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ORIGINAL RESEARCH

Analysis of the Cold-Responsive Transcriptome in the Mature Pollen of *Arabidopsis*

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Abstract Mature pollen is very sensitive to cold stress in chilling-sensitive plants. To understand the genetic regulation of tolerance to cold stress, we analyzed the transcript expression profile in mature pollen of *Arabidopsis* using Affymetrix GeneChips containing ~24,000 genes. Expression of 2,127 genes was cold-regulated, of which 697 genes were upregulated and 1,430 genes were downregulated. Further analysis showed that a large number of signal transduction components were significantly affected by cold treatment, indicating extensive changes in the gene regulatory networks of mature pollen. Many cold-responsive genes encode transcription factors, suggesting a multitude of transcriptional cascades. A number of genes important for the biosynthesis or signaling of plant hormones, such as abscisic acid, auxin, and jasmonate, were regulated by cold stress, which is of potential importance in coordinating cold tolerance with pollen growth and development. In addition, 159 mature pollen-specific genes that might be involved in pollen viability were also cold-regulated. Expression of the cold-responsive transcripts identified by microarray analysis was confirmed by quantitative real-time PCR. Our study provides an overall picture of the cold-responsive transcriptome in *Arabidopsis* pollen and is valuable for understanding gene regulation in response to cold stress and the molecular mechanisms of cold tolerance in mature pollen.

Keywords *Arabidopsis* · Cold stress · Mature pollen · Microarray

Introduction

While temperature is clearly limiting factor for crops cultivated on marginal lands, crop productivity everywhere is often at the mercy of random environmental temperature fluctuations (Zinn et al. 2010). Certain stages are more sensitive to chilling than others in the life cycle of a plant. Seedlings appear to be more susceptible than plants at advanced stages of development (Lyons 1973), and the maturation of pollen is the most sensitive process in the entire life cycle of cold-sensitive plants (Sataka and Koike 1983; Patterson et al. 1987). Cold temperatures can induce pollen sterility, which may be due to disruption of sugar metabolism in the tapetum, ultimately abolishing starch accumulation in the pollen grains (Oliver et al. 2005). After cold stress, the pollen germination rate and seed production of *Arabidopsis* were significantly reduced (Lee and Lee 2003).

The growth of pollen tubes is required for fertilization in flowering plants. Because of this crucial function in the plant reproductive cycle, pollen has been the object of considerable molecular biological research. More and more investigations of processes underlying pollen development and function have been extended by studying pollen-related gene expression. *Arabidopsis* MS1 is involved in the

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development of pollen and the tapetum (Ito et al. 2007). *Arabidopsis* phosphatidylinositol 3-kinase is essential for vacuole reorganization and nuclear division during pollen development (Lee et al. 2008). The pollen-specific MIKC* (MADS DNA-binding domain, intervening domain, keratin-like domain, and c-terminal domain) class of MADS-domain transcription factors are required for pollen maturation and tube growth and could affect the expression of many genes specific to mature pollen (MP) in *Arabidopsis* (Verelst et al. 2007a, b; Adamczyk and Fernandez 2009).

The plant sexual reproduction has been long recognized as being highly stress-sensitive, with reproductive stress tolerance often a limiting trait in crop plant productivity (Charles and Harris 1972; Herrero and Johnson 1980). This aspect of stress sensitivity was recently reviewed by Barnabás et al. (2008), Hedhly et al. (2008), and Thakur et al. (2010). In *Arabidopsis*, cold-regulated genes have been estimated to constitute about 4–20% of the genome (Lee et al. 2005; Hannah et al. 2005). Significant progress has been made in the past decade in elucidating the transcriptional networks regulating cold acclimation. The *ICE1–CBF* transcriptional cascade plays a central role in the cold response pathway in *Arabidopsis* (Thomashow 1999; Chinnusamy et al. 2007). CBF transcription factors regulate cold-responsive (*COR*) genes by binding C-repeat/dehydration response elements (CRT/DRE) to their promoters (Stockinger et al. 1997; Fowler and Thomashow 2002). While temperature stress has been extensively studied and reviewed (Iba 2002; Yamaguchi-Shinozaki and Shinozaki 2006; Chinnusamy et al. 2007; Kotak et al. 2007; Wahid et al. 2007), most of the literature emphasizes insights from experimentally accessible tissues, such as leaves and roots. However, studies on sexual reproduction are often more difficult because gamete development and fertilization are complex processes that occur during a short window of time and are predominantly hidden within tissues of the flower (Zinn et al. 2010).

Gene expression profiles in pollen and gene regulation in response to cold stress provide valuable information for understanding why mature pollen is so sensitive to cold stress. Lee and Lee (2003) studied gene expression in response to cold stress using serial analysis of gene expression in pollen (covering 4,211 unique tags). In this study, the genome-wide transcript profile of cold-stressed mature pollen of *Arabidopsis* was analyzed using the Affymetrix ATH1-121501 microarray chip. Genes that exhibited 2-fold or greater differential expression after cold stress were analyzed, as some of these genes are likely to play roles in cold stress responses in mature pollen. The results suggested that a complicated regulatory mechanism is associated with determining the viability of mature pollen following cold stress.

Materials and Methods

Plant Growth Conditions and Pollen Collection

Wild-type (WT) *Arabidopsis thaliana* Col-0 plants were grown under standard greenhouse conditions, with temperature controlled at 22°C and a 16-h photoperiod with irradiance of about 120 μmol m^{-2} s^{-1}. Flowers were selected at developmental stage 13 based on the following developmental characteristics (Smyth et al. 1990): the 1-mm-long bud opened, the petals were visible between the sepals and elongated rapidly, the stigma was already receptive at this stage, and anthesis occurred. This stage continues for about 6 h.

At a fixed time in the morning, flowers at stage 13 were marked and the plants were moved into a growth chamber at 4°C under the same light conditions for 48 h. After cold treatment, mature pollen grains were harvested by shaking the flowers in 0.3 M mannitol, as described by Honys and Twell (2003). To remove flowers, the suspension was filtered through one layer of lens wiping paper at 4°C, then mature pollen were concentrated by centrifugation (1,420×*g*, 5 min, 4°C). Freshly harvested pollen from more than 100 flowers was ground with quartz sand. Total RNA was extracted using the RNAeasy Plant Mini Kit (QIAGEN, Valencia, CA, USA), and DNA was removed via an on-column DNase treatment. The purified RNA was stored at −80°C prior to use.

Pollen Viability and Seed Production Assay

Light and epifluorescence microscopy of DAPI-stained pollen were performed as described previously (Park et al. 1998). Pollen viability was assessed by staining mature pollen with fluorescein diacetate in vitro (Verelst et al. 2007b), and pollen germination assays were carried out in vivo as described previously with modification (Mori et al. 2006; Chhun et al. 2007). To determine the pollen germination ratio, flowers at early stage 12 (Smyth et al. 1990) were emasculated. After waiting 12–24 h to ensure that the stigma was receptive, an excess quantity of pollen from a donor plant was placed on the stigma. After 30 min, the pistils were excised and stained with aniline blue on a glass microscope slide for 30 min before observation under ultraviolet (UV) illumination with an Olympus BX51 microscope (Chhun et al. 2007). Germinated pollen grains were those grains attached to the stigma and in which a pollen tube was detected with UV fluorescence. To assess pollen tube elongation after cold treatment, the plants were moved to the greenhouse for 5 h, after which the pistils were removed, the stigma was fixed with acetic acid/ethanol (1:3, *v*/*v*) for 24 h, then softened with 8 M NaOH for 4 h. The stigma was washed in distilled water three

times, then stained with aniline blue on a glass microscope slide for 30 min prior to observation by UV fluorescence microscopy. Pollen grains attached to the stigma, and in which pollen tube growth in the style was detected, were classified as possessing elongated pollen tubes (Chhun et al. 2007).

