A Gain-of-Function Mutation in the Arabidopsis Disease Resistance Gene *RPP4* Confers Sensitivity to Low Temperature[1][W][OA]

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How plants adapt to low temperature is not well understood. To identify components involved in low-temperature signaling, we characterized the previously isolated *chilling-sensitive2* mutant (*chs2*) of Arabidopsis (*Arabidopsis thaliana*). This mutant grew normally at 22°C but showed phenotypes similar to activation of defense responses when shifted to temperatures below 16°C. These phenotypes include yellowish and collapsed leaves, increased electrolyte leakage, up-regulation of *PATHOGENESIS RELATED* genes, and accumulation of excess hydrogen peroxide and salicylic acid (SA). Moreover, the *chs2* mutant was seedling lethal when germinated at or shifted for more than 3 d to low temperatures of 4°C to 12°C. Map-based cloning revealed that a single amino acid substitution occurred in the TIR-NB-LRR (for Toll/Interleukin-1 receptor- nucleotide-binding Leucine-rich repeat)-type resistance (R) protein RPP4 (for Recognition of *Peronospora parasitica4*), which causes a deregulation of the R protein in a temperature-dependent manner. The *chs2* mutation led to an increase in the mutated *RPP4* mRNA transcript, activation of defense responses, and an induction of cell death at low temperatures. In addition, a *chs2* intragenic suppressor, in which the mutation occurs in the conserved NB domain, abolished defense responses at lower temperatures. Genetic analyses of *chs2* in combination with known SA pathway and immune signaling mutants indicate that the *chs2*-conferred temperature sensitivity requires *ENHANCED DISEASE SUSCEPTIBILITY1*, *REQUIRED FOR Mla12 RESISTANCE*, and *SUPPRESSOR OF G2 ALLELE OF skp1* but does not require *PHYTOALEXIN DEFICIENT4*, *NONEXPRESSOR OF PR GENES1*, or SA. This study reveals that an activated TIR-NB-LRR protein has a large impact on temperature sensitivity in plant growth and survival.

For optimal growth and survival, plants have evolved unique and sophisticated defense mechanisms against multiple stresses, both abiotic and biotic. Cold stress has a significant limiting effect on the geographic location of plants and on crop productivity (Guy, 1990). It can disrupt cellular homeostasis by altering the fatty acid composition of membrane lipids, which can deactivate membrane proteins and uncouple major physiological processes (Los and Murata, 2004). Plants respond and adapt to cold stress in many biochemical and physiological processes. A number of genes are involved in the DREB/CBF (for DRE-binding protein/C-repeat-binding factor)-dependent pathway to control cold acclimation (Gilmour et al., 1992, 2004), and DREB/CBF-independent pathways have been identified as important for cold responses as well (Xin and Browse, 1998; Dong et al., 2006; Lee et al., 2006; Xin et al., 2007; Zhu et al., 2008).

Plants have evolved at least two layers of defense mechanisms against pathogens. One of them is mediated by resistance (R) proteins. Interaction of an R protein with a specific pathogen avirulence protein triggers the hypersensitive response (HR), which is a form of programmed cell death that limits pathogen growth and spread (Scheel, 1998). Most of the characterized R proteins encode proteins with nucleotide-binding Leu-rich repeat (NB-LRR) domains. A well-conserved ARC (for Apaf-1, R protein, and CED4) domain is found just after the NB domain, and these two domains are often referred to as the NB-ARC domain. The NB-LRR proteins can be grouped into two main classes based on their N-terminal structure, which has either a Toll/Interleukin-1 receptor (TIR) domain or a coiled-coil domain (Meyers et al., 2003).

The Arabidopsis (*Arabidopsis thaliana*) *RPP5* (for *Recognition of Peronospora parasitica5*) locus in Columbia-0 (Col) is composed of seven TIR-NB-LRR class *R* genes, including *RPP4* and *SNC1* (for *Suppressor of npr1-1, constitutive 1*) genes (Noel et al., 1999). *RPP4* plays an important role in resistance to *Hyaloperonospora parasitica* through multiple signaling components, in-

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cluding *DETACHMENT 9* (*DTH9*; Mayda et al., 2000), *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1*; Aarts et al., 1998), *PHYTOALEXIN DEFICIENT4* (*PAD4*; Glazebrook et al., 1996), *NONEXPRESSOR OF PR GENES1* (*NPR1*; Cao et al., 1997), *NON-RACE-SPECIFIC DISEASE RESISTANCE1* (*NDR1*; Century et al., 1995), *Phenylalanine Ammonium Lyase* (*PAL*; Mauch-Mani and Slusarenko, 1996), *avrPphB SUSCEPTIBLE2* (*PBS2*) and *PBS3* (Warren et al., 1999), *SUPPRESSOR OF G2 ALLELE OF skp1* (*SGT1b*) and *REQUIRED FOR Mla12 RESISTANCE* (*RAR1*; Austin et al., 2002), *RPS5* (Warren et al., 1998), and *SALICYLIC ACID INDUCTION-DEFICIENT1* (*SID1*), *SID2*, and salicylic acid (SA; McDowell et al., 2000; van der Biezen et al., 2002). In addition, *RPP4* mediates disease resistance and basal defense against *H. parasitica* through the transcription factor *AtWRKY70* (Knoth et al., 2007). *SNC1* confers disease resistance and suppresses plant growth in a temperature-dependent manner when activated (Stokes and Richards, 2002; Zhang et al., 2003; Yang and Hua, 2004; Zhu et al., 2010). The *RPP5* locus *R* genes are coordinately regulated by transcriptional activation and RNA silencing (Yi and Richards, 2007).

Although the initial stimuli of cold and biotic stresses are obviously different, in many cases these signals are integrated into a unified scheme and trigger a common set of responses. For instance, cold and defense responses are shown to share common targets, such as *PATHOGENESIS-RELATED* (*PR*) genes, which not only play a role in pathogen resistance but also are induced by cold stress and promote freezing tolerance (Snider et al., 2000; Seo et al., 2008). Furthermore, cold and defense responses share common regulators, such as the SUMO E3 ligase SIZ1 (for SAP and Miz1; Lee et al., 2007; Miura et al., 2007), AtSR1/CAMTA3 (for Arabidopsis signal responsive/Calmodulin-binding transcription activator 3; Doherty et al., 2009; Du et al., 2009), and the transcriptional repressor of DREB protein DEAR1 (for DREB and EAR protein 1; Tsutsui et al., 2009). In addition, defense responses induced by a number of *R* genes are modulated by temperature, including *Mi* in tomato (*Solanum lycopersicum*; Hwang et al., 2000), *N* in tobacco (*Nicotiana tabacum*; Someya et al., 2004), and *RESISTANCE TO POWDERY MILDEW8*, *SUPPRESSOR OF SALICYLIC ACID INSENSITIVE4*, *SNC1*, and the *RPP1*-like TIR-NB-LRR cluster in Arabidopsis (Xiao et al., 2003; Yang and Hua, 2004; Zhou et al., 2008; Alcazar et al., 2009). A recent study revealed that the NB-LRR proteins function as temperature-sensitive components in plant immune responses (Zhu et al., 2010). Some of the defense signaling components, such as *PAD4*, *EDS1*, and SA, are also regulated by temperature (Clarke et al., 2004; Yang and Hua, 2004). Moreover, the plasma membrane-bound NAC transcription factor NTL6 is proteolytically activated by cold and in turn enters the nucleus, thereby inducing defense responses by binding to the promoter of *PR* genes (Seo et al., 2010). All of these findings support an extensive signaling network between cold stress and defense responses.

Here, we report the investigation of a cold-sensitive mechanism of *chilling-sensitive2* (*chs2*) in Arabidopsis. The *chs2* mutant exhibits HR-like cell death and consequent lethality under cold stress. Map-based cloning revealed that *CHS2* encodes the TIR-NB-LRR-type R protein RPP4. An amino acid substitution in the NB-ARC region leads to a temperature-dependent gain-of-function phenotype. This study reveals the involvement of an activated *R* gene in cold response, suggesting a contribution of defense responses to temperature sensitivity.

RESULTS

Morphological Phenotypes of the Chilling-Sensitive Mutant *chs2*

The *chs2-1* and *chs2-2* mutants were isolated as chilling sensitive from an ethane methyl sulfonate (EMS)-mutagenized pool of Arabidopsis (Schneider et al., 1995). We further characterized the mutant phenotypes of these two alleles. They resembled the wild type when grown in soil at 22°C; however, the leaves of these two mutants turned yellow and wilted 3 d after being shifted to low temperature of 4°C to 12°C, and they eventually died (Fig. 1A). When planted on Murashige and Skoog (MS) plates directly at 4°C, the *chs2* seedlings died shortly after germination (Fig. 1C). Given that these two alleles showed similar phenotypes, we chose *chs2-2* (referred as *chs2* hereafter) for further studies.

