RESEARCH PAPER

The lysine-rich motif of intrinsically disordered stress protein CDeT11-24 from Craterostigma plantagineum is responsible for phosphatidic acid binding and protection of enzymes from damaging effects caused by desiccation

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Abstract

The late embryogenesis abundant (LEA)-like protein CDeT11-24 is one of the major desiccation-related phosphoproteins of the resurrection plant Craterostigma plantagineum. In this study, it was shown that CDeT11-24 is mostly intrinsically disordered and protects two different enzymes, citrate synthase and lactate dehydrogenase, against damaging effects caused by desiccation. Lipid-binding assays revealed that CDeT11-24 is able to interact with phosphatidic acid, although electrostatic repulsion was expected due to the overall negative net charge of the protein under the tested physiological conditions. CDeT11-24 carries an N-terminal lysine-rich sequence, which is predicted to form an amphipathic α-helix. Analysis of the truncated CDeT11-24 protein identified this region to be responsible for both activities: enzyme protection and phosphatidic acid interaction. Possible functions of the CDeT11-24 protein are discussed in the context of desiccation tolerance.

Key words: desiccation tolerance, lipid binding, K-segment, molecular shield.

Introduction

Intrinsically disordered proteins (IDPs) have attracted increasing attention in recent years, and research has demonstrated that the phenomenon of protein disorder is widespread in nature (Dunker et al., 2008; Gsponer and Babu, 2009; Tompa, 2011). IDPs are characterized by the absence of a defined three-dimensional structure under physiological conditions. Lacking a rigid structure confers several advantages to IDPs, such as structural flexibility and functional diversity (Uversky and Dunker, 2010).

Structural data indicate that most late embryogenesis abundant (LEA) proteins in plants are IDPs (Hundertmark and Hincha, 2008). LEA proteins were first described in cotton seeds (Gossypium hirsutum; Dure et al., 1981). They are synthesized abundantly in plants during the late stages of embryogenesis or are induced in vegetative tissues by various types of osmotic stress (Bartels and Sunkar, 2005). However, some LEA proteins show a constitutive expression (Welin et al., 1994; Rorat et al., 2004). LEA protein expression is not restricted to plants, as some desiccation-related LEA proteins are also present in bacteria and desiccation-tolerant animals such as brine shrimp, nematodes or rotifers (Tunnacliffe and Wise, 2007; Hand et al.,...
The desiccation-related protein CDeT11-24 from the resurrection plant *Craterostigma plantagineum* (Bartels et al., 1990) is designated as LEA-like, as it shares features with LEA proteins such as extreme hydrophilicity, the absence of cysteine residues and the presence of a lysine-rich sequence element that shows similarity to the so-called K-segment of dehydrins (Velasco et al., 1998). This protein has been shown to accumulate in response to drought and abscisic acid (ABA), and was identified as one of the major phosphoproteins in desiccated tissues of *C. plantagineum* (Röhrig et al., 2006). Although CDeT11-24 accumulates very early at the onset of dehydration, phosphorylation occurs during the later stages of dehydration. This biphasic expression discriminates CDeT11-24 from other LEA proteins and implicates a requirement for the protein early after the onset of water stress and a functional reallocation by phosphorylation at the late stages of desiccation.

CDeT11-24-related sequences are found in different plant species (van den Dries et al., 2011). In the model plant *Arabidopsis thaliana*, the related genes rd29A and rd29B were originally identified by Yamaguchi-Shinozaki and Shinozaki (1993). Recently, Msanne et al. (2011) analysed their response to cold, drought, and salt treatment. The authors used reverse genetic approaches, including complementation of T-DNA knockout mutants to analyse chimaeric gene constructs in transgenic plants regarding their morphological and physiological responses. It was concluded that the RD29A and RD29B proteins may function as warning system for abiotic stress. However, no physiological role for CDeT11-24 and related proteins has been presented to date.

In this work, the CDeT11-24 protein structure and function was investigated by biochemical analysis as well as by *in vitro* protection assays. The structure of CDeT11-24 was studied with regard to its intrinsically disordered state. Evidence is presented for specific interaction of CDeT11-24 with phosphatidic acid (PA) and for the ability of CDeT11-24 to protect enzymes from the damaging effects of desiccation.

### Material and methods

#### Plant material

*C. plantagineum* Hochst. plants were propagated as described by Bartels et al. (1990).

#### Molecular techniques

Standard molecular techniques were performed as described by Sambrook et al. (1989). DNA sequencing was carried out by Macrogen Inc. (Seoul, Korea).

