RESEARCH ARTICLE

Chloroplast RNA-Binding Protein RBD1 Promotes Chilling Tolerance through 23S rRNA Processing in Arabidopsis

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Abstract

Plants have varying abilities to tolerate chilling (low but not freezing temperatures), and it is largely unknown how plants such as Arabidopsis thaliana achieve chilling tolerance. Here, we describe a genome-wide screen for genes important for chilling tolerance by their putative knockout mutants in Arabidopsis. Out of 11,000 T-DNA insertion mutant lines representing half of the genome, 54 lines associated with disruption of 49 genes had a drastic chilling sensitive phenotype. Sixteen of these genes encode proteins with chloroplast localization, suggesting a critical role of chloroplast function in chilling tolerance. Study of one of these proteins RBD1 with an RNA binding domain further reveals the importance of chloroplast translation in chilling tolerance.
in Arabidopsis. Among the proteins encoded by these 49 genes, 16 are annotated as having chloroplast localization, suggesting a critical role of chloroplast function in chilling tolerance. We further studied RBD1, one of the four RNA-binding proteins localized to chloroplast. RBD1 is only expressed in the green photosynthetic tissues and is localized to nucleoid of chloroplasts. Furthermore, RBD1 is found to be a regulator of 23S rRNA processing likely through direct binding to the precursor of 23S rRNA in a temperature dependent manner. Our study thus reveals the importance of chloroplast function especially protein translation in chilling tolerance at genome-wide scale and suggests an adaptive mechanism involving low temperature enhanced activities from proteins such as RBD1 in chilling tolerance.

Introduction

Low temperature inhibits plant growth in general and limits the geographical distribution of plants. Earlier studies have identified numerous physiological and cellular changes associated with chilling (more than 0°C) or freezing (less than 0°C) conditions, such as alterations in membrane composition, calcium signals, metabolite composition, photosynthesis, and protective molecules [1,2]. Most of these changes are thought to help plants to cope with low temperature stresses. Plants differ in their abilities to tolerate chilling stresses. Low temperature often inhibits photosynthesis and reduces carbon uptake and allocation to developing sink tissues [3,4]. Many tropical and subtropical plants including maize, rice, and tomato do not survive at 4°C because they cannot undergo photosynthesis and carbon metabolism under this condition. Arabidopsis, as well as some overwinter cereals, can grow at the low temperature due to their biochemical and physiological adaptations which may include acclimation of photosynthetic metabolism [5,6].

Translation in chloroplast appears to be especially sensitive to chilling stresses. Chilling slows down protein biosynthesis in plastids by eliciting frequent ribosome pausing in tomato [7]. The otherwise chilling tolerant Arabidopsis plants become chilling sensitive when they are defective in chloroplast ribosomal biogenesis and RNA processing [8–12]. For instances, loss of the translation elongation factor SVR3, the rRNA maturation factor NUS1, and chloroplast RNA binding proteins CP29A and CP31A, all lead to increased chilling sensitivity through affecting chloroplast biogenesis [9,10,13]. In addition, the loss of chloroplast ribosomal subunits reduces the ability of plants to recover from prolonged chilling periods [11,12].

Chloroplast function is carried out by genes coded mostly by the chloroplast genome [14]. Transcription of such genes relies on two plastid RNA polymerases: nucleus-encoded RNA polymerase (NEP) and plastid-encoded RNA polymerase (PEP) [15,16]. Chloroplast RNAs need to be processed to become functional rRNAs and mRNAs. Many of the processing factors for RNA cleavage, splicing, editing or stability are RNA-binding proteins [13,17–19]. They are all coded by the nuclear genome. One family has pentatricopeptide repeats (PPR) and it usually carries out specific RNA processing in chloroplasts [19]. Another family contains RNA recognition motif/RNA-binding domain/ribonucleoprotein (RRM/RBD/RNP) domain and these proteins, referred to as RNPs, are suspected to regulate larger sets of RNAs [20]. Among the chloroplast RNPs, CP31A and CP29A are associated with a large pool of chloroplast transcripts and influence their stability, processing, and splicing [13].

While chilling tolerance mechanism is not well understood, cold acclimation, an enhancement of freezing tolerance by a prior exposure to low non-freezing temperature, has been intensively studied in *Arabidopsis thaliana* [21–25]. Cold acclimation is mediated in part by C-
repeat binding factors (CBFs) which regulate the expression of a large number of Cold Responsive (COR) genes, some of which are thought to confer freezing tolerance. The upregulation of the CBF genes by low temperature is critical for this acclimation and is mainly modulated by ICE1 (Inducer of CBF expression 1), ICE2 and the three closely related CAMTAs (Calmodulin binding Transcription Activators) [26–29]. Although genes regulated by CBF proteins play vital roles in freezing tolerance, they only represent a small percentage of the COR genes. CBF independent regulations of cold acclimation or freezing tolerance have been found in Arabidopsis [30,31]. It is yet to be determined whether or not chilling tolerance and cold acclimation have shared mechanisms.

To have a better understanding of chilling tolerance in Arabidopsis, we carried out a chilling sensitive mutant screen on all available T-DNA insertion mutants that represent half of the total Arabidopsis genes. Interestingly, mutants defective in chloroplast localized proteins are overrepresented, indicating the importance of chloroplast function in chilling tolerance. Detailed characterization of one such mutant of a RNA binding protein supports a critical role of chloroplast translation in chilling tolerance.

**Results**

**Screening for chilling sensitive mutants from a large collection of T-DNA mutants**

In order to identify genes important for chilling tolerance in Arabidopsis at the genome scale, we analyzed 11,000 T-DNA insertion mutants covering half of the total Arabidopsis genes for chilling sensitive phenotypes. Each of them is putative homozygous T-DNA knockout or knockdown mutants in indexed genes generated by the SALK Institute [32] and available from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/). The use of these indexed knockout or knockdown lines over chemical mutagenized population enabled the assessment and verification of phenotypes under different growth conditions and allowed uncovering of conditional lethal mutants and avoiding temperature-sensitive alleles of essential genes. In this screen (S1 Fig), four mutant seeds of each line were germinated and grown side by side with the wild-type Col-0 on vertical plates under a 16 hours (h) light/day photoperiod first at 22°C for 8 days and then transferred to 4°C for two months. Lines showing abnormal growth phenotypes compared to the wild type only at 4°C but not 22°C were selected as chilling sensitive mutants. These phenotypes include albino, yellow or purple leaves, abnormal leaf shapes, smaller leaves, and shorter roots. Because not all of these T-DNA lines were homozygous for the T-DNA insertion mutations, we further analyzed those lines where not all four seedlings exhibited mutant phenotypes to exclude those whose mutant phenotypes are not correlated with the T-DNA insertion. From this screen, a total of 54 lines showed growth defects at 4°C but not 22°C and we defined these mutants as chilling sensitive (Fig 1A).

We then examined the genes indexed to be disrupted by T-DNA insertions in these lines. Those with insertion in the promoter region but not exon, intron, or UTRs were removed as they might not affect the function of the indexed gene and the phenotype is unlikely due to a defect in the gene. This leaves us with 49 chilling sensitive mutant lines where the function or expression of the indexed genes are disrupted. None of the 49 genes are in the characterized CBF cold acclimation pathway, and loss of function mutants of CBF1, CBF2, CBF3, ICE1, HOS1 and SIZ1 were not in the collection. Although causal genes for chilling sensitivity may not be the indexed gene in a small proportion of the lines, we decide to analyze functional categories of these candidate genes as a group to reveal potential important chilling tolerance mechanisms. Among proteins encoded by the 49 candidate genes, 16 were annotated as localized to the chloroplast (Table 1), 10 to the nucleus, 6 to the cytosol, 4 to the mitochondria, and...
the rest either to other locations or without localization information. There is thus a high representation of chloroplast related genes in these mutants, suggesting a critical role of chloroplast function in chilling tolerance.

