Integrating Membrane Transport with Male Gametophyte Development and Function through Transcriptomics[1][W]

Kevin W. Bock, David Honys, John M. Ward, Senthilkumar Padmanaban, Eric P. Nawrocki[2], Kendal D. Hirschi, David Twell, and Heven Sze*

Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, Maryland 20742–5815 (K.W.B., S.P., E.P.N., H.S.); Laboratory of Pollen Biology, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, 16502 Prague 6, Czech Republic (D.H.); Department of Plant Physiology, Charles University, 128 44 Praha 2, Czech Republic (D.H.); Department Plant Biology, University of Minnesota, St. Paul, Minnesota 55108 (J.M.W.); Department of Pediatrics and Department of Human and Molecular Genetics, Children's Nutrition Research Center, Baylor College of Medicine, Houston, Texas 77030 (K.D.H.); and Department of Biology, University of Leicester, Leicester LE1 7RH, United Kingdom (D.T.)

Male fertility depends on the proper development of the male gametophyte, successful pollen germination, tube growth, and delivery of the sperm cells to the ovule. Previous studies have shown that nutrients like boron, and ion gradients or currents of Ca^{2+}, H^+, and K^+ are critical for pollen tube growth. However, the molecular identities of transporters mediating these fluxes are mostly unknown. As a first step to integrate transport with pollen development and function, a genome-wide analysis of transporter genes expressed in the male gametophyte at four developmental stages was conducted. Approximately 1,269 genes encoding classified transporters were collected from the Arabidopsis (*Arabidopsis thaliana*) genome. Of 757 transporter genes expressed in pollen, 16% or 124 genes, including *AHA6*, *CNGC18*, *TIP1.3*, and *CHX08*, are specifically or preferentially expressed relative to sporophytic tissues. Some genes are highly expressed in microspores and bicellular pollen (*COPT3*, *STP2*, *OPT9*), while others are activated only in tricellular or mature pollen (*STP11*, *LHT7*). Analyses of entire gene families showed that a subset of genes, including those expressed in sporophytic tissues, was developmentally regulated during pollen maturation. Early and late expression patterns revealed by transcriptome analysis are supported by promoter::*β*-glucuronidase analyses of *CHX* genes and by other methods. Recent genetic studies based on a few transporters, including plasma membrane H^+ pump AHA3, Ca^{2+} pump ACA9, and K^+ channel SPIK, further support the expression patterns and the inferred functions revealed by our analyses. Thus, revealing the distinct expression patterns of specific transporters and unknown polytopic proteins during microgametogenesis provides new insights for strategic mutant analyses necessary to integrate the roles of transporters and potential receptors with male gametophyte development.

Male fertility in plants depends on several critical events, beginning with the proper development of the male gametophyte, the transfer of mature pollen to a compatible stigma, and subsequent germination and pollen tube growth through the style. When the pollen tube reaches the ovule, it ruptures and delivers two sperm cells that result in double fertilization of egg and central cells (Lord and Russell, 2002). Although the major events of male reproductive development are well known, the molecular and cellular bases of these processes are still poorly understood (Twell, 2002; McCormick, 2004). Microgametogenesis starts with a single haploid microspore that divides and develops into a mature pollen grain consisting of a large vegetative cell, plus one generative cell or two sperm cells. Pollen germination and tube growth are manifestations of a single vegetative cell as it carries and delivers the sperm cells to the ovule. Thus, the development and the physiology of the male gametophyte is an attractive model to study the molecular and cellular bases of development, stress tolerance, signal transduction, growth, and morphogenesis (Twell, 2002; McCormick, 2004).

It is well recognized that the transport of ions and metabolites is required not only for nutrient uptake and sorting, metabolism, and energy production during plant growth, but also is integrated with signaling, movement, development, and stress tolerance (e.g. Franklin-Tong, 1999; Schwacke et al., 1999). This idea is

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[2] Present address: Center for Genome Sciences, Washington University School of Medicine, Campus Box 8510, 4444 Forest Park Blvd., St. Louis, MO 63108.

* Corresponding author; e-mail hsze@umd.edu; fax 301–314–0981.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Heven Sze (hsze@umd.edu).

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especially well illustrated in the establishment and maintenance of polarity in the growing pollen tube (Weisenseel et al., 1975; Hepler et al., 2001). The current picture emerging is that growth depends on a tip-focused Ca^{2+} gradient and other ion fluxes (for review, see Holdaway-Clarke and Hepler, 2003). Cytosolic [Ca^{2+}] at the tip reaches as high as 5 μM and drops to approximately 0.1 μM away from the apex. However, the Ca^{2+} gradient and the tip-focused acidification are not static and undergo regular oscillations that appear to be in phase with tube growth. Moreover, Ca^{2+} and H^+ influx are maintained as the tube grows. These and other studies suggest that signaling networks modulate a variety of pumps, porters, and channels in time and in space to orchestrate the ion gradients, oscillations, and fluxes (Feijo et al., 2001). Despite this knowledge of pollen tube physiology, we know almost nothing about the molecular nature of the transporters involved and those required for the proper development of the male gametophyte.

To begin integrating transport activities with microgametogenesis and pollen tube growth, we wanted to identify membrane transporters that are used by the male gametophyte. The complete Arabidopsis (*Arabidopsis thaliana*) genome revealed the presence of more than 800 transporters (Arabidopsis Genome Initiative, 2000); however, the biological roles of most genes and their integration with the plant life cycle remain unknown. The availability of the pollen transcriptome using the whole-genome Arabidopsis ATH1 chip reveals for the first time all the genes expressed in the mature male gametophyte (Honys and Twell, 2004; Pina et al., 2005). Moreover, a unique dataset for four stages of male gametophyte development enables developmental analysis of gene expression (Honys and Twell, 2004). These resources present a golden opportunity to study the molecular and cellular bases of cell proliferation, tip growth, gene expression, cell communication, and signaling through analysis of developmental gene-expression profiles.

Although genome-wide transcriptome data are publicly available or published, the wealth of information is obscured by the immense datasets for several reasons. The datasets include genes that are not organized in a biologically relevant manner and often are not well annotated. The data are not normalized using the same method and sometimes show irreproducibility from one laboratory to another. Data-mining tools, though available (e.g. Genevestigator; Zimmermann et al., 2004), are inadequate for bulk analysis of functionally related genes. Therefore, data mining and analyses by experts in the field present a powerful approach to distill significant insights from the large datasets.

Here we have focused on membrane transporters and have identified all those expressed in the male gametophyte, as well as those that are preferentially transcribed in pollen. By following expression over different stages of microgametogenesis, we distinguish between early microspore and pollen-expressed versus late pollen-expressed genes. This analysis provides comprehensive information about which transporter genes within multigene families are developmentally regulated and insight into their potential roles in microspore proliferation, pollen maturation, or pollen tube growth. Recent genetic evidence of transporter function further supports the inferred functions revealed by our analyses. These results therefore generate working ideas for streamlining efforts to uncover the roles of many transporters and putative receptors not only in the male gametophyte but also in sporophytic tissues.

RESULTS

To conduct a meaningful and useful analysis of the pollen transcriptome for all transporters, it was necessary (1) to compile a comprehensive list of all known and putative transporters from Arabidopsis, and (2) to organize the genes according to their gene families and their transporter classification (TC). At the time this project was started, a comprehensive list was not available in the public databases, including the Arabidopsis Membrane Protein Library (AMPL; www.cbs.umn.edu/arabidopsis/), Aramemnon (aramemnon.botanik.uni-koeln.de/), and PlantsT (plantst.genomics.purdue.edu).

Identifying Transporter Genes in Arabidopsis and in Pollen

We used three databases to obtain a comprehensive list of genes encoding polytopic membrane proteins from the completed Arabidopsis genome (Arabidopsis Genome Initiative, 2000). The initial list obtained from AMPL included genes predicting proteins with two or more transmembrane spans (Ward, 2001). Other sequences were added or removed based upon the isospecific homolog clusters from release 3.0 of the Aramemnon membrane protein database (Schwacke et al., 2003) and protein family membership in the TC system at the PlantsT database (Tchieu et al., 2003). Genes encoding proteins with nontransport activities were removed from the list. The final number of transporters is still uncertain as a large number of genes encode proteins annotated as ''expressed'' or ''hypothetical.'' Many genes encoding unclassified proteins were retained for analysis as they could encode potential transporters. In a few cases, peripheral subunits of known multimeric transporter complexes were also included. The final master list of 1,751 sequences includes 1,269 transporters and 482 membrane proteins of unknown function (Supplemental Table I). For simplicity, we refer to this list as the ''master'' list of transporters and unknown polytopic proteins, though the list will be revised as functional studies uncover new transporters. Transporter genes are defined as those encoding proteins that have a TC number or are related to proteins with a TC number. This master sheet is an updated and comprehensive list of all known and potential transporters from Arabidopsis organized using TC system (http://www.tcdb.org/).

Table I. *Number of transporter genes expressed in the Arabidopsis male gametophyte during microgametogenesis*

Results are based on transcriptomic analyses of the male gametophyte over four stages, including uninucleate microspore (MS), bicellular (BC), tricellular (TC), and mature pollen grain (MP), using the Affymetrix ATH1 gene chip (Honys and Twell, 2004). Transporter genes are defined as those encoding proteins that have been classified with a TC number. Unknown genes encode unknown or unclassified polytopic proteins. # Pol-Sp (%) refers to number of pollen-specific genes and relative percentages. # Spec. (%) and # Pref. (%) refer to genes specifically or preferentially expressed in pollen, respectively.

Gene Category	No. Genes	% Genome	% ATH1 Chip	# Pol-Sp (%)
Total genes in Arabidopsis (July 2005 Arabidopsis Report)	30,700	100.0		
Total genes on ATH1 chip	22,591	73.6	100.0	
Genes expressed in male gametophyte	13,977	45.5	61.9	1,355 (9.7)
Genes expressed in:				
Microspore, MS (95%)	11,565		51.2	798 (6.9)
Bicellular, BC (77%)	11,909		52.7	857 (7.2)
Tricellular, TC (88%)	8,788		38.9	703 (8.0)
Mature, MP (98%–100%)	7,235		32.0	625 (8.6)
				# Sp + Pref (%)
Genes encoding transporters and unknown polytopic proteins (master)	1,751	100.0		
Total transporter and unknown protein genes on ATH1 chip	1,511	86.3	100.0	
Classified transporter	1,120			
Unknown or unclassified protein	391			
Transporter and unknown protein genes expressed in pollen	1,046	59.7	69.2	150 (14.3)
Classified transporter	757			124 (16.4)
Unknown or unclassified protein	289			26 (9.0)
Classified transporter genes expressed in pollen	Total	# Spec (%)	# Pref (%)	# Sp + Pref (%)
Microspore stage	609	21 (3.4)	62 (10.2)	83 (13.6)
Bicellular stage	639	29 (4.5)	68 (10.6)	97 (15.2)
Tricellular stage	525	41 (7.8)	68 (13.0)	109 (20.8)
Mature pollen grain	520	40 (7.7)	69 (13.3)	109 (21.0)
All stages	757	48 (6.3)	76 (10.0)	124 (16.4)

Developmental pollen transcriptome data were incorporated into the master list of transporter and unknown protein genes using Microsoft Office Access 2003 SP1, which extracted the normalized data from the pollen transcriptome of Honys and Twell (2004) and linked them to the corresponding genes. Unlike other studies that looked at the genome-wide transcriptome of mature pollen alone (Honys and Twell, 2003; Becker et al., 2003; Zimmermann et al., 2004; Pina et al., 2005), the dataset of Honys and Twell (2004) included expression of all genes at four stages of microgametogenesis, including microspores, bicellular pollen, tricellular pollen, and mature pollen. The expression level of each gene at any stage of pollen development was compared with datasets from 12 sporophytic organs or tissues. "Pollen preferential" was assigned to those genes showing at least a 3-fold increase for the maximum expression signal at any stage of pollen development relative to the highest level in any sporophytic tissue (Supplemental Table I). "Pollen specific" was assigned to genes that showed positive expression at any stage of pollen development and a normalized value of zero for all sporophytic tissues examined.