For the seed production assay, cold-treated plants were transferred to the standard greenhouse and grown for about 3 weeks until seeds had matured. All of the seeds in more than 30 siliques were counted.

GeneChip Analysis

An Affymetrix ATH1-121501 microarray chip covering approximately 24,000 genes was utilized. Preparation of cDNA from total RNA and hybridization to ATH1 Arabidopsis Genome Arrays (Affymetrix Inc., Santa Clara, CA) was performed by the Shanghai Jintai Biological Technology Co. Ltd., Shanghai, China, in accordance with the standard manufacturer's protocol (Affymetrix 2001). The resulting data files were normalized and analyzed with Expression Console (Affymetrix) and Partek Genomics Suite 6.4 (Partek Inc., St Louis, MO; http://www.partek.com), and further processed with Microsoft Excel and Access (Microsoft). The expression profiles of cold-treated and untreated mature pollen were compared. Only genes with consistently present calls in the three biological replicates were considered in our analyses. Using the default parameters for Partek Genomics Suite, detection *P* values and signal values were calculated for each probe set. The *P* values indicated whether the transcripts were reliably detected. For functional classification of the genes, the Affymetrix ATH1 chip annotation was used, based on data from the TIGR database (http://www.tigr.org).

To test the cold response of a subset of genes implicated in a specific biological process, we performed an unbiased gene ontology (GO) analysis using the Gene Ontology Enrichment Analysis Software Toolkit (Zheng and Wang 2008; http://omicslab.genetics.ac.cn/GOEAST/).

Quantitative Real-Time PCR Validation

To analyze and confirm the expression pattern of differentially expressed genes, gene-specific primers (see Table 1) were designed for four randomly selected genes. We constructed cDNA from 1 μg RNA in a 20 μl reaction volume using the First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). Each cDNA sample was diluted 1:20 with water, and 2 μl of this dilution was used as template for quantitative RT-PCR. Half-volume reactions (10 μl each) were performed with the Lightcycler FastStart DNA Master SYBR Green I Kit (Roche) on a Roche LightCycler 480 real-time PCR machine, in accordance with the manufacturer's instructions. *ACTIN2* (At3g18780) was used as a control.

Results

Effect of Cold Stress on Mature Pollen Viability

Under the standard greenhouse growth environment, nearly all of the mature pollen grains showed green fluorescence after staining with FDA. Cold stress distinctly reduced the percentage of live pollen grains (Fig. 1a, c). After 48 h of cold treatment at 4°C, only 43.4% of pollen grains were viable. In the absence of cold stress, about 90% pollen germination had occurred 30 min after artificial pollination (Fig. 1d). Cold stress reduced the percentage pollen germination to 60.1% and 39.2% for the 24 and 48 h cold treatments, respectively. Furthermore, at 5 h after self-pollination, the number of elongated pollen tubes per pistil was about 138.2±13.4 under the standard growth condition (Fig. 1b, e). Cold stress significantly reduced the number of elongated pollen tubes to 83.6±6.4 and 46.7±5.3 for the 24 and 48 h cold treatments, respectively. These results indicated that cold stress did not completely abolish pollen germination or tube elongation, but did severely disturb these processes.

To further quantify the reduction in fertility under cold stress, the number of mature seeds was quantified in

Table 1 List of quantitative RT-PCR primer sequences

Gene ID	Primer forward (5′⟶3′)	Primer reverse (5′⟶3′)
AT3G18780 (ACTIN2)	TGTGCCAATCTACGAGGGTTT	TTTCCCGCTCTGCTGTTGT
AT1G18750 (AGL65)	GAGCACAAGCAACAGGCAAG	CGGTAGGGGAAAACATAAGAAGG
AT2G03060 (AGL30)	CTCCTCCTCTTCCTCTTACTCTTCC	GGTTTGGTTGGTGTTGTCGTT
AT1G22130 (AGL104)	ACTTTCCTGACCAGAATAGACG	TCTGCCATTTGGAGTTGTTG
AT1G77980 (AGL66)	CAGCAACTCAAGGCTGAGAA	CACAAGTTTCGTACTCTTCCATTG
AT1G62320	TCCGCGGACAGTTGGAGTCG	ATTTCTCCCGCCACACCCGC
AT1G01770	GGCCACTGGCGGCTCTCAAA	AGGGTCGTAGCCGAGACCGC
AT4G17690	AGCGAACCAATCCGTCCCCG	CCGATTGTGTGCCCGCCACT
AT2G23970	ACAATGGTTACAGACGCGGTGAAGC	AGCAAGCAATGTGGCTGATTCAGGG