Among the 16 proteins with chloroplast localization, 4 were chloroplast RNP proteins and three were previously characterized: ORRM1 (AT3G20930), CP29A (AT3G53460) and CP31A (AT4G24770). Mutants of CP29A and CP31A were chilling sensitive and defective in processing various RNAs in chloroplast [13]. ORRM1 is required for plastid RNA editing in Arabidopsis and maize [33], but its role in chilling tolerance has not been analyzed. The other RRM/RBD/RNP coding gene, AT1G70200, was not characterized before, and we named it RBD1.

Mutants of all of these 4 genes showed bleaching in newly emerging leaves at 4°C. A mutant of another RNP coding gene AML1 (AT5G61960) also had a bleaching phenotype. AML1 is predicted to localize in the nucleus but it may also have a chloroplast localization signal from the RNA-binding protein RBD1 is important for 23S rRNA processing and chilling tolerance in Arabidopsis

Fig 1. Chilling sensitivity of the RNP mutants. (A) Shown are wild type Col-0, orrm1, and aml1 mutants grown for 2 months at 4°C on the plate under a 16 h light/day photoperiod. Representative chilling sensitive phenotypes are shown. (B) Shown are wild type Col-0, rbd1-1, orrm1, aml1, cp29a, and cp31a mutants grown at 22°C and 4°C. Top row: Plants grown for 3 weeks at 22°C under a 12 hour (h) light photoperiod. Bottom row: Plants grown for 3 weeks at 22°C, then shifted to 4°C for additional 4 weeks under a 12 h light photoperiod. (C) Shown are fresh weights of the 5 RNP mutants grown at 22°C and 4°C for 3 weeks and 10 weeks respectively under a 12 h light photoperiod.

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Table 1. Chilling sensitive mutants potentially defective in chloroplast-localized proteins.

| T-DNA line | Gene ID       | Insertion site | Notes                                                                 |
|------------|---------------|----------------|----------------------------------------------------------------------|
| SALK_003066C | AT3G53460     | exon           | CP29; RNA-binding family protein                                       |
| SALK_055951C | AT4G24770     | exon           | CP31; RNA-binding family protein                                       |
| SALK_067017C | AT5G52440     | 300-UTR5       | HCF106; Thylakoid membrane delta pH translocation pathway component protein |
| SALK_113136C | AT3G21300     | exon           | RNA methyltransferase family protein                                   |
| SALK_071288C | AT1G05140     | 300-UTR5       | Peptidase M50 family protein                                          |
| SALK_090222C | AT4G10030     | intron         | ABHD11; alpha/beta-Hydrolases superfamily protein                     |
| SALK_107644C | AT2G37230     | exon           | Tetratricopeptide repeat (TPR)-like superfamily protein               |
| SALK_018914C | AT3G22690     | exon           | YS1; TPR protein                                                      |
| SALK_072648C | AT3G20930     | exon           | ORRM1; a RIP and a RNA-binding family protein                         |
| SALK_074256C | AT5G23070     | exon           | ATTK1B; a thymidine kinase                                            |
| SALK_075943C | AT5G52370     | exon           | Unknown protein                                                       |
| SALK_023468C | AT2G01918     | exon           | PQL3; required for NDH activity.                                      |
| SALK_012657C | AT1G70200     | exon           | RNA-binding family protein                                             |
| SALK_108952C | AT4G28210     | 300-UTR5       | EMB1923; involved in embryo development                               |
| SALK_122911C | AT3G08920     | exon           | Rhodanese/Cell cycle control phosphatase family protein              |
| SALK_013094C | AT4G31690     | exon           | Transcriptional factor B3 family protein                              |

RNA-binding protein RBD1 is important for 23S rRNA processing and chilling tolerance in Arabidopsis [34], but its role in chilling tolerance was not analyzed. We therefore further analyzed chilling sensitive phenotypes of mutants of these 5 RNP coding genes. When grown on soil at the normal temperature 22°C, the rbd1, cp29a and cp31a mutants exhibited a wild-type phenotype, while orm1 and aml1 had a smaller size than the wild type Col-0 (Fig 1B). The aml1 had yellow cotyledons at germination but recovered in a few days, and it often had white strips on the leaves. Consistent with visual phenotypes, the rbd1, cp29a and cp31a mutants had a fresh weight similar to that of the wild type at 22°C, but orm1 and aml1 mutants displayed a 35% and 58% reduction compared to the wild type (Fig 1C). When shifted to 4°C after grown at 22°C for three weeks, new leaves emerged from all of these mutants were yellow (Fig 1B). When these mutants were grown constantly at 4°C in soil from germination, all their leaves were yellow and the plants were much smaller than the wild type with only 10% to 35% of the wild-type fresh weight (Fig 1C).

**RBD1** is required for chloroplast function especially under chilling stress conditions

We chose RBD1 for further analysis because there were no prior reports on this gene. The RBD1 gene encodes a protein of 538 amino acids (aa) containing one RNA recognition motif/RNA-binding domain (Fig 2A). We confirmed that the chilling sensitive phenotype observed in the mutant line SALK_041100 is due to the loss of the RBD1 function through analysis of additional RBD1 mutants and complementation test. The SALK_041100 line (named rbd-1) has the T-DNA inserted in the third exon of the RBD1 gene, while the T-DNA insertion line SALK_012657 (named rbd1-2) has an insertion in the 3'-UTR of RBD1 (Fig 2B). Both mutants were loss-of-function mutants of RBD1 as no full length transcripts could be amplified by Reverse Transcription (RT)-PCR (Fig 2C). The rbd1-2 mutant, like rbd1-1, produced yellow emerging leaves at 4°C (Fig 2D). In addition, RNA interference (RNAi) was used to reduce the expression of RBD1, and 13 of 15 RNAi transgenic lines produced yellow leaves under chilling conditions.
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Fig 2. Characterization of the rbd1 mutants. (A) Structure diagram of the RBD1 protein. It contains one RNA recognition motif/ RNA-binding domain between 188–267 amino acid (aa) predicted at SMART (http://smart.embl-heidelberg.de/). (B) Diagram of T-DNA insertion mutants of the RBD1 gene. Arrows indicate the translation start (ATG) and stop (TAA) codons. Triangles indicate the site of T-DNA insertion in the SALK_041100 (rbd1-1) and SALK_012657 (rbd1-2) mutants. 'a' and 'b' mark the regions for RT-PCR analysis of RBD1 gene expression. (C) Semi-quantitative RT-PCR analysis of RBD1 expression in rbd1 mutants. The Actin gene was used as a normalization control. Bands of each row are cut from the same gel. (D) Phenotype of rbd1 mutants, rbd1 RNAi and complementation lines. Top row: plants grown for 3 weeks at 22°C with 12h light /12h dark. Bottom row: plants grown for 3 weeks at 22°C, then shifted to 4°C for additional 4 weeks with 12h light /12h dark. (E) The rbd1-1 plants at 2 days of 22°C recovery after 3 weeks of 4°C treatment. (F) The accumulation of pigments in rbd1 mutants and complementation lines. Shown are contents of chlorophyll a, chlorophyll b and carotenoid from the fifth and sixth leaves of 2 weeks old plants grown at 22°C with constant light. Means ± SD, n = 3, * represents significant differences between means at P < 0.05 according to Student’s t test.

