Arabidopsis Cor15am Is a Chloroplast Stromal Protein That Has Cryoprotective Activity and Forms Oligomers$^{1[W][OA]}$

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Many plants acquire increased freezing tolerance when they are exposed to nonfreezing temperatures of a certain duration. This process is known as cold acclimation and allows plants to protect themselves from freezing injury. A wide variety of polypeptides are induced during cold acclimation, among which is one encoded by COR15A in Arabidopsis (Arabidopsis thaliana). Previous studies showed that the COR15A gene encodes a small, plastid-targeted polypeptide that is processed to a mature form called Cor15am. In this study, we examined the biochemical properties and activities of Cor15am in more detail. We provide evidence that Cor15am localizes almost exclusively to the chloroplast stroma. In addition, the cold-regulated accumulation of Cor15am is affected by chloroplast functionality. Both gel-filtration chromatography and protein cross-linking reveal that Cor15am forms oligomers in the stroma of chloroplasts. Although Cor15am accumulates in response to low temperature, cold acclimation is not a prerequisite for oligomerization of Cor15am. Structural analysis suggests that Cor15am is composed of both ordered and random structures, and can stay soluble with small structural change after boiling and freeze-thaw treatments. Recombinant Cor15am exhibits in vitro cryoprotection of a freeze-labile enzyme, L-lactate dehydrogenase. Furthermore, Cor15am is capable of associating with L-lactate dehydrogenase in vitro and with potential stromal substrates in vivo. On the basis of these results, we propose that Arabidopsis Cor15am is a cryoprotective protein that forms oligomers in the chloroplast stroma, and that direct association of Cor15am with its substrates is part of its cryoprotective mechanism.

When plants are exposed to nonfreezing temperatures of a certain duration, they increase their level of freezing tolerance. This process, known as cold acclimation, appears to involve an alteration of gene expression (Thomashow, 2001). Considerable effort has been directed toward uncovering the mechanisms regulating the expression of cold-regulated genes (Shinozaki et al., 2003). Such studies have revealed that CBF/DREB transcription factors are key regulators of cold-regulated gene expression (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998; Liu et al., 1998), although there may be additional regulatory pathways that remain to be characterized. Overexpression of CBF/DREB transcription factors increased the freezing tolerance of Arabidopsis (Arabidopsis thaliana; Jaglo-Ottosen et al., 1998; Liu et al., 1998). In these plants, the downstream target genes for CBF/DREB transcription factors were up-regulated without cold acclimation, resulting in increased freezing tolerance.

Although a number of target genes for CBF/DREB transcription factors have been identified (Maruyama et al., 2004), the biochemical activities of the polypeptides encoded by those genes are poorly understood. However, some of them can be assigned to known classes of proteins. For example, many cold-induced hydrophilic polypeptides belong to the hydrophilin family, which includes late embryogenesis abundant (LEA) proteins. In addition to being hydrophilic, hydrophilins and LEA proteins are known to have a high Gly content (Garay-Arroyo et al., 2000). A recent paper suggested that hydrophilins may protect enzymes against the effects of water limitation in vitro (Reyes et al., 2005), but it remains to be established whether they work similarly in vivo. Other classes of proteins, such as heat shock proteins (Wang et al., 2003) and antifreeze proteins (Griffith and Yaish, 2004), also appear to accumulate during cold acclimation and to help protect plants from low-temperature stress.

In Arabidopsis, a small hydrophilic polypeptide, designated as Cor15a, is induced and targeted to chloroplasts during cold acclimation (Lin and Thomashow, 2007). Previous studies showed that the COR15A gene encodes a small, plastid-targeted polypeptide that is processed to a mature form called Cor15am. In this study, we examined the biochemical properties and activities of Cor15am in more detail. We provide evidence that Cor15am localizes almost exclusively to the chloroplast stroma. In addition, the cold-regulated accumulation of Cor15am is affected by chloroplast functionality. Both gel-filtration chromatography and protein cross-linking reveal that Cor15am forms oligomers in the stroma of chloroplasts. Although Cor15am accumulates in response to low temperature, cold acclimation is not a prerequisite for oligomerization of Cor15am. Structural analysis suggests that Cor15am is composed of both ordered and random structures, and can stay soluble with small structural change after boiling and freeze-thaw treatments. Recombinant Cor15am exhibits in vitro cryoprotection of a freeze-labile enzyme, L-lactate dehydrogenase. Furthermore, Cor15am is capable of associating with L-lactate dehydrogenase in vitro and with potential stromal substrates in vivo. On the basis of these results, we propose that Arabidopsis Cor15am is a cryoprotective protein that forms oligomers in the chloroplast stroma, and that direct association of Cor15am with its substrates is part of its cryoprotective mechanism.

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RESULTS

Precise Localization of Cor15am within Chloroplasts

Cor15A was originally identified as a cold-regulated gene in Arabidopsis (Lin and Thomashow, 1992a). Consistent with the hydrophilicity of Cor15am, it has been proposed that this protein is localized to the soluble fraction of chloroplasts (Lin and Thomashow, 1992a). However, there is interesting but somewhat conflicting evidence provided by the same group that overexpression of Cor15am primarily affected membrane stability of chloroplasts and protoplasts (Steponkus et al., 1998), implying that Cor15am might associate with chloroplast membranes. Therefore, as a first step of our analysis, we examined the precise localization and behavior of Cor15am in chloroplasts.

First, we generated antibody against the mature form of Cor15a, Cor15am. To obtain antigen, Cor15am carrying a C-terminal His-tag was expressed in Escherichia coli (Figs. 1 and 2A). As shown in Figure 2B, anti-Cor15am serum reacted with Cor15am-His, whereas preimmune serum did not. Furthermore, the serum detected an approximately 10-kD protein in Arabidopsis protein extract only after cold acclimation (Fig. 2C), indicating that the antibody reacts with native Cor15am.

