A mutant CHS3 protein with TIR-NB-LRR-LIM domains modulates growth, cell death and freezing tolerance in a temperature-dependent manner in Arabidopsis

Haibian Yang, Yiting Shi, Jingyan Liu, Lin Guo, Xiaoyan Zhang and Shuhua Yang

1State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, and
2National Plant Gene Research Center, Beijing 100193, China

Received 9 March 2010; revised 1 April 2010; accepted 15 April 2010; published online 26 May 2010.
*For correspondence (fax: +86 10 62734838; e-mail yangshuhua@cau.edu.cn).

SUMMARY

Low temperature is one of environmental factors that restrict plant growth homeostasis and plant–pathogen interactions. Recent studies suggest a link between temperature responses and defense responses; however, the underlying molecular mechanisms remain unclear. In this study, the chilling sensitive 3 (chs3-1) mutant in Arabidopsis was characterized. chs3-1 plants showed arrested growth and chlorosis when grown at 16°C or when shifted from 22 to 4°C. chs3-1 plants also exhibited constitutively activated defense responses at 16°C, which were alleviated at a higher temperature (22°C). Map-based cloning of CHS3 revealed that it encodes an unconventional disease resistance (R) protein belonging to the TIR-NB-LRR class with a zinc-binding LIM domain (Lin-11, Isl-1 and Mec-3 domains) at the carboxyl terminus. The chs3-1 mutation in the conserved LIM-containing domain led to the constitutive activation of the TIR-NB-LRR domain. Consistently, the growth and defense phenotypes of chs3-1 plants were completely suppressed by eds1, sgt1b and rar1, partially by pad4 and nahG, but not by npr1 and ndr1. Intriguingly, chs3-1 plants grown at 16°C showed enhanced tolerance to freezing temperatures. This tolerance was correlated with growth defect and cell death phenotypes caused by activated defense responses. Other mutants with activated defense responses, including cpr1, cpr5 and slh1 also displayed enhanced freezing tolerance. These findings revealed a role of an unconventional mutant R gene in plant growth, defense response and cold stress, suggesting a mutual interaction between cold signaling and defense responses.

Keywords: CHS3, disease resistance (R) protein, low temperature, cell death, Arabidopsis.

INTRODUCTION

As sessile organisms, plants have evolved a variety of tolerance mechanisms by triggering a cascade of regulatory events via changes in gene expression, and subsequent biochemical and physiological modifications, to withstand environmental stresses, including low temperature and pathogen attack.

Advances have been made in understanding plant responses to low temperatures (Thomashow, 1999; Chinnusamy et al., 2007; Hua, 2009). Cold stress induces a transient increase in cytosolic Ca²⁺ levels and activates the expression of the C-repeat binding transcription factors CBF/DREB1 (Thomashow, 1999). CBF/DREB1 in turn triggers the expression of a subset of cold responsive (COR) genes (Gilmour et al., 1992). CBF3 is transcriptionally regulated by the transcription factors ICE1 (inducer of CBF expression 1) and MYB15 (Chinnusamy et al., 2003; Agarwal et al., 2006). Besides the ICE1-CBF-COR cascade, which is one of the primary cold signaling pathways involved in plant responses to cold stress (Chinnusamy et al., 2007), other important components have been identified for cold responses in CBF-independent pathways (Xin and Browse, 1998; Zhu et al., 2005, 2008; Wang et al., 2009).

Plants use multiple mechanisms to fight against pathogen infection. Disease resistance (R) gene-mediated defense is one of the major mechanisms. The majority of R proteins fall into five classes. The largest class includes those proteins containing NB-LRR (nucleotide binding domain leucine-rich repeats) domains with either a coiled-coil (CC) domain or a
TIR (Toll/Interleukin-1 receptor) domain at the amino terminus (Dangl and Jones, 2001). Of the 159 NB-LRR-related genes identified in the Arabidopsis genome, 83 belong to the TIR-NB-LRR subgroup, and 51 belong to the CC-NB-LRR subgroup. The remaining proteins either lack a TIR or CC domain, or contain additional domains (Meyers et al., 2003). ENHANCED DISEASE SUSCEPTIBILITY 1 (EDS1) and PHYTOALEXIN DEFICIENT 4 (PAD4) are two positive regulators of defense responses mediating the function of the TIR-NB-LRR type of R genes, as well as basal disease resistance (Glazebrook et al., 1996; Aarts et al., 1998). NDR1 (non-race-specific disease resistance) mediates CC-NB-LRR R gene-induced responses (Aarts et al., 1998; Glazebrook, 2001). SGT1 (suppressor of G2 allele of skp1) and RAR1 (required for Mla12 resistance) were identified to mediate various R genes in response to their corresponding pathogen effectors (Shirasu et al., 1999; Austin et al., 2002; Liu et al., 2002; Muskett et al., 2002; Tornero et al., 2002). However, the mode of action for SGT1 and RAR1 in plants is not clear. SGT1 and RAR1 are considered to be co-factors functioning in HSP90-mediated stabilization of pre-activated NB-LRR protein complexes (Bieri et al., 2004; Leister et al., 2005; Azevedo et al., 2006). In addition, plant SGT1 interacts with the LRR domains of certain NB-LRR proteins, and may assist in their proper folding (Bieri et al., 2004; Leister et al., 2005).