To get a better understanding of *chs2* in response to chilling, we examined the phenotypes of *chs2* plants by shifting them to cold conditions at different growth stages either in soil or on agar plates. The 22°C-grown *chs2* plants at every developmental stage tested were hypersensitive to cold stress both in soil and on MS plates (Fig. 1, B–D). All parts of the *chs2* plants including the rosette leaves, cauline leaves, stems, flowers, and siliques became yellow, collapsed, and then died quickly after cold exposure (Fig. 1B). It is noteworthy that the mutant grown at 16°C to 18°C showed dwarf stature with curly chlorotic leaves and short inflorescence internodes (Fig. 1E). With decreased temperature, the *chs2* mutant plants showed more severe growth defects, and they were lethal when temperature was below 12°C (Fig. 1F). Therefore, the *chs2* mutant is sensitive to low temperature throughout plant development, with lower temperature causing more severe growth defects.

Physiological Characteristics of *chs2* at Low Temperatures

Leakage of ions from cell membranes is a good index to measure chilling sensitivity in plants (Lyons, 1973). We carried out ion leakage assays to determine the extent of chilling injury to *chs2* plants. No obvious

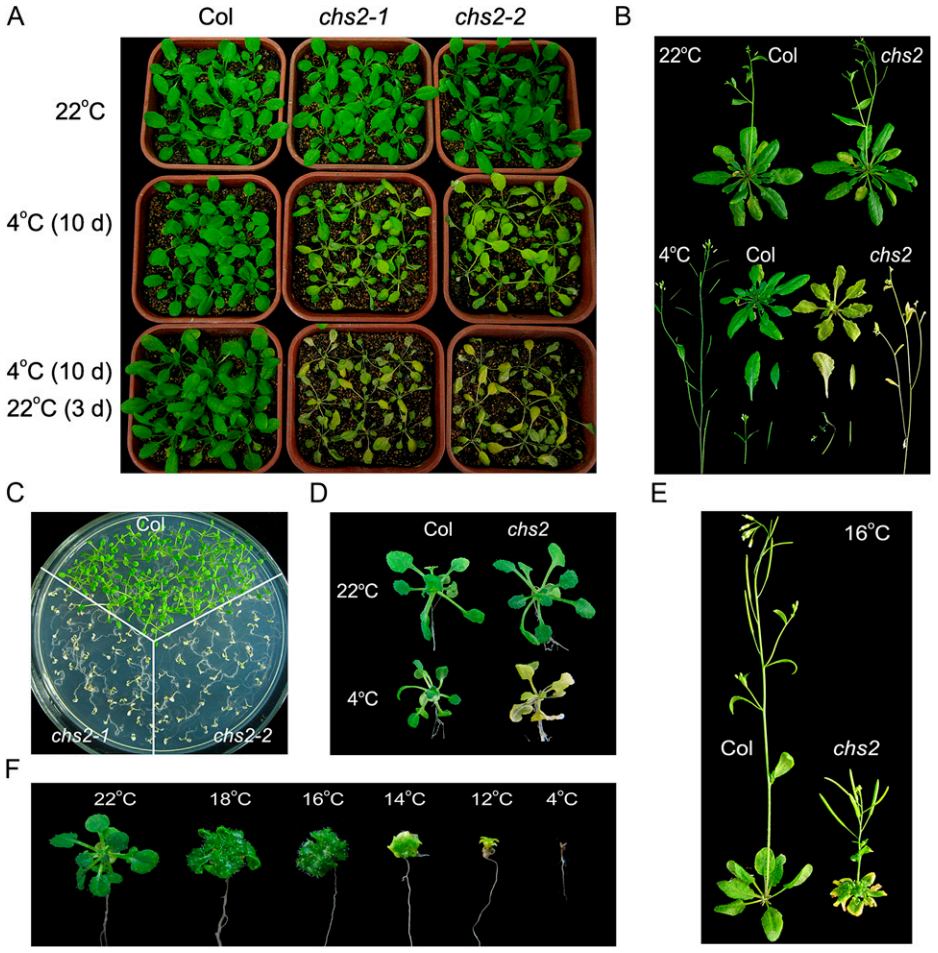

Figure 1. Cold sensitivity of *chs2* mutant plants. A, Phenotypes of wild-type Col and *chs2* plants grown in soil at 22°C for 4 weeks (top row), cold treated at 4°C for 10 d (middle row), followed by 22°C for 3 d (bottom row). B, Phenotypes of wild-type Col and *chs2* plants grown in soil at 22°C for 6 weeks (top) followed by cold treatment at 4°C for 10 d (bottom). C and D, Phenotypes of Col and *chs2* plants directly geminated on MS plates and grown at 4°C for 3 months (C) or grown at 22°C for 3 weeks and then transferred to 4°C for an additional 10 d (D). E, Phenotypes of wild-type Col and *chs2* plants grown in soil at 16°C for 7 weeks. F, Phenotypes of *chs2* plants grown on MS plates at the indicated temperatures for 4 weeks. Images are of representative plants.

changes in ion leakage were detected in wild-type leaves during cold treatment. However, ion leakage of *chs2* plants increased drastically following cold treatment (Fig. 2A). This result indicates that the cell membranes of *chs2* leaves are severely injured under cold stress, which is in agreement with the cold-sensitive phenotype of *chs2*.

Free Pro is an osmolyte considered to protect plants against cold stress (Xin and Browse, 1998; Nanjo et al., 1999). We investigated if the cold sensitivity of *chs2* is accompanied by reduced Pro levels. Indeed, the Pro content in *chs2* was much lower than in the wild type when treated at 4°C for 6 d (Fig. 2B), suggesting that less Pro accumulation in *chs2* might at least partly account for its cold sensitivity.

Chloroplasts Are Damaged in *chs2* Plants under Cold Stress

Because the *chs2* plants exhibited yellow leaves under cold stress (Fig. 1), we measured the chlorophyll content in the *chs2* mutant. The levels of chlorophyll *a* and chlorophyll *b* in cold-treated *chs2* plants were approximately 42% and 50% of those in the wild-type plants, respectively (Fig. 3A), implying that the chloroplasts in *chs2* are severely damaged under cold conditions.

The chloroplast morphology in cold-treated *chs2* plants was further examined using transmission electron microscopy. The mature chloroplasts of the *chs2* and wild-type plants at 22°C exhibited crescent-shaped and well-developed thylakoid membranes. Chloroplasts in cold-treated wild-type plants were similar to those in plants without cold treatment, but with larger starch granules, which is a normal response to cold stress. In contrast, cold-treated *chs2* chloroplasts were smaller and more spherical than those in the wild-type plants, and they contained fewer internal thylakoid membranes. Moreover, the starch grains in cold-treated *chs2* chloroplasts were either absent or reduced in size and number. The mutant chloroplasts also appeared to contain more plastoglobuli than wild-type chloroplasts (Fig. 3B). Thus, cold stress causes serious damage to the chloroplasts in *chs2* plants.

We then determined whether light had an effect on cold-induced phenotypes of *chs2*. Although the cold-induced phenotype of *chs2* was significantly delayed in the dark (Supplemental Fig. S1A), the plants eventually died. Accordingly, the degradation of chlorophyll *a* and *b* was also delayed in the dark (Supplemental Fig.