We separated cold-acclimated Arabidopsis chloroplasts into stroma, envelope, and thylakoid fractions by Suc density gradient centrifugation. Each fraction was resolved by SDS-PAGE, blotted onto nitrocellulose membrane, and probed with antibody against Cor15am. The purity of each fraction was confirmed using marker proteins such as the small subunit of Rubisco (SSU; stroma), Tic110 (envelope), and light-harvesting complex protein (LHCP; thylakoid). As shown in Figure 2D, Cor15am was found exclusively in the stromal fraction. As an additional control, we examined AccA protein, the carboxyl transferase α-subunit of acetyl-CoA carboxylase. AccA is a soluble stromal protein (Kozaki et al., 2000) but is also known to associate with envelope membranes (Shorrosh et al., 1996; Thelen and Ohlrogge, 2002; Ferro et al., 2003). Consistent with previous studies, AccA protein was found in the envelope fraction as well as in the stroma (Fig. 2D), supporting the idea that Cor15am does not stably associate with envelope membranes. The stromal localization of Cor15am was further examined by trypsin treatment of isolated intact chloroplasts. Trypsin permeates the outer membrane but not the inner membrane of intact chloroplasts (Schnell et al., 1994; Jackson et al., 1998). Cor15am was resistant to trypsin treatment, whereas the outer membrane component ToC75 was digested (Fig. 2E). This result indicates that Cor15am is indeed localized to the stroma and is not derived from the intermembrane space. Based on these data, we concluded that Cor15am is a soluble protein of the chloroplast stroma.

The Cold-Regulated Accumulation of Cor15am Is Affected by Chloroplast Functionality

Previous studies have shown that chloroplasts and light affect the expression of some cold-regulated genes in cereal plants (Chauvin et al., 1993; Crosatti et al., 1999; Dal Bosco et al., 2003). Likewise, it was shown that Cor15am accumulates in leaves but not in roots.
This distribution pattern implies that the presence of differentiated chloroplasts somehow affects the accumulation of Cor15am. To corroborate this line of evidence, we took advantage of an Arabidopsis mutant defective in functional chloroplasts. This mutant, plastid protein import2 (ppi2), lacks the protein import receptor Toc159 and exhibits an albino phenotype due to reduced accumulation of a set of proteins required for chloroplast differentiation (Bauer et al., 2000; Smith et al., 2004). Furthermore, the expression of nuclear genes encoding chloroplast proteins is compromised in ppi2. We first examined whether Cor15am accumulates to normal levels in ppi2 after cold acclimation. Total protein was extracted from wild-type and ppi2 shoots, before and after cold acclimation, and probed with Cor15am antibody. As a control, the samples were also probed with antibody against actin. As shown in Figure 3, the ppi2 mutant accumulated Cor15am to only approximately 30% of the level found in cold-acclimated wild type, suggesting that chloroplast functionality affects the accumulation of Cor15am in chloroplasts.

To further investigate the mechanism regulating Cor15am accumulation in chloroplasts, we measured the expression of the COR15A gene in the ppi2 mutant. Since ppi2 exhibits defects in functional chloroplasts, it was possible that the expression of COR15A was compromised, leading to the reduced accumulation of Cor15am. Indeed, real-time PCR analysis revealed that the cold-induced expression of COR15A in ppi2 was approximately 50% that of wild-type plants (Fig. 3B). These data indicate that the significant decrease in Cor15am accumulation in ppi2 plastids is in part attributable to a reduction of COR15A expression. Taken together, we conclude that the cold-regulated accumulation of Cor15am is affected by chloroplast functionality, and that this regulation occurs in part at the level of mRNA accumulation.
Recombinant Cor15am Protein Forms Oligomers

As a next step in our analysis, we wished to establish the biochemical properties of Cor15am. Cor15am runs at approximately 10 kD in SDS-PAGE, but in native-PAGE analysis both chloroplastic and recombinant Cor15am migrated with apparent molecular masses of about 70 kD (Gilmour et al., 1996). It was not known whether this behavior resulted from multimerization or from aberrant mobility of Cor15am. To distinguish between these two possibilities, we first performed chemical cross-linking analysis of purified recombinant protein. Recombinant Cor15am was cross-linked using glutaraldehyde, and a small aliquot was taken at each of several time points. The samples were resolved by SDS-PAGE and probed with anti-Cor15am antibody. In glutaraldehyde-treated samples, the strongest signal shifted to a higher \( M_r \) range, and this shift was time dependent (Fig. 4A). This phenomenon was also observed using other cross-linkers. When recombinant Cor15am was incubated with various concentrations of disuccinimidyl tartarate (DST) and dithiobisuccinimidyl propionate (DSP), we again observed three different complexes (Fig. 4, B and C). These results indicate that recombinant Cor15am forms homo-oligomers.

To further dissect the oligomeric state of Cor15am, we resolved recombinant Cor15am by gel-filtration chromatography. As shown in Figure 5B, the elution volumes of the \( M_r \) markers nicely fitted a regression line \( (r^2 = 0.999) \) under our experimental conditions. The major peak of recombinant Cor15am appeared to be at approximately 40 kD, accompanied by a minor peak at around approximately 20 kD (Fig. 5A). Since mature Cor15am-His is 10.4 kD, this result suggests that the majority of the recombinant Cor15am had formed homo-oligomers, most likely tetramers. This conclusion is consistent with the apparent molecular mass of complex 3 (C3 in Fig. 4) observed in cross-linking reactions, although we do not exclude the possible existence of other oligomeric forms, such as trimers and dimers. Taken together, we conclude that recombinant Cor15am forms homo-oligomers.

Cold Acclimation Is Not Required for the Formation of Cor15am Oligomers in the Chloroplast Stroma

The homo-oligomerization of recombinant Cor15am in vitro suggests that native Cor15am can form homo-oligomers in the chloroplast stroma. However, it is also possible that additional proteins are present in native Cor15am protein complexes and help protect chloroplasts from freezing injury. To test for the presence of other proteins, we performed gel-filtration chromatography on chloroplast stroma isolated from cold-acclimated Arabidopsis. The major peak of authentic Cor15am was centered around fractions 27 and 28 (Fig. 6), similar to that of recombinant Cor15am-His. This result suggests that a majority of Cor15am exists as homo-oligomers and does not stably associate with other proteins in the stroma.

