24 h before pollination. Pistils were collected at 0, 0.5, 3.5, and 8 HAP and immediately frozen in liquid nitrogen. Two pistils from each time point were collected and stained with 0.1% decolorized aniline blue (Martin, 1959) as a control for pollen tube growth. Unfertilized ovules were collected by the funiculus from dissected UP and immediately frozen in liquid nitrogen. To minimize biological variation, 20 pistils were collected from a minimum of 10 plants, and for ovule isolation, 50 pistils were used from about 30 plants to isolate approximately 1,500 ovules for each replicate experiment.

RNA Isolation, Target Synthesis, and Hybridization to Affymetrix GeneChips

Total RNA was extracted from tissues using the RNeasy Mini Plant Kit (Qiagen). Concentration and purity were determined by spectrophotometry, and integrity was confirmed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano assay (Agilent Technologies). RNA was processed for use on Affymetrix Arabidopsis ATH1 Genome Arrays, according to the manufacturer's Small Sample Labeling Protocol version II, described by Pina et al. (2005). Arrays were scanned in Affymetrix GeneChip scanner 2500.

GeneChip Data Analysis

We used the Arabidopsis ATH1 GeneChip representing 22,392 unique genes to obtain the transcriptional profiles of UP and unfertilized ovules and of pistils at 0.5, 3.5, and 8.0 HAP. Scanned arrays were analyzed first with Affymetrix MAS 5.0 software to obtain absent/present calls, and subsequent analysis was performed with dChip 1.3 (http://www.dchip.org; Wong Laboratory, Harvard University), applying the normalization parameters described by Pina et al. (2005). To ensure reliability of the analyses, each GeneChip experiment was performed with two biological replicates.

For the identification of reproductive organ-specific and -enriched genes, we combined our samples with data sets from leaves, seedlings, pollen, and siliques (Pina et al., 2005) to identify stage-specific reproductive organ-enriched genes.

Two main criteria were used to define with high stringency the set of genes showing differential expression between these experimental data sets. As an independent analysis method, significance analysis of microarrays (http://www-stat.stanford.edu/~tibs/SAM; Department of Statistics, Stanford University; Tusher et al., 2001; Storey, 2002, Leek et al., 2006) was used in the normalized signal intensities to detect significant differences in gene expression using pairwise comparisons, with a false discovery rate cutoff of 0.5% or less, to reveal genes showing differential gene expression. Next, to conservatively choose genes that showed consistent expression changes between tissues, we selected transcripts for which the absolute value of the LCB was above 1.2-fold, as discussed elsewhere (Pina et al., 2005; Johnston et al., 2007), to identify 18,413 genes showing significant expression changes for organ-enriched genes. Genes preferentially expressed in particular tissues were defined if present in only one of the tissues analyzed, based on comparisons of our samples with previous microarray data (Pina et al., 2005). Genes considered preferentially expressed were then uploaded on Genevestigator software (Zimmermann et al., 2004) and compared with high-quality arrays from the AtGenExpress database (Schmid et al., 2005), from samples representing different developmental stages of Arabidopsis. Samples containing carpels, flowers, seeds, and siliques were excluded. Using the Digital Northern tool, genes called present with $P < 0.05$ in at least two of the three replicates were excluded from the preferentially expressed organ data sets. Transcripts that fulfilled the first criterion but were called present in at least one other tissue were classified as enriched. The remaining genes were scored as expressed. However, for the ovule data sets, a careful analysis was performed to identify genes preferentially expressed in a particular organ due to a possible overlap in gene expression between the UP and ovule data sets. We hypothesized that highly expressed transcripts in ovule would be detected in UP with a relative dilution effect. To identify those genes, we estimated the relative contribution of ovule gene expression to UP, based on genes only called present in ovules (43 genes). The average expression value was calculated and compared with the value obtained for the same set of genes in UP. Based on this difference, the contribution of genes preferentially expressed in ovules was estimated at 46% ± 13%. Thus, ovule-enriched transcripts in which the ratio UP-ovule was less than the threshold (less than 46%) and called present in UP and ovules represent transcripts preferentially expressed in ovules (48 genes). For the time-course analysis, significant analysis of microarrays for time-course experiments (EDGE; Leek et al., 2006) and a false discovery rate of 0.5% or less were used to identify 5,225 genes with significant expression changes in the time-course data set. A more stringent value of LCB ≥ 1.5 was used for the time-course analysis to better ensure biological significance. The LCB index was used rather than "fold change" because LCB takes the spread of the data into account and thus is a more robust index for the prediction of transcriptional changes (Li and Hung Wong, 2001). The coefficient of variation obtained after statistical treatment was found to be less than 0.05 between each pair of biological replicate experiments, revealing consistently good quality of the data.