Several lines of evidence suggest that there is extensive interaction between cold and biotic stress signaling. Temperature is known to affect disease resistance to a variety of pathogens. Plant-pathogen interactions are regulated differently under various temperatures (Garrett et al., 2006). For example, temperature variation is known to modulate defense responses and cell death mediated by a number of R genes, including Mi in tomato (Hwang et al., 2000), N in tobacco (Someya et al., 2004) and SSI4, RPW8, SNC1 and the RPP1-like TIR-NB-LRR cluster in Arabidopsis (Xiao et al., 2003; Yang and Hua, 2004; Bombly et al., 2007; Zhou et al., 2008; Alcazar et al., 2009). The Arabidopsis SUMO E3 ligase SI2 (SAP and Miz1) has been implicated in responses to both temperature stress and plant defense signaling. SI2 is required for salicylic acid (SA) and PAD4-mediated R gene signaling, and confers plant innate immunity (Lee et al., 2007). SI2 also functions in basal thermotolerance independently of SA (Yoo et al., 2006). In addition, SI2 mediates sumoylation of ICE1 to regulate ICE1 activity (Lee et al., 2007). Thereby positively regulating CBF3-dependent cold signaling and freezing tolerance (Miura et al., 2007). Recent study further revealed that SI2 controls cold signaling through the regulation of SA accumulation (Miura and Ohta, 2009). The Arabidopsis transcription factor AtSR1/CAMTA3 (Arabidopsis thaliana signal responsive/calmodulin binding transcription activator 3) is involved in cold acclimation and SA-mediated plant immunity (Doherty et al., 2009; Du et al., 2009). AtSR1/CAMTA3 interacts with the promoter of EDS1 and represses its expression, and thus negatively regulates SA-mediated plant defense responses (Du et al., 2009). AtSR1/CAMTA3 also positively regulates CBF2 expression via binding to a 27-bp promoter fragment, leading to freezing tolerance (Doherty et al., 2009). A transcriptional repressor of the DREB protein, DEAR1 (DREB and EAR protein 1), functions in both plant defense signaling pathway and cold stress responses (Tsutsui et al., 2009). Additionally, a plasma membrane-bound NAC transcription factor NTL6 is shown to integrate cold signals into plant defense responses (Seo et al., 2009).

Here, we present the characterization of a temperature-sensitive 3 (chs3) mutant and the cloning of the CHS3 gene. The chs3-1 mutant exhibited chilling-sensitive phenotypes, including arrested growth and chlorosis, when grown at 16°C or shifted from 22 to 4°C. Intriguingly, the chs3-1 plant pre-grown at the chilling temperature of 16°C showed enhanced tolerance to freezing stress. In addition, chs3-1 exhibited dwarfism and activation of defense responses at 16°C, but not at 22°C. CHS3 encodes a unique protein with a TIR-NB-LRR portion followed by an LIM (Lin-11, Isl-1 and Mec-3 domains)-containing domain. These results indicate a role of the mutant non-conventional R-like CHS3 protein in modulating growth homeostasis and defense response. Our data thus suggest the prevalence of interplay among cold signaling, biotic stress signaling, and growth homeostasis.

RESULTS

Characterization of the chilling sensitive mutant chs3-1

The chs3-1 mutant was initially isolated from a pool of Arabidopsis EMS-mutagenized lines grown at chilling temperatures (Schneider et al., 1995). The mutant was morphologically indistinguishable from the wild type when grown at a permissive temperature (22°C), either in soil or on agar plates (Figure 1a-c). However, when plants grown at 22°C plants were incubated at 4°C for more than 3 days, chs3-1 plant growth was arrested compared with the wild type. The older leaves turned yellow and eventually died, whereas the emerging leaves showed water-soaked and curly phenotypes (Figure 1c).

To examine the extent of chilling injury to chs3-1 plants, we performed ion leakage assays on 2-week-old plants grown at 22°C and cold-treated for various lengths of time. No changes in electrolyte leakage were observed in wild-type leaves during cold treatments. In contrast, electrolyte leakage of chs3-1 plants was significantly increased following cold treatment (Figure 1e). These results suggest that the mutant was more susceptible to cold stress.

When germinated and grown at chilling temperatures (10–16°C; we used 16°C as chilling temperature for the rest of the studies), chs3-1 plants showed developmental defects, including dwarfism, with small and curly leaves and early senescence of the cotyledons (Figure 1a,b). Propidium iodide staining analysis showed that the size and shape of
pavement cells were altered in chs3-1 plants at 16°C (Figure 1d). These cells were much smaller and the lobes of the cells were less extended than those of the wild type. Scanning electron microscopy revealed similar phenotypes (Figure S1a). The growth defect appears to be restricted to leaves and stems, as no obvious defects in root growth were observed in chs3-1 plants at either 22 or 16°C (Figure S1b).

