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RESEARCH PAPER

Male gametophyte-specific WRKY34 transcription factor mediates cold sensitivity of mature pollen in *Arabidopsis*

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Abstract

Mature pollen is very sensitive to cold stress in chilling-sensitive plants. Plant *WRKY* DNA-binding transcription factors are key regulators in plant responses to abiotic and biotic stresses. Previous studies have suggested that *WRKY34* (At4g26440) gene might be involved in pollen viability, although the mechanism involved is unclear. In this study, it is shown that cold treatment increased *WRKY34* expression in the wild type, and promoter-GUS analysis revealed that *WRKY34* expression is pollen-specific. Enhanced green fluorescent protein-tagged WRKY34 was localized in the nuclei. Pollen harbouring the *wrky34* allele showed higher viability than pollen with the *WRKY34* allele after cold treatment. Further functional analysis indicated that the WRKY34 transcription factor was involved in pollen development regulated by the pollen-specific MIKC* class of MADS-domain transcription factors under cold stress, and cold-insensitivity of mature *wrky34* pollen might be partly attributable to the enhanced expression of transcriptional activator *CBF*s in the mutants. Thus, the WRKY34 transcription factor negatively mediated cold sensitivity of mature *Arabidopsis* pollen and might be involved in the *CBF* signal cascade in mature pollen.

Key words: *Arabidopsis*, cold stress, pollen, transcription factor, *WRKY34*.

Introduction

Temperature is one of the most important environmental factors affecting plant development and crop productivity. Certain stages of the plant life cycle are more sensitive to chilling than others. Seedlings appear to be more susceptible than plants at advanced stages of development (Lyons, 1973), and pollen maturation is the most sensitive process in the entire life cycle of cold-sensitive plants (Sataka and Koike, 1983; Patterson *et al.*, 1987). Cold stress significantly reduces the pollen germination rate and seed production of *Arabidopsis* (Lee and Lee, 2003).

Because of its crucial function in the plant reproductive cycle, pollen has been the focus of considerable cytological, biochemical, and molecular research. In recent years, investigation of processes underlying pollen development and function has been extended by studying pollen-related gene expression. For example, 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 grains in *Arabidopsis* (Verelst *et al.*, 2007*a*, *b*; Adamczyk and Fernandez, 2009). *Arabidopsis* phosphatidylinositol 3-kinase is essential for vacuole reorganization and nuclear division during pollen development (Lee *et al.*, 2008). *Arabidopsis* MS1 is involved in the development of pollen and the tapetum (Ito *et al.*, 2007). *RPG1* encodes a plasma membrane protein and is required for exine pattern formation of microspores in *Arabidopsis* (Guan *et al.*, 2008). The FLP1 protein is likely to play a role in the synthesis of the components of tryphine and sporopollenin of the exine (Ariizumi *et al.*, 2003).

Most plants, such as *Arabidopsis*, develop tolerance to freezing after exposure to low, non-freezing temperatures. This adaptive process, known as cold acclimation, involves

profound changes in biochemistry, physiology, and plant transcriptome. In *Arabidopsis*, cold-regulated genes have been estimated to constitute about 4–20% of the genome (Lee *et al.*, 2005; Hannah *et al.*, 2005). Considerable 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 *COR* (*COLD RESPONSIVE*) genes by binding C-repeat/dehydration response elements (CRT/DRE) to their promoters (Stockinger *et al.*, 1997; Fowler and Thomashow, 2002). CBFs regulate the expression of genes involved in phosphoinositide metabolism, transcription, osmolyte biosynthesis, ROS detoxification, membrane transport, hormone metabolism, and signalling, and many other genes with known or presumed cellular protective functions (Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Lee *et al.*, 2005). Over-expression of *CBF1*, *CBF2*, and *CBF3* causes phenotypes associated with freezing tolerance, such as increased proline and sugar concentrations and transcriptional activation of *COR* genes (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2004). Many important genes in the cold-response pathway have been studied in detail, such as *ICE1*, *ZAT12*, *HOS9*, and *MYB15* (Chinnusamy *et al.*, 2003; Lee *et al.*, 2005; Agarwal *et al.*, 2006; Benedict *et al.*, 2006). Although cold stress responses in whole plants have been extensively studied, the effect of cold stress on the development and function of plant organs, especially that of pollen, has received less attention.

The family of plant-specific WRKY transcription factors comprises over 70 members in *Arabidopsis* (Eulgem *et al.*, 2000; Dong *et al.*, 2003; Eulgem and Somssich, 2007). WRKY proteins typically contain one or two domains composed of about 60 amino acids with the conserved amino acid sequence WRKYGQK, together with a novel zinc-finger motif. The WRKY domain shows a high binding affinity to the TTGACC/T W-box sequence (Ulker and Somssich, 2004).

Accumulating evidence has demonstrated that *WRKY* genes are involved in regulating plant responses to biotic stresses. A majority of studies on *WRKY* genes address their involvement in disease responses and salicylic acid (SA)-mediated defence (Dellagi *et al.*, 2000; Eulgem *et al.*, 2000; Asai *et al.*, 2002; Zheng *et al.*, 2007; Lai *et al.*, 2008). In addition, *WRKY* genes are involved in plant responses to wounding (Hara *et al.*, 2000) and low Pi stress (Chen *et al.*, 2009). Although most WRKY proteins studied thus far have been implicated in regulating biotic stress responses, some *WRKY* genes regulate plant responses to freezing, oxidative stress, drought, salinity, cold, and heat (Huang and Duman, 2002; Seki *et al.*, 2002; Rizhsky *et al.*, 2004; Li *et al.*, 2009; Qiu and Yu, 2009). In addition, increasing evidence indicates that WRKY proteins are key regulators in certain developmental processes. Some *WRKY* genes regulate biosynthesis of anthocyanins (Johnson *et al.*, 2002), starch (Sun *et al.*, 2003), and sesquiterpenes (Xu *et al.*, 2004). Other *WRKY* genes may regulate embryogenesis (Lagace and Matton, 2004), seed size (Luo *et al.*, 2005), seed coat and trichome development (Johnson *et al.*, 2002; Ishida *et al.*, 2007), senescence (Robatzek and Somssich, 2001; Miao and Zentgraf, 2007; Jing *et al.*, 2009), and seed germination and post-germination arrest of development by abscisic acid (Jiang and Yu, 2009).

Based on the number of WRKY domains and the pattern of the zinc-finger motif, WRKY proteins can be divided into three different groups in *Arabidopsis* (Eulgem *et al.*, 2000). *WRKY34* is a group I WRKY family transcription factor (Chinnusamy *et al.*, 2007). The promoter of *WRKY34* is male gametophyte-specific (Honys *et al.*, 2006). In the present study, expression and subcellular localization of *WRKY34* in response to cold stress was investigated in the wild type (WT) and *wrky34* mutants using promoter–GUS analysis and quantitative RT-PCR. The objective was to elucidate the roles of the *WRKY34* transcription factor in mediating the cold sensitivity of mature *Arabidopsis* pollen grains.

Materials and methods

Plant growth conditions and pollen collection

Seeds of *wrky34-2* (SAIL_1284_C01), *agl65* (SALK_009651C), *agl66* (SALK_072108), and *agl104* (SALK_066443C) were obtained from the Arabidopsis Biological Resource Center (ABRC). Wild-type *A. thaliana* Col-0 plants and the mutants were grown in a greenhouse with temperature controlled at 22 °C and a 16 h photoperiod (photosynthetically active radiation about 120 μmol m^{-2} s^{-1}). For practical reasons the different genotypes were grown, harvested, and processed in separate batches, each concurrently with WT (control) plants.

Flowers at developmental stage 13 (Smyth *et al.*, 1990) were marked at a fixed time in the morning. For the cold treatment, the plants were transferred to a growth chamber, maintained at 4 °C under the same light conditions as in the greenhouse, for 24 h or 48 h. After cold treatment, mature pollen grains were harvested from the marked flowers by shaking in 0.3 M mannitol, as described by Honys and Twell (2003). The freshly harvested pollen was ground with quartz sand and total RNAs were extracted using the RNAeasy Plant Mini Kit (Qiagen, Valencia, CA). DNA was removed via an on-column DNAse treatment, and the RNA extracts were stored at –80 °C prior to use.