Figure 4. In vitro protein cross-linking analysis of recombinant Cor15am. A, Recombinant Cor15am-His was cross-linked with glutaraldehyde for the times indicated. B and C, Cross-linking reactions with DST (B) and DSP (C) were done at the indicated concentrations of cross-linker for 30 min. All reactions were stopped by adding 2\( \times \) SDS-PAGE sample buffer and then analyzed by immunoblot using antibody against Cor15am. C1, C2, and C3 indicate the positions of cross-linked Cor15am complexes, and M corresponds to the monomer.

Figure 5. Gel-filtration chromatography of recombinant Cor15am. A, Purified recombinant Cor15am-His protein was resolved by gel-filtration chromatography on a Sephacyr S-300 HR column equilibrated with TES buffer. Proteins were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. The molecular masses of the standard proteins are indicated by arrows. B, Estimation of the molecular mass of the Cor15am protein complex. The regression line \( (r^2 = 0.999) \) was drawn using the data for the standard proteins in A, and Cor15am-His was then plotted onto the line.
Although we established that both recombinant and authentic Cor15am can form oligomers, it was unclear whether in vivo oligomerization of Cor15am requires cold acclimation. Of particular interest, Arabidopsis constitutively overexpressing COR15A still showed increased levels of Cor15am in response to cold acclimation (Artus et al., 1996), suggesting that there may be regulation of Cor15am accumulation at the protein level. For example, cold acclimation might be required for efficient oligomerization of Cor15am in the stroma, and Cor15am may not accumulate in untreated plants because it is labile in its monomeric form. To test this hypothesis, we generated transgenic Arabidopsis plants constitutively expressing the COR15A-PROTEIN A fusion gene. As a negative control, we also generated Arabidopsis plants overexpressing protein A (pA) fused to the transit peptide of Rubisco (preSSU-pA).

To further test whether cold acclimation is a prerequisite for oligomerization of Cor15am in vivo, we isolated the stroma of chloroplasts from nonacclimated plants overexpressing Cor15a-pA. Gel-filtration chromatography revealed that the apparent molecular mass of the Cor15a-pA complex from nonacclimated plants was similar to that from acclimated plants (Fig. 7D). Therefore, we concluded that Cor15am forms oligomers as soon as it is imported into chloroplasts.

Structural Analysis of Cor15am

Previous literatures (Lin and Thomashow, 1992a; Steponkus et al., 1998) and the predicted secondary structure of Cor15am (Supplemental Fig. S1) suggested that it was predominantly α-helical. This, along with the observation that Cor15am forms oligomers, distinguishes it from other cold-inducible hydrophilic proteins.
proteins such as hydrophilins and LEA proteins, which are usually monomeric and highly unstructured (Garay-Arroyo et al., 2000). To obtain experimental verification of the predicted secondary structure of Cor15am, we examined recombinant Cor15am using CD spectroscopy. The recombinant protein exhibited a strong negative peak at around 205 nm, accompanied by a minor peak between 220 and 225 nm (Fig. 8A). Analytical program for protein-secondary structure (SSE-338W type; JUSCO) estimated that Cor15am contained a certain amount of ordered secondary structures as well as random structure (Fig. 8B). This was in contrast to the estimation by prediction programs (Supplemental Fig. S1).

Previous studies showed that Cor15am stayed soluble after boiling (Lin et al., 1990; Lin and Thomashow, 1992a). Therefore, we tested whether Cor15am can still retain the observed structure after boiling as well as a freeze-thaw cycle. As shown in Figure 8A, Cor15am exhibited small structural change. The amount of β-strand seemed to be affected particularly (Fig. 8B), although the functional significance of this structural change remains to be characterized. We conclude that Cor15am contains a certain amount of ordered secondary structures as well as random structure and can stay soluble with small structural change after temperature stress.

Cor15am Protects Lactate Dehydrogenase from Freeze-Inactivation

The structural stability of Cor15am at extreme temperatures is consistent with a previously proposed role in protecting proteins from temperature stress. This hypothesis was originally proposed many years ago based on the fact that in vitro translated Cor15a, the precursor of Cor15am, had potent cryoprotective activity (Lin and Thomashow, 1992b). However, because cleavage of transit peptides occurs cotranslationally during protein import into chloroplasts (Schnell and Blobel, 1993), it is unlikely that chloroplast-targeted, functional Cor15am still retains the transit peptide that was present in the previous in vitro assay. In addition, the Cor15a protein used in the previous study was electroeluted from an SDS-PAGE gel and precipitated with acetone (Lin and Thomashow, 1992b). Therefore, it is also possible that the structure of the Cor15a in this preparation was altered from its native state. Furthermore, the hypothesis that Cor15a’s main function is to protect proteins from temperature extremes has been displaced by an alternative proposal (Steponkus et al., 1998) in recent years. Because of this, it remained unclear whether Cor15am, the mature counterpart of Cor15a, indeed has cryoprotective activity. To resolve this issue, we evaluated the cryoprotective activity of our purified Cor15am by testing its ability to protect 1-lactate dehydrogenase (LDH) against freeze-inactivation. Equivalent concentrations of bovine serum albumin (BSA) and Suc were used as positive and negative controls, respectively. As shown in Figure 9, 20 μg/mL Cor15am-His was sufficient to completely protect LDH from freeze-inactivation, whereas the same concentration of BSA only preserved 40% of the LDH activity after a freeze-thaw cycle. The cryoprotective activity of purified Cor15am was comparable to that of other cryoprotective proteins, such as PCA60 (Wisniewski et al., 1999) and WAP18 (Ukaji et al., 2004), but was not as potent as described previously (Lin and Thomashow, 1992b). These results strongly support the idea that Cor15am protects chloroplastic enzymes from low-temperature or freeze-induced inactivation.