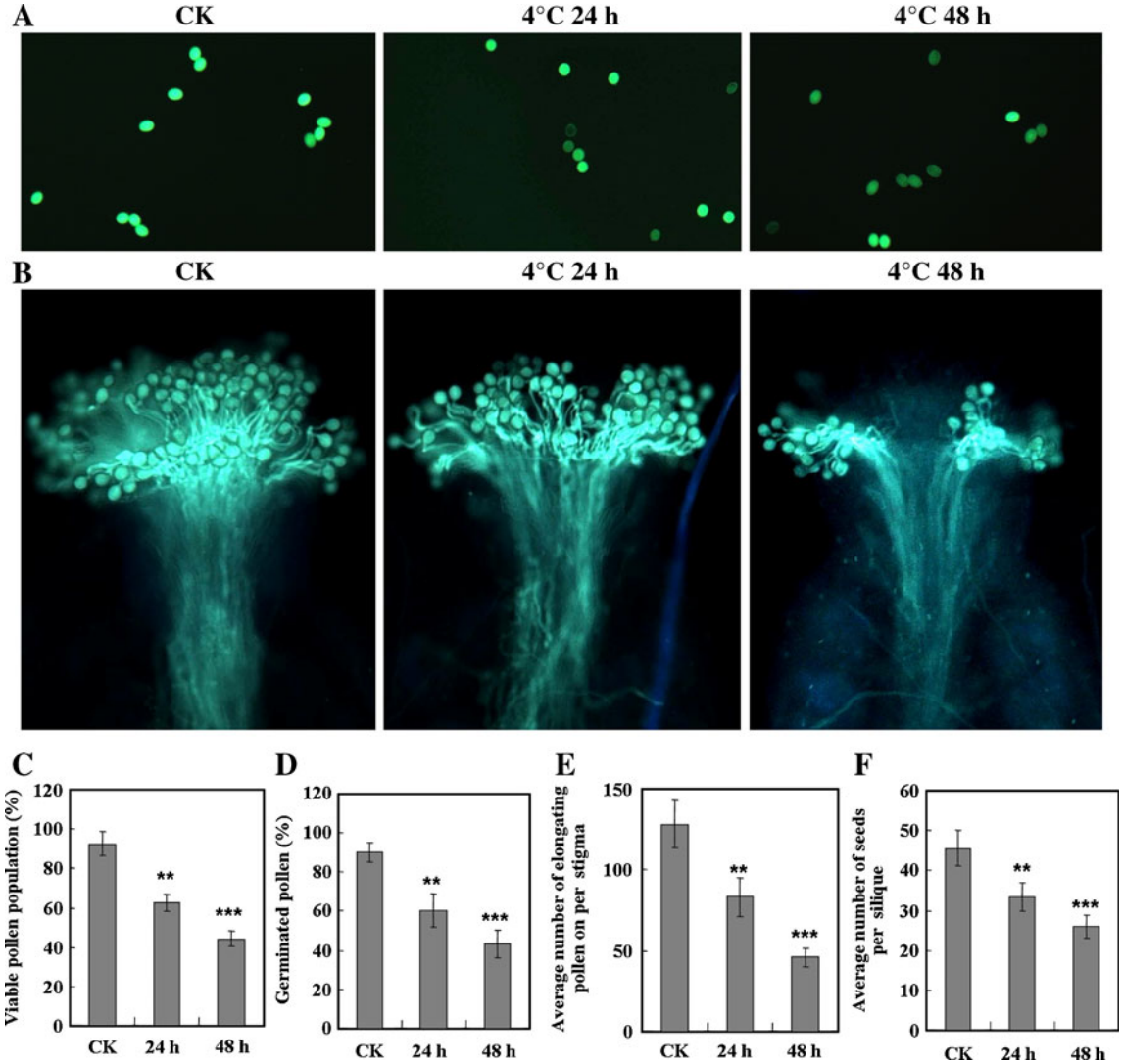

Fig 1 Effect of cold stress on pollen viability and seed product of *Arabidopsis*. **a** Fluorescence images of fluorescein diacetate (FDA)-stained mature pollen grains after cold treatment. **b** Aniline blue staining of germinated pollen in vivo. After cold treatment, elongated pollen on the stigma was significantly reduced. **c** Percentage of viable pollen grains determined by staining with FDA. In each replicate, over 100 pollen grains were counted. **d** Percentage of pollen germination in vivo. In each replicate, over 100 pollen grains were counted. **e** Average number of elongated pollen grains per stigma from self-fertilized plants. In each replicate, over 30 stigmas were counted. **f** Cold-treated mature pollen at stage 13 were transferred to normal conditions and grown until seeds had been produced. The number of seeds was counted. In each replicate, over 30 siliques were counted. *CK* indicates the mature pollen without cold treatment. *Error bars* indicate standard deviations of three independent biological replicates. Differences between the cold-stressed and unstressed plants are significant at the $0.01>P>0.001$ (**) or $P<0.001$ (***) levels

siliques that developed from flowers treated at 4°C for 24 or 48 h. As shown in Fig. 1f, unstressed plants produced about 46 seeds per silique, and this number was reduced in cold-treated plants. This indicated that cold stress considerably reduced the viability of mature pollen.

Gene Expression in Response to Cold Stress

When mature pollen was treated with 4°C for 48 h, 2,127 genes were differentially expressed by more than 2-fold. Among these genes, 697 were upregulated and 1,430 were downregulated (Table S1), which suggested that cold stress mainly downregulated gene expression in mature pollen.

The cold-regulated genes were categorized into 16 functional groups using the Functional Catalogue at http://mips.gsf.de/projects/funcat (Ruepp et al. 2004), with manual adjustment when necessary (Fig. 2). Of the induced genes, 50.9% had unknown functions (Fig. 2a), whereas the rest were primarily involved in metabolism (11.0%), signal transduction (6.4%), and transport facilitation (5.0%); only a low percentage had functions involving energy (0.8%) and cell organization (1.0%). A similar classification for the repressed genes was noted, for which the function of a

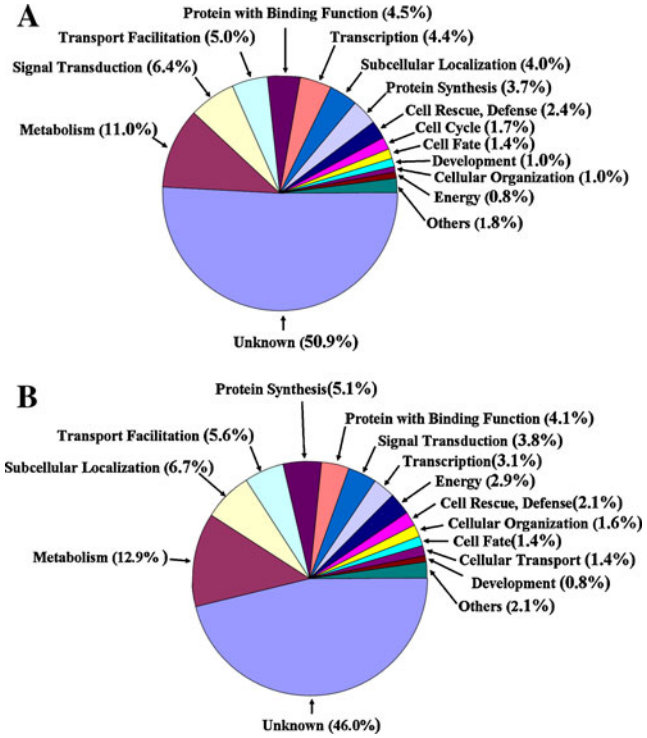

Fig 2 Functional classification of genes in which expression was changed >2-fold after cold treatment. **a** Cold upregulated genes. **b** Cold downregulated genes. Functional category is based on the Functional Catalogue at the website http://mips.gsf.de/projects/funcat, with manual adjustment when necessary

relatively large fraction (46.0%) was unknown and 12.9% of the remainder were involved in metabolism (Fig. 2b). Cell rescue and defense-related roles were assigned to 2.4% and 2.1% of the induced and repressed genes, respectively. Although genes lacking significant similarity to known genes or functions comprised the largest group of cold-regulated genes, further analysis focused on genes with sequence homology to known genes. Among such cold-regulated genes, there were 117 signal transduction genes (Table 2 and 3), 93 genes involved in transcription (Table 4 and 5), and 36 hormone-related genes (Table 6).

Subsequently, we compared our results with the reference dataset of Honys and Twell (2003), who characterized the transcriptome of four stages of pollen development: unicellular microspores (UNM), bicellular pollen (BCP), tricellular pollen (TCP), and mature pollen grains (MPG). Genes were classified as immature pollen (IP) if their expression in mature pollen decreased by at least 50% relative to the TCP stage. Similarly, genes were termed MP if their WT expression increased at least 50% in mature pollen, relative to the three immature stages (Verelst et al. 2007b). We found that of the 959 MP genes, 112 (11.6%) were downregulated and 47 were upregulated (Table S2). Therefore, transcription of a large portion of the MP genes might be involved in the cold response in mature pollen.

Among the genes upregulated in response to cold treatment, we found one GO term associated with the cold response, in which 19 genes were included (Table S3), and three GO terms associated with stress responses (Table 7). Of the genes downregulated after cold treatment, we found 11 GO terms associated with stress responses (Table 7), which implied that cross-talk occurred between the cold stress response pathway and other abiotic stress response pathways in mature pollen.

Overall, the microarray analysis demonstrated that cold stress could lead to extensive changes in the transcriptome of mature pollen, which may be responsible for the cold sensitivity of mature pollen.

Validation of Gene Expression

As shown in Fig. 3, the relative expression levels of four randomly selected genes varied in the untreated and cold-treated mature pollen. We also analyzed expression of the MIKC*-type transcription factors *AGL66*, *AGL104*, *AGL30*, and *AGL65*. Quantitative RT-PCR revealed that their expression was consistent with the results of the microarray analysis (Fig. 3; Table 4), thus confirming the reliability of the microarray analysis.

