Disruption of *Arabidopsis CHY1* Reveals an Important Role of Metabolic Status in Plant Cold Stress Signaling

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ABSTRACT To study cold signaling, we screened for *Arabidopsis* mutants with altered cold-induced transcription of a firefly luciferase reporter gene driven by the CBF3 promoter (*CBF3-LUC*). One mutant, *chy1-10*, displayed reduced cold-induction of *CBF3-LUC* luminescence. RNA gel blot analysis revealed that expression of endogenous *CBFs* also was reduced in the *chy1* mutant. *chy1-10* mutant plants are more sensitive to freezing treatment than wild-type after cold acclimation. Both the wild-type and *chy1* mutant plants are sensitive to darkness-induced starvation at warm temperatures, although *chy1* plants are slightly more sensitive. This dark-sensitivity is suppressed by cold temperature in the wild-type but not in *chy1*. Constitutive *CBF3* expression partially rescues the sensitivity of *chy1-10* plants to dark treatment in the cold. The *chy1* mutant accumulates higher levels of reactive oxygen species, and application of hydrogen peroxide can reduce cold-induction of *CBF3-LUC* in wild-type. Map-based cloning of the gene defective in the mutant revealed a nonsense mutation in *CHY1*, which encodes a peroxisomal β-hydroxyisobutyryl (HIBYL)–CoA hydrolase needed for valine catabolism and fatty acid β-oxidation. Our results suggest a role for peroxisomal metabolism in cold stress signaling, and plant tolerance to cold stress and darkness-induced starvation.

Key words: Cold stress; signal transduction; gene regulation; CHY1; β-hydroxyisobutyryl–CoA hydrolase.

INTRODUCTION

Throughout growth and development, plants are subjected to various environmental challenges, including interactions with microorganisms and changes in temperature, light intensity, and soil water potential. Unlike animals, plants cannot move but are forced to adapt to these biotic and abiotic stresses by metabolic modifications. For instance, plant acclimation to cold conditions involves complicated metabolic changes, including alterations in lipid composition, accumulation of compatible osmolytes such as sugars and proline, and changes in the expression of hundreds of genes (Thomashow, 1999). These changes underlie mechanisms used by plants from temperate regions to increase freezing tolerance following pre-exposure to low, non-freezing temperatures (0–10°C), namely cold acclimation (Levitt, 1980) or acquired freezing tolerance (Kaplan et al., 2004).

Low temperatures induce expression of diverse plant genes, known as *COR* (cold regulated), *KIN* (cold induced), *LTI* (low temperature induced), or *RD* (responsive to dehydration) genes (Medina et al., 1999; Nordin et al., 1993; Welin at al., 1995). Three transcription factors known as CBFs (CRT/DRE binding factor) or DREBs (DRE binding protein) can bind to *DRE*/*CRT* (dehydration-responsive element/C-repeat) *cis*-elements in promoters and activate transcription of the *COR*/*KIN*/*LTI*/*RD* genes (Stockinger et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). The *CBF* genes are transiently induced by low temperature, and this induction precedes that of *COR*/*KIN*/*LTI*/*RD* genes. Ectopic overexpression of *CBF1* or

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CBF3 in *Arabidopsis* results in constitutive expression of downstream cold-inducible genes, even at warm temperatures, elevated levels of proline and soluble sugars (Gilmour et al., 2000), and increased freezing tolerance (Gilmour et al., 2000; Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Liu et al., 1998). These studies demonstrate a critical role for the CBF regulon in cold acclimation, although it is not the only regulon contributing to plant cold responses (Xin and Browse, 1998; Zhu et al., 2005). Studies using a *cbf2* loss-of-function mutant suggest that CBF2 negatively regulates the expression of *CBF1* and *CBF3* in the cold (Novillo et al., 2004).

Cold stress causes dramatic changes to plant metabolism, as a result not only of general reductions in enzyme activities and reaction rates in the cold, but also active reconfiguration of the metabolome under the control of cold signaling (Cook et al., 2004; Kaplan et al., 2004; Levitt, 1980). The CBF regulon has a major role in the active reconfiguration of metabolite profiles (Cook et al., 2004). Whether and how metabolic status affects cold signaling and cold regulation of gene expression are not understood.

Forward genetic screening has been used to identify the upstream regulators of the *CBF* regulon. ICE1 is a positive regulator of *CBFs* and encodes a MYC-like bHLH transcriptional activator (Chinnusamy et al., 2003). The *ice1* mutation blocks *CBF3* expression, decreases expression of many downstream genes, and significantly reduces chilling and freezing tolerance (Chinnusamy et al., 2003). Conversely, the *Arabidopsis* HOS1 protein is a negative regulator of *CBF* genes. *CBFs* and their downstream *COR* genes show enhanced cold induction in *hos1* mutant plants (Ishitani et al., 1998). *HOS1* encodes a RING-finger ubiquitin E3 ligase that targets ICE1, a positive regulator of *CBF*s for ubiquitination and proteosomal degradation (Lee et al., 2001; Dong et al., 2006).

In this report, we present evidence supporting the importance of cellular metabolism in cold regulation and plant cold tolerance. In a screen for *Arabidopsis* mutants with deregulated expression of a luciferase reporter gene driven by the *CBF3* promoter (*CBF3-LUC*), we isolated a mutant, *chy1-10*, with reduced *CBF3-LUC* and endogenous *CBF3* expression in response to cold. *chy1* mutant plants are less tolerant to freezing stress compared to wild-type after cold acclimation. In addition, *chy1* is more sensitive than the wild-type to cold and dark treatment. Constitutive *CBF3* expression partially rescues the cold- and dark-sensitive phenotype of *chy1-10*. The defective gene was isolated by map-based cloning and encodes a peroxisomal β-hydroxyisobutyryl (HIBYL)–CoA hydrolase needed for fatty acid β-oxidation and valine catabolism. Our findings reveal a requirement for peroxisome function in the cold-induction of *CBF3*, for plant freezing tolerance, and survival following dark treatment, particularly in the cold. We suggest that metabolism is not passively regulated by cold stress, but that cellular metabolic status plays an important role in gene regulation in the cold. Peroxisomes are an important source of reactive oxygen species (ROS) (Corpas et al., 2001) and ROS can modulate the concentration of cytosolic free calcium (Price et al., 1994), which is known to be a second messenger that can activate cold-responsive gene expression (Catala et al., 2003; Knight et al., 1996; Monroy and Dhindsa, 1995). We provide evidence that metabolic status might be connected to cold regulation through ROS.

RESULTS

Isolation of *chy1-10*, a Mutant Defective in Cold Signaling

Transgenic *Arabidopsis* plants (ecotype Columbia) containing *CBF3-LUC* emit bioluminescence in response to low temperatures (Chinnusamy et al., 2003). *CBF3-LUC* plants (herein referred to as wild-type) were mutagenized with EMS and mutants with altered luminescence patterns in response to cold treatment were selected by luciferase imaging. We cloned the defective gene in one mutant, which revealed that the mutant is allelic (see below) to *chy1* (Zolman et al., 2001a). We therefore designated it *chy1-10*. Figure 1A shows luminescence images of *chy1-10* and wild-type seedlings with or without cold treatment (4°C) in the light. After 6 h of cold treatment, *chy1-10* plants showed only ~50% of the luminescence found in wild-type plants (Figure 1A and 1B).

To determine if the *chy1-10* mutation affects expression of endogenous *CBF*3, we extracted total RNA from mutant and wild-type seedlings treated with low temperature in the light. Consistent with the *CBF3-LUC* imaging result, cold induction of *CBF3* was reduced in *chy1-10* plants (Figure 1C). Wild-type plants showed *CBF3* induction after 1 h of cold treatment; expression peaked at 3 h. In contrast, *CBF3* induction in *chy1-10* was observed only after 3 h, and the expression level was substantially decreased. Reduced *CBF3* induction also was observed following cold and dark treatment of *chy1-10* (Figure 1D). In addition, levels of *CBF1* and *CBF2* mRNAs were somewhat lower in the *chy1-10* mutant than in wild-type (Figure 1D). We also examined induction of several *CBF* target genes following cold and dark treatment. Surprisingly, transcript levels of *COR15A*, *COR47A*, and *RD29A* in the *chy1-10* mutant were not lower than in wild-type (Figure 1D), supporting the hypothesis that the CBFs are not the sole transcriptional regulators of these target genes (Zhu et al., 2005).