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conditions (Fig 2D). Furthermore, when the full-length RBD1 cDNA driven by the cauliflower mosaic virus (CaMV) 35S promoter was transformed into the rbd1 mutants, the chilling sensitive phenotype was suppressed in 30 of 32 the transgenic lines (Fig 2D). Therefore, the loss of RBD1 function does confer chilling sensitivity.

We characterized the growth phenotypes of the rbd1-1 mutants in more detail. When plants were shifted from three weeks’ growth at 22°C to 4°C, the yellowing or bleaching became visible only after 2 weeks of chilling treatment and only happened in leaves emerged at 4°C. There is a gradient of yellowing decreasing from the youngest leaf to the oldest leaves (Fig 2D). This yellowing phenotype is reversible, when plants were moved from 4°C back to 22°C, the pale green appearance compared to the wild type when grown constantly at 22°C. Using spectrophotometer, we found that the two rbd1 mutants had a 10–20% reduction of chlorophyll a, chlorophyll b and carotenoids compared to the wild-type Col-0. This phenotype was not observed in the complemented lines (Fig 2F). Therefore, the rbd1 mutants are compromised in chloroplast function especially under chilling conditions.

Loss of RBD1 does not affect the expression of CBF or COR genes

Because the rbd1 mutants displayed chilling sensitivity, we analyzed whether chilling induction of the CBF and COR genes was altered in the rbd1 mutants. CBF1, CBF2, and CBF3 are induced by 4°C treatment at 6 hours to the same extent in Col-0 and rbd1-1 (Fig 3A). Similarly, these genes fall to the basal level after 3 weeks of 4°C treatment in both the wild type and the mutant (Fig 3A). COR15A, COR47, and KIN1 genes are regulated by the CBF genes, and they were induced by 4°C treatment at 6 hours and stayed elevated during the subsequent chilling treatment for four weeks (Fig 3B). The rbd1-1 mutant had the same induction kinetics and amplitudes of these three COR genes by 4°C treatment (Fig 3B). Therefore, the rbd1-1 mutant is not impaired in its capacity to induce the CBF or COR expression by 4°C and the chilling sensitivity of the mutants is likely independent of the CBF pathway.

The expression pattern of the RBD1 gene

The RBD1 gene is expressed in green tissues. Using RT-PCR analysis, RBD1 mRNA was detectable in leaves, stems, flowers and siliques but not in roots (Fig 4A). The expression pattern of RBD1 was further investigated in transgenic lines containing a β-glucuronidase (GUS) reporter gene fused to the RBD1 promoter. GUS expression was observed in all green tissues throughout development (Fig 4B). In 3-day-old seedlings, GUS staining was primarily detected in emerging cotyledons (Fig 4B). In 14-day-old seedlings, it was detected in most of the plant but not in roots, and was particularly strong in cotyledons (Fig 4B). GUS staining was also observed in the stems and siliques, but not in the matured seeds (Fig 4B). Within the flower, it was strong in the sepals and carpels, but not in the stamens and petals (Fig 4B). In addition, older green tissues usually had higher expression levels than younger green tissues, suggesting that RBD1 might contribute to the function of mature green tissues. Altogether, these expression patterns show that RBD1 is highly expressed in the photosynthetic tissues, which was in agreement with its putative roles in chloroplast function.

RBD1 is not induced by light according to the expression data from Arabidopsis eFP Browser (http://bbc.botany.utoronto.ca). It is not induced by chilling treatment either. RT-PCR revealed that RBD1 is expressed at the same level during the chilling treatment from day 0 to day 21 (Fig 3C). The RBD1 gene therefore might be constitutively expressed at the transcript level.
RNA-binding protein RBD1 is important for 23S rRNA processing and chilling tolerance in Arabidopsis

RBD1 is localized to the chloroplast

RBD1 is annotated as a chloroplast targeted protein, which could explain its expression in green tissues. To verify the subcellular localization of the RBD1 protein, we expressed GFP (Green fluorescent Protein) fusion of RBD1 under the 35S promoter (RBD1:GFP) in protoplasts isolated from wild-type Arabidopsis leaves and monitored fluorescence by confocal microscopy. While the GFP protein alone was present in the cytoplasm and nucleus, the RBD1:GFP fusion protein was found exclusively in chloroplasts (Fig 4C). In addition, the
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RBD1:GFP fusion protein was dispersed as small fluorescent particles within chloroplast (Fig 4C), which is reminiscent of nucleoid localization [35]. This localization pattern does not appear to be temperature-dependent. In both 22°C and 4°C, RBD1:GFP is only present in the nucleoid structure in chloroplast (S2 Fig).

The rbd1 mutants are defective in processing of 23S rRNA

The nucleoid localization pattern and the yellowish leaf mutant phenotype promoted us to investigate the role of RBD1 gene in chloroplast RNA processing because nucleoid is the site of rRNA processing and ribosome assembly [36]. We first analyzed total RNAs separated on formaldehyde denaturing agarose gel stained by ethidium bromide. Plastid rRNAs (23S, 16S, 5S, and 4.5S) and cytosolic rRNAs (25S, 18S, 5.8S, and 5S) can be easily observed on such gels because they are very abundant [37]. When the same amount of total RNAs was loaded on gel, rbd1-1 and rbd1-2 mutants showed significant reduced accumulation of the 1.1 and 1.3-kb species of the 23S rRNA compared to the wild type under chilling stress conditions, but exhibited almost the same amount of rRNAs as wild type at 22°C (Fig 5A).
RNA gel blot hybridization was used to further analyze the defects in 23S rRNA processing. The 23S rRNA with a full length of 2.9-kb is cleaved internally at two sites, yielding fragments of 0.5, 1.1, and 1.3-kb in wild type [38]. With a probe detecting the 3'-end fragments, we found that 4°C treatment did not affect the processing of 23S rRNA transcripts in the wild type (Fig 5B). In contrast, the chilling treated rbd1 mutants accumulated the partially processed 2.9-kb and the 2.4-kb of 23S rRNA at a much higher level than the wild type, while the fully processed product of 1.1-kb rRNA was greatly reduced (Fig 5B).

Consistent with a defect in processing of 23S rRNA which is a component of the chloroplast ribosome, the rbd1 mutants had a reduction in chloroplast translated protein. When total leaf proteins were analyzed on a SDS polyacrylamide gel, the chloroplast Rubisco Large Subunit (RbcL) protein, which is synthesized in chloroplast, was found to have a drastic reduction in
The rbd1 mutants exhibit a defect in 23S rRNA processing earlier than other defects

We further analyzed the transcripts of 9 chloroplast RNAs in the rbd1 mutants under normal and chilling conditions to assess how broad a role the RBD1 gene might play in chloroplast RNA regulation. They are 16S rRNA, PEP transcribed ndhF, psaA, rbcL, psbB, psbF and petB, NEP transcribed ycf3, and PEP and NEP transcribed rps4. Among these, 16S rRNA, ndhF, psbB, petB, ycf3 and rps4 need to be processed to become mature transcripts.

