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A DEAD Box RNA Helicase Is Essential for mRNA Export and Important for Development and Stress Responses in Arabidopsis

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An *Arabidopsis thaliana* mutant, *cryophyte*, was isolated and found to have an enhanced cold stress-induction of the master regulator of cold tolerance, C-repeat binding factor 2 (*CBF2*), and its downstream target genes. The mutant is more tolerant to chilling and freezing stresses but is more sensitive to heat stress. Under warm but not cold growth temperatures, the mutant has a reduced stature and flowers earlier. Under long day conditions, flowering of the mutant is insensitive to vernalization. The mutant is also hypersensitive to the phytohormone abscisic acid. The mutation was found in a DEAD box RNA helicase gene that is identical to the previously identified low expression of osmotically responsive genes 4 (*LOS4*) locus, which was defined by the *los4-1* mutation that reduces cold regulation of *CBFs* and their target genes and renders Arabidopsis plants chilling sensitive. We show evidence suggesting that the CRYOPHYTE/LOS4 protein may be enriched in the nuclear rim. In situ poly(A) hybridization indicates that the export of poly(A)$^+$ RNAs is blocked in the *cryophyte*/*los4-2* mutant at warm or high temperatures but not at low temperatures, whereas the *los4-1* mutation weakens mRNA export at both low and warm temperatures. These results demonstrate an important role of the CRYOPHYTE/LOS4 RNA helicase in mRNA export, plant development, and stress responses.

INTRODUCTION

Low temperature is one of the most common environmental factors influencing the growth, development, and survival of plants. The expression of many genes in plants is regulated by low temperatures (Thomashow, 1999). Some of these genes have in their promoters one or several copies of the *DRE*/*CRT* *cis*-element, which has the core sequence CCGAC (Yamaguchi-Shinozaki and Shinozaki, 1994). A small family of cold-inducible transcription factors known as C-repeat binding factors (*CBFs*) (Stockinger et al., 1997; Thomashow, 1999) binds to this element and activates transcription of the downstream cold responsive genes. Therefore, there is a transcriptional cascade leading to the expression of the *DRE*/*CRT* class of genes under cold stress.

We reported previously that the *Arabidopsis thaliana* low expression of osmotically responsive genes 4 (*LOS4*) locus is important for cold responsive gene expression and for chilling and freezing tolerance (Gong et al., 2002). A recessive mutation, *los4-1*, impairs cold-induced transcript accumulation of *CBF* genes and renders plants more sensitive to chilling and freezing stresses. Ectopic expression of *CBF3* rescues the chilling and freezing sensitive phenotypes of *los4-1* mutant plants, suggesting an important role of the CBF regulon in not only freezing tolerance but also chilling tolerance. *LOS4* encodes a putative DEAD box RNA helicase (Gong et al., 2002). Whether LOS4 indeed has RNA helicase activity and its cellular function are unclear.

RNA helicases refer to enzymes that use energy derived from the hydrolysis of a nucleotide triphosphate to unwind double-stranded RNAs (de la Cruz et al., 1999). RNA helicases have been implicated in every step of RNA metabolism, including nuclear transcription, pre-mRNA splicing, ribosome biogenesis, nucleocytoplasmic transport, translation, RNA decay, and organellar gene expression (de la Cruz et al., 1999; Tanner and Linder, 2001; Lorsch, 2002). The DEAD box RNA helicases compose the largest subfamily of RNA helicases. BLAST searches with LOS4 identified more than 50 different DEAD box RNA helicase genes in the Arabidopsis genome. Because RNA molecules are prone to forming stable nonfunctional secondary structures, their proper function requires RNA chaperones (Lorsch, 2002; Mohr et al., 2002). DEAD box RNA helicases are prominent candidates for RNA chaperones because these proteins can use energy derived from ATP hydrolysis to actively disrupt misfolded RNA structures so that correct folding can occur (Tanner and Linder, 2001; Lorsch, 2002). The function of the superfamily of RNA helicases in plants is poorly understood. One exception is

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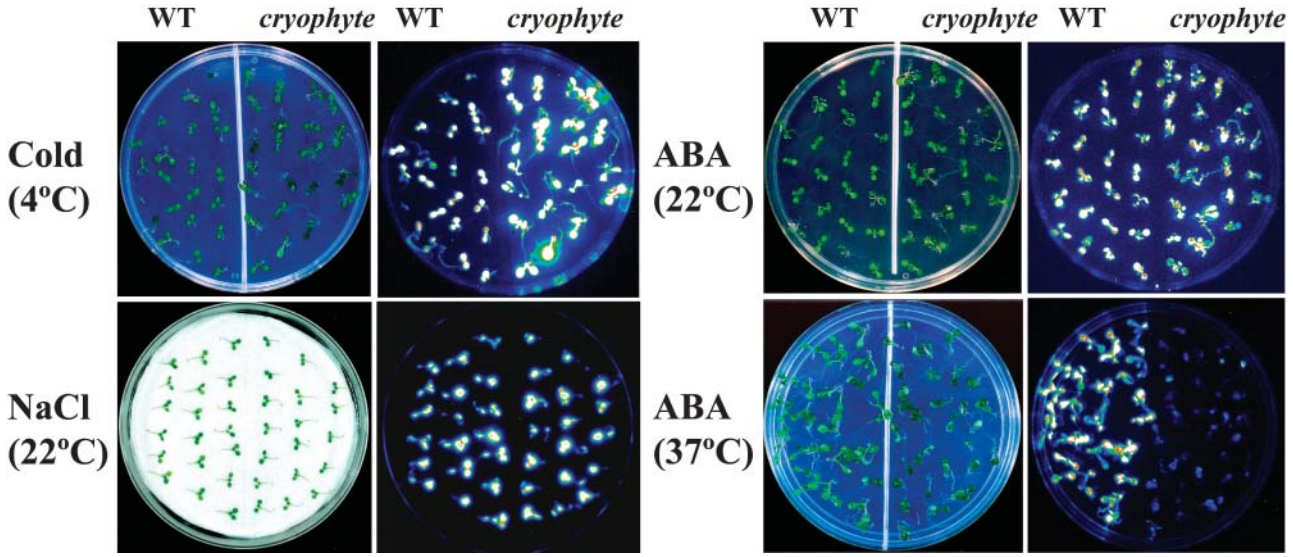

Figure 1. *RD29A-LUC* Expression in Wild-Type and *cryophyte* Mutant Plants in Response to Cold, ABA, or Osmotic Stress.

Pictures and luminescence images for wild-type (left) and *cryophyte* mutant (right) seedlings growing in MS agar plates were taken after treatments at 4°C for 24 h or with 300 mM NaCl at 22°C for 5 h or 100 μM ABA at 22 or 37°C for 3 h.

CARPEL FACTORY/DICER-LIKE 1, which is a DExH box helicase shown to be critical for the biogenesis of microRNAs and plant development (Jacobsen et al., 1999; Park et al., 2002).

In this study, we report the characterization of a novel Arabidopsis mutant (*cryophyte*) and the identification of the corresponding gene. The mutant was isolated as having an enhanced cold induction of *CBF2* and its downstream genes. Compared with the wild type, the mutant plants flower earlier and are smaller in size. We isolated the corresponding gene via positional cloning and found that the gene encodes a DEAD box RNA helicase identical to LOS4. *cryophyte* was thus designated as *los4-2*. We found that the LOS4 protein has an RNA-dependent ATPase activity. In situ poly(A) hybridization studies show that the export of poly(A)$^+$ RNAs is blocked in the *los4-2* mutant at warm and high temperatures. Consistent with its role in mRNA export, the LOS4 protein appears highly enriched at the nuclear rim. The underlying mechanisms of LOS4 function in stress responses and plant development are discussed.