**The chs3-1 mutation enhanced freezing tolerance**

The survival rates of the wild-type and chs3-1 seedlings in response to freezing were further examined. Seedlings grown on agar plates at either 22 or 16°C were subjected to a −6°C treatment for 2 or 4 h without cold acclimation, and were returned to normal growth conditions for 4 days before the survival rate was measured. The chs3-1 and wild-type seedlings grown at 22°C did not show significant differences in freezing tolerance at −6°C (data not shown). In contrast, the chs3-1 seedlings grown at 16°C were unexpectedly more tolerant to freezing than the wild-type seedlings (Figure 2a,b). The survival rates for chs3-1 plants grown at 16°C and frozen at −6°C for 2 h were 75, 74 and 65% in three independent experiments, whereas wild-type plants had survival rates of 40, 42 and 35%, respectively. Furthermore, the survival rates of chs3-1 plants frozen at −6°C for 4 h were 39, 40 and 49%, and wild-type plants had survival rates of 9, 12 and 14%, respectively. We also examined the survival rate of cold-acclimated chs3-1 plants in response to freezing. chs3-1 plants grown at either 22 or 16°C did not show enhanced tolerance to freezing, compared with the wild-type plants after 3 days of cold treatment at 4°C (data not shown). We reason that 4°C treatment already caused severe injuries to chs3-1 plants,
exhibiting yellowish and wilted leaves, and membrane damage (Figure 1c,e).

Accumulation of free proline (Pro) during cold stress is thought to protect plants (Xin and Browse, 1998). To test whether chs3-1 had altered Pro accumulation, we examined Pro content in wild-type and chs3-1 plants grown at 22°C, 16°C, or shifted from 22 to 4°C. At 22°C, the levels of free Pro were comparable between wild-type and chs3-1 plants. An increase in Pro content was observed in wild-type plants grown at 16°C, and in those shifted from 22 to 4°C, as compared with those grown at 22°C. There was less Pro accumulation in chs3-1 plants than in wild-type plants after the shift from 22 to 4°C (Figure 2c). This finding is consistent with the chilling-sensitive phenotype of chs3-1 plants grown at 22°C following 4°C treatment. However, chs3-1 plants grown at 16°C accumulated more Pro than wild-type plants (Figure 2d). This correlates with the freezing-tolerant phenotype of chs3-1 plants grown at 16°C.

As chs3-1 plants had an altered response to cold stress, we asked whether the expression of cold stress-responsive genes in the CBF pathway was altered in chs3-1 plants. Real-time RT-PCR analysis showed that although cold induction of CBF1 was slightly less in chs3-1 plants than in wild-type plants grown at 22°C, the induction of CBF2, CBF3 and their target genes, including RD29A and COR47, was similar in chs3-1 and wild-type plants grown at 22°C (Figure S2a). For plants grown at 16°C, the CBF genes were induced rapidly and peaked at 3 h in both wild-type and chs3-1 plants, and the induction was decreased after 6 h of cold treatment. No significant differences of CBF genes were observed between wild-type and chs3-1 plants at 3 and 6 h, although an increased induction of CBF genes was detected in chs3-1 plants at 12 h compared with wild-type plants. Cold induction of RD29A and COR47 was higher than wild-type plants at 12 and 24 h of cold treatment (Figure S2b). Therefore, the mutant chs3 could be involved in freezing stress signaling, largely independent of the CBF pathway.

Cell death and defense response in chs3-1 plants at chilling temperatures

We found that chs3-1 plants also exhibited chlorotic and spontaneous lesion phenotypes when grown at 16°C (Figure 1). To further confirm this finding, Trypan blue was used to stain dead cells or cells with damaged cell membranes. No obvious staining was observed in wild-type or chs3-1 plants grown at 22°C. In contrast, extensive cell death was detected in chs3-1 plants grown at 16°C (Figure 3a). The accumulation of hydrogen peroxide (H₂O₂) was subsequently analyzed by 3,3′-diaminobenzidine (DAB) staining. Strong staining was observed in chs3-1 plants grown at 16°C, but not in wild-type plants under the same conditions (Figure 3a). This result indicates that chs3-1 plants accumulate higher levels of H₂O₂ than wild-type plants.

We used RNA blot analysis further to determine the expression of several genes known to be involved in defense responses in chs3-1 plants grown at both 16 and 22°C. PR1 and PR2 transcripts were expressed at high levels in chs3-1 plants grown at 16°C, but not at 22°C. Expression levels of PAD4, EDS1 and GST2 were also much higher in chs3-1 plants grown at 16°C rather than those grown at 22°C (Figure 3b). Together, these data suggest that chs3-1 plants...
have constitutively activated defense responses when grown at 16°C, but not at 22°C.

As the induction of PR1 and PR2 gene expression generally requires SA signaling, the endogenous content of SA in chs3-1 plants was examined. The levels of both free SA and SA glucoside (SAG) were comparable in chs3-1 and wild-type plants grown at 22°C (Figure 3c). In contrast, the levels of SA and SAG were 23- and 45-fold higher, respectively, in chs3-1 plants than in wild-type plants grown at 16°C. chs3-1 plants shifted from 22 to 4°C accumulated approximately fivefold higher levels of both forms of SA than similarly treated wild-type plants. These data demonstrate that an SA-dependent defense pathway was activated in chs3-1 plants grown at 16°C, resulting in the hypersensitive response (HR)-like cell death phenotype.