Of 1,751 total transporter and unknown protein-encoding genes in the Arabidopsis genome, 1,511 were on the ATH1 chip, and 1,046 genes (or 69%) were expressed in developing or mature pollen (Table I). This value seems surprisingly high considering that these genes are expressed by one or two cell types, yet it is consistent with the previous estimation that 62% (or 13,977 genes) of the genome on the ATH1 chip (22,591 genes) is expressed in developing or mature pollen (Honys and Twell, 2004). The total number will increase slightly as some genes are absent from the ATH1 chip or were undetected due to very low expression. Nearly 10% (1,355) of all pollen-expressed genes are specific to the male gametophyte. The list of 1,046 genes expressed in the male gametophyte includes 757 genes that encode channels (11%), porters (61%), or pumps (19%), according to the TC system, plus 289 genes of unknown identity. Approximately 14% of the pollen-expressed transporter and unknown protein genes (1,046) are specifically or preferentially expressed in the male gametophyte using the criteria described above. When genes encoding classified transporters are considered independently, transporters make up 7.1% of the genes expressed in mature pollen relative to 5.3% of the total genes expressed in microspores or bicellular pollen. Furthermore, 13.6% of transporters are specifically or preferentially expressed in microspores; remarkably, the proportion of specific plus preferentially expressed transporter genes increases to approximately 21% (or 109 genes) in tricellular and mature pollen (Table I). Thus, transporter genes as a whole, including pollen-specific ones, are overrepresented as pollen matures.

Transporters Specifically or Preferentially Expressed in Developing Pollen

Table II shows a working list of 124 transporter genes that are defined as specifically or preferentially

Table II. *Genes that are specifically or preferentially expressed in pollen include classified transporters and unknown membrane proteins*

Genes are identified by their AGI number, TC number (TC Code), and family or AMPL family number when they have no TC number. TC codes are accompanied by a superscript "a" when a gene was tentatively classified by us. Protein descriptions were compiled from the Aramemnon protein database. Given protein names are listed if available (Protein). To identify genes that show specific (Spec) or preferential expression in pollen, the maximum expression from the four pollen stages (MaxP); the maximum expression from 12 sporophytic tissues (MaxS); and the ratio MaxP/MaxS (Fold) are shown. Genes showing exclusive expression in any stage of pollen development are designated as "S" in the Pol column. Expressed genes showing at least 3-fold higher expression than the highest value in a sporophytic tissue are designated as preferential ("P"). Asterisks denote genes that were also analyzed as pollen selective or enriched by Pina et al. (2005). Cluster numbers (Clus) refer to male gametophyte expression patterns described previously (Honys and Twell, 2004).

AGI Name	TC Code	Family	Protein Description	Protein	Pol	MaxP	MaxS	Fold	Clus
At4g32500	1.A.1	VIC	Putative potassium channel	AtAKT5	P	479	54	8.9	25
At2g25600	1.A.1	VIC	Potassium channel	AtSPIK/AKT6	S*	1,852	0	Spec	1
At1g15990	1.A.1	VIC	Putative cyclic nucleotide and calmodulin-regulated ion channel	AtCNGC07	S*	426	0	Spec	2
At1g19780	1.A.1	VIC	Putative cyclic nucleotide and calmodulin-regulated ion channel	AtCNGC08	S*	1,153	0	Spec	2
At3g48010	1.A.1	VIC	Putative cyclic nucleotide and calmodulin-regulated ion channel	AtCNGC16	S*	420	0	Spec	2
At5g14870	1.A.1	VIC	Putative cyclic nucleotide and calmodulin-regulated ion channel	AtCNGC18	P*	1,099	95	11.5	2
At5g60010	1.A.20	cytB	Respiratory burst oxidase family	AtRbohH	P	1,033	67	15.5	2
At3g45810	1.A.20	cytB	Respiratory burst oxidase family	AtRbohJ	S	543	0	Spec	2
At5g09710	1.A.35	MIT	Putative magnesium transporter	At5g09710	P	407	78	5.2	2
At4g28580	1.A.35	MIT	Putative magnesium transporter	AtMGT05	S	997	0	Spec	29
At3g09550	1.A.4[a]	TRP-CC	Ankyrin repeat protein family	At3g09550	P	670	94	7.2	3
At5g60070	1.A.4[a]	TRP-CC	Ankyrin repeat protein family	At5g60070	S	361	0	Spec	20
At5g37810	1.A.8	MIP	NOD26-like intrinsic protein	AtNIP4.1	P*	1,796	18	97.5	5
At4g01470	1.A.8	MIP	Putative tonoplast intrinsic protein 3 gamma	AtTIP1.3	P*	4,787	27	176.7	18
At3g47440	1.A.8	MIP	Putative tonoplast intrinsic protein	AtTIP5.1	S*	1,616	0	Spec	3
At2g30290	1.B.33	OmpIP	Spot 3 protein and vacuolar sorting receptor	AtELP4	S	793	0	Spec	18
At1g07340	2.A.1.1	MFS	Monosaccharide-H^+ symporter, gametophyte specific	AtSTP02	P	3,403	60	57.1	29
At3g05960	2.A.1.1	MFS	Monosaccharide-H^+ symporter, pollen specific	AtSTP06	P	1,423	51	27.8	22
At5g26250	2.A.1.1	MFS	Monosaccharide-H^+ symporter	AtSTP08	S	433	0	Spec	22
At1g50310	2.A.1.1	MFS	Monosaccharide-H^+ symporter, Glc specific	AtSTP09	P*	1,923	165	11.7	22
At5g23270	2.A.1.1	MFS	Monosaccharide-H^+ symporter, pollen tube specific	AtSTP11	P*	3,814	140	27.3	1
At2g16120	2.A.1.1	MFS	Putative monosaccharide-proton symporter	AtPLT1	S	1,005	0	Spec	22
At4g16480	2.A.1.1	MFS	Putative inositol/polyol (cyclic)-proton symporter	AtINT4	P*	1,688	327	5.2	22
At3g03090	2.A.1.1	MFS	Xylose transporter homolog	At3g03090	P*	1,880	287	6.6	1
At3g05150	2.A.1.1	MFS	Glc transporter family, AtERD6 homolog	At3g05150	P*	2,510	320	7.9	1
At3g51490	2.A.1.1	MFS	Putative monosaccharide transporter, sensing protein	Atmssp3	P	1,756	178	9.9	27
At1g79360	2.A.1.19	MFS	Sugar transporter family	At1g79360	P	727	117	6.2	18
At3g13050	2.A.1.19	MFS	Singleton, sugar-substrate symporter	At3g13050	P*	1,082	119	9.1	2
At3g20660	2.A.1.19	MFS	Sugar transporter family, organic cation transporter related	At3g20660	P*	757	197	3.8	3
At5g43340	2.A.1.9	MFS	Phosphate transporter	AtPHT6	S	1,622	0	Spec	29
At1g74780	2.A.1.x[a]	MFS	Nodulin-like anion transport proteins	At1g74780	P	1,752	506	3.5	1
At3g01630	2.A.1.x[a]	MFS	Nodulin-like anion transport proteins	At3g01630	S	1,838	0	Spec	1
At1g72130	2.A.17	POT	Proton-dependent oligopeptide transport family	At1g72130	P	114	35	3.3	39
At5g01180	2.A.17	POT	Proton-dependent oligopeptide transport family	At5g01180	P	809	89	9.1	19
At5g19640	2.A.17	POT	Proton-dependent oligopeptide transport family	At5g19640	S	504	0	Spec	27
At5g28470	2.A.17	POT	Proton-dependent oligopeptide transport family	At5g28470	S*	1,648	0	Spec	25
At1g24400	2.A.18	AAAP	Acidic and neutral amino acid transporter	AtLHT2	P	2,380	124	19.1	5
At1g67640	2.A.18	AAAP	Putative Lys/His transporter	AtLHT5	P*	696	16	43.2	–
At4g35180	2.A.18	AAAP	Putative Lys/His transporter	AtLHT7	P*	5,640	477	11.8	1
At1g71680	2.A.18	AAAP	Putative Lys/His transporter	AtLHT8	S	4,104	0	Spec	20
At5g02180	2.A.18	AAAP	Amino acid transporter family	At5g02180	P*	1,315	270	4.9	2
At5g15240	2.A.18	AAAP	Aromatic and neutral amino acid transporter protein; ANT1 homolog	At5g15240	P	565	98	5.8	29
At5g17400	2.A.29	MC	Putative mitochondrial adenylate translocator	At5g17400	P	3,550	312	11.4	1
At5g13490	2.A.29	MC	Putative mitochondrial adenylate translocator	AtAAC2	P	3,215	905	3.6	6
At1g14560	2.A.29	MC	Adenylate translocator (brittle-1)-like family	At1g14560	P*	998	273	3.7	2
At3g53940	2.A.29	MC	Adenylate translocator (brittle-1)-like family	At3g53940	S	213	0	Spec	29

