

Transcriptome Analyses Show Changes in Gene Expression to Accompany Pollen Germination and Tube Growth in *Arabidopsis*^{1[W][OA]}

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Pollen germination, along with pollen tube growth, is an essential process for the reproduction of flowering plants. The germinating pollen with tip-growth characteristics provides an ideal model system for the study of cell growth and morphogenesis. As an essential step toward a detailed understanding of this important process, the objective of this study was to comprehensively analyze the transcriptome changes during pollen germination and pollen tube growth. Using Affymetrix *Arabidopsis* (*Arabidopsis thaliana*) ATH1 Genome Arrays, this study is, to our knowledge, the first to show the changes in the transcriptome from desiccated mature pollen grains to hydrated pollen grains and then to pollen tubes of *Arabidopsis*. The number of expressed genes, either for total expressed genes or for specifically expressed genes, increased significantly from desiccated mature pollen to hydrated pollen and again to growing pollen tubes, which is consistent with the finding that pollen germination and tube growth were significantly inhibited *in vitro* by a transcriptional inhibitor. The results of Gene Ontology analyses showed that expression of genes related to cell rescue, transcription, signal transduction, and cellular transport was significantly changed, especially for up-regulation, during pollen germination and tube growth. In particular, genes of the calmodulin/calmodulin-like protein, cation/hydrogen exchanger, and heat shock protein families showed the most significant changes during pollen germination and tube growth. These results demonstrate that the overall transcription of genes, both in the number of expressed genes and in the levels of transcription, was increased. Furthermore, the appearance of many novel transcripts during pollen germination as well as tube growth indicates that these newly expressed genes may function in this complex process.

The primary function of pollen and the pollen tube is to deliver sperms to egg apparatus for double fertilization that is required for sexual reproduction in flowering plants. Pollen germination (PG) and pollen tube growth (PTG) are continuous and highly polarized processes with tip-growth characteristics; thus, they provide an ideal model system for the study of cell growth and morphogenesis in a broader sense (Feijó et al., 2001, 2004).

Using biochemical assays and mutant analysis, there were only a limited number of genes identified as being involving in pollen development, PG, and PTG, as well as in the interaction between pollen and female tissues (Scott et al., 1991; McCormick, 1993, 2004). In order to move toward a comprehensive understanding of molecular regulatory mechanisms

for PG and PTG, further analysis has been needed of the potential functions for a number of genes that are expressed in pollen as well as in PG and PTG. Several previous studies have analyzed the transcriptional profiles of *Arabidopsis* (*Arabidopsis thaliana*) pollen on a more global scale by application of either Affymetrix GeneChips or serial analysis of gene expression (Becker et al., 2003; Honys and Twell, 2003, 2004; Lee and Lee, 2003; Pina et al., 2005). Affymetrix *Arabidopsis* 8 K GeneChips (representing about 8,000 genes) and ATH1 Genome Arrays (representing 22,591 genes) were used to compare the transcriptome differences between pollen grains and vegetative tissues (Becker et al., 2003; Honys and Twell, 2003) and to analyze the transcriptional changes during microgametogenesis and reproductive development in *Arabidopsis* (Hennig et al., 2004; Honys and Twell, 2004). Lee and Lee (2003) used the serial analysis of gene expression methods to profile the transcriptome in pollen under normal and chilling conditions. These studies revealed a high proportion of specifically and differentially expressed genes in pollen transcriptome (for review, see da Costa-Nunes and Grossniklaus, 2003).

Honys and Twell (2004) showed that there was a decline in the number of diverse transcripts accompanied by pollen maturity and an increase in the proportion of male gametophyte-specific transcripts. Pina et al. (2005) reported that the transcriptome of pollen grains was smaller and unique from any other vegetative tissue. Genes in functional categories of signaling,

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vesicle transport, and cytoskeleton were proportionally overrepresented in the pollen grain transcriptome, suggesting their commitment to PG and PTG (Pina et al., 2005). Although those previous studies drew the outline of the main characteristics of the pollen or male gametophyte transcriptome (Becker and Feijó, 2007; Grennan, 2007), the possible dynamic changes of the pollen transcriptome during PG and PTG remain unclear.

A number of very dynamic cellular events occur during PG and PTG, including calcium oscillation, vesicle transport, ion fluxes, cell wall biosynthesis, cytoskeleton dynamics, et cetera (for review, see Hepler et al., 2001). Thus, one may ask whether there are transcriptional changes during PG and PTG. Although it was previously proposed that the mature pollen grains might already contain most of the transcripts for PG and PTG in some plant species (Mascarenhas, 1989; Guyon et al., 2000), one may speculate that there may be transcriptional changes during the transition from mature pollen to germinating and germinated pollen. A more detailed and comprehensive transcriptome analysis during PG and PTG may reveal additional novel genes functionally involved in these processes. In this study, *in vitro* PG assays showed that the inhibition of transcription resulted in significant decreases in both PG and PTG rates. By application of Affymetrix Arabidopsis ATH1 Genome Arrays, the transcriptional changes during PG and PTG were further analyzed. Our results demonstrate that the overall transcription was increased along with PG and PTG, suggesting that some newly transcribed and/or transcriptionally changed genes may play roles in the regulation of PG and PTG.

RESULTS

Inhibition of PG and PTG by the Transcription Inhibitor Actinomycin D

To test whether ongoing transcription plays any role in PG and PTG, the transcription inhibitor actinomycin D (Act D) was used during the *in vitro* PG assays. The results presented in Figure 1 clearly show that both PG and PTG were inhibited in a dose-dependent manner by the addition of Act D. As shown in Figure 1, treatment with $1 \mu\text{g mL}^{-1}$ Act D significantly inhibited both PG and PTG, and this inhibition increased to 44% and 50% for PG and PTG, respectively, when the concentration of Act D was increased to $100 \mu\text{g mL}^{-1}$. The inhibitory effects of Act D on PG and PTG shown in this study are similar to those in a previous report by Honys and Twell (2004), except for some differences in concentration dependence and the sensitivity of pollen to Act D (see details in "Discussion").