To examine the defense responses of chs3-1 seedlings grown at 22 or 16°C, resistance to a virulent pathogenic strain of Pseudomonas syringae pv. tomato (Pst) DC3000 was analyzed. Comparable bacterial growth was observed 3 days after inoculation in the wild-type and chs3-1 plants grown at 22°C. In contrast, chs3-1 plants grown at 16°C showed enhanced resistance against Pst DC3000, as compared with wild-type plants (Figure 3d). Therefore, general defense responses were probably activated in chs3-1 plants in a temperature-dependent manner.

**Positional cloning of the CHS3 gene**

chs3-1 was previously shown to be recessive in the Col background (Schneider et al., 1995), which was verified by our study. To identify the CHS3 gene, we crossed chs3-1 plants with Landsberg erecta (Ler) plants to generate a segregating population. Interestingly, the F1 plants from the cross showed an intermediate growth defect phenotype. Among the F2 progeny, the chilling-sensitive mutant phenotype segregated in a 1:2:1 ratio (dwarf:intermediate:wild type, 37:77:30; χ² = 1.38; P > 0.5), indicating the semi-dominant trait in Ler or mixed Ler and Col background (Figure S6). F2 offspring showing dwarf morphology at 16°C

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were chosen for mapping. The chs3-1 mutation was mapped to the top of chromosome V between markers nga151a and nga139 (Figure 4a, Table S2). Subsequent mapping refined the CHS3 locus to a 70-kb region between markers MPI7-2 and MCM23 (Figure 4a). DNA sequencing analysis of chs3-1 plants revealed a single G-to-A nucleotide substitution in the ninth intron–exon junction of At5g17890, which probably results in abnormal splicing and a truncated protein (Figures 4b and S3). RT-PCR analysis confirmed that the mis-spliced transcript was expressed in chs3-1 plants (Figure 4c).

To confirm that CHS3 is At5g17890, a wild-type copy of At5g17890 under the control of its own promoter was transformed into chs3-1. Among the 15 independent T1 transgenic plants obtained, all resembled wild-type plants when grown at 16°C (Figure 4d). Furthermore, both the cell death phenotype and elevated expression of PR genes were suppressed in these transgenic plants grown at 16°C (Figure 4e,f). Therefore, CHS3 is indeed At5g17890.

CHS3 is predicted to encode a 1613-amino acid protein that was previously named DA1-related protein 4 (DAR4) (Li et al., 2008). CHS3/DAR4 contains a TIR domain and an NB-LRR domain at the N terminus, and an LIM domain (Lin-11, Isl-1 and Mec-3) (Freyd et al., 1990) followed by an LIM-associated unknown-function domain at the C terminus (Figure S3). For simplicity, the two domains at the C terminus are referred to as the LIM-containing domain hereafter.
BLAST analysis revealed that the N-terminal portion was highly homologous with the TIR-NB-LRR proteins encoded by At4g36140, WRKY16, SLH1 (RRS-1) and At3g51580. The LIM-containing domain was similar to several other predicted proteins, including the Arabidopsis DA1 and DAR proteins (Li et al., 2008) (Figure S4). The chs3-1 mutation was located within the C-terminal LIM-containing region (Figure S3).

**chs3-1 is a gain-of-function mutation**

To identify the nature of the chs3-1 mutation, we isolated three T-DNA insertion lines of At5g17890, specifically, SALK_047971, SALK_143259 and SALK_036320 (TAIR). These lines were termed chs3-2, chs3-3 and chs3-4, and the T-DNA insertions were located at the fourth, fifth and 13th exons of CHS3, respectively (Figures 4b and S5). RT-PCR analysis showed that CHS3 mRNA expression was abolished in all three alleles of chs3 (Figure 4c), presumably causing a loss of CHS3 function. However, none of the mutant plants displayed obvious defects when grown at 16°C, suggesting that chs3-1 might be a gain-of-function mutant.

A genetic screen for suppressors of chs3-1 was performed to identify revertant mutations in CHS3 and additional components in the CHS3 pathway. M2 plants derived from EMS-mutagenized chs3-1 seeds were screened for mutants presenting wild-type morphology (Figure 4d). Two suppressors of chs3-1, designated chs3-r1 and chs3-r2, respectively, were mapped to the original CHS3 locus, and sequencing analysis revealed second-site mutations in CHS3 (Figure S3). Mutations of G201D and D278N in chs3-r1 and chs3-r2 were located in the P-loop/kinase 1 and kinase 2 motif of the CHS3 NB domain, respectively. These two conserved motifs are shown to be critical for the function of several plant NB-LRR proteins, including Rx, RPS2 and RPS5 (Tao et al., 2000; Bendahmane et al., 2002; Ade et al., 2007). Therefore, mutations of chs3-r1 and chs3-r2 possibly abolish CHS3 gene function. This result further supports that chs3-1 is a gain-of-function mutation.