Cor15am Is Capable of Associating with Other Proteins Both in Vitro and in Vivo

To further unravel the molecular mechanism by which Cor15am protects freeze-labile enzymes, we examined whether Cor15am could directly associate with LDH. A mixture of Cor15am and LDH was treated with DSP to stabilize any interactions between proteins, and then the Cor15am complexes were affinity purified using anti-Cor15am IgG Sepharose. As shown in Figure 10A, LDH was copurified with Cor15am, indicating that Cor15am can associate with the proteins it cryoprotects.

Figure 8. Circular dichroism spectra of Cor15am. A, The purified Cor15am protein was dialyzed against 50 mM HEPES-KOH buffer and the CD spectrum was measured using a spectropolarimeter. The same procedure was repeated with samples that were either boiled or freeze-thawed before the measurement. B, Estimation of secondary structural elements by analytical program for protein-secondary structure (SSE-338W type; JUSCO).
The direct association between Cor15am and LDH suggested that Cor15am may associate with stromal proteins. To test this hypothesis, we purified Cor15am-substrate complexes from the stroma of chloroplasts. We used a cross-linker in these experiments because Cor15am did not seem to associate stably with other proteins in the stroma (Fig. 6). Intact chloroplasts isolated from wild-type plants before and after cold acclimation were treated with DSP to chemically cross-link Cor15am with potential substrates. After lysis of chloroplasts, the stromal fraction was subjected to immunofinity chromatography. DSP is a cleavable cross-linker, and the disulfide bonds between Cor15am and associated proteins were cleaved by adding dithiothreitol (DTT) before electrophoresis. As shown in Figure 10, B and C, Cor15am was only detected in the eluate of the acclimated plants. This result is consistent with the cold inducibility of Cor15am. In addition, we observed that certain other proteins were significantly enriched in the eluate of the acclimated plants (Fig. 10B, arrows and arrowheads). Among those proteins, two appeared to consistently copurify with Cor15am: the large and small subunits of Rubisco (Fig. 10B, arrows). The consistent association of Cor15am with Rubisco suggests that the latter is a substrate for Cor15am’s cryoprotective activity. The amounts of other proteins enriched in the eluate of acclimated plants differed in each experiment (Fig. 10B, indicated by arrowheads). Despite this variability, we do not exclude the possibility that these proteins are also substrates of Cor15am. The validity of the interaction between Cor15am and Rubisco was further confirmed using transgenic lines expressing fusion proteins. When we performed similar experiments...
immunoaffinity chromatography using stroma isolated from Cor15a-pA-expressing plants, Rubisco was copurified with Cor15am-pA (Fig. 10D, lane 2). The amount of Rubisco in the Cor15am-pA eluate was much more than that in the negative control (Fig. 10D, lane 1), indicating that the interaction between Rubisco and Cor15am-pA is mediated by the Cor15am portion of the fusion protein. Based on these results, we concluded that Cor15am can associate with its substrates in vivo as well as in vitro, and that Rubisco is one of the potential substrates for Cor15am in the stroma of chloroplasts.

DISCUSSION

Hundreds of cold-regulated genes have been identified so far from a number of plant species. However, only a small number of polypeptides have been characterized at the molecular level, and, therefore, the biochemical activities of cold-regulated proteins remain largely unclear. In this study, we have examined in detail the biochemical properties of a cold-regulated protein, Arabidopsis Cor15am. We demonstrated that Cor15am is a chloroplast stromal protein (Fig. 2). In addition, we showed that the normal accumulation of Cor15am was prevented in the absence of functional chloroplasts (Fig. 3). We also obtained evidence that Cor15am forms oligomers (Figs. 4–6), most likely tetramers, both in vitro and in vivo. In transgenic plants expressing a Cor15am fusion protein constitutively, the formation of Cor15am oligomers did not depend on cold acclimation (Fig. 7). Finally, structural and biochemical analysis revealed that Cor15am is a cryoprotective protein containing ordered structures (Figs. 8 and 9) and is capable of associating directly with potential substrates (Fig. 10). Taken together, these data establish that Cor15am is a cryoprotective protein that contains ordered structures and forms oligomers in the stroma of chloroplasts.

We observed that Cor15am was localized exclusively to the stroma of chloroplasts. This raises the question of how Cor15am protects the chloroplast envelope from freezing injury (Steponkus et al., 1998). This role for Cor15am was originally proposed based on the observation that Cor15am altered the lamellar-to-hexagonal II phase transition temperature of liposomes in vitro (Steponkus et al., 1998). However, it is hard to imagine that Cor15am could protect the chloroplast envelope if it does not associate with this membrane. Although it is possible that other membrane-associated proteins mediate an interaction between Cor15am and the envelope, such proteins have not been identified yet. Alternatively, Cor15am may function primarily in the stroma. In this scenario, Cor15am would protect enzymes from inactivation or activate enzymes to maintain or increase chloroplast metabolic activity during cold acclimation. This hypothesis is supported by the fact that purified Cor15am has cryoprotective activity in a standard in vitro cryoprotection assay (Fig. 9). The cryoprotective activity of Cor15am is stronger than BSA and similar to other cryoprotective proteins (Wisniewski et al., 1999; Ukaji et al., 2004). Therefore, Cor15am is likely to protect chloroplastic enzymes from freeze- or chilling-induced inactivation.