Arabidopsis Pollen Collection and *in Vitro* PG and PTG Assays in Liquid Medium

To investigate the changes of the Arabidopsis pollen transcriptome during PG and PTG, pollen samples

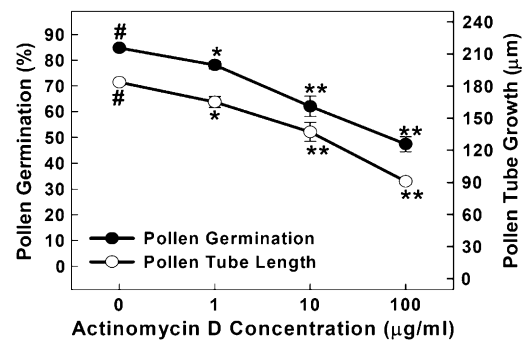


Figure 1. Effects of Act D on Arabidopsis PG and PTG. The experiments were repeated three times, and each treatment in one experiment had four replicates. Each data point is presented as mean \pm SE ($n = 4$). #, Data point for the control; *, significantly different from the control at $P < 0.05$ by Student's *t* test; **, significantly different from the control at $P < 0.01$ by Student's *t* test.

from three different stages, desiccated mature pollen grains (MP), hydrated pollen grains (HP), and pollen tubes (PT), were collected for RNA extraction. The transitions from MP to HP and from HP to PT were regarded as PG and PTG, respectively. MP was collected from freshly anther-dehisced flowers using a vacuum, as described previously (Johnson-Brousseau and McCormick, 2004). In order to get enough HP and PT samples, a "thin liquid layer" germination method was developed (Fig. 2, A–C; see "Materials and Methods" for details). Using this method, the PG rate was approximately 70% (usually between 70% and 75%), and, more importantly, a large amount of hydrated pollen grains and pollen tubes can be processed at the same time so that sufficient HP and PT could be collected for RNA extraction. The viability of HP was assayed by fluorescein diacetate (FDA) staining. In our experiments, more than 90% of HP grains were positive for FDA staining, indicating the high viability of the cultured pollen grains (Fig. 2, D–F).

Genes Expressed in MP, HP, and PT

The Arabidopsis ATH1 Genome Array, containing 22,591 probe sets representing 73.6% genome coverage of Arabidopsis (Bock et al., 2006), was used to investigate the dynamics of transcription throughout PG and PTG. The microarrays were hybridized with the fragmented cRNA made from total RNA of MP, HP, and PT, respectively. Two biological replicates derived from independent plant populations were performed to ensure reproducibility and reliability. Raw data sets were normalized and analyzed using GCOS software (see "Materials and Methods" for details). By conducting real-time PCR analyses for 24 selected genes, most of which showed similar expression patterns compared with the ATH1 microarray data, the validity of the microarray data was further confirmed (Fig. 3). The raw data sets (CEL) and the normalized expression data sets have been deposited in the Gene Ex-

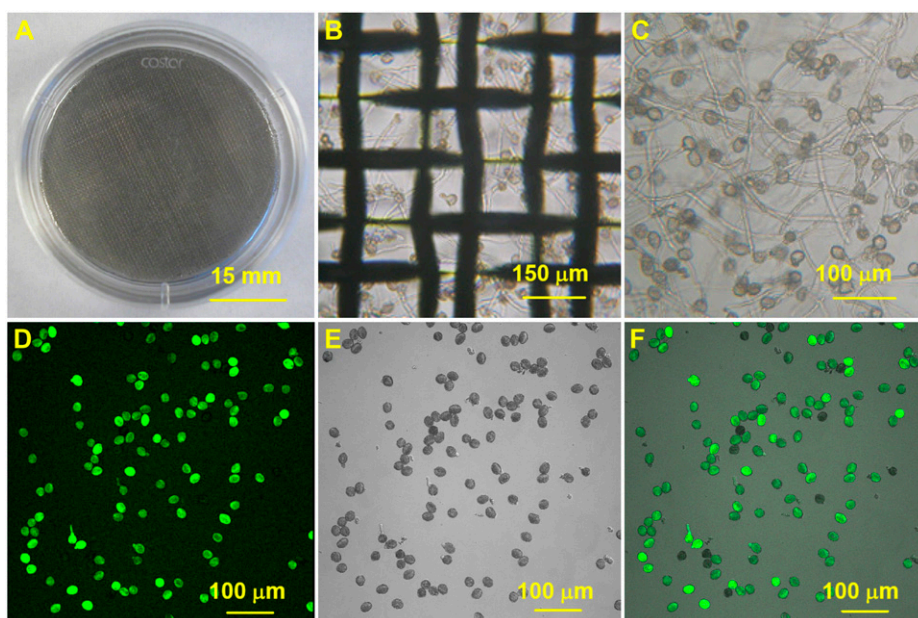


Figure 2. Large-scale in vitro PG in liquid medium and pollen viability assay. A to C show the thin liquid layer method for PG and PTG. A, A 35-mm dish with a steel-wire net (80 μm) in it. The pollen grain suspension with 30 μL of basic medium was spread on the steel-wire net. (B) The pollen tubes under the steel-wire net after 4 h of incubation. (C) The collected pollen tubes after filtering through a 50- μm nylon mesh. D to F show FDA staining of the hydrated pollen grains after incubation in liquid medium for 45 min. D, FDA fluorescence image. E, Bright-field image. F, Merged image of D and E.

pression Omnibus (GSE6696) at the National Center for Biotechnology Information (Edgar et al., 2002; www.ncbi.nlm.nih.gov/geo/).

Our microarray data showed that 3,945 genes gave present calls in MP (representing 17.5% of the unigene targets on the ATH1 microarray), 4,637 genes were expressed in HP (20.5%), and 4,892 genes were expressed in PT (21.7%; Table I). In total, 5,640 genes (representing 25.0% of the unigene targets on the ATH1 microarray) were present in at least one of the three stages (MP, HP, and PT). The numbers of detected transcripts are similar to those in previous studies that used the same microarray chips (Honys and Twell, 2004; Pina et al., 2005). Among these genes, 3,440 (15.2%) genes were consistently expressed in all three stages (Table I; Supplemental Table S1) and another 2,200 genes were preferentially expressed in one or two stages. Further analysis of these 2,200 genes showed that 163 genes were specifically expressed in MP (4.1% of the expressed genes in MP), 352 genes in HP (7.6% of the expressed genes in HP), and 731 genes in PT (14.9% of the expressed genes in PT) (Table I).

The genes that showed significant changes in expression levels during PG and PTG were analyzed. The genes that changed by more than 1.6-fold ($P < 0.01$) in both biological replicates during PG or PTG are listed in Supplemental Table S2 (see “Materials and Methods” for details). During PG, 222 genes were up-regulated, which was much more than were down-regulated (104 genes; Table I; Supplemental Table S2). Compared with PG, the overall change in transcription during PTG was more significant. There were 804 genes that were up-regulated and 686 genes that were down-regulated during PTG (Table I; Supplemental Table S2).