Competition experiments and plant genotyping

To determine whether cold stress affects the segregation ratio of progeny from self-fertilized *WRKY34/wrky34* heterozygotes produced by crosses between *wrky34* mutants and the WT, and because *WRKY34* was expressed only in pollen, plants with flowers at stage 13 were treated with cold stress; flowers from untreated greenhouse-grown plants were used as the control. After cold treatment, the *WRKY34/wrky34* plants were transferred to the greenhouse and grown until the seeds were harvested. The seeds were germinated on Murashige and Skoog medium, and genomic DNA was isolated from the seedlings and used as a template for PCR amplification of DNA fragments corresponding to the WT and the T-DNA insertion loci. The number of plants with the *WRKY34/wrky34* and *wrky34/wrky34* genotype (w34), and the number of plants with the *WRKY34/wrky34* and *WRKY34/WRKY34* genotype (W34), were counted. To quantify the fertility of flowers exposed to cold stress further, the number of seeds in siliques from flowers that were treated at 4 °C for 24 h or 48 h was scored.

To determine which gametophyte was responsible for the abnormal segregation ratio, reciprocal crosses between cold-treated heterozygous and unstressed WT plants were performed. The F_1 progeny was analysed by PCR-based genotyping.

All experiments were configured such that the expected transmission was always 50% and differences in the frequency of recombination between loci would have no impact on the outcome. In PCR-based genotyping, the presence of the T-DNA insertion allele was detected using the LB primer derived from the T-DNA sequence (5′-AAACGTCCGCAATGTGTTAT-3′) and a locus-specific primer. Presence of the WT allele was detected using the following gene-specific primers: LP1 (5′-GCGAGATCCGGGTTTAATGCACCG-3′) and RP1 (5′-GCATGTCTTGGCCAGTACCGGATG-3′) for *wrky34-1*, and LP2 (5′-GTCATTGGGCACAGCACTGTCT-3′) and RP2 (5′-ACTTGGGCTCAAGCTGAAACGTG-3′) for *wrky34-2*.

Pollen phenotyping

Light and epifluorescence microscopic examination of DAPI-stained pollen, including image capture and processing, were performed as described previously (Park *et al.*, 1998). To characterize phenotypically mature pollen of the *wrky34* homozygous mutants under cold stress, the pollen viability of homozygous plants was analysed. Evaluation of mature pollen viability by staining with fluorescein diacetate (FDA) *in vitro* (Verelst *et al.*, 2007*b*) and pollen germination assays *in vivo* were performed as described previously with some modifications (Mori *et al.*, 2006; Chhun *et al.*, 2007).

To determine the pollen germination frequency *in vivo*, flowers at the beginning of stage 12 (Smyth *et al.*, 1990) were emasculated. After waiting 12–24 h to ensure that the stigma was receptive, a saturating amount of pollen from a donor plant was placed on the stigma. After 30 min, the pistils were removed and directly stained with aniline blue on a glass slide for 30 min before observation with an Olympus BX51 ultraviolet (UV) epifluorescent microscope (Chhun *et al.*, 2007). Pollen grains attached to the stigma and in which a pollen tube was detected with UV fluorescence were classified as having germinated.

To detect pollen tube elongation *in vivo*, after cold treatment the plants were moved to the greenhouse for 5 h, after which pistils were excised and the stigma fixed in acetic acid:ethanol (1:3, v/v) for 24 h, then softened with 8 M NaOH for 24 h. The stigma was washed in distilled water three times, and then stained with aniline blue on a glass slide for 30 min prior to observation by UV microscopy. Pollen grains attached to the stigma and in which pollen tube growth in the style was detected by UV fluorescence were classified as possessing elongated pollen tubes (Mayer and Gottsberger, 2000; Chhun *et al.*, 2007).

Generation of 35S::WRKY34 and plant transformation

To produce transgenic plants, the *WRKY34* full-length cDNA was cloned into the pOCA30 vector, which contains the modified CaMV 35S promoter. The recombinant plasmid was introduced into *Agrobacterium tumefaciens* GV3101 and used to transform *A. thaliana* using the floral dip method (Clough and Bent, 1998). The transformed lines were selected for resistance to kanamycin (50 μg ml^{-1}). Northern blot analysis was used for further confirmation of their transgenic identity. High levels of homozygous lines (T_3 generation) were obtained from independent transformants.

Construction of promoter::GUS reporters and GUS staining

The 1.5 kb promoter fragments of *WRKY34* were amplified using ExTaq (TaKaRa) and the gene-specific primers. The resulting fragments were subcloned upstream of the GUS reporter gene in the pCAMBIA1300 Ti-derived binary vector (CAMBIA) and introduced into WT *Arabidopsis* plants. Transformation and GUS staining was performed as described by Yang *et al.* (2003).

Subcellular localization of WRKY34

The *WRKY34* full-length cDNA was linked to the pBluscript KS II vector, and the enhanced green fluorescent reporter gene (*eGFP*) was subcloned behind the *WRKY34* cDNA. The *WRKY34–eGFP* fragment was cloned by ligation into the sites behind the CaMV 35S promoter of pOCA30. The plasmid was isolated using Qiagen kits (Valencia, CA, USA), and concentrated to about 1 μg μl^{-1}. The recombinant plasmid was introduced into *A. tumefaciens* GV3101 and used to transform *Nicotiana benthamiana* epidermal cells. A plasmid containing *eGFP* alone was concentrated as above and bombarded in parallel as a control. Transformation of *N. benthamiana* epidermal cells and localization of the protein were performed essentially as described previously (Asai *et al.*, 2008).

Quantitative real-time PCR

cDNA was obtained from 1 μg total 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-reactions (10 μl each) were performed with the Lightcycler FastStart DNA Master SYBR Green I Kit (Roche, Mannheim, Germany) on a Roche LightCycler 480 real-time PCR machine, according to the manufacturer's instructions. *ACT2* (AT3G18780) was used as a control. Gene-specific primers used to detect transcripts are listed in Supplementary Table S1 at *JXB* online.

MIKC*-type transcription factors are known to be important regulators during pollen maturation and tube growth in *Arabidopsis*, and are negative regulators of the *WRKY34* gene (Verelst *et al.*, 2007*b*). The effect of these genes (*AGL66*, *AGL104*, and *AGL65*) on *WRKY34* expression under cold stress, and whether the expression of MIKC* genes is affected by *WRKY34* and cold stress, was investigated in the present study. Quantitative RT-PCR was used to measure mRNA transcript levels for the five pollen-expressed MIKC* genes in mature pollen of both the WT and *wrky34* mutants.

The effect of *WRKY34* knockdown on cold regulation of *CBF* transcription factors was also examined, as *WRKY34* is a cold-responsive transcription factor and the transcriptional activator *CBF* cold-response pathway plays a prominent role in cold acclimation (Stockinger *et al.*, 1997; Thomashow, 1999; Fowler and Thomashow, 2002; Chinnusamy *et al.*, 2007).

Results

Expression and subcellular localization of WRKY34

In total, 37 T_2 plants were analysed for GUS activity in inflorescences, bud clusters, and flowers. In 35 plants, GUS activity was only detected in the male gametophyte (Fig. 1A, B), and was observed in mature pollen grains from flowers at stage 13 (Fig. 1C, D). In seedlings of ~20 lines of T_2 plants for each line, no *GUS* expression was observed in seedlings, leaves, roots, and stamen vascular tissue (data not shown). Measurement of *WRKY34* mRNAs in WT plant organs by quantitative RT-PCR showed that *WRKY34* mRNAs were strongly detected in male gametophytic tissues, and barely detected in seedling, leaves, petioles, stems, and roots (Fig. 1F).