In an attempt to further clarify the mechanism by which Cor15am protects enzymes from inactivation, we examined the interaction between Cor15am and its substrates by cross-linking. As shown in Figure 10A, Cor15am is capable of associating with LDH in vitro. Furthermore, we identified Rubisco as a potential substrate in the stroma by immunoaffinity chromatography of stromal samples cross-linked in vivo (Fig. 10, B–D). Intriguingly, previous studies have shown that Rubisco activity increases during cold acclimation (Holaday et al., 1992; Strand et al., 1999). This increase in activity was attributed to both activation of Rubisco and a substantial increase in Rubisco protein levels (Holaday et al., 1992; Strand et al., 1999). Therefore, the protective activity of Cor15am, by preventing cold-inactivation of Rubisco, might contribute to the increase of Rubisco activity during cold acclimation. It is also possible that the presence of Cor15am allows Rubisco to accumulate to higher levels in the stroma of acclimated chloroplasts. Whether or not Cor15am has substrate proteins other than Rubisco in the stroma remains to be examined. Nevertheless, we speculate that Cor15am is likely to protect a wide variety of substrates. This proposal is supported by the fact that Cor15am protects LDH, a cytosolic protein, from inactivation (Fig. 9). Since Rubisco is the most abundant enzyme in the stroma of chloroplasts, Cor15am would be expected to associate predominantly with Rubisco. Nevertheless, other enzymes involved in sugar metabolism are possible targets because sugars appear to have important roles during cold acclimation (Stitt and Hurry, 2002).

Structural analysis revealed that Cor15am contains a certain amount of ordered secondary structures, such as $\alpha$-helices (Fig. 8). Furthermore, Cor15am also appears to form oligomers (Figs. 4–6), most likely tetramers. This feature distinguishes Cor15am from other cold-regulated proteins, such as hydrophilins and LEA-type proteins, which are also hydrophilic and boiling-stable. For example, LEA-type proteins have a mostly unfolded structure (Garay-Arroyo et al., 2000; Wang et al., 2003) and only a few of them have been found to form oligomers (Wang et al., 2002, 2003). This structural difference suggests that Cor15am protects freeze-labile enzymes via a different mechanism than hydrophilins and LEA proteins.

We speculate that Cor15am may exist as homo-oligomers under normal conditions. However, Cor15am may associate with other proteins under conditions where proteins are unstable. Another example of a stress-responsive, boiling-stable protein that forms homo-oligomers is SP1 isolated from aspen (Populus spp.; Wang et al., 2002). The crystal structure of SP1 revealed that this protein forms stable dimers and is
likely to form dodecamers as the biologically active unit (Dgany et al., 2004). As we observed for Cor15am, SP1 also contains α-helices and β-strands (Dgany et al., 2004). Those ordered structures in SP1 appear to be involved in protein-protein interaction (Dgany et al., 2004). Although it remains to be determined whether SP1 has protective activity, the mode of action of SP1 and Cor15am seems to resemble that of small heat shock proteins rather than that of LEA-type proteins.

Previous studies have shown that chloroplasts and light affect the expression of some cold-regulated genes in cereal plants (Chauvin et al., 1993; Crosatti et al., 1999; Dal Bosco et al., 2003). In Arabidopsis, a number of cold-regulated genes have been identified (Maruyama et al., 2004), but few proteins whose accumulation depends on functional chloroplasts have been reported. Given the fact that Cor15am accumulated in leaves but not roots (Lin and Thomashow, 1992a), we speculated that chloroplast functionality might also affect the expression of COR15A. In this study, we demonstrated that the normal accumulation of Cor15am in ppi2 mutant was significantly impaired, and that this was in part due to a defect in COR15A mRNA expression (Fig. 3). This result suggests that chloroplasts affect cold-regulated gene expression in Arabidopsis as well as in cereal. Although the mechanism by which chloroplasts influence the expression of COR15A remains to be characterized, such regulation has been shown to occur at the posttranscriptional level in barley (Hordeum vulgare). The promoter of the COR14b gene was active in the albino mutant of barley, which accumulates less than 5% of the wild-type levels of COR14b mRNA (Dal Bosco et al., 2003), suggesting that the chloroplast control of COR14b expression occurs posttranscriptionally. Because COR14b still exhibited chloroplast-regulated accumulation when expressed in Arabidopsis (Crosatti et al., 1999), Arabidopsis may have regulatory mechanisms similar to those in barley. It would be of great interest to investigate the molecular mechanism by which chloroplasts influence the expression of cold-regulated genes at the posttranscriptional level.

In summary, we demonstrated that Cor15am is almost exclusively located in the stroma of chloroplasts. Cor15am contains a certain amount of ordered secondary structures as well as random structure. Unlike most other hydrophilic and stress-inducible proteins, Cor15am is capable of forming oligomers, although cold acclimation is not a prerequisite for oligomerization. Cor15am also has the ability to protect a freeze-labile enzyme from inactivation and to directly associate with potential substrates. This evidence suggests a novel cryoprotective mechanism: that Cor15am protects enzymes in the chloroplast stroma from low- or freezing-temperature stresses. Although we were not able to obtain direct evidence for it, an intriguing hypothesis is that Cor15am can associate not only with freeze-labile stromal enzymes, but also with envelope membranes under certain conditions (Steponkus et al., 1998). If Cor15am indeed has multiple protective activities, it may be possible to provide broad and robust protection of plant cells from freeze-induced damage simply by overexpressing this single gene (Artus et al., 1996). Further investigation should provide insight into the molecular basis for Cor15am-mediated protection of the chloroplast envelope.

MATERIALS AND METHODS

Construction of Plant Transformation Vectors and Arabidopsis Transformation

All constructs used in this study are summarized in Figure 1. The COR15A cDNA was amplified from Arabidopsis (Arabidopsis thaliana) cDNA by RT-PCR using primers that introduced a 5′ NcoI site and a 3′ Xhol site. The cDNA was inserted into the NcoI and Xhol sites of pET21d to generate pET-Cor15a-His. For construction of pCAMBIA-Cor15a-pA, the COR15A cDNA was first subcloned into the NcoI and EcoR1 sites of pET21d-TEV-pA to generate pET21d-Cor15a-pA. This plasmid was then digested with NcoI and Xhol and the insert subcloned into the NcoI and Xhol sites of pCAMBIA3300.1 to generate pCAMBIA-Cor15a-pA. For expression in Escherichia coli, the mature portion of Cor15a, designated as Cor15am, was amplified by PCR using pET-Cor15a-His as the template. The amplified fragment was subcloned into pET21d to generate pET-Cor15a-His.

