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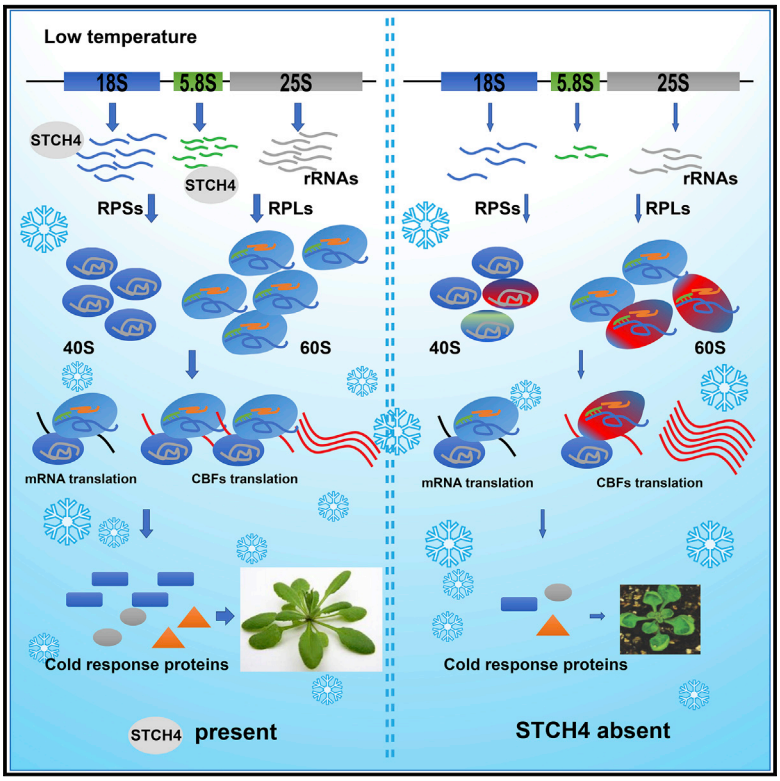
STCH4/REIL2 Confers Cold Stress Tolerance in *Arabidopsis* by Promoting rRNA Processing and CBF Protein Translation

Graphical Abstract



Highlights

- STCH4 maintains rRNA processing under low temperatures
- STCH4 promotes CBF translation under low temperatures
- Overexpression of STCH4 enhances cold stress tolerance
- STCH4 may be required for ribosome remodeling under low temperatures

Authors

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In Brief

Yu et al. show that the ribosomal biogenesis factor STCH4 confers cold stress tolerance in *Arabidopsis* by maintaining rRNA processing and promoting CBF protein translation. The ribosome is likely a site for cold stress response.

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STCH4/REIL2 Confers Cold Stress Tolerance in *Arabidopsis* by Promoting rRNA Processing and CBF Protein Translation

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SUMMARY

Plants respond to cold stress by inducing the expression of transcription factors that regulate downstream genes to confer tolerance to freezing. We screened an *Arabidopsis* transfer DNA (T-DNA) insertion library and identified a cold-hypersensitive mutant, which we named *stch4* (*sensitive to chilling 4*). *STCH4/REIL2* encodes a ribosomal biogenesis factor that is upregulated upon cold stress. Overexpression of *STCH4* confers chilling and freezing tolerance in *Arabidopsis*. The *stch4* mutation reduces CBF protein levels and thus delayed the induction of C-repeat-binding factor (CBF) regulon genes. Ribosomal RNA processing is reduced in *stch4* mutants, especially under cold stress. STCH4 associates with multiple ribosomal proteins, and these interactions are modulated by cold stress. These results suggest that the ribosome is a regulatory node for cold stress responses and that STCH4 promotes an altered ribosomal composition and functions in low temperatures to facilitate the translation of proteins important for plant growth and survival under cold stress.

INTRODUCTION

Plants frequently encounter chilling or freezing temperatures during the growing season, which adversely affects their growth, development, and productivity (Chinnusamy et al., 2007). Plants cope with cold stress by activating a set of transcription factors that regulate downstream genes, leading to physiological and biochemical adjustments at the cellular and whole-plant levels. In the past two decades, the molecular mechanisms of cold stress responses have been extensively studied in *Arabidopsis*. The cold-responsive C-repeat-binding factor (CBF) transcription factors, including CBF1, CBF2, and CBF3 (also known as DREB1b, DREB1c, and DREB1a), play central roles in plant cold acclimation (Jaglo-Ottosen et al., 1998; Zhao et al., 2015). The CBF proteins recognize and bind to the C-repeat/dehydration responsive element (CRT/DRE) motif in the promoters of many cold-responsive (COR) genes, such as *RD29a* and *COR15a*, and promote the transcription of these downstream genes (Zhao et al., 2015).

The three CBF genes are rapidly and transiently upregulated by cold stress. A set of transcription factors, including ICE1, MYB15, ZAT12, and CAMTA, control the transcriptional upregulation of *CBF* genes upon cold stress (Agarwal et al., 2006; Chinnusamy et al., 2003; Fowler et al., 2005; Doherty et al., 2009; Kim et al., 2013a). The kinase OST1 phosphorylates and stabilizes ICE1, which is important for the expression of the *CBF* genes and downstream genes (Ding et al., 2015). OST1 also phosphorylates the β-subunits of the nascent polypeptide-associated complex (NAC), BTF3 and BTF3L, which enhances the interaction between BTF3s and the CBFs to promote the stability of CBF proteins under cold stress (Ding et al., 2018).

Ribosomes, as the site for protein translation, are essential for all living organisms. The biogenesis of ribosomes is a vital and tightly regulated cellular process. Ribosome biogenesis requires the coordinated activity of all three RNA polymerases and many (>200) transiently associated ribosome biogenesis factors (RBFs) (Weis et al., 2015). Many RBFs coordinate the processing of primary ribosomal RNA (rRNA) transcripts, in which three of the four imbedded rRNAs initiate ribosome biogenesis (Weis et al., 2015). While pre-rRNA processing is well understood in yeasts and mammals, a detailed processing scheme for plant pre-rRNA processing is still lacking.

The cytoplasmic zinc-finger protein Rei1 in yeast is a ribosome biogenesis factor, and yeast lacking Rei1 is sensitive to cold. Rei1 triggers the release of Arx1 from the 60S ribosome subunit during cytoplasmic maturation. In Rei1 yeast mutant cells, Arx1 is continuously associated with the 60S subunit and fails to be recycled to the nucleus (Hung and Johnson, 2006). Rei1, Alb1, and Arx1 form a complex at the peptide exit tunnel of the 60S ribosome subunit, so the release of Alb1-Arx1 is essential for the downstream maturation steps of the 60S subunit in the cytoplasm (Greber et al., 2012, 2016; Hung and Johnson., 2006). Reh1 (for REI1 homolog1) is the paralog of Rei1 and functions redundantly with Rei1. In the absence of Rei1, Reh1 interacts with and stabilizes the 60S ribosome (Parnell and Bass, 2009).

The *Arabidopsis* homologs of Rei1 and Reh1, known as REIL1 and REIL2, are required for the accumulation of the 60S ribosome during cold acclimation and are important for plant growth in the cold (Schmidt et al., 2013, 2014; Beine-Golovchuk et al., 2018). Despite their critical roles in plant growth under low temperature conditions, the mechanisms by which the REIL proteins regulate cold responses in *Arabidopsis* are poorly understood.

In this study, we conducted a genetic screen for chilling sensitive mutants from an *Arabidopsis* transfer DNA (T-DNA) insertion mutant library and identified *stch4* (*sensitive to chilling 4*). *STCH4* was identified as the gene *REIL2* (Schmidt et al., 2013). We show that STCH4 functions differently from the yeast Rei1, and STCH4 modulates rRNA processing and affects CBF protein accumulation in *Arabidopsis*. Our results suggest that STCH4 functions as a ribosome biogenesis factor to modulate ribosome formation under cold stress conditions and triggers cold stress tolerance by regulating translation of early cold-responsive transcription factors, such as CBF proteins.

RESULTS

Isolation of the Chilling Sensitive Mutant *stch4*

To identify genes that are critical for cold stress tolerance, we screened the T-DNA insertion mutant library of *Arabidopsis thaliana* for mutants with chilling sensitive phenotypes (http://abrc.osu.edu/). We identified and subsequently characterized a mutant showing hypersensitivity to chilling stress that we named *stch4*. The T-DNA insertion in this mutant line was annotated to be in the gene *REIL2* (*At2g24500*) (Schmidt et al., 2013), which encodes a homolog of the yeast C2H2 zinc-finger protein Rei1 (Hung and Johnson., 2006).

We obtained *stch4-1* (salk_040068) and *stch4-2* (GK-116C10); each has a T-DNA insertion in the second exon of the gene (Figure S1A). Genotyping of the mutants confirmed that both mutant alleles were homozygous (Figure S1B). Both *stch4-1* and *stch4-2* lack *STCH4* transcripts and are thus knockout mutants (Figure S1C). Although mutations in ribosomal protein genes often lead to lethal or severe developmental phenotypes (Byrne, 2009; Weis et al., 2015), the *stch4* mutants did not show any growth or developmental phenotypes under normal growth temperatures. *Stch4* mutant seedlings grown under normal growth conditions (22°C) were similar to Col-0 wild-type seedlings in size and fresh weight (Figure S1F). However, when 7-day-old seedlings grown at 22°C were transferred to 4°C and allowed to grow for 50 days, the *stch4* mutant showed retarded growth in shoots and roots compared to the wild type (Figure 1A), consistent with previous findings (Schmidt et al., 2013, 2014; Beine-Golovchuk et al., 2018). We introduced a p*STCH4*: *STCH4-3xFLAG* transgene into the *stch4-1* mutant that was expressed at wild-type levels and rescued the chilling-sensitive phenotype of the mutant (Figure S2).

We evaluated the freezing tolerance of *stch4* mutants in electrolyte leakage assays. Ten-day-old seedlings with or without cold acclimation at 4°C for 7 days were subjected to freezing treatments. For the non-acclimated seedlings, 50% ion leakage (LT50) occurred at −3.8°C for the *stch4* mutants and −4.7°C for Col-0 wild-type seedlings (Figure 1B). For the cold-acclimated seedlings, the LT50 decreased to −6.0°C for the *stch4* mutants and to −7.9°C for the Col-0 wild type. Thus, cold acclimation increased the freezing tolerance for all seedlings, though less for the *stch4* mutants than for the Col-0 wild type (Figure 1B). The *stch4* mutant plants also showed a reduced survival rate under freezing temperatures compared to the Col-0 wild type, regardless of cold acclimation (Figure 1C). In contrast, Beine-Golovchuk et al. (2018) reported that the *reil1-1reil2-1* double mutant did not display differences from the Col-0 wild type in response to freezing conditions.