In contrast to that of 23S rRNA, the processing of 16S rRNA was only slightly affected in the rbd1 mutants compared to Col-0 at 4°C but not 22°C (S3B Fig). The transcripts for ndhF, psaA and rbcL showed decreased levels under chilling stress compared to the wild-type plant, but displayed similar levels to wild type at 22°C (S3A Fig). For psbB, psbF and petB, low temperature down regulated their transcripts levels, but the transcripts were at similar levels in the rbd1 mutants and wild type at 22°C and 4°C (S3B Fig). The transcripts of ycf3-ex2 and rps4 showed an over-accumulation under chilling condition in the rbd1 mutants compared to the wild type, but exhibited the same levels at 22°C (S3C Fig). In all, the loss of RBD1 mainly alters processing of 23S rRNAs but not other chloroplast RNAs we analyzed. It also reduces the expression level of some PEP transcribed genes, but increases the expression of some NEP transcribed genes at chilling temperature.

The leaf yellowing phenotype in the rbd1 mutants likely results from an accumulative defect over time because it only became visible two weeks after plants were shifted from 22°C to 4°C. To identify earlier defects in the mutants, we monitored the molecular events after the plants were shifted from 22°C to 4°C at 0 hour (h), 6 h, 1 day (d), 3 d, 7 d, 14 d, 21 d and 28 d. Among the 6 genes that showed altered processing and expression in the mutants, 23S rRNA was the first to show defects. Its processed 1.1-kb transcript was significantly lower than the wild type after 7 days of 4°C treatment (S4A Fig). The transcript of psaA showed defects in the rbd1 mutants after 2 weeks of cold treatment (S4B Fig). The transcripts of the rest ndhF, rbcL, ycf3-ex2 and rps4 genes began to show significant change after 3 or 4 weeks of chilling treatment (S4 Fig). Therefore, the rbd1 mutants exhibit a defect in 23S rRNA processing very early on (S4B, S4C and S4D Fig), which would lead to reduced chloroplast translation and bleaching phenotype under chilling conditions.

RBD1 binds to 23S rRNA transcripts in a temperature dependent manner

To determine whether or not RBD1 protein is associated with the 23S rRNA and the association is temperature regulated, we performed RNA co-immunoprecipitation (IP) assay at both 22°C and 4°C. RBD1 was fused to the GFP (RBD1:GFP) and expressed under the strong 35S promoter in Arabidopsis protoplasts. As a control, the signal peptide of ORRM1 that targets the protein to chloroplast was fused with GFP (sORRM1:GFP) for protoplast expression as well. After transformation, the protoplasts were incubated at 22°C for 8 h before being split for
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Further incubation at 22°C and 4°C respectively for 12 h to assess temperature dependency of binding. Total proteins from protoplasts were then IPed by the anti-GFP antibodies, and the co-IPed RNAs (after DNase I treatment) were reverse transcribed and detected by quantitative Real Time-PCR. We analyzed four transcripts: 23S rRNA, 16S rRNA, psbF, and rbcL and found binding only to 23S rRNA (Fig 6). Three regions of the 23S rRNA precursor were analyzed: 5' end, middle part, and 3' end, residing in three different processed RNAs. There was an 8, 11 and 6 fold increase of PCR products for the three regions of 23S rRNA precursor in the RBD1:GFP sample compared to the sORRM:GFP sample at 22°C (Fig 6A). Furthermore, the fold increase for the three regions were 23, 39 and 19 fold in the RBD1:GFP sample compared to the sORRM:GFP at 4°C (Fig 6B). Therefore, RBD1 binds to three regions of 23S rRNA.

![Fig 6. RBD1 binds to the 23S rRNA in a temperature dependent manner.](image-url)

(A) Shown are relative amounts of 23S rRNA transcripts co-immunoprecipitated (co-IPed) from RBD1 versus sORRM1 analyzed by qRT-PCR. Primer pairs for 5'-terminal (5'), middle part (M), and 3'-terminal (3') of 23S rRNA were respectively used to amplify RNAs from immuno-precipitates after reverse transcription. DNase I was used to treat the precipitated samples before reverse transcription (RT), and samples not subject to RT were used as control in PCR to ensure there is no genomic DNA contamination. (B) Shown are relative amounts of transcripts of 16S rRNA, psbF and rbcL co-IPed from RBD1 versus sORRM1 analyzed by qRT-PCR. The GFP fusions of RBD1 (RBD1:GFP) and the signal peptide of ORRM1 (sORRM1:GFP) were expressed in protoplasts of wild-type Arabidopsis leaves at 22°C and 4°C, and anti-GFP antibodies were used to precipitate the GFP fusions. '-' and '+' mean no antibodies and added antibodies respectively.

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Mutants of the 5 RNP genes have both specific and shared defects

We further compared the molecular defects among the 5 RNP mutants after 4 weeks of 4°C treatment. The rbd1 mutants had the most drastic defect in 23S rRNA processing among the 5 RNP mutants. By RNA gel staining, reduction of processed 23S rRNAs compared to the wild type was the most pronounced in the rbd1-1 mutant (Fig 7A). By RNA blotting, the processed 1.1-kb transcript was found to decrease in all of these 5 RNP mutants, with the most severe reduction happening in rbd1-1. The partially processed 2.9- and 2.4-kb transcripts were over-accumulated in these mutants at 4°C except for orrm1 (Fig 7B). Among the 5 RNP mutants, the rbd1-1 mutant exhibited the highest ratio of partially processed transcripts (2.4- and 2.9-kb) over processed transcripts (1.1-kb). Consistent with the observed 23S rRNAs processing defect in the 5 RNP mutants, the accumulation of RbcL protein at 4°C was reduced to different extent, with an approximately 20% to 40% reduction compared to the wild type (Fig 7C). In contrast to the RbcL protein, TOC75, a cytosol synthesize chloroplast outer membrane protein [40–42], showed an increased amount of 20% to 40% at 4°C in the 5 RNP mutants compared to the wild type (Fig 7D). In addition, rbd1-1 showed the highest sensitivity to spectinomycin followed by aml1 among the 5 RNP mutants assayed (Fig 7E). At 3mg/L concentration, all rbd1-1 seedlings turned white and the aml1 mutant was pale yellow, while the other three mutants stayed green similarly to the wild type. These results indicate that loss of RBD1 has a larger effect on 23S rRNA processing compared to other mutants, which may impact chloroplast translation more than the others. It also shows that the ORRM1 mutation has the least impact on rRNA processing.