All pCAMBIA constructs were introduced into Arabidopsis (ecotype Columbia) via Agrobacterium tumefaciens-mediated transformation by the floral dip method (Clough and Bent, 1998).

RNA Extraction and Real-Time PCR Analysis

Total RNA was isolated using a RNAiso kit (TaKaRa) from the aerial tissues of Arabidopsis grown on 0.5× Murashige and Skoog plates supplemented with 1% Suc, before or after cold acclimation. COR15A mRNA was quantified with a LightCycler PCR machine (Roche), using a One Step SYBR RT-PCR kit (TaKaRa) and gene-specific primers. The transcript levels of COR15A were normalized to those of 18S ribosomal RNA.

Expression of Cor15am-His in E. coli

For bacterial expression, pET21d-Cor15am-His was transformed into E. coli BL21(DE3). Expression was induced with 1 mM IPTG for 3 h at 37°C and the soluble fraction was recovered. The Cor15am-His protein was purified under nondenaturing conditions using His-bind resin (Novagen).

Antibodies and Immunoblotting

Rabbit polyclonal antibody against Cor15am was produced using Cor15am-His (Fig. 2A) as the antigen. Antibodies against Tic110 (Inaba et al., 2003, 2005), Toc75 (Hilbrunner et al., 2001), SSU (Sasaki et al., 1981), LHCP (Payan and Cline, 1991), and AccA (Kozaki et al., 2000) were analyzed by SDS-PAGE and immunoblotting after reisolation. Proteins were detected with a luminoimage analyzer (AE-6972C; ATTO) using chemiluminescence reagents. When necessary, the signal was quantified using Image acquisition software (CS Analyzer; ATTO).

Arabidopsis Chloroplast Isolation and Fractionation

Wild-type and transgenic Arabidopsis were grown on 0.5× Murashige and Skoog plates supplemented with 1% Suc. Chloroplasts were isolated from 14- to 18-d-old seedlings as described previously (Smith et al., 2002). For some experiments, plants were cold acclimated for 48 h prior to isolation of chloroplasts. Intact chloroplasts were treated with trypsin as described previously (Kouranov et al., 1998), and were analyzed by SDS-PAGE and immunoblotting after reisolation. Chloroplasts were fractionated into stroma, envelope, and thylakoid fractions as described previously (Smith et al., 2002), with some modification. Briefly, broken chloroplasts in 50 mM Tricine-KOH, 2 mM EDTA, and 1 mM DTT (TE/DTT) buffer containing 0.6 M Suc were diluted with 2 volumes of...
TE/DTT buffer to give 0.2 mM Suc in the final mixture. The diluted chloroplasts were homogenized with a Teflon homogenizer, layered onto Suc density step gradients (0.46 g/mL and 1.1 g/mL), and then separated by ultracentrifugation at 270,000 g for 90 min. The 0.2 mM Suc layer and 0.2 mL/0.46 mL interface layer were collected as the stroma, precipitated with 10% trichloroacetic acid, and dissolved in SDS-PAGE sample buffer. The 0.46 g/mL/1 mL interface layer, which corresponds to the envelope fraction, was diluted with 5 volumes of TE/DTT and recovered by ultracentrifugation at 270,000 g for 1 h. The resulting envelope pellet was directly dissolved in SDS-PAGE buffer. The pellet from the Suc density gradient centrifugation was directly dissolved in SDS-PAGE sample buffer and used as the thylakoid fraction.

**Cross-Linking Reactions**

For protein cross-linking using glutaraldehyde, 30 μM (on a monomer basis) recombinant Cor15am-His was incubated with 0.125% glutaraldehyde (Wako Chemical) at room temperature. A small aliquot of the sample was taken out at each of the indicated time points and then quenched by adding an equal volume of 2× SDS-PAGE sample buffer containing DTT and incubating for 15 min.

For cross-linking reactions using DST and DSP (PIERCe), 2 μM recombinant Cor15am-His was incubated with the indicated concentrations of cross-linker for 30 min at room temperature. The samples were then quenched by adding an equal volume of 2× SDS-PAGE sample buffer (minus DTT) and incubating for 15 min.

To examine the interaction between Cor15am and LDH (obtained from Nacalai Tesque), 0.8 μM LDH was incubated with 0.5 mM DSP in the presence or absence of Cor15am (10 μM) for 30 min at room temperature. The reaction was quenched by incubating with Gly at a final concentration of 50 mM for an additional 15 min. The quenched samples were mixed with an equal volume of HEGS buffer (50 mM HEPES-KOH, pH 7.5, 2 mM EDTA, 10% glycerol, and 250 mM NaCl). The samples were then analyzed by immunoaffinity chromatography, as described later.

For cross-linking of stromal proteins, isolated chloroplasts (700 μg of chlorophyll) were first incubated in the dark for 30 min to deplete the reducing potential. The depleted chloroplasts were then incubated with 2.5 mM DSP on ice for 15 min in the dark (Inaba et al., 2005). The reaction was quenched by incubating with Gly at a final concentration of 50 mM on ice for an additional 15 min. The quenched chloroplasts were stored at −80°C until extraction. To prepare stromal extracts, freeze-thawed chloroplasts were lysed in 5 volumes of TES buffer containing 15 mM Tricine-KOH, pH 7.5, 2 mM EDTA, and 250 mM NaCl and centrifuged at 100,000 g for 20 min. The supernatant was used for immunoaffinity chromatography.

**Gel-Filtration Chromatography**

To obtain the stromal fraction, chloroplasts (1 mg chlorophyll/mL) were lysed in 5 volumes of TES buffer containing 15 mM Tricine-KOH, pH 7.5, 2 mM EDTA, and 250 mM NaCl and centrifuged at 100,000 g for 20 min. The supernatant was resolved on a Sephacryl S-300 HR column equilibrated with 20 mM Tricine-KOH buffer (12.5 mM NaCl) containing 2% Triton X-100. The depleted chloroplasts were then incubated with 2.5 mM DSP on ice for 15 min. The quenched chloroplasts were stored at −80°C until extraction. To prepare stromal extracts, freeze-thawed chloroplasts were lysed in 5 volumes of TES buffer containing 15 mM Tricine-KOH, pH 7.5, 2 mM EDTA, and 250 mM NaCl. The lysed chloroplasts were centrifuged at 20,000 g for 20 min, and the supernatant was used for immunoaffinity chromatography.