To identify the coregulated genes, all of the detected transcripts (5,640 genes) were further analyzed and distributed into nine clusters (I–IX) according to their expression profiles (Supplemental Fig. S1; Supplemental Table S3). Cluster I contained 704 genes (representing 12.5% of the 5,640 expressed genes) that were exclusively up-regulated during PTG (from HP to PT), while cluster V contained 119 genes (2.1%) that were up-regulated during PG (from MP to HP). Cluster III had 630 genes (11.2%) that were down-regulated dur-

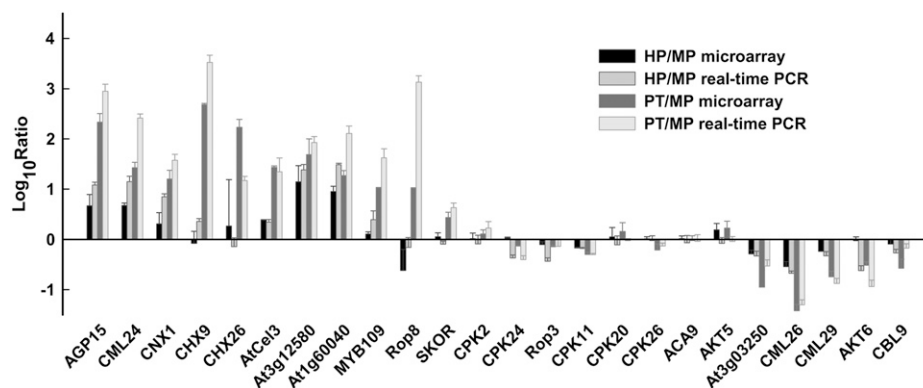


Figure 3. Confirmation of transcriptional profiles of the selected genes by real-time PCR. HP-MP and PT-MP ratios in microarray data of 24 tested genes were compared with the real-time PCR results. The sequences of the gene-specific primers used for PCR are listed in Supplemental Table S8.

Table 1. Numbers of expressed genes, specifically expressed genes, and transcriptionally changed genes during PG and PTG

The first column indicates the three stages (MP, HP, and PT) and two processes (PG and PTG). The "Number of Genes" and "Percentage" columns refer to the numbers of expressed genes, specifically expressed genes, and transcriptionally changed genes as well as the relative percentages.

Gene Category	No. of Genes	Percentage
Genes expressed in		Percentage in ATH1 array (22,591 genes)
MP	3,945	17.5
HP	4,637	20.5
PT	4,892	21.7
All three stages	3,440	15.2
At least one stage	5,640	25.0
Genes specifically expressed in		Percentage in respective stage
MP	163	4.1
HP	352	7.6
PT	731	14.9
Transcriptionally changed genes		Percentage in total expressed genes (5,640 genes)
Up-regulated in PG	222	3.9
Down-regulated in PG	104	1.8
Up-regulated in PTG	804	14.3
Down-regulated in PTG	686	12.2

ing PTG. The expression of genes in cluster II (3,980 genes) was not significantly changed in PG and PTG processes.

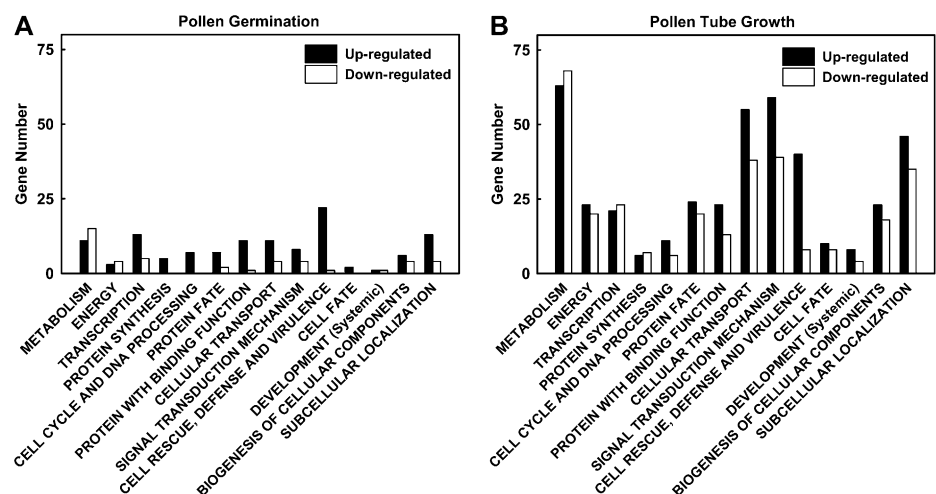
Gene Ontology Analysis and Family Analysis of the Transcriptionally Changed Genes during PG and PTG

To evaluate the potential functions of genes with altered transcription levels during PG and PTG, a Gene Ontology (GO) analysis was conducted using the FunCat Scheme version 2.0 web service in the Munich Information Center for Protein Sequences (Ruepp et al., 2004; <http://mips.gsf.de/projects/funcat>). The genes whose expression levels changed more than 1.6-fold during PG or PTG were analyzed, and they were finally classified into 14 main GO categories, as shown in Figure 4. Functional categories such as cell rescue, transcription, subcellular localization, metabolism, proteins with binding function, and cellular transport

were overrepresented during PG (Fig. 4A). During PTG, the up-regulated genes were mainly aggregated into the categories of metabolism, signal transduction, cellular transport, subcellular localization, cell rescue, and protein fate (Fig. 4B).

