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***Studying the Involvement of the Linker Protein CWLP
and its Homologue in
Cytoskeleton-plasma Membrane-Cell Wall Continuum
and in Drought***

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Abstract

The study has been focused on proline-rich proteins from the HyPRP family. Three proline-rich proteins have been characterized with the CWLP as the main objective. We showed that this unique protein is assembled in the plasma membrane (PM) and forms a continuum between the cell wall (CW) and cytosol via the PM. While spanning the PM, it is arranged in lipid rafts as CWLP-aquaporin complexes that recruit PP2A- β , as a part of PP2A enzyme, close to the aquaporin moiety where it dephosphorylates two crucial Ser residues and induces closure of the aquaporin water channels. The closure of water channels renders cells more tolerant to plasmolysis and plants to dehydration. This unique effect was observed not only in *Arabidopsis*, but also in potato plants over expressing the CWLP, suggesting a possible usage in crop plants as a valve that reduces loss of water or/and elevates cold resistance.

The CWLP is a member of the HyPRP protein family that all possess structurally similar 8CM domain, predicted to localize to PM lipid rafts. In this study, two additional highly homologous HyPRP proteins were also studied. The GPRP showed the same localization and its over expression increased tolerance to lack of water. However, the third one, PRP940, despite sharing high homology in the 8CM domain, is completely different and is assembled in parallel to cortical microtubules in the cell. Moreover, our data suggest that this protein is not involved in rendering plants resistant to lack of water.

We suggest implying CWLP as a tool for better regulation of water maintenance in crop plants.

Introduction – Scientific Background

The *Arabidopsis* Cell Wall Linker Protein (CWLP) belongs to the family of Hybrid Proline Rich Proteins (HyPRPs), a highly conserved protein family present in all species of higher plants analyzed thus far (Dvorakova *et al.*, 2007). HyPRPs contain the following three parts; N-terminal signal peptide, proline-rich domain (PRP) with repetitive elements of various length, and a C-terminal hydrophobic part containing four alpha helices with a conserved arrangement of eight cysteine residues (denoted as 8CM motif) (Jose-Estanyol *et al.*, 2004). This 8CM motif is not specific for HyPRPs, and has been identified in more than 500 plant proteins, including different types of lipid transfer proteins (LTPs), albumin 2S-like seed storage proteins, amylase inhibitors and trypsin inhibitors (Jose-Estanyol *et al.*, 2004).

The HyPRP family of *Arabidopsis thaliana* contains 29 genes arranged in clusters in the *Arabidopsis* chromosomes. They are divided into seven sub-groups according to their chromosomal clustering and protein sequence homology. The tandem chromosomal arrangement of *HyPRP* genes is not unique to *Arabidopsis* and is also found in the rice genome.

Drought, cold and salt stresses are all characterized by a decrease in environmental water availability to plants. Most plants encounter at least temporary decreases in water content at various stages of their life cycle and many also generate highly desiccation-tolerant structures, such as seeds and pollen (Ingram and Bartels, 1996).

Upon stress imposition, as a part of the "osmotic adjustment" process, the cellular concentration of small organic molecules ("osmolytes") increases. Proline (Pro) is the most common osmolyte in plants, being accumulated in response to various abiotic and biotic stresses (Szabados and Savoure, 2009). Concomitantly with the rise in cellular levels of free Pro during stress, an increase in the cellular content of certain Proline Rich Proteins (PRPs) occurs (Deutch and Winicov, 1995; Goodwin *et al.*, 1996; Garcia-Gomez *et al.*, 2000; Stein *et al.*, 2011).

Most of the *Arabidopsis* HyPRPs possess a PRP domain of 100 to 150 amino acid residues. Among them, five HyPRPs have relatively long and conserved proline-rich domains (up to 1500 amino acids), and therefore are considered as the "Conserved Type" subgroup, C-type (Dvorakova *et al.*, 2007). The C-type HyPRPs are found in both

Gymnosperms and Angiosperms, exhibiting less amino acid sequence divergence than the rest of HyPRPs, indicating constrained evolutionary selection, likely due to important, yet undefined, function/s (Dvorakova *et al.*, 2007).

Results - summary of activities and accomplishments of the research

In this study we have characterized highly homologous proteins from the C-type HyPRP sub-family shown in Fig. 1, possessing Pro-rich and highly conserved 8CM domains.