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

AGI Name	TC Code	Family	Protein Description	Protein	Pol	MaxP	MaxS	Fold	Clus
At5g48970	2.A.29	MC	Putative mitochondrial carrier	At5g48970	P	1,113	318	3.5	3
At1g74240	2.A.29	MC	Putative mitochondrial carrier	At1g74240	S	612	0	Spec	29
At5g25430	2.A.31	AE	Putative boron transporter	At5g25430	P*	1,497	46	32.2	1
At1g16380	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX01	S	788	0	Spec	2
At1g79400	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX02	P*	1,198	69	17.2	2
At5g22900	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX03	S	35	0	Spec	17
At1g08135	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX06b	P*	816	168	4.9	3
At2g28180	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX08	S*	3,086	0	Spec	1
At3g44930	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX10	S*	967	0	Spec	17
At2g30240	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX13	S*	731	0	Spec	20
At1g06970	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX14	S*	868	0	Spec	2
At2g13620	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX15	S*	1,585	0	Spec	2
At3g17630	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX19	P	3,133	107	29.2	22
At5g37060	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX24	S*	652	0	Spec	2
At5g58460	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX25	S*	497	0	Spec	19
At5g01690	2.A.37	CPA2	Putative cation/proton exchanger	AtCHX27	P*	1,084	268	4.1	2
At3g52080	2.A.37	CPA2	Singleton, putative cation/proton exchanger	AtCHX28	S*	597	0	Spec	2
At2g29410	2.A.4	CDF	Putative metal tolerance protein	AtMTPb1	P	1,110	68	16.4	2
At2g05760	2.A.40	NCS2	Putative nucleobase ascorbate transporter	AtNAT01	P	643	93	7.0	29
At2g41050	2.A.43	LCT	Putative lysosomal cystine transporter	At2g41050	P	320	53	6.0	6
At3g29060	2.A.47.2[a]	DASS	PHO1 homolog, EXS family	AtPHO1; H09	S	1,817	0	Spec	2
At4g28700	2.A.49	Amt	Putative ammonium transporter	AtAMT1.4	S	1,113	0	Spec	29
At1g22150	2.A.53	SulP	High-affinity sulfate transporter	AtSultr1.3	P	582	21	27.8	28
At5g19600	2.A.53	SulP	Putative sulfate transporter	AtSultr3.5	P	2,001	535	3.7	18
At4g18790	2.A.55	Nramp	Putative ion metal transporter	AtNRAMP5	P*	1,477	35	42.3	2
At4g05140	2.A.57	ENT	Putative equilibrative nucleoside transporter	AtENT5	S	198	0	Spec	20
At4g05110	2.A.57	ENT	Putative equilibrative nucleoside transporter	AtENT6	P	516	128	4.0	29
At1g61630	2.A.57	ENT	Putative equilibrative nucleoside transporter	AtENT7	P	1,610	16	99.0	29
At1g49810	2.A.62	NhaD	Putative sodium-proton antiporter	AtNHD2	P	109	11	10.2	39
At1g73700	2.A.66	MATE	Putative MATE-related efflux carrier	AtDTX17	P	855	149	5.7	25
At4g00350	2.A.66	MATE	Putative MATE-related efflux carrier	AtDTX34	S	2,127	0	Spec	2
At5g41000	2.A.67	OPT	Putative Fe(III)-phytosiderophore uptake mediator	AtYSL4	P	269	50	5.4	29
At1g65730	2.A.67	OPT	Putative Fe(III)-phytosiderophore uptake mediator	AtYSL7	P	725	117	6.2	27
At5g55930	2.A.67	OPT	Oligopeptide transporter; ISP4-like family	AtOPT1	P	7,114	265	26.9	1
At1g09930	2.A.67	OPT	Oligopeptide transporter; ISP4-like family	AtOPT2	S	392	0	Spec	6
At4g26590	2.A.67	OPT	Oligopeptide transporter; ISP4-like family	AtOPT5	S	340	0	Spec	2
At5g53520	2.A.67	OPT	Oligopeptide transporter; ISP4-like family	AtOPT8	S	219	0	Spec	4
At5g53510	2.A.67	OPT	Oligopeptide transporter; ISP4-like family	AtOPT9	P	2,772	74	37.4	29
At5g15100	2.A.69	AEC	Auxin efflux carrier protein family	AtPIN5	P*	1,058	52	20.5	6
At2g02810	2.A.7.11	DMT	UDP-Gal/UDP-Glc transporter	AtUTr1	P	1,909	416	4.6	1
At3g46180	2.A.7.11	DMT	Putative UDP-Gal/UDP-Glc transporter	AtUTr5	P	607	175	3.5	6
At1g12600	2.A.7.11	DMT	Hypothetical protein	At1g12600	P	710	47	15.3	22
At1g07290	2.A.7.13	DMT	Nucleotide sugar transporter	AtGONST2	S	20	0	Spec	37
At1g21070	2.A.7.15[a]	DMT	Putative phosphate translocator-homolog, KT group	At1g21070	P	646	205	3.2	2
At4g09810	2.A.7.15[a]	DMT	Putative phosphate translocator-homolog, KT group	At4g09810	P	571	179	3.2	25
At5g47470	2.A.7.4	DMT	Nodulin MtN21 family protein	At5g47470	P	1,542	73	21.2	2
At5g25400	2.A.7.9[a]	DMT	Putative phosphate translocator-homolog, KD group	At5g25400	P	728	33	21.9	18
At4g19960	2.A.72	KUP	Putative potassium transporter	AtKUP09	P	1,724	303	5.7	18
At1g23070	2.A.82[a]	OST	Hypothetical protein	At1g23070	S	343	0	Spec	28
At2g27240	2.A.85	ArAE	Similar to aluminum-activated malate transporter	At2g27240	S	35	0	Spec	17
At4g27420	3.A.1.205	ABC	Putative white-brown complex homolog	AtWBC09	S	967	0	Spec	29
At2g29940	3.A.1.205	ABC	Pleiotropic drug-resistance ABC transporter	AtPDR03	P	1,070	141	7.6	25
At5g60740	3.A.1.205	ABC	Putative white-brown complex homolog	AtWBC29	P*	1,304	68	19.2	2
At2g37010	3.A.1.205	ABC	Putative nonintrinsic ABC transporter	AtNAP12	S*	865	0	Spec	2
At5g61730	3.A.1.211	ABC	Putative ABC transporter	AtATH11	P	660	36	18.2	19
At5g61740	3.A.1.211	ABC	Putative ABC transporter	AtATH14	P	273	6	46.2	29
At5g61700	3.A.1.211	ABC	Putative ABC transporter	AtATH16	P*	1,262	83	15.2	2
At3g08560	3.A.2	F-ATP	V1-ATPase subunit E; peripheral or central stalk	AtVHA-E2	S	2,657	0	Spec	18
At4g25950	3.A.2	F-ATP	V1-ATPase subunit G; V1-V0 coupling subunit	AtVHA-G3	S	3,929	0	Spec	18
At2g07560	3.A.3	P-ATP	Putative PM P3A-type H^+-ATPase	AtAHA06	S*	8,417	0	Spec	18
At3g42640	3.A.3	P-ATP	Putative PM P3A-type H^+-ATPase	AtAHA08	P*	6,225	162	38.4	2

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

AGI Name	TC Code	Family	Protein Description	Protein	Pol	MaxP	MaxS	Fold	Clus
At1g80660	3.A.3	P-ATP	Putative PM P3A-type H^+-ATPase	AtAHA09	S*	6,379	0	Spec	18
At4g11730	3.A.3	P-ATP	Putative PM P3A-type H^+-ATPase	AtAHA12	S	224	0	Spec	29
At2g22950	3.A.3	P-ATP	Putative Ca^{2+}-transporting P2B-type ATPase	AtACA07	S*	2,530	0	Spec	18
At3g21180	3.A.3	P-ATP	Putative Ca^{2+}-transporting P2B-type ATPase	AtACA09	P*	2,781	349	8.0	3
At3g22910	3.A.3	P-ATP	Putative Ca^{2+}-transporting P2B-type ATPase	AtACA13	P	350	46	7.6	29
At1g54280	3.A.3	P-ATP	Putative aminophospholipid translocase, P4-type	AtALA06	P*	1,449	170	8.5	3
At3g13900	3.A.3	P-ATP	Putative aminophospholipid translocase, P4-type	AtALA07	P*	1,110	90	12.3	3
At1g26130	3.A.3	P-ATP	Putative aminophospholipid translocase, P4-type	AtALA12	P*	861	154	5.6	3
At1g20350	3.A.8	MPT	Mitochondrial inner membrane translocase component	AtTIM17-1	P	326	92	3.5	29
At1g16360	9.A.1	PLI	Membrane protein common family	At1g16360	P	1,770	89	19.9	2
At1g79450	9.A.1	PLI	Membrane protein common family	At1g79450	P	1,054	307	3.4	2
At5g59040	9.A.12	Ctr2	Copper transporter	AtCOPT3	S	3,973	0	Spec	29
At1g57550	9.B.12	SHP	Putative low temperature and salt responsive protein	At1g57550	S	2,456	0	Spec	18
At2g38905	9.B.12	SHP	Putative low temperature and salt responsive protein	At2g38905	P	2,591	128	20.3	25
At4g17790	9.B.27[a]	YdjX-Z	Expressed protein	At4g17790	P	4,996	810	6.2	1
At1g12450	9.B.27[a]	YdjX-Z	Expressed protein	At1g12450	P	1,812	258	7.0	1
At1g03270	9.B.37[a]	HCC	Expressed protein	At1g03270	P	210	61	3.5	5
At4g30850		658	Putative adiponectin receptor (ADIPOR)	At4g30850	P	3,456	668	5.2	1
At2g33670		U26	Seven-transmembrane MLO protein	AtMLO05	P	1,331	35	37.6	19
At1g42560		U26	Seven-transmembrane MLO protein	AtMLO09	S	1,116	0	Spec	20
At3g20300		359	Putative olfactory receptor	At3g20300	P	4,306	214	20.1	1
At4g25040		106	Integral membrane protein family	At4g25040	S	1,537	0	Spec	22
At2g40990		204	DHHC-type Zn-finger domain-containing protein	At2g40990	S	1,962	0	Spec	1
At4g24630		204	DHHC-type Zn-finger domain-containing protein	At4g24630	P	1,791	220	8.2	1
At5g05070		204	DHHC-type Zn-finger domain-containing protein	At5g05070	S	1,127	0	Spec	1
At2g33640		204.2	DHHC-type Zn-finger domain-containing protein	At2g33640	P	532	16	33.6	29
At5g39650		236	Expressed protein	At5g39650	S	1,057	0	Spec	2
At1g52580		304	Rhomboid family; putative sugar transporter	At1g52580	P*	3,649	7	553.7	1
At2g29050		304	Rhomboid family; putative sugar transporter	At2g29050	P*	2,281	251	9.1	1
At3g59070		442	Putative auxin-induced protein; cytochrome b561	At3g59070	P	231	41	5.6	29
At1g18520		478	Senescence-associated protein family	At1g18520	P	2,247	39	58.0	25
At2g03840		478	Senescence-associated protein family	At2g03840	P	1,161	91	12.8	1
At5g57810		478	Senescence-associated protein related	At5g57810	S	2,121	0	Spec	26
At5g22790		498	Expressed protein; chloroplast inner membrane	At5g22790	S	367	0	Spec	29
At1g10090		534	Expressed protein	At1g10090	S	34	0	Spec	17
At3g54510		534	ERD4 protein-related	At3g54510	P	558	132	4.2	19
At4g02900		534	ERD4 protein related	At4g02900	P	584	129	4.5	2
At5g49250		555	Hypothetical protein	At5g49250	S	317	0	Spec	37
At1g19970		625	ER lumen protein-retaining receptor family	At1g19970	P	748	188	4.0	5
At4g10850		U05	Nodulin MtN3 family protein	At4g10850	P	40	7	5.8	17
At5g40260		U05	Nodulin MtN3 family protein	At5g40260	P	5,322	40	133.5	29
At5g62850		U05	Nodulin MtN3 family protein	At5g62850	P	2,796	15	181.4	20
At3g09760		U12	Putative RING-domain protein	At3g09760	P	1,870	212	8.8	1

expressed in developing pollen. The list includes members of well known transport families, such as P-type H^+ pumps and Ca^{2+} pumps, several ABC transporters, and only two vacuolar H^+-ATPase subunits, E2 (At3g08560) and G3 (At4g25950). H^+-coupled cotransporters include members of the monosaccharide/H^+ symporter (STP), amino acid transporter (AAAP), and putative oligopeptide and nitrate transporter (OPT and POT) families. Channels include two K^+ channels of the voltage-gated ion channel (VIC) family, four members of the cyclic nucleotide and calmodulin-regulated ion channel (CNGC) subfamily, and two putative tonoplast water channels of the major integral protein (MIP) family (Table II). Anion transporters include a putative borate transporter, sulfate transporters, and phosphate translocators. Although this working list may include genes that are expressed in sporophytic tissues (e.g. meristem cells) not included in our analysis, the table highlights those that have potential functions in pollen development. The transporters are diverse and in general include a few members of each gene family. However, a striking presence is the CPA2 family of putative cation/proton antiporters, in which 14 of 28 genes are specifically or preferentially expressed in pollen.