When chs3-1 was crossed with chs3-2, chs3-3 or chs3-4, all F1 progeny exhibited a weaker version of the chs3-1 phenotype (Figure 4g). When the construct overexpressing CHS3 containing the chs3-1 point mutation (35S:chs3) was transformed into chs3-2 plants, five of the 14 T1 transgenic plants showed chs3-1-like phenotypes to varying extents (data not shown). These data indicate that chs3-1 is semi-dominant in the chs3-2 background. Similarly, chs3-1 showed a semi-dominant phenotype when crossed with Ler plants (Figure S6). BLAST analysis revealed no gene identical to CHS3 in the Ler ecotype, indicating that CHS3 is ecotype-specific.

It is noteworthy that transgenic plants overexpressing wild-type CHS3 or CHS3 containing the chs3-1 point mutation in the Col background exhibited a wild-type phenotype (data not shown). This result is in line with the finding that chs3-1 is recessive in Col.

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Figure 5. Overexpression of the LIM domain of CHS3 suppresses the chs3-1 phenotype.

Col, chs3-1 and transgenic plants overexpressing the LIM domain of CHS3 driven by the Super promoter in chs3-1 (chs3/Super:CHS3-LIM) were grown at 16°C for 3 weeks.

(a) RT-PCR analysis of CHS3-LIM in Col, chs3-1 and three independent transgenic lines of chs3-1/Super:CHS3-LIM. The β-tubulin gene TUB8 was used as a control.
(b) Phenotypes of the same genotypes described in (a).
(c) Trypan blue staining of the same genotypes as described in (a).
(d) Relative mRNA levels of PR1 and PR2 genes in the same genotypes as described in (a), assayed by real-time PCR. Data represent means of three replicates ± SD. Similar results were observed in three independent experiments.

Overexpression of the LIM domain of CHS3 suppresses the chs3-1 phenotype

To further dissect the role of CHS3, we transformed the C-terminal LIM fragments into chs3-1 under the control of the Super promoter (Super:CHS3-LIM). Figure 5(a) shows that the CHS3-LIM was indeed overexpressed in the transgenic plants harboring Super:CHS3-LIM. Strikingly, these transgenic plants showed suppression of chs3-1 morphological phenotypes to varying degrees (Figure 5b).

In accordance with the growth phenotype, overexpression of CHS3-LIM fully or partly reversed the cell death phenotype, and elevated PR expression in chs3-1 plants.
(Figure 5c,d). This result suggests that the R-like activity in chs3-1 is activated by the chs3-1 mutation, whereas the introduced wild-type LiM domain interferes with the TIR-NB-LRR domain and suppresses its activity, thus complementing chs3-1 defective phenotypes.

The chs3-1 phenotype is partially dependent on SA

To determine whether increased levels of SA in chs3-1 plants were required for the activation of the downstream defense pathway, chs3-1 plants expressing the nahG gene encoding an enzyme that degrades SA to catechol were generated. At 16°C, chs3-1 nahG plants were largely similar to wild-type plants in morphology, albeit with slightly smaller leaves (Figure 6a). Formation of cell death lesions and accumulation of H$_2$O$_2$ were markedly suppressed (Figure 6b,c), and the expression of PR genes and SA accumulation were restored to wild-type levels in chs3-1 nahG plants (Figures 6d and 7a). To further confirm the SA dependency of the chs3-1 phenotype, we crossed chs3-1 with eds5-1 and sid2-1, two mutants with defects in SA biosynthesis (Nawrath and Metraux, 1999; Wildermuth et al., 2001), and to npr1, a mutant defective in SA signaling (Durrant and Dong, 2004). The stunted phenotype of chs3-1 and the upregulation of PR expression were partially suppressed by eds5-1, but the cell death phenotype was not substantially affected (Figure 6). The sid2-1 and npr1-1 mutations abolished PR1 gene upregulation and resistance to Pst DC3000, but did not revert the growth defect or cell death phenotype of chs3-1 to the wild-type phenotype (Figures 6 and 7). Therefore, growth defects and defense activation in chs3-1 plants are partly dependent on SA.

The chs3-1 phenotype is dependent on EDS1, SGT1b and RAR1

As CHS3 contains the TIR-NB-LRR domains, we crossed chs3-1 with pad4-1, eds1-1 and ndr1-1 mutants to test whether EDS1, PAD4 and NDR1 are required for CHS3-mediated resistance responses. We found that the chs3-1 eds1-1 double mutant was indistinguishable from the wild type at 16°C. The double mutant had no cell-death phenotype or constitutive PR1 gene expression. It also did not over-accumulate SA, and was not more resistant to the Pst DC3000 pathogen than the wild type (Figures 6 and 7). The pad4-1 mutation partially suppressed the chs3-1 phenotype at 16°C. The morphological defects in chs3-1 plants were partially rescued, and the expression of the PR1 gene was also greatly reduced by pad4-1. However, chs3-1 pad4-1 plants still exhibited chlorosis, accumulated more SA and were more resistant to Pst DC3000 than wild-type plants (Figures 6 and 7). The chs3-1 ndr1-1 double mutant was essentially the same as chs3-1 in both morphological and defense-associated phenotypes (Figure 6). Thus, the chs3-1 phenotype is completely dependent on EDS1 and partially dependent on PAD4, but independent of NDR1.