The *chy1-10* mutant plants were backcrossed to *CBF3-LUC* wild-type, and the resulting F1 seedlings showed wild-type *CBF3-LUC* luminescence after 6 h of cold treatment. The F2 population from the self-fertilized F1 plant segregated in ~3:1 ratio of wild-type to mutant (data not shown), indicating that the mutant defect is caused by a recessive mutation in a single nuclear gene. All subsequent characterization was performed using *chy1-10* mutant plants backcrossed to wild-type four times to remove potential unlinked mutations.

chy1-10 Is Defective in Acquired Freezing Tolerance

Because the *chy1-10* mutant shows reduced cold induction of the *CBF3* gene, which is important for freezing tolerance, we examined the effect of cold acclimation on whole plant

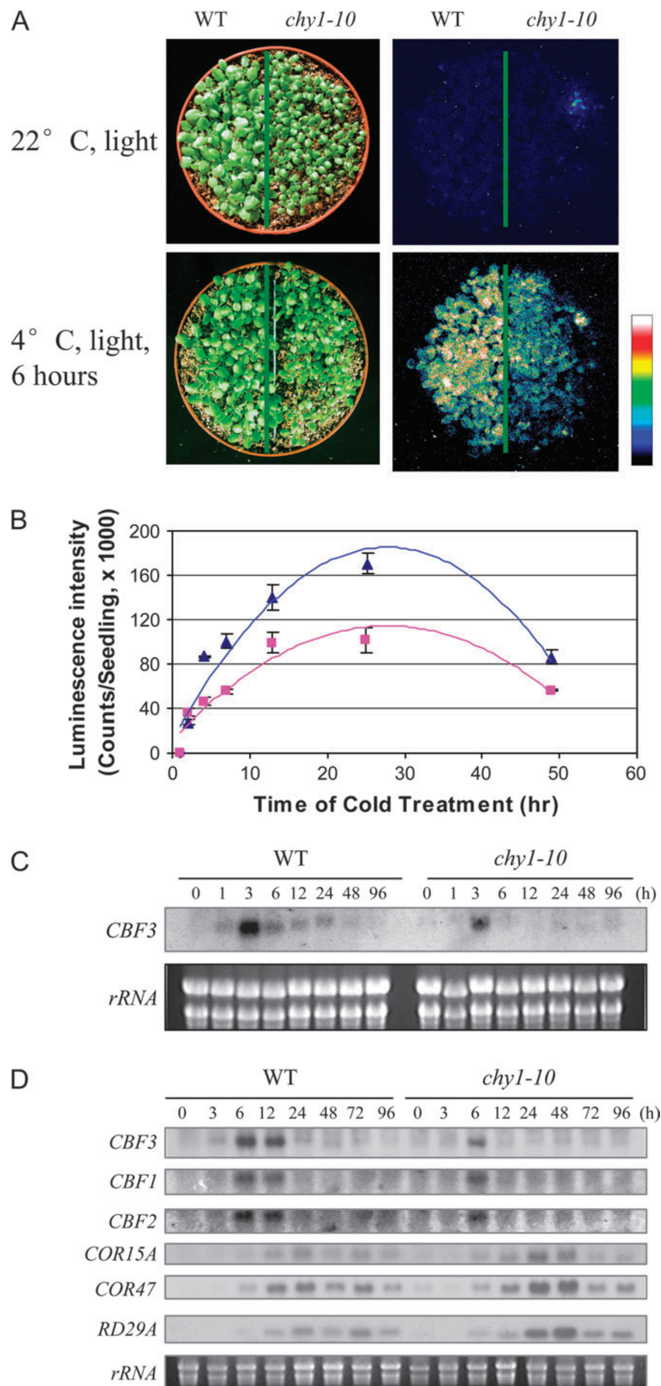


Figure 1. The *chy1-10* Mutation Alters Cold Regulated Gene Expression.

(A) Luminescence images of wild-type (left) and *chy1-10* (right) plants taken with or without cold treatment at 4°C in the light for 6 h. The color scale at the right shows the luminescence intensity from dark blue (lowest) to white (highest).

(B) Time course of luminescence intensity in wild-type (triangles) and *chy1-10* (squares) plants during cold treatment in the light. Shown are the mean values ± standard deviation ($n = 20$).

(C) Northern blot analysis showing reduced induction of *CBF3* in 2-week-old WT and *chy1-10* mutant plants following cold treatment in the light for the indicated time (h). A photo of the ethidium bromide-stained rRNA is included as a loading control.

(D) RNA was prepared from WT and *chy1-10* seedlings treated at 4°C in the dark for the indicated times. Gene probes used for RNA gel blot hybridization are indicated at left.

freezing tolerance and leaf electrolyte leakage. Ten-day-old *chy1-10* and wild-type seedlings grown on agar plates were cold acclimated at 4°C in the light for 4 d, subjected to freezing treatment (see Methods), then returned to normal growth conditions for 2 d and examined. The *chy1-10* mutant was less freezing-tolerant than wild-type at temperatures below –5°C (Figure 2A and 2B). For example, only ~46% of *chy1-10* plants survived freezing at –8°C, whereas ~69% of wild-type plants survived (Figure 2B). To assay the effect of cold acclimation on leaf electrolyte leakage, wild-type and *chy1-10* plants were treated at 4°C in the light for 4 d, then the leaves were excised and subjected to freezing conditions followed by electrolyte leakage assays. *chy1-10* leaves showed higher levels of electrolyte leakage than wild-type at most freezing temperatures (Figure 2C). In contrast, without cold acclimation, wild-type and *chy1-10* leaves showed similar increases in electrolyte leakage (Figure 2D). These results show that the mutant plants are defective in acquired freezing tolerance.

The *chy1-10* Mutation Disrupts a Peroxisomal HIBYL–CoA Hydrolase

We used a map-based cloning strategy to identify the mutated gene in *chy1-10* (see Methods). Initial mapping localized *chy1-10* to the bottom of chromosome 5, between the markers MBK5 and K9I9 (Figure 3A). Subsequent mapping using new markers delimited *chy1-10* to an approximately 50-kb region on two BAC clones, K14B20 and K2A18, between markers K14B20-56k and K2A18-24k. Candidate genes in this region were sequenced from *chy1-10* mutant plants. This analysis revealed a single nucleotide substitution in At5g65940 (K14B20.11) in the mutant: a G-to-A change at position 34121 of the BAC clone, corresponding to position 1771 of the gene, creating a premature stop codon that would truncate the encoded enzyme (Figure 3B). No mutations were found in any other sequenced genes.

CHY1 encodes a peroxisomal β-hydroxyisobutyryl (HIBYL)–CoA hydrolase that hydrolyzes HIBYL–CoA to β-hydroxyisobutyrate and CoA during Val catabolism (Zolman et al., 2001a). The *Arabidopsis* CHY1 protein is 43% identical to a mammalian HIBYL-CoA hydrolase that functionally complements the *Arabidopsis chy1* mutant when targeted to the peroxisome (Zolman et al, 2001a). Five *chy1* mutant alleles had been previously described. *chy1-1* has a G-to-A mutation at position 518 that alters the 3′-splice site of the fourth intron; *chy1-2* has a 34-bp deletion from positions 1084–1118, which alters the 5′-splice site of the eighth intron; and *chy1-3* has a G-to-A mutation at position 407, changing a conserved Gly to Ser (Zolman et al., 2001a) (Figure 3B). Two additional *chy1* mutant

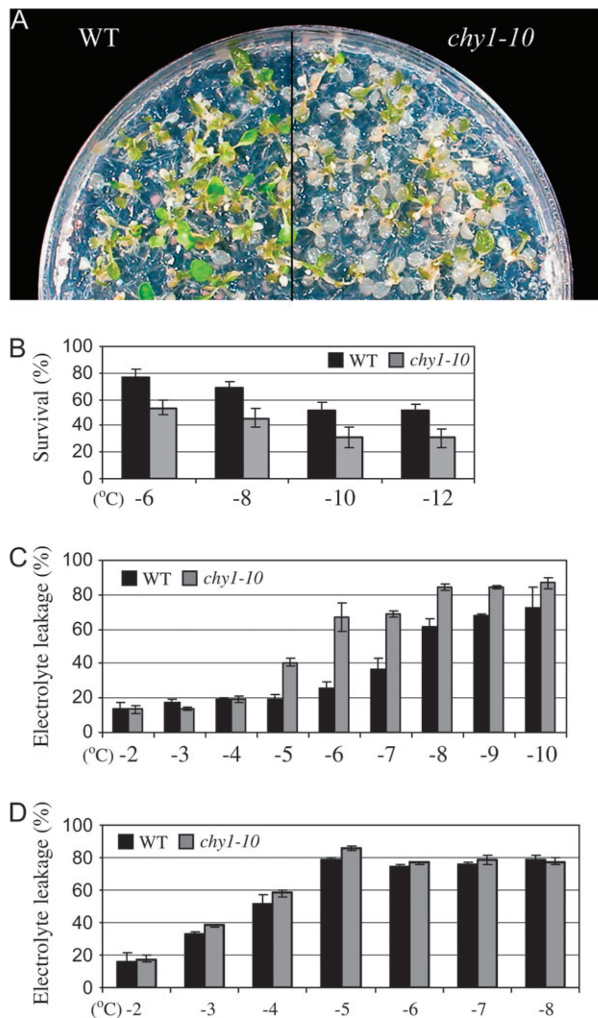


Figure 2. Freezing Sensitivity of *chy1-10* Mutant Plants.