1. The Cell Wall Linker Protein (CWLP)

The *Arabidopsis* Cell Wall Linker Protein (*AtCWLP*, *At3g22120*) is a member of the C-type sub-group of HyPRPs. Previously, by using a yeast-based screening of *Arabidopsis thaliana* cDNA library aiming at the identification of plasma membrane (PM) proteins, the CWLP cDNA was isolated and identified bioinformatically as a HyPRP protein (Figure 2). The presence of many Lys residues in the N-terminal part of the PRP domain led to the assumption that these basic residues interact with acidic moieties of the cell wall (CW) polymers, linking the CW with the PM. The 8CM domain was predicted to be localized to the PM with a ten amino acid moiety protruding to the cytosol (Figure. 2). Close homology with the cold-stress induced BnCWLP (85% identity) (Goodwin *et al.*, 1996), and high similarity of its 8CM domain with that of the salt-induced Alafalfa MsPRP2 (Deutch and Winicov, 1995) have led to the speculation that the hydrophobic 8CM domain plays an important role in strengthening the PM during osmotic and cold stresses (Weyman *et al.*, 2006).

1.1. CWLP expression in Arabidopsis

To elucidate the pattern of *CWLP* expression during plant development, the 2 kb promoter region, including 25 bp 5' UTR, was cloned upstream to the GUS coding region. T3 homozygous plants harboring the *CWLPp*-GUS cassette were examined for GUS expression using X-Gluc staining (Figure 3A). According to the pattern of GUS expression the *CWLP* promoter is active in almost all aerial tissues (cotyledons, leaves, stems, flowers, siliques) but not in roots, except for a strong expression in the apices of main and lateral roots.

1.2. Cellular localization of CWLP

Bioinformatic analysis predicted a N-terminus signal peptide (24 amino acid residues) (Goo *et al.*, 1999), which may target the *CWLP* protein to the endomembrane system, PM or outside to the CW. Three additional domains were identified; the PRP domain composed of the N-terminal part of Lys-Pro rich repeats and the C-terminal part, rich in Pro, Tyr and Val residues. The second domain is a 8CM domain, suggested to be a transmembrane domain. The third one is a C-terminal moiety protruding to the cytosol.

Figure 3. CWLP characterization

A Native expression in *Arabidopsis*: GUS staining of transgenic *Arabidopsis* plants harboring the CWLP promoter-GUS transcriptional fusion. The presence of putative consensus sequences in AtCWLP promoter is shown in the upper part. AtCWLP is expressed in all aerial tissues and in primary and lateral root tips. 1- seven-d-old seedling with cotyledons and first two leaves. 2- rosette leaf. 2- root of 1-d-old seedling. 3- single flower of mature 4-week-old plant. 4- stamen. 5- mature (left) and young (right)

siliques. 6 to 8- lateral roots of 7-d-old seedlings at three different growth stages. Gus activity is limited to root tips. Bars = 1mm (in 1 to 5) or 0.1mm (6 to 8).

B-C *CWLP is localized to the plasma membrane and bridges between cytosol and cell wall with its C-terminal and N-terminal ends, respectively. Confocal microscopy of Arabidopsis thaliana leaves stably over expressing CWLP fused to GFP either at the C-terminal (B) or N-terminal (C) end of the protein.*

Propidium Iodide and FM4-64 were used to stain the cell wall (CW) and plasma membrane (PM), respectively. Sections indicated in white bars were scanned and fluorescence of CWLP (green), or CW and PM (red) are shown in lower panels. Schematic representation of expression cassettes used for the transformations are shown above the images. SP – signal peptide, PRD – proline rich domain, 8CM/TM – eight-cysteine motif/ transmembrane domain.

D *CWLP is present in the cytosolic fraction, Triton X-100 soluble and detergent-resistant membrane fractions. Transgenic Arabidopsis seedlings (15-d-old), over expressing either CWLP-GFP or the control GFP-ROP6^{CA}, were subjected to differential detergent extraction. Equal volumes of cytosolic (Cyt.), Triton X-100 soluble (TSM) and detergent resistant membrane (DRM) fractions were analyzed by western blotting using anti-GFP antibody.*

E *CWLP is glycosylated in Arabidopsis. CWLP-GFP, over expressed in transgenic Arabidopsis seedlings, was immunoprecipitated using 5.5 µg polyclonal anti-GFP antibody per ml of diluted cell extract. The immunoprecipitated CWLP-GFP was separated on 10% a PA gel and blotted onto a nitrocellulose membrane. Half of the blot was stained for glycosylation (left) and the other half (right) was probed with monoclonal mouse anti-GFP Ab.*

To assess AtCWLP cellular localization *in planta*, transgenic *Arabidopsis* plants constitutively overexpressing CWLP-GFP (Figure 3 B) or GFP-CWLP (Figure 3 C) were generated. Detached leaves were stained for 5 to 10 min with 1.5mM Propidium Iodide (PI), or with 8µM FM4-64, for CW and PM staining, respectively. Figure 3 B (upper panel) shows that the GFP is arranged in clusters protruding to the cytosol at the inner side of the PM, when the cell wall (CW) (left) or PM (right) are stained in red. These

observations indicate that the CWLP is assembled in the PM in clusters with the C-terminal end protruding to the cytosol. The fluorescence patterns of GFP, PI (Figure 3 B, lower left panel) and FM4-64 (Fig. 3 B, lower right panel) show distinct peaks for each fluorophore confirming the bioinformatics prediction that the CWLP has a cytosolic C-terminal protruding end.