RESULTS

Identification of the *cryophyte* Mutant

A genetic screen was performed to isolate Arabidopsis mutants with deregulated expression of the luciferase reporter gene under control of the cold, abscisic acid (ABA), and osmotic stress responsive *RD29A* promoter (Ishitani et al., 1997). A group of mutants were found to have enhanced *RD29A-LUC* expression primarily in response to cold but not to ABA or osmotic stress. One of these mutants, designated as *cryophyte* (for its increased cold tolerance, see below) was chosen for detailed characterization. Figure 1 shows a comparison of luminescence images of *cryophyte* and wild-type seedlings under cold, ABA, and high salt treatments. Quantitation of the luminescence intensities indicated that *RD29A-LUC* expression in *cryophyte* mutant plants is higher than that in the wild type specifically under cold treatment (data not shown). The *RD29A-LUC* expression is virtually the same in *cryophyte* plants in response to NaCl and ABA as in the wild type (Figure 1).

RD29A-LUC expression is activated by ABA, cold, and osmotic stress but not by heat. To examine the effect of heat stress on the luminescence phenotype, we combined ABA and heat (37°C) treatments. After 3 h of ABA treatment at 37°C, *RD29A-LUC* expression was determined by luciferase imaging. The heat shock treatment did not significantly affect the luminescence response to ABA in wild-type plants (Figure 1). However, the heat treatment virtually abolished the luminescence response to ABA in *cryophyte* mutant plants at 37°C (Figure 1). We noticed that most of the *cryophyte* mutant plants appeared damaged by the heat treatment, whereas no damage was seen in the wild-type plants.

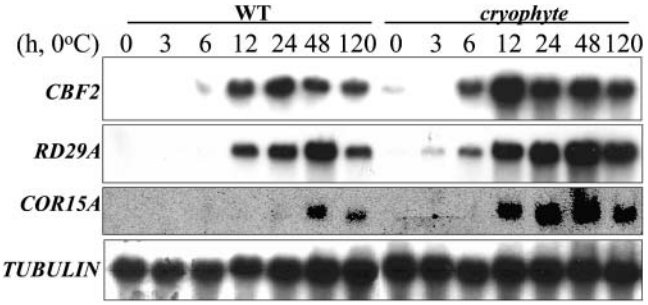

Figure 2. RNA Gel Blot Analysis of Cold Responsive Genes.

Ten-day-old seedlings grown on MS medium were treated at 0°C for the indicated time periods. Total RNA was extracted after the treatments and subjected to RNA gel blot analysis. *TUBULIN* was used as a loading control.

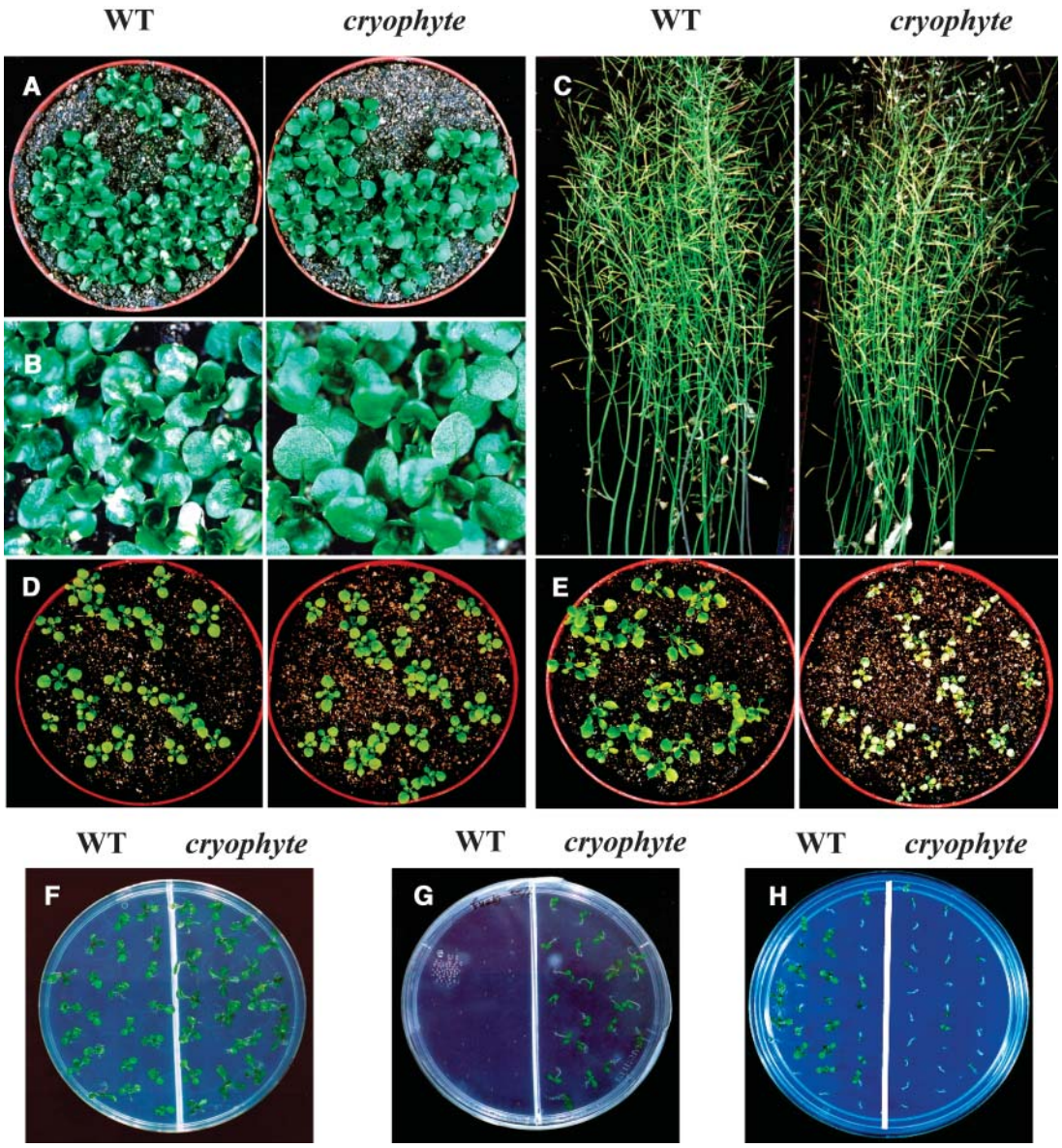

Figure 3. Responses of the *cryophyte* Mutant to Low or High Temperatures and ABA.

(A) Plants were grown in a cold room (4°C) under continuous light for 2 months. Left panel, wild-type plants; right panel, *cryophyte* mutant plants.
(B) A close-up picture of the plants in **(A)**. Left panel, the wild type; right panel, *cryophyte* mutant. Notice that chilling damage as indicated by leaf necrosis (white areas) was only observed in wild-type plants but not in mutant plants.
(C) A comparison of the mature wild-type and *cryophyte* mutant plants that had been grown at 4°C.
(D) Wild-type and *cryophyte* mutant seedlings grown at 22°C for 10 d.
(E) Seedlings in **(D)** were transferred to high temperature (30°C) and incubated for 3 d.
(F) A comparison of wild-type and *cryophyte* mutant plants grown in normal conditions (22°C) on MS medium for 10 d.
(G) and **(H)** The germination of wild-type and *cryophyte* mutant seeds at 30°C **(G)** and on a medium containing 0.1 μM ABA (22°C) **(H)**.

Homozygous *cryophyte* mutant plants were backcrossed with the wild type. The resulting F1 plants all exhibited a wild-type luminescence phenotype. The F2 progeny from the selfed F1 segregated ~3:1 for the wild type/mutant (data not shown). These results indicate that the *cryophyte* mutant is caused by a recessive mutation in a single nuclear gene.