Pina et al. (2005) sorted the main gene families of Arabidopsis, and these gene families were further grouped according to their functional classes of signaling, transcription, transporter/channel, cell wall, cytoskeleton, vesicle trafficking, cell cycle, and small RNA pathways. To further analyze the potential functions of the genes with altered transcription during PG and PTG, the gene family tables organized by Pina et al. (2005) were used. In addition, in order to focus on transporter/channel-related genes, a comprehensive list of genes encoding membrane proteins from the Arabidopsis genome (Bock et al., 2006) was also applied. The contents of these two tables were integrated and used to identify the important functional cate-

Figure 4. Functional category distribution of transcriptionally changed genes in PG and PTG. The FunCat Scheme version 2.0 Web service at the Munich Information Center for Protein Sequences was used to analyze the GO categories of transcriptionally changed genes in PG (A) and PTG (B). The numbers of changed genes in 14 main biological categories are represented.



gories and gene families that correlated with the processes of PG or PTG. Supplemental Table S4 showed that the categories of cell wall, transcription, and signaling were overrepresented during PG, whereas the overrepresented categories during PTG were transporters, signaling, and cell wall. To further reveal the differences between PG and PTG, the user-driven tool MapMan (Thimm et al., 2004) was applied to display the diagrams of various pathways related to these processes. The results showed that genes in some functional categories or gene families (such as abiotic stress, divalent cation transport, G-proteins, etc.) were significantly changed in their transcription levels during PG or PTG (Supplemental Fig. S2; Supplemental Table S5). The integrated results suggested remarkable functional differences between PG and PTG. The function terms of cell rescue and transcription were overrepresented in PG, and genes in the categories of transport and signaling were preferentially expressed in PTG. Furthermore, genes in the CaM/CML (for calmodulin/calmodulin-like protein), CHX (for cation/hydrogen exchanger), and Hsp (for heat shock protein) families were significantly changed, mainly up-regulated during PG and PTG (Tables II–IV; Supplemental Table S7), which suggested their roles in these processes.

Members of the CaM/CML, CHX, and Hsp Gene Families Functioned during PG and PTG

The Arabidopsis genome contains seven *CaM* genes and 50 additional *CML* genes (McCormack and Braam, 2003; McCormack et al., 2005). There are probe sets for six *CaM* genes and 48 *CML* genes included in the Arabidopsis ATH1 Genome Array (Table II), and five *CaM* genes and 19 *CML* genes were detected during PG and PTG in our experiments (Supplemental Table S6). As shown in Tables III and IV and Supplemental Table S7, some *CaM* and *CML* genes significantly increased in transcription levels during PG or PTG. Among these genes, four *CML* genes (*CML39*, *CML49*, *CML3*, and *CML16*) were up-regulated during PG and 12 genes (*CaM7*, *CML49*, *CML3*, *CML16*, etc.) increased their transcription during PTG (Tables II–IV; Supplemental Table S7). It should be noted that some other *CaM/CML* genes (*CaM2*, *CaM3*, *CML6*, *CML13*, *CML25*, *CML28*, etc.) were highly expressed during PG and PTG (Supplemental Table S6).

Along with the related KEA subfamily genes, the CHX family was predicted to comprise the CPA2 (for cation/proton antiporter) family (Maser et al., 2001). The Arabidopsis genome contains 28 *CHX* genes (*AtCHX1–AtCHX28*; Sze et al., 2004), and 25 *CHX* genes are included in the Affymetrix ATH1 array (Table II). Our data showed that 18 *AtCHX* genes were detected during PG and PTG, and the numbers of *AtCHX* genes expressed at the three stages (MP, HP, and PT) were 11, 11, and 17, respectively (Table II; Supplemental Table S6). Seven members of this family were specifically expressed in PT (Supplemental Table S6). It should be mentioned that the transcriptional levels of nine *CHX* genes were specifically or preferentially up-regulated during PTG (Tables II and IV; Supplemental Table S7), but no *CHX* genes were up-regulated during PG.

The Affymetrix Arabidopsis ATH1 Genome Array contains probe sets for 180 of 219 genes of Hsps and Hsp-related proteins in the Arabidopsis genome (Thimm et al., 2004; Table II). Our microarray data showed that the transcription of 85 genes of this family was detectable during PG and PTG (Supplemental Table S6). The number of expressed *Hsp* genes increased from MP (46 genes) to HP (73 genes) and PT (77 genes; Table II). There were 19 and 18 genes up-regulated in PG and PTG, respectively, and 10 of them overlapped (Tables II–IV; Supplemental Table S7). It should be emphasized that many genes dramatically increased their transcription during PG, and the transcriptional changes of some genes were even over 50-fold (Table III; Supplemental Table S7). On the other hand, there was no *Hsp* gene down-regulated in this process.

DISCUSSION

Transcriptional Effects on PG and PTG in Arabidopsis

Honys and Twell (2004) previously reported that Act D had an inhibitory effect on both PG and PTG in Arabidopsis (ecotype Landsberg *erecta*) and that the inhibition could reach 20% to 30% at concentrations between 0.1 and 1 $\mu\text{g mL}^{-1}$ Act D. Using a different ecotype (Columbia) of Arabidopsis, we found similar inhibitory effects of Act D on PG and PTG. We observed a significant dose-dependent inhibition of PG

Table II. Numbers of *CaM/CML*, *CHX*, and *Hsp* genes expressed and transcriptionally changed during PG and PTG

The first column indicates the name of the gene family. The gene number in the column "Genome" represents the total number for each gene family in the Arabidopsis genome, and the number in the column "ATH1" shows the gene number included in the ATH1 chip for each family. The following three columns show the numbers of expressed genes in three different stages (MP, HP, and PT). The four right columns refer to the numbers of transcriptionally up-regulated (Up) and down-regulated (Down) genes during PG and PTG. For more details, see Supplemental Table S6 and S7.

Gene Family	Genome	ATH1	MP	HP	PT	Up PG	Down PG	Up PTG	Down PTG
CaM	7	6	5	5	4	0	0	1	0
CML	50	48	14	17	19	4	2	11	3
CHX	28	25	11	11	17	0	2	9	3
Hsp	219	180	46	73	77	19	0	18	9

Table III. *Transcriptionally up-regulated genes of CML and Hsp families during PG*

The first three columns show the gene family name, Affymetrix probe set, and The Arabidopsis Information Resource locus (Arabidopsis Genome Initiative identifier [AGI ID]) assigned to the corresponding probe. The gene annotation is given in the fourth column. For more details, see "Materials and Methods" and Supplemental Table S7.