When GFP is fused to the N-terminal end (Figure 3 C), GFP fluorescence overlaps that of PI, which stains the CW (Figure 3 C left panel, upper and lower photos). Correlatively, GFP fluorescence appears between the two peaks of FM4-64 fluorescence, serving here as PM marker (Figure 3 C right panel, upper and lower photos). These data suggest that the CWLP forms a continuum between the CW and the cytosol through the PM, strengthening the hypothesized role of CWLP (Goo *et al.*, 1999) in bridging the three entities.

1.3. CWLP membrane localization

Membrane microdomains, playing important roles in protein sorting, signal transduction, or infection by pathogens, have been termed “lipid rafts” (Owen *et al.*, 2012). They are formed by the dynamic clustering of sphingolipids and sterols and are characterized by their insolubility in nonionic detergents at low temperature (detergent resistant membranes, DRM). In this study, the possible DRM localization of CWLP-GFP was examined. *Arabidopsis* seedlings (15-d-old) were subjected to differential detergent extraction (Sorek *et al.*, 2007). Following separation of soluble (cytosolic) and membrane fractions, the membrane fraction was incubated with 1% Triton X-100-containing buffer. After separation of Triton X-100 soluble membranes (TSM) by centrifugation, the precipitated proteins were further extracted with 1% Triton X-100 and 0.5% SDS-containing buffer. The detergent resistant fraction was collected after centrifugation and all the different fractions were analyzed by western blotting using anti-GFP Ab as a probe. Transgenic *Arabidopsis* seedlings, expressing GFP fused constitutively an active plant Rho-related GTPase (ROP6^{CA}), which is localized to DRMs (Sorek *et al.*, 2007), was used as a positive control (Figure 3 D, right lane). Figure 3D shows that CWLP is present both in DRMs and in TSMs and to a lesser extent in the cytosolic fraction. This data suggest that CWLP is localized to lipid rafts (DRM) of the PM which are in a

dynamic equilibrium with the PM soluble fraction (TSM) (Owen *et al.*, 2012).

1.4. AtCWLP is glycosylated

Post-translational processing of CWLP was postulated because the observed MW of CWLP-GFP (~ 90 kDa), determined by SDS-PAGE, differed from the expected (~ 57 kDa) MW (Figure 3 D). Furthermore, bioinformatic analysis of CWLP by DictyOGlyc software, that predicts putative O-glycosylation sites, revealed several possible hydroxylation sites in the proline-rich domain. Therefore, the possible presence of sugar moieties on the CWLP molecule was studied. Fig. 3 E confirms that the overexpressed CWLP-GFP is glycosylated, in *Arabidopsis* seedlings. The immunoprecipitated CWLP-GFP is detected at ~ 90 kDa, both in the western blot and by using the Glycoprotein Detection Kit (Figure 3 E, right and left panel, respectively). This kit, based on modified Periodic Acid-Schiff staining, specifically detects sugar moieties. The glycosylation of IgG heavy chain, used for immunodetection of CWLP-GFP in the western blot, is shown at ~ 55 kDa and serves as a positive control.

1.5. CWLP overexpression increases cellular tolerance and microtubule stability under water shortage conditions

Exposure of plant cells to hypertonic environment results in plasmolysis. The loss of water from protoplasts through the PM, due to lower water potential in the surrounding, causes protoplast shrinkage that disconnects the PM from the CW, except for a few maintained adhesion sites, termed as "hechtian strands" (Lang *et al.*, 2004). This process is accompanied by a collapse of the microtubule net of the cytoskeleton (Fig. 4, A to C). Plasmolysis is a reversible process, and upon re-exposure to high external water potential extensive water uptake occurs accompanied by recovery of cytoskeleton normal structure and turgor pressure and reattachment of the PM to the CW. Hence, plasmolysis is a naturally occurring process in plant cells when exposed to water shortage during transient drought, high salinity and freezing.

Since the CWLP was predicted to serve as a CW-PM adhesion protein, the effect of the high level of CWLP molecules on protoplast shrinkage during plasmolysis has been studied using plants that over express CWLP, CWLP-GFP or CWLP-HA (Figure 4).