PCA and hierarchical clustering of time-course experiments was performed using Partek Genomics Suite 6.07 (Partek). For hierarchical clustering analysis, Pearson's dissimilarity was used to calculate row dissimilarity and Ward's method was used for row clustering. To group genes with similar expression patterns, clustering analysis was performed using the Self-Organizing Tree Algorithm (SOTA; Herrero et al., 2001), available as a Web-based resource in GEPAS (for Gene Expression Profile Analysis Suite 1.1; http://gepas.bioinfo.cipf.es/; Herrero et al., 2003). Annotations for the 22,325 genes represented on the Arabidopsis ATH1 Genome Array are based on Gene Ontology annotations of The Arabidopsis Information Resource release 8. Genes were classified into functional categories using the Munich Information Center for Protein Sequences functional classification scheme using Virtual Plant 1.0 (http://virtualplant.bio.nyu.edu/cgi-bin/vpweb2/).

Time-course regulation of auxin and ethylene-related transcripts were represented using an adapted version of the auxin (Lau et al., 2008) and ethylene (Olmedo et al., 2006) pathways and displayed using MAPMAN application software (Thimm et al., 2004).

RT-PCR Analysis

Nonhybridized complementary RNA from one replicate of each sample used for the time-course analysis (UP and pistils at 0.5HAP, 3.5HAP, and 8.0HAP) and additionally from ovules, pollen, silique, leaf, and seedling samples were reverse transcribed using the SuperScript II system (Invitrogen)

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according to the manufacturer's instructions with random primers in a 20-μL reaction. Five nanograms of each template cDNA was used in a standard PCR of 35 cycles. The sequences of all primers and the description of the genes tested by RT-PCR are available in Supplemental Table S2.

GUS Staining

Twelve genes represented in the time-course clusters were selected according to significant variation of gene expression pattern and availability in the collection of *DsE* and *DsG* trap lines at Cold Spring Harbor Laboratory (http://genetrap.cshl.org/; Sundaresan et al., 1995). The tagged genes were confirmed by TAIL-PCR using degenerated and *Ds*-specific primers as described by Liu et al. (1995; Supplemental Table S2). Time-course pollinations were performed as described before for the microarray experiments, and the GUS expression pattern was monitored in dissected pistils at 0, 3.5, and 8.0 HAP. For GUS staining, the samples were processed as described previously by Sundaresan et al. (1995) but excluding the potassium ferricyanide and ferrocyanide agents.

All microarray data were submitted to Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the series accession number GSE27281.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Time-course regulation of hormone-related transcripts.

Supplemental Table S1. Functional classification of gene clusters.

Supplemental Table S2. List of primers used for RT-PCR analysis and *Ds* insertion lines.

Supplemental File S1. Reproductive tissue expression profile.

Supplemental File S2. Expression profile of genes regulated during pollen-pistil interactions.

Supplemental File S3. Clustering analysis of 1,373 genes regulated during pollen-pistil interactions.

Supplemental File S4. List of regulated genes predicted to encode secreted proteins.