We investigated whether the *stch4* mutants were sensitive to other abiotic stresses. The *stch4* mutants and Col-0 wild type showed similar responses to abscisic acid (ABA), NaCl, and mannitol treatment (Figure S1D). In addition, *STCH4* transcript levels increased in response to cold stress (Figure 1E), but not ABA, mannitol, or NaCl treatment (Figure S1E). Together, these data suggest that STCH4 has a preferential function in cold stress tolerance. *STCH4* mRNA levels were comparable in Col-0 wild-type plants and the *cbf1/2/3* triple mutants (Zhao et al., 2016) with or without cold stress treatment (Figure 1D), indicating that cold-induced expression of the *STCH4* gene does not require the core CBF regulatory pathway.

STCH4 promoter-GUS analysis revealed GUS activity in various tissues, with stronger expression in the vascular tissues, root tip, and anther (Figure S3D). To evaluate the subcellular localization of the STCH4 protein, we generated transgenic *Arabidopsis* plants expressing a STCH4-GFP fusion under the control of the *STCH4* promoter (Figure S3B). We detected GFP fluorescence in both the cytoplasm and nucleus, which was not altered by cold stress (Figure S3A). STCH4-GFP expressed under the control of the 35S promoter in tobacco leaves was also localized to the cytoplasm and nucleus (Figure S3C). This subcellular localization of STCH4 is distinct from the exclusively cytosolic localization of Rei1 protein in yeast (Greber et al., 2016; Hung and Johnson, 2006) and suggests that the STCH4 protein functions in both the nucleus and the cytoplasm.

Overexpression of *STCH4* Confers Increased Cold Tolerance

Cold-induced expression of *STCH4* and cold hypersensitivity of the *stch4* mutant suggests that *STCH4* functions in the cold stress response. To test whether overexpression of *STCH4* improves cold tolerance, we introduced a 35S promoter::*STCH4* transgene into *stch4* mutant plants. The overexpression lines displayed significantly higher *STCH4* transcripts levels than the Col-0 wild type or the *stch4* mutants (Figure 2B). Under normal

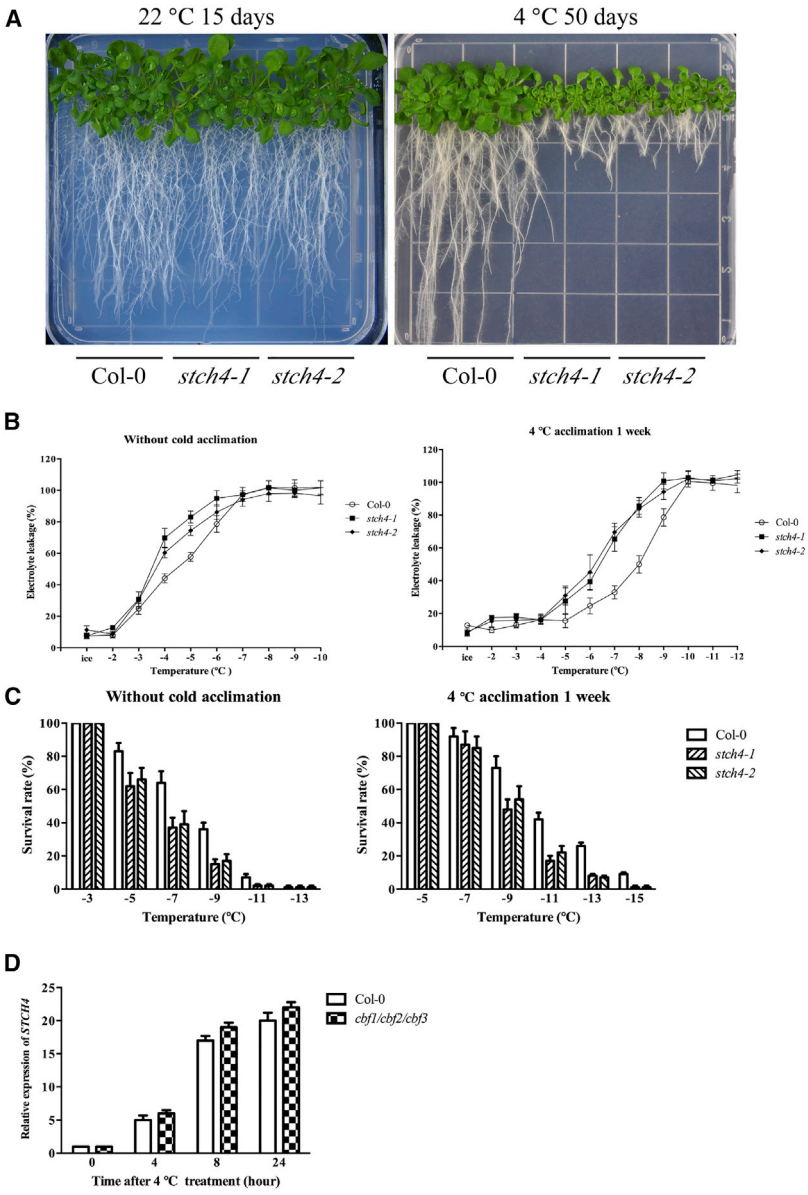

Figure 1. *stch4* Mutants Show Hypersensitivity to Chilling and Freezing Stress

(A) The chilling sensitive phenotype of the *stch4* mutants. This experiment was repeated 10 times with similar results.

(B) Freezing-induced electrolyte leakage from *stch4-1*, *stch4-2*, and Col-0 wild-type leaves with (right panel) and without (left panel) cold acclimation. The values represent the mean ± SD (n = 7). This experiment was repeated 4 times with a similar pattern.

(C) Quantitative analysis of plant survival rate under freezing temperatures with (right panel) and without (left panel) cold acclimation. The values represent the mean ± SD (n = 10). This experiment was repeated 4 times with a similar pattern.

(D) Cold-induced expression of *STCH4* is not dependent on CBFs. The values represent the mean ± SD (n = 3). See also Figures S1–S3.

conditions, all three genotypes showed similar growth. However, at 4°C, the overexpression lines showed enhanced growth compared to the Col-0 wild type, and the *stch4* mutants exhibited hypersensitivity to cold, as expected (Figures 2A and S4). *STCH4*-overexpressing plants showed an improved tolerance to freezing compared to the Col-0 wild type, as measured by the electrolyte leakage assay (Figure 2C) and survival rates (Figure 2D). Thus, *STCH4* is not only required, but also sufficient for chilling and freezing tolerance in *Arabidopsis*.

STCH4 Affects the Expression of Cold-Inducible Genes

To examine the effects of *stch4* mutations on cold-stress-inducible gene expression, we used northern blot analyses to examine the cold-induced expression of well-known cold-responsive genes, including *COR15a*, *COR47*, *RD29a*, and *CBFs*. Induction of the late-responsive genes *COR15a* and *RD29a* was delayed in the *stch4* mutant compared to the Col-0 wild type (Figure 3A). Unexpectedly, early-responsive transcription factor genes such as *CBF1-3*, *ZAT10*, *ZAT12*, and *ZF*, which are thought to control the late-responsive genes (Park et al., 2015), displayed increased transcript levels in the *stch4* mutant compared to the Col-0 wild type during cold stress (Figure 3B).

To further understand the role of STCH4 in cold-responsive gene regulation, we performed RNA sequencing (RNA-seq). We analyzed the transcriptomes of *stch4* and the Col-0 wild type after 0 h, 4 h (early), or 24 h (late) of cold treatment. We used a fold change ≥ 2 and a false discovery rate (FDR; Benjamini-Hochberg adjusted p value) $\leq$ 0.01 (Love et al., 2014) to define differentially expressed genes (DEGs).

After 4 h of cold treatment, we observed 1,022 and 949 upregulated genes in *stch4-1* and the Col-0 wild type, respectively. Of these 949 cold-induced genes in the Col-0 wild-type, 162 were not upregulated in *stch4-1* mutants and may represent STCH4-modulated, cold-inducible genes (Figure 3C). In addition, 436 and 417 genes were downregulated after 4 h of cold treatment in the *stch4* mutant and Col-0 wild type, respectively, and 179 genes that were downregulated in the Col-0 wild type failed to be downregulated in the *stch4* mutants (Figure 3C).

After 24 h of cold treatment, 2,588 and 2,812 genes were upregulated in *stch4* mutants and the Col-0 wild type, respectively (Figure 3C). Among the 2,812 cold-inducible genes in the wild type, 533 genes were not induced in the *stch4* mutants. In addition, 2,760 and 2,613 genes were downregulated after 24 h of cold treatment in the *stch4* mutant and Col-0 wild type, respectively, and 545 genes that were downregulated in Col-0 were not downregulated in the *stch4* mutant (Figure 3C).