Detached leaves of 10-d-old seedlings of Wild Type (WT), and plants over expressing CWLP-HA or CWLP-GFP (denoted as CWLP-GFP-OE and CWLP-HA-OE, respectively) were exposed to 30% glycerol for 20 min. This treatment was followed by a freeze shuttering procedure that cracks the cells (Wasteneys *et al.*, 1997), allowing cellular penetration of anti-CWLP, anti-HA, or anti-tubulin primary antibodies and their detection by fluorophore-labeled secondary antibodies (Cy3 identifies CWLP and HA while Alexa- fluor 488 marks tubulin). The original GFP fluorescence could not be detected in CWLP-GFP-OE cells after the freeze shuttering and immunostaining treatments. After the reaction, leaves of both lines were mounted in Elvanol solution on glass slides and examined by confocal microscope. WT epidermal cells showed expected plasmolytic features typified by protoplast shrinkage and collapsing of cytoskeleton microtubule structure, which was well observed by anti-tubulin antibodies (Figure 4 A to C). By contrast, no plasmolysis was observed in the similarly treated CWLP-GFP-OE (Figure 4 D to G) or CWLP-HA-OE (Figure 4 H to K) plants. In these plants, the plasma membrane remained attached to the CW, and the microtubule net maintained its normal arrangement (Figure 4D to 2K). CWLP clustering pattern, detected by red-marked antibodies on the peripheral areas of the cells (Figure 4 F and J), is similar to the observed GFP fluorescence pattern in CWLP-GFP-OE plants (Figure 3B). Yellow spots that were observed in the merged images (Figure 4 G and K) between green-labeled tubulin and red-labeled CWLP suggested a possible direct or indirect connection between the proteins, which further raised the possible involvement of CWLP in maintaining cytoskeleton structure during plasmolysis (Figure 4 G and K). This plasmolysis-tolerance was also observed in living leaf cells of CWLP-GFP-OE seedlings treated with 0.6M sorbitol for 30 minutes. However, root cells of the same plants did not show CWLP-GFP clustering organization, observed in leaves, and were plasmolysis-sensitive (data not shown). Therefore, it is suggested that in roots, where the native CWLP is not expressed (Figure 3 A), additional yet undefined PM elements are missing, which are essential for proper assembly of the CWLP. Furthermore, the fusion of the CWLP either to GFP or HA does not interfere with the native CWLP function of protecting leaf cells against water loss, thus CWLP maintains its function when a short (HA) or long (GFP) moiety is fused to its C-terminal end.

Figure 4. Stability of the cytoskeleton structure in detached leaves of CWLP-O.E *Arabidopsis* plants exposed to plasmolysing conditions.

Images show epidermis cells of 14-d-old seedlings of WT, CWLP-GFP-OE and CWLP-HA-OE plants. Detached leaves exposed to 30% glycerol for 20 minutes, underwent freeze shuttering process, and were then probed with specific primary antibodies (anti CWLP, anti Tubulin and anti HA) and secondary fluorescent antibodies containing Cy3 (red marker) for CWLP and HA and Alexa fluor 488 (green marker) for tubulin idetection. All images are Z-stack multi focal layers taken by confocal microscope.

(A-C) Disrupted structure of the cytoskeleton of plasmolysed WT cells demonstrated by transmittance image (A), tubulin fluorescence (B) and merged image of both (C). Arrows indicate the gap between of the protoplast and CW resulting from the plasmolysis process.

(D-G) Intact cytoskeleton of 30% glycerol treated non-plasmolysed CWLP-GFP-OE cells demonstrated by a transmittance (D), tubulin fluorescence (E), CWLP-GFP fluorescence (F) and combined image of the three (G).

(H-K) Intact cytoskeleton of 30% glycerol-treated non-plasmolysed CWLP-HA-OE cells demonstrated by transmittance image (H), tubulin fluorescence image (I), CWLP-HA fluorescence (J) and combined image of the three (K).

CW- Cell wall, P- Protoplast, Bars = 10 μ m. Antibodies used for specific protein detection are mentioned on the upper right side of each image.

1.6. The C-terminal and N-terminal ends of the CWLP are essential for its function

We hypothesized that the cytoplasmic protruding C-terminal end of the CWLP is mediating the linkage between the PM and cytosolic components, whereas the external N-terminal PRP domain is involved in interaction with the CW, as had been suggested by Goodwin *et al.* (1996). As CWLP-GFP over expression in *Arabidopsis* seedlings renders them more tolerant to plasmolysis (Figure 4 D to K), the maintenance of this function has served as a tool for analyzing deletion mutants. Transgenic plants constitutively over expressing either C-terminal deleted CWLP, lacking the last eight amino acid residues, or N-terminal deleted mutant, lacking 133 amino acid residues downstream to the signal peptide (Δ 25-158), but still containing the GFP tag (denoted as N-del-CWLP-GFP-OE), were generated. Deletion of either C-terminal moiety (Figure 5 B) or N-terminal Pro- and Lys-rich part (25-158, Figure 5 C), abolished the tolerance to plasmolysis acquired only by over expressing the full length CWLP-GFP (Figure 5 A). Hence deletion of either N-terminal PRP or C-terminal cytosolic protruding end cancels the tolerance to plasmolysis, confirming the need for full-length CWLP for increased tolerance to water loss. It also indicates that the CWLP forms a continuum between the cytosol and the CW through the plasma membrane.