(A) Decreased survival of *chy1-10* mutant plants 2 d after freezing treatment at –8°C following a 4-d cold acclimation.

(B) Quantification of wild-type and *chy1-10* survival 2 d after freezing treatment following a 4-d cold acclimation. Data shown are mean values with standard deviation (*n* = 8).

(C) Comparison of leaf electrolyte leakage after freezing treatments at the indicted temperatures (see Methods) following a 4-d cold acclimation at 4°C in the light. Data shown are mean values with standard errors (*n* = 10).

(D) Comparison of leaf electrolyte leakage after freezing treatments at the indicated temperatures without cold acclimation. Data shown are mean values with standard deviation (*n* = 10).

alleles (*dbr5-1* and *dbr5-2*) have G-to-A substitutions at the last base of introns 6 and 11, respectively, disrupting the corresponding 3′ splice sites (Lange et al., 2004).

The original *chy1* alleles were isolated as resistant to inhibition of root elongation and promotion of lateral root formation by the auxin precursor indole-3-butyric acid (IBA) (Zolman et al., 2001a) or the synthetic compound 2,4-dichlorophenoxybutyric acid (2,4-DB) (Lange et al., 2004). *chy1* mutants also exhibit developmental phenotypes indicative of defects in peroxisomal fatty acid β-oxidation, including reduced hypocotyl elongation in the dark in the absence of exogenous sucrose (Lange et al., 2004; Zolman et al., 2001a) and slowed seed storage lipid catabolism during germination (Lange et al., 2004; Zolman et al., 2000). We found that *chy1-10* displays the same IBA resistance and sucrose dependence as the original *chy1* alleles (Figure 4A and 4B). We also compared *CBF3* expression in the cold and light and observed reduced expression of *CBF3* in the *chy1-1* and *chy1-3* mutant alleles (Figure 4C).

To confirm that the HIBYL–CoA hydrolase defect causes the reduced cold-induction of *CBF3-LUC* in *chy1-10*, a 35S-*CHY1* overexpression construct (Zolman et al., 2001a) was introduced into *chy1-10* plants via *Agrobacterium*-mediated transformation. T2 progeny of 50 Basta-resistant transformants were subjected to luminescence imaging and found to have wild-type luminescence patterns in the cold (Supplemental Figure 1). This result showed that the HIBYL–CoA hydrolase gene rescued the luminescence phenotype of *chy1-10* mutant plants. In addition, we crossed *chy1-1* and *chy1-3* with *chy1-10* and analyzed the resulting F1 progeny by luminescence imaging for expression of the *CBF3-LUC* transgene. As expected, *CBF3-LUC* expression in both *chy1-10/chy1-1* and *chy1-10/chy1-3* F1 plants was significantly reduced in response to cold treatment compared to wild-type (data not shown).

Together, these results indicate that *chy1-10* is allelic to *chy1-1* and *chy1-3*, and that all of the examined alleles disrupt HIBYL–CoA hydrolase activity similarly.

Sensitivity of *chy1* to Darkness-Induced Starvation

As mutations in *CHY1* disrupt peroxisomal fatty acid β-oxidation required for plant growth during early seedling development in the absence of exogenous sugar (Lange et al., 2004; Zolman et al., 2001a), we examined the sensitivity of *chy1-10* plants to darkness-induced starvation. Leaf senescence and seedling injury occurred rapidly in the dark at room temperature in both wild-type and *chy1-10* plants. Interestingly, the mutant appeared more sensitive than wild-type to the dark treatment (Figure 5A). Measurement of leaf injury using electrolyte leakage confirmed the rapid damage suffered by both wild-type and the *chy1* mutant during dark treatment at room temperature and the increased sensitivity of *chy1-10* to these conditions (Figure 5B).

Due to the impaired cold-regulated *CBF3* expression in the mutant (Figure 1C and 4C), we analyzed the performance of the *chy1-10* mutant in the cold and dark. The damage caused by dark (Figure 5) was prevented by cold temperature in the wild-type but not in the mutant (Figure 6A). After treatment at 4°C in the dark for 10 d, *chy1-10* mutant plants were severely damaged and died, whereas the same treatment did not significantly damage wild-type plants (Figure 6A). We examined the *chy1-1* and *chy1-3* mutant alleles under the cold and dark treatment, and observed the same phenotype as in *chy1-10* (Figure 7).

To examine the extent of *chy1-10* leaf injury, leaves excised from soil-grown plants treated at 4°C in the dark for different

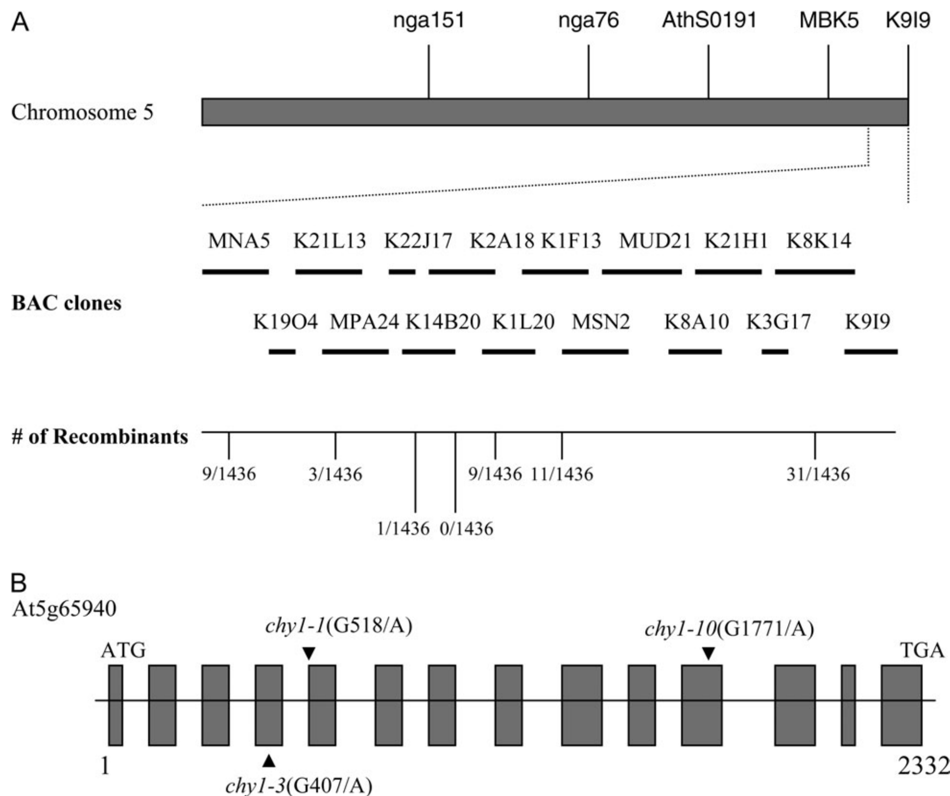


Figure 3. Positional Cloning of *chy1-10*.

(A) Genetic mapping with PCR-based markers positioned *chy1-10* on the BAC clone K14B20. The number of recombination events out of the total number of chromosomes examined is indicated.

(B) Sequence analysis of *chy1-10* identified a single nucleotide change (G1771 to A1771) that creates a premature stop codon in At5g65940 (K14B20.11). Mutations previously found in *chy1-1* and *chy1-3* (Zolman et al., 2001a) also are indicated.

time periods were assayed for electrolyte leakage (Ishitani et al., 1998). Wild-type leaves showed little increase in electrolyte leakage during a 12-d cold treatment in darkness (Figure 6B). In contrast, *chy1-10* leaves showed a dramatic increase in electrolyte leakage beginning after 8 d of cold treatment in the dark. After a 12-d dark treatment, approximately 80% of cellular electrolytes in *chy1-10* leaves were lost (Figure 6B).