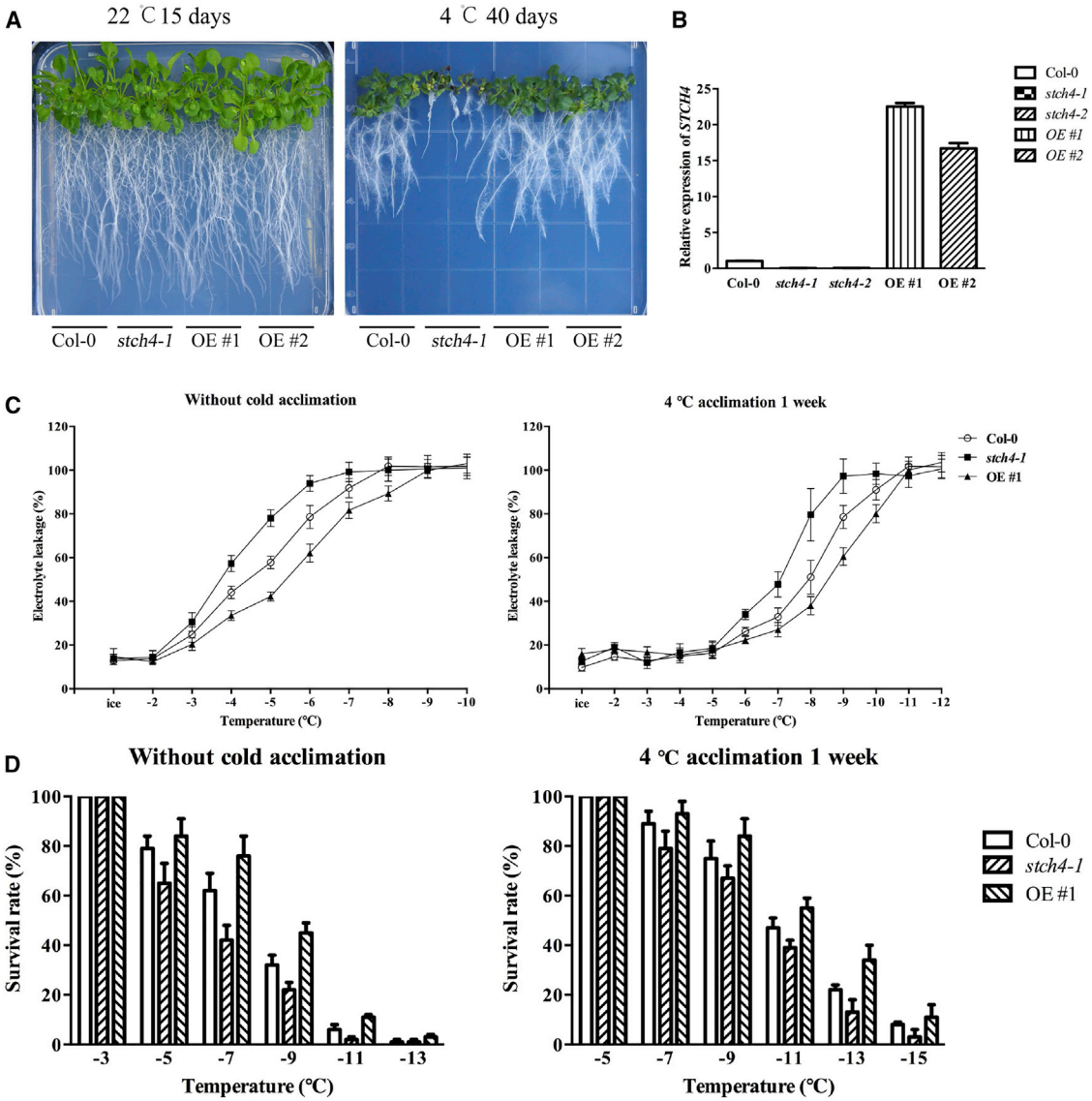

Figure 2. Overexpression of STCH4 in Transgenic Plants Increases Chilling and Freezing Tolerance

(A) Chilling sensitivity test in the Col-0 wild-type, *stch4-1*, and two independent *STCH4* overexpression lines. This experiment was repeated 10 times with similar results.

(B) Expression levels of *STCH4* determined by qRT-PCR. The values represent the mean ± SD (n = 3).

(C) Freezing-induced electrolyte leakage in leaves with (right panel) and without (left panel) cold acclimation. The values represent the mean ± SD (n = 7). This experiment was repeated 4 times with a similar pattern.

(D) Quantitative analysis of the plant survival rate after treatments with freezing temperatures with (right panel) and without (left panel) cold acclimation. The values represent the mean ± SD (n = 10). This experiment was repeated 4 times with a similar pattern.

See also Figure S4.

We further analyzed the genes that were upregulated by at least 2-fold after 4 or 24 h of cold treatment in the Col-0 wild type (3,761 genes). We compared the expression of these 3,761 genes in *stch4-1* and Col-0 before and during cold treatment. We found that 585 of the 3,761 genes showed elevated cold induction in the *stch4-1* mutants compared to Col-0. Of these, about 75% were induced early, after 4 h of cold treatment, but declined at 24 h of cold treatment (Figure 3E). On the other hand, 632 of the 3,761 cold-inducible genes showed decreased cold induction in the *stch4* mutant compared to the Col-0 wild type, which included both early and late cold-responsive genes (Figure 3D).

Of the 585 genes with elevated cold induction in the *stch4* mutant, 58 encode transcription factors and may represent the first-wave early cold-responsive genes in the cold response pathway (Park et al., 2015) modulated by STCH4. We generated a heatmap of selected genes encoding transcription factors. Most transcription factor genes displayed a higher expression

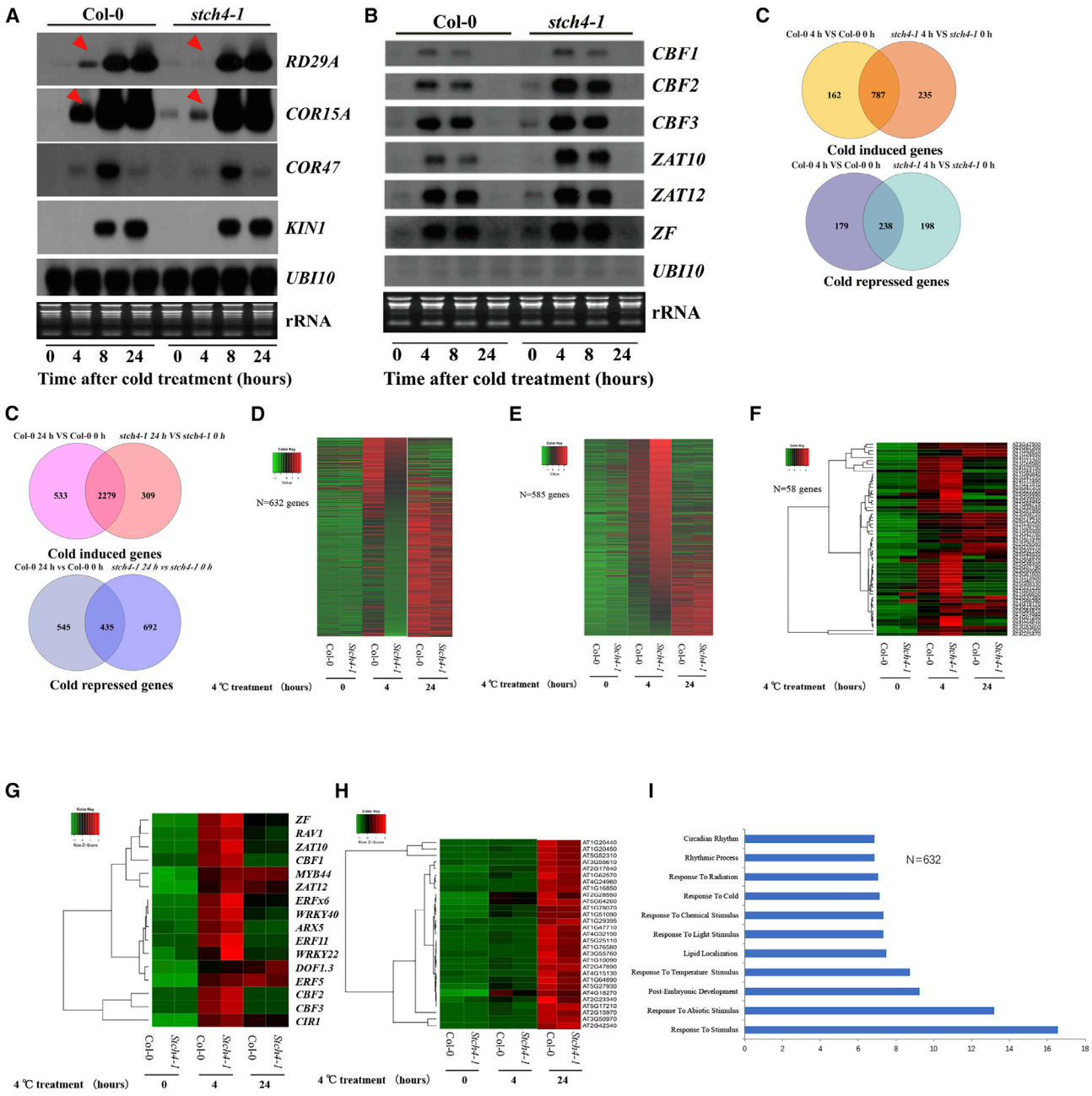

Figure 3. Gene Expression Profiling and Analysis

(A) Northern blotting showing the steady-state transcript levels of several cold-inducible marker genes. The red arrows indicate reduced transcript levels in the *stch4-1* mutants after 4 h of low temperature treatment. This experiment was repeated 2 times with similar results.

(B) Northern blotting showing increased expression of the cold-responsive transcription factor genes.

(C) Venn diagrams showing the numbers of the cold-regulated genes, overlapping and non-overlapping, between the *stch4-1* mutant and Col-0 wild-type after 4 h and 24 h of 4°C cold treatment.

(D) Heatmap of cold-induced genes that are expressed lower in the *stch4-1* mutant than in Col-0 (n = 632).

(E) Heatmap of cold-induced genes that are expressed higher in the *stch4-1* mutant than in Col-0 (n = 585).

(F) Heatmap showing the transcript levels of the cold-responsive transcription factors whose expressions are higher in the *stch4-1* mutant than in the Col-0 wild-type after 4 h of cold treatment.

(G) Heatmap showing the transcript levels of the first-wave cold-responsive transcription factors whose expression levels are higher in the *stch4-1* mutant than in the Col-0 wild-type after 4 h of cold treatment.

(H) Heatmap of the CBF regulon genes showing decreased expression levels in the *stch4* mutant compared with the Col-0 wild-type after cold treatment for 4 or 24 h.

(I) GO analysis of the cold-inducible genes whose expression decreased in the *stch4-1* mutant in response to cold treatment.

in the *stch4* mutant at 4 h, but not at 24 h, of cold treatment (Figure 3F). Park et al. (2015) identified 30 transcription factors that are quickly induced by low temperatures, and these genes were named ''first-wave cold-inducible transcription factors.'' We analyzed 16 of the 30 genes classified as ''first-wave cold-inducible transcription factors'' and found that they showed higher expression in the *stch4* mutants than the Col-0 wild type after 4 h of cold treatment (Figure 3G), consistent with the northern blot results (Figure 3B).

Increased expression of first-wave cold-inducible transcription factors was previously associated with a stronger induction of downstream genes, such as the CBF regulon genes regulated by CBF proteins (Jia et al., 2016; Zhao et al., 2016). However, the CBF regulon genes showed a decreased induction in the cold-treated *stch4* mutant compared to the Col-0 wild type (Figure 3H), even though the induction of early-response genes such as *CBF1-3* was higher in the *stch4* mutant than in the Col-0 wild type (Figures 3B and 3G). We performed Gene Ontology (GO) analysis of the 632 cold-inducible genes that showed a reduced cold induction in the *stch4* mutants and found genes involved in ''response to stimulus,'' ''response to abiotic stimulus,'' and ''response to cold'' (Figure 3I).

Overall, our transcriptome analyses revealed that a loss of STCH4 increases the cold induction of the first-wave transcription factors and decreases the cold induction of the downstream late-response genes. These data suggest that STCH4 is required for the protein accumulation of the first-wave transcription factors, such as the CBF proteins. STCH4/REIL2 has been implicated in ribosome biogenesis (Beine-Golovchuk et al., 2018). We therefore hypothesized that the *stch4* mutation may cause reduced ribosome biogenesis and thus impaired translation of the first-wave transcription factors. Reduced translation may increase the transcript levels of these transcription factors through feedback regulation in the *stch4* mutant plants.