Gene Family	Probe Set	AGI ID	Annotation FC P Value
CML	259866_at	At1g76640	Calmodulin-related protein, putative (CML39)
	259064_at	At3g07490	Calcium-binding protein, putative (CML3)
	259137_at	At3g10300	Calcium-binding EF hand family protein (CML49)
	256755_at	At3g25600	Calmodulin, putative (CML16)
Hsp	260248_at	At1g74310	Heat shock protein 101 (HSP101)
	250351_at	At5g12030	17.7-kD class II heat shock protein 17.6A (HSP17.7-CII)
	252515_at	At3g46230	17.4-kD class I heat shock protein (HSP17.4-CI)
	248045_at	At5g56030	Heat shock protein 81-2 (HSP81-2)
	263150_at	At1g54050	17.4-kD class III heat shock protein (HSP17.4-CIII)
	248332_at	At5g52640	Heat shock protein 81-1 (HSP81-1)/heat shock protein 83 (HSP83)
	266294_at	At2g29500	17.6-kD class I small heat shock protein (HSP17.6B-CI)
	260978_at	At1g53540	17.6-kD class I small heat shock protein (HSP17.6C-CI; amino acids 1–156)
	247691_at	At5g59720	18.1-kD class I heat shock protein (HSP18.1-CI)
	261838_at	At1g16030	Heat shock protein 70, putative/HSP70, putative
	262911_s_at	At1g59860	17.8-kD class I heat shock protein (HSP17.8-CI)
	250296_at	At5g12020	17.6-kD class II heat shock protein (HSP17.6-CII)
	263374_at	At2g20560	DNAJ heat shock family protein
	256999_at	At3g14200	DNAJ heat shock N-terminal domain-containing protein
	256245_at	At3g12580	Heat shock protein 70, putative/HSP70, putative
	255811_at	At4g10250	22.0-kD ER small heat shock protein (HSP22.0-ER)
	248043_s_at	At5g56000	Heat shock protein 81-4 (HSP81-4)
	252670_at	At3g44110	DNAJ heat shock protein, putative (J3)
	258984_at	At3g08970	DNAJ heat shock N-terminal domain-containing protein

and PTG by Act D when its concentration was increased from 1 to 100 $\mu\text{g mL}^{-1}$ (Fig. 1). The differences in concentration dependence and sensitivity of PG and PTG to Act D in these two independent experiments may have resulted from different ecotypes of Arabidopsis, different media, or different methods used in PG assays. Honys and Twell (2004) used liquid medium (Hodgkin, 1983; Derksen et al., 2002), in which two ecotypes of Arabidopsis pollen showed different and relatively low germination rates, ranging from 64% (Landsberg *erecta*) to 76% (Columbia; Lalanne et al., 2004). Using the modified solid medium and methods (see "Materials and Methods" for details), we usually observed an approximately 85% germination rate for Arabidopsis (Columbia) pollen in our experiments (Fig. 1). The different germination rates in these two independent experiments may result in different sensitivities of PG and PTG to Act D.

Ylstra and McCormick (1999) showed that Act D inhibited transcription in developing tobacco (*Nicotiana tabacum*) pollen in a concentration-dependent manner, with a concentration range from 0 to 200 $\mu\text{g mL}^{-1}$, and 98.6% of de novo RNA synthesis was inhibited by 100 $\mu\text{g mL}^{-1}$ Act D with no decrease in pollen viability. In our in vitro PG assays, we observed that 100 $\mu\text{g mL}^{-1}$ Act D inhibited PG and PTG of Arabidopsis by 44% and 50%, respectively (Fig. 1). Meanwhile, the viability of pollen tubes was not remarkably decreased. Pollen tubes treated with 100 $\mu\text{g mL}^{-1}$ Act D still kept growing slowly, although their growth rates decreased dramatically. The length

of pollen tubes reached $85 \pm 2 \mu\text{m}$ at 4.5 h and $113 \pm 1 \mu\text{m}$ at 6 h when the concentration of Act D was 100 $\mu\text{g mL}^{-1}$. All of these findings indicated that the effects of Act D on PG and PTG resulted from the inhibition of transcription but not from the decrease in pollen viability. It is suggested that transcription, especially of newly transcribed genes and/or the transcriptionally increased genes, is potentially important for the regulation of PG and PTG in Arabidopsis.

A New Method for the Collection of Germinating Pollen and Pollen Tubes for RNA Extraction

Although Pereira et al. (2005) reported a method to obtain RNA samples from Arabidopsis pollen tubes for reverse transcription PCR analysis, the amount of RNA was not sufficient for a microarray experiment. We developed a thin liquid layer in vitro PG method in this study, by which Arabidopsis pollen grains can germinate well in vitro (germination rate can reach 70% or higher) and a large amount of germinated pollen can be collected for microarray experiments. Recently, Boavida and McCormick (2007) also optimized a liquid medium and developed a method to isolate large amounts of Arabidopsis pollen tubes. Both methods may further facilitate future pollen transcriptomic and proteomic analyses.

A pollen grain with the emergence of a pollen tube may be considered to be entering the germination process. Given that transcriptional change is an extremely rapid process, it should appear before the

Table IV. *Transcriptionally up-regulated genes of CML/CaM, CHX, and Hsp families during PTG*

See Table III for the definition of each column. For more details, see Supplemental Table S7.

Gene Family	Probe Set	AGI ID	Annotation FC P Value	
CaM and CML	252713_at	At3g43810	Calmodulin-7 (CAM7)	
	249583_at	At5g37770	Calmodulin-related protein 2, touch-induced (TCH2; CML24)	
	259137_at	At3g10300	Calcium-binding EF hand family protein (CML49)	
	254487_at	At4g20780	Calcium-binding protein, putative (CML42)	
	259064_at	At3g07490	Calcium-binding protein, putative (CML3)	
	256755_at	At3g25600	Calmodulin, putative (CML16)	
	252206_at	At3g50360	Caltractin/centrin (CML20)	
	254782_at	At4g12860	Calcium-binding protein, putative (CML2)	
	259866_at	At1g76640	Calmodulin-related protein, putative (CML39)	
	253963_at	At4g26470	Calcium-binding EF hand family protein (CML21)	
	262639_at	At1g62820	Calmodulin, putative (CML14)	
	255772_at	At1g18530	Calmodulin, putative (CML15)	
	CHX	249884_at	At5g22910	Cation/hydrogen exchanger, putative (CHX9)
		246337_at	At3g44910	Cation/hydrogen exchanger, putative (CHX12)
266161_at		At2g28170	Cation/hydrogen exchanger, putative (CHX7)	
249883_at		At5g22900	Cation/hydrogen exchanger, putative (CHX3)	
246336_at		At3g44900	Cation/hydrogen exchanger, putative (CHX4)	
263469_at		At2g31910	Cation/hydrogen exchanger, putative (CHX21)	
251101_at		At5g01680	Cation/hydrogen exchanger, putative (CHX26)	
258408_at		At3g17630	Cation/hydrogen exchanger, putative (CHX19)	
263720_at		At2g13620	Cation/hydrogen exchanger, putative (CHX15)	
Hsp		264865_at	At1g24120	DNAJ heat shock protein, putative
	263754_at	At2g21510	DNAJ heat shock N-terminal domain-containing protein	
	258979_at	At3g09440	Heat shock cognate 70-kD protein 3 (HSC70-3; HSP70-3)	
	256245_at	At3g12580	Heat shock protein 70, putative/HSP70, putative	
	262911_s_at	At1g59860	17.8-kD class I heat shock protein (HSP17.8-CI)	
	248043_s_at	At5g56000	Heat shock protein 81-4 (HSP81-4)	
	250351_at	At5g12030	17.7-kD class II heat shock protein 17.6A (HSP17.7-CII)	
	263150_at	At1g54050	17.4-kD class III heat shock protein (HSP17.4-CIII)	
	261838_at	At1g16030	Heat shock protein 70, putative/HSP70, putative	
	248616_at	At5g49580	DNAJ heat shock N-terminal domain-containing protein	
	250296_at	At5g12020	17.6-kD class II heat shock protein (HSP17.6-CII)	
	266294_at	At2g29500	17.6-kD class I small heat shock protein (HSP17.6B-CI)	
	260978_at	At1g53540	17.6-kD class I small heat shock protein (HSP17.6C-CI; amino acids 1–156)	
	261458_at	At1g21080	DNAJ heat shock N-terminal domain-containing protein	
	249148_at	At5g43260	Chaperone protein DNAJ-related	
	250934_at	At5g03030	DNAJ heat shock N-terminal domain-containing protein	
	266858_at	At2g26890	DNAJ heat shock N-terminal domain-containing protein	
	255811_at	At4g10250	22.0-kD ER small heat shock protein (HSP22.0-ER)	