We also exposed 50 transgenic lines of *chy1-10* plants overexpressing *CHY1* (see above) to 4°C in the dark for 10 d. All survived similarly to the wild-type (data not shown) and had only minor electrolyte leakage (Figure 6C), indicating that the HIBYL–CoA hydrolase gene rescued the *chy1-10* mutant phenotypes. These results show that cold treatment can prevent darkness-induced injury in wild-type but not in *chy1* mutant plants, consistent with an important role of *CHY1* in not only dark, but also cold responses.

CBF3 Overexpression in *chy1-10* Partially Rescues the Mutant Defects in Cold and Dark Responses

The *chy1-10* mutation substantially decreases *CBF3* expression (Figure 1). We hypothesized that reduced *CBF3* expression may be partly responsible for some of the mutant phenotypes, and that enhancing *CBF3* expression in *chy1-10* mutant plants may suppress the defects. To test this hypothesis, *CBF3* was expressed behind a strong constitutive promoter (Gong et al., 2002) in *chy1-10* mutant plants. Thirty-five independent transgenic lines were obtained from *Agrobacterium*-mediated transformation and 10 were selected for detailed characterization.

RNA blot analysis demonstrated *CBF3* overexpression in all of the transgenic lines under normal growth conditions, when endogenous *CBF3* mRNA is not detected (Figure 8A). Interestingly, constitutive expression of *CBF3* significantly increased the survival of *chy1-10* plants in the cold and dark (Figure 8B). A representative homozygous T3 population after cold treatment in the dark for 10 d is shown in Figure 8C. These results are consistent with the possibility that the mutant survival phenotypes can be attributed at least in part to altered *CBF3* expression, and the CBF regulon has an important function in chilling tolerance in the dark.

Accumulation of Reactive Oxygen Species in *chy1-10*

The peroxisome is an important source of reactive oxygen species (ROS) (Corpas et al., 2001). ROS can modulate calcium signaling in plants (Price et al., 1994), and calcium is a second messenger that can mediate cold regulation of gene expression (Catala et al., 2003; Knight et al., 1996; Monroy and

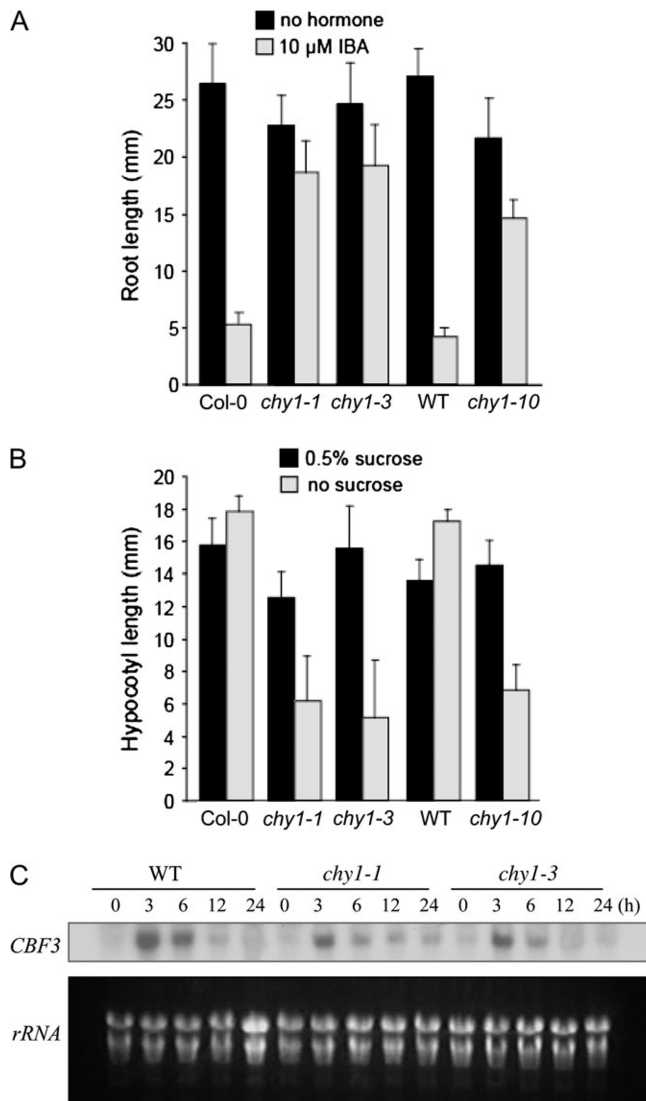


Figure 4. Altered Phenotypic Responses and Impaired Induction of *CBF3* in *chy1* Mutant Plants.

(A) Root elongation on IBA. Roots of 8-day-old seedlings grown without hormone (black bars) or on 10 μM IBA (gray bars) were measured as described (Zolman et al., 2000). *chy1-1* and *chy1-3* are in the Col-0 background; *chy1-10* is in the WT background. Error bars indicate the standard deviation of the means ($n > 12$).

(B) Hypocotyl elongation in the dark in the absence of sucrose. Hypocotyl lengths of 5-day-old seedlings grown in the dark on medium supplemented with 0.5% sucrose (black bars) compared to sucrose-free medium (gray bars). Error bars indicate the standard deviation of the means ($n > 12$).

(C) Northern blot analysis shows impaired induction of *CBF3* in 2-week-old *chy1-1* and *chy1-3* mutant plants following cold treatment in the light for the indicated times. A photo of the ethidium bromide-stained rRNA is included as a loading control.

Dhindsa, 1995). We assayed wild-type and *chy1-10* plants for ROS levels using nitroblue tetrazolium (NBT) staining for superoxide and 3,3′-diaminobenzidine (DAB) staining for hydrogen peroxide (Lee et al., 2002a). Without cold treatment, wild-type leaves showed minimal NBT or DAB staining, indicating low superoxide and hydrogen peroxide levels (Figure 9A). After a 2-d cold treatment in the dark, substantial superoxide staining was detected in both wild-type and *chy1-10*, but the level in the mutant appeared higher than in the wild-type (Figure 9A). Interestingly, DAB staining was elevated in *chy1-10* leaves even without cold and dark treatment, suggesting a high basal level of hydrogen peroxide in the mutant (Figure 9A). After the cold and dark treatment, high levels of hydrogen peroxide were detected in both the wild-type and mutant (Figure 9A).

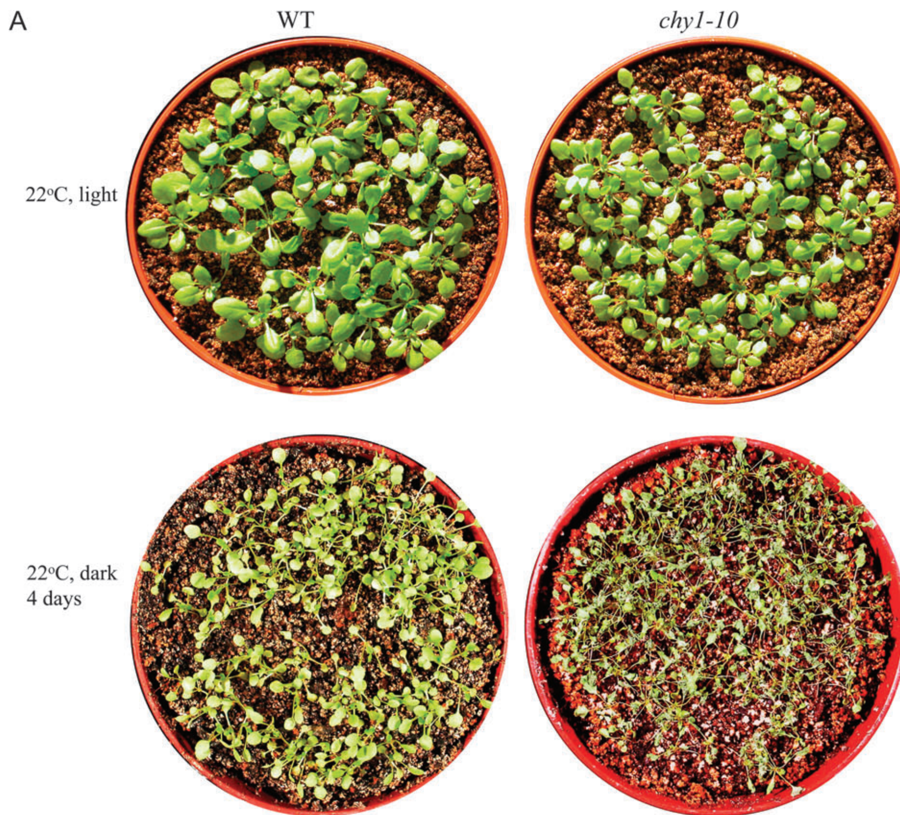
We also examined the level of reactive oxygen species in root tissues of *chy1-10* mutants using CM-H2DCF diacetate acetyl ester fluorescence imaging (Shin and Schachtman, 2004). Consistent with the DAB staining, this assay showed higher basal levels of ROS in *chy1-10* than in the wild-type (Figure 9B). After the cold and dark treatment, the levels of reactive oxygen species in *chy1-10* again were substantially higher than those in the wild-type. These results suggest that disruption of the HIBYL–CoA hydrolase causes accumulation of reactive oxygen species.