Twenty-six of the genes specifically or preferentially expressed in pollen either are not classified as transporters in the TC system or are not annotated (Table II). Examples include the DHHC-type zinc (Zn)-finger domain-containing proteins, rhomboid family proteins,

nodulin MtN3-like proteins, and unknown expressed proteins.

Coexpression of Pollen-Specific Genes Late or Early in Development

Strong clues to function can be inferred from the pattern of expression during microgametogenesis. Nearly half (64 out of 150) of the pollen-specific and pollen-preferential transporter genes are coexpressed late in pollen development, first appearing at the tricellular stage or in the mature pollen (Fig. 1, A and B; Supplemental Fig. 1A). These expression patterns belong to clusters 1, 2, and 3, according to the coexpression groupings of Honys and Twell (2004). This group includes *SPIK* (At2g25600); several CNGCs, including *CNGC18* (At5g14870), *AtRbohH* (At5g60010), *AHA08* (At3g42640), *ACA9* (At3g21180), *OPT1* (At5g55930), *STP11* (At5g23270), *TIP5.1* (At3g47440), and *BOR1* homolog (At5g25430); and several CHXs, including *CHX08* (At2g28180). Many unclassified or unknown genes are also expressed late in pollen development. Thirty-one genes belonging to clusters 18 to 22 show a similar late pollen-expression pattern, except that the level of message peaked in tricellular stage and dropped dramatically in mature pollen (Fig. 1C; Supplemental Fig. 1C). This group included genes encoding plasma membrane (PM)-localized H^+ pumps (*AHA6*, At2g07560; *AHA9*, At1g80660), autoinhibited calcium pump (*ACA7*, At2g22950), vacuolar H^+-ATPase subunits (*VHA-E* and *VHA-G*), putative tonoplast water channel (*TIP1.3*, At4g01470), monosaccharide/H^+ symporter (*STP9*, At1g50310), putative receptor *MLO9* (At1g42560), cation/proton exchangers (*CHX13*, At2g30240; *CHX19*, At3g17630), and putative K^+ transporter (*KUP9*, At4g19960).

In contrast, a group of only 22 pollen-specific and pollen-preferential genes showed peak expression in the microspore and bicellular pollen, with expression low or undetectable in tricellular or mature pollen (Cluster 29 of Honys and Twell, 2004; Fig. 1D). These genes are referred to as early pollen-expressed genes. This group included putative ABC transporter *WBC09* (At4g27420), ammonium transporter (*Amt1.4*, At4g28700), copper transporter (*COPT3*, At5g59040), magnesium transporter (*MGT5*, At4g28580), *STP2* (At1g07340), *OPT9* (At5g53510), phosphate transporter (*AtPHT6*/*AtPHT1-6*, At5g43340), *ACA13* (At3g22910), and *AHA12* (At4g11730). Approximately 23 other genes showed variations of other expression patterns (Fig. 1, B and D).

Identifying Additional Transporter Genes with Roles in Pollen Biology

Over 80% of pollen-expressed genes are expressed in sporophytic tissues, so we examined the expression pattern of all members of a transporter gene family over the four pollen developmental stages. In most cases, this approach revealed differential expression of additional genes within each family during microgametogenesis, regardless of whether they were expressed highly in sporophyte. In many cases, the pollen-specific or -preferential genes identified in Table II are also the most highly expressed members of their gene families at a certain developmental stage (e.g. *CNGC18*, *BOR1* homolog, *CHX08*).

Here we highlight a few gene families that show distinct increases or decreases in expression during microgametogenesis. For instance, *AHA6*, *AHA8*, and *AHA9* of the PM H^+-ATPase family are late pollen-specific genes; however, AHA3 (At5g57350), known to function in phloem, is highly expressed in the early stages of pollen development (Fig. 2, F.1) when other AHA genes show little or no expression. These results show that discrete members of the AHA family are developmentally regulated during microspore proliferation and pollen maturation.

Among autoinhibited Ca^{2+}-pumping ATPases, *ACA2* (At4g37640), *ACA7*, and *ACA9* are late pollen-expressed genes, though only *ACA9* expression is especially high in mature pollen (Fig. 2, F.2). In contrast, *ACA10* (At4g29900) and *ACA13* are early pollen-expressed genes. Multiple ACAs are likely localized at different subcellular membranes, including the endoplasmic reticulum (ER) ACA2, the vacuolar ACA4, and a PM ACA8 (Sze et al., 2000). Curiously, ER-type Ca^{2+} pumps (*ECA1–4*) are expressed in pollen but do not display differential patterns of expression (Fig. 2F).

Two K^+ channels, *SPIK* and *SKOR* (At3g02850), are highly expressed late in pollen development, though *SKOR*, an outward-rectifying channel, is also expressed in the stele (Fig. 2, A.1). *AKT5* (At4g32500) is constitutively expressed. Except for two cyclic nucleotide and calmodulin-regulated ion channels that are expressed early in pollen development, most of those (*CNGC7*, At1g15990; *CNGC8*, At1g19780; *CNGC16*, At3g48010; and *CNGC18*) activated late in pollen development are also preferentially or specifically expressed in the gametophyte (Fig. 2, A.2). Several putative Cl^- channels are expressed in pollen at all stages, though only *CLCc* (At5g49890) showed enhanced expression in the mature pollen grain (Fig. 2A). Interestingly, only six of more than 30 MIPs are highly expressed in the male gametophyte (Fig. 2, A.3). Three of these genes (*TIP1.3*; *TIP5.1*, At3g47440; *NIP4.1*, At5g37810) are also pollen specific, indicating that expression of aquaporins in pollen is under strict regulation by the gametophytic program.

The expression of monosaccharide/H^+ symporters of the STP family is particularly striking during microgametogenesis. *STP2* is an early pollen-expressed gene, whereas *STP11* is expressed late in pollen maturation. *STP4* (At3g19930), *STP6* (At3g05960), and *STP9* are coexpressed late in pollen development, yet their expression profiles are distinct from *STP11* (Fig. 2, B.1). All of these, except for *STP4*, are specifically or preferentially expressed in pollen.

Fourteen members of the cation/proton exchanger (CHX) gene family are expressed late in pollen

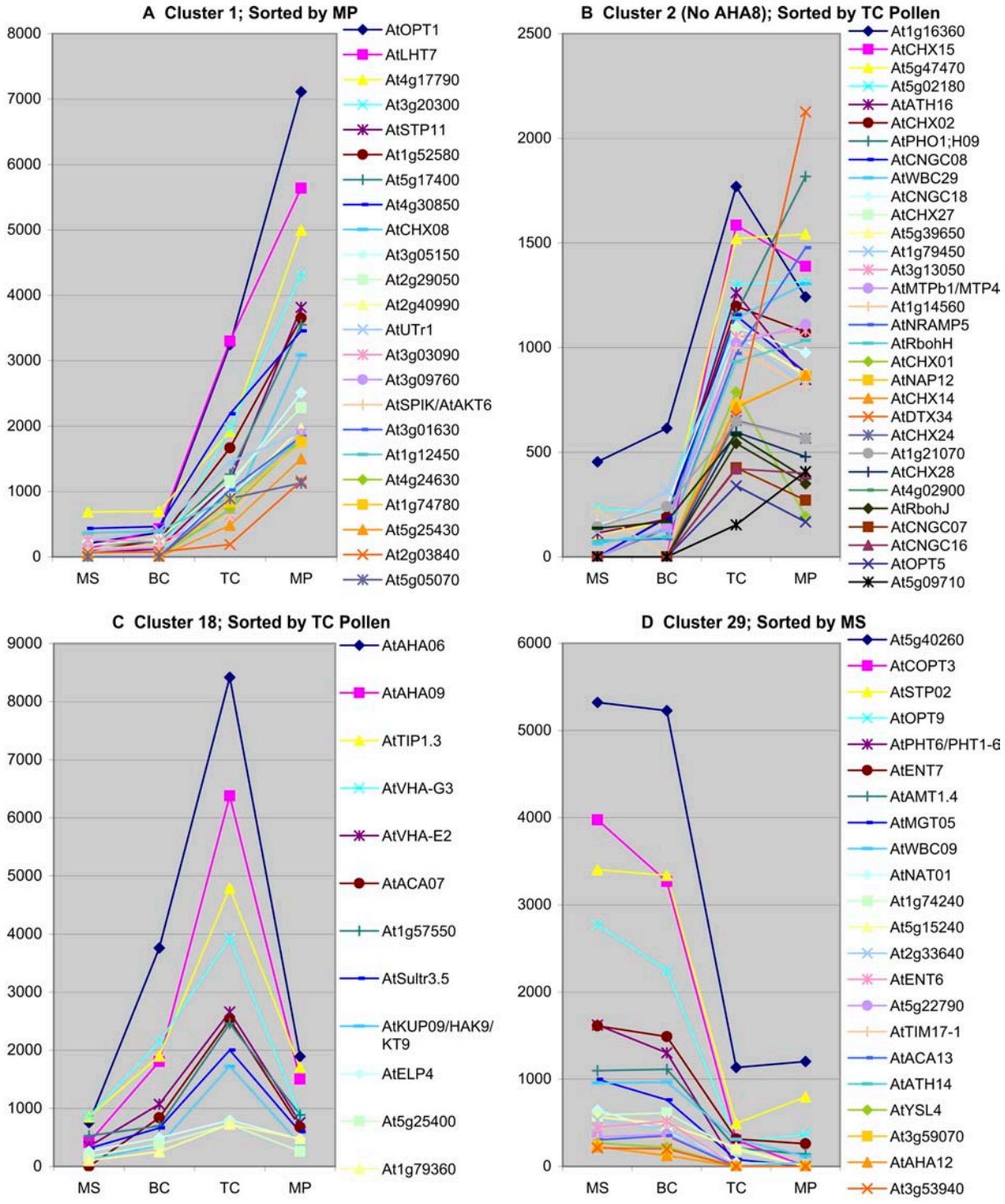

Figure 1. Coexpression of genes encoding transporters revealed many genes are expressed either early or late during microgametogenesis. Shown is the relative expression of each gene at the four stages of pollen development: microspore (MS), bicellular (BC), tricellular (TC), and mature pollen (MP). Protein names are provided when available; all other genes are listed by their Arabidopsis Genome Initiative (AGI) names. Data are taken from Supplemental Table I. A, Coexpression of 23 transporter genes late in pollen development (Cluster 1). B, Coexpression of 32 genes late in development in Cluster 2. *AHA8* was omitted as its expression peaked at 6,225. C, Coexpression of 12 genes showing peak levels at the tricellular stage (Cluster 18). D, Early pollen-expressed genes are corepressed as pollen matures (Cluster 29).