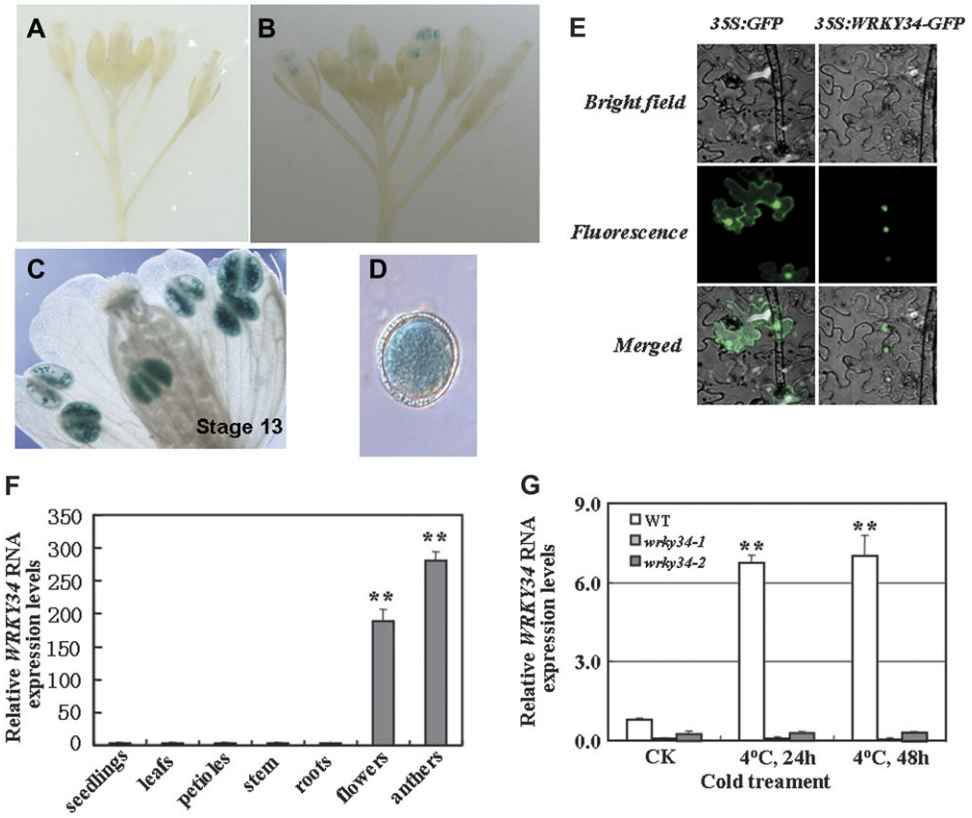

Fig. 1. Expression patterns and subcellular localization of WRKY34. (A) A negative control non-transgenic wild-type (WT) inflorescence, showing no GUS activity. (B) A transgenic inflorescence, showing GUS staining in the anthers. (C) Transgenic mature pollen grains with GUS staining at the floral developmental stage 13. (D) Transgenic mature pollen grains with GUS staining. (E) The subcellular localization of the *WRKY34–GFP* fusion protein in *Nicotiana benthamiana* epidermal cells. Images in the left column show the control plasmid expressing only the green fluorescent protein (GFP) and those in the right column show the *WRKY34–GFP* fusion protein expressed in *N. benthamiana* epidermal cells. The cells were examined with brightfield (top) and UV fluorescence (middle) microscopy, and as a merged image (bottom) showing either the diffused (control plasmid) or the nuclear localization of the proteins. (F) Quantitative RT-PCR comparison of the *WRKY34* mRNA expression levels in different tissues from the WT plants grown at 22 °C. (G) Quantitative RT-PCR comparison of *WRKY34* RNA expression levels in cold-treated (4 °C) and untreated (22 °C) mature pollen of the WT and *wrky34* mutant. After cold treatment, *WRKY34* RNA expression levels significantly increased in the WT, and showed no distinct change in the *wrky34* mutants. Error bars indicate standard deviations of three independent biological samples. Differences between the untreated and treated plants with cold stress are significant at $P < 0.01$ (**).

As shown in Fig. 1, the fluorescence signal of the WRKY34-eGFP construct was detected in the nuclei of the *Nicotiana benthamiana* cells (Fig. 1E).

Quantitative RT-PCR revealed that *WRKY34* transcription in mature pollen increased by more than 7-fold after 24 h or 48 h cold stress compared to the control (Fig. 1G).

Identification of homozygous wrky34 *mutants*

Characterization of two homozygous *wrky34* mutants is shown in Fig. 2. Mature pollen of the *wrky34-1* (SALK_133019) mutant, which contained T-DNA at the second exon (Fig. 2A), produced no *WRKY34* transcripts, in contrast to the WT (Fig. 2B). In the *wrky34-2* (SAIL_1284_C01) mutant, which contained T-DNA at position –316 relative to the ATG of *WRKY34* (Fig. 2A), *WRKY34* expression in mature pollen was significantly reduced (Fig. 2B). *WRKY34* transcripts were also detected in the *wrky34* mutants after cold treatment and the level of *WRKY34* transcripts for both mutants did not change distinctly in response to cold stress (Fig. 1G). The *wrky34* mutant lines were sensitive to kanamycin since it harboured the T-DNA, which necessitated PCR-based analyses of the genotypes. No morphological abnormalities were observed in the *wrky34* mutants after growth at 22 °C (data not shown).

Segregation ratios in progeny of self-fertilized WRKY34/wrky34 *heterozygous mutants harbouring a T-DNA insertion allele showed distorted segregation ratios after cold stress*

The ratio of w34 (*WRKY34*/*wrky34* and *wrky34*/*wrky34*) genotypes to W34 (*WRKY34*/*wrky34* and *WRKY34*/*WRKY34*) genotypes in the progeny from greenhouse-grown *WRKY34*/*wrky34* heterozygous mutants was accorded with a predicted ratio of 1:1 (Table 1). For progeny from the cold-treated heterozygous mutant plants,

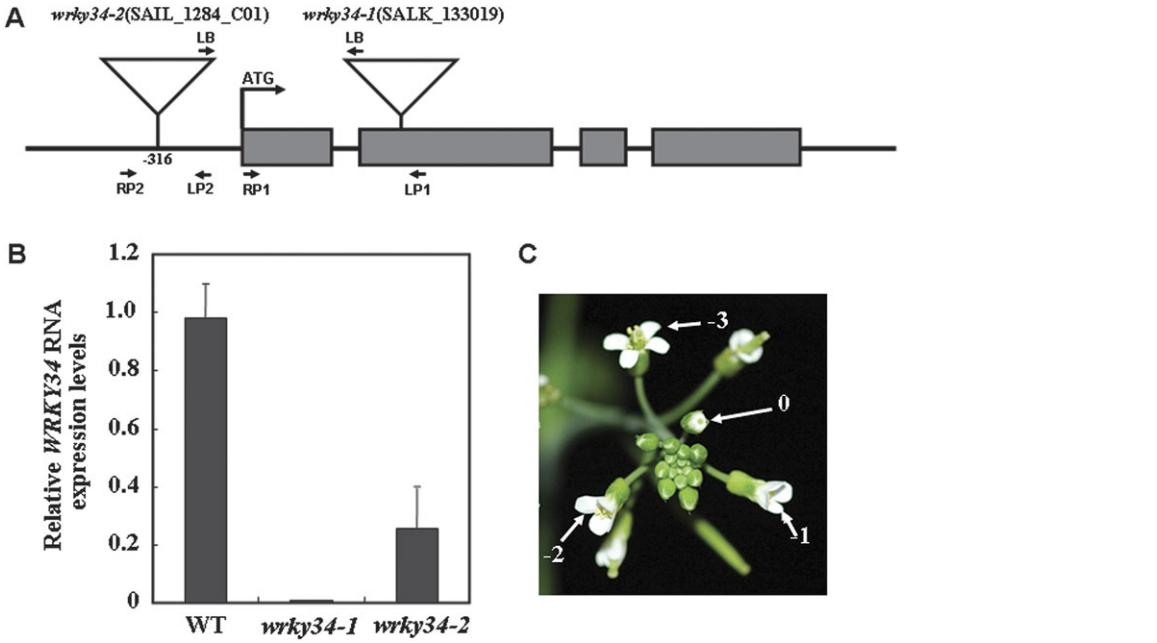

Fig. 2. Characterization of *wrky34-1* and *wrky-2* mutants, and developmental stage of the inflorescence at the time of cold treatment. (A) Exon and intron structure of the *Arabidopsis WRKY34* gene, showing the locations of the T-DNA insertion sites in both mutants. Boxes indicate exons. (B) Relative *WRKY34* RNA expression in mature pollen of the *wrky34* mutants. Error bars indicate standard deviations of three independent determinations. Three independent experiments were shown by re-extracting RNA from other samples. Each experiment was also executed three times. (C) Flowers at stage 13 at the time of cold treatment are numbered '0'; flowers at more advanced stages are indicated by negative numbers.