#### Preparation of recombinant CDeT11-24 protein

The CDeT11-24 gene sequence (GenBank accession no. JQ067608) was isolated from a Uni-ZAP XR library (Stratagene; La Jolla, CA, USA) prepared from desiccated (relative water content <2%) *C. plantagineum* leaves using the primers CDeT11-24Ndel (5′-ATATCTATGTGAACTCGCATGACCCTGC-3′) and CDeT11-24Xhol (5′-ATAATCTCGACCTGAGTGATGATCCTGTG-3′). The Ndel/Xhol fragment was cloned into the expression vector pET28 (Novagen; Darmstadt, Germany), yielding an N-terminal 6His-tag fusion protein (6His-CDeT11-24). The vector (pET28-6His-CDeT11-24) was transformed into *Escherichia coli* strain BL21(DE3) (Amersham Pharmacia Biotech; Piscataway, NJ, USA). Expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. Induction was carried out at 28 °C for 3 h. Metal ion affinity chromatography was performed as described by Kirch and Röhrig (2010) with an additional heat-treatment step. For this purpose, the bacterial lysate was incubated for 10 min in a water bath at 100 °C, followed by centrifugation (10 min at 20 000 g, 4 °C) prior to further purification.

For modification of the pET28-6His-CDeT11-24 vector, site-directed mutagenesis PCR was carried out using the primers pET28-Ndelf (5′-GGGATATACATTATGAGCGACGCCCATC-3′) and pET28-Ndelr (5′-GATGGCTGCTGATATGATATCTCC-3′). After removal of the 6His-tag sequence by Ndel digestion, the vector was ligated and transformed into *E. coli* BL21(DE3) yielding the plasmid pET28-CDeT11-24. CDeT11-24 expression was induced with 1 mM IPTG for 3 h at 37 °C. The bacteria were harvested by centrifugation and the resulting pellet was dissolved in 20 mM MES (pH 5.5) and incubated for 10 min in a water bath at 100 °C. The extract was centrifuged (20 000 g, 10 min, 4 °C) and the resulting supernatant loaded on an ion-exchange Mono S column (GE Healthcare; Uppsala, Sweden). Elution was carried out at a flow rate of 1 ml min-1 with 25 mM MES (pH 5.5) using a linear gradient of 0–500 mM NaCl. The CDeT11-24 protein was eluted at approximately 180 mM NaCl with an estimated purity of >95%.

For deletion of the CDeT11-24 lysine-rich sequence element, site-directed mutagenesis was performed on the pET28-CDeT11-24 plasmid with the primer pair CDeT11-24-Ncoif (5′-GGATATACATTATGAGCGACGCCCATC-3′) and CDeT11-24-Ncoir (5′-GGATATACATTATGAGCGACGCCCATC-3′). The resulting plasmid pET28-CDeT11-24 was digested with Xhol. The empty vector was transformed into *E. coli* BL21(DE3) to yield the plasmid pET28-ΔK-CDeT11-24. Expression and purification of ΔK-CDeT11-24 protein was performed as described above for CDeT11-24.

#### Isolation of CDeT11-24 protein

The purification of CDeT11-24 protein from dried plant leaves was performed by immunoaffinity chromatography as described by van den Dries et al. (2011).

#### Isolation of *C. plantagineum* heat-stable proteins

Dried leaves of *C. plantagineum* were ground in liquid nitrogen and mixed with 100 mM Tris/HCl (pH 7.5), 100 mM NaCl, 5 mM dithiothreitol and 1:100-diluted Protease Inhibitor Cocktail (Sigma-Aldrich; St Louis, MO, USA). After incubation on ice (15 min), the solution was placed for 10 min at 100 °C in a water bath and subsequently chilled briefly on ice. The heat-stable proteins were recovered in the supernatant after centrifugation (14 000 g, 10 min, 4 °C).
Bioinformatic analysis

Disorder prediction was performed using the meta-predictor PONDR-FIT (Xue et al., 2010). Helical wheel projection and database analysis was done using HELIQUEST (Gautier et al., 2008).

Circular dichroism (CD) analysis

CD measurements were carried out using a J810 spectropolarimeter (JASCO; Tokyo, Japan). The scan rate was 20 nm min⁻¹ at 0.2 nm resolution and 20 mdeg sensitivity. All samples were mixed 1 h prior to the CD run and centrifuged at 12 000 g for 2 min before filling the cuvettes. The protein concentration was 0.2 mg ml⁻¹ and 0.1 µg µl⁻¹ was added to the liposomes and incubated for 7 unless indicated otherwise) at a concentration of 2 µg µl⁻¹, shaken and 20 mdeg sensitivity. All samples were mixed 1 h prior to the CD run and centrifuged at 12 000 g.