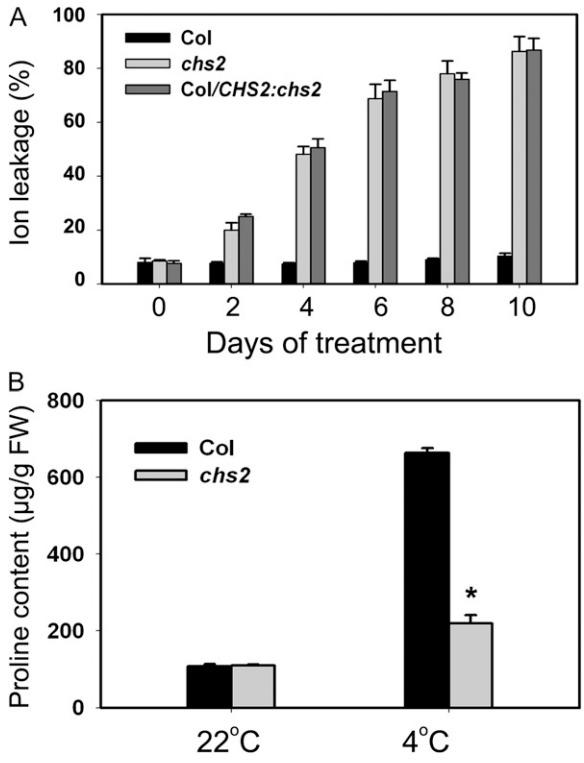

Figure 2. Physiological analysis of *chs2* mutant plants. A, Ion leakage assay in *chs2* plants. Plants grown at 22°C for 3 weeks were then treated at 4°C for the indicated times. The data represent means of three replicates ± SD. Similar results were observed in three independent experiments. B, Pro content in *chs2* plants. Plants grown at 22°C for 3 weeks were treated at 4°C for 6 d. The data represent means of four replicates ± SD. * $P < 0.01$ (*t* test), significant difference from Col. Similar results were observed in three independent experiments. FW, Fresh weight.

S1B). These results demonstrate that light accelerates the *chs2*-conferred phenotype, but low temperature triggers this phenotype in the absence of light.

The *chs2* Mutation Causes Reactive Oxidative Species Accumulation and Imbalanced Reactive Oxidative Species-Scavenging Network under Cold Stress

Low temperature can perturb electron transport chains and cause the production of reactive oxidative species (ROS; Fryer et al., 2002; Hideg et al., 2002; Pfannschmidt et al., 2003). Therefore, we examined hydrogen peroxide (H_2O_2) accumulation in *chs2* plants under cold conditions by 3,3′-diaminobenzidine (DAB) staining. Strong staining was detected in cold-treated *chs2* plants but not in wild-type plants (Fig. 4A), indicating that the mutant plants accumulated more H_2O_2 than the wild-type plants. Under light, chloroplast is the main site of ROS generation; consistently, DAB precipitates were mostly present in the chloroplasts. Therefore, the *chs2*-induced phenotypes under cold stress might be caused by the impairment of normal chloroplast function and by the overgeneration of ROS in the chloroplasts.

When subjected to low temperature, plants accumulate excess H_2O_2 (O'Kane et al., 1996), which in turn induces the expression of genes associated with oxidative stress (Iba, 2002; Mittler et al., 2004; Rizhsky et al., 2004). More H_2O_2 accumulation in *chs2* was observed under cold conditions (Fig. 4A). Therefore, we examined the expression of several genes encoding ROS-detoxification enzymes, including copper/zinc superoxide dismutase (CSD), ascorbate peroxidase (APX), and catalase (CAT), in cold-treated *chs2* plants. No obvious differences in expression of *CSD1*, *APX1*, or *CAT1* were detected between wild-type and *chs2* plants at 22°C. In contrast, the expression of these genes was substantially elevated in *chs2* plants relative to wild-type plants under cold stress (Supplemental Fig. S2B). The zinc-finger protein ZAT12 plays a crucial role in oxidative and abiotic stress signaling (Rizhsky et al., 2004; Davletova et al., 2005). In addition, ferritin protein nanocages are essential for protecting cells against oxidative damage (Ravet et al., 2009). We found that *ZAT12* and *FER1* were also significantly up-regulated in cold-treated *chs2* plants relative to wild-type plants (Supplemental Fig. S2B). Therefore, the chilling sensitivity of *chs2* might result from an imbalance of ROS detoxification and consequent impairment of oxidative signaling.

The Expression of Cold-Regulated Genes Is Not Affected in *chs2*

We further examined whether the *chs2* mutation affects the induction of cold-regulated genes. The *CBF1* to *CBF3* genes were rapidly induced in *chs2* and wild-type plants 3 h after exposure to cold, and their target genes *RD29A* and *COR47* were substantially induced at 6 to 12 h after cold treatment. No significant difference in expression of these genes was observed between *chs2* and wild-type plants (Supplemental Fig. S3). Therefore, the *chs2* gene appears not to affect the *CBF* pathway.

chs2 Constitutively Activates Defense Responses under Cold Conditions

Leaves in cold-treated *chs2* plants turned yellow, lost turgor pressure, and collapsed (Fig. 1), resembling the pathogen-induced HR cell death response. Extensive cell death did occur in cold-treated *chs2* plants but not in wild-type plants, as revealed by trypan blue staining (Fig. 4B). Furthermore, *PR1* and *PR2* were highly expressed in *chs2* plants under cold stress (Fig. 4C). Consistently, cold-treated *chs2* plants harboring a *PR1*:*GUS* construct showed stronger staining of GUS than wild-type *PR1*:*GUS* transgenic plants (Fig. 4D).

Because high *PR* gene expression is often associated with elevated levels of SA, the endogenous SA levels in *chs2* were examined. The levels of both free SA and total SA in *chs2* were comparable to those in wild-type

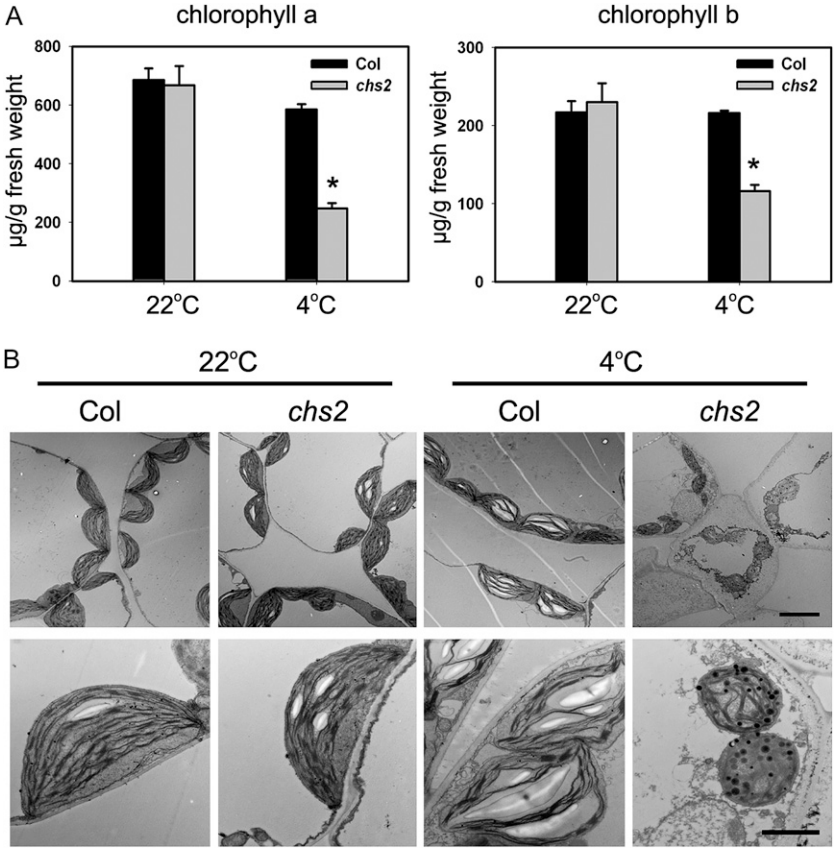

Figure 3. The effect of the *chs2* mutation on chloroplast development under cold stress. Wild-type Col and *chs2* plants were grown at 22°C for 3 weeks and then treated at 4°C for 10 d. A, Chlorophyll content of Col and *chs2* seedlings. The data represent means of four replicates ± SD. * $P < 0.01$ (*t* test), significant difference from Col. Similar results were observed in three independent experiments. B, Transmission electron microscopy of plastids from *chs2* plants. Bar = 5 μm (top row) and 2 μm (bottom row). Images are of representative plants.

plants grown at 22°C. However, cold-treated *chs2* plants accumulated approximately 22- and 65-fold higher levels of SA and total SA, respectively, than wild-type plants (Fig. 4E). Thus, *chs2* plants constitutively activate defense responses under cold stress.

A Mutation in *RPP4* Is Responsible for the Chilling-Sensitive Phenotype

The *chs2* mutant was previously shown to contain a dominant mutation in a single nuclear locus (Schneider et al., 1995). To identify the *chs2* mutation, *chs2-2* was crossed with Landsberg *erecta* (L*er*) to generate a mapping population. Given that the *chs2* mutation is dominant, wild-type-looking seedlings were chosen for mapping from the segregating F2 population after cold treatment. The *chs2* mutation was initially mapped to the middle of chromosome IV. Approximately 3,000 plants were then selected for fine mapping. The *chs2* mutation was narrowed to a 145-kb region containing the *RPP5* cluster region (Fig. 5A). To identify the molecular lesion in *chs2-2*, all of the annotated genes in this region were amplified from *chs2-2* and sequenced. Only one nucleotide substitution of C to T was found in the second exon of *At4g16860* (*RPP4* or *ColA*) in *chs2-2*, resulting in a Ser-to-Phe change at residue 389 (Fig. 5B). The same mutation was found in *chs2-1*.