The *cryophyte* Mutant Shows Enhanced Cold Responsive Gene Expression and Increased Chilling and Freezing Tolerance

RNA gel blot analysis was performed to determine whether the *cryophyte* mutation affects the expression of endogenous stress responsive genes in the cold. As shown in Figure 2, the level of

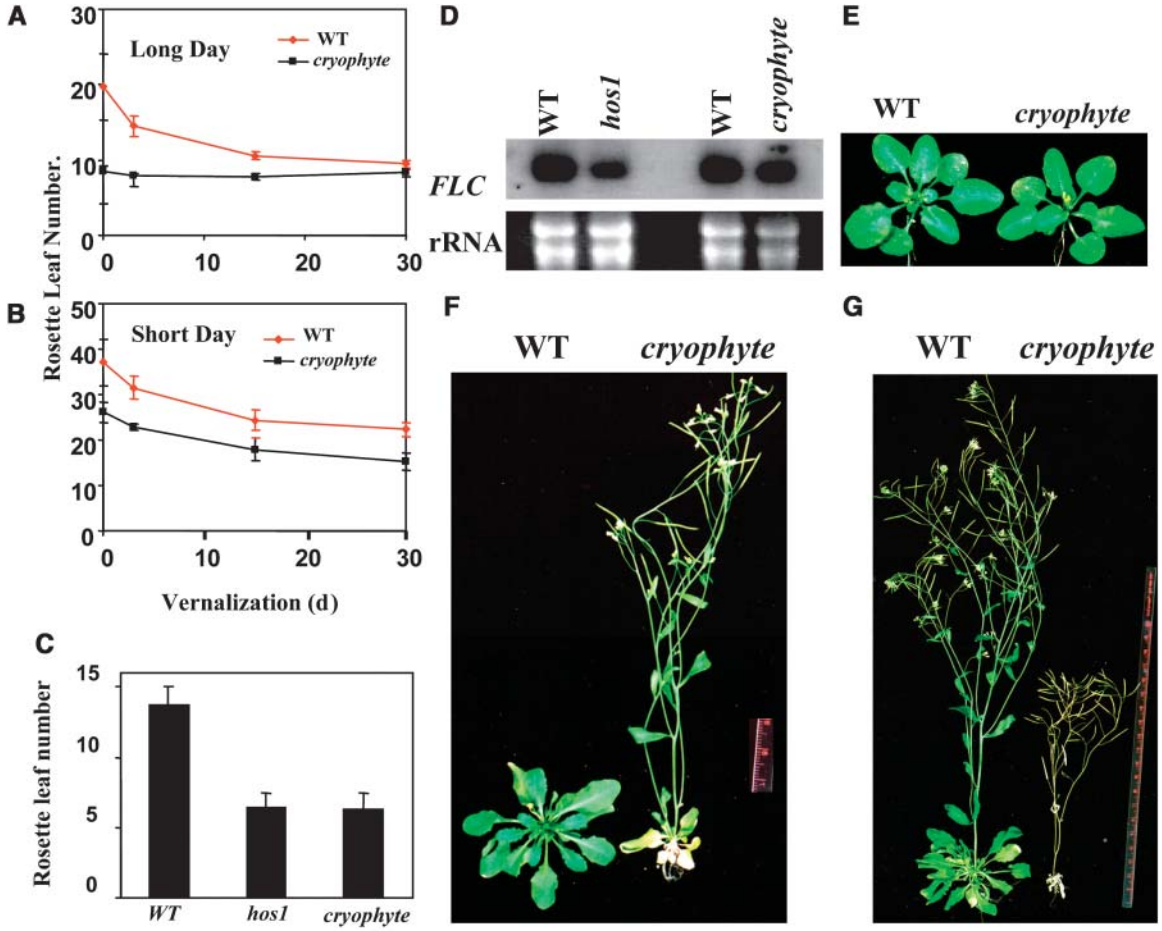

Figure 4. Vernalization Effect on Flowering Time in *cryophyte* Mutant Plants.

(A) and **(B)** Wild-type and *cryophyte* mutant plants with various time period of vernalization treatment at 4°C were grown under long day (16 h light, 8 h dark) condition **(A)** or under short day (8 h light, 16 h dark) **(B)** at 22°C. Flowering time is indicated by the total number of rosette leaves at the time of first flower bud emergence (means and SE, $n = 15$).

(C) Comparisons of flowering time in wild-type, *hos1*, and *cryophyte* mutant plants grown at 22°C without vernalization treatment.

(D) *FLC* expression in *hos1* and the *cryophyte* mutant. Total RNA was isolated from wild-type, *hos1*, and *cryophyte* mutant seedlings grown on MS medium for 2 weeks. Twenty micrograms of total RNA were loaded per lane. The rRNAs were used as a loading control.

(E) A comparison of wild-type and mutant plants grown under normal conditions (22°C) before flowering.

(F) *cryophyte* mutant plants flower earlier.

(G) Size comparison between a mature wild-type and a *cryophyte* mutant plant grown under normal conditions (22°C). Refer to Figure 3C for growth comparison at 4°C.

CBF2 expression is higher in the mutant than that in wild-type plants under cold treatment. Consistent with the pattern of *RD29A-LUC* expression, cold-induced expression of the endogenous *RD29A* gene is higher in the *cryophyte* mutant than that in wild-type plants. Similarly, expression of another cold responsive gene, *COR15A*, is also higher in the *cryophyte* mutant under cold treatment (Figure 2).

Because *cryophyte* mutant plants exhibit an enhanced expression of cold responsive genes (Figures 1 and 2), we tested the effect of cold treatment on their growth and development. The seeds of the *cryophyte* mutant and the wild type were planted in soil and allowed to germinate at 22°C. After germination, the plants were placed in a cold room (4°C) and were grown under continuous light. As shown in Figures 3A and 3B, *cryophyte* mutant plants grew slightly but noticeably better than did wild-type plants at 4°C. Under the conditions used in this study, many wild-type plants showed chilling damage as evidenced by leaf necrosis (Figures 3A and 3B). However, the mutant leaves did not exhibit such necrosis (Figures 3A and 3B). We also tested the freezing resistance of the mutant and wild-type plants. Ten-day-old seedlings on nutrient agar plates were treated for 4 d in a cold room (4°C) under light. The seedlings were transferred to a temperature-controlled freezer (Gong et al., 2002). After a freezing treatment at −9°C for 3 h, the plates were removed and incubated at room temperature for 2 d. We found that 10 ± 1% of wild-type plants and 56 ± 3% of the *cryophyte*

mutant plants survived the freezing treatment (50 to 60 seedlings per experiment; data represent average and standard deviation of three experiments). The results suggest that the *cryophyte* mutant plants are more tolerant to both chilling and freezing stresses.

Responses of the *cryophyte* Mutant to High Temperatures and ABA

The luminescence response to ABA is greatly reduced at high temperatures in *cryophyte* mutant seedlings but not in wild-type plants (Figure 1). Therefore, we tested the effect of the *cryophyte* mutation on plant growth at high temperatures. The mutant and wild-type plants that had been grown at 22°C for 10 d were transferred to 30°C. At the time when the seedlings were transferred, there was no apparent difference between the mutant and wild-type plants (Figure 3D). After a 1-d incubation at 30°C, the leaves of *cryophyte* mutant plants began to die. After 3 d at 30°C, the mutant plants died completely and could not recover after being transferred back to 22°C (Figure 3E). By contrast, wild-type plants grew quite well at 30°C.

Furthermore, we found that the *cryophyte* mutant plants were completely killed by incubation at 28°C for 5 d or at 26°C for 7 d. When the mutant plants were treated at 28°C for 3 d and then transferred back to 22°C, approximately half of the mutant plants survived but grew poorly and did not produce any seeds (data not shown). None of the wild-type plants appeared to be adversely affected by the treatments at 26, 28, or 30°C.