Discussion

In this genome-wide chilling sensitivity screen, we identified 54 T-DNA insertion lines that exhibited a chilling sensitive phenotype in Arabidopsis. Although we have not tested if all results from disruption of the gene that the T-DNA is inserted in, we expect that most of them are. Interestingly, a large proportion of these genes are related to chloroplast function, indicating that it is one of the weakest links in chilling tolerance. Earlier studies have implicated a strong association of compromised chloroplast function with chilling tolerance, and this study supports this notion at the genome wide scale. Wild-type Arabidopsis plants usually survive at low temperatures such as 4°C but mutants with compromised chloroplast function are sensitive to chilling similarly to the subtropical and tropical plants. To what extent difference in chilling tolerance among different plants is due to difference in chloroplast function at low temperatures is worth studying in the future. This screen also suggests that chilling tolerance and cold acclimation may not use identical mechanisms in Arabidopsis. Mutants of the known key regulators of the CBF pathway, which are unfortunately not present in the collection, are not known or reported to have chilling sensitive phenotypes. Mutants identified in the chilling sensitive screen here were not isolated as defective in the CBF pathways mutants. Indeed, the rbd1-1 mutant is not defective in cold induction of the CBF pathway, suggesting that the induction of the CBF pathway may not require optimal chloroplast function and chilling sensitivity can result from CBF independent defect. Nevertheless, chilling tolerance can be connected with the CBF pathway. In Arabidopsis, a chilling sensitive mutant crlk1 (calmodulin receptor like kinase 1) is chilling sensitive and is also delayed in CBF induction [43]. In rice, a chilling
Fig 7. Analyses of chloroplast translation in the 5 RNP mutants. (A) Total RNAs extracted from the 5 RNP mutants were fractionated on 1.2% formaldehyde gels and stained with ethidium bromide. (B) RNA blotting analysis of 23S rRNA processing in the 5 RNP mutants. Total RNAs extracted from leaves were fractionated on 1.2% formaldehyde gels and hybridized to a probe described in Fig 5B. Equal loading was controlled by the cytosolic 25S rRNA (25S) stained with ethidium bromide. (C) Ponceau S Staining of total proteins from Col-0, rbd1-1, aml1, orrm1, cp29a and cp31a after SDS-PAGE separation. Accumulation of RbcL is indicated by an arrow. (D) TOC75 protein was detected by immune-blotting using specific monoclonal antibodies, loading control is shown in Fig 7C. (E) Shown are Col-0, rbd1-1, aml1, orrm1, cp29a, and cp31a mutants grown on solid media in the presence or absence of spectinomycin (spec) at 3 mg/L for 10 days. Plants were grown for 3 weeks at normal condition followed by 4°C for 4 weeks (A and B) or 2 weeks (C and D), and tissues were collected from five newly emerged leaves. Marked numbers indicate the relative amount in the mutant compared to that in Col-0 quantified by Image J.

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sensitive mutant cold1 is defective in CBF induction [44]. It is possible that chilling sensitivity can result from defects in multiple processes and some might be shared with the CBF pathway.

The chloroplast localized proteins critical for chilling tolerance are involved in multiple aspects of chloroplast function, including transcript regulation and maturation, chloroplast development, protein transportation and secretion, as well as photosynthesis. For instance, YSI is required for editing of rpoB transcripts and chloroplast development during early growth [45]. PQL3 is required for NDH activity in photosynthetic electron transport chain [46]. ATTK1B plays an important role in plant growth and development through the nucleotide salvage pathway [47]. Thus, the high proportion of chloroplast related genes in these mutants indicated a key role of chloroplast function in chilling tolerance in Arabidopsis.

We found in this study that RBD1, one of the chloroplast localized proteins identified in the chilling sensitive screen, is involved in 23S rRNA processing. The highly conserved rrr operon on chloroplast genomes encodes the 23S, 16S, 5S and 4.5S rRNAs and three tRNAs [48]. The primary precursor is initially processed to generate tRNAs, precursors of 23S, 16S, 5S and 4.5S rRNA by a series of endo- and exonucleolytic cleavages [49, 50]. The partially processed 23S rRNA is then excised at two sites named the hidden breaks [38] (Fig 5B). Several molecules have been found to be involved in this final processing step. The CSP41b endonuclease and the RH39 RNA helicase are shown to be involved in processing the 23S rRNA at the 1st and 2nd hidden breaks respectively [36, 38, 51]. The RNase E endonuclease as well as the PNPase and RNase R exonucleases may also be involved in the final processing of 23S rRNA because their loss of function mutants accumulate incompletely processed 2.9-kb and 2.4-kb of the 23S rRNA, although their main function is to excise the 23S-4.5S species to generate 23S and 4.5S rRNAs [50, 52, 53]. Our study identifies RBD1 as a new regulator of 23S rRNA processing. Loss of RBD1 causes over-accumulation of the partially processed 2.9-kb and the 2.4-kb of 23S rRNA and reduction of the fully processed 1.1-kb species while the total amount of 23S rRNA does not change significantly (Fig 5B). The RBD1 protein is localized to nucleoid where the processing happens (Fig 4C), and it binds to the 23S rRNA but not other RNAs tested (Fig 6). This indicates that RBD1 functions in the last step of 23S rRNA processing, and it may facilitate the processing by guiding the endonucleases to the hidden breaks. Further refining its RNA binding sequences and determining its interaction with processing enzymes will reveal more its mode of action. Compared to the other 4 RNP mutants with similar chilling sensitive phenotypes, the rbd1-1 mutant exhibited the strongest 23S rRNA processing defect (Fig 7B). It has additional chloroplast RNA defects, but they did not occur at normal growth temperature and were induced at a much later stage after plants were chilling treated (S3 and S4 Figs).

Because RBD1 does not appear to have enzymatic activities, it might interact with an RNase to bring it to the location for processing via its specific binding to the target RNA. Therefore, RBD1 is a positive facilitator of 23S rRNA processing. This function of RBD1 is more important at low temperatures than at normal temperatures. The rbd1 mutants show a slight defect at normal growth temperature but very strong defect at 4°C (Fig 5B and S4A Fig). Interestingly, RBD1 exhibited a higher association with the 23S rRNA at 4°C than at 22°C, suggesting a regulation of its activity by temperature. The enhanced binding of RBD1 to the precursor could compensate for a less efficient binding of the processing enzyme RNase to 23S rRNA, so that chloroplast translation machinery can be efficiently produced at low temperatures as well.

It is still yet to be determined how chloroplast function, especially translation, is the weak link in chilling tolerance. Likely, RNAs in chloroplast form more non- or less- functional secondary structures under chilling temperatures. Such structures of rRNAs may compromise the assembly or function of the translation machinery, while those of mRNAs may reduce their translation efficiency. RNA binding proteins such as RBD1 and other RNPs may reduce the
formation of such non- or less-functional structures and thus become more critical under chilling conditions.

Our study also indicates that various processes of RNA metabolism and protein translation in chloroplasts are inter connected. Different primary defects can have ripple effects leading to a similar chilling sensitive phenotype at later stages. For instance, RBD1 is closely associated with processing of the 23S rRNA transcripts. The primary defect of rbd1 mutants is likely the reduced processing and accumulation of mature 23S rRNA products. This will lead to defect of plastid ribosome especially under chilling conditions and subsequently the translation defect in the chloroplasts. Since the core subunits of PEP are synthesized on plastid ribosomes, the rbd1 mutants are expected to have reduced PEP level which will lead to a reduction in PEP-dependent mRNAs. As a compensation, the NEP dependent genes might become overexpressed [54]. Indeed, the chilling-treated rbd1 mutants had reduced transcript levels of the PEP-dependent genes tested such as ndhF, psaA and rbcL, while NEP-dependent genes such as ycf3 had higher expression. rps4, transcribed by both PEP (in green tissue) and NEP (in white tissue) [55], had an overexpression in rbd1 mutants under chilling condition (S3 Fig). The slight deficiency in 16S rRNA processing might be an indirect effect of losing the RBD1 function because abnormal ribosome assembly have been shown to indirectly affect RNA processing [56,57]. Interestingly, the rbd1 mutants had a reduced level of chloroplast synthesized RbcL protein, but increased level of cytosol translated TOC75 protein (Fig 7D), suggesting a compensation at the translation level as well.