The *chs2* mutant is a dominant mutation, suggesting a gain-of-function substitution. To determine whether the *chs2* phenotype was caused by the *chs2* mutation, a 12-kb genomic fragment including the complete *chs2* gene under the control of its own promoter (*CHS2*:*chs2*) was transformed into wild-type Col. Thirty-two out of 35 T1-independent transgenic lines showed all the *chs2*-conferred phenotypes under cold stress, including seedling lethality (Fig. 5C), high ion leakage (Fig. 2A), elevated *PR1* expression (Fig. 5E), and extensive cell death (Fig. 5F). These data indicate that mutated *chs2* recapitulates all the *chs2*-conferred phenotypes and therefore that *CHS2* is *RPP4*. *RPP4* encodes a TIR-NB-LRR-class R protein with high similarity to *SNC1* (74% amino acid identity and 78% similarity). The Ser-389 residue in *chs2* is very close to the putative GxP or GLPL motif in the ARC domain, which is conserved in many NB-LRR proteins (Rafiqi et al., 2009). This finding hence supports the importance of the ARC domain for the normal activity of R proteins.

The *chs2-s1* Mutation Suppresses the Chilling Sensitivity of *chs2*

To further confirm that the mutation in *RPP4* is responsible for the *chs2* phenotype, we carried out a genetic suppressor screen in the *chs2* background. M2 plants derived from EMS-mutagenized *chs2* seeds were screened for mutants displaying wild-type morphology under cold stress. One such mutant, named *chs2-s1* (for *chs2 suppressor 1*), was isolated (Fig. 5D).

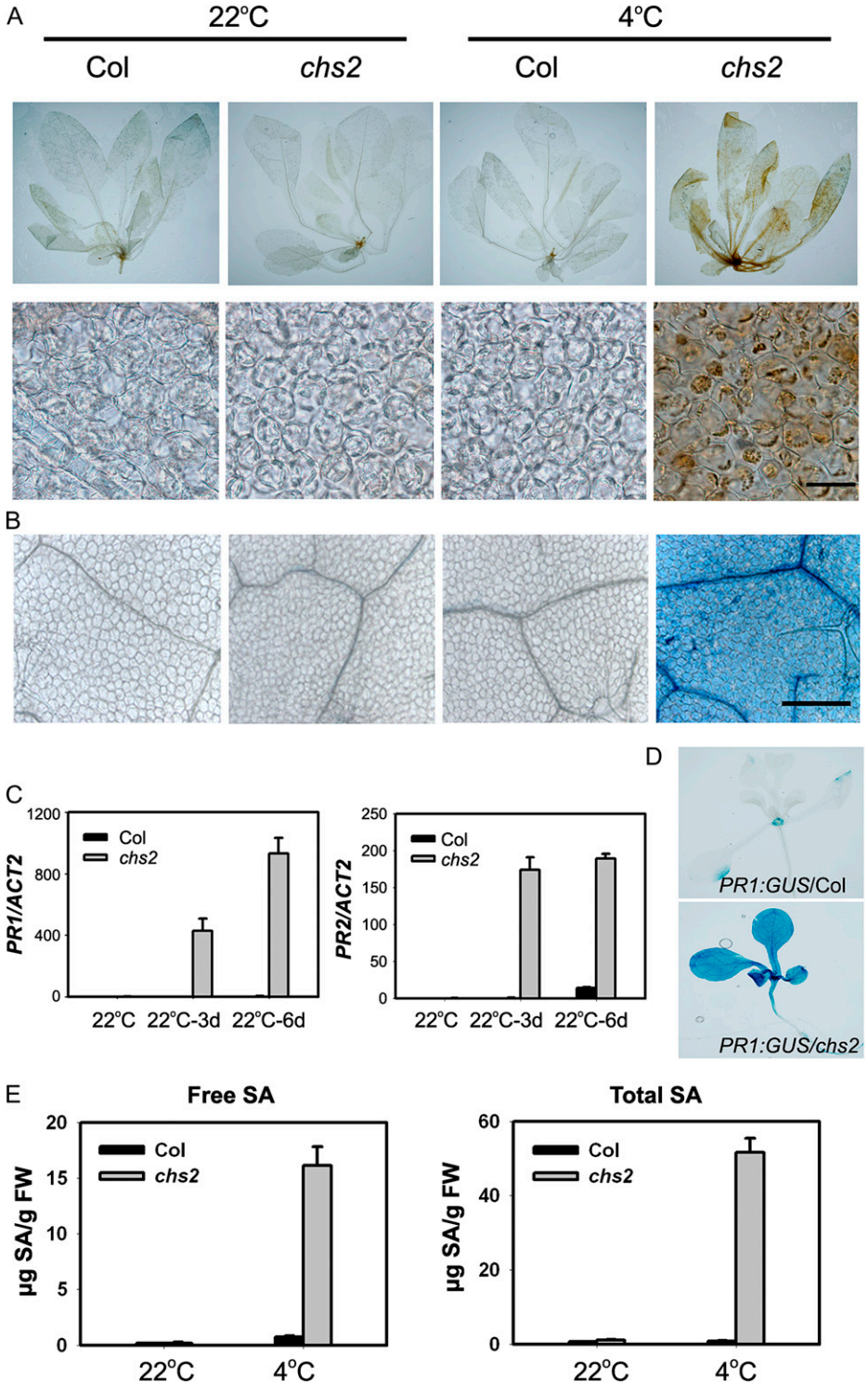

Figure 4. *chs2* constitutively activates defense responses to cold. Wild-type Col and *chs2* plants were grown at 22°C for 3 weeks and then treated at 4°C for 6 d. For A, B, and E, 20 plants were tested for each genotype. Images are of representative plants from one of three independent experiments. A, H_2O_2 accumulation in *chs2* plants stained by DAB. Bar = 20 μm. B, Cold-induced cell death in *chs2* plants. Detached leaves were stained with trypan blue. Bar = 100 μm. Images are of representative plants. C, Expression of *PR1* and *PR2* in wild-type and *chs2* plants by real-time RT-PCR. The data represent means of three replicates ± SD. Similar results were observed in three independent experiments. D, GUS analysis of *PR1* in *chs2* plants. *PR1:GUS* transgenic plants were crossed with *chs2* plants. The F2 homozygous lines were used for GUS staining analysis. Images are of representative plants. E, SA accumulation in *chs2* under cold conditions. Three-week-old 22°C-grown plants were treated at 4°C for 6 d. The data represent means of three replicates ± SD. Similar results were observed in three independent experiments. FW, Fresh weight.

PR1 gene expression and the cell death phenotype were significantly suppressed in *chs2-s1* (Fig. 5, E and F). This mutation was mapped to the original *RPP4* locus. Sequencing analysis revealed a second point mutation of E to K at amino acid position 300 in *chs2-s1*, which resides close to the Walker B/Kinase 2 motif of the RPP4 NB domain (Fig. 5B). This motif was shown to be important for the function of NB-LRR proteins, and mutations in or close to this conserved motif might abrogate the activity of NB-LRR proteins (Bendahmane et al., 2002).

RPP4 Expression in *chs2* at Different Conditions

To elucidate the physiological function of *RPP4*, we examined its organ-specific expression in Arabidopsis.