The *stch4* Mutation Reduces the Accumulation of CBF Proteins

To test whether STCH4 may participate in protein translation, we first examined protein synthesis in the Col-0 wild type and *stch4* mutant seedlings with or without cold treatment. We found that general protein synthesis was not affected in the *stch4* mutant at 22°C and 4°C (Figure 4A). As expected, protein translation clearly decreased when seedlings were transferred to a low temperature.

To further investigate protein translation during cold treatment, we examined *de novo* protein synthesis under low temperatures by labeling newly synthesized proteins with L-Azidohomoalanine (AHA), as previously described (Glenn et al., 2017), and performed mass spectrometry. We identified a total of 3,652 proteins with the AHA tag in both Col-0 and *stch4-1* mutant plants, and 1,570 showed a significant change in abundance between *stch4-1* and Col-0. Among these differentially translated proteins, 1,307 showed a reduced abundance in the *stch4-1* mutant, whereas 263 showed an increased abundance (Data S2). GO enrichment analysis of the significantly decreased proteins in *stch4-1* revealed that 37 proteins are cold-stress-related proteins (Figure 4E), including LOS1, LOS2, STA1, and SAL1/FRY1/HOS2 (Guo et al., 2002; Lee et al., 1999, 2002, 2006; Xiong et al., 2004). Although the protein levels were lower in the *stch4-1* mutant, their corresponding transcript levels were not significantly different between *stch4-1* and Col-0, based on the RNA-seq data (Figure 4E). We did not detect AHA-tagged CBF proteins in this assay, possibly due to their low abundance. In addition to cold-stress-related proteins, many other proteins involved in diverse cellular functions showed differential translation in *stch4-1* mutants (Data S2), some of which might also be important for cellular functions under cold conditions.

Unlike *los1-1* (a cold-sensitive allele of the translation elongation factor 1a gene) mutant plants that are defective in overall protein synthesis in the cold (Guo et al., 2002), *stch4-1* mutant plants showed defective synthesis of only a subset of proteins (Figures 4A and 4E). More than half of the *de novo* synthesized proteins were not significantly different between *stch4-1* and Col-0, indicating that STCH4 has a selective effect on protein synthesis.

To investigate CBF protein levels, we generated transgenic *Arabidopsis* Col-0 and *stch4-1* plants harboring a CBF-GUS reporter under the control of the endogenous *CBF* promoter for each of the three homologs (p*CBF1*:*CBF1*:*GUS*, p*CBF2*:*CBF2*:*GUS*, or p*CBF3*:*CBF3*:*GUS*). We selected the transgenic plants with a single copy of the transgene in the Col-0 background (Col-0) and crossed these lines with the *stch4-1* mutant to introduce the transgene into the *stch4* mutant background (Figure S5B). During cold treatment, the CBF-GUS transcript levels were higher in the *stch4* mutant compared to in the wild type (Figure S5C), but the CBF-GUS protein levels were lower in the *stch4* mutant than in the wild type, inferred from GUS staining (Figure 4B) and activity (Figure 4C). The levels of endogenous CBF proteins were also substantially reduced in cold-treated *stch4* mutants compared to in the Col-0 wild type (Figure 4D), whereas the levels of *CBF* transcripts were relatively higher in the *stch4* mutants than in the Col-0 wild type under the same treatment conditions (Figure S5A). These results are consistent with STCH4 modulating the translation of a subset of proteins, such as CBFs, under cold stress conditions, although we cannot exclude an effect on protein stability regulation. The increased transcript levels of the first-wave transcription factors in response to cold may be a consequence of feedback regulation due to reduced levels of these proteins in the *stch4-1* mutant.

The *stch4* Mutation Causes Defective rRNA Processing under Low Temperature

The primary transcript of rRNA is sequentially processed by specific enzymes to produce mature 5.8S, 18S, and 25S rRNA (Figure 5A). It was recently shown that the *reil1-1reil2-1* mutant shows a delayed accumulation of 25S and 18S rRNA under cold conditions (Beine-Golovchuk et al., 2018). We designed specific probes to detect various rRNA intermediates by northern analysis (Figure 5A). We first analyzed how temperature affects rRNA processing in wild-type Col-0. We transferred 2-week-old Col-0 seedlings from 22°C to 4°C, 7°C, 15°C, 27°C, and 32°C for 24 h and found that cold stress negatively affected the formation of processed rRNA species (Figure 5B), which suggests that ribosome biogenesis is inhibited in response to low temperatures.

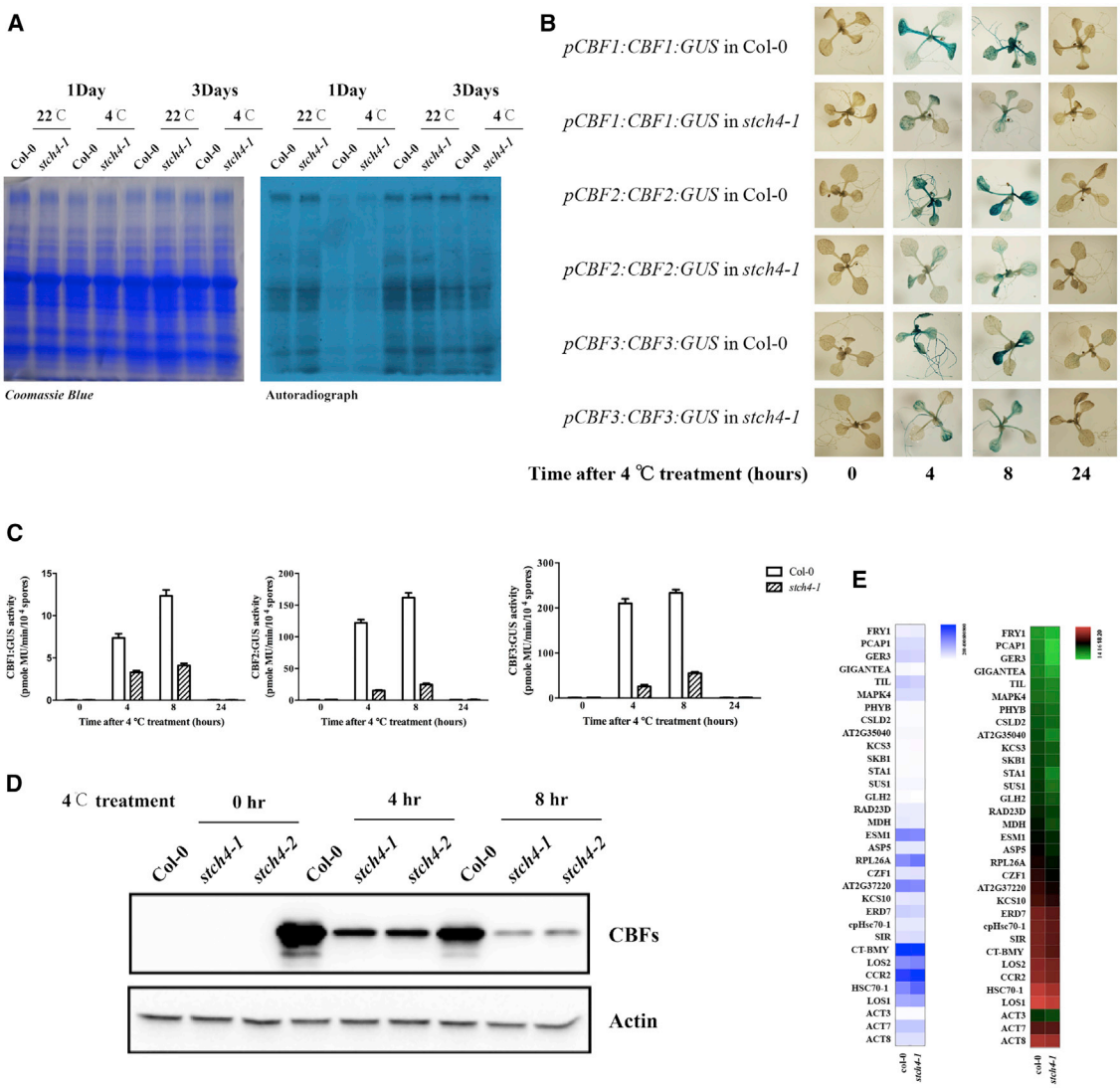

Figure 4. The *stch4* Mutation Reduces CBF Protein Levels under Cold Stress

(A) Total protein synthesis in the *stch4* mutant is comparable to the Col-0 wild-type under both normal and cold stress conditions. This experiment was repeated 3 times with similar results.

(B) GUS staining assay shows reduced production of the CBF-GUS fusion protein in the *stch4-1* mutant after cold treatment.

(C) GUS activity assay indicates reduced production of the CBF-GUS fusion protein in the *stch4-1* mutant. The values represent the mean ± SD (n = 5).

(D) Western blotting shows reduced protein levels of native CBF proteins in the *stch4* mutants compared to in the Col-0 wild-type. Actin served as a loading control using an anti-actin antibody. This experiment was repeated 3 times with similar results.

(E) Heatmap showing protein abundance decrease of cold-response genes (right panel) but without transcript level change (left panel). ACT3/7/8 were used as controls.

See also Data S2 and Figure S5.