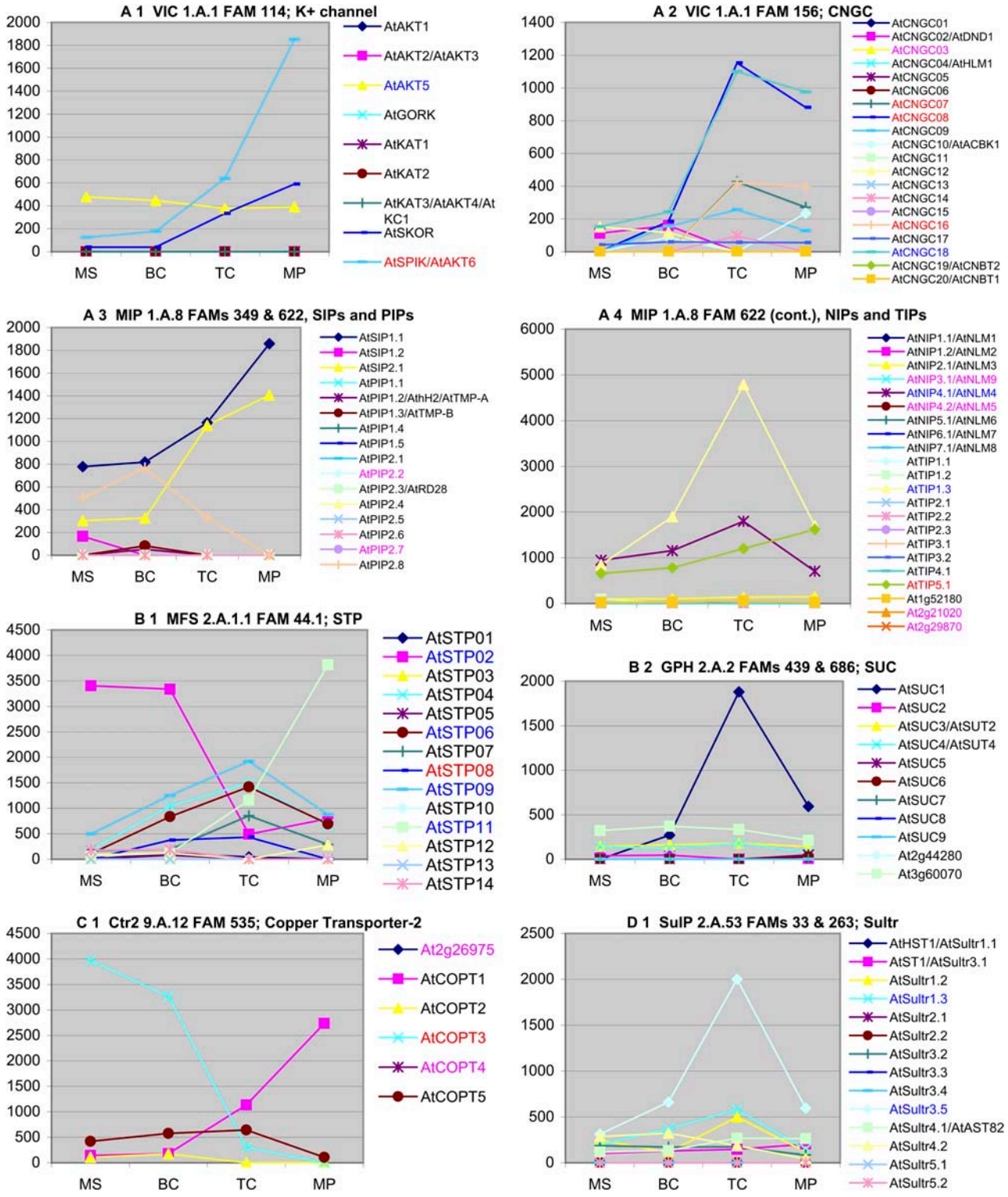

Figure 2. Discrete subsets of genes within selected gene families are expressed in male gametophyte in a developmentally regulated manner. Relative expression of all genes within a gene family was monitored at the microspore (MS), bicellular (BC), tricellular (TC), and mature pollen (MP) stages. Protein names are provided when available; all other genes are listed by their AGI numbers. Gene names in red and blue indicate those that are either specifically or preferentially expressed in pollen, respectively. Genes that do not have unique probes on the ATH1 gene chip are highlighted in pink. Data are taken from Supplemental Table I. A, Channels. A.1, VIC K^+ channel, TC number 1.A.1, FAM number 114; A.2, VIC CNGC, TC number 1.A.1, FAM number 156; A.3, MIP family, TC number 1.A.8, FAM numbers 349 and 622; A.4, MIP family (continued), TC number 1.A.8, FAM number 622. B, Sugar transporters. B.1, MFS monosaccharide-proton symporter (STP), TC number 2.A.1.1,

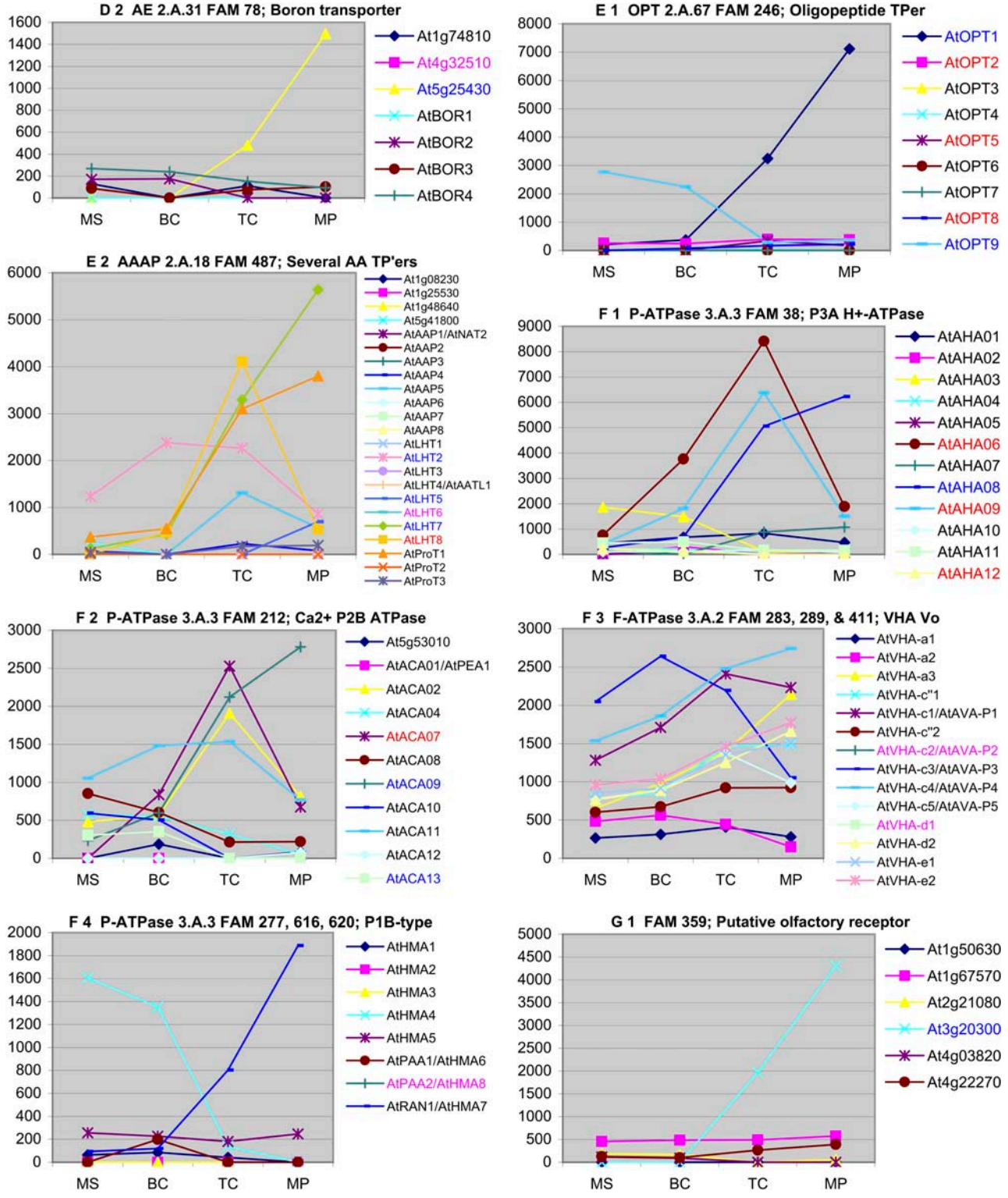

Figure 2. (*Continued.*)
FAM number 44.1; B.2, GPH Suc-proton symporter/Suc transporter (SUC), TC number 2.A.2, FAM numbers 439 and 686. C, Cation transporters. C.1, Ctr2 copper transporter, TC number 9.A.12, FAM number 535. D, Anion transporters. D.1, SulP sulfate permease, TC number 2.A.53, FAM numbers 33 and 263; D.2, AE boron transporter, TC number 2.A.31, FAM number 78. E, Nitrogen-containing compound and amino acid transporters. E.1, OPT oligopeptide transporter, TC number 2.A.67, FAM number 246; E.2, AAAP amino acid/auxin permease, TC number 2.A.18, FAM number 487. F, Pumps. F.1, P-ATPase PM P3A-type H^+-ATPase, TC number 3.A.3, FAM number 38; F.2, P-ATPase calmodulin-regulated Ca^{2+}-transporting P2B-type ATPase, TC number 3.A.3, FAM number 212; F.3, V-ATPase Vo membrane sector, TC number 3.A.2, FAM numbers 283, 289, and 411; F.4, P-ATPase heavy metal-transporting P1B-type ATPase, TC number 3.A.3, FAM numbers 277, 616, and 620. G, Unclassified proteins. G.1, Putative olfactory receptor, FAM number 359. FAM, AMPL family.

development (Supplemental Fig. 2C), raising questions about their roles and multiplicity. It is possible they are localized to different intracellular compartments and are modulated by different signaling molecules. A few *CHX* genes are preferentially expressed in vegetative tissues, such as *CHX17* (At4g23700) in the root (Sze et al., 2004). So it was surprising to find that only *CHX17* appears to be expressed at the microspore and bicellular pollen stages from transcriptomic data. This low level gametophytic expression was questioned until it was verified by promoter::β-glucuronidase (GUS) staining (Fig. 3).

Among cation transporters, Cu^{2+} transporter *COPT3* is expressed early in microspore development, whereas *COPT1* (At5g59030) appears late in pollen maturation (Fig. 2, C.1). *COPT1* is also highly expressed in root tips, trichomes, and guard cells (Sancenon et al., 2004). A heavy-metal pump with specificity for Zn^{2+} and Cd^{2+}, *HMA4* (At2g19110), is expressed early in pollen development (Fig. 2, F.4). In contrast, the Cu^{2+} pump *RAN1/HMA7* (At5g44790; Fig. 2, F.4) is switched on later in tricellular or mature pollen stages. These and other results (Supplemental Fig. 2) strongly suggest that specific members of each gene family play distinct roles in microgametogenesis and in postpollination events.

Several amino acid transporter genes that are specific or preferentially expressed in pollen (*LHT7*, At4g35180; *LHT8*, At1g71680) appear late in development (Fig. 2, E.1). Interestingly, *AtProT1* (At2g39890), a Pro/H^+ symporter with specificity for betaine and γ-aminobutyrate, is also highly expressed late in pollen development. Pro is the most abundant amino acid in mature tomato (*Lycopersicon esculentum*) pollen, suggesting that AtProT1, like LeProT1, may accumulate compatible osmolytes to tolerate dessication as pollen matures (Schwacke et al., 1999). Among genes in the oligopeptide transporter family, *OPT9* (At5g53510) expression is exclusively high in microspores and bicellular pollen, in contrast to *OPT1* expression in tricellular and mature pollen grains. This pattern suggests these two pollen-specific *OPT* genes are differentially regulated to transport oligopeptides in the proliferating microspore or in mature pollen (Fig. 2, E.1).