Discussion

One aim of functional genome analysis is to understand the temporal and spatial expression patterns of all genes with roles in the developmental processes of an organism and to understand how they function in response to biotic and abiotic stresses, such as pathological conditions and environmental stresses. In the present work, microarray analysis was employed to analyze the gene regulation pattern in response to cold stress in mature pollen of *Arabidopsis*. Because pollen plays a critical role in sexual reproduction of plants, it is highly likely that the reduced fertility was induced by stress originating from defective functioning of pollen (Zinn et al. 2010). Although cold stress responses in plants have been extensively studied, there have been few investigations into the effect of cold stress on pollen development and function. In this study, the comparative microarray profiles of transcripts from mature pollen grains before and after cold treatment provide clues to the function of those genes whose contribution to cold acclimation of mature pollen.

Cold Regulation of Signal Transduction Components

In our study, cold stress induced the downregulation of eight calcium-binding genes (Table 3), and the GO terms for response to cadmium ions (GO:0046686) and

Table 2 Genes upregulated during cold stress involved with signal transduction

Category	AGI ID	Fold change	Name
Calcium-binding	AT5G04220	3.27	Calcium- and lipid-binding protein
Protein phosphatase	AT3G62260	2.71	Putative protein phosphoprotein phosphatase
	AT4G31860	2.62	Protein phosphatase 2C
	AT1G03445	2.62	Putative serine/threonine protein phosphatase
	AT1G07160	2.35	Proteinphosphatase 2C
	AT3G17090	2.05	Protein phosphatase 2C
Protein kinase	AT5G01810	11.81	Serine/threonine protein kinase ATPK10
	AT1G63500	9.75	Protein kinase, putative
	AT1G71830	7.65	Protein kinase, putative
	AT2G32510	6.65	Putative protein kinase
	AT2G38490	4.26	Putative protein kinase
	AT4G18710	4.13	Shaggy-like protein kinase
	AT5G14640	3.88	Protein kinase MSK3
	AT3G02880	3.78	Putative protein kinase
	AT2G02710	3.24	Putative receptor-like protein kinase
	AT1G07870	3.1	Protein kinase, putative
	AT5G67080	3.07	Protein kinase-like protein
	AT4G32660	2.46	Protein kinase AME3
	AT4G38470	2.38	Protein kinase-like protein protein kinase 6
	AT1G74490	2.41	Putative protein kinase
	AT2G39660	2.39	Putative protein kinase
	AT5G55910	2.19	Serine threonine-specific protein kinase ATPK64
	AT2G17090	2.17	Putative protein kinase
	AT3G59350	2.21	Protein kinase
	AT3G18810	2.06	Protein kinase, putative
Receptor-like kinase	AT1G71830	7.65	Putative receptor protein kinase
	AT2G33170	6.62	Putative receptor-like protein kinase
	AT4G29450	5.13	Serine/threonine-specific receptor protein kinase
	AT1G19090	4.26	Receptor-like serine/threonine kinase, putative
	AT2G02710	3.24	Putative receptor-like protein kinase
	AT1G49270	2.62	Putative receptor-like protein kinase
	AT5G35390	2.62	Putative receptor-like protein kinase
	AT5G45840	2.44	Receptor protein kinase
	AT3G51550	2.31	Receptor protein kinase
	AT3G14840	2.24	Receptor-like serine/threonine kinase, putative
	AT1G66150	2.21	Receptor protein kinase TMK1, putative
	AT1G17540	2.19	Receptor-like serine threonine kinase, putative
	AT3G18810	2.06	Putative somatic embryogenesis receptor-like kinase
Response regulator	AT5G24470	11.65	Putative two-component response regulator protein
	AT3G11020	2.47	DREB2B transcription factor
	AT2G40940	2.21	Ethylene response sensor ERS
GTP-related	AT2G21880	20.94	Putative RAS superfamily GTP-binding protein
	AT1G52280	3.9	GTP-binding protein RAB7D, putative
	AT2G23460	2.34	Putative GTP-binding protein

calcium-binding (GO:0005509) were associated with genes downregulated by cold stress (Table 2, Table S3, Fig. 4). It is widely accepted that calmodulin or a calmodulin-like protein gene family are involved in the regulation of mature pollen viability (Taylor and Hepler 1997; Franklin-Tong 1999), and Ca^{2+}-binding proteins are the main signaling components during cold stress. Thus, cold-induced inhibition of Ca^{2+} signaling components might be responsible for the cold sensitivity of mature pollen.

Table 3 Genes downregulated by cold stress involved with signal transduction

Category	AGI ID	Fold change	Name
Calcium-binding	AT5G10660	−2.06	Vacuolar calcium-binding protein
	AT3G51860	−2.15	Ca^{2+}/H^{+}-exchanging protein
	AT3G22910	−2.87	Calmodulin-stimulated calcium atpase, putative
	AT5G51050	−3.13	Calcium-binding transporter-like protein
	AT1G09210	−7.36	Putative calcium-binding protein
	AT4G37640	−10.84	Plasma membrane-type calcium atpase (aca2)
	AT5G17480	−14.17	Calcium-binding protein
	AT4G20780	−22.79	Calcium-binding protein
Protein phosphatase	AT5G19280	−2.03	Kinase-associated protein phosphatase
	AT1G78200	−2.23	Putative protein phosphatase 2C
	AT3G55050	−2.23	Protein phosphatase 2C
	AT3G16800	−2.51	Protein phosphatase, putative
	AT1G54450	−2.55	Protein phosphatase 2A, putative
	AT3G51470	−2.57	Protein phosphatase 2C
	AT1G13460	−2.6	Protein phosphatase 2A, putative
	AT1G72770	−2.7	Protein phosphatase 2C
	AT5G27930	−3.01	Protein phosphatase
	AT3G15260	−3.37	Putative protein phosphatase 2C
	AT3G23610	−4.34	Dual-specificity protein phosphatase
	AT1G69960	−4.35	Serine/threonine protein phosphatase 2A
	AT3G05640	−21.29	Putative protein phosphatase 2C
Protein kinase	AT1G73460	−2.03	Serine/threonine protein kinase
	AT3G56050	−2.07	Putative serine threonine-specific protein kinase NPK15
	AT1G73670	−2.1	Putative MAP kinase
	AT4G27940	−2.15	Putative protein kinase LePK7
	AT5G35410	−2.18	Serine/threonine protein kinase SOS2
	AT4G32250	−2.27	Putative protein mixed-lineage protein kinase
	AT4G31230	−2.43	Protein kinase
	AT5G08590	−2.62	Serine/threonine protein kinase
	AT4G09570	−2.77	Calmodulin-domain protein kinase CDPK4
	AT4G35500	−2.93	Protein kinase
	AT3G22420	−3.06	Putative protein kinase
	AT3G51990	−3.38	Putative serine/threonine protein kinase
	AT1G64628	−3.6	Protein kinase, putative
	AT5G25110	−3.49	Serine/threonine protein kinase
	AT2G38910	−3.74	Putative calcium-dependent protein kinase
	AT5G08160	−3.96	Serine/threonine protein kinase
	AT1G29230	−4.25	Protein kinase PK4
	AT1G48480	−5.56	Protein kinase, putative
	AT1G08650	−6.83	Putative calcium-dependent protein kinase
	AT2G16750	−10.35	Putative protein kinase
	AT1G78980	−11.55	Leucine-rich repeat protein kinase, putative
	AT1G49740	−12.51	Unknown protein-like protein kinase
	AT1G79250	−13.21	Serine/threonine protein kinase, putative
Receptor-like kinase	AT5G61350	−2.18	Receptor-like protein kinase precursor
	AT1G36380	−2.18	Hypothetical protein
	AT1G29330	−2.22	ER lumen protein-retaining receptor
	AT4G04510	−2.28	Putative receptor-like protein kinase
	AT2G30290	−2.26	Putative vacuolar sorting receptor