To begin to investigate whether ROS may influence cold responsive gene expression, we tested the effect of exogenous application of H_2O_2 on *CBF3-LUC* expression. Soil-grown *CBF3-LUC* seedlings were sprayed with 5 mM H_2O_2 or an H_2O control, held at 22°C for 5 h, transferred to 0°C for 12 h, and then imaged for luciferase expression. We found that exogenous application of hydrogen peroxide significantly reduced cold-regulated expression of *CBF3-LUC* both in the wild-type and in the *chy1-10* mutant (Figure 10A and 10B).

DISCUSSION

In this study, a forward genetics screen led to the unexpected finding that disruption of a peroxisomal metabolic enzyme impairs cold regulated expression of *CBF3*, which encodes a key transcription factor that controls plant freezing tolerance (Stockinger et al., 1997; Yamaguchi-Shinozaki and Shinozaki, 1994). The *chy1* mutation impairs the ability of the mutant plants to tolerate freezing stress following cold acclimation and to survive dark treatment, especially in the cold. Although mutations in *CHY1* previously had been shown to confer developmental defects on seedlings grown in the absence of exogenous sucrose and decreased catabolism of seed storage lipids during germination (Lange et al., 2004; Zolman et al., 2000, 2001a), the importance of CHY1 for plant responses to cold stress and starvation in the dark was not known. Our results suggest a role for peroxisomal metabolism in cold-responsive gene expression and plant responses to cold and dark stresses.

Several lines of evidence support a role for CHY1 in cold signaling and cold tolerance. First, *chy1* mutant plants show reduced expression of *CBF3-LUC* and endogenous *CBFs* in response to cold treatment (Figure 1). Second, the mutant plants are defective in cold acclimation, as revealed by freezing



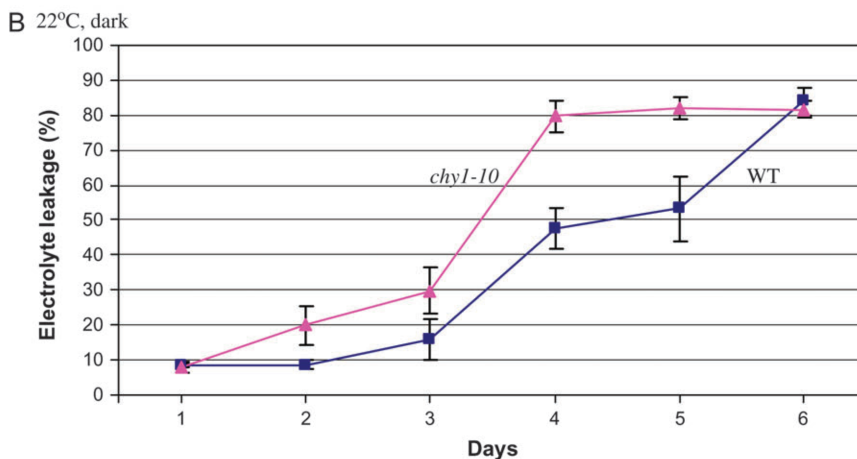


Figure 5. Sensitivity of Wild-Type and *chy1-10* Mutant Plants to Darkness-Induced Starvation at Room Temperature.

(A) Fourteen-day-old wild-type and *chy1-10* mutant plants grown at 22°C were shifted to the dark at room temperature for 4 d. Plants were examined 2 d after return to normal growth conditions.

(B) Ion leakage was assayed using 14-day-old soil-grown plants treated in the dark at room temperature for the indicated number of days. Data shown are mean values with standard deviation (n = 10).

tolerance assays (Figure 2). Third, cold treatment can prevent darkness-induced injury in the wild-type but not in *chy1* mutant plants (Figure 6). Fourth, ectopic expression of *CBF3* can partially rescue the *chy1* mutant sensitivity to the cold and dark (Figure 8), although the partial rescue of mutant defects might be a general effect of *CBF3* overexpression. CHY1 also has a role in plant survival during darkness-induced starvation, as the *chy1* mutant has reduced survival and greater electrolyte leakage in darkness even at normal growth temperatures, although both wild-type and mutant plants are more sensitive to dark treatment at room temperature than in the cold (Figure 5).

Interestingly, although *chy1-10* plants are impaired in cold induction of *CBF3-LUC* in the light (Figure 1) as well as in the dark (data not shown), *chy1-10* plants are chilling sensitive in the dark but not in the light (data not shown). Light also alleviates the cold sensitivity of *los4-1* mutant plants, which were similarly identified as having decreased *CBF* expression (Gong et al., 2002). *los4-1* mutant plants, which are defective in an RNA helicase, are severely damaged after cold treatment for 2 weeks in the dark but not in the light. Although photosynthesis is inefficient at low temperatures (Stitt and Hurry, 2002), light may activate certain cold-tolerance mechanisms, including changes in gene expression. For example, light-induced

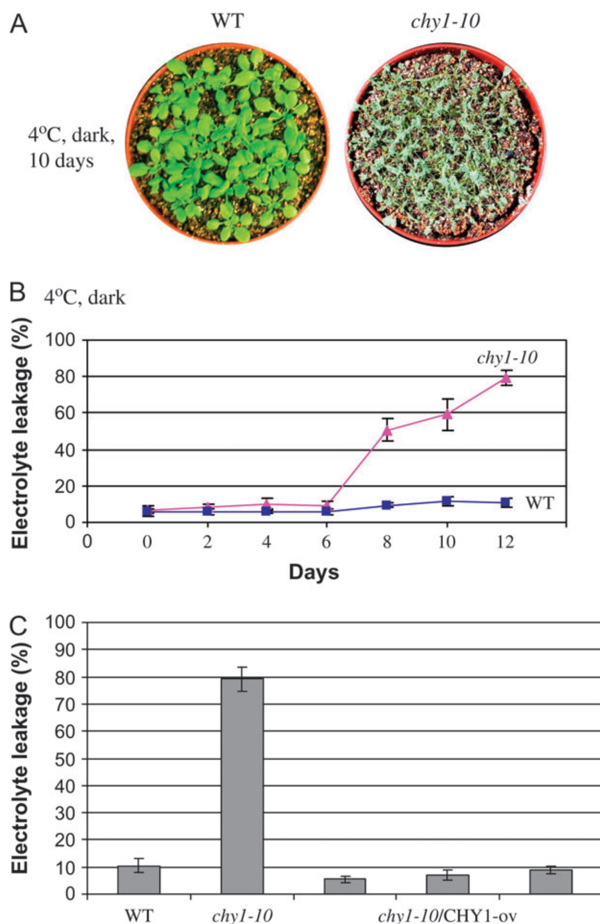


Figure 6. Chilling Sensitivity of *chy1-10* Mutant Plants in the Dark at 4°C.

(A) Fourteen-day-old wild-type and *chy1-10* mutant plants grown at 22°C were shifted to 4°C and grown in the dark for 10 d.

(B) Ion leakage was assayed using wild-type and *chy1-10* leaves from 14-day-old plants grown in soil at 22°C and then shifted to 4°C in the dark for the indicated number of days. Data shown are mean values with standard deviation ($n = 10$).

(C) Leaf ion leakage in 14-day-old WT, *chy1-10*, and three transgenic lines of *chy1-10* transformed with *35S-CHY1* (*chy1-10*/CHY1-ov) after cold treatment at 4°C for 10 d in the dark. Data shown are mean values with standard deviation ($n > 10$).

proline and sucrose accumulation may help protect cells from cold injury (Gong et al., 2002). Indeed, applying a 3% sucrose solution to leaves of soil-grown *chy1-10* plants or incubating *chy1* seedlings on medium supplemented with 1% sucrose rescued the mutant plants from darkness damage at either 4 or 22°C (data not shown). The protective effects of applied sucrose are consistent with either osmotic protection from chilling damage or alleviation of carbon starvation in the dark.