Lactate dehydrogenase (LDH) desiccation assay

LDH (EC 1.1.1.27) from rabbit muscle (Sigma-Aldrich; St Louis, MO, USA) was dialysed overnight in 2 × 500 ml of water. LDH (100 µg ml⁻¹) alone or in combination with 200 µg ml⁻¹ 6His-CDeT11-24 protein, 200 µg ml⁻¹ CDeT11-24 protein, 200 µg ml⁻¹ ΔK-CDeT11-24 protein, 200 µg ml⁻¹ BSA or 0.2 M trehalose was desiccated in a vacuum centrifuge. The molar ratio of the proteanct protein CDeT11-24 to enzyme was approximately 2:1. After the first dehydration cycle, samples were rehydrated with 20 µl of distilled water and 2 µl was used for the LDH activity assay. Dried samples derived from the second and third cycle were dissolved in 18 or 16 µl of distilled water, respectively. For determination of LDH activity, 2 µl of the LDH sample was added to a quartz cuvette containing 500 µl of LDH assay buffer (200 mM NADH, 2 mM pyruvate). Absorption was monitored using a spectrophotometer at 340 nm every 5 s for 1 min.

Citicrute synthase desiccation assay

A desiccation assay with citrate synthase (CS; EC 4.1.3.7) from porcine heart (Sigma-Aldrich; St Louis, MO, USA) was performed essentially as described above for LDH. CS alone (20 µl) at a concentration of 120 µg ml⁻¹ or in combination with 240 µg ml⁻¹ 6His-CDeT11-24 protein, 240 µg ml⁻¹ CDeT11-24 protein, 240 µg ml⁻¹ ΔK-CDeT11-24 protein (molar ratio of CDeT11-24 to enzyme in each assay of approximately 1.6:1), 240 µg ml⁻¹ BSA or 0.2 M trehalose was desiccated in a vacuum centrifuge. The CS activity assay was performed as described by Sreere (1969). An aliquot of 2 µl of the CS sample was added to a cuvette containing 500 µl of CS assay buffer (50 mM Tris/HCl (pH 8.0), 2 mM EDTA, 100 µM oxalacetic acid, 100 µM 5,5'-dithiobis(2-nitrobenzoic acid), 95 µM acetyl CoA). Absorption was measured at 412 nm every 5 s for 1 min.

Lipid–protein overlay assay

Lipids (5 µg) dissolved in chloroform were spotted onto a nitrocellulose membrane. The following lipids were used: monogalactosyldiacylglycerol, digalactosyldiacylglycerol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, phosphatidylcholine (PC), PA, and cardiolipin (all from Sigma-Aldrich; St Louis, MO, USA). Subsequently, the membrane was dried for 1 h at room temperature and then saturated for 1 h with Tris-buffered saline supplemented with Tween 20 [TBST: 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 0.1% (v/v) Tween 20] containing 5% (w/v) BSA. C. plantagineum heat extract or recombinant CDeT11-24 protein was added at a concentration of 1 µg ml⁻¹ and the membrane was incubated overnight with gentle shaking at 4 °C. The membrane was then washed three times for 10 min each with TBST followed by incubation with 1:5000-diluted rabbit anti-CDeT11-24 polyclonal antibody (Velasco et al., 2008) in TBST, supplemented with 4% (w/v) milk powder. After 1 h, the membrane was washed again with TBST and incubated for 45 min with peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich; St Louis, MO, USA) at 1:5000 dilution in TBST/4% (w/v) milk powder. After three further washes with TBST, the membrane was incubated for 1 h in ECL Western blotting detection reagent (GE Healthcare; Uppsala, Sweden). Signals were detected in a Las-1000 luminescent imager (FujiFilm; Tokyo, Japan).