Table 3 (continued)

Category	AGI ID	Fold change	Name
	AT2G25790	−2.42	Putative receptor-like protein kinase
	AT5G65280	−2.82	G protein-coupled receptor-like protein
	AT3G25040	−2.83	ER lumen protein-retaining receptor, putative
	AT3G15610	−3.08	Similar to serine/threonine kinase receptor
	AT5G27210	−3.37	Putative receptor protein
	AT1G72460	−4.52	Putative receptor-like protein kinase
	AT1G77280	−4.97	Hypothetical receptor-like protein kinase
	AT1G19970	−11.09	ER lumen protein-retaining receptor
	AT4G25390	−12.77	Receptor kinase
Response regulator	AT1G53290	−2.09	Hypothetical protein
	AT4G23690	−6.33	Putative disease resistance response protein
GTP-related	AT2G17800	−2.09	Putative GTP-binding protein
	AT4G02080	−2.2	SAR1/GTP-binding secretory factor
	AT5G10260	−2.23	GTP-binding protein
	AT2G44610	−2.17	Putative small GTP-binding protein
	AT3G16620	−2.43	Putative GTP-binding protein
	AT2G22290	−2.5	Putative GTP-binding protein
	AT1G56330	−2.7	GTP-binding protein SAR1B
	AT5G59840	−2.84	GTP-binding protein
	AT3G07270	−3.08	GTP cyclohydrolase I
	AT1G43890	−3.43	GTP-binding protein RAB1Y
	AT3G12160	−3.45	Ras-related GTP-binding protein
	AT1G09180	−4.88	Putative GTP-binding protein
	AT5G59150	−4.88	GTP-binding protein rab11

Protein phosphorylation and dephosphorylation have been implicated in cold signal transduction. Indeed, protein kinases and phosphatases with altered expression formed the largest group of genes (a total of 60 genes among 123 cold-regulated signaling genes; Table 2 and 3). In addition, many protein phosphatases showed differential expression (Table 2 and 3), suggesting that posttranslational modification occurred during the cold response of mature pollen.

Twenty-seven genes for receptor-like kinases (RLKs) were regulated by cold stress (Table 2 and 3). Among the cold-regulated *RLKs*, one *RLK* has been studied in detail. The leucine-rich repeat transmembrane protein kinase, *RKL1* (At1g48480), was predominantly expressed in stomatal cells and was induced by wounding, pathogen attack, and salicylic acid (Tarutani et al. 2004a, b). Recent work indicates that RPK1 plays an important role in abscisic acid (ABA) signal transduction (Osakabe et al. 2005). These results suggest that cold signal transduction in mature pollen grains shared, at least in part, some common pathways with other biotic, abiotic, and ABA stress signaling through these RLKs.

Intriguingly, the pseudoresponse regulator, *APRR5* (At5g24470), was strongly upregulated (Table 2). Pseudo-response regulators are proteins that lack the conserved aspartame residue that in typical response regulators is the phosphorylation target of the upstream kinase in two-component systems (Hwang et al. 2002). These response regulators of two-component systems have been implicated in cytokinin signaling (Hwang and Sheen 2001; Kiba et al. 2002). Thus, this observation indicated an interaction between cytokinin signaling and cold response in mature pollen.

Cold stress also altered expression of 16 GTPase genes, including two Rab GTPases (Table 2 and 3), and GO terms for small GTPase-mediated signal transduction (GO:0007264) and GTP-binding (GO:0005525) were associated with the genes downregulated by cold stress (Table S3). Although no in planta function for these genes has been established in *Arabidopsis*, Rab GTPases are known to function in intracellular membrane trafficking (Zerial and McBride 2001). Thus, cold regulation of GTPases indicates that active, or inhibition of, membrane trafficking might take place in response to cold stress in mature pollen.

Cold Regulation of Transcription Factors

When classified by their characteristic DNA-binding domains, the 93 cold-regulated transcription factors fall into eight families (Table 4 and 5). Transcription factors

Table 4 Genes upregulated during cold stress involved in transcription

Category	AGI ID	Fold change	Name
AP2	AT1G53910	5.39	AP2 domain-containing protein, putative
	AT3G14230	2.41	AP2 domain-containing DNA-binding protein
	AT3G16280	2.04	Putative AP2 domain transcription factor
	AT5G61590	19.66	AP2 domain-containing transcription factor
	AT3G11020	2.47	DREB2B transcription factor
Myb	AT4G26930	27.76	Putative myb-related protein
	AT3G01530	7.73	Putative transcription factor
	AT4G37260	4.97	MYB-related protein
	AT2G46830	4.13	MYB-related transcription factor CCA1
	AT5G67300	3.78	MYB-related protein
	AT5G61620	3.22	transcriptional activator
	AT1G01380	2.29	Myb homolog CPC, putative
	AT1G26580	2.16	Putative MYBfamily transcription factor
	AT5G16880	2.09	TOM target of myb1
bHLH	AT3G47640	3.27	bHLH family protein
	AT2G35940	2.67	Bel1-like homeodomain1 BLH1
WRKY	AT4G26440	7.02	Transcription factor WRKY34
	AT2G30250	2.77	Transcription factor WRKY25
MADS	AT5G39750	3.19	Putative protein MADS-box protein 2
bZIP	AT1G13600	8.31	bZIP transcription factor
	AT5G28770	4.8	bZIP transcription factor
	AT3G58120	3.15	Putative protein, transcription activator
	AT5G49450	2.52	Putative protein
NAC	AT1G69490	3.87	Unknown protein, similar to NAC domain protein NAM
	AT5G39610	21.52	NAM/CUC2-like protein CUC2
	AT4G28530	5.26	NAM/CUC2-like protein NAM
	AT1G12260	3.21	Unknown protein similar to NAM
	AT3G04070	2.87	NAM-like protein
Zn	AT4G29190	9.75	Putative protein zinc finger transcription factor
	AT5G57660	5.41	CONSTANS-like B-box zinc finger protein
	AT5G41400	3.5	RING zinc finger protein
	AT2G31380	2.96	Putative CONSTANS
	AT5G04240	2.81	Zinc finger protein
	AT4G38960	2.88	Putative zinc finger protein
	AT2G21320	2.59	Putative CONSTANS
	AT1G73760	2.47	Putative RING zinc finger protein
	AT4G08590	2.14	Putative zinc finger protein
	AT5G04340	2.04	Putative c2h2 zinc finger transcription factor

with Zn finger or Myb domains comprised the two major families, accounting for 27 genes and 16 genes, respectively, of the 94 cold-regulated transcription factors. Transcription factors with a Zn finger domain are involved in plant stress resistance and in hormone signal transduction in *A. thaliana* (Sakamoto et al. 2004; Ciftci-Yilmaz et al. 2007) and rice (Liu et al. 2007). We also found that five basic helix-loop-helix (bHLH) transcription factors were cold-regulated (Table 4 and 5). As Myb and bHLH proteins often interact with each other to control transcription (Ramsay and Glover 2005), the differential expression of *Myb* and *bHLH* indicated that the regulation of some cold-responsive genes might be achieved by modulation of the ratio of these partner factors.