observation of any morphological changes. In our experiment, we collected pollen grains at 45 min after they were incubated in liquid medium as the HP sample. At this time, about 15% to 20% of pollen grains showed the emergence of pollen tubes (Fig. 2, D–F). Although most of the pollen grains did not exhibit significant morphological changes (the emergence of a pollen tube) at this stage, it is reasonable to speculate that those hydrated pollen grains may have initiated germination-related gene transcription. Taking this into account, the RNA extracted at this stage should contain and represent the transcriptome information for PG.

Increased Transcription during PG and PTG

Our data showed that the total number of transcribed genes gradually increased from MP to HP to

PT (Table I), suggesting that newly initiated transcription occurs during PG and PTG. This result is consistent with the observation that PG and PTG were inhibited by the transcription inhibitor Act D (Fig. 1). Honys and Twell (2004) reported a dramatic decrease in the number of transcripts expressed during microgametogenesis in Arabidopsis, which may be associated with reduced pollen activity during pollen maturation. Subsequently, the desiccated mature pollen becomes “silent” or “resting” at the end of microgametogenesis. However, some unique or male gametophyte-specific transcripts were selectively activated during pollen maturation (Honys and Twell, 2004). In particular, a set of de novo synthesized transcription factors emerged at the late stage of microgametogenesis, suggesting ongoing transcription after pollen maturation (Honys and Twell, 2004). In this study, microarray data clearly showed that a

large number of genes initiated transcription during PG and/or PTG (Table I). It is assumed that potential functions of these genes may be to prepare essential materials and energy for PTG and to increase the physiological and biochemical activities of germinating pollen and pollen tubes. In addition, a specific analysis showed that the proportion of specifically expressed genes was remarkably increased from MP to HP to PT (Table I), which suggested that PTG may require many cellular components or regulatory factors for its dynamic activity.

Besides newly transcribed genes, many other genes already present in MP significantly increased their transcriptional levels during PG or PTG. Analysis of these transcriptionally changed genes (changed by more than 1.6-fold) demonstrated that up-regulated genes were dominant during both PG and PTG compared with down-regulated genes (Table I). These results suggest that the newly transcribed genes as well as the up-regulated genes might play important roles in PG or PTG.

Genes That May Be Functionally Involved in PG and PTG

Because overall transcription was significantly increased in this study, further analyses were concentrated on the functional distribution of up-regulated genes. The results derived from both FunCat 2.0 and MapMan analyses showed that the cell rescue-related (or stress/abiotic/heat-related) genes, mainly *Hsps*, were overrepresented among the up-regulated genes during PG (Fig. 4A; Supplemental Fig. S2; Supplemental Table S5). Meanwhile, many genes in the transcription category (transcription factors or transcription-related regulators) were also markedly up-regulated during PG (Fig. 4A; Supplemental Tables S4 and S5). The genes in these two categories (cell rescue and transcription) were assumed to sense or raise germination signals, activate pollen from the resting (silent) stage, and mobilize materials and energy for PG.

In contrast to PG, PTG had a distinct gene function distribution. Signaling-related genes and transporter genes were dominantly transcribed and/or up-regulated during PTG (Fig. 4B; Supplemental Tables S4 and S5). These findings are consistent with previous reports that genes in these two categories are involved in the regulation of polarized tip growth of pollen tubes (for review, see Hepler et al., 2001). It has been demonstrated that growing pollen tubes are regulated under a complex signal cross talk for polarized growth, and plenty of transporters supply sufficient materials for the rapid growth of pollen tubes (Hepler et al., 2001; Holdaway-Clarke and Hepler, 2003).

Members of several gene families (CaM/CML, CHX, and Hsp) from different functional categories (signaling, transport, and cell rescue) seem to be involved in the regulation of PG and/or PTG (Tables II–IV; Supplemental Tables S6 and S7). It is well accepted that cytosolic free calcium is a key messenger

in the regulation of PG and PTG (for review, see Steer and Steer, 1989; Taylor and Hepler, 1997; Franklin-Tong, 1999). Ca^{2+} -dependent modulation of cellular activities occurs via intracellular Ca^{2+} -binding proteins or known Ca^{2+} sensors, such as CaMs and CMLs. Previous studies showed that some CaMs or CaM-related proteins had similar localization with cytosol-free calcium in growing pollen tubes (Tirlapur et al., 1994; Rato et al., 2004) and had essential roles in the regulation of PG (Golovkin and Reddy, 2003). Our results showed that some CaMs or CMLs were up-regulated or highly transcribed during PG or PTG, suggesting their potential roles in Ca^{2+} signaling pathways for the regulation of PG and/or PTG in Arabidopsis. Further functional characterization of these CaM/CML genes may increase our understanding of the Ca^{2+} signaling mechanism during PG and PTG.