Liposome-binding assay

Liposome-binding assays were performed as described previously (Dubots et al., 2010). Lipids were dissolved in chloroform and dried. The resulting lipid films were then suspended in MNT buffer (20 mM MES, 30 mM Tris-HCl, 100 mM NaCl, 1 mM dithiothreitol, at pH 7 unless indicated otherwise) at a concentration of 2 µg µl⁻¹, shaken for 1 h at 37 °C and 250 r.p.m. and subsequently vortexed for 5 min. After centrifugation (20 000 g, 10 min) the supernatant was removed and 0.1 µg µl⁻¹ CDeT11-24 protein in MNT was added to the liposome pellet. Incubation was carried out for 30 min at 30 °C. The liposomes were then harvested by centrifugation at 20 000 g for 10 min. Proteins of the resulting pellet and the corresponding supernatant were separated on a 12% SDS-polyacrylamide gel (Laemmli, 1970) and visualized by Coomassie blue staining.

Light microscopy of liposomes

Liposomes with a PA concentration of 2 µg µl⁻¹ in MNT buffer (pH 7) were produced as described above. CDeT11-24 protein at a concentration of 0.1 µg µl⁻¹ was added to the liposomes and incubated for 30 min at 30 °C. An aliquot (5 µl) of this suspension was transferred to a glass slide. The liposomes were then examined (40× magnification) with an Eclipse 80i microscope (Nikon; Tokyo, Japan). Images were documented with a DS-2MV camera (Nikon; Tokyo, Japan).

Results

Structural analysis

Several independent observations indicate that the CDeT11-24 protein is an IDP. The protein contains predominantly hydrophilic amino acids and is negatively charged with a predicted pI of 4.7 (Velasco et al., 1998). Extensive hydrophobic motifs, which drive protein folding, are missing. The CDeT11-24 protein remained soluble in heat-treated protein extracts of dried C. plantagineum tissues (Fig. 1A). Solubility at high temperatures is also characteristic for IDPs (Tompa, 2002). In silico analysis of the CDeT11-24 amino acid sequence with the disorder meta-predictor PONDR-FIT (Xue et al., 2010) predicted a mostly disordered structure. To compare recombinant 6His-CDeT11-24 protein with the native protein, CDeT11-24 was purified from dehydrated tissue of C. plantagineum by immunoaffinity chromatography. CDeT11-24 protein extracted from plant cells showed a CD spectrum similar to the spectrum of recombinant protein (Fig. 1C), indicating that there was no significant difference between native and recombinant proteins with regard to this property. Small differences around 220 nm could be explained by the contribution of low-level contaminants from the purification process.
It was demonstrated that intrinsically disordered LEA proteins change their secondary structure during dehydration into a more structured form (Goyal et al., 2003; Li and He, 2009; Popova et al., 2011). Therefore, the capability of the protein to adopt helical structures in the presence of trifluoroethanol (TFE), a helix-promoting solvent, was tested. Comparative CD spectra with TFE-titrated 6His-CDeT11-24 showed that increasing concentrations of TFE induced a reduction of the overall random coil, whereas the proportion of α-helical content increased (Fig. 1D, 1E). The transition midpoint of titration was around 20% TFE (Fig. 1E), which is similar to that of other disordered proteins (Mouillon et al., 2008).

**The lysine-rich sequence element of CDeT11-24**

Despite the overall negative net charge, CDeT11-24 is characterized by a local positively charged lysine-rich sequence motif in the N-terminal part of the protein (aa 24–45). The core sequence motif (EKKSMLAKVKEKAK) shows similarities to the canonical K-segment (EKKGIMDKIKEKLPG), which is characteristic of class 2 LEA proteins, the so-called dehydrins, and which is postulated to form an amphiphatic helix (Close, 1996; Eriksson et al., 2011). Multiple sequence alignments of CDeT11-24 homologues showed that the lysine-rich motif is conserved within plant proteins (Fig. 2A). α-Helical wheel projection of the CDeT11-24 lysine-rich sequence element using HELIQUEST showed that two different positively charged lysine motifs and a stretch of hydrophobic amino acids are arranged at different faces of the helical model (Fig. 2B). Such amphiphatic helices are known to interact with cellular membranes (Drin and Antonny, 2010). Recently, it was shown that K-segments of different dehydrins bind to anionic phospholipids (Koag et al., 2003, 2009; Eriksson et al., 2011). Database analysis for α-helices with physico-chemical properties similar to the lysine-rich sequence element from CDeT11-24 resulted in matches of 99% with proteins predicted as lipid-binding proteins (data not shown).