In the present study, 13 genes belonging to the AP2 family were cold-regulated (Table 4 and 5). This indicated that AP2 transcription factors might play a role in the cold response of mature pollen, a finding supported by the fact that DREBs/CBFs are in the AP2/ERF family and are important for the activation of many cold-responsive genes

Table 5 Genes downregulated during cold stress involved in transcription

Category	AGI ID	Fold change	Name
AP2	AT1G53170	−2.42	AP2 domain, putative
	AT3G20310	−2.19	Ethylene-responsive element-binding factor, putative
	AT3G59970	−2.38	Putative methylenetetrahydrofolate reductase
	AT3G11930	−3.28	Ethylene-responsive protein, putative
	AT3G24500	−20.49	Ethylene-responsive transcriptional coactivator, putative
	AT5G05410	−3.5	DREB2A
Myb	AT1G54260	−2.02	Hypothetical protein
	AT1G74840	−2.26	Myb-related transcription activator, putative
	AT5G04760	−2.31	I-box binding factor
	AT3G55730	−3.33	MYB transcription factor
	AT3G12560	−4.15	Myb family transcription factor, putative
	AT3G27810	−5.08	MYB family transcription factor Atmyb3, putative
	AT3G27810	−5.08	Myb protein identical to ATMYB3
bHLH	AT3G23210	−2.58	Basic helix-loop-helix (bHLH) family protein
	AT2G42300	−3.19	Basic helix-loop-helix (bHLH) family protein
	AT1G61660	−3.52	Basic helix-loop-helix (bHLH) family protein
HSF	AT1G32330	−2.26	Heat shock transcription factor HSF8, putative
	AT2G26150	−2.39	Putative heat shock transcription factor
	AT1G67970	−3.54	Putative heat shock transcription factor
MADS	AT2G34440	−2.34	MADS-box protein (AGL29)
	AT3G57230	−2.44	MADS-box transcription factor
	AT1G22130	−2.72	AGL104 (AGAMOUS-LIKE 104), transcription factor
	AT1G18750	−2.1	AGL66 (AGAMOUS-LIKE 66), transcription factor
	AT1G18750	−2.1	AGL65 (AGAMOUS-LIKE 65), transcription factor
	AT2G03060	−4.76	AGL30 DNA-binding, transcription factor
bZIP	AT1G58110	−2.17	bZIP transcription factor
	AT2G22850	−2.97	Putative embryo-abundant protein
	AT2G46270	−3.11	G-box-binding bZIP transcription factor
	AT4G34590	−3.38	bZIP transcription factor ATB2
	AT1G06850	−4.27	bZip DNA-binding protein, putative
	AT3G01470	−5.52	Homeobox-leucine zipper protein
	AT1G75388	−2.44	bZIP transcription factor ATB2, putative
NAC	AT1G01720	−2.41	NAC domain protein, putative
	AT5G04410	−2.56	Putative protein NAC2
	AT3G49530	−3.32	NAC2-like protein NAC2
	AT1G52890	−2.02	NAM-like protein
	AT1G52880	−5.12	NAM-like protein
	AT1G61110	−36.93	NAM protein, putative
Zn	AT3G07650	−2.09	Unknown protein similar to zinc finger protein
	AT3G43430	−2.11	Putative protein RING-H2 zinc finger protein ATL4
	AT1G10560	−2.16	Putative zinc-binding protein
	AT2G29410	−2.28	Putative zinc transporter
	AT5G10650	−2.36	Pspzf zinc finger protein
	AT1G74410	−2.34	Putative RING zinc finger protein
	AT2G15580	−2.46	Putative RING-H2 zinc finger protein
	AT5G43030	−2.57	CHP-rich zinc finger protein-like
	AT2G27580	−2.67	Putative zinc finger protein
	AT3G21890	−3.47	Zinc finger protein, putative
	AT2G23780	−3.49	Putative RING zinc finger protein

Table 5 (continued)

Category	AGI ID	Fold change	Name
	AT5G37340	−5.59	Zinc finger protein
	AT1G63840	−5.71	Putative RING zinc finger protein
	AT2G17730	−6.23	Unknown protein similar to C3HC4-type zinc fingers
	AT5G04390	−7.01	Zinc finger transcription factor
	AT1G68190	−9.62	Putative zinc finger protein
	AT1G70810	−22.17	Unknown protein similar to zinc finger and C2 domain protein

Table 6 Cold-regulated genes with hormone-related roles

Category	AGI ID	Fold change	Name
Upregulated			
Auxin	AT2G33830	81.00	Putative auxin-regulated protein
	AT1G59500	2.83	Auxin-regulated protein GH3, putative
	AT5G48220	2.19	Indole-3-glycerol phosphate synthase
ABA	AT5G45340	2.35	Cytochrome P450
Ethylene	AT5G61590	19.66	Ethylene-responsive element-binding factor
	AT1G73730	2.77	Ethylene-insensitive3-like3 EIL3
	AT2G40940	2.21	Ethylene response sensor ERS
	AT1G09740	2.20	Putative ER6 protein
Jasmonate	AT3G15500	6.19	Putative jasmonic acid regulatory protein
	AT4G16690	23.58	Methyl jasmonate esterase
GA	AT1G30040	2.16	ATGA2OX2 gibberellin 2-beta-dioxygenase
Brassinosteroid	AT4G18710	4.13	BIN2 brassinosteroid-insensitive 2
Downregulated			
Auxin	AT4G22620	−2.16	Auxin-responsive family protein
	AT1G14020	−2.59	Putative growth regulator protein
	AT1G51760	−3.71	Auxin conjugate hydrolase ILL5
	AT4G13790	−4.71	SAUR-AC
	AT5G44300	−5.46	Auxin-repressed protein
	AT2G36910	−2.11	Putative ABC transporter
ABA	AT3G53880	−2.93	Reductase-like protein abscisic acid-activated protein
	AT2G31750	−2.87	Putative glucosyltransferase
	AT4G34131	−2.80	Glucosyltransferase
	AT3G23050	−2.44	Indoleacetic acid-inducible gene (IAA7)
	AT5G23350	−7.24	Putative protein
	AT5G53820	−31.60	ABA-inducible protein
	AT3G02480	−18.43	ABA-responsive protein
Ethylene	AT3G20310	−2.19	Ethylene-responsive element-binding factor
	AT3G11930	−3.28	Ethylene-responsive protein, putative
	AT3G24500	−20.49	Ethylene-responsive transcriptional coactivator, putative
	AT1G05710	−2.76	Ethylene-responsive protein, putative
Jasmonate	AT3G15510	−3.00	Putative jasmonic acid regulatory protein
	AT5G13220	−2.46	Jasmonate-zim-domain protein 10
	AT1G19180	−4.04	Jasmonate-zim-domain protein 1
	AT1G72450	−5.27	Jasmonate-zim-domain protein 6
GA	AT5G11740	−2.76	Gibberellin-responsive protein CRG16
	AT5G59845	−6.79	Gibberellin-regulated family protein
Salicylic acid	AT4G34131	−2.80	Glucosyltransferase