Table 1. Segregation analysis of progeny of self-fertilized heterozygous *WRKY34/wrky34* plants with cold treatment

The inheritance of *wrky*34 was analysed using PCR-based genotyping. w34 is the number of plants containing the *wrky34* allele (plants with the *WRKY34/wrky34* and *wrky34/wrky34* genotypes), W34 is the the number of plants containing the *WRKY34* allele (plants with the *WRKY34/wrky34* and *WRKY34/WRKY34* genotypes), Ratio is w34:W34. The χ^2 test was used to compare the observed ratios with a predicted ratio of 1:1. The untreated greenhouse-grown plants were used as the control.

Parent (self-fertilized)	Cold treatment	w34	W34	Ratio	χ^2
WRKY34/wrky34-1	Control	163	147	1.11:1	0.8 (*P >0.05*)
WRKY34/wrky34-1	24 h, 4°C	153	115	1.33:1	5.4 (*P <0.05*)
WRKY34/wrky34-1	48 h, 4°C	161	80	2.01:1	27.2 (*P <0.01*)
WRKY34/wrky34-2	Control	161	151	1.07:1	0.3 (*P >0.05*)
WRKY34/wrky34-2	24 h, 4°C	149	114	1.31:1	4.7 (*P <0.05*)
WRKY34/wrky34-2	48 h, 4°C	156	85	1.84:1	20.9 (*P <0.01*)

the w34 to W34 ratio for both lines differed significantly from 1:1 (χ^2 test, P <0.01). The ratios of w34 to W34 plants were 1.33:1 and 1.31:1 for *WRKY34/wrky34-1* and *WRKY34/wrky34-2*, respectively, after 24 h cold treatment; divergence from the 1:1 was even greater after 48 h cold treatment (Table 1). Thus, among the progeny, plants harbouring a *wrky34* allele were significantly more frequent than plants harbouring a *WRKY34* allele (the WT), which indicated that differential gametophytic viability existed after exposure to cold stress.

Pollen harbouring the wrky34 *allele showed higher viability than that with the* WRKY34 *allele after cold stress*

When the *WRKY34/wrky34* heterozygote with or without cold treatment was used as the maternal parent in reciprocal crosses between untreated WT and cold-treated heterozygous plants, the ratios for WT and heterozygous plants did not differ significantly from 1:1 (P >0.05; Table 2). These results indicated that the *wrky34* mutation did not affect female gametophytic viability. When the *WRKY34/wrky34* heterozygote without cold treatment was the paternal parent, the ratios also accorded with 1:1, indicating that the *wrky34* mutation did not affect male gametophytic viability in the absence of cold treatment.

By contrast, when the *WRKY34/wrky34* heterozygote with cold treatment was the paternal parent, the ratios of WT and heterozygous plants in the *WRKY34/wrky34-1* and *WRKY34/wrky34-2* progeny populations were 0.26:1 and 0.31:1, respectively, and differed significantly from 1:1 (P <0.01; Table 2). Thus, male gametophytes with a *wrky34* allele exhibited greater competitive ability than gametophytes with a *WRKY34* allele after cold treatment.

Cold sensitivity of mature pollen in wrky34 *mutants*

Without cold treatment, nearly all of the mature pollen from homozygous WT and *wrky34* plants exhibited green fluorescence after staining with FDA. After cold treatment, the percentage of viable pollen grains from both mutants

Table 2. Analysis of the genetic transmission of *WRKY34/wrky34* after Cold treatment

The inheritance of *wrky34* was analysed by genotyping the F_1 progeny of the specified crosses. Ratio is the observed wild-type (WT) to heterozygous frequencies. The WT parental plants were grown at 22 °C without cold treatment. The χ^2 test was used to compare the observed ratios with a predicted ratio of 1:1. The untreated greenhouse-grown parental plants were used as the control.

Cold treatment	Parentage (female×male)	Wild type	Heterozygous	Ratio	χ^2
Control	*WRKY34/wrky34-1*×WT	90	95	0.95:1	0.05 (*P>0.05*)
Control	WT×*WRKY34/wrky34-1*	101	99	1.02:1	0.01 (*P>0.05*)
48 h, 4 °C	*WRKY34/wrky34-1*×WT	77	84	0.92:1	0.12 (*P>0.05*)
48 h, 4 °C	WT×*WRKY34/wrky34-1*	22	97	0.23:1	11.2 (*P<0.01*)
Control	*WRKY34/wrky34-2*×WT	88	95	0.93:1	0.11 (*P>0.05*)
Control	WT×*WRKY34/wrky34-2*	94	98	0.96:1	0.04 *P>0.05*)
48 h, 4 °C	*WRKY34/wrky34-2*×WT	79	83	0.95:1	0.05 (*P>0.05*)
48 h, 4 °C	WT×*WRKY34/wrky34-2*	33	107	0.31:1	8.11 (*P<0.01*)

was distinctly higher than that of WT (P <0.05; Fig. 3A). After 48 h cold treatment, the percentage of viable pollen grains was 44.3%, 62.5%, and 62.8% in WT, *wrky34-1*, and *wrky34-2* plants, respectively.

Without cold stress, the percentage pollen germination for the *wrky34* mutants was indistinguishable from that of WT pollen at 30 min after artificial pollination (Fig. 3B). After 24 h cold treatment, the percentage of germinated pollen grains was higher in the mutants than in the WT: 76.9% and 71.8% in *wrky34-1* and *wrky34-2*, respectively, compared with 57.2% in the WT (P <0.05). With 48 h cold stress, the difference between the *wrky34* mutants and WT was enhanced (P <0.01; Fig. 3B).

At 5 h after self-pollination, the number of elongated pollen tubes per stigma did not differ significantly between the WT and *wrky34* mutants in the absence of cold treatment (Fig. 3C, D). After cold treatment, the number of elongated pollen tubes per stigma in the *wrky34* mutants was considerably higher than that of the WT. With 48 h cold treatment, the number of elongated pollen tubes per stigma was 46.2±5.3, 96.1±9.5, and 85.7±10.3 in the WT, *wrky34-1*, and *wrky34-2* plants, respectively (P <0.01; Fig. 3C, D). These data indicate that cold stress did not completely abolish pollen germination or pollen tube elongation, but more severely inhibited these processes in the WT than in the *wrky34* mutants.

The average seed number per silique in all unstressed plants was about 46 (Fig. 3E, F). The seed number was severely reduced in WT plants, but only slightly reduced in *wrky34* plants, treated at 4 °C for 24 h (Fig. 3E). With 48 h cold stress, the average seed number per silique was 25.9±2.9, 34.8±2.6, and 33.8±3.1 for the WT, *wrky34-1* and *wrky34-2*, respectively (Fig. 3E). In the mature siliques of *wrky34* plants exposed to cold stress, the gaps in the silique were fewer than those in the WT (Fig. 3F). Moreover, when unstressed WT pollen was crossed onto cold-stressed stigmas of WT and *wrky34* mutant plants, no differences in seed set were recorded (data not shown). This indicated that reduced fertility of the plants was primarily due to defective pollen, and that cold stress more strongly affected pollen of the WT than the *wrky34* mutant. Although exposure of mature pollen to cold stress resulted in reduced seed set, the seed set of *wrky34* mutants was significantly higher than that of the WT, which indirectly indicated that the mature pollen of homozygous *wrky34* mutants is less sensitive to cold stress. It also showed that even without competing pollen, *wrky34* mutant pollen was more tolerant to cold stress and had a greater chance of achieving successful fertilization than WT pollen after cold treatment.