**Lipid binding of CDeT11-24**

To test possible lipid binding, protein–lipid overlay assays were performed with different lipids immobilized on a nitrocellulose membrane and CDeT11-24 protein as probe. First, heat-extracted native CDeT11-24 protein was examined in binding assays. Fig. 3A shows that uncharged lipids such as monogalactosyldiacylglycerol and digalactosyldiacylglycerol did not bind native CDeT11-24. However, CDeT11-24 bound to the phospholipid monoester PA. Weak binding was also

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**Fig. 1.** CDeT11-24 heat solubility and secondary structure. (A) Total proteins (TP) and heat-extracted proteins (HE) from *C. plantagineum* dried leaves were separated on a 12% SDS-polyacrylamide gel and visualized by Coomassie blue staining. The CDeT11-24 protein is indicated. (B) In silico disorder analysis of the CDeT11-24 protein sequence using PONDR-FIT. (C) CD spectra of recombinant 6His-CDeT11-24 (solid line) and plant-derived CDeT11-24 (dashed line). (D) CD spectra of 6His-CDeT11-24 titrated with different TFE concentrations. (E) Change in 6His-CDeT11-24 α-helical content as function of TFE concentration. Structural content was calculated by CDNN software.
observed with cardiolipin, which is a phospholipid containing two phosphodiester groups. All other phospholipids tested (phosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol and PC), which contained only one phosphodiester group, did not bind to CDeT11-24. These data suggested that CDeT11-24 shows a phosphomonoester group specificity. Recombinant 6His-CDeT11-24 protein was also tested for lipid binding. Binding experiments using CDeT11-24 with a 6His-tag digested with protease ArgC showed that the 6His-tag motif was also able to bind PA (see Supplementary Method, Fig. S1, and Table S1 in JXB online). Therefore, recombinant CDeT11-24 protein without His-tag (CDeT11-24) was generated and purified. CDeT11-24 protein was able to interact specifically with PA, confirming the result obtained with the native plant protein (Fig. 3A). CDeT11-24 protein carrying a deletion of the K-like segment (∆K-CDeT11-24) was not able to bind PA (Fig. 3A), indicating that the lysine-rich sequence motif of the protein is responsible for the interaction with PA.

Protein binding was also characterized using PA-containing liposomes as an affinity matrix. CDeT11-24 and liposomes were incubated in the presence of buffered solutions with different pH values and subsequently centrifuged. The protein was precipitated with PA lipid vesicles in the pH range of 5–9 (Fig. 3B). Only weak binding was detected at pH 9, indicating a pH-dependent interaction. pH 7 was used for all following binding assays.
Buffer concentrations up to 400 mM NaCl were tested without affecting CDeT11-24 lipid binding (data not shown), indicating a strong interaction.

As the concentration of PA in intracellular membranes undergoes a dynamic change and increases in plants as a response to water deficit (Katagiri et al., 2001), different PC/PA concentrations were tested for CDeT11-24 binding (Fig. 3C). The results showed that CDeT11-24–liposome binding was dependent on the concentration of PA and was significantly reduced at a PC-to-PA ratio of 75:25%. No CDeT11-24 binding was detected with liposomes containing only PC. Strongly reduced binding was observed when the ΔK-CDeT11-24 protein was used for the assays (Fig. 3D). However, residual activity indicated that, in addition to the lysine-rich sequence element, another part of the protein interacts weakly with liposomes.

Further evidence for CDeT11-24–PA binding comes from the formation of aggregates, which were detected after addition of CDeT11-24 to PA-containing vesicles. These aggregates were visible under a light microscope and were not observed without protein or when ΔK-CDeT11-24 was added to the vesicles (Fig. 3E). As CDeT11-24 contains only one lysine-rich sequence element, such an association requires either the formation of lipid-bound protein multimers or the presence of a second lipid-binding domain.

**Enzyme desiccation assays**

The function of CDeT11-24 has been correlated with the protection of cells against damage induced by desiccation stress (Velasco et al., 1998). Desiccation-induced damage of cells affects protein activities. Therefore, CDeT11-24 was tested for the ability to prevent dehydration-induced loss of enzymatic activities using *in vitro* dehydration stress assays in the presence of the two enzymes, CS and LDH (Goyal et al., 2005; Haaning et al., 2008). CS lost 50% of its initial activity after one cycle of dehydration/rehydration and enzymatic activity dropped to approximately 10% after three cycles in comparison with the untreated control (Fig. 4A). In the presence of recombinant CDeT11-24 proteins, CS activity remained at more than 80% for 6His-CDeT11-24 and at 70% for CDeT11-24, even after three cycles of dehydration and rehydration. The protective effect exceeded the effect caused by the disaccharide trehalose, which was used as a reference (Crowe et al., 2001). Nevertheless, when ΔK-CDeT11-24 was used in the assays, no significant protection was observed, indicating that the K-like segment is responsible for this protein activity. BSA was used as a control for proteins in the dehydration assays. Although BSA showed protection after one dehydration/rehydration cycle, the degree of protection was much less after two and three cycles than with CDeT11-24 or trehalose (Fig. 4A).