1.7. The effect of tubulin- and actin-depolymerizing agents on CWLP-GFP localization

We next analyzed the correlation between maintenance of intact microtubule and microfilament organization and CWLP assembly in the PM. We could not find any correlation between the casein kinase 1-like 6 microtubule-binding domain (CKL6-CTD-mCherry) that interacts and thereby decorates microtubules (Ben-Nissan *et al.*, 2008) and the clusters of CWLP-GFP (Figure 6 A). Moreover, no correlation was found between the fluorescence pattern of the actin-binding domain of fimbrin 1 fused to DsRed (ABD2-DsRed) that decorates actin microfilaments, and CWLP-GFP fluorescence (Figure 6 B).

Figure 5. The C- and N-termini of the CWLP are essential for its function.

Plasmolysis tolerance assays were conducted by exposing detached leaves to 0.6M sorbitol for 30 minutes. PI staining was used as a red cell wall marker in A and C. The corresponding gene cassettes expressed in plants are depicted above the pictures. Epidermis cells of A. Full length CWLP, B; C-del CWLP ($\Delta 326-334$), and C; N-del CWLP ($\Delta 25-158$) are shown. FM4-64 dye was applied for 5 minutes prior the microscopic analysis. CW- Cell wall, P- protoplast. Proline-rich part (PR)- dark blue, 8CM domain – green, cytoplasmic protruding moiety- dark green.

Yellow arrows indicate the gap between CW and PM.

Merged fluorescence and transmittance images of single confocal slices are shown in A and C. In B, transmittance image is displayed. Bar = 50 μm in A; and 20 μm in B.

However, to further examine the need for intact tubulin or actin cytoskeleton function, we examined the effect of oryzalin and latrunculin B (LatB) that cause de-polymerization of microtubules and microfilaments, respectively, on the clustering of CWLP-GFP in the PM (Kandasamy and Meagher, 1999). Fourteen-d-old seedlings over expressing either tubulin $\alpha 6$ -GFP, mTalin-GFP, which interacts with microfilaments (Kost *et al.*, 1998), or

CWLP-GFP, were treated as follows: tubulin α 6-GFP and CWLP-GFP were incubated without or with 5 μ M oryzalin for 10 min (Figure 6 C) or 25 μ M LatB for 2 h (Figure 6 D), and then FM4-64 was added to the solution for additional 5 min before examining by confocal microscopy. Oryzalin treatment destroyed the microtubule net (Figure 6 C, upper left panel) but did not interfere with CWLP-GFP clustering in the PM (Figure 6 C, lower left panel). In the non-treated plants over expressing mTalin-GFP, actin microfilaments showed uniform cytoplasmic organization decorated by mTalin-GFP (Figure 6 D, upper left panel). However, following LatB treatment, actin microfibers almost disappeared leaving unidentified round-shaped GFP-containing structures in the cytoplasm, probably due to microfilament destruction (Figure 6 D, upper right panel). Correlatively, the organization and localization of CWLP-GFP molecules were affected by LatB treatment (Figure 6 D lower right panel)). Instead of the normal scattered clusters at the inner side of the PM (Figure 6 D lower left panel)), the CWLP appeared in the periphery of giant vesicular structures, which require further study to clarify whether they belong to interruption of the assembly process or to the endocytosis process causing enhanced CWLP turnover.

To further support the need for intact actin cytoskeleton for proper CWLP-GFP assembly in the PM, we examined the effect of over expressed *Arabidopsis* myosin XI-K tail domain, which blocks regular actin-myosin cytoskeleton trafficking (Avisar *et al.*, 2009), on the assembly of CWLP-GFP clusters in the PM. The blockage in actin-myosin trafficking changed the assembly of CWLP-GFP in the PM, and instead of defined clustering, a diffuse appearance of CWLP-GFP in the PM was observed (data not shown), further supporting the notion that normal functioning of the actin-myosin cytoskeleton is required for proper assembly of the CWLP clusters.

Figure 6. Correlation between CWLP-GFP and cytoskeleton components measured in transgenic *Arabidopsis* seedlings.

A - no direct overlapping of microtubules, decorated by CLK6-CTD-mCherry and CWLP-GFP. Spinning-disc confocal microscope image of epidermis cells.

B - no direct overlapping of microfilaments, decorated by Fimbrin 1-ABD2-DsCherry, and CWLP-GFP. Spinning disc confocal microscope image of epidermis cells.

C. No effect of oryzalin, a microtubulin depolymerization drug, on tubulin α 6-GFP (upper panel) and CWLP-GFP assembly (lower panel).