We tested the effect of heat stress on the germination of *cryophyte* mutant seeds. Seeds were plated on MS agar medium and after 2 d of cold stratification were incubated at 30°C in the dark or under continuous light in a growth chamber. None of the wild-type seeds germinated at 30°C under either dark or light conditions. However, 100% of *cryophyte* mutant seeds germinated at 30°C in both dark and light. Figure 3G shows the germination of *cryophyte* mutant seeds under light at 30°C. At 22°C, there was no difference in the germination rate between the mutant and wild-type seeds (Figure 3F). After germination at 30 or 22°C, very young *cryophyte* seedlings could survive for approximately 1 week at 30°C, although they were not able to continue to grow and develop at the high temperature. In the end, the young mutant seedlings became yellowish and died at 30°C. These results suggest that the *cryophyte* mutation may have impaired the production of a germination inhibitor under high temperatures. In addition, the mutation appears to have less impact on the development of younger seedlings compared with older ones under high temperatures.

We also tested the germination of *cryophyte* mutant seeds in response to ABA treatment. The mutant and wild-type seeds were planted on MS medium containing 0.1 μM ABA and grown under continuous light at 22°C. ABA inhibited the germination of both the mutant and wild-type seeds. However, the germination

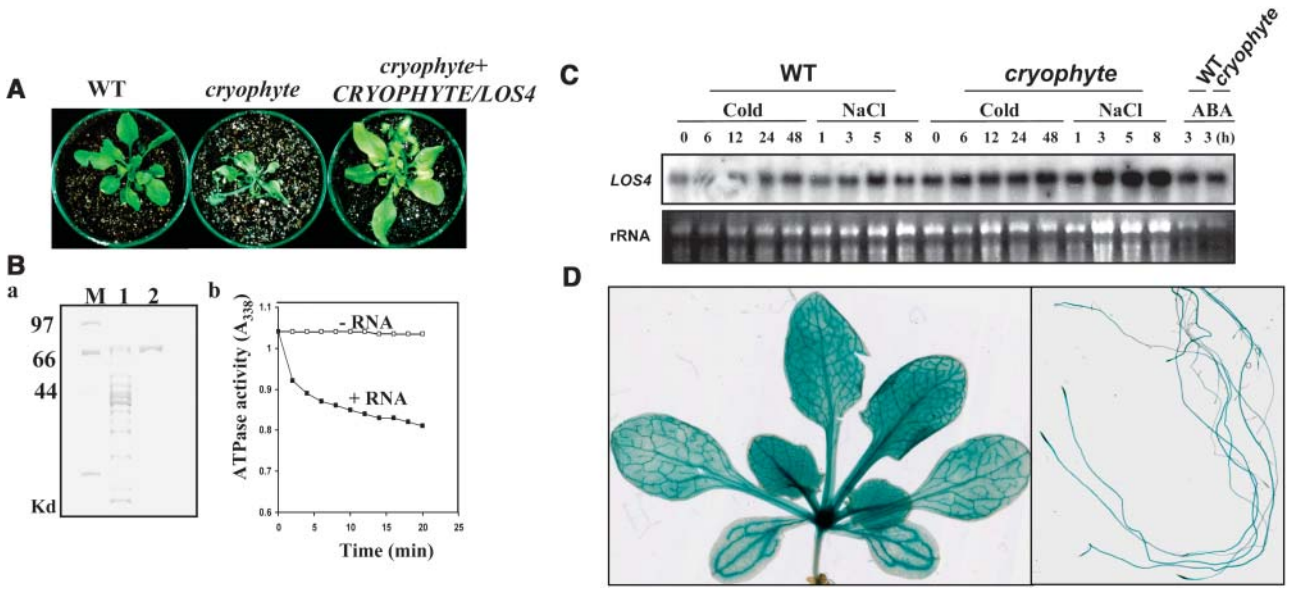

Figure 5. Complementation of the *cryophyte*/*los4-2* Mutant and Characterization of the *LOS4* Gene and Gene Product.

(A) Complementation of *los4-2* mutant by the *LOS4* gene. Plants were treated at 30°C for 3 d.

(B) Purification and RNA-dependent ATPase activity of the LOS4 protein. (a) Purification of His-LOS4 fusion protein. Lane 1, total proteins from isopropyl-1-thio-β-D-galactopyranoside–induced *E. coli* cells; lane 2, His-LOS4 protein purified by nickel column affinity chromatography. Proteins were resolved by SDS-PAGE and stained with Coomassie blue. M, protein size markers. (b) The ATPase activity of LOS4 was measured as a decline in absorbance at 338 nm (Iost et al., 1999).

(C) Expression of the *LOS4* gene is not regulated by stresses.

(D) *LOS4* promoter-GUS expression pattern. Left, shoot; right, root.

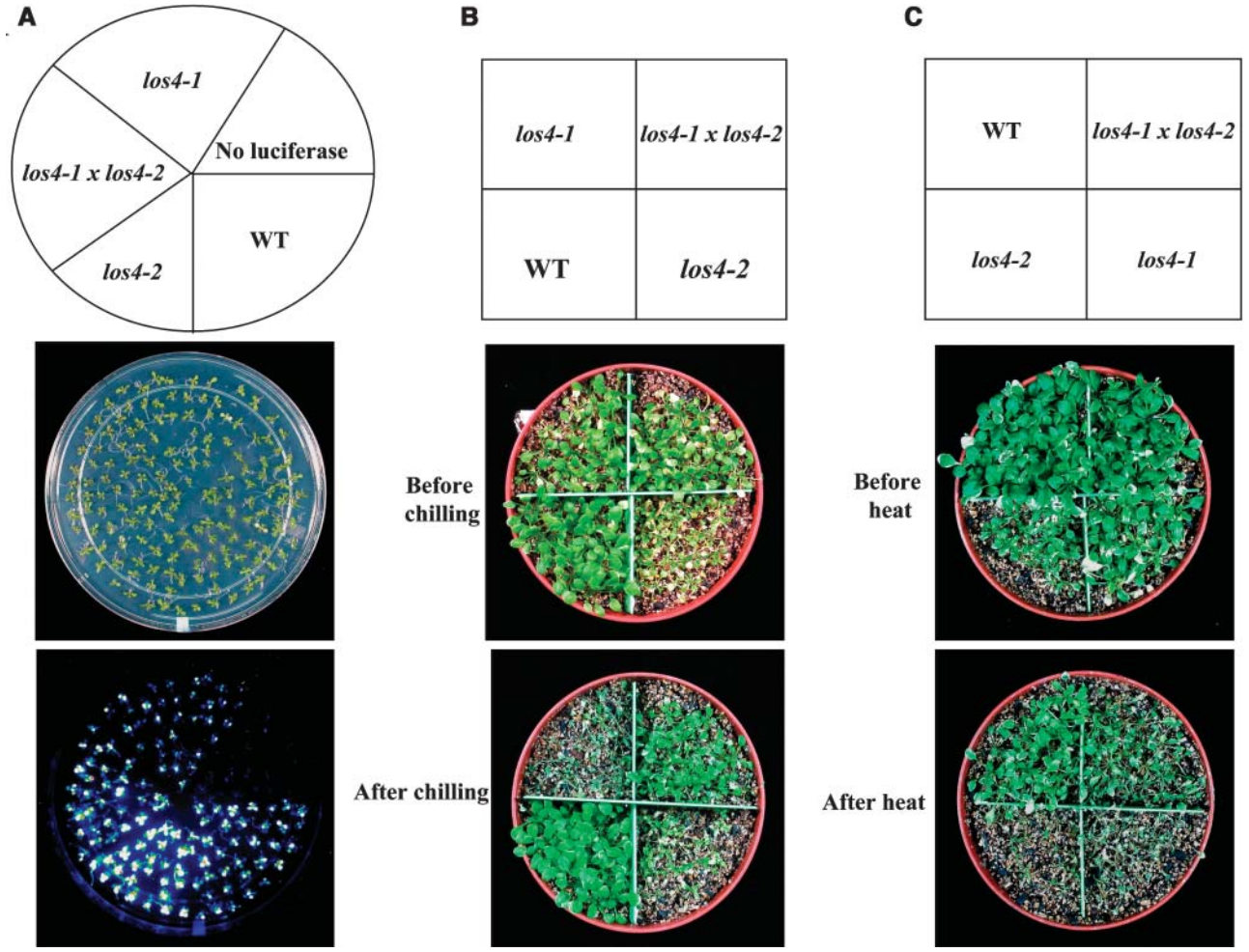

Figure 6. Phenotype of *los4-1*/*los4-2* Compound Heterozygote Plants.