Together, this study reveals the importance of chloroplast RNA-binding proteins in chilling tolerance, and further studies will further enhance our understanding of molecular mechanisms of chilling tolerance and its variations in different plant species.

Materials and Methods

Plant materials and growth conditions

Arabidopsis T-DNA insertion lines were obtained from Arabidopsis Biological Resource Center with the stock numbers CS27941, CS27942 and CS27943. Four seeds of each line were sterilized and sowed on 0.5× MS (Murashige and Skoog, Sigma) solid medium with 1.2% agar and 2% sucrose. Plants were grown on vertical plates under a 16 h-light/d photoperiod for 8 days before being transferred to 4°C for two months. When using soil, plants were grown under a 12h-light photoperiod with light intensity at 100 μmol m⁻² sec⁻¹ and relative humidity at 50–70%. For spectinomycin treatment, seeds were sterilized and planted on 0.5×MS medium containing 0.8% agar and 2% sucrose with 0–3 mg/L spectinomycin under the conditions described above.

Plasmid construction

For complementing the rbd1 mutants, a full-length At1g70200 cDNA was amplified and cloned into PMDC32 [58] to generate the construct of p35S::RBD1. For the promoter reporter construct pRBD1::GUS, a 1.5-kb sequence upstream of the RBD1 translation start site was amplified and cloned into pCAMBIA1300 vector (CAMBIA, http://www.cambia.org). To generate a GFP-tagged RBD1 fusion protein (RBD1:GFP) for transient expression in protoplasts, the coding region of the RBD1 was amplified from the cDNA and cloned into the pSA-T6-EGFP-N1 vector [59].

Generation of transgenic plants

Agrobacterium tumefaciens stains of GV3101 carrying the resulting constructs were used to transform plants by standard floral dipping [60]. Primary transformants were selected on
0.5×MS (Murashige and Skoog, Sigma) medium containing 0.8% agar and 2% sucrose with 25 mg/L hygromycin for selection.

**Protoplast transformation**

Protoplast isolation and transformation were carried out as previously described [61]. In brief, protoplasts were generated from 14-day-old wild-type Arabidopsis seedlings grown on plates with 12h light/12h darkness photoperiod and transformed with the plasmid DNAs. Protoplasts were then analyzed for GFP signals or for RNA co-IP from 12 hours to 48 hours after transformation.

**Histochemical GUS staining**

GUS staining was performed as described previously [62]. Briefly, tissues were stained with X-Gluc staining buffer (5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 100 mM sodium phosphate buffer, pH 7.0, and 0.005% Triton X-100 and 2 mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid) for 1–2 hours at 37°C, followed by incubating in 70% ethanol to remove chlorophyll.

**Real-time quantitative PCR and gene expression**

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. cDNAs were synthesized from total RNA by using AffinityScript QPCR cDNA Synthesis Kit (Agilent Technologies). Real-time quantitative PCR was performed on the BIO-RAD PCR System using iQSYBR GREEN SuperMix (BIO-RAD). Actin was used as a control.

**RNA gel blot analyses**

Total RNA was extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. For transcript analysis, five youngest visible leaves were harvested for RNA extraction. Ten micrograms of RNA per sample were separated on an agarose gel containing 1.2% formaldehyde and then transferred to uncharged nylon membranes (HybondN; GE Healthcare). The blots were UV cross-linked (150 mJ/cm²) and hybridized with gene specific, ³²P labeled, single strand DNA probes.

**Protein analysis**

Arabidopsis leaves were quick frozen in liquid nitrogen and homogenized in extraction buffer (50 mM Tris-HCl, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 10% Glycerol, 5 mM DTT, 0.25% Triton-X 100, 2% PVPP, 1 mM PMSF). After centrifugation at 14,000 rpm for 10 min twice, the supernatants were mixed with loading buffer, boiled and loaded onto 12% SDS polyacrylamide gel. The proteins were visualized with Coomassie Blue staining of the gel or Ponceau S staining of the transferred blot.

**RNA co-immunoprecipitation**

After transformation, the protoplasts were incubated at 22°C for 8 h before half of each sample was transferred to 4°C and the other half to 22°C for 12 h. Protoplasts were then disrupted in 500 µL protoplast disruption buffer (0.3M sorbitol, 20 mM Tricine-KOH (pH 8.4), 10 mM EDTA, 10 mM NaHCO₃ and 0.1% BSA) and incubated on the ice for 30 minutes with several inversion, then were centrifuged at 300×g for 2 minutes. Poured off the supernatant, chloroplasts pellet were disrupted in 200 µL chloroplast disruption buffer (2 mM DTT, 200 mM KOAC, 30 mM HEPES, pH 8.0, 10 mM MgOAc, and 2 mg/ml proteinase inhibitor cocktail)
and incubated on the ice for 30 minutes with occasional roughly vortex, then were centrifuged at 16,000×g for 30 minutes at 4°C. The supernatant was diluted with one volume of Co-IP buffer (150 mM NaCl, 20 mM Tris-HCl, 1 mM EDTA, 5 mM MgCl₂, 1.1% Triton X-100, 100U/ml RNase inhibitor, 1 mM PMSF and 2mg/ml proteinase inhibitor cocktail) and incubated with 10 μg of GFP antibody (mouse IgG2a, Invitrogen) for 6 h with rotation, followed by 2 h rotation with 50 μL Dynabeads Protein G (Invitrogen) at 4°C. Beads containing the IPed protein and its bound RNAs were collected with a magnet, and supernatants were recovered and pellets were washed three times with Co-IP buffer. After the last wash, pellets were resuspended in 200μL Co-IP buffer. Resuspension was then extracted with TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Total RNA was treated by DNase I (Promega) to remove DNA contamination, and RT-minus control was performed to confirm complete removal of DNA in the following steps. cDNAs were synthesized from total RNA by using Superscript III reverse transcriptase (Invitrogen). Real-time quantitative PCR was performed on the BIO-RAD PCR System using iQSYBR GREEN SuperMix (BIO-RAD).

Supporting Information

S1 Fig. Flow chart of screening chilling sensitive mutants.
(TIF)

S2 Fig. Analysis of RBD1 localization at 22°C and 4°C. Shown are confocal microscope images of protoplasts expressing RBD1:GFP at 22°C and 4°C. Left panels show the GFP signals, middle panels show the chlorophyll signals, and the right panels show the merged signals.
(TIF)

S3 Fig. Analysis of accumulation and processing of chloroplast transcripts in rbd1 mutants at 22°C and 4°C. Shown are transcripts with reduced (A), similar (B) and increased (C) expression level after chilling treatment relative to wild-type controls. Plants were grown for 3 weeks at normal condition followed by 4°C for 4 weeks, and tissues were collected from five newly emerged leaves. Marked numbers indicate the relative amount in the mutant compared to that in Col-0 quantified by Image J.
(TIF)

S4 Fig. Time-course analyses of chloroplast transcripts accumulation and processing in rbd1 mutants at chilling condition. (A) Shown are transcripts with processing defects after chilling treatment compared to the wild type. (B-C) Shown are transcripts with reduced (B) and increased (C) expression level after chilling treatment relative to wild-type controls. (D) Schematic diagram of the chloroplast transcript defects at different chilling treatment time points. For A, B, and C, plants were grown for 3 weeks at normal condition followed by 4°C for 4 weeks, and tissues were collected from five newly emerged leaves.
(TIF)

S1 Table. Primers used in this study.
(DOC)

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Author Contributions
Conceived and designed the experiments: JH ShuaiW JKZ GB. Performed the experiments: ShuaiW GB ShuW LY FY YW. Analyzed the data: ShuaiW GB JH. Contributed reagents/materials/analysis tools: ShuaiW GB ShuW FY YW. Wrote the paper: ShuaiW JH.