LDH activity was also protected during dehydration in the presence of the CDeT11-24 proteins (Fig. 4B). In these assays, ΔK-CDeT11-24 and BSA showed no protection and the enzymatic activities corresponded to the values of the control assays where LDH was used alone.

**Structural analysis of truncated CDeT11-24**

We then analysed whether deletion of the K-like segment altered the protein structure (Fig. 5). CD analysis of ΔK-CDeT11-24 revealed no differences in the helical content between the truncated protein and the corresponding full-sized recombinant CDeT11-24 (Fig. 5A). The CD spectra were similar to the spectra of 6His-CDeT11-24 and the native CDeT11-24 purified from plant tissue (see Fig. 1C). TFE titration of AK-CDeT11-24 (Fig. 5C) led to a decrease in random coil structure and an increase in the α-helical content similar to the full-sized protein (Fig. 5B). These data indicated that deletion of the K-like segment did not alter the overall structure of CDeT11-24. Nevertheless, it is likely that the K-like segment could form an amphiphatic α-helix in the presence of TFE, but contribution of this small epitope to the total helical content would be minor and might not be detectable by CD spectroscopy.

**Discussion**

Our results showed that the CDeT11-24 protein mostly assumes a disordered structure. Stretches of the protein with a predicted ordered structure (Fig. 1A) coincided with a conserved domain (aa 183–383, ProDom entry PD010085; see Röhrig et al., 2006), which is present in the CDeT11-24 protein and its stress-associated related proteins in spinach (CAP160) or *A. thaliana* (RD29A and RD29B). CDeT11-24 protein is mostly unstructured under physiological conditions, but in the presence of TFE, it seems to fold increasingly into α-helical structures. Such structural flexibility is a typical feature of some LEA proteins and IDPs (Tunnacliffe and Wise, 2007; Uversky and Dunker, 2010), which agrees with a function of these proteins as molecular shields. In an increasingly crowded cellular environment following water loss, the interaction with different proteins is required to fulfill this protective function. Many IDPs are known to be involved in various interactions with multiple binding partners (Uversky, 2011). In the case of CDeT11-24, such a binding diversity may be facilitated by ionic interactions with charged residues or with target-induced secondary structures. Furthermore, CDeT11-24 contains several stretches with predicted coiled-coil structures (Röhrig et al., 2006). These regions may additionally allow multiple sets of protein–protein interactions with itself and other proteins such as dehydrins.

The finding that the CDeT11-24 protein protects the enzymes CS and LDH *in vitro* against desiccation damage supports a function of the protein as a molecular shield, which has been described previously for LEA proteins (Reyes et al., 2005; Boucher et al., 2010). This effect could be explained by the interaction of CDeT11-24 with these enzymes, preventing drying-induced activity loss by acting as a molecular shield or possibly as molecular chaperone (Goyal et al., 2005; Chakrabortee et al., 2007, 2010). Nevertheless, a function of CDeT11-24 as a molecular shield by direct interaction with client proteins was not demonstrated experimentally in this work and remains to be elucidated.

Deletion analysis showed that the protective function of CDeT11-24 was dependent on the presence of the K-like segment in the N-terminal part of the protein, which demonstrated the specificity of this protein activity. As TFE titrations and CD spectroscopy showed that the truncated ΔK-CDeT11-24 exhibits a similar α-helical content to the full-sized protein, it seems unlikely that a functional loss is caused by a structural change of the whole protein due to the deletion. Thus, enzyme protection...
is more likely to be mediated by the lysine-rich sequence itself, which may facilitate protein-protein interactions via the amphiphatic helix or by ionic interactions. These data are in agreement with those of Reyes et al. (2008), who demonstrated that the K-segments are responsible for the protective function of hydrophilins in freeze-thaw experiments. The removal of K-segments from dehydrins ERD10 and Rdhn5 affected these proteins in their ability to protect LDH.