(A) Cold-induced *RD29A-LUC* expression in the *los4-1*/*los4-2* compound heterozygote. Top panel, diagram of the genotypes used; middle panel, picture of the seedlings; bottom panel, *RD29A-LUC* expression (luminescence image).
(B) and **(C)** Sensitivity to chilling and heat stress, respectively. The wild type, *los4-1*, *los4-2*, and Columbia ecotype without the *RD29A-LUC* transgene were used as controls.

of *cryophyte* mutant seeds was much more inhibited by ABA than that of wild-type seeds (Figure 3H). The germination of *cryophyte* mutant seeds on 0.1 μM ABA medium was delayed by approximately 2 d compared with that of wild-type seeds. Postgermination seedling development in the mutant was also more inhibited by ABA than in the wild type (Figure 3H).

cryophyte Mutant Plants Flower Earlier at Normal Growth Temperatures

Under our regular growth conditions (16 h light at 22°C and 8 h dark at 18°C), *cryophyte* mutant plants flowered ~18 d after imbibition, as compared with 25 d for wild-type plants. Under short day conditions (8 h light at 22°C and 16 h dark at 18°C), the mutant also flowered earlier than the wild type. Because leaf number (LN) at the first flower bud emergence is a more accurate indicator of flowering time, we determined the LN values for the mutant and wild-type plants (Figures 4A to 4C). The results show that under both long day and short day conditions, the mutant had significantly lower LN values than the wild type.

Vernalization treatment (i.e., long period of cold) can significantly influence the time to flower in Arabidopsis. The *hos1* mutant, which also shows an enhanced cold induction of *RD29A-LUC*, flowers earlier, and the mutant appears constitutively vernalized (Ishitani et al., 1998). We tested whether the early flowering phenotype of the *cryophyte* mutant is related to vernalization. Interestingly, *cryophyte* mutant plants were not responsive to vernalization treatments under long day conditions (Figure 4A). By contrast, wild-type plants flowered earlier in response to vernalization (Figure 4A). However, under short day conditions, both the mutant and wild-type plants were responsive to vernalization (Figure 4B).

In the absence of vernalization, the *hos1* mutant (Ishitani et al., 1998) flowers at approximately the same time as the *cryophyte* mutant (Figure 4C). In *hos1* mutant plants, there is a constitutive reduction in the expression of *FLC*, a negative regulator of vernalization (Lee et al., 2001). We determined *FLC* expression in the *cryophyte* mutant plants and found that the mutation has no substantial effect on *FLC* expression (Figure 4D). The result suggests that the *cryophyte* mutation promotes plant flowering possibly through a vernalization-independent pathway.

The sizes of the *cryophyte* mutant and wild-type plants are similar before the mutant plants bolt (Figure 4E). However, mature *cryophyte* mutant plants are much smaller than wild-type plants. Whereas wild-type plants typically reach a height of 35 to 45 cm, the mutant plants usually do not grow taller than 20 cm (Figure 4G). The mutant plants produce fewer siliques, and the mutant siliques are shorter than that in wild-type plants.

At 4°C (continuous light), *cryophyte* mutant plants grow as big and produce as many seeds as wild-type plants (Figure 3C). In addition, the mutant does not flower earlier compared with the wild type at 4°C.

Cloning and Characterization of the *CRYOPHYTE* Gene

We isolated the *cryophyte* mutation using a map-based cloning strategy. The mutation was mapped to chromosome III between a simple sequence length polymorphism (SSLP) marker on F8J2 and an SSLP marker on F4P12. Because the *LOS4* gene (i.e., *T4D2.40*) is located in this region (Gong et al., 2002), we sequenced the *LOS4*/*T4D2.40* gene from the *cryophyte* mutant. Remarkably, sequence comparison revealed a G-to-A change in the second exon in the *LOS4*/*T4D2.40* gene from *cryophyte* mutant plants. A binary construct containing wild-type *LOS4*/*T4D2.40* gene (Gong et al., 2002) was then introduced into *cryophyte* mutant plants. Analysis of the resulting transgenic plants showed that wild-type *LOS4*/*T4D2.40* gene complemented the *cryophyte* mutant in the heat sensitive phenotype (Figure 5A) and other phenotypes, including the enhanced *RD29A-LUC* expression in the cold (data not shown). Therefore, the *cryophyte* mutation is allelic to *los4-1* and was designated subsequently as *los4-2*.

The *los4-2* mutation would change Glu-94 to Lys, whereas the *los4-1* mutation changes Gly-364 to Arg. LOS4 is most closely related to Dbp5p/Rat8p (Gong et al., 2002) in yeast, showing 39% identity and 62% similarity over 411 amino acids. The LOS4 open reading frame was fused with a His-tag and expressed in *Escherichia coli*. The recombinant His-LOS4 protein showed an RNA-dependent ATPase activity intrinsic to RNA helicases (Figure 5B). For unknown reasons, we were not able to obtain soluble proteins corresponding to the *los4-1* or *los4-2* mutant versions.

To determine if the expression of *LOS4* is modulated by cold, high salt, or ABA, we performed RNA gel blot analysis using total RNA extracted from 2-week-old wild-type and *los4-2* seedlings. As shown in Figure 5C, the *LOS4* transcript is present constitutively, and the level is not substantially affected by any of the treatments. An analysis of transgenic Arabidopsis plants expressing a *LOS4* promoter-β-glucuronidase (GUS) reporter gene indicated that *LOS4* is ubiquitously expressed in all plant parts, such as leaves, roots (Figure 5D), and other tissues (data not shown).

Genetic allelism test was performed to determine the interaction between the *los4-1* and *los4-2* mutations. As shown in Figure 6A, under cold treatment (0°C for 24 h), F1 plants resulting from a cross between the *los4-1* and *los4-2* mutants show a level of *RD29A-LUC* expression similar to that of wild-type plants, which is higher than that of *los4-1* but lower than that of *los4-2*. When 20-d-old plants grown under normal conditions were treated with chilling stress (4°C, 2 weeks), the F1 plants as well as the wild type and *los4-2*, but not *los4-1*, survived (Figure 6B). However, under heat treatment (30°C, 2 d), only the wild-type plants survived, and the F1 plants and the two parents were killed (Figure 6C). The results suggest that the *los4-1* and *los4-2* mutations complement each other in their transheterozygote for phenotypes that are opposite in the two mutants. Consistent with their allelic nature, for phenotypes that are similar in the two mutants (i.e., heat sensitivity), the two mutations do not complement each other.