Supplemental File S5. Transcription factors regulated during the time course represented by gene families.
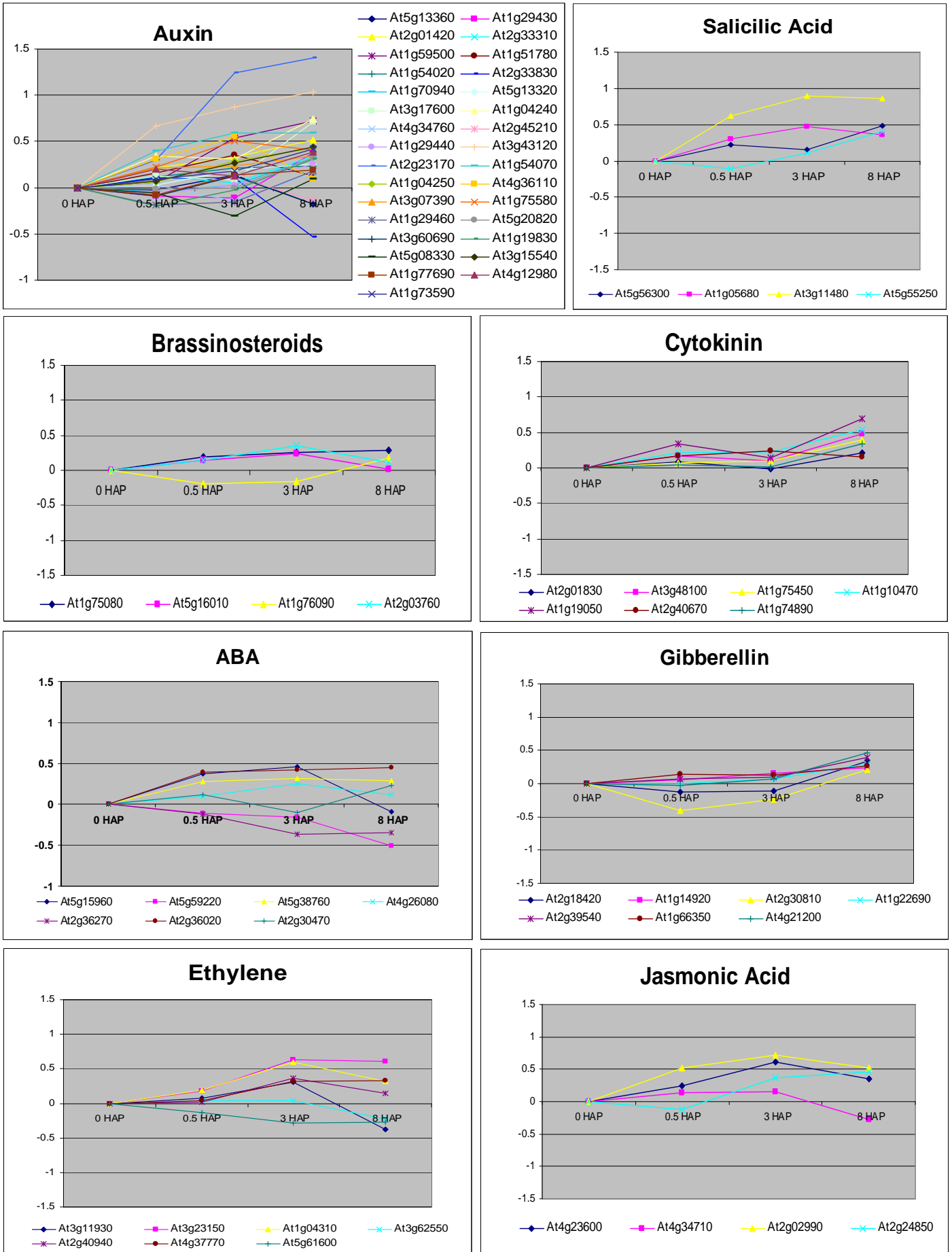
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A