Peroxisomes are respiratory microbodies bound by a single membrane. Peroxisomal matrix proteins are encoded in the nucleus, synthesized on free ribosomes, and post-translationally imported into the organelle. In the early 1960s, when peroxisomes were first characterized from mammalian tissues, their main function was thought to be removal of toxic hydrogen peroxide. Peroxisomes are now known to be involved in a range of important cellular functions in eukaryotic cells. In plants, peroxisomes are important for fatty acid β-oxidation, the glyoxylate cycle, and photorespiration (reviewed by Olsen, 1998). As a result, peroxisome-defective mutants have seedling establishment defects attributed to decreased seed storage oil utilization during germination (Hayashi et al., 1998; Zolman et al., 2000). Analysis of peroxisome-defective mutants also has revealed that the naturally occurring auxin IBA is converted to the active auxin IAA in the peroxisome, and that this process is important for lateral root formation in developing seedlings (Zolman et al., 2000, 2001b, 2005; Zolman and Bartel, 2004). Moreover, biosynthesis of the defense hormone jasmonic acid requires three rounds of peroxisomal β-oxidation (reviewed by Creelman and Mullet, 1997). Peroxisomal processes also include metabolism of the branched-chain amino acids (Zolman et al., 2001a), propionate, and isobutyrate (Lucas et al., 2007). In addition, a peroxisome biogenesis protein can influence photomorphogenesis (Hu et al., 2002). The dominant *ted3* mutation in a peroxisomal protein (PEX2) suppresses the pleiotropic de-etiolated mutant phenotypes of *det1* and *cop1*. Although the precise mechanism is unclear, peroxisomes may release signaling molecules that affect the expression of nuclear genes controlling photomorphogenesis (Hu et al., 2002).

The original *chy1* mutant alleles were isolated based on resistance to the inhibitory effects of exogenous IBA on root elongation (Zolman et al., 2001a). These mutants exhibit developmental defects in the absence of exogenous sucrose (Zolman et al., 2001a) and catabolize seed storage lipids poorly during germination (Zolman et al., 2000), indicating defects in peroxisomal fatty acid β-oxidation. Two additional *chy1* alleles (*dbr5-1* and *dbr5-2*) were isolated in a screen for 2,4-DB resistant mutants (Lange et al., 2004). *CHY1* encodes HIBYL–CoA hydrolase, which is involved in Val catabolism in humans; a human HIBYL–CoA hydrolase cDNA modified to contain a peroxisomal targeting signal can functionally complement the *chy1* mutant (Zolman et al., 2001a). As expected, *chy1* mutants show reduced levels of Val catabolism (Lange et al., 2004). *chy1* mutants also show enhanced sensitivity to exogenous isobutyrate and propionate (Lucas et al., 2007).

The *CHY1*-encoded HIBYL–CoA hydrolase is involved in catabolism of Val and isobutyrate. During this process, methylacrylyl–CoA is converted to HIBYL–CoA in a reversible reaction. A peculiarity in these pathways is that CHY1 hydrolyzes the CoA thioester, which is later reformed. Disruption of this hydrolysis is hypothesized to cause HIBYL–CoA accumulation and shift the equilibrium from HIBYL–CoA back to methylacrylyl–CoA. Methylacrylyl–CoA is toxic, reacting rapidly with nucleophiles such as cysteine, cysteamine, and glutathione; this conjugation may covalently inactivate enzymes with active site nucleophiles (Brown et al., 1982; Dearfield et al., 1991). Indeed, activity of the β-oxidation enzyme 3-ketoacyl–CoA thiolase is significantly decreased in *chy1*

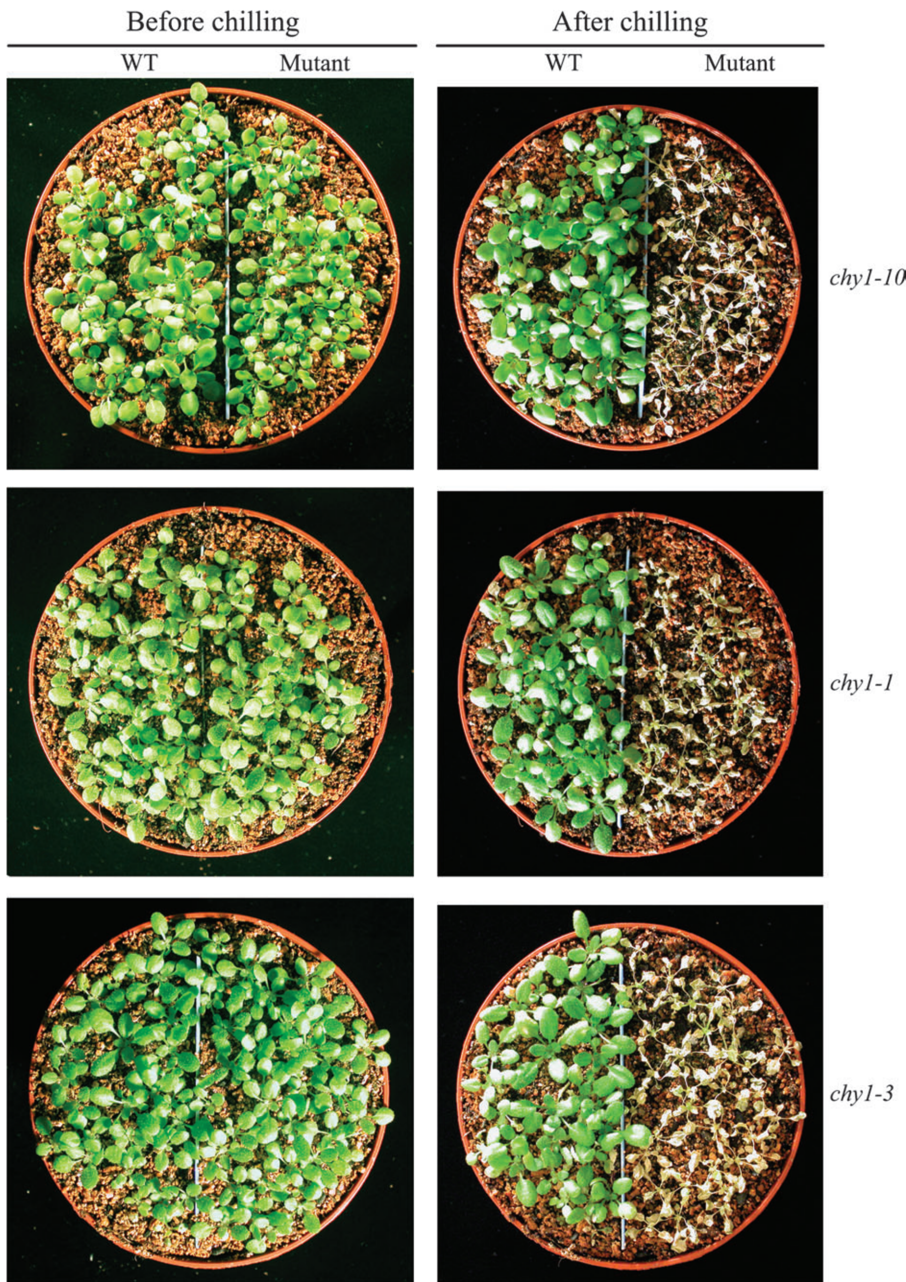


Figure 7. *chy1-1* and *chy1-3* Mutant Plants Are Chilling Sensitive in the Dark.

Fourteen-day-old *chy1-1* and *chy1-3* mutant plants grown at 22°C were shifted to 4°C and grown for 10 d in the dark.

mutants (Lange et al., 2004); the IBA-response phenotype and sucrose dependence of *chy1* mutants is likely an indirect effect of methylacrylyl–CoA accumulation leading to thiolase inactivation. In addition, methylacrylyl–CoA may disrupt cellular membranes in *chy1* mutants, perhaps causing accumulation of reactive oxygen species, electrolyte leakage and impairing cold-induced gene expression. Alternatively, methylacrylyl–CoA may be sequestered in the peroxisome and the ensuing damage may be limited to this organelle. Peroxisomal defects including reduced β-oxidation may disrupt a peroxisome-derived signal that affects cold-induced nuclear gene expression, in a manner analogous to the effect of the *ted3* mutation on nuclear gene expression (Hu et al., 2002). It also is possible that potential alterations in auxin response or homeostasis in the *chy1* mutant may contribute to the impaired cold stress tolerance of the mutant.