To investigate a potential role for SGT1b and RAR1 in CHS3-mediated signaling, chs3-1 sgt1b (eta3) and chs3-1 rar1-20 double mutants were generated and analyzed. Both chs3-1 sgt1b and chs3-1 rar1-20 double mutants displayed wild-type-like phenotypes at 16°C (Figure 6a). Spontaneous cell death and constitutive expression of the PR1 genes were abolished in both double mutants (Figure 6b–d). The levels of free SA and SAG in chs3-1 sgt1b and chs3-1 rar1-20 were reduced to wild-type levels (Figure 7a). Moreover, chs3-conferring resistance to Pst DC3000 was nearly abolished in the double mutants (Figure 7b). Taken together, these results indicate that the chs3-1 phenotype requires both SGT1b and RAR1.

Freezing tolerance of chs3-1 is associated with its growth defect and cell death phenotype

To investigate the relationship among growth defect, defense response and freezing tolerance of chs3-1, the phenotypes of the double mutants under freezing conditions were analyzed. chs3-1 eds1, chs3-1 sgt1b and chs3-1 rar1 plants resembled wild-type plants in terms of morphology and cell death phenotypes. These double-mutant plants also exhibited a similar freezing sensitivity to wild-type plants. chs3 pad4 and chs3 nahG plants were of intermediate size and showed a moderate cell death phenotype, yet their freezing sensitivity was similar to that of wild-type plants. In contrast, chs3-1 sid2-1 plants exhibited severe growth defects and an extensive cell death phenotype, as did chs3-1 plants. Accordingly, chs3-1 sid2-1 plants displayed similar enhanced freezing tolerance to chs3-1 plants (Figure 8a). These results suggest that the freezing tolerance of chs3-1 plants is mainly correlated with the morphological defect and cell death phenotype.

Next, we further examined whether other mutants grown in conditions where defense responses are activated (Figure S7) had altered responses to freezing temperatures. These mutants include bon-1-1 (Hua et al., 2001; Yang and Hua, 2004), snc1-1 (Li et al., 2001), cpri-1-1 (Bowling et al., 1994), cpri5-2 (Bowling et al., 1997) and slh1 (Noutoshi et al., 2005). As shown in Figure 8b, cpri1-1, cpri5-2 and slh1 plants were more tolerant to freezing than wild-type plants, whereas bon-1-1 and snc1-1 mutants showed no obvious differences in the freezing sensitivity to the wild type. This suggests it may not be a general phenomenon that defense leads to freezing tolerance, but it is also not a specific phenomenon of chs3-1.

DISCUSSION

In this study, we characterized a previously reported chilling-sensitive chs3-1 mutant that displays growth arrest and chlorosis when grown at chilling temperatures of 10–16°C, or when shifted from 22 to 4°C. chs3-1 plants also exhibit temperature-dependent defense-associated phenotypes. Surprisingly, we found that chs3-1 plants grown at a chilling
temperature of 16°C showed enhanced tolerance when exposed to freezing temperatures.

**CHS3 encodes an unusual TIR-NB-LRR-LIM-type R protein**

*CHS3* encodes an unconventional R-like protein with N-terminal TIR-NB-LRR domains and a C-terminal LIM-containing domain. A single amino acid substitution in *chs3-1* causes constitutive activation of defense responses reminiscent of *R*-activated phenotypes at 16°C, including programmed cell death, elevated expression of *PR* genes, accumulation of SA and resistance to the bacterial pathogen *Pst* DC3000. Therefore, *CHS3* is likely to exhibit R-like protein activity.

Figure 6. Phenotypes of *chs3* double mutants grown at 16°C. Wild-type plants, *chs3-1* plants and double mutants were grown at 16°C for 3 weeks.

(a) Growth phenotypes of the double mutants. Representative plants are shown.

(b, c) Trypan blue (b) and 3,3′-diaminobenzidine (DAB) (c) staining of the leaves from the double mutants. Scale bar: 100 μm.

(d) RNA blot analysis of the *PR1* gene in the double mutants. rRNA transcripts were used as controls.
The R-like activity of chs3 has a genetic requirement similar to TIR-NB-LRR-type R genes. chs3 activity is partially dependent on SA, but independent of NPR1. It is also fully dependent on EDS1 and partially dependent on PAD4, but not NDR1, which is consistent with most of the TIR-type R genes. Furthermore, RAR1 and SGT1b are required for chs3 activity, similar to most of the TIR- and CC-type R proteins (Shirasu et al., 1999; Austin et al., 2002; Liu et al., 2002; Tornero et al., 2002). Hence, chs3 induces a resistance signaling pathway similar to the conventional R proteins.