The LOS4 Protein May Be Enriched around the Nuclear Envelope

In the elongation zone of transgenic Arabidopsis roots expressing a translational fusion between LOS4 and the green fluorescence protein (GFP), the GFP signal appeared to be in both the cytoplasm and nucleus (Gong et al., 2002). However, because the nuclei in the elongating root cells are pressed by large vacuoles to the cell periphery, it was difficult to determine whether the GFP signal associated with the nucleus resides inside the nucleus or at the nuclear envelope. Because meristematic cells at the root tip have more prominent nuclei, we examined under high magnification these cells in LOS4-GFP–expressing plants to determine whether LOS4-GFP is inside the nucleus or at the nuclear rim. Confocal images show that in the root tip cells, the green fluorescence signal appears highly enriched around the nuclear envelope (Figure 7A). In addition,

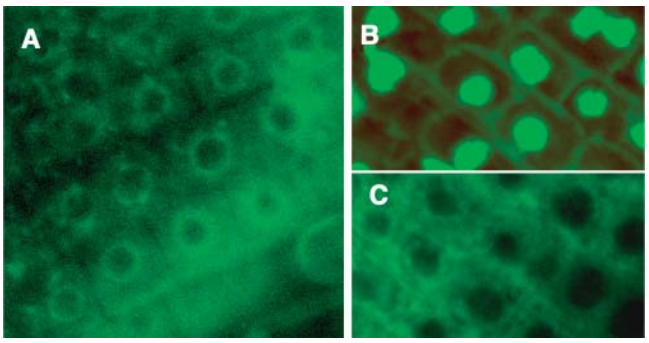

Figure 7. LOS4-GFP Fusion Protein Is Localized in the Cytoplasm and Enriched at the Nuclear Rim.

(A) LOS4-GFP localization.
(B) A known nuclear-localized GFP fusion protein as a control.
(C) A known cytoplasmically localized GFP-fusion protein as a control.

the GFP signal is also clearly present in the cytoplasm (Figure 7A). As controls, root meristematic cells expressing a known GFP fusion protein localized inside nuclei (GFP fused to a transcription factor, transgenic Arabidopsis line N7) and a known GFP fusion protein localized in the cytoplasm (GFP fused to an acidic ribosomal protein, transgeneic Arabidopsis line Q1) (Cutler et al., 2000) are shown in Figures 7B and 7C.

LOS4 Is Essential for mRNA Export

The apparent enrichment of LOS4 at the nuclear rim suggests that it may be involved in mRNA export. To determine the potential role of LOS4 in mRNA export, we performed in situ hybridization to localize poly(A) signals in wild-type and *los4-2* mutant plants. Leaves of *los4-2* and wild-type plants grown under normal conditions (22°C) for 2 weeks in soil were hybridized with a 45-mer oligo(dT) probe end-labeled with fluoresceine. As shown in Figure 8A (b), *los4-2* leaf cells accumulated much stronger poly(A) signals in nuclei than did wild-type cells at 22°C. Stronger nuclear poly(A) signals were also observed in *los4-2* roots and hypocotyls (data not shown), indicating that the entire mutant plant is defective in mRNA export.

los4-2 mutant plants cannot survive high temperature treatments (Figure 3E). If the heat sensitivity is caused by a more severe defect in mRNA export at high temperatures, then increased poly(A) signals might be seen in the cell nuclei of the

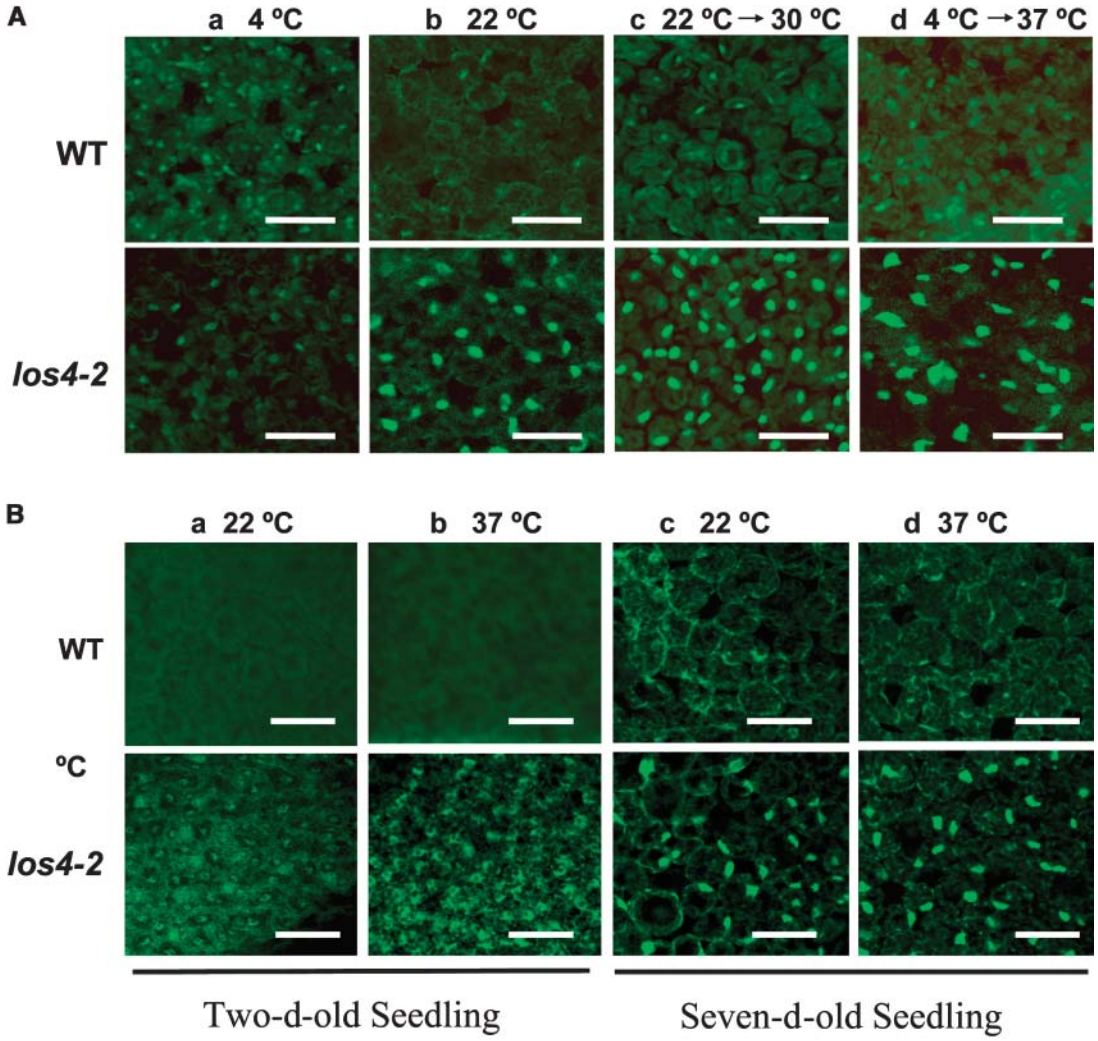

Figure 8. Defects of *cryophyte*/*los4-2* Plants in mRNA Export.

(A) Poly(A) RNA export is blocked in *los4-2* cells at warm and high temperatures. Wild-type and *los4-2* mutant plants were grown at 4°C for 2 months or 22°C for 2 weeks. In situ hybridization with fluoresceine-labeled oligo(dT) probe was performed with seedlings growing at 4°C (a) or 22°C (b). (c) Plants transferred from 22 to 30°C and kept at 30°C for 2 h. (d) Plants transferred from 4 to 37°C and kept at 37°C for 30 min. Bars = 40 μm.