Our results indicate that the desiccation-related CDeT11-24 protein from C. plantagineum binds in vitro to PA via the lysine-rich sequence element of the protein. PA is a multifunctional stress-signalling phospholipid, and its formation is triggered in plants in response to various stress factors such as drought, salinity, cold, wounding or pathogen infection (Testerink and Munnik, 2005). For interaction of the dehydrin K-segment with anionic lipid vesicles, an electrostatic nature of the interaction has been postulated, and dehydrin binding was not restricted to a certain class of phospholipids (Koag et al., 2009). Such an ionic interaction with charged lipids was not observed for CDeT11-24, as the protein did not bind to zwitterionic lipids and anionic lipids, which contain one single phosphodiester group. Our results indicate that binding of CDeT11-24 is specific to the phospholipid PA. The specificity can be explained by the properties of the phosphomonoester group of the molecule, a unique feature of this lipid. This phosphate exists in mono-anionic and di-anionic form and has two pKₐ values. Recently, an electrostatic/hydrogen bond switch model has been proposed as a basis for the specific interaction of proteins with PA (Kooijman et al., 2007). This interaction requires several basic amino acids such as lysine and arginine in the binding domain. NMR spectroscopy

**Fig. 4.** Enzyme desiccation assays. (A) CS activity before and after repeated cycles of dehydration/rehydration. The concentrations used were: CS, 120 µg ml⁻¹; BSA, 240 µg ml⁻¹; 0.2M trehalose; 6His-CDeT11-24, 240 µg ml⁻¹; CDeT11-24, 240 µg ml⁻¹; ∆K-CDeT11-24, 240 µg ml⁻¹. The molar ratio of CDeT11-24 to CS in each assay was approximately 1.6:1. (B) LDH activity before and after repeated cycles of dehydration/rehydration. The concentrations used were: LDH , 100 µg ml⁻¹; BSA, 200 µg ml⁻¹; 0.2M trehalose; 6His-CDeT11-24, 200 µg ml⁻¹; CDeT11-24, 200 µg ml⁻¹; AK-CDeT11-24, 200 µg ml⁻¹. The molar ratio of CDeT11-24 to LDH was approximately 2:1. Bars represent the mean of three repetitions ±standard deviation. Statistical significance was determined by Student’s t-test. Results significantly different from those for CS or LDH alone are indicated by *, P<0.05 and **, P<0.01.
showed that, upon binding at constant physiological pH, consecutive lysine residues increase the negative charges of the phosphate from mono-anionic under physiological pH to di-anionic by forming intermolecular hydrogen bonds. It was postulated that this increase in PA charge together with hydrogen bond formation results in cooperative binding and thus in a docking of the protein-binding domain on the PA molecule.

Several PA-binding proteins have been identified so far (Stace and Ktistakis, 2006), but no consistent interacting sequence motif was identified. Thus, affinity to PA cannot be predicted from the protein sequence, but comparisons strengthen the relevance of basic amino acids for the interaction (Testerink and Munnik, 2005). Although the CDeT11-24 protein has a global negative charge, the lysine-rich sequence element located at the N-terminus of the protein with a pI of 10.4 exhibits a locally restricted environment with positive charges. Several consecutive lysine residues are present in this motif and α-helical wheel projection generates two clusters of lysine residues exposed at different faces of this helix. Such strong basic domains may facilitate a cooperative interaction according to the electrostatic/hydrogen bond model (Kooijman and Burger, 2009; Kooijman and Testerink, 2010). Helical wheel projection of the CDeT11-24 lysine-rich sequence also revealed a region with non-polar amino acids, which might be involved in hydrophobic interactions with the acyl chain of the PA molecule, which has been described as an attractive partner for such a hydrophobic interaction due to its cone-shaped structure (Kooijman et al., 2003).

Both the CDeT11-24 protein and PA are related to the desiccation process. In fully turgent plants, the PA concentration in the plant membrane is low (approximately 1%) but increases upon water stress (Katagiri et al., 2001). This accumulation is due to the enzymatic activity of phospholipase D, which is induced by dehydration and ABA (Frank et al., 2000; Katagiri et al., 2001). The CDeT11-24 protein accumulates in cells under the same conditions as PA. The stress protein may then be partially recruited by PA to the membrane via the N-terminal lysine-rich sequence. Binding of CDeT11-24 to membranes seems to be PA-concentration dependent, as indicated by the in vitro studies (Fig. 3C). However, it must be expected that only a certain proportion of the CDeT11-24 protein binds to cellular membranes, as CDeT11-24 has been localized in immunohistochemical studies within the cytoplasm (Velasco et al., 1998). A possible explanation for this discrepancy with the in vitro data is that membrane-bound CDeT11-24 may not have been detected due to an excess of the cytoplasmic protein. Both forms—PA-bound and cytoplasmic CDeT11-24 protein—might interact with different proteins in the ongoing process of drying cells to prevent denaturation and thus functional loss of proteins. Another hypothesis for the function of the CDeT11-24 protein could be that binding to PA displaces other proteins from their interaction with the phospholipid to regulate or fine-tune signalling pathways (Fig. 6). Several PA-binding plant proteins have been identified recently using affinity chromatography and mass spectrometry (Testerink et al., 2004). Identified proteins with known signalling function were 14-3-3 proteins, SnRK2 serine/threonine protein kinase and the regulatory subunit RCN1 of phosphatase 2A (PP2A). The best-understood signalling function is the binding of PA to type 2C protein phosphatases (PP2C, for example ABI1), which negatively regulates ABA signalling.