Effect of WRKY34 *over-expression on fertility of mature pollen*

Most of the T_3 transgenic lines showing a high level of *WRKY34* transcription were almost sterile (data not shown). Two of these lines were selected to elucidate whether reduction in male gametophytic fertility caused the sterility. *WRKY34* expression in *35S:WRKY34* plants was detected using quantitative RT-PCR with gene-specific primers. In mature pollen, the levels of *WRKY34* transcripts in the transgenic plants, *35S::WRKY34-1* and *35S::WRKY34-2*, were 15-fold and 21-fold higher than that in vector transgenic plants, respectively (Fig. 4B), and both transformants were almost sterile (Fig. 4A). Scanning electron microscopic examination of the pollen surface and staining of the nuclei of mature pollen did not reveal obvious differences compared to that of the vector control (see Supplementary Fig. S1 at *JXB* online).

Pollen viability and pollen tube growth of the *35S::WRKY34-1* and *35S::WRKY34-2* plants were examined, using the vector transgenic plants as the control. Less than 10% of the pollen grains was viable (Fig. 4C, D), and only a few pollen tubes elongated on the stigma of *35S::WRKY34* plants (Fig. 4E, F). Male and female fertility of *35S::WRKY34* plants were also investigated by performing reciprocal crosses between homozygous and vector transgenic plants. In vector transgenic plants pollinated by mature pollen of *35S::WRKY34*, and self-fertilized *35S::WRKY34* plants, the fertilization aborted (see Supplementary Fig. S2 at *JXB* online). By contrast, fertilization was successful when the pistil of *35S::WRKY34* was pollinated with mature pollen of the vector transgenic plant (see Supplementary Fig. S2 at *JXB* online). These results indicated that the sterility of *35S::WRKY34* plants was caused by defective pollen. Thus, over-expression of *WRKY34* in mature pollen of transgenic plants greatly reduced fertility.

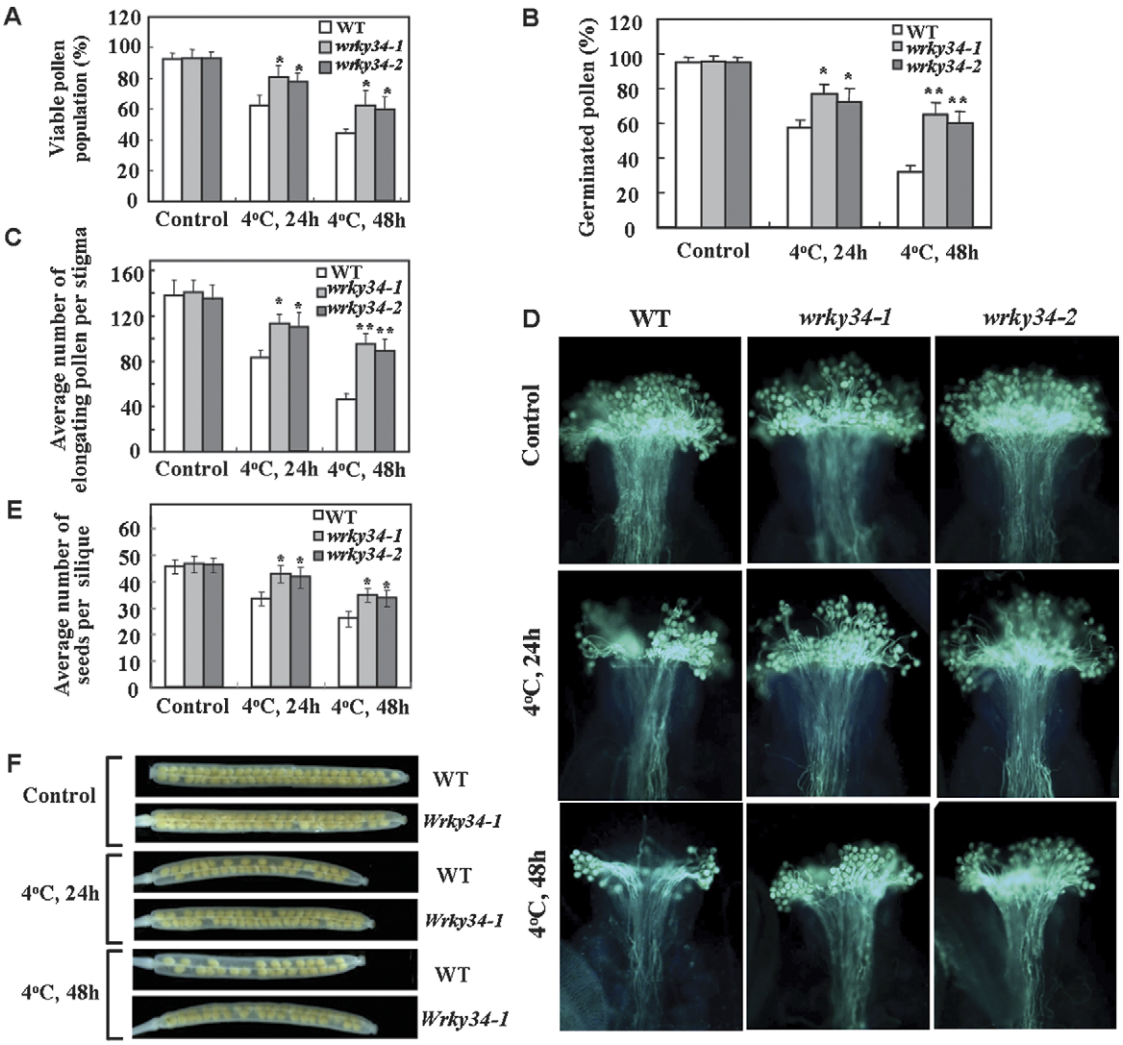

Fig. 3. Mature pollen viability and seed set of *wrky34-1* and *wrky34-2* plants compared with the wild-type (WT) after cold treatment. (A) The percentage of viable pollen grains determined by staining with fluorescein diacetate. In each replicate, over 100 pollen grains were counted. (B) Percentage pollen germination *in vivo*. In each replicate, more than 100 pollen grains were counted. (C) Average number of elongated pollen tubes per stigma from self-fertilized plants. In each replicate, more than 30 stigmas were counted. (D) Germinated pollen *in vivo* stained with aniline blue. After cold treatment, elongated pollen tubes on the stigma were more frequent in the *wrky34* mutants than the WT. (E) Seed set by flowers cold-treated at stage 13 and subsequently transferred to normal growing conditions until seeds had matured. The number of seeds per silique was counted. (F) Representative examples of seed set within mature siliques from cold-stressed wild-type and *wrky34-1* plants. Because the seed production difference between the *wrky34-1* and *wrky34-2* was slight, a representative example of *wrky34-1* seed sets was used to present the *wrky34-1* and *wrky34-2*. Error bars indicate standard deviations of three replicate experiments. In each replicate, over 30 siliques were sampled. Differences between the WT and *wrky34* mutants after cold stress were significant at $P < 0.05$ (*) or $P < 0.01$ (**). The untreated greenhouse-grown plants were used as the control.

Expression analysis of WRKY34 *transcripts in three* MIKC* *gene mutants under cold stress*

In the absence of cold stress, expression of *WRKY34* in the *agl66*, *agl65*, and *agl104* mutants did not differ significantly from that of the WT (Fig. 5). After cold treatment, the level of *WRKY34* in *agl66*, *agl65*, and *agl104* was 1.2, 1.9, and 1.6 times, respectively, that in the WT. On the other hand, cold treatment led to a 6.7, 8.0, 12.8, and 10.9-fold increase in accumulation of *WRKY34* in the WT, *agl66*, *agl65*, and *agl104*, respectively (Fig. 5). Thus, mutation of *AGL65*, *AGL66*, and *AGL104* enhanced the cold-induced expression of *WRKY34*. Previous studies have shown that MIKC* proteins exhibit functional redundancy, and a single MIKC* mutation does not significantly affect their function (Verelst *et al.*, 2007*a*, *b*; Adamczyk and Fernandez, 2009). This might explain why *WRKY34* expression in *agl66*, *agl65*, and *agl104* showed no obvious difference from the WT without cold treatment.