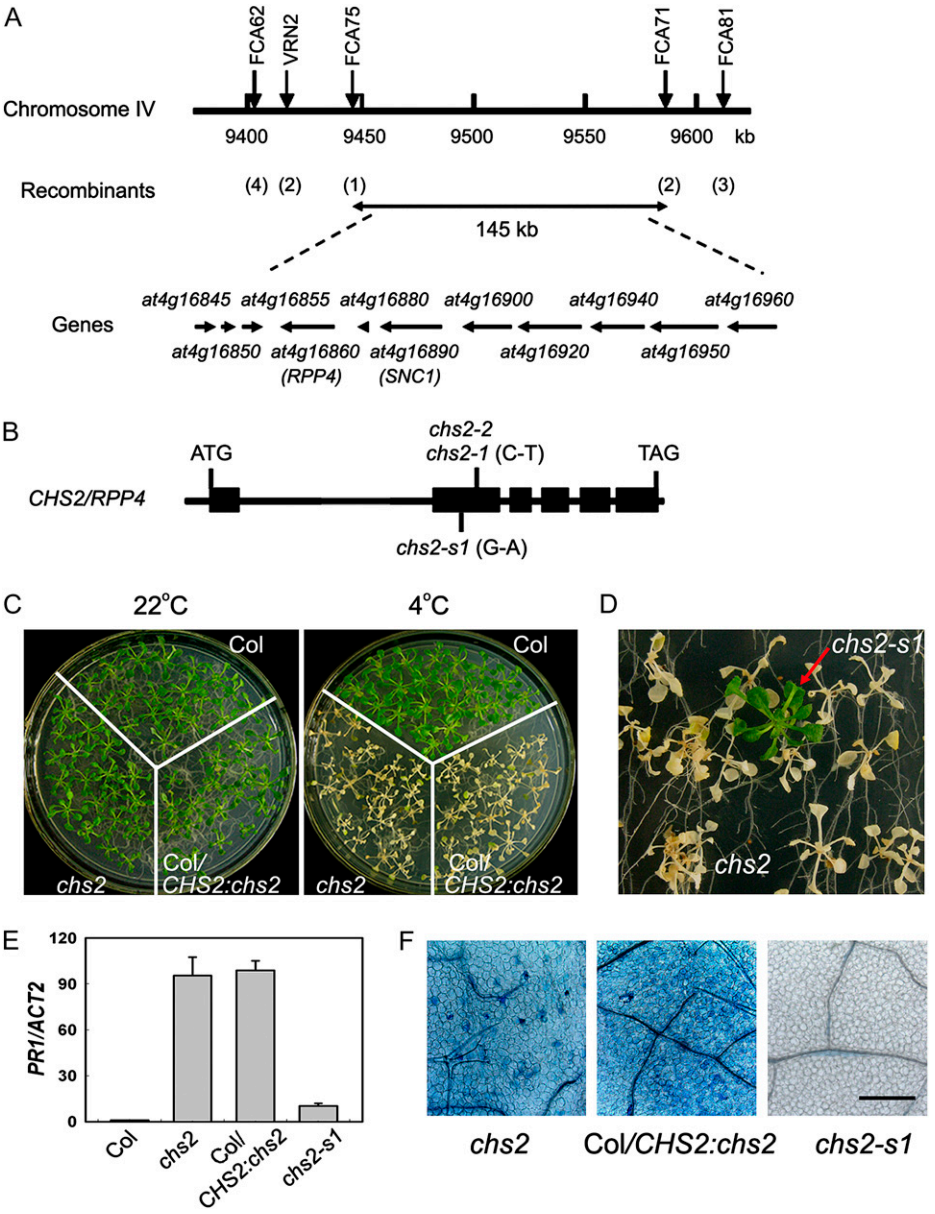

Figure 5. Map-based cloning of *CHS2*. A, A genetic map of the *CHS2* locus on chromosome IV. Positions of the markers used for mapping are indicated above the line. The corresponding nucleotide positions are numbered in kilobases below the line. The number of recombinants is indicated in parentheses. Predicted genes are shown by arrows indicating the direction of transcription. B, A schematic diagram of the genomic structure of the *CHS2* gene. Boxes and lines indicate exons and introns, respectively. The nucleotide substitutions in *chs2* and *chs2-s1* are shown. C, Complementation of the *chs2* mutant. Wild-type Col, *chs2*, and Col transformed with a genomic clone containing the mutated *chs2* (Col/*CHS2*:*chs2*) were grown at 22°C for 2 weeks (left) and then treated at 4°C for 10 d (right). D, Screening of the *chs2* suppressor *chs2-s1*. EMS-mutagenized *chs2* plants were grown at 22°C for 2 weeks and then treated at 4°C for 10 d. E, *PR1* gene expression in Col, *chs2*, Col/*CHS2*:*chs2*, and *chs2-s1* plants treated at 4°C for 6 d by real-time RT-PCR. The data represent means of three replicates ± SD. Similar results were observed in three independent experiments. F, Trypan blue staining of the leaves from *chs2*, Col/*CHS2*:*chs2*, and *chs2-s1* plants. Bar = 100 μm.

Transgenic plants harboring a GUS reporter gene driven by the *RPP4* promoter were generated and analyzed. GUS staining revealed that *RPP4* was expressed at low levels in leaves, stems, flowers, and siliques, and it was barely expressed in roots (Fig. 6A). This result is in agreement with public data from Genevestigator (https://www.genevestigator.com/gv/index.jsp) and was validated by quantitative reverse transcription (RT)-PCR analysis (Fig. 6B).

RPP4 was expressed at relatively low levels in the plants, consistent with the low steady-state expression levels of *R* genes under normal conditions. However, *R* genes can be induced by certain stimuli such as pathogens and SA. Therefore, we investigated whether *RPP4* expression was responsive to various stimuli. The expression of *RPP4* was not induced by the oxidative inducer methyl viologen in either wild-type Col or *chs2* plants (Fig. 6C). However, we found that *RPP4* in the wild-type Col background was induced by benzothiadiazole (an SA analog) and cold. Strikingly, cold stress dramatically enhanced the induction of the mutated *RPP4* in *chs2* (Fig. 6C; Supplemental Fig. S4A). Cold-induced overexpression could be a consequence of feedback regulation upon *R* gene activation, which might account for the phenotypes of *chs2* mutants under cold stress.

To test if overexpression of wild-type *RPP4* would recapitulate the *chs2* phenotype, we generated transgenic lines expressing wild-type *RPP4* driven either by its native promoter (*RPP4*:*RPP4*) or by the cauliflower mosaic virus 35S promoter (*35S*:*RPP4*), and we analyzed their phenotypes under cold conditions. Interestingly, neither the *RPP4*:*RPP4* nor *35S*:*RPP4* transgenic line, in which *RPP4* was indeed overex-