References
1. Theocharis A, Clement C, Barka EA (2012) Physiological and molecular changes in plants grown at low temperatures. Planta 235: 1091–1105. doi: 10.1007/s00425-012-1641-y PMID: 22526498
2. Lyons JM (1973) Chilling Injury in Plants. Annual Review of Plant Physiology and Plant Molecular Biology 24: 445–466.
3. Holaday AS, Martindale W, Aired R, Brooks AL, Leegood RC (1992) Changes in Activities of Enzymes of Carbon Metabolism in Leaves during Exposure of Plants to Low Temperature. Plant Physiol 98: 1105–1114. PMID: 16668733
4. Somersalo S, Krause GH (1990) Reversible photoinhibition of unhardened and cold-acclimated spinach leaves at chilling temperatures. Planta 180: 181–187. doi: 10.1007/BF00193993 PMID: 24201942
5. Huner NP, Oquist G, Hurry VM, Krol M, Falek S, et al. (1993) Photosynthesis, photoinhibition and low temperature acclimation in cold tolerant plants. Photosynth Res 37: 19–39. doi: 10.1007/BF02185436 PMID: 24317651
6. Stitt M, Hurry V (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. Current Opinion in Plant Biology 5: 199–206. PMID: 11960736
7. Grennan AK, Vijayan P, Feldmann KA, Browse JA (1998) Chloroplast development at low temperatures requires a homolog of DIM1, a yeast gene encoding the 18S rRNA dimethylase. Plant Cell 10: 699–711. PMID: 9596631
8. Liu X, Rodermel SR, Yu F (2010) A var2 leaf variegation suppressor locus, SUPPRESSOR OF VARIATION3, encodes a putative chloroplast translation elongation factor that is important for chloroplast development in the cold. BMC Plant Biol 10: 287. doi: 10.1186/1471-2229-10-287 PMID: 21187014
9. Kusumi K, Sakata C, Nakamura T, Kawasaki S, Yoshimura A, et al. (2011) A plastid protein NUS1 is essential for build-up of the genetic system for early chloroplast development under cold stress conditions. Plant J 68: 1039–1050. doi: 10.1111/j.1365-313X.2011.04755.x PMID: 21981410
10. Rogalski M, Schottler MA, Thiele W, Schulze WX, Bock R (2008) Rpl33, a nonessential plastid-encoded ribosomal protein in tobacco, is required under cold stress conditions. Plant Cell 20: 2221–2237. doi: 10.1105/tpc.108.063992 PMID: 18757552
11. Fleischmann TT, Scharff LB, Alkatib S, Hasdorf S, Schottler MA, et al. (2011) Nonessential plastid-encoded ribosomal proteins in tobacco: a developmental role for plastid translation and implications for reductive genome evolution. Plant J 23: 3137–3155. doi: 10.1105/tpc.111.088906 PMID: 21934145
12. Kupsch C, Ruwe H, Gusewski S, Tillich M, Small I, et al. (2012) Arabidopsis chloroplast RNA binding proteins CP31A and CP29A associate with large transcript pools and confer cold stress tolerance by influencing multiple chloroplast RNA processing steps. Plant Cell 24: 4266–4280. doi: 10.1105/tpc.112.103002 PMID: 23110894
13. Wakasugi T, Tsuzuki T, Suguri M (2001) The genomics of land plant chloroplasts: Gene content and alteration of genomic information by RNA editing. Photosynth Res 70: 107–118. PMID: 16228365
14. Hedtke B, Borner T, Weihe A (1997) Mitochondrial and chloroplast phage-type RNA polymerases in Arabidopsis. Science 277: 809–811. PMID: 9242608
15. Hess WR, Borner T (1999) Organellar RNA polymerases of higher plants. Int Rev Cytol 190: 1–59. PMID: 10331238
16. Stern DB, Goldschmidt-Clermont M, Hanson MR (2010) Chloroplast RNA metabolism. Annu Rev Plant Biol 61: 125–155. doi: 10.1146/annurev-arplant-042809-112242 PMID: 20192740
17. Tillich M, Beick S, Schmitz-Linneweber C (2010) Chloroplast RNA-binding proteins: repair and regulation of chloroplast transcripts. RNA Biol 7: 172–178. PMID: 20215878
18. Jacobs J, Kuck U (2011) Function of chloroplast RNA-binding proteins. Cell Mol Life Sci 68: 735–748. doi: 10.1007/s00018-010-0523-3 PMID: 20848156
19. Lorkovic ZJ, Bart A (2002) Genome analysis: RNA recognition motif (RRM) and K homology (KH) domain RNA-binding proteins from the flowering plant Arabidopsis thaliana. Nucleic Acids Res 30: 629–635. PMID: 11809873
21. Chinnusamy V, Zhu JK, Sunkar R (2010) Gene regulation during cold stress acclimation in plants. Methods Mol Biol 639: 39–55. doi: 10.1007/978-1-60761-702-0_3 PMID: 20387039

22. Larkindale J, Vierling E (2008) Core genome responses involved in acclimation to high temperature. Plant Physiol 146: 748–761. PMID: 18055584

23. Saidi Y, Finka A, Goloubinoff P (2011) Heat perception and signalling in plants: a tortuous path to thermostolerance. New Phytol 190: 556–565. doi: 10.1111/j.1469-8137.2010.03571.x PMID: 21138439

24. Hua J (2009) From freezing to scorching, transcriptional responses to temperature variations in plants. Curr Opin Plant Biol 12: 568–573. doi: 10.1016/j.pbi.2009.07.012 PMID: 19716335

25. Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol 50: 571–599.

26. Chinnusamy V, Ohta M, Kanrar S, Lee BH, Hong X, et al. (2003) ICE1: a regulator of cold-induced transcriptome and freezing tolerance in Arabidopsis. Genes Dev 17: 1043–1054. PMID: 12672693

27. Fursova OV, Pogorelokov GV, Tarasov VA (2009) Identification of ICE2, a gene involved in cold acclimation which determines freezing tolerance in Arabidopsis thaliana. Gene 429: 98–103. doi: 10.1016/j.gene.2008.10.016 PMID: 19026725

28. Doherty CJ, Van Buskirk HA, Myers SJ, Thomashow MF (2009) Roles for Arabidopsis CAMTA Transcription Factors in Cold-Regulated Gene Expression and Freezing Tolerance. Plant Cell 21: 972–984. doi: 10.1105/tpc.108.063958 PMID: 19270186

29. Kim Y, Park S, Gilmour SJ, Thomashow MF (2013) Roles of CAMTA transcription factors and salicylic acid in configuring the low-temperature transcriptome and freezing tolerance of Arabidopsis. Plant J 75: 364–376. doi: 10.1111/tpj.12005 PMID: 23581962

30. Park S, Lee CM, Doherty CJ, Gilmour SJ, Kim Y, et al. (2015) Regulation of the Arabidopsis CBF regulon by a complex low-temperature regulatory network. Plant J 82: 193–207. doi: 10.1111/tpj.12796 PMID: 25736223