Auxin

At5g13360	At1g29430
At2g01420	At2g33310
At1g59500	At1g51780
At1g54020	At2g33830
At1g70940	At5g13320
At3g17600	At1g04240
At4g34760	At2g45210
At1g29440	At3g43120
At2g23170	At1g54070
At1g04250	At4g36110
At3g07390	At1g75580
At1g29460	At5g20820
At3g60690	At1g19830
At5g08330	At3g15540
At1g77690	At4g12980
At1g73590	

Salicilic Acid

At5g56300, At1g05680, At3g11480, At5g55250

Brassinosteroids

At1g75080, At5g16010, At1g76090, At2g03760

Cytokinin

At2g01830, At3g48100, At1g75450, At1g10470, At1g19050, At2g40670, At1g74890

ABA

At5g15960, At5g59220, At5g38760, At4g26080, At2g36270, At2g36020, At2g30470

Gibberellin

At2g18420, At1g14920, At2g30810, At1g22690, At2g39540, At1g66350, At4g21200

Ethylene

At3g11930, At3g23150, At1g04310, At3g62550, At2g40940, At4g37770, At5g61600

Jasmonic Acid

At4g23600, At4g34710, At2g02990, At2g24850

Supplemental Figure S1. Kinetics of regulation of hormone-related transcripts during pollen-pistil interactions