Possible mode of action of CHS3

CHS3 is an unusual R-like protein in that it contains a plant-specific LIM-containing domain at its C terminus. In animals, LIM domains are widely present in proteins with diverse cellular roles as regulators of gene expression, cytoarchitecture, cell adhesion, cell motility and signal transduction (Kadrmas and Beckerle, 2004). In Arabidopsis, 14 genes encode LIM domain-containing proteins, which are divided into two subfamilies. One subfamily is highly homologous with LIM proteins in animals, and is reported to bind and bundle F-actin (Thomas et al., 2007). The other subfamily includes DA1 and DAR proteins (with CHS3 being DAR4) and is specific to plants (Li et al., 2008). The sequence of the LIM-containing domain in the latter subfamily is also highly conserved. The mutated protein encoded by da1 has a negative effect on DA1 and DAR1 protein. Overexpression of the DA1-mutated cDNA recapitulated the da1 mutant phenotype (Li et al., 2008). The biochemical function of these proteins is poorly understood. However, mutations of DA1 and CHS3/DAR4 were both found in the conserved LIM-containing domain, and loss-of-function mutants of these two genes have no obvious phenotypes, implying that they may function through similar biochemical mechanisms. The LIM-containing domain of these proteins is likely to be critical for their functions.

SLH1 is another unconventional R-like protein with TIR-NB-LRR domains at the N terminus and a WRKY domain at the C terminus (Noutoshi et al., 2005). In the slh1 mutant, a 3-bp insertion in the WRKY domain impairs the SLH1 DNA binding activity, and leads to constitutive defense activation. It is postulated that the resistance signaling activity of SLH1 is negatively regulated by its WRKY domain, which may act as a transcriptional repressor or as a guardee, as defined in the ‘guard hypothesis’ (Noutoshi et al., 2005). In the latter model, the SLH1 WRKY domain mimics a virulence target of Avr (avirulence) products, and forms an intramolecular ‘guardee’ of the TIR-NB-LRR portion of the protein (Noutoshi et al., 2005). CHS3 and SLH1 share similarities in the following aspects: the domain arrangements are similar, with both having TIR-NB-LRR at the N terminus and an extra domain at the C terminus; the wild-type copies of CHS3 and SLH1 could complement chs3-1 and slh1 mutant phenotypes, respectively; neither of their T-DNA null mutants show defective phenotypes, whereas F1 plants from the crosses of chs3-1 or slh1 with their corresponding null mutants display growth defects, as does chs3-1 or slh1.

Figure 7. Salicylic acid (SA) accumulation and pathogen resistance of the double mutants. Wild-type Col, chs3-1 and double mutants were grown at 18°C for 3 weeks.
(a) SA accumulation in the double mutants. Data represent means of three replicates ± SD. Similar results were observed in three independent experiments.
(b) Pathogen resistance of the double mutants. Data represent means of four replicates ± SD. *P < 0.01 (Student’s t-test), comparing pathogen resistance for each genotype versus the wild-type Col under the same assay conditions. Similar results were observed in four independent experiments.

The R-like activity of chs3 has a genetic requirement similar to TIR-NB-LRR-type R genes. chs3 activity is partially dependent on SA, but independent of NPR1. It is also fully dependent on EDS1 and partially dependent on PAD4, but not NDR1, which is consistent with most of the TIR-type R genes. Furthermore, RAR1 and SGT1b are required for chs3 activity, similar to most of the TIR- and CC-type R proteins (Shirasu et al., 1999; Austin et al., 2002; Liu et al., 2002; Tornero et al., 2002). Hence, chs3 induces a resistance signaling pathway similar to the conventional R proteins.
defective phenotypes. The recessive nature of the chs3-1 mutant is probably linked to the model that the wild-type CHS3 and mutated chs3 proteins form a heterodimer, thus suppresses chs3 activity in the heterozygous plants. Further study of the interaction of the TIR-NB-LRR region and the LIM-containing domain of the CHS3 and chs3 proteins will help to elucidate the intramolecular interaction of CHS3 and its role in regulating defense responses.

**Interaction between cold stress and defense signaling pathways**

The chs3-1 mutant exhibits temperature-sensitive phenotypes, including growth inhibition and chlorosis at chilling temperature. The chs3-induced phenotypes at chilling temperature are the result of activated defense responses mediated by the mutated unconventional R-like gene chs3. This finding is consistent with previous studies showing that a number of mutants with defense activation, including bon1-1, bap1, snc1-1, mpk4, mkk1/2 and ssi4, exhibit retarded growth dependently on temperature (Yang and Hua, 2004; Ichimura et al., 2006; Yang et al., 2006a; Gao et al., 2008; Qiu et al., 2008; Zhou et al., 2008; Gou et al., 2009). Together, these results support the notions that defense responses have a large impact on plant growth, and that defense responses are temperature modulated. In addition, our data suggest that different R-like genes have different temperature-sensitive ranges. For example, some mutants with activated R-like proteins, such as bon1-1, snc1-1 and ssi4, have growth defects at 22°C, but not 28°C (Yang and Hua, 2004; Zhou et al., 2008), whereas chs3-1 and several incompatible lines with enhanced disease resistance (Alcazar et al., 2009) show growth defects at 16°C, but not at 22°C.