In this work, we demonstrate a connection between peroxisomal metabolism, cold-regulated *CBF3* expression, and cold tolerance. *chy1* mutants have reduced *CBF* expression levels. However, interestingly, the levels of three cold-regulated transcripts that are activated by CBFs, including *COR15A*, *COR47A*, and *RD29A*, were not lower in *chy1-10* than wild-type. Mutations that either decrease (Lee et al., 2002a, 2002b) or increase (Zhu et al., 2005) *RD29A* expression in the cold

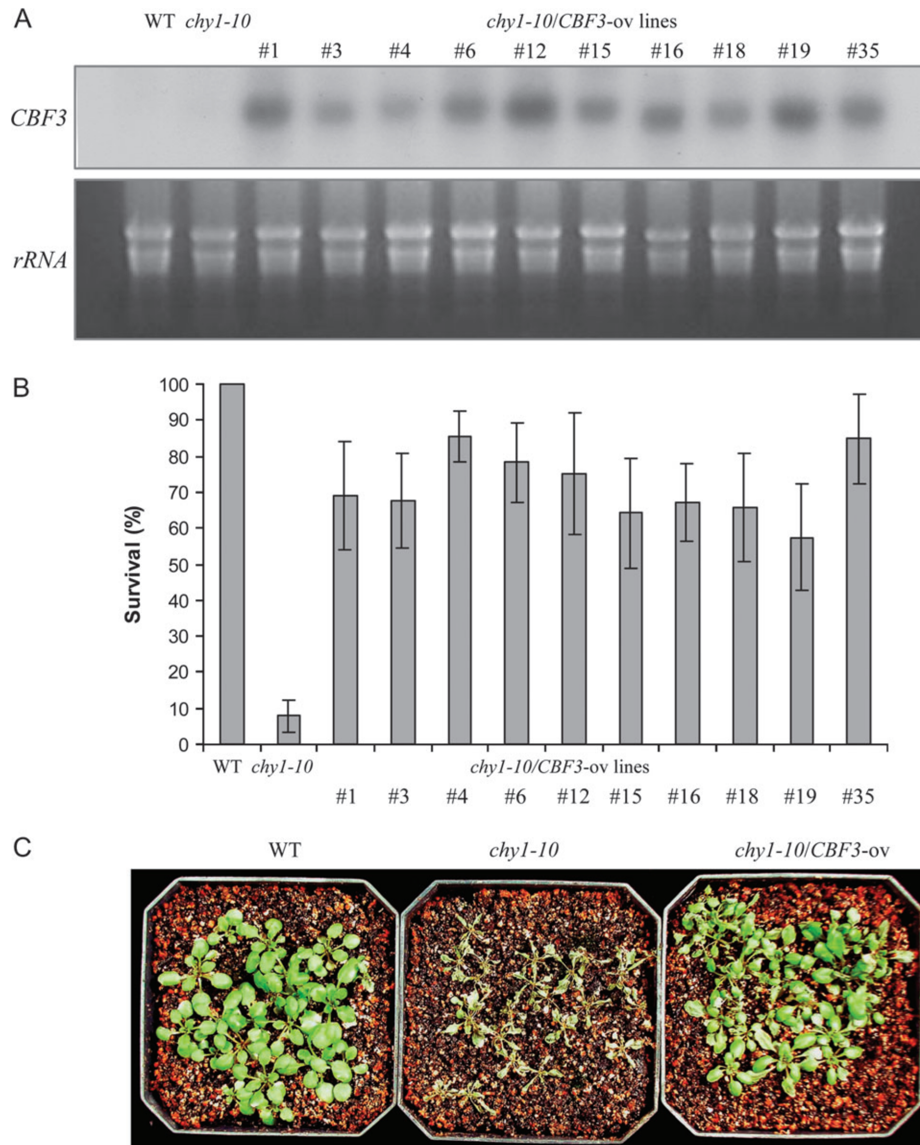


Figure 8. Ectopic Expression of *CBF3* Suppresses the Chilling-Sensitivity of *chy1-10* Mutant Plants.

(A) Northern blot of *CBF3* expression in WT, *chy1-10*, and *chy1-10* lines transformed with a *CBF3* overexpression construct grown without cold treatment. A photo of the ethidium bromide stained rRNA is included as a loading control.

(B) *chy1-10* mutant plants overexpressing *CBF3* (CBF3-ov) were chilling resistant. Comparison of survival after cold treatment at 4°C for 10 d in the dark. Data shown are mean values from three experiments with standard deviation (*n* = 15).

C) Fourteen-day-old WT, *chy1-10*, and *chy1-10*/CBF3-ov (#35) were transferred to 4°C for 10 d in the dark.

without changing the expression of *CBFs* have been reported. These observations, together with our findings reported here, suggest that the *CBF* genes are not the sole transcriptional regulators for *RD29A* and several other *COR/RD* genes. The increased expression of *RD29A* and other *COR/RD* genes in *chy1* despite reduced *CBF* levels implies that the *chy1* mutation enhances a CBF-independent pathway for the activation of the *COR/RD* genes. The effect of decreased induction of CBFs on cold tolerance in the *chy1* mutant plants may be mediated by CBF target genes other than the *COR/RD* genes examined here. Alternatively, the peroxisomal defect in *chyl* may affect CBF-independent pathways for cold tolerance. Further examination of *chy1* and other peroxisome-defective mutants may clarify the connection between reduced *CBF* levels, downstream gene expression, and changes in cold and freezing responses.

Our study has uncovered a new role for plant peroxisomes in responses to cold and dark treatment. Low temperatures have large impacts on cellular metabolism (Levitt, 1980). We suggest that integration of metabolic status (e.g. peroxisomal activities) affects plant adaptive responses to cold treatment. Our results suggest the possibility that metabolic status might be

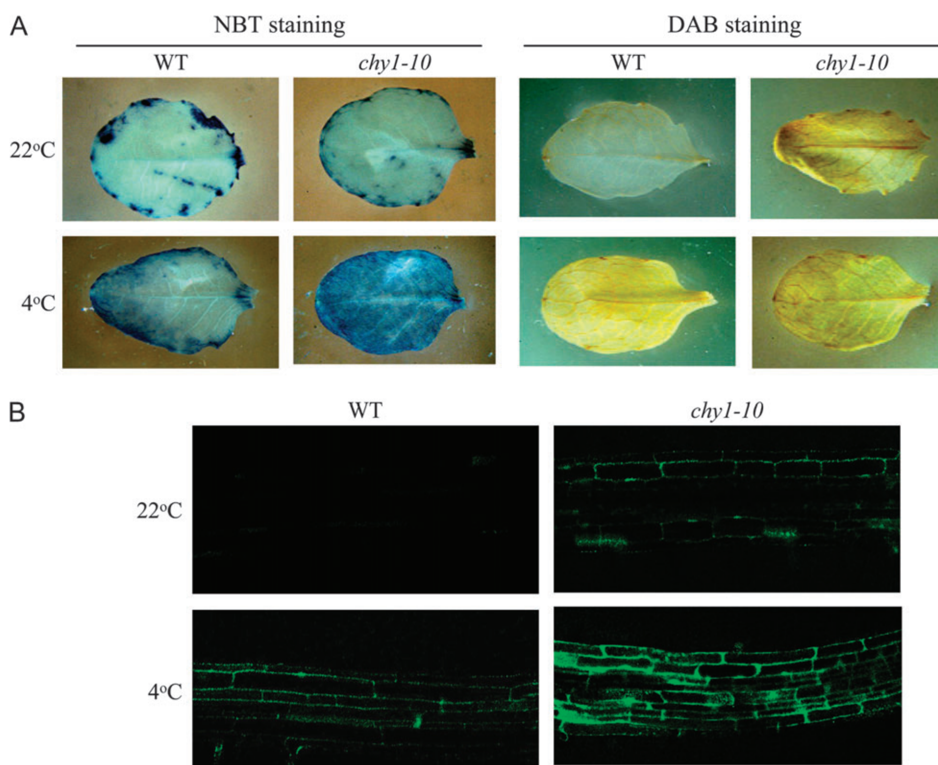


Figure 9. Detection of Reactive Oxygen Species in WT and *chy1-10* Mutant Plants.