(B) Poly(A) RNA export is less affected in very young *los4-2* seedlings than in older seedlings. The poly(A) RNA distribution in 2-d-old wild-type and *los4-2* plants that were grown at 22°C (a) and subjected to a 1-h incubation at 37°C (b) or in 7-d-old wild-type and *los4-2* plants that were grown at 22°C (c) and subjected to a 1-h incubation at 37°C (d). Bars = 40 μm.

mutant. After being shifted to 30°C for 2 h, *los4-2* mutant plants produced even stronger poly(A) signals in nuclei compared with those that were not heat shocked (Figure 8A, c). By contrast, wild-type plants did not show a substantial increase in nuclear poly(A) signals after the shift to 30°C. There was no obvious difference in poly(A) signal strength in cells between *los4-2* and wild-type plants when they were grown at 4°C (Figure 8A, a). After the plants were moved from 4 to 37°C for 30 min, *los4-2* but not wild-type plants quickly accumulated poly(A) RNA in the nuclei (Figure 8A, d). These results suggest that the *los4-2* mutant protein is defective in its mRNA export function at normal and high temperatures but is still fully functional at low temperatures.

Very young *los4-2* seedlings are more tolerant to heat stress relative to older mutant plants (Figure 3H; data not shown). Two-day-old and 7-d-old *los4-2* and wild-type seedlings grown under normal conditions were fixed and used for in situ hybridization. As shown in Figure 8B (a and c), nuclear poly(A) signals in the 2-d-old *los4-2* seedlings are much weaker than those in the 7-d-old *los4-2* seedlings. After a 37°C treatment for 60 min, the nuclear poly(A) signals in the 2-d-old *los4-2* seedlings are still much weaker than those in the 7-d-old *los4-2* seedlings (Figure 8B, b and d). No significant nuclear poly(A) signal was detected in either the 2-d-old or 7-d-old wild-type seedlings under any of the treatment conditions. These results suggest that LOS4 is less critical for mRNA export at very early stages of Arabidopsis development.

We tested whether the *los4-1* mutation also affects mRNA export. *los4-1* mutant plants do not show any morphological difference with wild-type plants under normal growth conditions, but *los4-1* mutant plants are more sensitive to chilling (Gong et al., 2002) and heat stress (Figure 6C), and the chilling sensitivity is more severe in the dark than in the light (Gong et al., 2002). We compared the nuclear poly(A) signals between *los4-1* and wild-type plants at different temperatures. *los4-1* mutant plants show an accumulation of poly(A) signals in the nuclei compared with wild-type plants at 22°C (Figure 9A) as well as 4°C (Figures 9B and 9C). There are no apparent differences in the nuclear poly(A) signals between the dark- and light-treated mutant plants at 4°C (Figures 9B and 9C). These results show that the *los4-1* mutation also affects mRNA export.

DISCUSSION

The allelic *cryophyte*/*los4-2* and *los4-1* mutations both affect cold responses but in opposite ways. The *los4-2* mutation enhances cold induction of *CBFs* and their downstream target genes, whereas *los4-1* reduces the expression of these genes in the cold (Gong et al., 2002). Consistent with their gene regulation phenotypes, *los4-1* mutant plants are sensitive to chilling stress, whereas *los4-2* plants appear to be more resistant to chilling than the wild type. The *los4-2* mutation disrupts mRNA export at warm and high temperatures but not at low temperatures. By contrast, the *los4-1* mutation impairs mRNA export at warm and low temperatures. Therefore, *los4-2* appears to be a heat-sensitive allele that may even enhance mRNA export at low temperatures, whereas *los4-1* appears to be a constitutive allele that affects mRNA export at both low and warm temperatures.

The heat sensitive phenotype of *los4-2* plants is likely a consequence of the severe mRNA export defect in the mutant at high temperatures. It is possible that the cold responsive gene regulation and cold tolerance/sensitivity phenotypes of the *los4-1* and *los4-2* plants are also caused by the RNA export defects in the mutants. Our RNA gel blot results suggested that the mutations changed the total cellular levels of several cold responsive gene transcripts (Figure 2; Gong et al., 2002). It is

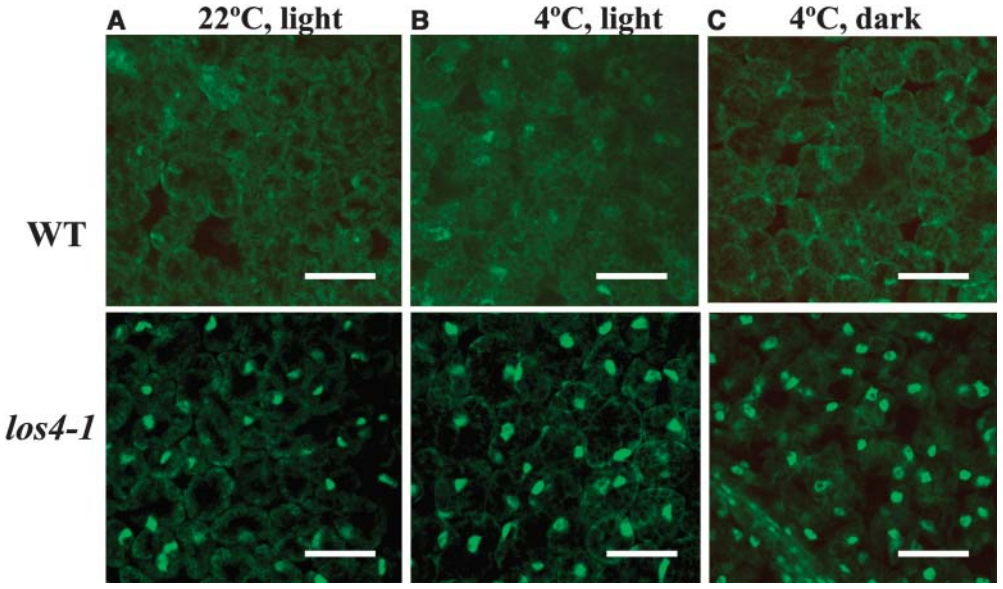

Figure 9. Defects in mRNA Export in *los4-1* Plants.

Poly(A) RNA distribution in 2-week-old wild-type and *los4-1* mutant plants that were grown at 22°C **(A)** and subjected to a 2-d treatment at 4°C under light **(B)** or dark **(C)**. Bars = 40 μm.

possible that the RNA export defects affect cold responses by directly influencing the export of *CBF* mRNAs. Alternatively, an early component(s) in cold signaling may be affected by the mRNA export activity of LOS4.

It is also possible that LOS4 may play a more specific role in cold responses, and this may be unrelated to its function in mRNA export. A more specific role of LOS4 in cold responsive gene regulation is suggested by the fact that although RNA export is significantly impaired at 22°C in *los4-2* and *los4-1*, *RD29A-LUC* induction by ABA or salt stress at 22°C is not different between these mutants and the wild type. The RNA export defect in *los4-1* even at low temperatures is much less severe than that in *los4-2* at 22°C. Nevertheless, the expression of *RD29A-LUC* and other cold responsive genes is impaired in *los4-1* at low temperatures (Gong et al., 2002). Furthermore, the expression of several cold responsive genes is different between *los4-2* and the wild type, despite the fact that there is no obvious RNA export defect in the mutant at cold temperatures. It has been suggested that certain RNAs can be temperature sensors because temperature influences the extent of RNA secondary structure (Morita et al., 1999). Indeed, an RNA thermosensor has been found and shown to control the expression of virulence genes in *Listeria monocytogenes* (Johansson et al., 2002). RNA helicases are capable of unwinding double stranded RNAs and thus control the extent of secondary structure of RNAs. Therefore, it is conceivable that LOS4 as an RNA helicase may be directly involved in temperature sensing, and the *los4-1* and *los4-2* mis-sense mutations may somehow affect the RNA sensor(s) in opposite ways. The *los4-1* and *los4-2* lesions seem to affect different functional domains of the protein, as indicated by the fact that the two mutations complemented each other for the *RD29A-LUC* expression phenotype.