31. Xin Z, Mandaokar A, Chen J, Last RL, Browae J (2007) Arabidopsis ESK1 encodes a novel regulator of freezing tolerance. Plant J 49: 786–799. PMID: 17316173

32. Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, et al. (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653–657. PMID: 12893945

33. Sun T, Germain A, Giloteaux L, Hammani K, Barkan A, et al. (2013) An RNA recognition motif-containing protein is required for plastid RNA editing in Arabidopsis and maize. Proc Natl Acad Sci U S A 110: E1169–1178. doi: 10.1073/pnas.1220162110 PMID: 23487777

34. Kaur J, Sebastian J, Siddiqui I (2006) The Arabidopsis-mei2-like genes play a role in meiosis and vegetative growth in Arabidopsis. Plant Cell 18: 545–559. PMID: 16473967

35. Sato N, Albriex C, Joyard J, Douce R, Kuroiwa T (1993) Detection and characterization of a plastid envelope DNA-binding protein which may anchor plastid nucleoids. EMBO J 12: 39. doi: 10.1007/978-1-60761-702-0_3 PMID: 20387039

36. Bohne AV (2014) The nucleoid as a site of rRNA processing and ribosome assembly. Front Plant Sci 5: 257. doi: 10.3389/fpls.2014.00257 PMID: 24926303

37. Leaver CJ, Ingle J (1971) The molecular integrity of chloroplast ribosomal ribonucleic acid. Biochem J 123: 235–243. PMID: 5001777

38. Nishimura K, Ashida H, Ogawa T, Yokota A (2010) A DEAD box protein is required for formation of a hidden break in Arabidopsis chloroplast 23S rRNA. Plant J 63: 766–777. doi: 10.1111/j.1365-313X.2010.04276.x PMID: 20561259

39. Vazquez D (1979) Inhibitors of protein biosynthesis. Mol Biol Biochem Biophys 30: i-x, 1–312. PMID: 8440245

40. Perry SE, Keegstra K (1994) Envelope membrane proteins that interact with chloroplast precursor proteins. Plant Cell 6: 93–105. PMID: 8130644

41. Schnell DJ, Kessler F, Blobel G (1994) Isolation of components of the chloroplast protein import machinery. Science 266: 1007–1012. PMID: 7973649

42. Hinnah SC, Hill K, Wagner R, Schlicher T, Soll J (1997) Reconstitution of a chloroplast protein import channel. EMBO J 16: 7351–7360. PMID: 9405364

43. Yang T, Shad Ali G, Yang L, Du L, Reddy AS, et al. (2010) Calcium/calmodulin-regulated receptor-like kinase CRLK1 interacts with MEKK1 in plants. Plant Signal Behav 5: 991–994. doi: 10.4172/psb.1000085 PMID: 20724845

44. Ma Y, Dai X, Xu Y, Luo W, Zheng X, et al. (2015) COLD1 confers chilling tolerance in rice. Cell 160: 1209–1221. doi: 10.1016/j.cell.2015.01.046 PMID: 25726666

45. Zhou W, Cheng Y, Yap A, Chateaukeeper AL, Delannoy E, et al. (2009) The Arabidopsis gene YS1 encoding a DYW protein is required for editing of rpoB transcripts and the rapid development of
chloroplasts during early growth. Plant J 58: 82–96. doi: 10.1111/j.1365-313X.2008.03766.x PMID: 19054358

46. Yabuta S, Ifuku K, Takabayashi A, Ishihara S, Ido K, et al. (2010) Three PsbQ-like proteins are required for the function of the chloroplast NAD(P)H dehydrogenase complex in Arabidopsis. Plant Cell Physiol 51: 866–876. doi: 10.1093/pcp/pcq060 PMID: 20430763

47. Xu J, Zhang L, Yang DL, Li Q, He Z (2015) Thymidine kinases share a conserved function for nucleotide salvage and play an essential role in Arabidopsis thaliana growth and development. New Phytol 208: 1089–1103. doi: 10.1111/nph.13530 PMID: 26139575

48. Harris EH, Boynton JE, Gillham NW (1994) Chloroplast ribosomes and protein synthesis. Microbiol Rev 58: 700–754. PMID: 7854253

49. Bellaoui M, Keddie JS, Gruissem W (2003) DCL is a plant-specific protein required for plastid ribosomal RNA processing and embryo development. Plant Mol Biol 53: 531–543. PMID: 15010617

50. Bollenbach TJ, Lange H, Gutierrez R, Erhardt M, Stern DB, et al. (2005) RNR1, a 3'–5' exoribonuclease belonging to the RNR superfamily, catalyzes 3' maturation of chloroplast ribosomal RNAs in Arabidopsis thaliana. Nucleic Acids Res 33: 2751–2763. PMID: 15891117

51. Beligni MV, Mayfield SP (2008) Arabidopsis thaliana mutants reveal a role for CSP41a and CSP41b, two ribosome-associated endonucleases, in chloroplast ribosomal RNA metabolism. Plant Mol Biol 67: 389–401. doi: 10.1007/s11103-008-9328-2 PMID: 18398686

52. Stoppel R, Manavski N, Schein A, Schuster G, Teubner M, et al. (2012) RHON1 is a novel ribonucleic acid-binding protein that supports RNase E function in the Arabidopsis chloroplast. Nucleic Acids Res 40: 8593–8606. PMID: 22735703

53. Walter M, Kiliar J, Kudla J (2002) PNPass activity determines the efficiency of mRNA 3’-end processing, the degradation of rRNA and the extent of polyadenylation in chloroplasts. EMBO J 21: 6905–6914. PMID: 12486011

54. Hajdukiewicz PT, Allison LA, Maliga P (1997) The two RNA polymerases encoded by the nuclear and the plastid compartments transcribe distinct groups of genes in tobacco plastids. EMBO J 16: 4041–4048. PMID: 9233813

55. Swiatecka-Hagenbruch M, Liere K, Borner T (2007) High diversity of plastidial promoters in Arabidopsis thaliana. Mol Genet Genomics 277: 725–734. PMID: 17333279

56. Barkan A (2011) Expression of plastid genes: organelle-specific elaborations on a prokaryotic scaffold. Plant Physiol 155: 1520–1532. doi: 10.1104/pp.110.171231 PMID: 21346173

57. Prkryl J, Watkins KP, Friso G, van Wijk KJ. Barkan A (2008) A member of the Whirly family is a multi-functional RNA- and DNA-binding protein that is essential for chloroplast biogenesis. Nucleic Acids Res 36: 5152–5165. doi: 10.1093/nar/gkn492 PMID: 18676978

58. Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in plants. Plant Physiol 133: 462–469. PMID: 14555774

59. Tzfira T, Tian GW, Lacroix B, Vyas S, Li J, et al. (2005) pSAT vectors: a modular series of plasmids for autofluorescent protein tagging and expression of multiple genes in plants. Plant Mol Biol 57: 503–516. PMID: 15821977

60. Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735–743. PMID: 10069079

61. Zhai Z, Jung HI, Vatamaniuk OK (2009) Isolation of protoplasts from tissues of 14-day-old seedlings of Arabidopsis thaliana. J Vis Exp.

62. Wang Y, Hua J (2009) A moderate decrease in temperature induces COR15a expression through the CBF signaling cascade and enhances freezing tolerance. Plant J 60: 340–349. doi: 10.1111/j.1365-313X.2009.03959.x PMID: 19563440