Compared to the Col-0 wild-type, the *stch4* mutant had lower levels of the intermediate processing products, including 32S, 27S (27SA, 27SB), pre-18S, and pre-5.8S (7S, 5′-5.8S and 6S) rRNA, even under normal growth conditions (compare the 0 time point between the Col-0 wild type and the *stch4* mutant in Figure 5C). After 24 h of 4°C treatment, rRNA processing in the *stch4* mutant showed a more severe reduction in processed rRNA intermediates than in the Col-0 wild type. The transcripts of 32S, 27S (27SA, 27SB), pre-18S, and pre-5.8S (7S, 5′-5.8S and 6S) rRNA in the *stch4* mutant were barely detectable (Figure 5C). Quantitative analysis of the northern blots revealed that 5-5.8S rRNA levels substantially decreased within 4 h of cold treatment in the *stch4* mutant but remained relatively steady for at least 8 h of cold treatment in the Col-0 wild type (Figure 5D). In contrast, quantitative analysis of the 35S primary transcripts of the rRNA gene revealed that rRNA transcription was not affected by the *stch4* mutation at any temperature analyzed (Figure 5D). These results suggest that

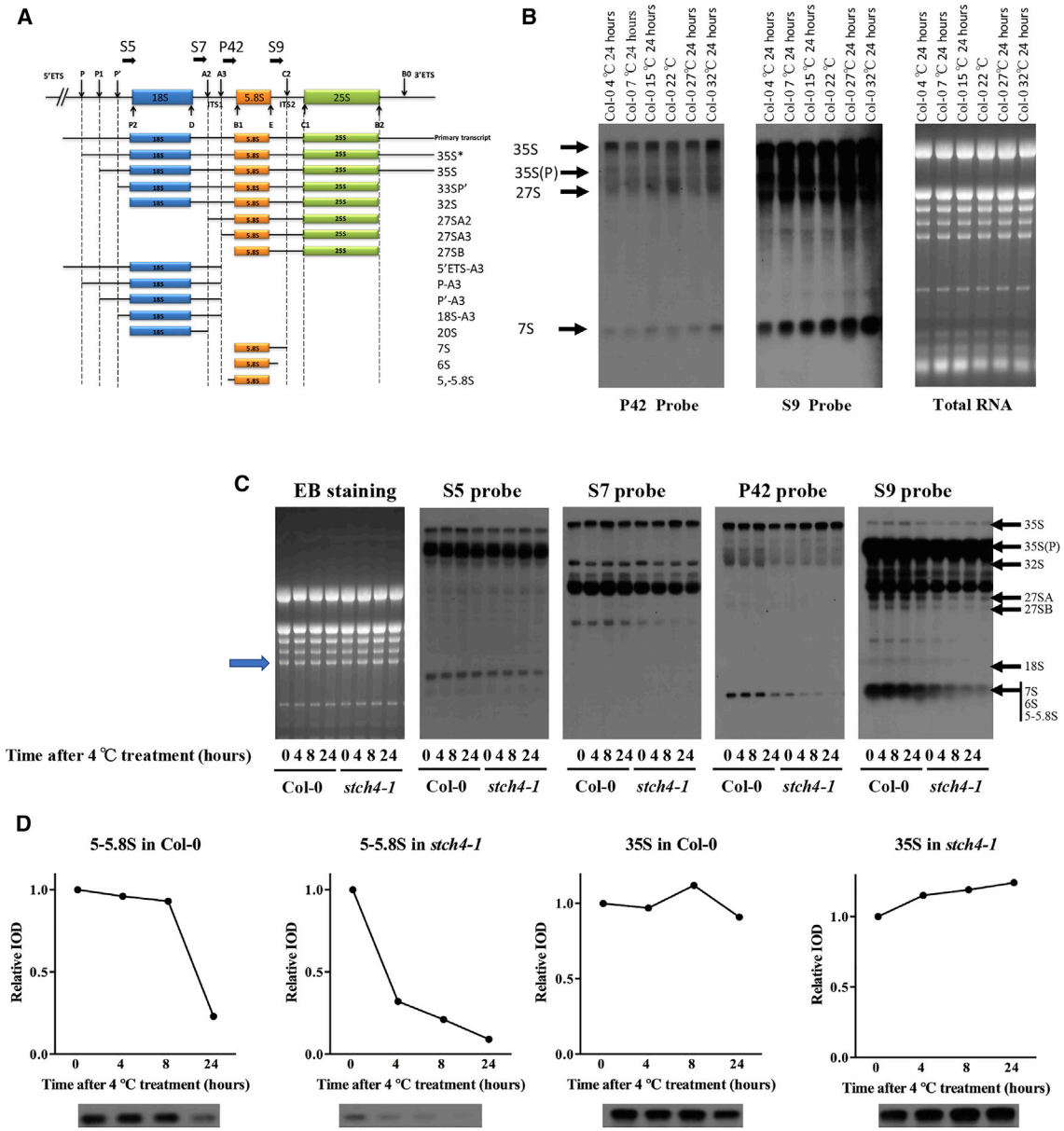

Figure 5. STCH4 Is Required for Proper rRNA Processing

(A) Diagram showing rRNA processing.

(B) Northern blotting shows that rRNA processing is sensitive to temperature changes in the Col-0 wild-type.

(C) Northern blotting shows rRNA processing is affected by the *stch4* mutation, especially under low temperatures.

(D) IOD analysis quantifies northern blot bands. The above experiments were repeated 3 times with similar results.

See also Figure S6.

STCH4 plays an important role in rRNA processing, especially at low temperatures.

Processing pre-rRNA in the nucleolus requires the U3 complex (Grandi et al., 2002). We examined U3 small nuclear RNA (snRNA) to determine whether STCH4 affects U3 snRNA formation. The abundance of U3, U14, and snoR30 snRNA was unaltered by the *stch4* mutation at normal and cold temperatures (Figure S6). These results suggest that the STCH4 modulation of rRNA processing does not arise from altered U3 snRNA.

Temperature-Dependent Association of STCH4 with Ribosomal Proteins

To determine whether the STCH4 protein associates with ribosomal proteins, we introduced the p*STCH4: STCH4-3xFLAG* construct into *stch4* mutant plants and conducted immunoprecipitation-mass spectrometry (IP-MS) analysis. The STCH4-3xFLAG transgene was expressed at similar levels as *STCH4* in Col-0, as assessed by qRT-PCR (Figure S2B), and rescued the chilling sensitive phenotype of the *stch4-1* mutant (Figures

A

GO Information			CM		ID:394712213		ID:963645474	
GO Term	Onto	Description	1	2	Ajusted Pvalue	Num	Ajusted Pvalue	Num
GO:0003735	F	structural constituent of ribosome			1.8e-13	27	3.6e-33	77
GO:0008135	F	translation factor activity, nucleic acid binding			0.0042	7	9.7e-07	21
GO:0016853	F	isomerase activity			0.0076	8	1.2e-10	32
GO:0005840	C	ribosome			4.9e-16	29	1.8e-37	83
GO:0030529	C	ribonucleoprotein complex			1.3e-14	31	2.1e-31	86
GO:0033279	C	ribosomal subunit			2.1e-13	23	2.5e-31	66
GO:0022625	C	cytosolic large ribosomal subunit			2.8e-07	11	1.4e-13	28
GO:0015935	C	small ribosomal subunit			3e-07	11	6.2e-16	31
GO:0015934	C	large ribosomal subunit			7.2e-07	12	1.2e-15	35
GO:0022627	C	cytosolic small ribosomal subunit			3.6e-06	9	2e-11	23
GO:0042254	P	ribosome biogenesis			---	---	2.4e-06	23
GO:0022613	P	ribonucleoprotein complex biogenesis			---	---	5.1e-06	23
GO:0006418	P	tRNA aminoacylation for protein translation			---	---	1.2e-05	13
GO:0004812	F	aminoacyl-tRNA ligase activity			---	---	6.1e-05	12

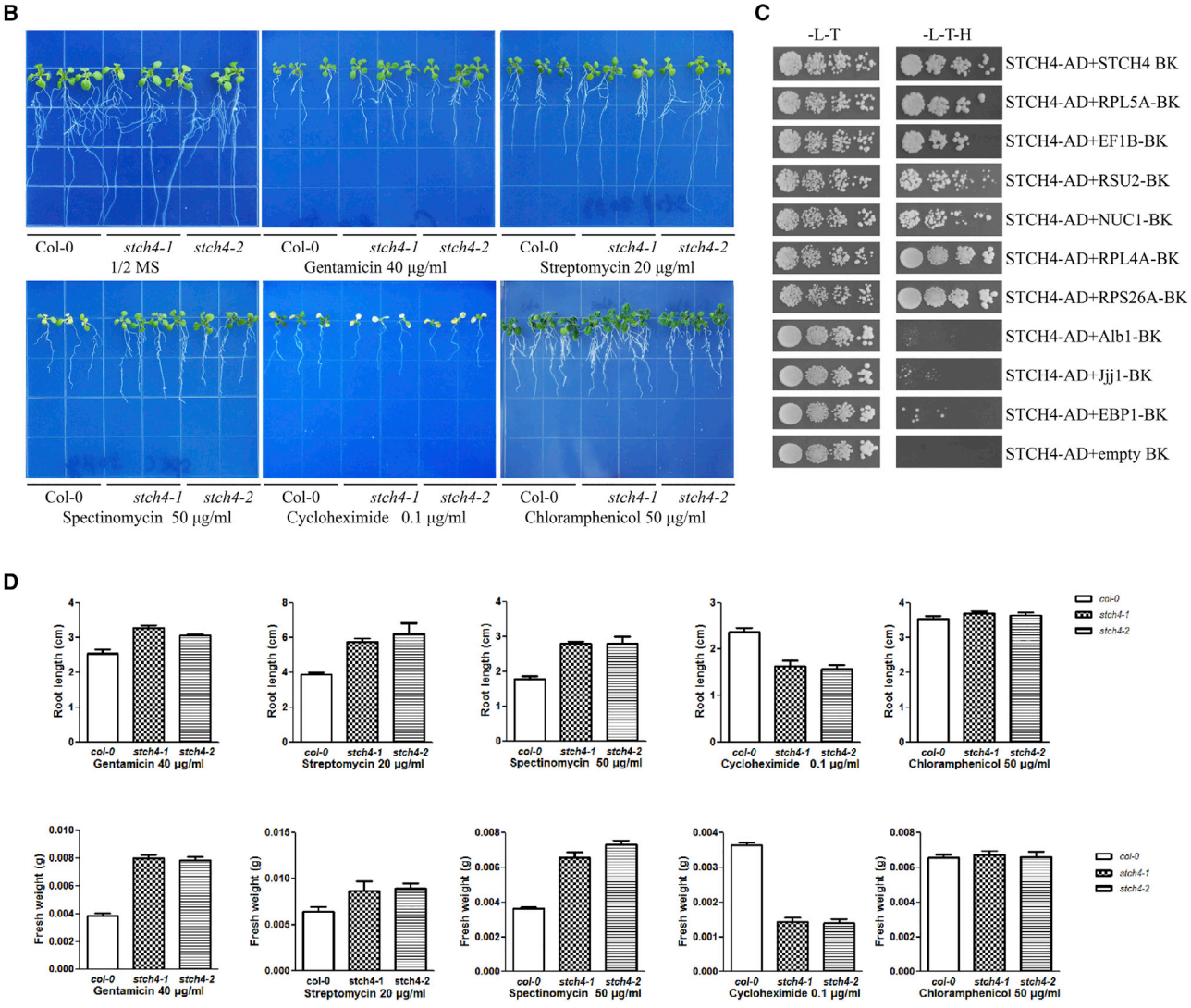

Figure 6. STCH4 Is Associated with Ribosomal Biogenesis Proteins

(A) IP-MS analysis shows the association of STCH4 with ribosomal proteins and the association under cold stress. Onto, ontology; F, molecular function; C, cellular component; P, biological process; CM, color map. 1 and 2 indicate the strength of STCH4 interacting with the identified proteins in the categories under room temperature and cold stress conditions, respectively. Num, number of identified proteins.