AUXIN
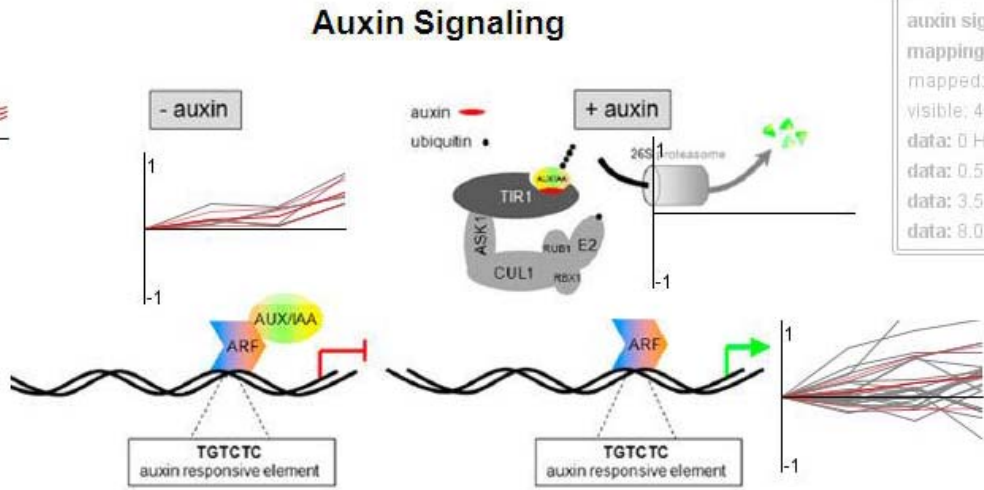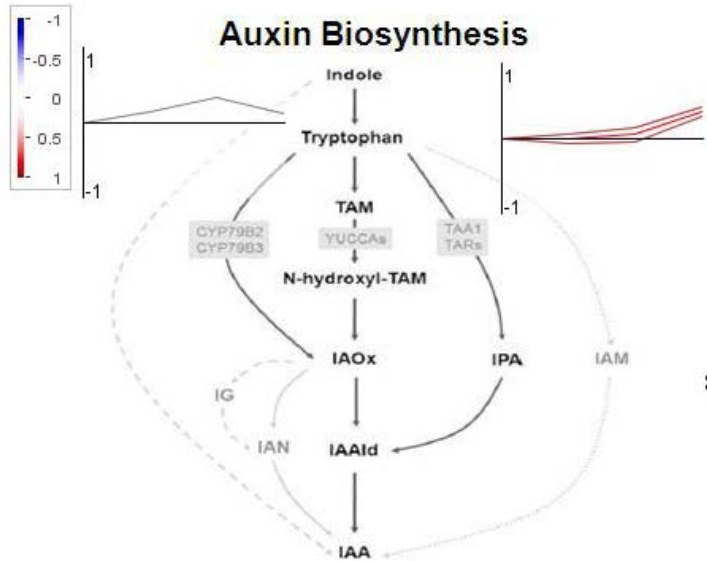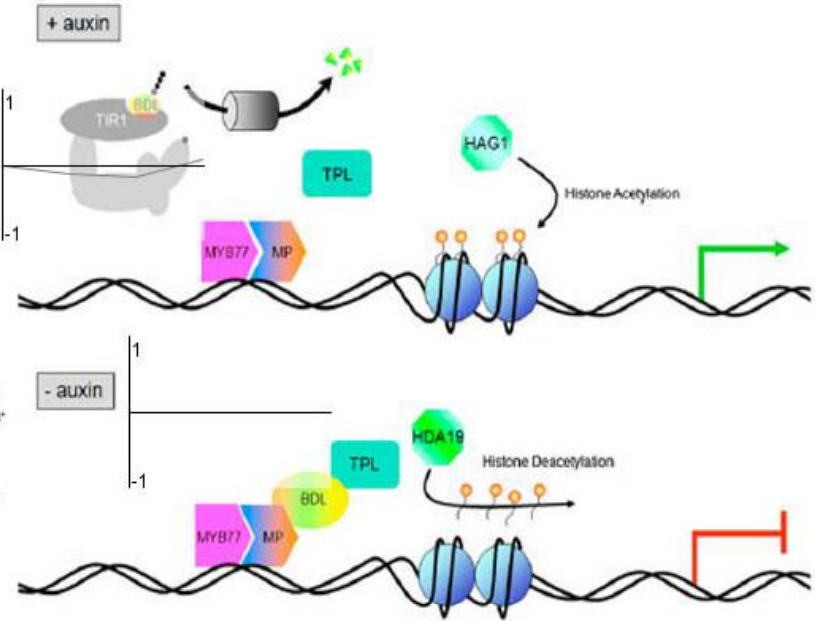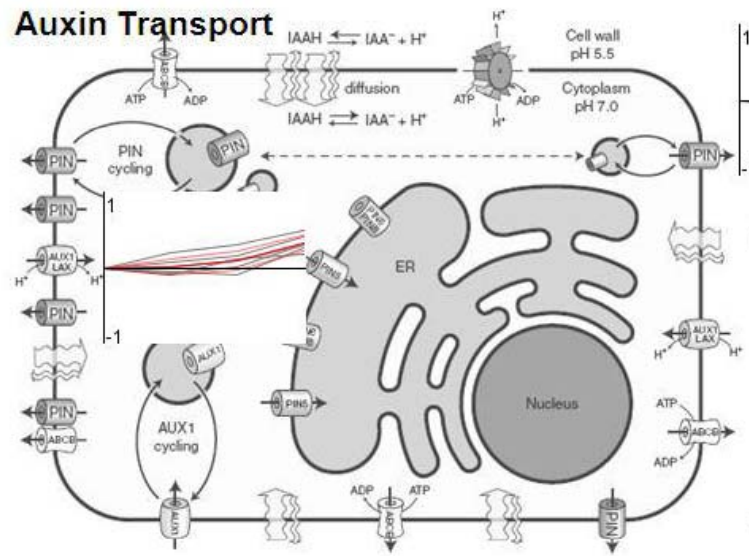
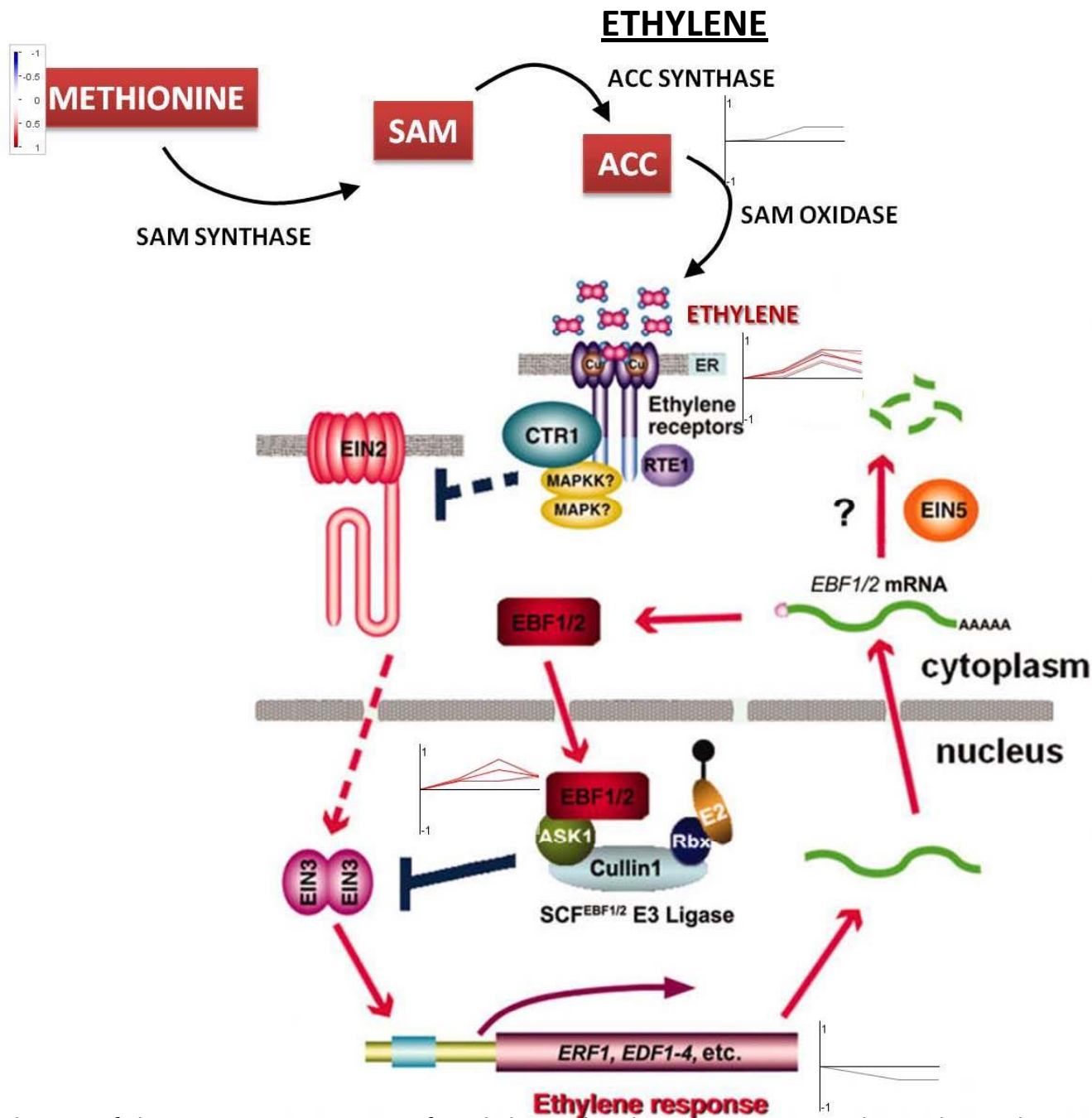Visualization of changes in gene expression for AUXIN related transcripts organized according with gene function using MAPMAN software.
Adapted from Lau *et al.* (2008)

(Thimm et al, 2004)

ETHYLENE

Visualization of changes in gene expression for Ethylene related transcripts organized according with gene function using MAPMAN software.
Adapted from Olmedo *et al.* (2006)