In contrast to the chilling sensitivity of chs3-1 plants shifted from 22 to 4°C, chs3-1 plants grown at 16°C showed enhanced tolerance to freezing temperatures. The greater accumulation of the Pro osmolyte in chs3-1 plants at 16°C contributes, at least in part, to the increased freezing tolerance of chs3-1. However, the enhanced freezing tolerance appears to be largely independent of the CBF pathway, because the induction of CBF genes is not dramatically altered by the chs3 mutation. This finding of an R-like gene implicated in freezing tolerance suggests a link between cold stress and defense signaling pathways or co-regulation of cold response and defense responses by chs3.

The freezing tolerance of chs3-1 appears to be correlated with growth defect and cell death phenotypes caused by activated defense responses. It is not clear what role, if any, SA plays in the freezing tolerance of chs3-1 plants. chs3-1 nahG plants are freezing sensitive, but chs3-1 sid2 plants remain tolerant to freezing. Two SA-accumulating mutants, siz1 and atsr1/camta3, are freezing sensitive, rather than freezing tolerant (Lee et al., 2007; Doherty et al., 2009; Du et al., 2009). In this study, we demonstrate that some mutants with defense activation and greater SA accumulation,
including cpr1-1, cpr5-2 and slh1 mutants, display enhanced tolerance to freezing, whereas others such as bon1-1 and snc1-1 are not freezing tolerant. This suggests that although there may not be a general connection between defense and freezing tolerance, it is not a specific phenomenon of chs3-1. The interaction between freezing tolerance and defense response signaling pathways needs to be investigated further.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Arabidopsis thaliana plants in the Columbia (Col), Landsberg erecta (Ler) and Nossen (No) backgrounds were used in this study. pad4-1, sid2-1, sgt1b, rar1-20, snc1-1, cpr1-1, bon1-1 and cpr5-2 are in the Col background. eds1-1 is in theWs background, and slh1 is in the No background. The chs3-1 (Schneider et al., 1995) (stock number CS 8000), chs3-2 (SALK_047971), chs3-3 (SALK_143259) and chs3-4 (SALK_036320) mutants from the Col background were obtained from ABRC (Arabidopsis Biological Resource Center, http://arabidopsis.org).

Plants were grown at 22 or 16°C under a 16-h light/8-h dark photoperiod at 100 μmol m⁻² sec⁻¹ with 50–70% relative humidity. Arabidopsis seeds were either directly sown on soil or grown on agar.

Aldrich, http://www.sigmaaldrich.com) with 2% sucrose and 0.8% agar.

Electrolyte leakage and proline content assays

Two-week-old plants grown at 22°C and 3-week-old plants grown at 16°C on a 0.8% agar plate were placed in a freezing chamber (RuMED4001) set to −1°C and programmed to cool at −1°C per hour. Petri dishes of plants were removed after exposed to −6°C for 1, 2, 3 and 4 h. After a freezing treatment, plants were incubated at 4°C in the dark for 12 h, and then transferred to light at 22°C. The survival of the seedlings was scored visually after 4 days.

Salicylic acid measurement and pathogen resistance assay

Free SA and SAG were extracted and measured from 2-week-old plants grown at 22°C and 3-week-old plants grown at 16°C according to methods described previously (Li et al., 1999). Bacterial infection of Arabidopsis was performed as described by Bowling et al. (1994).

Histochemistry

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

Quantitative RT-PCR

Total RNA isolated from 3-week-old plants was treated with RNase-free Dnase I (Promega, http://www.promega.com) at 37°C for 1 h, and then used for the first-strand cDNA synthesis (Promega). Real-time PCR was performed using SYBR Green PCR Master Mix kit (Takara, http://www.takara-bio.com). Analysis was performed using the Applied Biosystems PRISM 7500 Real-time PCR system. Relative expression levels were calculated as described by Miura et al. (2007).

ACKNOWLEDGEMENTS

We thank Jian Hua and Hao Yu for their critical reading and editing of the manuscript. We thank Jeffery L. Dangl, Julia Dewdney, Xinnian Dong, Jane E. Parker, Kazuo Shinozaki, Brain J. Staskawicz and the ABRC for mutant seeds. This work was supported by grants from National Natural Science Foundation of China (grant nos 3070181 and 90817007), the National Key Basic Research Program of China (grant no. 2009CB119100), the Ministry of Agriculture of China for transgenic research (grant no. 2008ZX08009-003) and the Ministry of Science and Technology of China (grant no 2007AA021402).

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Phenotype of the chs3-1 mutant grown at 16°C.
Figure S2. Relative mRNA levels of cold-responsive genes in chs3-1.
Figure S3. Derived amino acid sequence of CHS3.
Figure S4. Phylogenetic trees of CHS3 and its homologs in Arabidopsis.
Figure S5. Characterization of T-DNA insertions in the chs3 mutants.
Figure S6. Phenotype of chs3-1 in the Ler background.
Figure S7. RT-PCR analysis of PR1 transcripts in the defense response mutants.

Table S1. Gene-specific primers used in this study.
Table S2. Molecular markers used for the map-based cloning of chs3-1.
REFERENCES


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