Our results suggest that the LOS4 protein may be enriched at the nuclear rim and is essential for mRNA export. The role of DEAD box RNA helicases in development or stress responses in multicellular organisms has not been investigated previously. Analysis of the mRNA export defective mutant *cryophyte*/*los4-2* has provided an uncommon opportunity to understand the contribution of mRNA export to higher plant development and stress responses. The seeds of *los4-2* plants could germinate at high temperatures that are inhibitory to the germination of wild-type seeds. It is possible that high temperatures induce a germination inhibitor in the wild type, but this inhibitor cannot be made in the *los4-2* mutant because of its severe defect in mRNA export. Recent studies have revealed an intriguing connection between RNA metabolism and ABA signaling in plants (Hugouvieux et al., 2001; Xiong et al., 2001). The ABA-hypersensitive mutations, *sad1* and *abh1*, were found to be in genes encoding an Sm-like RNA splicing/export/degradation factor and an mRNA cap binding protein, respectively (Hugouvieux et al., 2001; Xiong et al., 2001). However, the exact mechanistic connection between RNA metabolism and ABA sensitivity remains to be elucidated. The germination of *los4-2* seeds is more sensitive to ABA inhibition (Figure 3H). It is possible that *los4-2* mutation influences the RNA export of some gene(s) that are involved in ABA signal transduction.

Early flowering is another dramatic and interesting phenotype of *los4-2* mutant plants. Vernalization accelerates flowering by reducing the expression of *FLC*, a repressor of flowering (Sheldon et al., 2000). We have shown previously that the early flowering phenotype of *hos1* plants correlates with reduced *FLC* expression in the mutant (Lee et al., 2001). In response to vernalization treatment, flowering of *hos1* is further accelerated (Ishitani et al., 1998). By contrast, *los4-2* mutant plants do not show a substantial reduction in *FLC* expression (Figure 4D). Furthermore, flowering of the *los4-2* mutant plants are not responsive to vernalization under long day conditions (Figure 4A). Under the same conditions, the *hos1* mutant flowers just as early as *los4-2* (Figure 4C), but *hos1* can still respond to vernalization by flowering even earlier (Ishitani et al., 1998). Two vernalization mutants reported previously, *vrn1* and *vrn2*, are defective in the memory or maintenance of vernalization-induced *FLC* down-regulation (Gendall et al., 2001; Levy et al., 2002). The vernalization insensitivity under long day conditions and the early flowering phenotype of *los4-2* plants may be consequences of an mRNA export defect in the mutant. The RNA export of *FLC* or other flowering repressors may be defective in *los4-2* mutant plants.

METHODS

Plant Materials, Growth Conditions, and RNA Analysis

Plant materials, growth conditions, and RNA analysis are as described by Gong et al. (2002). Luminescence imaging was as described (Ishitani et al., 1997). For the RNA gel blot analysis of the *LOS4* gene, the full-length *LOS4* cDNA was used as a probe.

Genetic Mapping

The *cryophyte* mutant in the C24 background was crossed with wild-type *Arabidopsis thaliana* in the Columbia background. The F2 population was screened for mutants with higher luminescence expression at 4°C. DNA samples were prepared from single leaves of mutant F2 plants. Using SSLP markers, the mutation was initially mapped to chromosome 3 between SSLP markers nga162 and nga112. The same markers for mapping *los4-1* were used to narrow down the *cryophyte* mutation to the same region as the *LOS4* locus.

DNA Constructs and Plant Transformation

The construct for *los4-1* complementation (Gong et al., 2002) was used for *cryophyte* mutant complementation. For the LOS4-GFP fusion construct, *LOS4* cDNA was amplified using the forward primer, 5′-CATCTAGAATGGCGGATACGGTAGAGAAAGTTCCCACC-3′ (underline indicates *Xba*I), and the reverse primer, 5′-GCGGATCCACTCGTCCAGCAGGCCAGCTTCCTTTAATGC-3′ (underline indicates *Bam*HI site for GFP in-frame fusion). The PCR product was digested with *Xba*I and *Bam*HI and ligated to pBIN35S-GFP vector double digested with the same two enzymes. For the *LOS4* promoter-GUS fusion construct, PCR was conducted using the forward primer, 5′-ATGTCGACCATGATCAGTAATATCTTTGCATCCTC-3′ (underline indicates *Sal*I site), and the reverse primer, 5′-AGGGATCCCAAATTCATCTTAACTTCTCCATTGATTCG-3′ (underline indicates *Bam*HI site). The final length of the PCR fragment was 1149 bp. The PCR products were digested with *Bam*HI and *Sal*I and cloned into pBI101 vector. The resulting vectors were transferred into *Agrobacterium tumefaciens* strain GV3101. These strains were then used to transform wild-type Arabidopsis (ecotype Columbia-0, *gl1*). Transformants were selected on an MS medium (Sigma-Aldrich, St. Louis, MO) containing 35 mg/L of kanamycin and were transferred to soil to set

seeds. Histochemical assays of *GUS* expression were performed as described (Lee et al., 2001).

ATPase Activity Assay of LOS4 RNA Helicase

The PCR product amplified using a forward primer, 5′-CGGGATCC-GATGGCGGATACGGTAGAGAAAGTTCCCACC-3′ (*Bam*HI site is underlined), and a reverse primer, 5′-AACTGCAGCTCGTCCAGCAGGC-CAGCTTCCTTTAATGC-3′ (underline indicates *Pst*I site), was cloned into pETblue-2 to make a His-tagged His-LOS4 fusion protein. The protein was purified on a His-affinity column. The ATPase activity of LOS4 RNA helicase was determined as described by Iost et al. (1999).

Poly(A) RNA in Situ Localization Assay

Poly(A) RNA in situ hybridization was conducted essentially as described by Engler et al. (1994) with minor modifications. Samples were taken from equivalent portions of young leaves at similar developmental stages from the wild type and mutants and were fixed in glass vials by adding 10 mL of fixation cocktail containing a mixture of 50% fixation buffer (120 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, 2.7 mM KCl, 0.1% Tween 20, 80 mM EGTA, 5% formaldehyde, and 10% DMSO) and 50% heptane. The samples were gently shaken for 30 min at room temperature. After dehydration twice for 5 min in absolute methanol and three times for 5 min in absolute ethanol, the samples were incubated for 30 min in 1:1 ethanol:xylene and then washed twice for 5 min with absolute ethanol, twice for 5 min with absolute methanol, and once for 5 min with 1:1 methanol:fixation buffer without 5% formaldehyde. The samples were postfixed in the fixation buffer for 30 min at room temperature. After fixation, the samples were rinsed twice with fixation buffer without 5% formaldehyde and once with 1 mL of perfect Hyb Plus hybridization buffer (Sigma-Aldrich; H-7033). Each glass vial was then added with 1 mL of hybridization buffer and prehybridized in an incubator for 1 h at 50°C. After prehybridization, 5 pmol of 45-mer oligo(dT) labeled with one molecule of fluoresceine at the 5′-end (synthesized by MWG Company, High Point, NC) was added into each glass vial and hybridized at 50°C in darkness for more than 8 h. After hybridization, the samples were washed once for 60 min in 2× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.1% SDS at 50°C and once for 20 min in 0.2× SSC, 0.1% SDS at 50°C in darkness. The samples were observed immediately using a Bio-Rad MRC-1024 confocal laser-scanning microscope (Hercules, CA) with a 488-nm excitation laser and a 522/DF35 emission filter. All samples were observed at the same conditions, including using the same 40× objective (bar = 40 μm), same laser strength. Approximately 10 to 12 optical sections in 0.8-mm steps were collected and projected with an imaging software package. Each experiment was repeated at least three times, and similar results were obtained.

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