Table 7 Significantly enriched GO terms related to cold stress

GO ID	GO term	*q*	*m*	*t*	*k*	*P* value
Upregulated genes						
GO:0009409	Response to cold	19	226	22,765	697	0.0047
GO:0006950	Response to stress	86	1,931	22,765	697	0.0264
GO:0009651	Response to salt stress	23	345	22,765	697	0.0266
GO:0009628	Response to abiotic stimulus	55	1,110	22,765	697	0.0266
Downregulated genes						
GO:0046686	Response to cadmium ion	87	326	22,765	1,430	0.0000
GO:0009651	Response to salt stress	61	345	22,765	1,430	0.0000
GO:0009628	Response to abiotic stimulus	134	1,110	22,765	1,430	0.0000
GO:0006950	Response to stress	199	1,931	22,765	1,430	0.0000
GO:0006979	Response to oxidative stress	44	277	22,765	1,430	0.0000
GO:0009408	Response to heat	26	122	22,765	1,430	0.0000
GO:0009266	Response to temperature stimulus	43	341	22,765	1,430	0.0003
GO:0009415	Response to water	24	169	22,765	1,430	0.0033
GO:0042542	Response to hydrogen peroxide	18	114	22,765	1,430	0.0049
GO:0000302	Response to reactive oxygen species	20	135	22,765	1,430	0.0059
GO:0009414	Response to water deprivation	21	159	22,765	1,430	0.0198

q number of probes associated with the listed GO ID in the test dataset, *m* number of probes associated with the listed GO ID on the array, *k* total number of probes in the test dataset, *t* total number of probes on the array with GO annotation

(Fowler and Thomashow 2002). Our study revealed that GO terms, which included 19 cold-responsive genes, were among the genes upregulated by cold stress in mature pollen (Table 7 and Table S2). However, there is no direct evidence that the function of these genes is related to cold stress, and many proteins thought to contribute to cold acclimation, such as COR, lipid transfer proteins, and α-amylase, were not induced in mature pollen of *Arabidopsis*. The *ICE-CBF* cold response pathway has a prominent role in cold acclimation, and its transcriptional network in *Arabidopsis* has been well investigated (Thomashow 1999; Chinnusamy et al. 2007). However, we did not identify *CBF*s in the genes upregulated in response to cold stress, which might be a result of downregulation of calcium signaling cascade components (Chinnusamy et al. 2007; Fig. 4). These results are consistent with a previous study in suggesting that inability to induce expression of genes important in cold acclimation might be responsible for the cold sensitivity of pollen (Lee and Lee 2003).

Our microarray data revealed that basic leucine zipper (bZIP) transcription factors were cold-regulated (Table 4 and 5). bZIP transcription factors play a role in plant pathogen responses, light signaling, and ABA and abiotic stress signaling (Jakoby et al. 2002).

Expression of 11 transcription factors with NAC domain genes were cold-regulated (Table 4 and 5). Plant-specific NAC family transcription factors have a conserved NAC domain at the N-terminal of the protein (Olsen et al. 2005) and have been implicated in plant development (Xie et al. 2000; Takada et al. 2001; Vroemen et al. 2003). It was recently reported that several NAC transcription factors are also involved in biotic and abiotic stress responses (Fujita et al. 2004; Tran et al. 2004).

Interestingly, two WRKY transcription factors were upregulated (Table 4), and three heat shock transcription factors (HSFs) were downregulated (Table 5). WRKY plant transcription factors have been implicated in both biotic and abiotic stress responses (Seki et al. 2002; Rizhsky et al. 2002; Qiu and Yu 2009), while HSFs have been reported in heat-stress responses (Eulgem et al. 2000). These results suggested that several HSFs and WRKY factors could be involved in the cold response of mature pollen.

Cold Regulation of Hormone-Related Genes

Plant hormones are crucial regulators of growth and development. Plants growing under cold stress display growth and development patterns different from those growing under normal growth conditions, which might have to do with an altered hormone homeostasis and/or signal transduction in cold-stressed plants. To investigate this possibility, we inspected the expression pattern of hormone-related genes after cold treatment.

ABA is an important stress hormone that mediates abiotic stress signal transduction and tolerance. ABA accumulates in response to abiotic stress, such as drought and salt (Xiong and Zhu 2003; Cho et al. 2009). Cold stress also increases the endogenous ABA content in plants but to a much lesser extent (Lang et al. 1994). Because ABA biosynthesis is mainly regulated at the transcriptional level (Xiong and Zhu 2003), it is of interest to know if ABA biosynthesis genes are regulated by cold. According to our

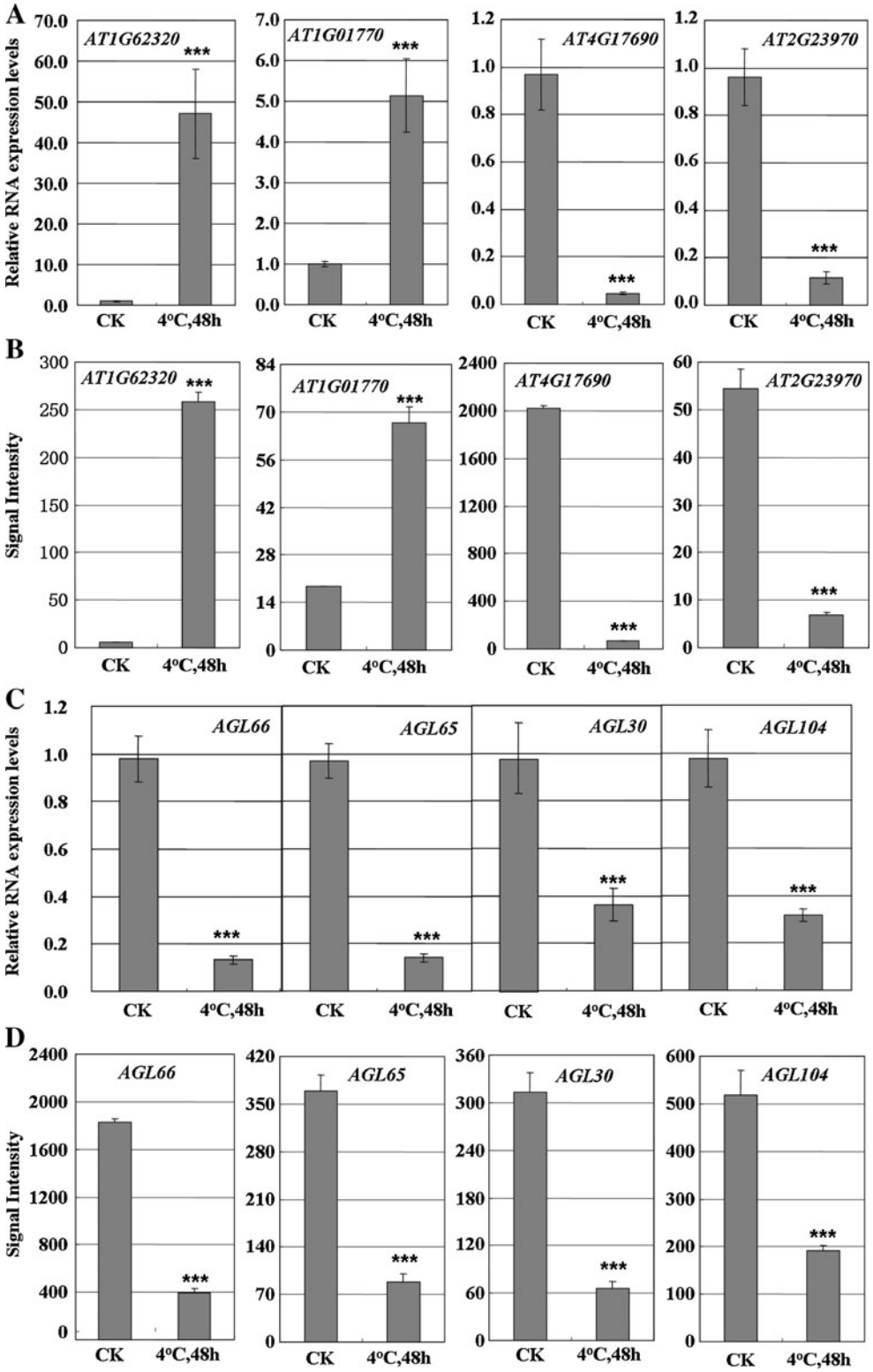

Fig 3 Quantitative RT-PCR analysis. **a** The analysis of four random selected genes. **b** The expression pattern of four random selected genes were detected by the Affymetrix chip. **c** The analysis of *AGL66*, *AGL65*, *AGL30*, and *AGL104*. *ACTIN2* gene was used as reference. **d** The expression pattern of f *AGL66*, *AGL65*, *AGL30*, and *AGL104* were detected by the Affymetrix chip. *CK* indicates the sample without cold treatment. *Error bars* indicate standard deviations of three independent biological replicates. Differences between the cold-stressed and unstressed samples are significant at the $0.01>P>0.001$ (**) or $P<0.001$ (***) levels