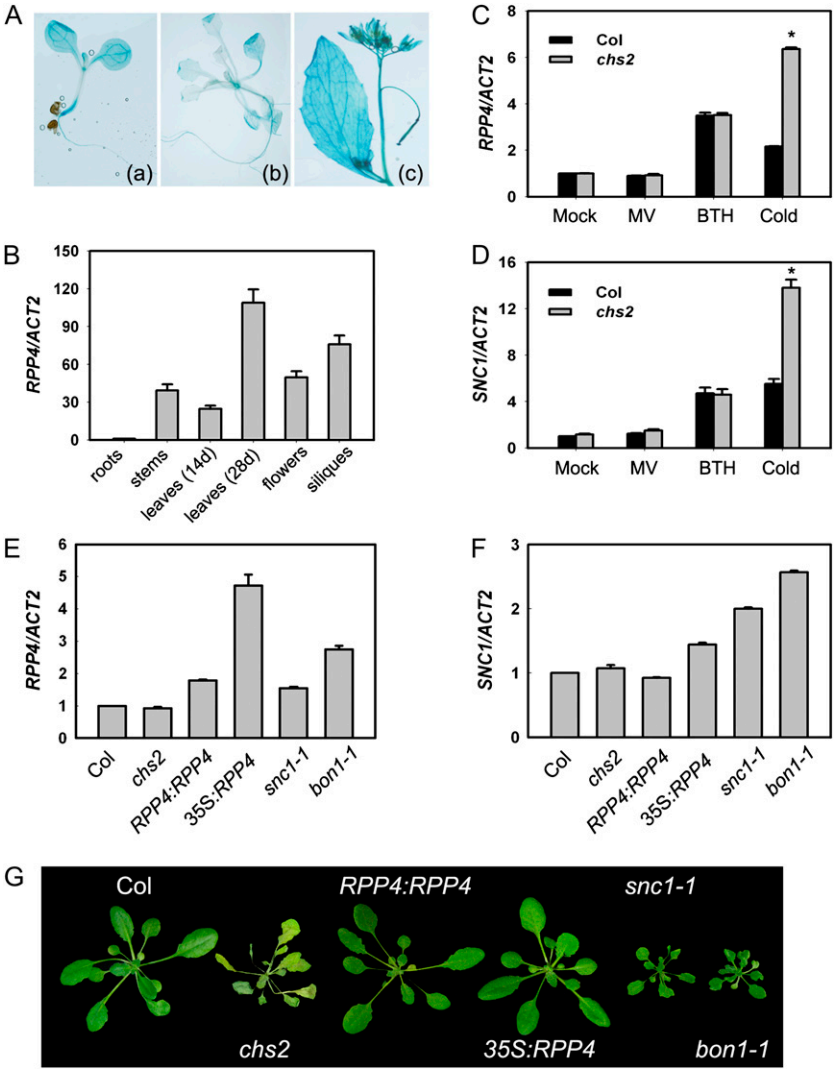

Figure 6. Expression of *RPP4* and *SNC1* in *chs2*. A and B, Expression of *RPP4* by GUS staining (A) and by real-time PCR (B). Total RNA was extracted from various tissues. The data represent means of three replicates ± SD. C and D, Expression of *RPP4* (C) and *SNC1* (D) under various treatments. Total RNA was extracted from plants treated with cold (4°C), methyl viologen (MV; 5 μM), or benzothiadiazole (BTH; 0.5 mM) for 24 h. E and F, Expression of *RPP4* (E) and *SNC1* (F) in 2-week-old 22°C-grown plants (Col, *chs2*, *RPP4:RPP4*, *35S:RPP4*, *snc1-1*, and *bon1-1*) by real-time PCR. The data represent means of three replicates ± SD. * $P < 0.01$ (*t* test), significant difference from Col. All experiments were repeated three times with similar results. G, Phenotypes of the plants (Col, *chs2*, *RPP4:RPP4*, *35S:RPP4*, *snc1-1*, and *bon1-1*) grown in soil at 22°C for 4 weeks and then cold treated at 4°C for 10 d.

pressed (Fig. 6E), exhibited *chs2*-like phenotypes at 4°C (Fig. 6G). Therefore, the *chs2*-conferred phenotypes are not simply caused by constitutive expression of *RPP4* but rather by the amino acid substitution in *chs2*. All of these data indicate that *chs2* is a gain-of-function mutant and that cold-induced overexpression of the mutated *RPP4* gene is required for the *chs2* phenotype.

chs2-Induced Chilling Sensitivity Is Independent of *SNC1*

Since the *RPP5* locus *R* genes are coordinately regulated (Yi and Richards, 2007), we examined the expression of *SNC1*, a close homolog of *RPP4*, in the *chs2* mutant. Similar expression patterns of *SNC1* induction were found in wild-type Col and *chs2* plants (Fig. 6D). *SNC1* expression was induced by benzothiadiazole and cold stress in both genotypes. Moreover, *chs2* plants accumulated higher levels of the *SNC1* transcript than did cold-treated Col plants (Fig. 6D; Supplemental Fig. S4B).

To determine whether up-regulation of *SNC1* also contributes to the *chs2* phenotype, we tested the cold sensitivity of *snc1-1* and *bon1-1* plants, in which *SNC1* is activated or derepressed (Yang and Hua, 2004; Li et al., 2007; Fig. 6F). Neither of them showed a *chs2*-like lethal phenotype at cold stress (Fig. 6G). In addition, we transformed the *CHS2:chs2* clone into *snc1-11* loss-of-function mutant plants. All 10 independent transgenic lines displayed a *chs2*-like chilling-sensitive phenotype (data not shown), indicating that the *chs2* mutation confers a *chs2* phenotype independent of *SNC1*.

chs2-Induced Chilling Sensitivity Is Independent of SA and NPR1

Because *chs2* plants accumulated high levels of free SA and total SA after cold treatment (Fig. 4E), we then determined whether activation of the SA pathway is

necessary for the *chs2* phenotype by crossing *chs2* with the SA-deficient mutant *sid2-2* (Wildermuth et al., 2001). The *chs2 sid2* double mutants exhibited chilling sensitivity and extensive cell death phenotypes similar to those of *chs2* (Fig. 7, A and C). As expected, the levels of SA and total SA in the *chs2 sid2* double mutants were reduced to a wild-type level under cold stress (Fig. 8). Therefore, the *chs2*-conferred chilling-sensitive phenotype does not require SA.

NPR1 is a master regulator of SA signaling and plant immunity (Cao et al., 1994). To examine the requirement for *NPR1* in *chs2*-mediated signaling, a *chs2 npr1* double mutant was generated and then characterized. The loss of *NPR1* function, while significantly reducing *PR1* expression, did not abrogate the *chs2*-mediated cold-sensitive morphology, cell death, or the accumulation of SA at low temperature (Figs. 7 and 8), indicating that *NPR1* is dispensable for the *chs2*-conferred phenotype.

chs2-Induced Chilling Sensitivity Requires Multiple Signaling Components

To assess whether defense signaling components (including *EDS1*, *PAD4*, *SGT1b*, and *RAR1*) are involved in the *chs2*-mediated temperature signaling pathway, we first examined *RPP4* expression in *eds1-2* (Col; Bartsch et al., 2006), *pad4-1* (Jirage et al., 1999), *rar1-20* (Muskett et al., 2002), and *sgt1b/eta3* (Gray et al., 2003) mutants. *RPP4* expression was slightly down-regulated by *eds1* and *pad4* but not by *rar1* or *sgt1b* (Supplemental Fig. S5). We also generated double mutants of *chs2* with *eds1-1* (Parker et al., 1996), *pad4-1*, *rar1-20*, and *sgt1b/eta3* for further analyses.

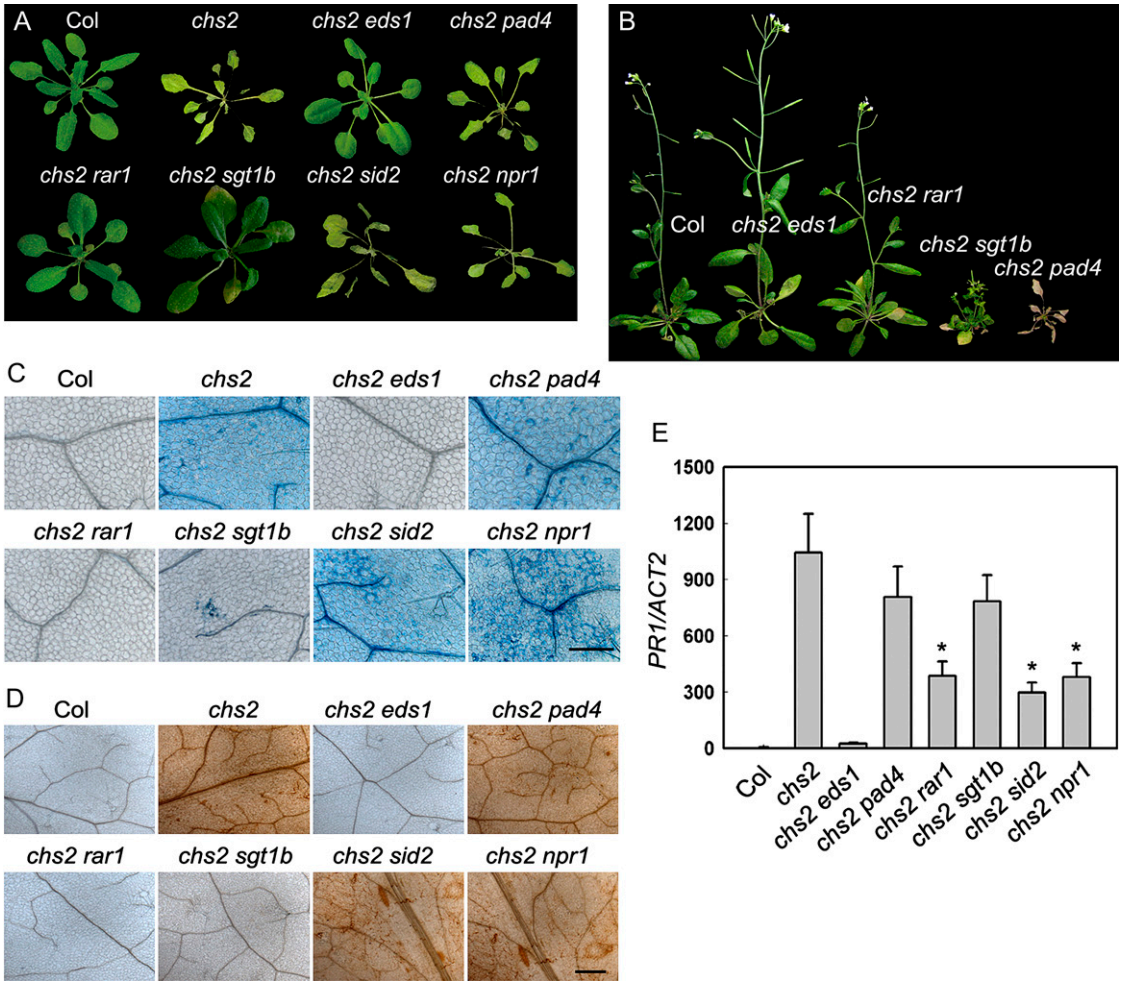

Figure 7. Phenotypes of the *chs2* double mutants under cold conditions. Three-week-old 22°C-grown plants were treated at 4°C for 6 d (C–E), 14 d (A), or 5 weeks (B). A and B, Growth phenotypes of the double mutants under cold conditions. Representative plants are shown. C, Trypan blue staining of the leaves from the double mutants. Bar = 100 μm. Note that the photographs of 4°C-treated Col and *chs2* plants stained with trypan blue are identical to those shown in Figure 2B. D, DAB staining of the leaves from the double mutants. Bar = 100 μm. E, *PR1* gene expression in the double mutants by real-time PCR. The data represent means of three replicates ± SD. * $P < 0.01$ (*t* test), significant difference from *chs2*. All experiments were repeated three times with similar results.