(legend continued on next page)

S2A and S2D), indicating that the FLAG-tagged STCH4 protein is functional in transgenic plants. We detected the STCH4-3xFLAG protein in the transgenic plants with an anti-FLAG antibody (Figure S2C), and we used the anti-FLAG antibody for IP-MS analysis of 2-week-old Col-0 wild-type and STCH4-3xFLAG complementation seedlings with or without cold treatment at 4°C for 4 h. Proteins that were identified in the complementation line but not the Col-0 negative control in at least two of three replicates were considered putative STCH4-interacting proteins. These proteins were grouped into STCH4-interacting proteins under room temperature and STCH4-interacting proteins under cold stress conditions (Data S1).

We conducted GO analysis of the STCH4-interacting proteins identified in untreated plants and cold-treated plants (Figure 6A). STCH4 associated with ribosomal proteins, including proteins in both large and small ribosomal subunits, under both conditions. Interestingly, we detected STCH4-associated proteins only present in cold-treated plants in four GO terms including the GO term "ribosome biogenesis." In addition, STCH4-associated proteins in several other GO terms were enriched in cold-treated plants (Figure 6A). These results suggest that cold stress alters STCH4-protein interactions.

We performed yeast two-hybrid assays to confirm some of the interactions identified by IP-MS. This analysis showed that STCH4 directly interacts with RPL5A, EF1B, RSU2, and NUC1, which are important ribosomal factors in rRNA processing, and with RPL4A and RPS26A, which are components of large and small ribosome subunits. Unexpectedly, STCH4 did not interact with AtAlb1, AtJjj1, or AtEBP1, the *Arabidopsis* proteins homologous to yeast Alb1, Jjj1, and Arx1, respectively. Moreover, the localization of the *Arabidopsis* AtEBP1 and AtTIF6, whose homologs in yeast are mislocalized in the *rei1* mutant, was not affected by the *stch4* mutation (Figure S3E). These results suggest that the *Arabidopsis* STCH4 protein is functionally distinct from yeast Rei1, even though these two proteins are homologs and are both important for ribosome biogenesis and chilling tolerance.

We also tested whether the *stch4* mutation affects ribosome functions by evaluating the sensitivity of the *stch4* mutant to protein translation inhibitors. Previous studies demonstrated that ribosome biogenesis mutants, such as *apum23* and *rpl4*, exhibit an altered sensitivity to translation inhibitors targeting ribosomes because these mutations cause aberrant ribosome structure and function (Abbasi et al., 2010; Rosado et al., 2010). The *stch4* mutants showed an apparent resistance to several aminoglycoside antibiotics, including gentamycin, streptomycin, and spectinomycin, compared with the Col-0 wild-type plants (Figures 6B and 6D). Aminoglycoside antibiotics target the aminoacyl-tRNA binding site (A site) in the ribosome during translation (Tsai et al., 2013). However, the *stch4* mutant did not show an altered sensitivity to chloramphenicol, an antibiotic known to prevent protein chain elongation by blocking peptidyl transferase activity (Hang et al., 2014). Cycloheximide is a eukaryotic protein translation inhibitor that interferes with the translocation step. The *stch4* mutant showed a sensitivity to cycloheximide that was similar to the wild type (Figures 6B and 6D). These results suggest that the *stch4* mutation may affect the formation of the A site on the ribosome under low temperatures.

DISCUSSION

The ribosome is often thought to contain structurally unvarying components and equally translate different mRNA, given its essential cellular functions. However, this premise has recently been challenged by several important findings that suggest the ribosome is dynamic, not static, in its structural formation and mRNA translation (Simsek et al., 2017). Kondrashov et al. (2011) demonstrated that ribosomal protein L38 regulates the formation of a ribosome complex that selectively translates Hox mRNA to modulate tissue patterning in mice. This finding suggests that altering ribosome constituents is a pivotal mechanism to control gene expression. Recent studies from that laboratory found that the heterogeneity of ribosomes with distinct selectivity for translating different mRNA is a genome-wide phenomenon and plays important roles in the post-transcriptional regulation of gene expression (Simsek et al., 2017; Shi et al., 2017). Therefore, the ribosome likely shows cell-type-specific functions to preferentially translate proteins essential for that particular cell type.

Whether and how ribosomes may change in response to stress conditions is currently not clear. It is plausible that stress like high or low temperatures may affect rRNA folding during processing, which may influence the proteins associated with the ribosome. In addition, stress conditions may induce the production of specific ribosomal protein factors or ribosomal proteins that could alter ribosomal composition. Beine-Golovchuk et al. (2018) found that REIL proteins promote the accumulation of the cytosolic 60S ribosome to influence ribosome remodeling in response to low temperatures, which supports the notion that ribosomes may contribute to temperature perception in plants. In our study, we found that cold stress affected rRNA processing, and the *stch4* mutation caused a severe reduction in 5′-5.8S rRNA levels, especially under cold temperatures (Figure 5). 5′-5.8S rRNA is critical for 60S ribosome maturation. In response to low temperatures, STCH4 may contribute to ribosomal remodeling by modulating rRNA production. In addition, STCH4 may recruit distinct ribosomal proteins to remodel the ribosome after exposure to low temperatures. This conclusion is supported by our observation that STCH4 is associated with ribosomal proteins and that STCH4-protein interactions are altered in response to cold stress (Figures 6A and 6C).

The *stch4* mutant did not show growth and developmental defects under normal growth conditions but exhibited hypersensitivity to low temperature stress. This result suggests that STCH4 is likely required for the translation of proteins critical for cold

(B) Sensitivity of the *stch4* mutant to antibiotics. This experiment was repeated 3 times with similar results.
(C) Yeast two-hybrid assay of STCH4 interaction with some ribosome biogenesis factors.
(D) Root length and fresh weight of the Col 0 wild-type and *stch4-1* and *stch4-2* mutants grown in 1/2 MS medium supplemented with different antibiotics. The values represent the mean ± SD (n = 7).
See also Data S1 and Figure S2.

stress responses. Indeed, general protein production was not affected by the *stch4* mutation, but the protein levels of the first-wave cold-inducible CBF transcription factors were clearly impaired (Figure 4). Together, these findings suggest that STCH4 functions as a ribosomal biogenesis factor and promotes ribosomal formation that may favor the translation of cold-inducible transcription factor mRNA, thus conferring cold stress tolerance, though we cannot rule out the contribution of protein degradation to the accumulation of CBF proteins in the *stch4* mutant. Our *de novo* protein synthesis assay also identified 37 cold-related proteins significantly reduced in the *stch4* mutant, which may contribute to the cold hypersensitive phenotype of the *stch4* mutant (Figure 4E). In addition, we detected a number of proteins not induced by cold showing a significant reduction in the *stch4* mutant. Some of these proteins may also function in cellular processes important for plants to function in the cold.

Ribosome biogenesis is initiated in the nucleolus, where rRNA genes are actively transcribed into a pre-rRNA that is subsequently processed to form mature rRNA. The formation of ribosomal subunits undergoes several assembling, remodeling, and rRNA processing steps in the nucleolus and nucleoplasm before entry into the cytoplasm. During rRNA processing, early-binding ribosomal proteins are recruited to associate with rRNA (Gerhardy et al., 2014). In eukaryotic cells, mutations in ribosomal proteins or ribosome assembly factors can cause aberrant pre-rRNA processing and genetic diseases in humans (Simsek et al., 2017). The *Arabidopsis* STCH4 protein is likely an early-binding ribosomal protein involved in pre-rRNA processing. By modulating rRNA processing, STCH4 may alter the processing patterns of rRNA intermediates, which could change the proteins associated with rRNA during rRNA processing and in mature ribosomes. STCH4-mediated changes in ribosomes seem critical for plants to cope with low temperature stress, as shown by the hypersensitivity of the *stch4* mutant to cold stress.

In addition to associating with specific ribosomal proteins under cold conditions, STCH4 may affect cold acclimation by direct interactions with cold-responsive proteins. Our IP-MS analysis revealed that STCH4 associates with cold-responsive proteins like COR and KIN upon cold treatment (Data S1). These proteins have been implicated in cold acclimation, and overexpression of COR genes enhances cold tolerance (Artus et al., 1996; Steponkus et al., 1998). The association of STCH4 with cold-responsive proteins suggests that STCH4 may cooperate with COR and KIN proteins to mediate cold acclimation in plants. We also found that STCH4 can interact with CSDP1 under cold conditions (Data S1). CSDP1 was reported to bind the 5′ UTR region of mRNA at low temperatures to disrupt the secondary structure of mRNA, which improves translation under low temperatures (Juntawong et al., 2013). STCH4 may work with CSDP1 to enhance protein translation and promote cold acclimation.

The *Arabidopsis* STCH4 is homologous to the yeast Rei1. In yeast, Rei1 is a cytosolic protein that functions in the cytoplasmic maturation of the 60S ribosome subunit by promoting the release of several ribosomal proteins. Rei1 interacts with ribosome-associated proteins, such as Alb1, Jjj1, Tif6, and Arx1 (Greber et al., 2012; Meyer et al., 2010), only in the cytoplasm and promotes the formation of the translationally active 60S subunit. However, the STCH4 protein is localized in both the cytosol and nucleus (Figure S3), which suggests that STCH4 not only functions in the cytoplasm, but also plays a role in the nuclear assembly process of ribosomes. Thus, *Arabidopsis* STCH4 may have diverged from yeast Rei1 and evolved to fulfill additional or distinct functions in ribosome biogenesis. In fact, the *stch4* mutation affects pre-rRNA processing (Figure 5), which occurs in the nucleolus. The STCH4 protein did not interact with proteins homologous to yeast Alb1, Jjj1, and Arx1 (AtEBP1) (Figure 6C). This finding differs from those of Schmidt et al. (2014), who showed REIL proteins could interact with *Arabidopsis* Arx1 and Jjj1 in a yeast two-hybrid assay. Our IP-MS analysis also did not identify the protein homologs of the yeast Alb1, Jjj1, and ARX1 in the anti-FLAG-STCH4 immuno-precipitated samples. Instead, we found that the *Arabidopsis* STCH4 interacts with several other ribosomal proteins, including RPL5A, EF1B, RSU2, NUC1, RPL4A, and RPS26A, that are associated with either the large or small subunit (Figure 6C). Ribosome biogenesis, especially pre-rRNA processing, in yeast and mammals has been well studied, but the detailed process in plants is still lacking (Weis et al., 2015). Our findings of STCH4's function in pre-rRNA processing and its interaction with other ribosomal proteins and non-ribosomal cold-responsive proteins will further the study and understanding of ribosomal biogenesis and cold stress response in plants.