Expression analysis of MIKC gene transcripts in* wrky34 *mutants under cold stress*

In untreated WT and *wrky34* mutant plants, the levels of MIKC* transcripts showed no significant difference between the WT and *wrky34* mutants. After cold treatment,

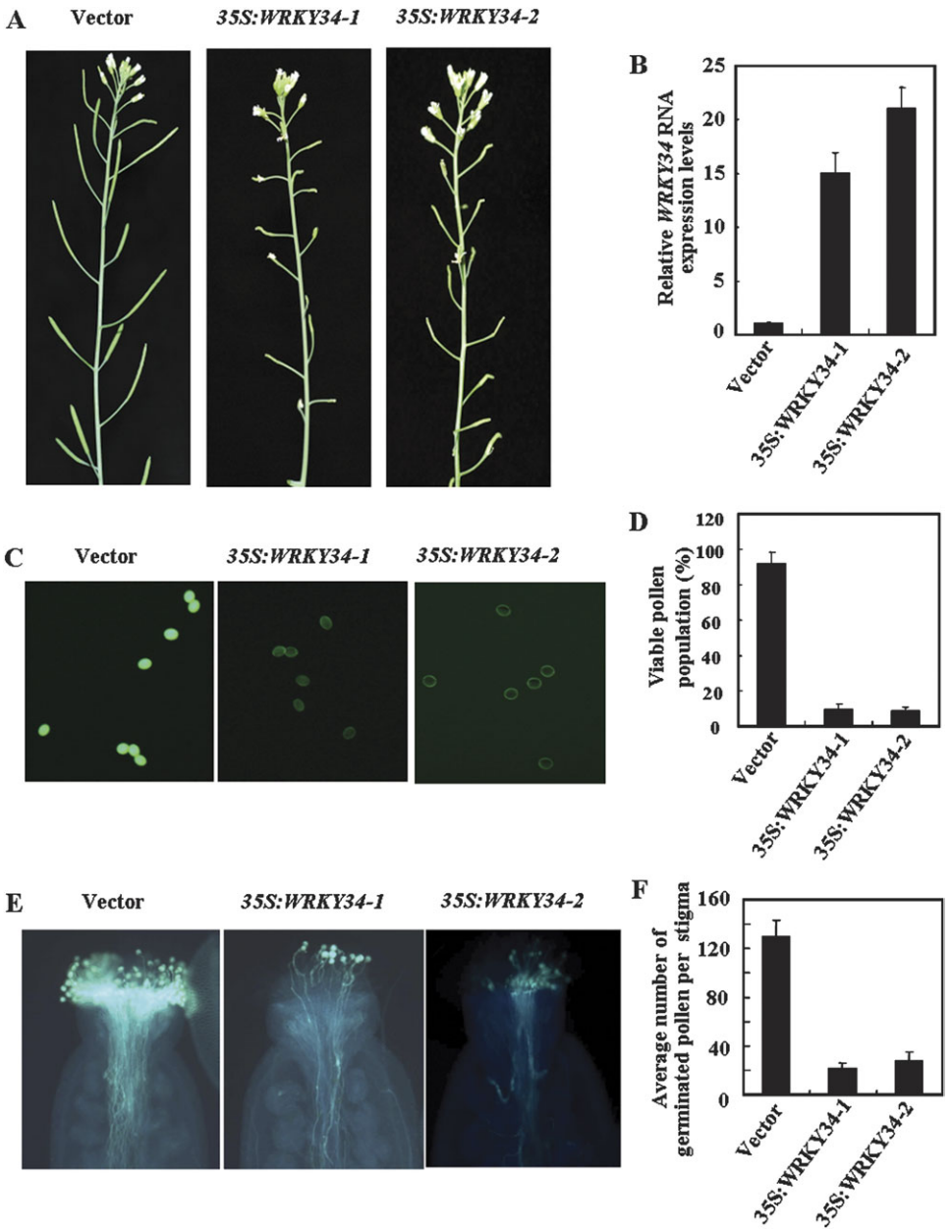

Fig. 4. Phenotype of *35S::WRKY34* compared with the vector transgenic plant (control). (A) Inflorescence of a vector transgenic, *35S::WRKY34-1* and *35S::WRKY34-2* plants under normal growing conditions. (B) Relative *WRKY34* RNA expression levels in mature pollen of *35S::WRKY34-1* and *35S::WRKY34-2* plants. (C) Fluorescence of mature pollen grains from vector transgenic plant, *35S::WRKY34-1* and *35S::WRKY34-2* plants stained with fluoroscein diacetate (FDA). (D) The proportion of viable pollen grains as determined by staining with FDA. (E) Elongated pollen tubes from vector transgenic, *35S::WRKY34-1* and *35S::WRKY34-2* plants stained with aniline blue. (F) Average number of elongated pollen tubes per stigma of *35S::WRKY34-1* and *35S::WRKY34-2* plants. Error bars indicate standard deviations of three independent biological samples.

expression of these genes decreased considerably in both the WT and *wrky34* mutants (Fig. 6). Cold treatment strongly reduced accumulation of *AGL66*, *AGL65*, *AGL104*, *AGL30*, and *AGL94* transcripts in all plants, and the accumulation of transcripts of each gene in cold-treated *wrky34* mutants did not differ significantly from that in the WT. In addition, expression of the five MIKC* genes in *35S:WRKY34* transgenic plants did not differ significantly from that of vector transgenic plants (see Supplementary Fig. S3 at *JXB* online). Thus, the expression of MIKC* genes was independent of *WRKY34* expression.

Effect of WRKY34 *on expression of transcriptional activator* CBF *genes*

Without cold treatment, expression of *CBF1*, *CBF2*, and *CBF3* increased about 2-fold in *wrky34* mutants relative to that in the WT (Fig. 7A). Quantitative RT-PCR revealed that *CBF*s transcription was induced by cold treatment in

wrky34 mutants and reached a maximum level after about 24 h of cold stress, and thereafter began to decrease slightly (Fig. 7A). *CBF* transcription in mature pollen of the WT was not induced by cold treatment when compared to *wrky34* mutants. In mature pollen of *wrky34* mutants, 24 h cold treatment led to a 10-fold increase in the accumulation of *CBF3* transcripts, and a 3.4-fold increase in the accumulation of *CBF1* transcripts, which was consistent with microarray analysis (Table 3). In addition, five *COR* genes, which are target genes of CBFs (Lee *et al.*, 2002; Maruyama *et al.*, 2004), displayed greater induction in mature pollen of *wrky34* mutants than that in WT following cold treatment (Fig. 7B).

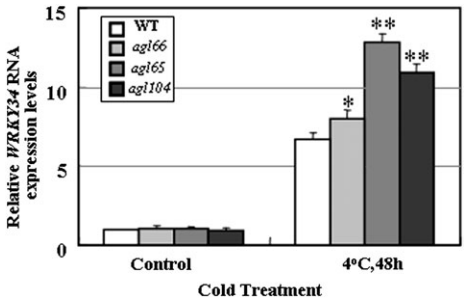

Fig. 5. *WRKY34* RNA expression in *agl66*, *agl65*, and *agl104* mutants. *WRKY34* RNA expression was determined in RNA extracted from mature pollen after treatment. Error bars indicate standard deviations of determinations from three independent RNA extracts. Differences between the wild-type (WT) and *wrky34* mutants after cold treatment were significant at $P < 0.05$ (*) or $P < 0.01$ (**).The untreated greenhouse-grown plants were used as the control.