It is important to point out that many genes do not show any specific pattern of expression. Examples include the vacuolar H^+-pumping ATPase subunits (*VHA*; Fig. 2, F.3), H^+-pumping pyrophosphatases (*AVP1–3*), and the mitochondrial carrier (MC) family proteins. These proteins probably support essential cellular processes, such as energy production and vesicle trafficking during microgametogenesis. Expression patterns of most other genes are presented in Supplemental Figure 2.

Promoter::GUS Activity Confirms Late and Early Pollen-Expression Patterns

We verified microarray results by analyzing promoter::GUS activity of several *CHX* genes during flower development. The upstream regulatory regions were transcriptionally fused with the GUS gene and introduced into Arabidopsis plants by floral dip. Transgenic plants were analyzed for promoter activity in flowers at stages 9 to 14, which spans the period from microspore development to mature pollen (Bowman, 1994). All the flower stages shown for each gene were derived from a single inflorescence stained for GUS activity under the same conditions.

CHX24 promoter-driven GUS is active in pollen of flowers at stages 12 through 14; however, stage 10 flowers showed no GUS staining (Fig. 3). These results agree with the microarray data that show that *CHX24* (At5g37060) is preferentially expressed in tricellular and mature pollen (Supplemental Table I). Therefore, *CHX24* is classified as a late pollen-expressed gene. Similar results were observed for CHX06a (At1g08140) and CHX08 (data not shown). By contrast, we detected *CHX17* promoter activity in the anther of stage 10 flowers, but not in pollen of stage 12 to 13 flowers. These results parallel those found in the whole-genome microarray, which indicated low levels of expression in the microspore stage. CHX17 transcript was undetectable in total RNA isolated from mature pollen (Sze et al., 2004). These results support the conclusion that *CHX17* is an early pollen-expressed gene.

Comparison of Transcriptome Analyses and Published Functional Studies

To determine if the developmental pollen transcriptome can provide useful insights into transporter gene function, we compared microarray expression data for several genes that have been functionally characterized by other groups (summarized in Table III). Both *STP2* and *AHA3* are early pollen-expressed genes based on the microarray data (Fig. 2, B.1 and F.1). *STP2* is pollen specific, though *AHA3* is not. Each belongs to a sizable gene family with more than 10 members; however, other members of the family show little or no expression at the microspore stage, suggesting a primary role of STP2 and AHA3 in microgametogenesis. STP2 was shown to be a monosaccharide/H^+ symporter on the PM. Although a *stp2* knockout mutant has not been reported, the expression pattern of *STP2* clearly points to a role in microspore nutrition. In situ hybridization and immunolocalization show RNA and protein are expressed in the microspore at the beginning of callose degradation before tetrad release. STP2 was suggested to function in sugar import after the microspore is symplastically cut off from tapetal cells (Truernit et al., 1999). *AHA3* promoter::GUS staining confirmed the microarray data that *AHA3* is expressed in microspores. Strikingly, heterozygous *AHA3/aha3* mutants produce pollen with a 1:1 ratio of full round pollen and smaller, misshapen grains (Robertson et al., 2004). Results would support a model in which the PM AHA3 produces a proton gradient that drives the uptake of sugars and other nutrients required to support the developing microspore.

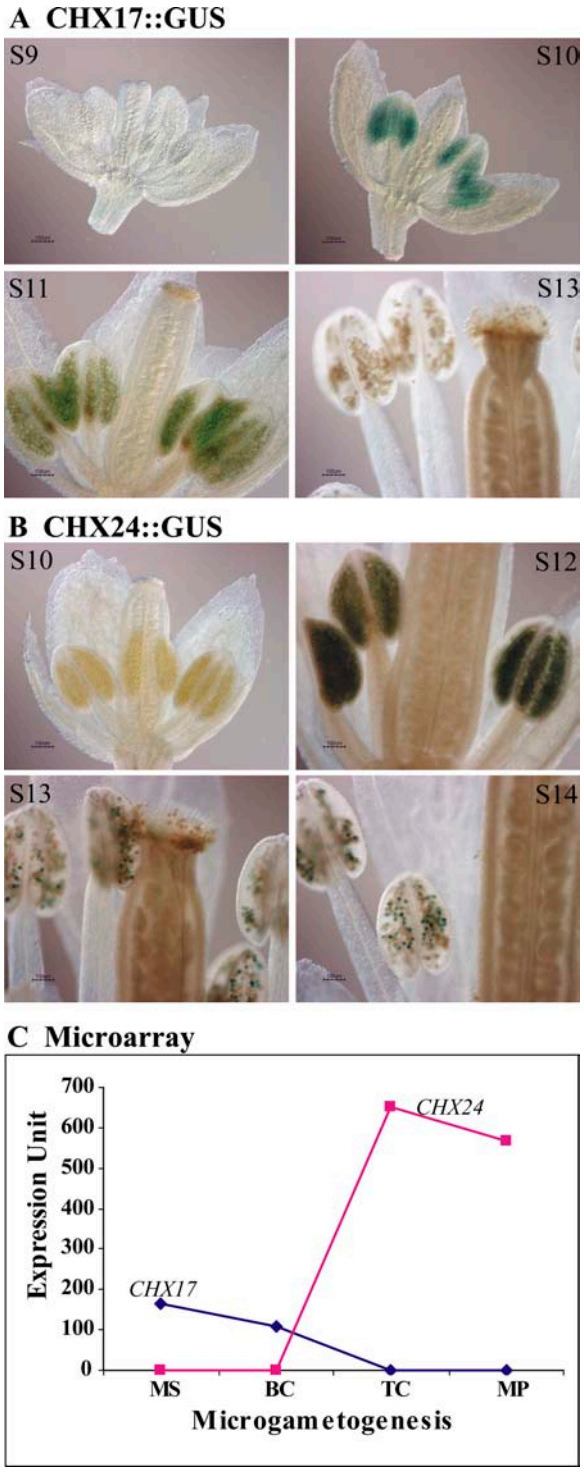

Figure 3. Differential expression of *CHX* genes in developing pollen is confirmed by promoter::GUS analyses of the inflorescence. A, *CHX17* promoter::GUS. Flowers at different stages (S9–S13) were taken from one inflorescence that was stained for GUS activity. B, *CHX24* promoter::GUS. Flowers at different stages (S10–S14) were taken from one inflorescence stained for GUS activity. C, Relative expression of *CHX17* and *CHX24* in male gametophyte as revealed by ATH1 gene chip. MS, BC, TC, and MP refer to the microspore, bicellular, tricellular, and mature pollen stages. Data were taken from Supplemental Table I.

STP11, *SPIK*, and *ACA9* genes are specifically or preferentially expressed in pollen late in development according to microarray analyses (Supplemental Table I). Schneidereit et al. (2005) showed that *STP11* promoter::green fluorescent protein is active in the pollen tube. Furthermore, specific antibodies showed STP11 protein is exclusively found in the pollen tube. STP11 is a high-affinity, broad-spectrum monosaccharide/H^+ symporter localized at the PM and is thought to supply monosaccharides to the growing pollen tube. Mouline et al. (2002) showed that Shaker K^+ channel, *SPIK/AKT6*, is highly expressed in the grain and tubes according to promoter::GUS and reverse transcription-PCR analyses of pollen RNA. Moreover, protoplast of homozygous *spik-1* mutant grain showed reduced K_{in}^+ channel activity after hyperpolarization relative to wild-type pollen. Knockout mutants also showed decreased pollen tube growth, lending support to the electrophysiological results that SPIK channels provide the main pathway for K^+ uptake during pollen tube growth. Recently, the Ca^{2+}-ATPase ACA9 protein fused to yellow fluorescent protein was localized to the PM of pollen tubes (Schiott et al., 2004). Significantly, pollen from homozygous *aca9* plants showed reduced tube growth in vivo and a high frequency of aborted fertilization. As PM-bound Ca^{2+} pumps extrude Ca^{2+} to the walls, perturbation of extracellular [Ca^{2+}] plus possibly disruption of [Ca^{2+}] dynamics in the cytosol could lead to decreased tube elongation and reduced seed set.

Together, these few examples demonstrate that some early pollen-expressed genes (e.g. *AHA3*) are critical for development of the microspore to the bicellular stage. Interestingly, some late pollen-expressed genes (*STP11*, *ACA9*), with high levels of RNA in mature pollen grains, are expressed as proteins later in the growing pollen tube. These observations support the idea that late pollen-expressed genes influence postpollination events, such as tube growth, fertilization, and seed set.

DISCUSSION

The male gametophyte presents a simple model for systems biology studies, as its short life span from a microspore to a pollen tube depends on nearly all the major cellular processes of a living organism. Key processes from nutrition, cell division, growth, development, and signaling can be studied as the microspore divides and differentiates to form a mature pollen grain that germinates to deliver sperms to the ovule. A first step in systems biology requires the identification and localization of all the functional components at different stages (Minorsky, 2003). We have presented the first genome-wide analyses of membrane transporters expressed in the male gametophyte at four developmental stages. This type of transcriptomic analysis is particularly significant as nearly all transcripts can be considered expressed during the ontogeny of essentially a single cell type. We have assumed

Table III. *Comparing pollen transcriptome analyses with functional studies of transport genes expressed at different stages of pollen development*

"Microarray" column indicates gene expression predominantly detected in microspore (1), bicellular (2), tricellular (3), and/or mature (4) pollen as determined by whole-genome pollen transcriptome (Honys and Twell, 2004). "Expression" refers to RNA and/or protein expression as detected by other methods, such as promoter::GUS, in situ, or immunostaining. "Function" refers to analyses of pollen development or tube growth determined using single mutants (ko), if indicated.

Gene	Microarray	Expression	Function	Reference
AtSTP2	1, 2	In situ, immunostain in microspores; mRNA or protein not found in the mature pollen or germinating pollen	Uptake of hexose and pentose across the PM; suggests a role in import of sugars after microspore is symplastically cut off from tapetal cells	Truernit et al. (1999)
AtSTP11	3, 4	Protein exclusively in pollen tube	Supplies monosaccharides to growing pollen tube	Schneidereit et al. (2004)
ACA9	3, 4	Protein localized to PM of pollen tube	Ca^{2+} extrusion pump important for Ca^{2+} homeostasis; ko mutant: reduced tube growth, fertilization, less seed set	Schiott et al. (2004)
SPIK	3, 4	Promoter::GUS in pollen grain and tube	K^+ uptake for pollen tube growth; ko mutant: impaired pollen tube growth, decreased pollen competitive ability	Mouline et al. (2002)
AHA3	1, 2	Promoter::GUS in late microspore and cell undergoing mitosis	PM H^+ extrusion pump generates driving force for nutrient uptake; ko mutant: male gametophytic lethal	Robertson et al. (2004)
VHA-A	1, 2, 3, 4	Gametophyte and sporophytic tissues	T-DNA mutant is male gametophytic lethal; essential for male gametophyte development	Dettmer et al. (2005)
RAN1	3, 4	Pollen and sporophytic tissues	Cu^{2+} pump needed for male gametophyte function	Woeste and Kieber (2000)

that most of the pollen-expressed genes represent messages found in the microspore and the vegetative cell, though it remains possible that a minor fraction of messages expressed late in pollen development is the product of generative or sperm cells (Engel et al., 2003). A systematic identification of transporters is an important first step to discover how transport of ions and metabolites is integrated with the diverse phases of pollen development. We show that the expression of genes encoding known and putative membrane transporters are overrepresented in mature pollen relative to the microspore, underscoring the significance of transporters in the maturation and progamic phases of pollen development (Honys and Twell, 2004; Pina et al., 2005).