microarray data, only one of the known ABA synthesis genes was cold-regulated, suggesting that ABA biosynthesis is not a major event induced by cold stress, a finding consistent with previous studies (Lang et al. 1994; Lee et al. 2005). Three ABA catabolism genes were cold-regulated. ABA 8′-hydroxylase (At5g45340) was upregulated, and two ABA glucosyltransferases (At2g31750 and At4g34131) were downregulated (Table 6). The ABA-induced genes *At5g53820* and *At3g02480* were strongly repressed by cold stress, which suggested that ABA content in mature pollen

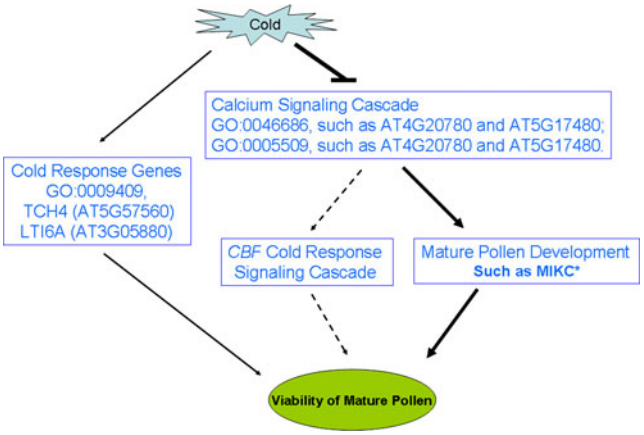

Fig 4 Proposed model for the cold response in mature pollen. *Down arrow* indicates promotion; *line without an arrow* indicates repression

might be reduced greatly under cold stress. As described above, a bZIP transcription factor could bind to the ABA-responsive element in ABA-responsive promoters (Choi et al. 2000).

We noted that the expression of nine genes with auxin-related roles was affected by cold stress. Six of these genes were downregulated in response to cold stress, while three genes were upregulated (Table 6). For example, a putative auxin-regulated gene (At2g33830) was induced by 76-fold after cold treatment. This suggests that cold may change endogenous auxin content or interfere with auxin sensitivity in mature pollen. In particular, the expression of one auxin-related gene (At4g13790), belonging to the *SAUR* gene family, was downregulated in response to cold. *SAUR* gene transcripts are very unstable (Hagen and Guilfoyle 2002). Thus, cold stress might either prevent the stabilization of the *SAUR* gene transcripts or decrease their transcription.

Jasmonic acid (JA) is required for stamen and pollen maturation in *Arabidopsis*. *Arabidopsis* mutants defective in JA synthesis, including *fad3*, *fad7*, and *fad8* (Marchler-Bauer et al. 2003), are male sterile, as is the JA-perception mutant *coi1* (Feys et al. 1994). The JA synthesis mutants can be restored to fertility by application of exogenous JA (Stintzi and Browse 2000). A putative transcription factor, ZIM, that was found to contain a 27-amino-acid motif previously identified as a domain of unknown function, increases by 6- to 40-fold in stamens following JA treatment (Browse 2009). Our data showed that three ZIM genes and one JA-regulated gene were significantly down-regulated after cold treatment (Table 6), suggesting that JA content decreases in cold-treated pollen. In addition, JA synthesis in developing *Arabidopsis* flower buds is dependent on auxin signaling (Nagpal et al. 2005; Cecchetti et al. 2008). Therefore, cross-talk between auxin and the JA signal pathway may take place during cold treatment of mature pollen.

Ethylene response factors (ERFs) played important roles in regulating plant biotic and abiotic stress tolerance. Our results showed that eight ethylene response factors regulated by cold may play roles in the cold response of mature pollen (Table 6). The role of these genes in the cold response of mature pollen is an interesting research topic that needs further exploration.

Gibberellins (GAs) are involved in much plant developmental processes, including seed development, stem elongation, flowering, and fruit development (Richards et al. 2001). Many GA-related genes have been isolated and characterized (Hedden and Kamiya 1997). Gibberellins function not only to promote the growth of plant organs, but also to induce phase transitions during plant development. Under cold conditions, plants grow more slowly and some even show growth defects or damage. Some of these cold-induced growth changes might be attributed to the slowing of metabolic activities in the cold (Kubien et al. 2003). In this study, gibberellin 2-oxygenase (*GA2OX2*) was upregulated, whereas two GA-regulated genes were significantly downregulated (Table 6), which suggested that GA homeostasis was disrupted by cold stress in mature pollen.

Cold Regulation of Mature Pollen-Specific Genes

Mature pollen grains contain mRNAs whose protein products appear to function during the late-maturation stages of pollen germination and tube growth (Mascarenhas 1975). In the present study, we discovered that cold stress significantly repressed the expression of many MP genes that may play a role in pollen viability (Table S2). Even though more than 600 transcription factors are expressed throughout pollen development (Verelst et al. 2007b), very few have been functionally characterized so far. In our results, the MIKC* class of MADS-domain transcription factors, including *AGL66*, *AGL65*, *AGL30*, and *AGL104*, which are known to be important regulators of pollen germination and tube growth (Verelst et al. 2007a, b; Adamczyk and Fernandez 2009), were significantly down-regulated by cold stress (Fig. 3c; Table 5), and thus might contribute to the reduced fertility of mature pollen.

Based on our microarray results and GO enrichment analysis, we propose the following model for the cold response of mature pollen (Fig. 4). When mature pollen is exposed to cold stress, expression of calcium signaling cascade components is inhibited and the translation products decreased the expression of genes involved in mature pollen development and activation of *CBF* cold response pathway, leading to sensitive to cold stress. In addition, some of cold response genes independent on *CBF* cold response pathway, might be involved in the cold response of mature pollen.

In summary, we have identified a large number of genes strongly expressed during the cold response in mature pollen. Our results indicated that a calcium signaling cascade might play a key role in the cold response, affecting the downstream transcriptional regulation of *COR* genes and mature pollen development, and which might be responsible for the cold sensitivity of mature pollen (Fig. 4). These data will facilitate a rapid and targeted reverse genetics approach for identifying key mediators of cold tolerance in mature pollen. The differentially expressed genes may play key roles in cold stress tolerance and related gene regulatory networks in mature pollen. The results suggest that the regulation of cold stress tolerance of mature pollen was associated with the late-maturation stages of pollen germination and tube growth. Our findings provide a foundation for further experiments to explore the network of gene regulation in response to cold stress and to determine the function of cold-responsive genes in the cold tolerance of mature pollen through mutant analysis and other molecular and cell biological approaches.

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