Discussion

In the present study, the *wrky34* allele was identified as a novel mutation that affects mature pollen viability under cold stress. Genetic analysis showed that mature pollen of the *wrky34-1* and *wrky34-2* mutants was less sensitive to cold stress compared with that of the WT. *WRKY34* is expressed in the early stages of male gametophyte development and low levels of transcripts are present in mature pollen (Verelst *et al.*, 2007*b*; Honys *et al.*, 2006). Without cold stress, knock-down of WRKY34 function did not affect pollen viability. Cold stress significantly induced *WRKY34* expression in the WT, and pollen harbouring the *wrky34* allele showed greater competitiveness than pollen containing the *WRKY34* allele after cold treatment (Tables 1, 2). Mature pollen of *wrky34* mutants showed higher cold-stress tolerance than that of the WT following cold treatment (Fig. 3), and over-expression of *WRKY34* conferred much reduced fertility on mature pollen even under normal growing conditions (Fig. 4). All of these results

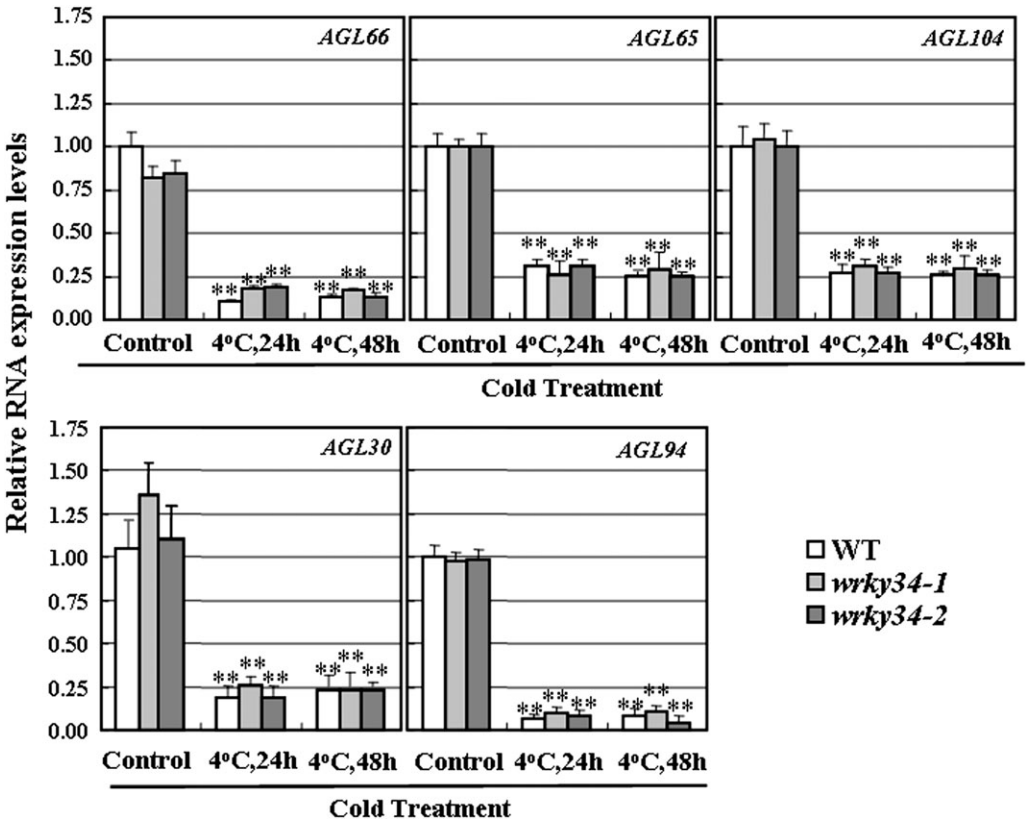

Fig. 6. RNA levels of MIKC* genes in mature pollen of *wrky34* mutants and wild-type (WT) plants. MIKC* RNA expression was determined in RNA extracted from mature pollen after treatment with 4 °C for 24 h or 48 h. Error bars indicate standard deviations from three independent RNA extracts. Differences between the untreated and cold-stressed plants were significant at $P < 0.01$ (**).

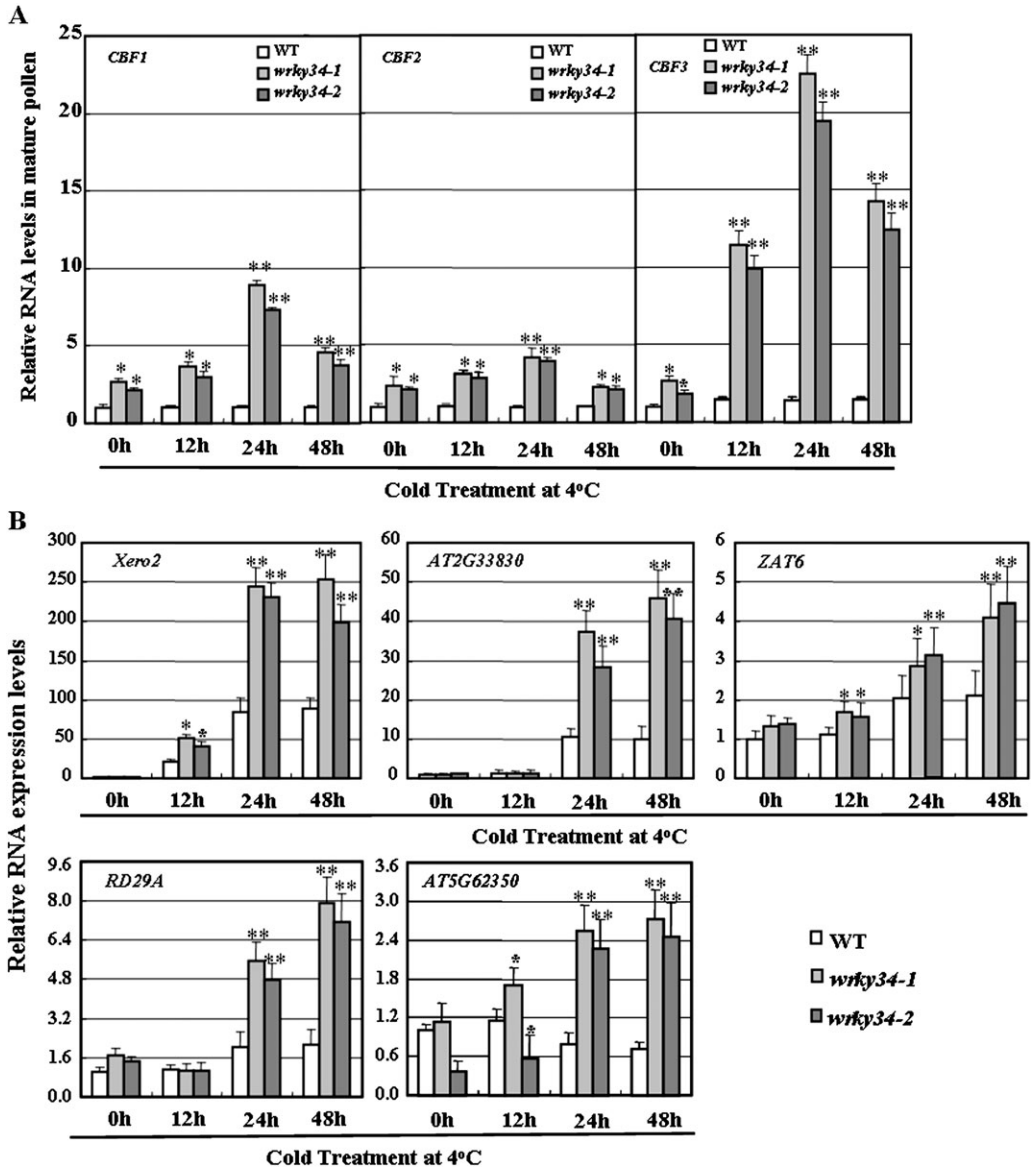

Fig. 7. Expressionof *COR* RNA in mature pollen of wild-type (WT) and *wrky34* plants. (A) Relative RNA levels for *CBF*1, *CBF*2, and *CBF*3. (B) Relative RNA levels for five *COR* genes. Relative RNA levels were analysed using gene-specific primers by real-time PCR. Error bars indicate standard deviations of determinations from three independent RNA extracts. Differences between the wild-type (WT) and *wrky34* mutants after cold treatment were significant at $P < 0.05$ (*) or $P < 0.01$ (**).

suggest that *WRKY34* functions as a negative regulator of cold sensitivity in mature pollen.