Validity of Pollen Transcriptome Analyses

Microarray results of Arabidopsis plants often yield variable results in different studies, so the transporter genes that we identified as specifically or preferentially expressed in pollen (Table II) were compared with those identified recently in mature pollen alone by Pina et al. (2005). Instead of using Gene Ontology terms, which collected 671 transport genes (Pina et al., 2005), we compiled a list of 1,269 classified transporter genes based on gene family classification obtained from three transport databases (AMPL, Aramemnon, and PlantsT), published papers, and investigator Web sites. The criterion used to define pollen-specific genes was similar in both studies, though the methods differed. Exclusive calculation of detection calls in biological replicates were used in this study, whereas Pina et al. (2005) scored genes as expressed when a gene gave a positive detection call in at least one of several replicates. Furthermore, we assigned genes as preferentially expressed in pollen if they showed at least 3-fold higher expression relative to the highest level in any sporophytic tissue, rather than the 1.2-fold minimum used by Pina et al. (2005) for pollen-enriched genes. Thus, approximately 43 of 94 pollen-selective and/or -enriched genes identified by Pina et al. (2005) were absent from our Table II, probably due to the small number of sporophyte datasets used (four arrays and two arrays from siliques). We used a large number of sporophyte datasets consisting of 75 microarrays (Supplemental Table I) from 12 tissues; consequently, our estimate of pollen-specific or -preferential genes is decreased. By analyzing only mature pollen, Pina et al. (2005) missed the early pollen-specific genes. The different methods used to select pollen-specific or pollen-enriched genes probably override variations resulting from the different algorithms (i.e. MAS4 or MAS5 detection calls) used to compute the normalized expression levels of the pollen transcriptome data and from use of two different ecotypes, Columbia (Col-0) and Landsberg *erecta* (L*er*). Regardless of the methods used, 51 genes overlapped in both studies as specific or enriched in mature pollen (see asterisk in Table II).

Other lines of evidence provide strong support for the validity of the normalized pollen transcriptome results used in our analyses. First, a portion of the pollen-preferential genes has been verified by PCR amplification of reverse-transcribed messages isolated from mature pollen and by promoter::GUS analyses (Honys and Twell, 2003; Sze et al., 2004). Second, we

showed that promoter activity of the *CHX17* and *CHX24* genes correspond to early and late expressed genes, respectively, which is consistent with the coexpression clusters of the pollen transcriptome. Third, differential expression of discrete genes in pollen seen in microarrays confirms previous studies by others. For instance, STP2 mRNA and protein were localized in microspores by in situ hybridization and immunohistochemical staining but not in tricellular or mature pollen (Truernit et al., 1999). In contrast, STP11 protein is exclusively in the pollen tube, but not in pollen grains (Schneidereit et al., 2005). As transcriptomic results indicated STP11 peaks at the mature pollen stage, these results support the idea that many messages are stored until pollen germination. Moreover, *AHA3* promoter-driven GUS activity occurred in early and vacuolated microspores, as well as microspores undergoing the first mitosis, but was absent from the mature grains (Robertson et al., 2004), consistent with the early pollen expression of *AHA3* in transcriptome data. Together, these results verify the developmentally regulated expression seen in the pollen transcriptome for genes encoding channel, cotransporters, and pumps, and emphasize the value of microarray data derived from highly purified populations of viable spores at defined stages (Honys and Twell, 2004).

Distinct Functions of Early and Late Genes

Although the functions of only a few of the transporter genes have been studied in pollen so far, the results strongly indicate that genes specifically or preferentially expressed in pollen (Table II) serve critical roles for pollen maturation or pollen tube growth. Examples include (1) knockout mutants of an inward-rectifying K^+ channel, SPIK/AKT6, which showed decreased pollen tube growth (Mouline et al., 2002); (2) impaired Ca^{2+} efflux from the pollen tube decreased pollen tube growth, fertility, and seed set in homozygous *aca9* mutants (Schiott et al., 2004); (3) a monosaccharide/H^+ symporter that was exclusively expressed in pollen tube PM (Schneidereit et al., 2005); and (4) loss of function in a late pollen-expressed Cu^{2+} pump gene, *RAN1*, resulted in male gametophyte infertility (Woeste and Kieber, 2000). These few examples (Table III) show that late pollen-expressed genes play critical roles in K^+ and monosaccharide nutrient uptake for tube growth, and that maintaining Cu^{2+} homeostasis, extracellular [Ca^{2+}], and cytosolic Ca^{2+} dynamics are important for tube growth, fertilization, and seed set.

If so, other genes showing specific or preferential expression in pollen from Table II are promising candidates for detailed functional studies. Ca^{2+} gradients and oscillations accompany tip growth, suggesting that putative Ca^{2+} channels, like CNGC proteins, and Ca^{2+} pumps, like ACA7, are involved. Given the role of pH oscillation in tube growth (Messerli and Robinson, 1998; Feijo et al., 1999), the specific roles of PM H^+ pumps (e.g. AHA6, AHA8, and AHA9), H^+-coupled cotransporters (e.g. CHX), and anion channels (e.g. CLC) that alter pH and/or membrane potential across the PM or intracellular compartments are particularly exciting. Boron is essential for in vitro pollen germination; thus, At5g25430 (Table II), a gene related to BOR1 (Takano et al., 2002), is a prime candidate for a pollen tube boron exporter. The roles of tonoplast water channels, such as TIP1.3 and 5.1, in pollen grain dessication and tube growth also need to be investigated.

In addition, transporter genes expressed in sporophytic tissues are also crucial, especially when they are selectively expressed in pollen relative to other members of the family at a developmental stage. For instance, SUC1 (At1g71880) is a PM-localized H^+/Suc symporter widely expressed in vegetative organs. Yet transcriptome results show it is the only member of the *SUC* family highly expressed at the tricellular pollen stage (Fig. 2, B.2). Pollen expression was shown previously by in situ hybridization and promoter::GUS (Stadler et al., 1999). *hap3* mutant pollen germinates but the pollen tube fails to exit the style (Johnson et al., 2004), suggesting a critical role of SUC1 in accumulating Suc for pollen tube growth. The PM proton pump AHA3, known to reside in phloem companion cells (DeWitt and Sussman, 1995), is selectively expressed in microspores. Only half the microspores from heterozygous *aha3* mutants develop into mature pollen grains. This example suggests that a distinct role of the PM AHA3 is to produce a proton gradient to drive uptake of sugars and other nutrients (Robertson et al., 2004) to nourish developing microspores.

Many other transporter genes expressed early in pollen development are most likely important for microspore proliferation or cell division. For instance, *STP2* is preferentially expressed in microspores and bicellular pollen, and this H^+-coupled monosaccharide symporter protein appears at the beginning of callose degradation when the microspores are released from the tetrad (Truernit et al., 1999). Other genes corepressed with *STP2* identified in our study include *WBC09*, *COPT3*, and *OPT9*, as well as other non-pollen-specific genes.

However, functional analyses using mutants may depend on the absence of similar transporters and the sensitivity of the functional assay. We have begun testing single homozygous *chx* mutants containing a single T-DNA insertion. So far, there are little or no obvious phenotypic changes in flowering, in vitro pollen germination, or seed set. This may not be surprising given the multiplicity of *CHX* genes in mature pollen, so double mutants are being tested. A voltage-independent K^+ channel, KCO4/TPK4 (At1g02510), was recently localized to the PM of pollen tubes. The channel is modulated by Ca^{2+} and pH and is thought to have roles in K^+ homeostasis and membrane voltage control. However, knockout mutants did not show any apparent growth phenotype, though the ratio of the instantaneous to steady-state current was slightly lower in mutant than in wild type (Becker et al., 2004).

Constitutively Expressed Transporter Genes

Notably, many genes are constitutively expressed during microgametogenesis, suggesting they serve essential needs at all stages and have high turnover rates. For instance, many transporters in the MC family are highly expressed at all stages, most likely to supply a constant need for energy and metabolites. Proton pumps, V-ATPase and pyrophosphatase, acidify endomembrane compartments of the secretory system, including the vacuole, Golgi, and intracellular vesicles (Sze et al., 1999), so are probably required through all stages of gametophyte development. Several ion channels, including putative mechanosensitive channels (MscS) and putative Glu ionotropic channels (GLR), appear to be expressed at all stages. Multiple genes encoding nearly identical (e.g. *VHA-c1–c5*) or homologous proteins with similar membrane localization may compensate for defects in one gene. However, a mutant of a single gene is predicted to compromise male fertility or cause male gametophyte lethality as seen with vacuolar H^+-ATPase peripheral subunit A, *vha-A*, mutant (Dettmer et al., 2005) and with *mia* mutants of a P(5) P-type cation-pumping ATPase (Jakobsen et al., 2005). Our analysis showed that several singletons are expressed at all stages of pollen development. One example is TPC1, the vacuolar Ca^{2+}-activated calcium channel known from electrophysiological studies as the slow vacuolar channel (Peiter et al., 2005). These transporters are probably integrated with male gametophyte development through signaling networks that modulate their transport activity in a temporal and spatial manner.

Unknown Proteins and Potential Receptors in Pollen

In the process of classifying proteins on the master list, more than 100 proteins that were previously unclassified or labeled as hypothetical or expressed have been tentatively assigned as members of a protein family based on results of BLASTP at the Transport Classification Database (TCDB) Web site (see ''Materials and Methods'' and Supplemental Table I). However, there are still 482 predicted proteins (out of 1,751) that are unclassified within the TC system. Approximately 300 of the unknown proteins are expressed in pollen, and 9% (26 genes) are specifically or preferentially expressed in pollen. These represent novel polytopic proteins with potentially unique roles in pollen tube guidance or growth. For instance, At3g20300 predicts a protein with approximately seven transmembrane domains. According to the Universal Protein Resource (UniProt; http://www.pir.uniprot.org/), it is 23% similar and 12% identical to olfactory receptors from Drosophila (DmOR83c, or Locus Tag CG15581; Vosshall et al., 1999). A homolog in rice (*Oryza sativa*), Os08g41000, has an e value of 2e-43 and 45% similarity with At3g20300. Regardless of whether these act as odorant receptors, the pollen tube depends on diverse chemical cues to guide its path to the ovule, raising the exciting possibility that several unknown proteins, such as At3g20300, the ADIPOR-like At4g30850, and MLO05 (At2g33670) and MLO09 (At1g42560; Devoto et al., 2003), encode novel receptors in the male gametophyte.

SUMMARY

Transcriptomic analysis of all transporter genes is an important first step to identify transport proteins that participate in pollen development, postpollination events, or both. Our analysis has revealed many specific transporter genes, including (1) those that are specifically or preferentially expressed in the male gametophyte relative to the sporophyte; (2) gene(s) preferentially expressed in pollen relative to other members of its gene family; and (3) those that are expressed early or late in pollen development. Expression and phenotypic analyses of a few transport genes support the idea that early and late pollen-expressed genes have distinct functions in pollen development, maturation, or postpollination events. Thus, these findings provide the groundwork to streamline functional studies of many specific transporter genes and an opportunity to discover the functions of unknown proteins, including putative membrane receptors. Transport of ions and metabolites is intimately connected with signal transduction, cell wall metabolism, cytoskeleton rearrangement, and vesicle trafficking. Thus, this type of analysis, coupled with studies to determine transport activity, protein spatial distribution, and interacting partners, will provide insights toward understanding the systems biology of the male gametophyte.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotypes Col-0 and L*er* were used in this study. Wild-type and transgenic seeds were sterilized according to published procedures (Boyes et al., 2001). In general, growth conditions in the light incubators were as follows: light/dark cycle 16 h/8 h, light intensity 150 μmol s^{-1} m^{-2} photosynthetically active radiation, and temperature 22°C/20°C.