The CBF regulon is important for cold acclimation in plants (Jaglo-Ottosen et al., 1998). Mutant analyses have demonstrated that the early cold-inducible *CBF* genes, *CBF1*, *CBF2*, and *CBF3*, are critical for cold acclimation and freezing tolerance and have redundant functions in upregulating downstream genes such as the *COR* genes (Jia et al., 2016; Zhao et al., 2016). Paradoxically, although the *stch4* mutant is hypersensitive to cold stress, the transcript levels of some of the first-wave transcription factors including *CBF*s are higher in the *stch4* mutant than in the wild type (Figure 3). These intriguing observations, together with the observation that downstream genes such as *RD29a* and *COR15a* are downregulated in the *stch4* mutant (Figure 3), suggest that the translation of the *CBF* transcripts may be impaired in the mutant. Indeed, we found that the *stch4* mutation caused a reduced accumulation of CBF proteins. Thus, increased transcript levels of *CBF* genes may occur following impaired feedback suppression of *CBF* gene transcription given fewer CBF proteins in the mutant. Interestingly, Beine-Golovchuk et al. (2018) found that the expression levels of *CBF*s and downstream genes, such as *COR15B*, *COR15A*, and *KIN2*, are higher in the *reil1-1reil2-1* mutant under normal temperatures, but relatively lower or unchanged under cold treatment for 1 day, 1 week, or 3 weeks. This discrepancy could arise from different cold treatment duration. In our case, we measured gene expression after cold treatment for 4 or 24 h, while Beine-Golovchuk et al. (2018) analyzed gene expression after cold treatment for 1 day, 1 week, or 3 weeks.

A previously reported *Arabidopsis* mutant, *los1-1*, also showed increased transcript levels of *CBF* genes but decreased expression of the downstream genes under cold conditions (Guo et al., 2002). The *los1-1* mutant, which is hypersensitive to

chilling and freezing temperatures, is a cold-sensitive allele of the *LOS1* gene encoding the translation elongation factor 2, important for general protein translation. It was proposed that increased transcript levels of the *CBF* genes in the *los1* mutant may occur from feedback regulation (Guo et al., 2002). Therefore, both the *stch4* and *los1-1* mutations may impair protein synthesis under cold stress conditions, resulting in more *CBF* transcripts but less *COR* mRNA, which causes increased chilling and freezing sensitivity. The important difference seems to be that the *stch4-1* and *stch4-2* mutants are knockout alleles, and our results suggest that STCH4 is required for CBF protein accumulation under low temperature conditions, whereas *los1-1* is a temperature-sensitive allele, and LOS1 is required for mRNA translation not only in the cold, but presumably also under normal growth conditions. Interestingly, the abundance of LOS1 in the *stch4-1* mutant decreased, which suggests that STCH4 and LOS1 may work in the same cold response pathway to modulate ribosomes and protein translation under cold stress conditions.

Overall, our results suggest that the ribosome serves as an important regulatory site for gene expression in response to cold stress. Under cold stress conditions, the ribosome undergoes remodeling. STCH4 is a ribosome biogenesis factor that accumulates under cold conditions and is critical for this cold-induced remodeling. This ribosome remodeling is important for rRNA processing under cold stress and perhaps alters ribosome selectivity for certain mRNA, such as the *CBF* mRNA, to enhance cold tolerance. Future work is needed to determine whether unique features in the 5′ or 3′ UTRs of the *stch4*-affected cold-response-related genes including *CBF*s may be recognized by specific ribosome-associated proteins under low temperatures.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2019.12.012.

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AUTHOR CONTRIBUTIONS

Conception and design, J.-K.Z., H.Y., H.S., and B.-h.L; Performed research, H.Y. and X.K.; RNA-seq analysis, H.H. and W.W.; Writing, review, and/or revision of the manuscript, H.Y., H.S., J.-K.Z., and X.K.; Technical or material support, D.-J.Y. and J.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-FLAG	Sigma-Aldrich	Cat # P2983; RRID: AB_439685
Anti-GFP	Sigma-Aldrich	Cat # G1546; RRID: AB_1079024
Anti-ACTIN	Sigma-Aldrich	Cat # ab197345
Anti-CBFs	Donated by Yun DJ Lab	N/A
Anti-FLAG tag agarose beads	Sigma-Aldrich	Cat # A2220
Bacterial and Virus Strains		
Agrobacterium tumefaciens GV3101	Intact Genomics	Cat # 1282-12
Yeast strain AH109	Clontech	Cat # 630498
Chemicals, Peptides, and Recombinant Proteins		
T4-PNK	New England BioLabs	Cat # M0201S
(γ-32P) dCTP	PerkinElmer	Cat # BLU013H250UC
(γ-32P) dATP	PerkinElmer	Cat # BLU012H250UC
Trizol	Invitrogen	Cat # 15596018
2x real-time PCR mix	Takara	Cat # RR820Q
EXPRE35S35S Labeling Mix	PerkinElmer	Cat # NEG072002MC
X-gluc	Thermo Scientific	Cat # R0851
protease inhibitor cocktail	Sigma-Aldrich	Cat # P9599
Hind III	New England BioLabs	Cat # R3104S
Cycloheximide	Sigma-Aldrich	Cat # C7698-1G
Streptomycin	Sigma-Aldrich	Cat # P4333-20ML
Spectinomycin	Sigma-Aldrich	Cat # S0692-1ML
Gentamicin	Sigma-Aldrich	Cat # G1272-10ML
Chloramphenicol	Sigma-Aldrich	Cat # C1919-5G
Critical Commercial Assays		
hybridization buffer	Sigma-Aldrich	Cat # H7033
Ladderman labeling kit	Takara	Cat # 6046
TURBO DNA-free Kit	Invitrogen	Cat # AM1907
Click-IT AHA (L-Azidohomoalanine)	Invitrogen	Cat # C10102
DBCO Agarose	Click Chemistry Tools	Cat # 1034-2
Deposited Data		
RNA-seq data	This paper	GEO: GSE141304
Experimental Models: Organisms/Strains		
N. benthamiana		N/A
Arabidopsis thaliana Col-0 wild-type	ABRC	CS1092
stch4-1	ABRC	SALK_040068
stch4-2	ABRC	GK_116C10
pSTCH4::STCH4-3xFLAG/ stch4-1	This paper	N/A
pSTCH4::STCH4-GFP/ stch4-1	This paper	N/A
p35S::STCH4-3xFLAG/ stch4-1	This paper	N/A
pCBF1::CBF1-GUS/Col-0	This paper	N/A
pCBF2::CBF2-GUS/Col-0	This paper	N/A
pCBF3::CBF3-GUS/Col-0	This paper	N/A
pCBF1::CBF1-GUS/ stch4-1	This paper	N/A

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
pCBF2::CBF2-GUS/ stch4-1	This paper	N/A
pCBF3::CBF3-GUS/ stch4-1	This paper	N/A
Oligonucleotides		
Primers for cloning, see Table S1	This paper	N/A
Primers for genotyping and qRT-PCR, see Table S1	This paper	N/A
Probes for Northern blotting, see Table S1	This paper	N/A
Recombinant DNA		
pCAMBIA1305-pSTCH4::STCH4-3xFLAG	This paper	N/A
pCAMBIA1305-pSTCH4::STCH4-GFP	This paper	N/A
pCAMBIA1305-35S::STCH4-3xFLAG	This paper	N/A
pCAMBIA1305-35S::STCH4-GFP	This paper	N/A
pCAMBIA1305-pCBF1::CBF1-GUS	This paper	N/A
pCAMBIA1305-pCBF2::CBF2-GUS	This paper	N/A
pCAMBIA1305-pCBF3::CBF3-GUS	This paper	N/A
Software and Algorithms		
Trimmomatic v0.32	Bolger et al., 2014	N/A
PRINSEQ v0.20.4	Schmieder and Edwards, 2011	N/A
TopHat v2.0.13	Kim et al., 2013b	N/A
Cufflinks v2.2.1	Trapnell et al., 2013	N/A
Cuffmerge	Trapnell et al., 2013	N/A
Cuffdiff	Trapnell et al., 2013	N/A
AgriGO	Du et al., 2010; Tian et al., 2017	N/A

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jian-Kang Zhu (jkzhu@sibs.ac.cn). All materials are available after completion of the respective material transfer agreements.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Arabidopsis thaliana and growth conditions

The *stch4-1* (SALK_040068) and *stch4-2* (GK_116C10) seeds were obtained from the *Arabidopsis* Biological Resource Center (ABRC). Genotyping of SALK_040068 and GK_116C10 lines was conducted using PCR with the primers listed in Table S1. *Arabidopsis thaliana* Columbia-0 (Col-0, wild-type) and the mutant plants were grown in soil under a cycle of 16 hours light (light intensity ~130 mol μm-2 s-1.) and 8 hours dark at 22°C.

Nicotiana benthamiana and growth conditions

Nicotiana benthamiana was grown in greenhouses in soil under a 16-hr light/8-hr dark photoperiod at 22°C.

METHOD DETAILS

Stress treatments

For the chilling assay, surface-sterilized seeds were stratified at 4°C for 3 days and plated on 1/2 MS medium with 0.5% Agar for germination and growth at 22°C for 5-6 days. The plates were then transferred to 4°C culture chamber for cold stress treatment for up to 2 months. For the freezing sensitivity test, both survival rate assay and iron leakage assay were performed. For the survival rate assay, 14-day-old seedlings grown on 1/2 MS plates with 0.3% phytagel were cold-acclimated at 4°C for 48 hours in dark. The plates were then put on ice and placed in a freezing chamber for freezing treatment at −1°C for 16 hours in dark. Ice chips were sprinkled on the plants before the chamber reached −1°C. The freezing chamber was programed to decrease 2°C per hour and was kept at each designed temperature for 2 hours until the temperature reached −11°C and stayed at this temperature for 2 hours. The plates were taken out after treatment at desired temperatures and transferred to 4°C for gentle thawing in dark. The plates

were then transferred to 22°C growth chamber with the setting of long day condition. Survival of the seedlings was recorded after 2 days recovery. For the electrolyte leakage assay, plants were grown at 22°C in soil under an 8-h or 16-h photoperiod with a light intensity of about 130 mol μm-2 s-1. Cold acclimation was performed by treating the plants at 4°C under an 8-h or 16-h photoperiod at about 40 μm-2 s-1 for 7 days. Freezing treatment and electrolyte leakage assay were carried out as previously described (Lee and Zhu, 2010).

For the sensitivity of plants to NaCl, ABA, mannitol, and antibiotics, 7-day-old seedlings grown in 1/2 MS plates were transferred to 1/2 MS agar medium containing different concentrations of NaCl, ABA, mannitol, or antibiotics. The seedlings were cultured under an 8-h or 16-h photoperiod with a light intensity of about 130 mol μm-2 s-1, and after 10 days, the fresh weight and root length were measured, and the pictures were taken.