Previous studies have shown that H^+ (Feijó et al., 1999, 2001) and K^+ (Feijó et al., 1995; Fan and Wu, 2000; Fan et al., 2001) fluxes across the pollen or pollen tube plasma membrane are critical for PTG and/or PG, but the molecular identification of transporters mediating the H^+ and K^+ fluxes in pollen and/or pollen tubes remains unclear. Sze et al. (2004) demonstrated that there were 18 genes of the CHX family that were specifically or preferentially expressed in the male gametophyte. In addition, by analyzing promoter::GUS activities of AtCHXs, they suggested that some AtCHXs may be functionally involved in osmotic adjustment and K^+ homeostasis during mature pollen desiccation and rehydration (Sze et al., 2004; Bock et al., 2006). Our data show that seven AtCHX genes were specifically expressed in PT during PG and PTG, and nine genes were preferentially up-regulated during PTG (Table IV; Supplemental Tables S6 and S7). Further attention should be given to analysis of these AtCHXs for their possible involvement in regulating the local pH gradient immediately adjacent to the PM of pollen tubes, in adjusting ion homeostasis, as well as in maintaining turgor and membrane potential of pollen tubes (for review, see Holdaway-Clarke and Hepler, 2003).

Hsps belong to a large family that has been classified into several groups, including Hsp60, Hsp70, Hsp90, Hsp100, and the small Hsps (for review, see Vierling, 1991; Winter and Sinibaldi, 1991; Miernyk, 1999). It is well known that *Hsps* are significantly induced when cells or organisms are exposed to supraoptimal temperatures or other stresses. Although some previous studies failed to detect heat shock responses in microspores or mature pollen in several species of plants, such as maize (*Zea mays*; Dupuis and Dumas, 1990; Young et al., 2001), rape (*Brassica napus*; Young et al., 2004), and tobacco (Volkov et al., 2005), other studies have shown that many *Hsps* are expressed in microspores and mature pollen even at normal temperature in maize (Magnard et al., 1996), Arabidopsis (Haralampidis et al., 2002), and tobacco (Volkov et al., 2005). The data presented in this study showed that the transcription of many *Hsps* and related genes was significantly changed (mainly up-regulated) dur-

ing PG and/or PTG, although most of them had undetectable levels of transcription in desiccated mature pollen grains (Tables III and IV; Supplemental Tables S6 and S7). It is further proposed that these Hsps may function as molecular chaperones (for review, see Miernyk, 1999) involved in the regulation of protein modification (such as protein folding) during PG and PTG, which is associated with the rapid protein synthesis and high physiological activity in germinating pollen and pollen tubes.

In addition to the genes that showed significant transcriptional changes during PG and PTG, as discussed above, some other genes were constitutively transcribed at constant and high levels during PG and PTG, such as *ROP1* (Li et al., 1999; Gu et al., 2003), *ACA9* (Schiott et al., 2004), and *VGD1* (Jiang et al., 2005). These genes have all been reported to play essential roles in the regulation of PG and PTG, suggesting that the constantly and highly transcribed genes are also important for the regulation of PG and PTG.

CONCLUSION

The data presented in this study provide, to our knowledge for the first time, a genome-wide view of the dynamic changes in the transcriptome during the transition from mature pollen grains to germinating pollen and to growing pollen tubes in Arabidopsis. Overall, transcription is increased during PG and PTG in terms of both the total number of transcribed genes and the transcriptional levels of some genes. A number of genes were significantly up-regulated, and, more importantly, some new groups of genes were preferentially transcribed during PG and/or PTG. The GO analysis of these up-regulated genes revealed a function skew toward cell rescue, transcription, signal transduction, and cellular transport during PG and PTG. Most of the functional categories correlate well with the known cellular dynamic activities in germinating pollen and/or growing pollen tubes. The members of the *CaM/CML*, *CHX*, and *Hsp* gene families, whose transcription was remarkably regulated during PG and PTG, were further analyzed and discussed in detail. This work provides a comprehensive transcriptional overview toward the further understanding of molecular networks for the regulation of PG and PTG.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (*Arabidopsis thaliana* ecotype Columbia) plants were grown in mixed soil in a growth chamber. The light intensity was 120 to 150 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for a 16-h daily light period, and day and night temperatures were $22^\circ\text{C} \pm 2^\circ\text{C}$ and $18^\circ\text{C} \pm 2^\circ\text{C}$, respectively. Plants were watered once every 5 d with tap water, and the relative humidity in the growth chamber was kept near 70%.

In Vitro PG Assay

In vitro Arabidopsis PG experiments were conducted as described previously (Fan et al., 2001), except that the basic medium was slightly modified.

Briefly, the basic medium was composed of 1 mM KCl, 5 mM CaCl_2 , 0.8 mM MgSO_4 , 1.5 mM boric acid, 1% (w/v) agarose, 19.8% (w/v) Suc, 0.05% (w/v) lactalbumin hydrolysate, 10 μM myo-inositol, and 5 mM MES, and the pH was adjusted to 5.8 with Tris. The heated solution (100°C , 2 min) was poured into small petri dishes (1.5 mL per dish; dish diameter was 35 mm) and cooled down to form a medium layer. For assays with the addition of Act D, the stock solution of Act D was added after the temperature of the medium dropped to below 60°C . The dehisced anthers were carefully dipped onto the surface of the medium to make pollen grains stick. The dishes were incubated for 4.5 h in a climate chamber ($25^\circ\text{C} \pm 0.2^\circ\text{C}$, 100% relative humidity), frozen at -20°C for 3 min to quickly terminate the PG and PTG, and kept on ice for counting the PG rate and measuring pollen tube length. Pollen grains with emerging tubes equivalent to or longer than their diameters were considered germinated. An approximately 85% germination rate for Arabidopsis (Columbia) pollen was usually observed (Fig. 1). All of the experiments were repeated three times, and four replicates (dishes) were carried out for each treatment in every experiment. For each replicate or each dish, there were more than 400 pollen grains counted in order to calculate the PG rate, and approximately 80 pollen tubes were measured for length.