D. Latrunculin B (LatB), a microfilament depolymerization drug, causes disappearance of the microfilament net (upper right panel) and interferes with the proper assembly of CWLP-GFP leading to the appearance of giant vesicular structures with a peripheral arrangement of the CWLP-GFP clusters (indicated by a white arrow).

1.8. CWLP over expression confers freezing tolerance to Arabidopsis plants

Previously it was reported that *BnCWLP*, the canola orthologue of *AtCWLP*, is specifically induced by cold treatment, but not by heat, drought, exogenous ABA or wounding (Goodwin *et al.*, 1996). Since *AtCWLP* is a close homologue of *BnCWLP*, we examined its role in cold stress. WT plants were exposed to 4°C for four days (96 h) and then recovered at 25°C in a growth chamber. Although no cold-induced phenotypic damage could be visualized, a gradual elevation of *CWLP* transcript levels and cellular free proline was observed (Figure 7 A and 7 B, respectively). Whereas the increase in transcript levels is slow and takes about 72 h, the reduction to normal levels occurs within 2 h of recovery at 25°C (Fig. 7A), indicating a rapid breakdown. These results indicated that *CWLP* is synthesized in response to cold stress. To estimate whether or not, increased levels of *CWLP* are required for elevated cold tolerance, we compared freezing tolerance of WT, *CWLP*-GFP-OE, N-del-*CWLP*-GFP-OE, C-del-*CWLP*-OE and *cwlp* (a K/O T-DNA mutant in the coding region) plants, by measuring the temperature at which 50% electrolyte leakage occurs (LT_{50}) due to freezing-induced cellular damage (McKhann *et al.*, 2008). Detached leaves of 42-d-old nonacclimated plants (NA) and acclimated ones, pre-exposed to 14 days at 4°C (denoted as ACC), were exposed to temperature range of -1°C to -22°C, and then thawed under highly controlled conditions. LT_{50} values were calculated for the various lines of the NA and ACC treatments. Whereas all the non-acclimated plants from the various lines were equally sensitive to freezing (Figure 7 C), the LT_{50} of the acclimated *CWLP*-GFP-OE plants was 1.5°C lower than that of the other acclimated lines (Figure 7 C and D), suggesting that over expression of *CWLP* contributes to cold stress tolerance. However, the natural cold-induced synthesis of *CWLP* is not enough for acquiring freezing tolerance and only a higher level of *CWLP* molecules can cope better with -22°C.

Figure 7. CWLP overexpression confers freezing tolerance to CWLP-GFP expressing plants.

A. Gradual increase in CWLP transcription induced by coldness. RNA blot of AtCWLP transcripts extracted from leaves during exposure to 4°C for 96 h and 6h of recovery at 25°C. Ten μ g of total-RNA were loaded in each lane. Full length (1 kb) CWLP coding region was used as probe. rRNA was detected using 1 kb fragment of the tomato 26S rRNA and served as a loading control.

B Leaf free Pro concentration (μ mol/gr FW) before the cold stress ($t = 0$), during three time points of coldness (48h, 72h, 96h) and after 2h recovery.

C Comparison of electrolyte leakage (LT_{50}) caused by gradually reduced temperatures in detached leaves from five different plant lines: CWLP-GFP-OE, Wild type (Col.), *cwlp* (CWLP T-DNA K/O mutant), C-del CWLP-OE and N-del CWLP-GFP-OE (as described previously). The plants were exposed to increased freezing stress in the range between -1°C to -22°C after pre-treatment of 14 d at 4°C (defined as acclimated -ACC). Non-acclimated plants of all lines were equally sensitive.

D Increased freezing tolerance calculated from the data measured in (C).

1.9. CWLP over expression confers tolerance to water shortage

In light of increased tolerance to cellular plasmolysis of CWLP-GFP over expressing plants, the sensitivity to dehydration was estimated. CWLP-GFP-O.E plants, not irrigated for 21 days, showed relative tolerance compared to WT plants (Figure 8). WT and CWLP-GFP-OE plants were planted together in the same soil-containing boxes to prevent independent and local effects that could happen to plants grown in separate pots (Figure 8 A). The boxes were occasionally rotated in the growth chamber to prevent position effects. The number of dehydrated plants, which lost turgor pressure, was estimated every second day. Ten days after water withholding, 70% of WT and only 20% of the CWLP-GFP-OE plants wilted (Figure 8 B and 8 C). Two days later, a 10% increase in the number of dehydrated plants occurred simultaneously in both lines. In the final observation, 21 days after last irrigation, more than 90% of WT plants but only 65% of the CWLP-GFP-OE plants were dehydrated (Figure 8 B and 8 C). The relative water content at this time decreased below 50% in WT plants while it remained above 60% in CWLP-GFP-OE plants (Figure 8 D). On the 21st day of the stress all the plants were watered and all recovered with no significant difference in recovery rate. Similar drought tolerance was observed in potato plants over expressing CWLP-HA (Figure 9). These results confirm that over expression of CWLP increases whole plant tolerance to water shortage.