**CD Spectroscopy**

CD spectra were determined as described previously (Miyazaki et al., 2004) for 60 μM recombinant Cor15am-His in 50 mM HEPES-KOH buffer, pH 7.5, using a J-720 spectropolarimeter (JUSCO). For some experiments, the protein sample was either boiled or subjected to a freeze-thaw treatment before the measurement. Secondary structural elements were estimated by analytical program for protein-secondary structure (SSE-338W type; JUSCO).

**Cryoprotection Assay**

The cryoprotective activity of Cor15am was assayed as described previously (Lin and Thomashow, 1992b). Briefly, the freeze-labile enzyme LDH (obtained from Nacalai Tesque) was diluted to 12.5 μg/mL in 10 mM KPO4, pH 7.5. Fifty microliters of this solution was mixed with an equal volume of 2× cryoprotectant solution and then frozen at −22°C for 48 h. The samples were thawed at room temperature and the LDH activity was measured using a spectrophotometer (Hitachi).

**Imunoaffinity Chromatography**

For immunoaffinity chromatography of wild-type plants, anti-Cor15am IgG was affinity purified using a Cor15am antigen column. For immunoaffinity chromatography of transgenic plants expressing PA fusions, anti-PA IgG (obtained from Sigma) was affinity purified using PA Sepharose (GE Healthcare). The purified IgG was dialyzed against phosphate buffer containing 150 mM NaCl and then coupled to CNBr-activated Sepharose (GE Healthcare) according to the manufacturer’s recommendations.

The cross-linked samples were applied to the antibody column and the column was washed with 20 volumes of TEGS buffer (15 mM Tricine-KOH, pH 7.5, 2 mM EDTA, 10% glycerol, and 250 mM NaCl) containing Triton X-100. The bound proteins were eluted with 0.2 M Gly-HCl, pH 2.2. Cross-links between Cor15am and its substrates were cleaved by adding SDS-PAGE sample buffer containing DTT. After SDS-PAGE, the samples were analyzed by either western blotting or Coomassie Brilliant Blue staining.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Secondary structure prediction of Cor15am.

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**LITERATURE CITED**

Artus NN, Uemura M, Steponkus PL, Gilmour SJ, Lin C, Thomashow MF. (1996) Constitutive expression of the cold-regulated Arabidopsis thaliana COR15a gene affects both chloroplast and protoplast freezing tolerance. Proc Natl Acad Sci USA 93: 13404–13409

Bauer J, Chen K, Hilbunner A, Wehrl E, Eugster M, Schnell D, Kessler F. (2000) The major protein import receptor of plastids is essential for chloroplast biogenesis. Nature 403: 203–207

Chauvin LP, Houde M, Sarhan F. (1993) A leaf-specific gene stimulated by light during acclimation to low temperature. Plant Mol Biol 23: 255–265

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

Crosatti C, Polverino de Laureto P, Bassi R, Cattivelli L. (1999) The interaction between cold and light controls the expression of the cold-regulated barley gene cor14b and the accumulation of the corresponding protein. Plant Physiol 119: 671–680

Dal Bosco C, Busconi M, Govoni C, Baldi P, Stanca AM, Crosatti C, Bassi R, Cattivelli L. (2003) cor gene expression in barley mutants affected in chloroplast development and photosynthetic electron transport. Plant Physiol 131: 793–802

Dgany O, Gonzalez A, Sofer O, Wang W, Zolotnitsky G, Wolf A, Shoham Y, Altman A, Wolf SG, Shoseyov O, et al. (2004) The structural basis of the thermostability of SP1, a novel plant (Populus tremula) boiling stable protein. J Biol Chem 279: 51516–51523

Ferro M, Salvi D, Brugiere S, Miras S, Kowalski S, Louwagie M, Garin J, Jourd J, Rolland N. (2003) Proteomics of the chloroplast envelope membranes from Arabidopsis thaliana. Mol Cell Proteomics 2: 325–345

Garay-Arroyo A, Colmenero-Flores JM, Garcia Rubio A, Covarrubias AA. (2000) Highly hydrophilic proteins in prokaryotes and eukaryotes are common during conditions of water deficit. J Biol Chem 275: 5668–5674
Plant Physiol. Vol. 144, 2007 523

Gilmour SJ, Lin C, Thomashow MF (1996) Purification and properties of Arabidopsis thaliana COR (cold-regulated) gene polyepitides COR15am and COR6.6 expressed in Escherichia coli. Plant Physiol 111: 293–299

Griffith M, Yaish MW (2004) Antifreeze proteins in overwintering plants: a tale of two activities. Trends Plant Sci 9: 399–405

Hilbrunner A, Bauer J, Vidi PA, Infanger S, Weibel P, Hohwy M, Kessler F (2001) Targeting of an abundant cytosolic form of the protein import receptor at Toc139 to the outer chloroplast membrane. J Cell Biol 154: 309–316

Holaday AS, Martindale W, Alred 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

Inaba T, Alvarez-Huerta M, Li M, Bauer J, Ewers C, Kessler F, Schnell DJ (2005) Arabidopsis thc110 is essential for the assembly and function of the protein import machinery of plastids. Plant Cell 17: 1482–1496

Inaba T, Li M, Alvarez-Huerta M, Kessler F, Schnell DJ (2003) AtTic110 functions as a scaffold for coordinating the stromal events of protein import into chloroplasts. J Biol Chem 278: 38617–38627

Jackson DT, Froehlich JE, Keegstra K (1989) The hydrophilic domain of Tic110, an inner envelope membrane component of the chloroplastic protein translocation apparatus, faces the stromal compartment. J Biol Chem 273: 16583–16588

Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, Schabenberger O, Thomashow MF (1998) Arabidopsis CBF1 overexpression induces COR genes and enhances freezing tolerance. Science 280: 104–106

Kouranov A, Chen X, Fuks B, Schnell DJ (1998) Tic20 and Tic22 are new components of the protein import apparatus at the chloroplast inner envelope membrane. J Cell Biol 143: 991–1002

Kozaki A, Kamada K, Nagano Y, Iguchi H, Sasaki Y (2000) Recombinant carboxyltransferase responsive to redox of plastid acetyl-CoA carboxylase. J Biol Chem 275: 10702–10708

Lin C, Guo WW, Everson E, Thomashow MF (1990) Cold acclimation in Arabidopsis and wheat: a response associated with expression of related genes encoding ‘boiling-stable’ polyepitides. Plant Physiol 94: 1078–1083

Lin C, Thomashow MF (1992a) A cold-regulated Arabidopsis gene encodes a polyepitide having potent cryoprotective activity. Biochem Biophys Res Commun 183: 1103–1108

Lin C, Thomashow MF (1992a) DNA sequence analysis of a complementary DNA for cold-regulated Arabidopsis gene cor15 and characterization of the COR15 polyepitide. Plant Physiol 99: 519–525

Liu Q, Kasuga M, Sakuma Y, Abe H, Miura S, Yamaguchi-Shinozaki K, Shinozaki K (1998) Two transcription factors, DREB1 and DREB2, with a hydrophilic domain of chloroplast protein import machinery. Science 280: 309–316

Miyazaki T, Sagawa R, Homma T, Noguchi S, Harada T, Komatsuda A, Ohtani H, Waku H, Sawada K, Okata M, et al (2004) Identification of cold-inducible downstream genes of the Arabidopsis DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J 38: 982–993

Miyazaki T, Sagawa R, Homma T, Noguchi S, Harada T, Komatsuda A, Ohtani H, Waku H, Sawada K, Okata M, et al (2004) 73-kDa molecular chaperone HSP73 is a direct target of antibiotic gentamicin. J Biol Chem 279: 17295–17300

Payan LA, Cline K (1991) A stromal protein factor maintains the solubility and insertion competence of an imported thylakoid membrane protein. J Cell Biol 112: 603–613

Reyes JL, Rodrigo MJ, Colmenero-Flores JM, Gil JV, Garay-Arroyo A, Campos F, Salamini F, Bartels D, Covarrubias AA (2005) Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects in vitro. Plant Cell Environ 28: 709–718

Sasaki Y, Ishiye M, Sakihama T, Kamikubo T (1981) Light-induced increase of mRNA activity coding for the small subunit of ribulose-1,5-bisphosphate carboxylase. J Biol Chem 256: 2315–2320

Schnell DJ, Blobel G (1993) Identification of intermediates in the pathway of protein import into chloroplasts and their localization to envelope contact sites. J Cell Biol 120: 103–115

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

Shinozaki K, Yamaguchi-Shinozaki K, Seki M (2003) Regulatory network of gene expression in the drought and cold stress responses. Curr Opin Plant Biol 6: 410–417

Shorrosh BS, Savage LJ, Soll J, Ohlrogge JB (1996) The pea chloroplast membrane-associated protein, IEP96, is a subunit of acetyl-CoA carboxylase. Plant J 18: 261–268

Smith MD, Fitzpatrick LM, Keegstra K, Schnell DJ (2002) In vitro analysis of chloroplast protein import. In JS Bonifacino, M Dasso, JB Harford, J Lippincott-Schwartz, KM Yamada, eds, Current Protocols in Cell Biology, Ed Suppl 14. John Wiley and Sons, New York, pp. 11.16.11–11.21.16

Smith MD, Rounds CM, Wang F, Chen K, Afitlhile M, Schnell DJ (2004) AtToc159 is a selective transit peptide receptor for the import of nucleus-encoded chloroplast proteins. J Cell Biol 165: 323–334

Stepenkos PL, Uemura M, Joseph RA, Gilmour SJ, Thomashow MF (1998) Mode of action of the COR15a gene on the freezing tolerance of Arabidopsis thaliana. Proc Natl Acad Sci USA 95: 14570–14575

Stitt M, Hurry V (2002) A plant for all seasons: alterations in photosynthetic carbon metabolism during cold acclimation in Arabidopsis. Curr Opin Plant Biol 5: 199–206

Stockinger EJ, Gilmour SJ, Thomashow MF (1997) Arabidopsis thaliana CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 94: 1035–1040

Strand A, Hurry V, Henkes N, Gustafsson P, Gardestrom P, Stitt M (1999) Acclimation of Arabidopsis leaves developing at low temperatures. Increasing cytoplasmic volume accompanies increased activities of enzymes in the Calvin cycle and in the sucrose-biosynthesis pathway. Plant Physiol 119: 1387–1398

Thelen JJ, Ohlrogge JB (2002) The multisubunit acetyl-CoA carboxylase is strongly associated with the chloroplast envelope through non-ionic interactions to the carboxyltransferase subunits. Arch Biochem Biophys 400: 245–257

Thomashow MF (2001) So what’s new in the field of plant cold acclimation? Lots! Plant Physiol 125: 89–93

Ukaji N, Kuwabara C, Takezawa D, Arakawa K, Fujikawa S (2004) Accumulation of pathogenesis-related (PR) 10/Bet v 1 protein homologues in mulberry (Morus bombycis Koidz.) tree during winter. Plant Cell Environ 27: 1112–1121

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. Planta 218: 1–14

Wang WX, Pelah D, Albergam T, Shoseyov O, Altman A (2002) Characterization of SPI, a stress-responsive, boiling soluble, homo-oligomeric protein from aspen. Plant Physiol 130: 865–875

Wisniewski M, Webb R, Balsamo R, Close TJ, Yu XM, Griffith M (1999) Purification, immobilization, cryoprotective, and antifreeze activity of PCA60: a dehydrin from peach (Prunus persica). Physiol Plant 105: 600–608