(A) Wild-type and *chy1-10* leaves stained with NBT (dark blue) for superoxide or DAB (brown) for hydrogen peroxide after incubation at 22°C or following cold treatment at 4°C for 2 d.

(B) Detection of reactive oxygen species in WT and *chy1-10* roots using CM-H2DCF diacetate, acetyl ester fluorescence imaging of seedlings grown at 22°C or following cold treatment for 2 d.

connected to cold regulation through ROS. The elucidation of the precise molecular mechanisms underlying the effect of peroxisomes on cold-regulated gene expression will require further investigation.

METHODS

Plant Materials and Mutant Isolation

Construction of *CBF3-LUC* and generation of transgenic *Arabidopsis* plants was previously described (Chinnusamy et al., 2003). *Arabidopsis thaliana* ecotype Columbia (with the *glabrous1* mutation) containing *CBF3-LUC* (hereafter referred to as wild-type) was mutagenized using EMS (ethyl methanesulfonate). M2 seedlings were screened for mutants defective in cold-regulated *CBF3-LUC* expression by luminescence imaging as described (Chinnusamy et al., 2003). Mutants were backcrossed to wild-type four times to eliminate unlinked mutations.

Gene Expression Analysis

Two-week-old wild-type and mutant plants grown in soil were treated at low temperature (0–4°C) in the light (20 μmol m^{-2} s^{-1}) or dark. Total RNA was extracted from whole seedlings (above-ground parts) and RNA analysis was performed using gene-specific probes as described (Ishitani et al., 1998).

Electrolyte Leakage and Freezing Tolerance Assays

For chilling tolerance, plants grown in soil in a growth chamber with 16 h light at 22°C, 8 h dark at 18°C, and 75% relative humidity for 2 weeks were transferred to 4°C in dark or constant light. Plants were photographed 24 h after transfer from 4°C to room temperature.

The freezing tolerance assay was carried out as described (Chinnusamy et al., 2003). Ten-day-old seedlings of wild-type and *chy1-10* grown on 0.7% agar media were cold acclimated at 4°C in the light for 4 d. These plates were placed on ice in darkness in a freezing chamber set to –1°C for 16 h. Ice chips were sprinkled on the plants before the chamber was programmed to cool at –1°C per hour. Petri dishes of plants were removed after being frozen at the desired temperature for 2 h, thawed at 4°C overnight, and then transferred to a growth chamber at 22°C. Survival of the seedlings was scored visually after 2 d.

The electrolyte leakage test was performed to compare membrane integrity and chilling sensitivity. Two-week-old plants grown in soil were treated at 4 or 22°C in the dark. Several rosette leaves from treated and untreated plants were

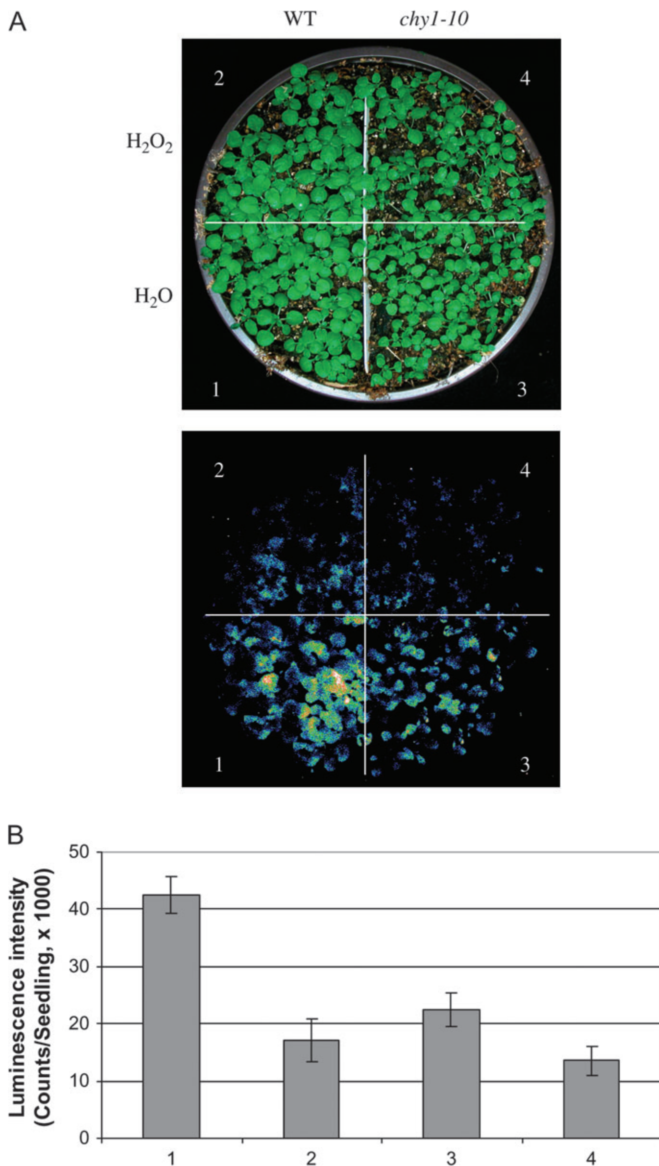


Figure 10. Effect of Hydrogen Peroxide on Cold-Regulated Expression of *CBF3-LUC*.

(A) Luminescence images of *CBF3-LUC* plants. *CBF3-LUC* seedlings of the WT or *chy1-10* genotype were sprayed with H_2O (lower panel) or 5 mM H_2O_2 (upper panel). Luminescence images of the seedling were taken following cold treatment at 0°C for 12 h.
(B) Quantification of luminescence intensity of the *CBF3-LUC* seedlings treated with or without 5 mM hydrogen peroxide. Data shown are mean values with standard deviation (n = 10).

detached and transferred to tubes with 10 mL deionized water. The conductivity of the solution was measured after shaking overnight at room temperature. After measurement, the samples were autoclaved for 30 min. After shaking at room temperature for two additional hours, the conductivity of the solution was measured again. The percent electrolyte leakage was calculated as the percentage conductivity before versus after autoclaving (Lee et al., 2002b). The leaf electrolyte leakage assay after freezing treatments was carried out as described previously (Ishitani et al., 1998).

Map-Based Cloning of *chy1-10*

For recombination mapping, the *chy1-10* mutant in the *Arabidopsis* Columbia ecotype was crossed with wild-type plants of the L*er* ecotype. F1 plants from the cross were self-fertilized, and the resulting F2 seeds were collected. A total of 718 homozygous *chy1-10* mutants were selected from the segregating F2 population using the chilling-sensitive phenotype after cold treatment. Genomic DNA extracted from these seedlings was used for PCR-based mapping. Initial mapping linked the mutation to the markers MBK5 and K9I9 on the bottom of chromosome 5. New mapping markers on MNA5, MPA24, K14B20, K1L20, MSN2, and K8K14 BAC clones were developed based on insertion/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 mutant and wild-type plants and sequenced to identify the mutation.

Overexpression of *CHY1* (HIBYL–CoA Hydrolase) and *CBF3* cDNAs

For *chy1-10* complementation, the *CHY1* cDNA cloned behind the 35S promoter in a binary vector (Zolman et al., 2001a) was introduced into homozygous *chy1-10* mutant plants by *Agrobacterium tumefaciens* strain GV3101 transformation. T2 transgenic lines resistant to Basta (glufosinate ammonium) were selected and analyzed. The cloning of *CBF3* downstream of the strong constitutive super promoter was described (Gong et al., 2002). *Agrobacterium* strain GV3101 containing this construct was used to transform *chy1-10* mutant plants. Hygromycin-resistant transgenic plants were selected and T2 progeny were examined.

Detection of Reactive Oxygen Species

For superoxide detection, leaves detached from 2-week-old plants were vacuum-infiltrated with 0.1 mg mL^{-1} nitroblue tetrazolium (NBT) in 25 mM Hepes buffer, pH 7.6. After 2 h incubation at room temperature in the dark, samples were transferred to 80% ethanol and treated at 70°C for 10 min. For hydrogen peroxide staining, leaves were vacuum-infiltrated with 0.1 mg mL^{-1} 3,3′-diaminobenzidine (DAB) in 50 mM Tris-acetate buffer, pH 5.0. Samples were incubated for 24 h at room temperature in the dark before transferring to 80% ethanol. For chilling treatment, plants were placed at 4°C in the dark for 2 d before staining. Detection of the reactive oxygen species in seedling roots by CM-H2DCF diacetate, acetyl ester staining was as described (Shin and Schachtman, 2004). Fluorescence imaging was performed using confocal microscopy (Leica TCS SP2).

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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