Genome-Wide Microarray Analyses of Pollen

Spore Isolation

Mature pollen was isolated according to Honys and Twell (2003). Isolated spores from three stages of immature male gametophyte were obtained from Arabidopsis ecotype L*er* as described before (Honys and Twell, 2004; Sze et al., 2004). Briefly, mixed spores from inflorescences removed of open flowers were separated with two sequential Percoll step gradients. The relative purity and viability of isolated fractions was determined by light and fluorescence microscopy using 4′,6-diamino-phenylindole and fluorescein 3′,6′-diacetate staining.

DNA-Chip Hybridization

Briefly, total RNA extracted from spores at each developmental stage using the RNeasy plant kit (Qiagen) was used to synthesize double-stranded cDNA.

The cDNA was then transcribed in vitro to obtain biotin-labeled complementary RNA in the presence of biotinylated UTP and CTP as described (Honys and Twell, 2004). Arabidopsis ATH1 genome arrays were hybridized with biotinylated target complementary RNA for 16 h at 45°C. Microarrays were stained with Streptavidin-Phycoerythrin solution and scanned with an Agilent 2500A GeneArray scanner (Agilent Technologies).

Data Analysis

For gametophytic developmental stages, two biological replicates from independent plant populations were used to ensure reliability and reproducibility. Seventy-five sporophytic datasets (see supplemental "Materials and Methods" section), representing 12 vegetative tissues (seedlings, whole plants, shoots, leaves, guard cell-enriched leaf extracts, petioles, stems, hypocotyls, xylem, cork, root hair zone, and roots), from public baseline GeneChip experiments were downloaded from the NASCArray database (http://www.arabidopsis.info; Craigon et al., 2004) for comparison to the pollen transcriptome (Honys and Twell, 2004). Duplicates or triplicates were used in each experiment. All gametophytic and sporophytic datasets were normalized using the freely available sChip 1.3 software (http://www.dchip.org/) as described previously (Honys and Twell, 2004). The reliability was ensured by the normalization of all 75 arrays to the median probe intensity level and the use of normalized CEL intensities of all arrays for the calculation of model-based gene-expression values based on the Perfect Match-only model. For the treatment of biological replicates, an exclusive approach was used. If a specific gene had a Detection call "P" in all replicates of an experiment, then it was scored as expressed. When expression was absent in any of the replicates, a value of 0 was given to that gene for that particular dataset (Honys and Twell, 2004). A mean value was then calculated for sporophytic tissues for which multiple datasets were available. Suspension-cultured cell was not included as a sporophytic tissue in our analysis.

Analyses of Transporters in the Pollen Transcriptome

Identifying and Classifying Transporters

To obtain a comprehensive list of predicted membrane proteins in Arabidopsis, Ward (2001) assembled the AMPL (http://www.cbs.umn.edu/arabidopsis/) by utilizing a series of protein sequence analysis programs. Of the 27,288 predicted proteins available from The Institute for Genomic Research at the time, 4,752 were found to have at least two transmembrane domains as predicted by HMMTOP (Tusnady and Simon, 1998). The Needleman and Wunsch (1970) programming method was used to create a similarity matrix for all pairs of the 4,752 proteins (Ward, 2001). A Perl program called cluster.mem read the resulting matrix and extracted groups of protein sequences. These groups were collected into distinct protein families by a Perl program called cfoo5; 607 families were numbered using this method (Ward, 2001).

Proteins were then added or subtracted based upon three factors: (1) the presence or absence in isospecic homolog clusters from the Aramemnon membrane protein database, release 3.0 (http://aramemnon.botanik.uni-koeln.de/; Schwacke et al., 2003); (2) protein family membership in the TC system at the PlantsT database (Tchieu et al., 2003); and (3) additional transmembrane domain predictions, specifically ConPred_II and Aramemnon's consensus prediction (the latter combines the results of 16 programs). With exceptions made for single, truncated proteins of larger families, three transmembrane domains were chosen as a suitable threshold for separating putative transporters from membrane-associated proteins. This threshold was based upon predictions for known transporters. Using those approaches, a first master list of 1,604 proteins was obtained. This original list was then compared to a list of 2,286 membrane proteins from Aramemnon, which included 960 proteins classified by the TC system and 1,326 polytopic proteins (three or more transmembrane domains) that were unclassified. Using both the protein descriptions and isospecic homolog clusters from Aramemnon, 147 proteins were extracted from the Aramemnon list. The final master list (Supplemental Table I) of transporter proteins contains 1,751 sequences, including 482 that are labeled as unknown or without a family designation from either AMPL or Aramemnon.

Using AMPL protein family numbers as a base, proteins in the master worksheet were further classified using Aramemnon and Saier's TCDB (http://www.tcdb.org/; Busch and Saier, 2004). The Aramemnon database groups proteins as isospecic homologs when a minimum threshold of 20% similarity (and at least 20% coverage) is reached in alignments performed using the Smith-Waterman algorithm (FASTA 3 tools; Pearson, 1996; Schwacke et al., 2003). Occasionally, two or more family numbers from AMPL were combined (into one family number) if representative members of each family were grouped as isospecic homologs by Aramemnon with at least 25% similarity, the same threshold used by Ward (2001).

TC codes and descriptions were also originally obtained from Aramemnon for all proteins from the master worksheet. Unknown proteins listed as "unclassified" by Aramemnon were searched against protein sequences in the TCDB using BLASTP (default parameters) at the TCDB Web site. E values below e-20 were considered significant, and those proteins were classified as members of the family with the highest-scoring BLAST result. Proteins were nominated as putative members of a family (designated with a superscript "a" next to their TC code) when e values between e-04 and e-20 were achieved. All remaining unclassified proteins were blasted at UniProt (Bairoch et al., 2005), in order to gather information about putative functions from several resources, such as Inter-Pro-EMBL (http://www.ebi.ac.uk/interpro/), PFAM (http://www.sanger.ac.uk/Software/Pfam/), and Protein Information Resource (PIR; http://pir.georgetown.edu/). Revised information on the list of classified transporter genes from Arabidopsis and their expression in pollen will be available under Arabidopsis 2010 at http://www.life.umd.edu/CBMG/faculty/sze/lab/index.html.

Transporter Genes Expressed in Pollen

Expression data from the pollen and sporophyte transcriptomes were incorporated into the master sheet by creating a query in Microsoft Office Access 2003, SP1, which extracted columns of data from Honys and Twell's updated supplementary data file 1 (July 2005), and inserted this data into the corresponding rows for each gene in the master sheet. Some of the putative transporter genes were not included on the ATH1 chip and have no data shown. To identify genes with specific and preferential expression in the male gametophyte, the expression data were analyzed in Microsoft Office Excel 2003, SP1. First, maximum pollen ("MaxPollen") and maximum sporophytic ("MaxSpor") expression signals were extracted for each gene on the ATH1 chip. The MaxPollen:MaxSpor ratio was then calculated for each gene to determine the fold difference in expression between pollen and sporophytic tissues. Expression was defined as specific if an expression signal was present in any stage of the male gametophyte (MaxPollen > 0.00) and an expression signal was absent from all 12 sporophytic tissues (MaxSpor $= 0.00$). Preferential expression was defined as maximum pollen expression being at least 3 times greater than maximum sporophytic expression (MaxPollen:MaxSpor > 3.00).

The 3× increase in expression was arbitrarily chosen as a suitable cutoff to indicate genes with preferential expression in pollen for the following reasons. When the commonly used 10×, 5×, and 3× cutoffs were applied to determine pollen-preferential expression, the number of detected genes was 42, 72, and 93, respectively. However, when a 2× cutoff was used, the number of pollen-preferential transporters was 135, which is disproportionately high. Furthermore, due to cell-type heterogeneity in sporophytic tissues or organs, transcriptomic data of pollen and of complex sporophytic organs are not strictly comparable by statistical means, even when normalized with the best available method. Expression in pollen is mainly from a single cell type, whereas expression in organs includes multiple cell types. Thus, the relative transcript level from one cell type could be considerably diluted in sporophytic tissues. Given this uncertainty, pollen specific and pollen preferential used in this article should be viewed as relative working terms. The normalized data are provided in Supplemental Table I for other users to apply their own criteria for analyzing a subset of genes.

To group male gametophyte-expressed genes with similar expression patterns and therefore discover those most likely to be coregulated, all pollen-expressed genes were clustered using the EPCLUST program with a threshold value of 0.05 (Honys and Twell, 2004). This method yielded 39 unique clusters. Cluster numbers, from Honys and Twell's supplemental table I, were added to the master sheet by creating another query in Microsoft Office Access.

Promoter::GUS Reporter Analyses

To examine the precise gene-expression patterns of *AtCHX* members, promoter regions upstream of the ATG start codon were transcriptionally fused with GUS to generate the *CHX*::GUS reporters. Promoter fragments of *CHX17* and *CHX24* were amplified by PCR from Col-0 genomic DNA isolated from 3-week-old seedlings using Expand High Fidelity PCR system (Roche). The primers used to generate the 2,034-bp *CHX17* promoter region were

5′-CGCGTCGACTTCGTCAAGTGTCAACGAGCTTCATAG-3′ (appended *Sal*I site is underlined) and 5′-CGCGGATCCGCTTTAAAGATCTGACAAATGATGAAT-3′ (appended *Bam*HI site is underlined). The primers used to generate CHX24 (1,638 bp) are 5′-CGCGTCGACTCTGGAAAGTGTAGTAGTCATGCGTACCG-3′ (appended *Sal*I site is underlined) and 5′-CGCGGATCCGCAAACTAATCTTTCATAATAAGATTTGA-3′ (appended *Bam*HI site is underlined). The *Sal*I-*Bam*HI PCR fragments of *CHX17* and *CHX24* promoters were cloned into the plasmid pRITA I, and then subcloned into the binary vector pMLBart as described (Sze et al., 2004). Using the floral-dip method of Agrobacterium-mediated plant transformation (Clough and Bent, 1998), these binary vectors were transformed into Arabidopsis Col-0 ecotype plants. Transgenic progenies were selected in soil by spraying 0.05% phosphoinothricine (BASTA) on 1-week-old seedlings. Ten independent T1 lines for each construct were obtained, and at least five independent homozygous T2 lines for each construct were examined for GUS expression.

Histochemical assays for *GUS* activity in T2 or T3 generation of Arabidopsis transgenic plants were performed according to the protocol described previously (Sze et al., 2004). Inflorescence from 6- to 8-week transgenic plants were rinsed with staining buffer lacking 5-bromo-4-chloro-3-indolyl β-D-glucuronide (50 mM sodium phosphate, pH 7.2, 0.5 mM $K_4Fe[CN]_6$, 0.5 mM $K_3Fe[CN]_6$), and then incubated for 24 to 48 h at 37°C in staining buffer containing 1 mM 5-bromo-4-chloro-3-indolyl β-d-glucuronide. To clear chlorophyll from the green tissues, the stained tissues were incubated in 70% ethanol overnight. GUS staining patterns were recorded using a Nikon Eclipse E600 microscope (Nikon Instruments) equipped with a differential interference contrast lens. Images were processed using Adobe Photoshop software (version 5.5; Adobe Systems).

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