Molecular complementation and generation of transgenic plants

For molecular complementation, the coding sequence of STCH4 was PCR-amplified using the primers listed in Table S1 and cloned into the vector pCambia1305. The FLAG-tagged constructs were transferred into the Agrobacterium strain GV3101 for Agrobacterium-mediated floral dipping transformation of the stch4 mutants (the homozygous SALK_040068 plants). For STCH4 overexpression, the STCH4 coding sequence was PCR-amplified using the primers listed in Table S1 and cloned into the vector PGWB2. For STCH4 subcellular localization assay, the STHC4 CDS was PCR-amplified and cloned into the vector PGWB6 to create the STCH4-GFP fusion construct. To generate the CBF:GUS lines, CDSs of the CBF genes were PCR-amplified and cloned into the vector pCambia1301 to create CBF-GUS protein fusion. The constructs were transformed into *Arabidopsis* by using floral dipping method with the Agrobacterium strain GV3101.

RNA blot analysis

Total RNA was extracted from 2 g of 2-week-old seedlings with or without cold treatment using Trizol reagent (Invitrogen, USA). RNA samples (10 μg) were electrophoresed in 1.2% agarose/formaldehyde gel and transferred to a nylon membrane. For small RNA blot analysis, fractionated small RNAs (5 μg) were resolved in 6% Polyacrylamide-urea gel and transferred to a nylon membrane. The RNAs were crosslinked to the nylon membrane by UV light. For small RNA hybridization, DNA oligomer probes are listed in Table S1. The probes were labeled by T4-PNK (NEB, USA) with (γ-32P) dCTP. Hybridization was carried out at 37°C overnight in a hybridization buffer (Sigma, USA). The membranes were washed by SSC buffer and then exposed to X-ray films at −80°C overnight. For mRNA and rRNA hybridization, probes were amplified by PCR from a genomic DNA or cDNA templates and labeled by 9-mer random primers with (α-32P) dCTP using the Ladderman labeling kit (Takara, Japan) or directly synthesized as primers and labeled with (γ-32P) dATP using T4 PNK (NEB). Hybridization was performed at 56°C overnight in a hybridization buffer (Sigma, USA). The membranes were washed by SSC buffer and exposed to X-ray films at −80°C overnight.

RNA-seq analysis

14-day-old wild-type and stch4-1 seedlings grown on 1/2 MS medium (1/2 × MS salts, 2% sucrose, 0.6% agar, pH 5.7) were treated without or with 4°C for 4 or 24 hours for total RNA extraction. Total RNA was isolated with the Trizol reagent (Invitrogen, USA) and treated with TURBO DNA-free Kit (Ambion) to remove genomic DNA contaminants. RNA-seq libraries were constructed following the standard Illumina protocols. Three biological replicates for each sample were used for RNA-seq. Illumina sequencing was performed in the Shanghai Center for Plant Stress Biology with an Illumina HiSeq 2500 System.

For each sample, RNA-seq raw reads were trimmed using Trimmomatic v0.32 and PRINSEQ v0.20.4 (Bolger et al., 2014; Schmieder and Edwards, 2011). PRINSEQ was subsequently employed to mainly remove low-complexity reads. The remaining reads were then aligned to *A. thaliana* genome sequence and the reference-annotated genes (TAIR10) using TopHat v2.0.13 program (Kim et al., 2013b) with customized parameters specially for our RNA-seq libraries from plant *A. thaliana*. Transcriptomes were reconstructed for each sample by Cufflinks v2.2.1 (Trapnell et al., 2013). To obtain a high confidence of transcriptomes, novel constructed transcript was filtered out when the abundance was < 20% (default is 10%) of the most abundant isoform for each gene. All of the constructed transcriptomes were then merged with the reference-annotated transcripts using Cuffmerge (Trapnell et al., 2013) to yield comprehensive re-annotated gene transcripts including known and novel annotated transcripts in our RNA-seq samples. Subsequently, significantly differentially expressed genes were predicted by Cuffdiff (Trapnell et al., 2013) using twofold change and multiple test P value < 0.05. The GO analysis performed on differentially expressed genes by AgriGO (Du et al., 2010; Tian et al., 2017). The heat-map generated by R ggplot2 package.

Quantitative real-time PCR

Total RNA was extracted as described above. The first-strand cDNA was synthesized from 1 μg purified RNA using oligo-dT and SuperScript III Reverse Transcriptase. The 10x diluted cDNA samples were used as templates and quantitative real-time PCR was performed by using Bio-Rad CFX Manager with 2x real-time PCR mix (Takara, Japan). The ubiquitin 10 gene was used as an internal control. Each RT-qPCR assay had three independent biological samples and 3 technical replicates. RT-qPCR results were analyzed by ΔΔCT method.

Protein synthesis assay

Wild-type and stch4 mutant seeds were germinated and seedlings were grown on a 1/2 MS medium containing 1% sucrose and 0.6% agar. Two-week-old plants grown under 22°C were labeled with 250 μCi /mmol [35S]Met and [35S]Cys (EXPRE35S35S Labeling Mix, NEN) that was diluted to 1/2 MS liquid medium by directly placing plants onto filter paper soaked with 1/2 MS liquid medium containing 250 μCi /mmol [35S]Met and [35S]Cys. The plants were then incubated at 4°C or 22°C for 1 day or 3 days. Treated plants were then harvested and rinsed briefly in water, and total proteins were extracted by homogenization in a protein-loading buffer. The protein samples were denatured by boiling for 10 mins and separated in a 10% SDS-PAGE gel. The gel was stained or dried, and then exposed to an X-ray film.

GUS assay

X-gluc staining was performed as described by Sessions et al. (1999). The seedlings were stained with X-gluc solution for 4 hours at 37°C, and the samples were then bleached with 70% (v/v) ethanol. Pictures were taken with microscopy. For GUS activity assay, the fluorescence of 4-methylumbelliferone (4-MU), the product of GUS-catalyzed hydrolysis, was measured using the TECAN CENios system. The protein concentration in the supernatant was assessed by the Bradford method (Bradford, 1976). GUS activity was normalized with the protein concentration and calculated as pmol of 4-MU per milligram of soluble protein per minute.

Western blot analysis

Plant materials were frozen and ground into powder with liquid nitrogen. The ground powder (~50 mg) was resuspended in 100 μL of cold extraction buffer with 50 mM Tris-HCl, pH 8, and 50 mM NaCl supplemented with 1 μL of protease inhibitor cocktail (Sigma-Aldrich, P9599) before denaturing with SDS buffer. Proteins were separated using SDS-PAGE (7%–12% [w/v] acrylamide) and analyzed by western blotting using anti-CBFs antibodies (Donated by Yun DJ Lab), anti-ACT2 antibodies (Sigma), anti-GFP antibodies (Sigma) or anti-FLAG antibodies (Sigma).

Immunoprecipitation

Immunoprecipitation of STCH4-3xFLAG proteins from 14-day-old seedlings with or without cold treatment was performed as previously described (Schulze et al., 2010). Briefly, proteins were solubilized from plant powder using detergent buffer (25 mM Tris-HCl, pH 8, 150 mM NaCl, and 1% [w/v] NP-40 and protease inhibitor mixture). Anti-FLAG tag agarose beads (Sigma) were used to immunoprecipitate STCH4-3xFLAG from the protein extracts. The agarose beads were washed for 3 times with washing buffer, and the eluted proteins were analyzed by ''Proteomics and Metabolomics, Shanghai Center for Plant Stress Biology, Chinese Academy of Sciences'' using mass spectrometry.

Yeast two-hybrid assay

The coding sequences of RPL5A and other candidate genes that were identified by IP-MS as potential interactors of STCH4 were fused in-frame with the GAL4 DNA binding domain (BD) of the bait vector pGBKT7 (Clontech). The coding sequence of STCH4 was cloned in the prey vector pGADT7 (Clontech). The bait plasmid and the prey plasmid were co-transformed into the yeast strain AH109. The clones growing in the –L/-T liquid selection medium (Clontech) were diluted by a 10x series dilution and spotted onto –L/-T/-H medium (Clontech) to determine interactions between the two proteins.

Southern bolt analysis

For Southern blot analysis, genomic DNA was prepared following a previously described protocol (Watson and Thompson, 1986). The genomic DNA (10 μg) was digested with restriction endonucleases Hind III. Hybridization and washing were carried out according to the published procedure (Church and Gilbert, 1984). The probe of GUS was generated by PCR amplification and labeled with (α-32P) dCTP using the Ladderman labeling kit (Takara, Japan)

Protein localization assays

For localization of GFP and STCH4:GFP fusion proteins, transient protein expression assays were performed by Agrobacterium–mediated transfection (agroinfiltration) in N. benthamiana leaves (Liu et al., 2002). After 48 hours of incubation at 25°C in dark, cells expressing GFP or STCH4:GFP fusion proteins were observed and photographed by using Zeiss point scanning confocal LSM700 Upright microscope equipped with LSM imaging system.

Protoplast transformation

Transient expression in leaf mesophyll protoplasts isolated from stch4 mutant and Col-0 plants was performed using a polyethylene glycol transformation method as described previously (Yoo et al., 2007). The epidermal layers were removed by using 3M Magic tapes and the resulting leaf materials were digested by the cell wall digesting enzymes solution for 1 hour. The protoplasts were diluted by adding equal volume of W5 solution and then filtered through with a 70 μm nylon mesh. The collected protoplasts are precipitated by centrifuge at 1400 rpm, washed twice with W5 solution, and incubated on ice for 60-90 mins. The supernatant

was discarded, and the protoplasts were re-suspended in MMG solution. The protoplasts were then transformed using the PEG method. After transformation, the protoplasts were collected, resuspended with W5 buffer, and transferred to six-well plates for confocal microscopy.

Bioorthogonal Noncanonical Amino Acid Tagging (BONCAT) in plant

BONCAT was performed as described in a previous report (Glenn et al., 2017). In brief, to label the newly synthesized proteins, AHA was pulsed into 1-week-old seedlings by submerging the seedlings in the AHA-containing medium for 2 min. The medium was then discarded, and the seedlings were immediately placed at 4°C for 4 hours. Nascent proteins were enriched by DBCO-agarose beads and subjected to LC-MS/MS analysis.

QUANTIFICATION AND STATISTICAL ANALYSIS

In general, data are represented by mean ± SD. Biological repeats timers (n) are provided in each figure legend. One-way analysis of variance (ANOVA) was performed to check for statistically significant differences. For details of RNA-seq data analysis can be found in methods.

DATA AND CODE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE141304