Fig. 5. Secondary structure of ΔK-CDeT11-24. (A) Comparison of CD spectra of truncated ΔK-CDeT11-24 (solid line) with recombinant CDeT11-24 (dashed line). (B, C) CD spectra of CDeT11-24 (B) and ΔK-CDeT11-24 (C) titrated with different TFE concentrations. (D) α-Helical content as a function of TFE concentration: CDeT11-24, open circles; ΔK-CDeT11-24, triangles. Structural content was calculated using CDNN software.
In the early process of ABA signalling, AB1 is recruited to the membrane by binding to PA, triggering the ABA response (Zhang et al., 2004). Displacement of AB1 from PA binding by CDeT11-24 could provide a shut-off mechanism for the ABA response (Fig. 6). In addition to this hypothetical model, binding of PP2Cs to the AB1 receptor has already been demonstrated (Park et al., 2009; for review, see Cutler et al., 2010). In the course of ABA signalling, AB1 binding leads to a tethering of PP2Cs to the receptor and thus to inactivation of the phosphatase activity, which in turn triggers further signal transduction.

Phosphorylation and dephosphorylation of CDeT11-24 are tightly controlled and are part of the desiccation tolerance mechanism of *C. plantagineum* (Röhrig et al., 2006). Therefore, protein phosphorylation might regulate CDeT11-24 binding to PA, as a similar mechanism was recently demonstrated for the cold-induced stress protein LTI30 (Eriksson et al., 2011). The phosphorylation sites of LTI30 were identified directly within different K-segments or between them. Thus, phosphorylation provides an electrostatic switch for LTI30 binding to the negatively charged membrane by decreasing the net positive charge of the K-segment region. In contrast, for CDeT1124, no phosphorylation sites were identified in close proximity to the lysine-rich sequence element (Röhrig et al., 2006). However, as CDeT11-24 is highly negatively charged under physiological conditions, the introduction of additional charges by phosphate groups could exceed a certain threshold of electrostatic repulsion and may be a mechanism of regulating PA interactions. Another hypothesis is the possibility that phosphorylation regulates protein–protein interactions of CDeT11-24 with itself and other proteins. This hypothesis originated from the finding that phosphorylation sites coincide with predicted coiled-coil regions (Röhrig et al., 2006). However, regulation of CDeT11-24 function seems to be complex, as several different phosphorylation sites were identified on the protein (Röhrig et al., 2006).

In conclusion, CDeT11-24 may be a multifunctional stress protein that protects cells from the damaging effects of desiccation.

![Fig. 6. Hypothesis of the function of CDeT11-24 binding to PA in ABA signalling. (A) ABA induces phospholipase (PL) activity, which leads to an increase in intracellular PA and recruitment of PA-binding signalling proteins such as AB1 to the plasma membrane. (B) Intracellular reorganization of AB1 inactivates the protein phosphatase 2C and thus triggers the ABA response. (C) ABA signalling also induces expression of CDeT11-24. In addition to the ability to protect enzymes against desiccation damage, CDeT11-24 binding to PA may displace AB1 from the interaction with PA. Such activation of this negative-regulator protein may provide a mechanism for fine-tuning or shut-off of ABA signalling. Parts (A) and (B) were modified from Zhang et al., 2004.](image)

Nevertheless, the physiological significance of CDeT11-24 binding to PA has yet to be demonstrated before this protein can be considered a true PA target.

### Supplementary data

Following supplementary data can be reviewed on *JXB* online:

**Supplementary Method.** ArgC digest of 6His-CDeT11-24 and liposome binding of the digest products.

**Supplementary Figure S1.** Liposome-binding assays with ArgC digest products of 6His-CDeT11-24.

**Supplementary Table S1.** Mass spectrometry/mass spectrometry search results.

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