The drought tolerance observed in potato over expressing CWLP-GFP shows that high level of *Arabidopsis* CWLP molecules assembled in the PM of potato cells can increase dehydration tolerance and this capacity is not unique to *Arabidopsis*. Thus, expression of CWLP in other crop plants may increase their tolerance to short water shortages occurring throughout their development and might allow reduction in irrigation frequency.

1.10. The effect of NaCl on CWLP-GFP over expressing plants

The concept that dehydration resistance is also accompanied by tolerance to salinity was examined using the assay of elongation of roots in young *Arabidopsis* seedlings (Liu and Zhu, 1997). Germination and elongation of primary roots were measured in vertically oriented plates containing MS medium supplemented with 1% sucrose and 150 mM NaCl, illuminated 12 h a day with $60 \mu\text{mol}\cdot\text{s}^{-1}\cdot\text{m}^{-2}$. No difference in root elongation was

recorded among WT, CWLP-GFP, *cwlp* (K/O mutant), N-del-CWLP-GFP and C-del-CWLP seedlings (data not shown). These results were further confirmed by irrigation of mature plants grown in pots with 150 mM NaCl. WT and all 4 types of mutants did not differ in their response to salinity. Therefore, we conclude that over expression of CWLP-GFP does not confer salinity tolerance and is probably specific to availability of water molecules.

Figure 8. CWLP over expression increases tolerance to water shortage.

WT or CWLP-GFP-OE plants, grown for 3 months under 8h light/ 16h dark photoperiod at 25°C were exposed to drought by water withholding.

A WT (left) and CWLP-GFP-OE (right) plants after the last irrigation (zero time point).

B WT (left) and CWLP-GFP OE (right) plants after 21 days without irrigation.

C Percentage of dehydrated plants after 10, 12 and 21 days without irrigation, corresponding to the experiment shown in B. Black columns - Wild Type, Grey columns - CWLP-GFP-OE.

D Relative water content (RWC) of WT and CWLP-GFP-OE rosette leaves after 20 days without irrigation. The values represent the average of 15 leaves (3 leaves taken from each of 5 different plants).

Figure 9. Over expression of CWLP-GFP in potato increases tolerance to dehydration

A Western blot of total leaf proteins of Arabidopsis and potato plants over expressing CWLP-GFP probed with anti CWLP polyclonal antibodies. KO- T-DNA knockout mutant of CWLP. A1 and A10 - transgenic potato lines.

B Water loss from excised leaves. Comparison of WT, A2 and A10 lines.

C A2 line (CWLP-GFP) is more tolerant to water loss in comparison to WT, after 16 days water withholding.

D Comparison of leaves in turgor in WT (black columns) and A2 (gray) plants after 16, 1 and 21 d of water withholding. Insert shows leaf photos of 18-d-dehydrated leaves corresponding to the 18-d columns shown below.

1.11. Identification of Protein Phosphatase 2A- β ' regulatory subunits as CWLP interacting proteins

To gain insight into the mechanism underlying the observed increase in drought and freezing tolerance, we conducted Yeast Two hybrid screenings to find CWLP interacting proteins. Two approaches were undertaken 1) Yeast Two hybrid nuclear system to find

interaction with cytoplasmic proteins and 2) Split-Ubiquitin Yeast two hybrid system for interaction with membranal proteins. In the first system the full length CWLP, without the signal peptide, was used to screen an *Arabidopsis* cDNA library. Among the identified cytosolic proteins, four isoforms of the Protein Phosphatase 2A- β'' regulatory subunit (PP2A β'') gave the strongest interaction (Fig. 10 A). PP2A holoenzymes belong to the class of Ser/Thr phosphatases and constitute, in addition to the regulatory subunit, a catalytic and a structural subunit. To obtain further evidence that CWLP interacts with the PP2A β'' regulatory subunit, full length cDNA sequence of the most strongly interacting β'' subunit (*At5g28900*) was fused YFP and the construct (PP2A β'' -YFP) was transiently co expressed with CWLP, fused to HA (CWLP-HA), in *Nicotiana benthamiana* leaves. Total membrane proteins were isolated and co-immunoprecipitation (IP) was performed using polyclonal anti-GFP (also recognizes YFP) covalently bound to protein A-agarose. Proteins bound to PP2A β'' -YFP were detected in western blots using anti-HA antibody as probe. Fig. 10 B confirms the interaction between CWLP and the regulatory subunit of the phosphatase. A band of the expected size (~67 kDa) of CWLP-HA was clearly detected in the IP fraction (Fig. 10 B, right panel). When the membrane proteins were immunoprecipitated with protein A lacking conjugated antibody (control), no bands were detected. In the input fraction (before the IP step) we detected an additional band (~95 kD) that was often found to accompany the ~67 kD band in western blots of *N. benthamiana* protein extracts isolated after transient expression of CWLP-HA. Since the exact size of CWLP-HA cannot be predicted due to post-translational modifications, mostly glycosylation (see Figure 3 E), we could conclude that the ~95 kDa band represents an additional version of the protein which is more glycosylated. Still additional post-translational modifications that might occur remain to be determined. The blot was subsequently re-probed with anti-GFP monoclonal antibody to confirm the presence PP2A β'' -YFP, that together with CWLP-HA was immunoprecipitated by using anti GFP antibody that interacted with PP2A β'' -YFP (Fig.10, left panel). In addition to the expected band (~87 kDa), additional specific bands were observed, correlating in size with bands detected in input lane, indicating that these are specific PP2A β'' -YFP bands that underwent posttranslational modifications.