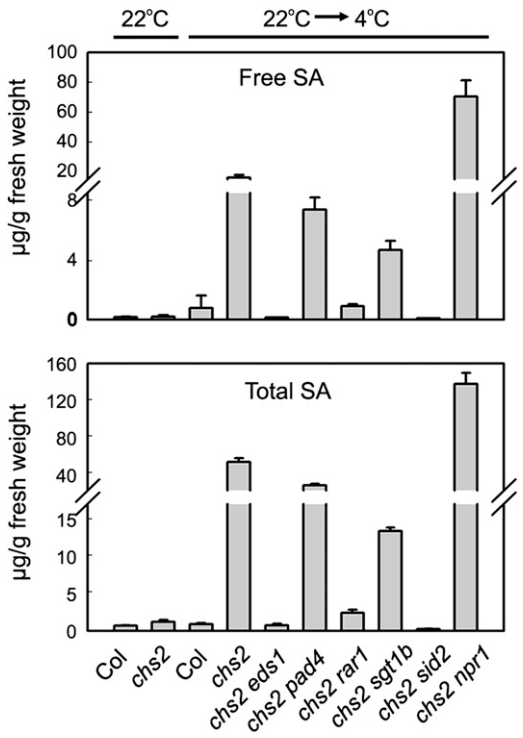

Figure 8. SA accumulation in the double mutants under cold conditions. Three-week-old 22°C-grown plants were treated at 4°C for 6 d. Shown are mean values of free and total SA amount in different genotypes of three replicates ± SD. Similar results were observed in three independent experiments.

Because *eds1-1* is in the Wassilewskija accession, which does not contain the *RPP4* gene, we compared the phenotypes of multiple *chs2/EDS1*and *chs2 eds1* lines from the F2 population of *chs2* crossed with *eds1-1* to eliminate potential effects of mixed background. Among 211 F2 progeny, all 12 lines of *chs2 eds1* and 25 lines of *chs2/+ eds1* showed wild-type-like morphology at 4°C (Fig. 7A). Extensive cell death, elevated *PR1* expression, and accumulation of H_2O_2 and SA under cold conditions were also totally suppressed in these *chs2 eds1* and *chs2/+ eds1* lines (Figs. 7 and 8). Moreover, all 14 *chs2 EDS1* lines and 26 *chs2/+EDS1* lines out of 211 F2 progeny we analyzed uniformly resembled *chs2* phenotypes (data not shown). These results indicate that *chs2* chilling sensitivity is dependent on *EDS1*.

The *chs2 pad4* double mutant resembled the *chs2* mutant in terms of morphology under cold, although the cold-induced lethal phenotype of *chs2 pad4* was delayed slightly compared with the *chs2* mutant (Fig. 7, A and B). Cell death, H_2O_2 accumulation, and *PR1* gene expression in the *chs2 pad4* double mutant were all comparable to those in *chs2* under cold stress (Figs. 7 and 8). Therefore, the *chs2*-conferred phenotypes are largely independent of *PAD4*.

RAR1 and SGT1b were previously identified as regulators of various *R* genes (Austin et al., 2002; Muskett et al., 2002). *rar1-20* completely suppressed *chs2* cold-induced lethality at 4°C (Fig. 7, A and B). In accordance with the morphological phenotype, cell death and H_2O_2 accumulation were abolished in *chs2 rar1-20* (Fig. 7, C and D). Cold-induced *PR1* expression was partially suppressed in the *chs2 rar1-20* double mutant (Fig. 7E). In addition, levels of SA in *chs2 rar1-20* were restored to wild-type levels (Fig. 8). Therefore, the *chs2*-conferred phenotype requires *RAR1*.

chs2 sgt1b double mutant plants largely resembled wild-type plants 3 to 6 d after cold treatment, when *chs2* started to exhibit a chilling defect. However, prolonged cold treatment (1–2 weeks) resulted in slightly yellow leaves in *chs2 sgt1b* (Fig. 7A). Moreover, *chs2 sgt1b* showed dwarfism with curly and chlorotic leaves after cold treatment for 5 weeks (Fig. 7B), which is characteristic of *chs2* grown at 16°C to 18°C (Fig. 1E). The cell death phenotype and H_2O_2 accumulation were partially suppressed by the *sgt1b* mutation (Fig. 7, C and D). *PR* expression was partially compromised in *chs2 sgt1b* plants (Fig. 7E). In addition, SA accumulation in *chs2 sgt1b* was drastically reduced to one-fourth level compared with *chs2* (Fig. 8). Taken together, these data indicate that the *chs2* phenotype is partially dependent on *SGT1b*.

DISCUSSION

The Chilling Sensitivity of *chs2* Is a Result of Activated Defense Responses

In this study, we characterized a previously reported chilling-sensitive mutant, *chs2*. This *chs2* mutant exhibits yellowish leaves, increased ion leakage, damaged chloroplasts, ROS accumulation, extensive cell death, and consequent lethality at chilling temperatures (below 12°C). To our surprise, all the morphological and cell death phenotypes of *chs2* under cold conditions are a result of the up-regulation of defense responses through the activated *R* gene *RPP4*. Chloroplast morphological change and ROS accumulation are observed in mutants showing cell death phenotypes (Tanaka et al., 2003; Dong et al., 2007; Hirashima et al., 2009). The accumulation of excess H_2O_2 in *chs2* is likely due to programmed cell death induced by the activated *RPP4* gene. This finding reveals a great impact of defense responses on cold sensitivity in plant growth and survival.

chs2 mutants contain a gain-of-function mutation (S389F) in the TIR-NB-LRR-type *R* gene *RPP4*. The S389F mutation is located in the NB-ARC1 domain of RPP4. The plant NB-ARC domain has been shown to be responsible for ATP binding and hydrolysis (Tameling et al., 2002; Ueda et al., 2006). The NB-ARC domain serves as a molecular switch for R protein activity, and its action is dependent on its nucleotide-binding state (ATP/ADP). Some R protein mutations affecting the ATP-binding domain will inactivate the protein (Dinesh-Kumar et al., 2000; Tao et al., 2000; Howles et al., 2005; Ueda et al., 2006; van Ooijen et al.,

2008); in contrast, reduced ATP hydrolysis with normal ATP binding can result in constitutive activation of some R proteins (Takken et al., 2006; Ade et al., 2007; van Ooijen et al., 2008). We hypothesize that the *chs2* mutation might interfere with ATP hydrolysis, thus causing a gain-of-function activity. It is possible that low temperature induces a conformational change within *chs2*, resulting in an active signaling state (on state) under cold conditions. In accordance with this study, a number of mutants with deregulated R-like proteins have been shown to have temperature-dependent autoimmune responses (Xiao et al., 2003; Yang and Hua, 2004; Zhou et al., 2008; Alcazar et al., 2009).

Temperature Sensitivity and *R* Genes

Many gain-of-function mutations of *R* genes confer temperature sensitivity. However, their temperature-sensitive ranges can be different. *RPP4* and *SNC1* are highly homologous in their predicted amino acid sequences; in addition, their gene structures are very similar, including their position at the *RPP5* locus and their numbers of exons and introns (van der Biezen et al., 2002). A gain-of-function *snc1-1* mutant shows a growth-defective phenotype and activated defense responses at 22°C but not at 28°C (Yang and Hua, 2004). Nevertheless, *snc1-1* can survive and set seeds even at temperatures of 4°C to 22°C. In contrast, *chs2* shows obvious defense activation at 16°C to 18°C and is lethal at temperatures below 12°C. As these *R* or *R*-like genes share downstream signaling components such as EDS1 (Li et al., 2001), the temperature sensitivity likely comes from *R* genes, as different mutants have different ranges of temperature sensitivity. This was demonstrated recently by altering the temperature sensitivity of defense responses through manipulating *R* genes. Specific missense mutations in *SNC1* and *N* genes could retain defense responses normally inhibited at elevated temperatures, and additional missense mutations in the SNC1 protein reverse the temperature sensitivity of defense responses (Zhu et al., 2010). Thus, differences in temperature sensitivity and sensitivity range are most likely due to varying temperature sensitivity in R protein, and different forms of NB-LRR proteins mediate temperature sensitivity in plant immune responses by conformationally transitioning between off and on states (Zhu et al., 2010).