Mature pollen of *wrky34* mutants is less sensitive to cold stress at floral developmental stage 13. At this stage, the pollen grains are mature, the stigma is receptive, and anthesis occurs (Smyth *et al.*, 1990). Mature pollen grains contain mRNAs whose protein products appear to function during the late-maturation stages of pollen tube growth and germination (Mascarenhas, 1975). Thus, differences in the gene expression profiles between *wrky34* and WT provide valuable information for understanding why mature pollen of *wrky34* mutants is more tolerant to cold stress relative to that of the WT.

In mature pollen, the MIKC* transcription factors, including *AGL66*, *AGL65*, *AGL30*, *AGL104*, and *AGL94*, are known to be important regulators of pollen germination and tube growth, and regulate gene expression by binding to the MEF2 motif (Verelst *et al.*, 2007*a*, *b*; Adamczyk and Fernandez, 2009). Two MEF2 motifs are present in the putative promoter of *WRKY34* (see Supplementary Fig. S4 at *JXB* online), and *WRKY34* expression in MIKC* double mutants is significantly increased, indicating that MIKC* genes are negative regulators of *WRKY34* expression (Verelst *et al.*, 2007*b*). Therefore, it is possible that MIKC* genes are involved in the cold response of mature pollen. Consistent with this hypothesis, expression of the five MIKC* genes was similarly decreased in both the WT and *wrky34* plants in response to cold treatment (Fig. 6), and expression of *WRKY34* in *agl66*, *agl65*, and *agl104* was significantly higher than that of the WT (Fig. 5), supporting the suggestion that *WRKY34* acts as a downstream gene of

Table 3. Microarray analysis of the *wrky34-1* mutant and wild-type (WT)

Relative RNA levels of cold-related and MPG-specific genes in microarray results. RNA was extracted from mature pollen of the *wrky34-1* mutant and wild-type plants after treatment at 4 °C for 48 h. Gene expression levels were also measured by qRT-PCR.

AGI	Annotation	*wrky34* mutants versus WT
Cold response pathway genes		
AT4G25480	*ATCBF3*: involved in response to low temperature and abscisic acid	5.3
AT4G25490	*ATCBF1*: involved in response to low temperature and abscisic acid	2.0
AT5G04340	*ZAT6*: C_2H_2 Zinc Finger 6, transcription factor/zinc ion binding	3.1
AT5G62350	MMI9.21: invertase/pectin methylesterase inhibitor family protein	4.2
AT3G50970	Xero2: Low tempature-induced 30	2.8
AT5G52310	*RD29A*: Low tempature-induced 78	3.4
AT2G33830	Dormancy/auxin associated family protein	3.7
TCG/MPG-specific genes		
AT3G02480	ABA-responsive protein-related	6.1
AT3G28770	Unknown protein	5.3
AT1G78980	*SRF5*:ATP binding/kinase/protein serine/threonine kinase	2.6
AT1G24110	Peroxidase activity, response to oxidative stress.	2.2

MIKC* transcription factors, and that cold stress affects mature pollen viability by inhibiting expression of MIKC* genes. These results also implied that the cold-sensitivity of mature pollen in the WT might result from the accumulation of *WRKY34* transcripts.

The MIKC* genes affect a large suite of tricellular and mature pollen grains (TCP/MPG) specific genes, including *WRKY34* (Honys and Twell, 2003; Verelst *et al.*, 2007*b*; Adamczyk and Fernandez, 2009). The mature pollen of *wrky34* mutants was cold-insensitive, which implied that *WRKY34* was involved in the regulation of MIKC* gene-mediated pollen viability under cold stress. To test this hypothesis, the microarray results with the reference dataset of Honys and Twell were compared (Honys and Twell, 2003). In the *wrky34-1* mutant, the expression levels of 147 MPG-specific genes, which are putative downstream genes of MIKC* transcription factors (Verelst *et al.*, 2007*b*; Adamczyk and Fernandez, 2009), were significantly higher than those of the WT (Table 3; see Supplementary Table S2 at *JXB* online). Four of these genes were analysed by qRT-PCR, which revealed that the transcript levels of *SRF5*, *AT3G02480*, *AT1G24110*, and *AT3G28770* displayed reduced inhibition in *wrky34* mutants than in WT following cold treatment (see Supplementary Fig. S5 at *JXB* online). Interestingly, the putative peroxidase gene *AT1G24110* showed greater induction in mature pollen of *wrky34* mutants than in the WT (see Supplementary Fig. S5 at *JXB* online). The enhanced expression of these genes in the *wrky34* mutants might be partly responsible for the lower decrease in pollen viability than in the WT under cold stress. All of these results suggested that WRKY34 and MIKC* transcription factors may be important regulators controlling the mature pollen response to cold stress.

The *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). The pathway includes activity of three transcription factors, namely CBF1, CBF2, and CBF3 (also known as DREB1b, c, and a, respectively), that are rapidly induced in response to low temperature followed by expression of the CBF-targeted genes (the CBF regulon) that act in concert to increase plant freezing tolerance (Stockinger *et al.*, 1997; Fowler and Thomashow, 2002; Maruyama *et al.*, 2004; Chinnusamy *et al.*, 2007). Therefore, it is possible that *CBF* genes might be associated with the cold tolerance of mature pollen in *wrky34* mutants. Surprisingly, *CBF*s were significantly induced by cold stress in *wrky34* mutants rather than in the WT, and disruption of *WRKY34* also enhanced induction of *CBF*-targeted genes (Fig. 7; Table 3), implying that the cold-induced expression of *WRKY34* in the WT might repress expression of cold-induced *CBF* genes in mature pollen. On the other hand, other reports have shown that WRKY proteins (ABF1 and ABF2) bind to the box2/W-box of the GA-regulated α-Amy2 promoter (Rushton *et al.*, 1995), and GaWRKY1 strongly activated the CAD1-A promoter by binding to the W-box (Xu *et al.*, 2004). A barley WRKY gene, *HvWRKY38*, and its rice (*Oryza sativa*) orthologue, *OsWRKY71*, act as transcriptional repressors of gibberellin-responsive genes in aleurone cells (Zou *et al.*, 2008). The putative 3 kb promoter zone of *CBF3* and *CBF1* has five and four TTGAC(C/T) W-boxes, respectively, which raise the possibility that WRKY34 regulates transcription of *CBF3* and *CBF1*. These results suggested that the cold-tolerance of mature pollen in the *wrky34* mutants was partly attributable to elevated expression of *CBF*s and *CBF*s-targeted genes. It was reported previously that poor expression of cold-responsive genes that play a role in stress tolerance might be why *Arabidopsis* pollen is cold-sensitive (Lee and Lee, 2003). These results might explain why the transcripts of cold-responsive genes do not accumulate in mature pollen of the WT.

In summary, this study confirmed that the WRKY34 transcription factor negatively mediates cold sensitivity in mature pollen of *Arabidopsis*. Pollen-specific MIKC* transcription factors were negative regulators of the transcripts of *WRKY34* during the cold response, and the WRKY34 transcription factor was involved in mature pollen

development regulated by the pollen-specific MIKC* class of MADS-domain transcription factors under cold stress. Cold insensitivity of mature *wrky34* pollen is partly attributable to the induction of *CBF*s and the up-regulation of *COR* genes in the mutants, indicating that the WRKY34 transcription factor might be involved in the *CBF* signal cascade in mature pollen. Elucidation of the physiological functions of WRKY34 requires further investigation.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. List of quantitative RT-PCR primer sequences.

Supplementary Table S2. Mircoarray data for expression of 147 mature pollen grains (MPG) specific genes.

Supplementary Fig. S1. Nuclear staining and pollen surface of vector and *35S:WRKY34* plants.

Supplementary Fig. S2. Example of the reciprocal crosses between *35S:WRKY34* and vector plants.

Supplementary Fig. S3. Levels of MIKC* RNA in mature pollen of vector and *35S:WRKY34* plants.

Supplementary Fig. S4. Positions of two MEF2 motifs in the putative promoter of WRKY34.

Supplementary Fig. S5. RNA levels of mature pollen specific genes after cold treatment.

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