Preparation and Collection of Pollen and Pollen Tube Material for RNA Extraction

The desiccated mature pollen grains were collected from freshly anther-dehisced flowers using a vacuum as described previously (Johnson-Brousseau and McCormick, 2004) and then transferred directly into liquid nitrogen for triturating to prevent pollen hydration. After this, the triturated pollen samples were transferred to extraction buffer for total RNA extraction. In order to get enough hydrated pollen grains and especially pollen tubes, the thin liquid layer germination method was developed (Fig. 2, A–C). First, the freshly anther-dehisced flowers were collected into 1.5-mL microfuge tubes filled with 800 μL of basic medium (the contents of this medium are the same as the basic medium for in vitro PG assays described above, and the concentration of Suc is modified to 15% [w/v]). The microfuge tubes were strongly vortexed for 2 min to release the mature pollen grains into the medium. The mixture was then transferred into new microfuge tubes and centrifuged at 11,000 rpm for 1 min. The pollen pellet was resuspended in 30 μL of the basic medium and subsequently cultured in petri dishes. We noticed that making a well-spread thin layer of pollen grains is a key point in getting high rates (70%–75%) of PG. In order to well spread a tiny drop of pollen grains on the petri dish (35 mm diameter) surface, a steel-wire net ($\varnothing 80 \mu\text{m}$) was placed (Fig. 2B) in the petri dish. The covered petri dishes were transferred into a chamber and incubated at 25°C for 45 min, and then the hydrated pollen grains were collected for total RNA extraction. For the collection of pollen tube samples, the pollen grains were incubated for 4 h, which resulted in an average pollen tube length of about 150 μm . After removing the steel-wire net, the culture mixture was collected and washed using the basic medium (19.8% [w/v] Suc). The pollen tubes were filtered using a 50- μm nylon mesh to remove the ungerminated pollen grains. The pollen tube samples were collected from the nylon mesh for total RNA extraction.

For the FDA staining assay, pollen grains were incubated for 45 min in the basic medium with the addition of 2 $\mu\text{g mL}^{-1}$ FDA. After washing out FDA with basic medium, the fluorescence of pollen grains was observed on a Zeiss LSM 510 META confocal microscope. The stock solution of FDA (5 mg mL^{-1}) was made using acetone.

RNA Extraction, Target Preparation, and Hybridization to Affymetrix GeneChips

The collected pollen grains or pollen tubes were transferred to liquid nitrogen for trituration. The triturated samples were transferred to TRIzol reagent (Invitrogen) for extraction of total RNA. RNA concentration, purity, and integrity were determined and confirmed using an Eppendorf Biophotometer and electrophoresis. The total RNA was further purified using the RNeasy Mini Kit (Qiagen). The purified RNA (8 μg) was used to generate first-strand cDNA in a reverse transcription reaction (One-Cycle Target Labeling and Control Reagents; Affymetrix). After second-strand synthesis, the double-stranded cDNAs were used to generate cRNA via an in vitro transcriptional reaction. The cRNA was labeled with biotin, and 20 μg of the labeled cRNA was fragmented. The size distribution of the cRNAs and fragmented cRNAs

was assessed using an Eppendorf Biophotometer and electrophoresis. Fragmented cRNA (15 μg) was added to 300 μL of hybridization solution, and 200 μL of this mixture was used for hybridization on Arabidopsis ATH1 Genome Arrays for 16 h at 45°C. The standard wash and double-stain protocols (EukGE-WS2v5-450) were applied using an Affymetrix GeneChip Fluidics Station 450. The arrays were scanned on an Affymetrix GeneChip scanner 3000.

GeneChip Data Analysis

To ensure reproducibility and reliability, two biological replicates were conducted and the RNAs that were used for microarray experiments were extracted from two independent plant populations. The scanned arrays were analyzed first with Affymetrix GCOS 1.0 (MAS 5.0) software to generate detection calls. All six data sets (three different samples from two biological replicates) were normalized using Affymetrix GCOS software, and the TGT value was set to 100. When analyzing the transcriptionally changed genes, the signal ratio between two stages was calculated to represent the fold change of this gene for its transcription in the corresponding process, and the fold change P value for each gene was obtained at the same time using GCOS software.

A gene was considered to be expressed when it had a detection call "P" in both replicates. A gene that had detection call P only in one stage (MP, HP, or PT) was defined as a specifically expressed gene in this stage. When determining the transcriptionally changed genes, the exclusive approach was used. A gene was considered to be transcriptionally changed when it was up-regulated or down-regulated more than 1.6-fold in both replicates ($\text{FC_rep1} > 1.6$ and $\text{FC_rep2} > 1.6$ or $\text{FC_rep1} < -1.6$ and $\text{FC_rep2} < -1.6$). A negative or positive value represents downward or upward regulation, respectively. Meanwhile, the fold change P values of this gene in two replicates must both be less than 0.01 ($\text{p-value_rep1} < 0.01$ and $\text{p-value_rep2} < 0.01$).

The FunCat Scheme version 2.0 Web service (<http://mips.gsf.de/projects/funcat>) and MapMan software were used to analyze the functional categories of genes with altered expression levels and the differences between the PG and PTG transcriptomes.

Microsoft Excel and Access were used to extract and manage the microarray data. The annotations of the 22,746 genes represented on the Arabidopsis ATH1 Genome Array were downloaded from NetAffx (<http://www.affymetrix.com/analysis/index.affx>).

Real-Time PCR Analysis

Total RNA extraction and purification were performed as described above. The RNA extract was treated with DNase I (Takara Bio) using standard protocols to eliminate genomic DNA. RNAs were reverse transcribed with SuperScript II RNase H Reverse Transcriptase (Invitrogen) using Random Hexamer Primer (Promega). The cDNA samples were diluted to 8 ng μL^{-1} . Triplicate quantitative assays were performed on 1 μL of each cDNA dilution using the Power SYBR Green PCR Master Mix (Applied Biosystems; P/N 4368577) with the Opticon II system according to the manufacturer's protocols (MJ Research). The gene-specific primers (Supplemental Table S5) were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). The amplification of 18S rRNA was used as an internal control to normalize the data.

Supplemental Data

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

Supplemental Figure S1. Cluster analysis of the expressed genes during PG and PTG.

Supplemental Figure S2. MapMan analysis of the difference in transcription between PG and PTG.

Supplemental Table S1. Genes with detectable expression during PG and PTG.

Supplemental Table S2. Genes with altered transcription (up-regulated or down-regulated more than 1.6-fold) during PG and PTG.

Supplemental Table S3. Cluster analysis results of the expressed genes during PG and PTG.

Supplemental Table S4. Results of functional category and gene family analyses of genes with altered expression levels during PG and PTG.

Supplemental Table S5. MapMan analysis of genes with altered expression levels during PG and PTG.

Supplemental Table S6. Expressed genes in the CaM/CML, CHX, and Hsp gene families.

Supplemental Table S7. Transcriptionally up-regulated genes of the CaM/CML, CHX, and Hsp families during PG and PTG.

Supplemental Table S8. Sequences of the specific primers for the genes tested in real-time PCR experiments.

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