RPP4 Regulates Cold Response and Defense Responses via Both Common and Distinct Signaling Mediators

Previous studies show that *RPP4* confers resistance to *H. parasitica*, which requires the action of multiple signaling components including *DTH9*, *EDS1*, *PAD4*, *NPR1*, *NDR1*, *PAL*, *PBS2*, *PBS3*, *SGT1b*, *RAR1*, *RPS5*, *SID1*, *SID2*, and SA. In this study, we found that *chs2* is dependent on *EDS1*, *SGT1*, and *RAR1* but is independent of *PAD4*, *NPR1*, and SA. This result indicates that the signaling components required for the temperature sensitivity of *chs2* mutants show similarities and differences with those required for *RPP4* function in pathogen resistance. The different genetic requirement of *chs2* and *RPP4* might be due to the nature of the mutation in the CHS2 protein. The molecular mechanism by which *chs2* regulates temperature-dependent cell death is still unknown, and the subcellular localization of RPP4 or CHS2 remains unclear. Further study on the protein localization, protein activities, and suppressors of *chs2* will shed more light on the function of RPP4 in the regulation of temperature-dependent cell death and the interconnected mechanisms of cold stress and defense signaling.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) plants of the accessions Col and Wassilewskija were used in this study. The *chs2-1* and *chs2-2* (Schneider et al., 1995) mutants were obtained from the Arabidopsis Biological Resource Center (ABRC; stock nos. CS6298 and CS6299). Plants were grown at 22°C or 4°C under a long-day (16 h of light/8 h of dark) photoperiod at 100 μmol m^{-2} s^{-1} with 50% to 70% relative humidity in soil or on MS medium (Sigma) containing 2% Suc and 0.8% agar.

Genetic Mapping and Cloning of the *CHS2* Gene

The *chs2-2* seeds were treated with 0.3% EMS for 8 h. Approximately 20,000 M2 plants (derived from 5,000 M1 seeds) were screened at 4°C for *chs2-s* mutants with a wild-type phenotype.

To map the *chs2-2* mutation, a homozygous *chs2-2* mutant (Col background) was crossed with L*er*. The F1 plants from the cross were self-fertilized, and the resulting F2 seeds were collected. The segregating F2 population seedlings with a wild-type phenotype were used for mapping. A total of 3,000 F2 plants were selected. Genomic DNA from these F2 plants was extracted and used for PCR-based mapping with simple sequence length polymorphism and derived cleaved-amplified polymorphic sequence (dCAPS) markers. Additional mapping markers were developed based on insertions/deletions identified from the Cereon Arabidopsis polymorphism and L*er* sequence collection (www.arabidopsis.org). Genomic DNA corresponding to candidate genes was PCR amplified from the mutant and sequenced to identify the mutation.

To map *chs2-s1* mutations, the F2 populations were derived from genetic crossing between the mutants (in Col) and L*er*. Bulked segregation analysis was performed with simple sequence length polymorphism, cleaved-amplified polymorphic sequence, and dCAPS markers.

Plasmid Construction and Plant Transformation

A 12-kb *Pst*I genomic fragment containing the *RPP4* promoter and coding region was cloned from bacterial artificial chromosome clone F5D3 (ABRC) into the binary vector pCAMBIA1300 (CAMBIA) to generate the *RPP4:RPP4* construct. A 1.0-kb *Eco*RV-*Eco*RI genomic fragment containing the *chs2* mutation was amplified by PCR using CHS2-1F and CHS2-1R from the genomic DNA of *chs2* plants and used to replace the wild-type fragment in *RPP4:RPP4* to generate the *CHS2:chs2* construct.

An 8.3-kb genomic fragment containing the *RPP4* coding region and 3′ untranslated region from *RPP4:RPP4* was cloned into the binary vector pGreen-0229 (Hellens et al., 2000) to generate the *35S:RPP4* construct.

For the *CHS2:GUS* fusion, a 1.46-kb genomic fragment upstream of the *CHS2* ATG start codon was amplified by PCR using the CHS2-p1F and CHS2-p1R primers (Supplemental Table S1) and fused with the GUS reporter gene in the binary vector pZPGUS2 (Diener et al., 2000).

Agrobacterium tumefaciens strain GV3101 carrying different constructs was used to transform wild-type (Col) plants via floral dip transformation (Clough and Bent, 1998).

Genetic Analysis

To generate double mutants, *chs2-2* was crossed to *eds1-1* (Parker et al., 1996), *pad4-1* (Jirage et al., 1999), *rar1-20* (Muskett et al., 2002), *sgt1b/eta3* (Gray et al., 2003), *sid2-2* (Wildermuth et al., 2001), and *npr1* (Durrant and Dong, 2004). The F2 progeny were specifically genotyped. Homozygosity of the *chs2* mutation was identified using dCAPS markers and the CHS2-2F and CHS2-2R primers (Supplemental Table S1).

Ion Leakage and Pro Content Assays

The electrolyte leakage test was performed as described previously (Lee et al., 2002). Three-week-old plants grown in soil under normal conditions were treated at 4°C for different periods of time. The percentage of electrolyte leakage was calculated as the percentage of conductivity before versus after autoclaving. Pro content was measured as described by Bates et al. (1972).

SA Measurement

Free SA and total SA were extracted and measured from 3-week-old plants grown at 22°C or treated at 4°C for 6 d as described with some modifications (Li et al., 1999). The last extracted residue was dissolved in acetonitrile and analyzed by HPLC using 5% acetate (pH 3.2) as the mobile phase.

Analysis of Chlorophyll, and Electron Microscopy

Total chlorophylls were determined as described previously (Huang et al., 2009). Sections of leaf tissue were prepared for electron microscopic analysis as described (Huang et al., 2009).

Histochemical Staining Assay

Trypan blue staining and DAB staining were performed as described previously (Bowling et al., 1997; Thordal-Christensen et al., 1997). Histochemical detection of GUS activity was performed as described previously (Yang et al., 2006).

Quantitative RT-PCR

Total RNA was isolated from 10-d-old seedlings on MS plates or 21-d-old seedlings in soil using TRIzol (Invitrogen) followed by treatment with RNase-free DNase I (Takara). Two micrograms of RNA was subjected to first-strand cDNA synthesis using Moloney murine leukemia virus reverse transcriptase (Promega) and an oligo$(dT)_{18}$ primer. The primers used for real-time PCR are listed in Supplemental Table S1. Real-time PCR was performed using SYBR Green PCR Master Mix (Takara). Analysis was performed using the Applied Biosystems PRISM 7500 real-time PCR system. The primer efficiencies were measured and the relative expression levels were calculated as described previously (Miura et al., 2007).

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *RPP4/CHS2*, At4g16860; *PAD4*, At3g52430; *EDS1*, At3g48090; *NPR1*, At1g64280; *SID2*, At1g74710; *NDR1*, At3g20600; *RAR1*, At5g51700; *SGT1b*, At4g11260; *SNC1*, At4g16890; *PR1*, At2g14610; *PR2*, At3g57260; *CBF1*, At4g25490; *CBF2*, At4g25470; *CBF3*, At4g25480; *RD29A*, At5g52310; *COR47*, At1g20440; *ZAT12*, At5g59820; *APX1*, At1g07890; *CAT1*, At1g20630; *FER1*, At5g01600; *CSD1*, At1g08830; *ACT2*, At3g18780.

Supplemental Data

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

Supplemental Figure S1. The effect of light on the *chs2* phenotype under cold stress.

Supplemental Figure S2. Expression of ROS-associated genes in *chs2* plants under cold stress.

Supplemental Figure S3. Relative mRNA levels of cold-responsive genes in *chs2*.

Supplemental Figure S4. Expression of *RPP4* and *SNC1* in *chs2* under cold stress.

Supplemental Figure S5. Expression of *RPP4* in *eds1*, *pad4*, *rar1*, and *sgt1b* mutants.

Supplemental Table S1. Gene-specific primers used in this study.

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