Figure 10. CWLP interacts with β'' regulatory subunits of PP2A.

A Y2H nuclear system using CWLP as bait. Schematic representations of bait and prey constructs used in the assay. Full length CWLP was cloned in pAS2-1 bait vector (Clontech). Arabidopsis cDNA library was cloned in pACT2 prey vector. Lower panel -

growth of yeast cells on X-gal containing medium. GF14 protein (At1g35160) served as negative control.

B Co-IP of CWLP-HA with PP2A β'' -YFP. Membrane proteins of *Nicotiana benthamiana* leaves, transiently co-expressing tagged CWLP-HA and PP2A β'' -YFP, were isolated and the PP2A β'' -YFP linked fractions were immunoprecipitated (IP) using rabbit anti-GFP polyclonal antibodies irreversibly cross-linked to protein A agarose beads. Input – total membrane proteins prior to IP. Control – protein A agarose without cross-linked antibody.

C Co-localization of CWLP-CFP and PP2A β'' -YFP transiently expressed in *N. benthamiana* leaves. Confocal microscopy analysis (left panel) and fluorescence measurement (right panel) of CWLP-CFP (green) and PP2A β'' -YFP (red) obtained from the numbered cross-sections indicated by yellow bars in the left panel.

The interaction between CWLP and PP2A β'' was further strengthened by studying the localization of the two fluorescently labeled proteins, which were transiently expressed in *N. benthamiana* leaves. Confocal microscopy of the injected leaves revealed co-localization of CWLP-CFP and PP2A β'' -YFP (Fig. 10 C, left panel). Overlapping peaks of fluorescence intensity observed in three different cross-sections confirmed that the proteins are co-localized to the inner side of the PM (Fig. 10 C, right panel). These results show that the CWLP, which is assembled in the PM, interacts with the PP2A- β'' regulatory subunit and is capable of recruiting it to the CWLP vicinity at the inner side of the PM.

1.12. Identification of Aquaporins as CWLP interacting proteins

Using the Split-Ubiquitin Y2H strategy, we identified three aquaporin proteins (PIP2;7, PIP2;1 and PIP1;3 as interactors (Figure 11 A). The strongest interaction with CWLP was observed with PIP2;7. To further confirm the results biochemically and in plant cells, the interaction between CWLP and PIP2;7 was examined by co-immunoprecipitation (co-IP) of CWLP-HA and PIP2;7-GFP using protein extracts from *N. benthamiana* leaves transiently expressing both proteins. Anti-GFP polyclonal antibodies, covalently bound to

protein A-agarose, were used to capture the complex. PIP2;7 is known to be located in the DRM fraction (PM lipid raft fraction). Since we showed earlier that CWLP is also present in DRMs (Figure 3 D), we again performed the co-IP on the total membrane fraction, thus reducing nonspecific binding. Our results confirm that CWLP, indeed, interacts with PIP2;7 (Figure 11). When precipitation with Protein A- agarose alone (w/o cross-linked antibodies) was used as control, no bands could be detected (data not shown). When probing with monoclonal anti-HA antibodies, two CWLP-HA bands (~67 and 95 kDa) were detected in the IP lane (Figure 11, right panel). These bands were also present in the DRM fraction before IP, and represent total CWLP-HA present prior to IP. Re-probing of the blot with anti-GFP monoclonal antibody, for confirming the presence of the GFP-PIP2;7, revealed several bands in the IP fraction (Fig. 11, left panel). Aquaporins are known to assembly as tetramers in the membrane (Maurel, 2007; Hove and Bhawe, 2011). We also detected this tendency to form higher oligomers. GFP-PIP2;7 dimers and trimers (~110 and 170 kD, respectively) were the major forms observed after IP (Fig. 11 B, left panel). This was also true when only the DRM fraction (used as control) was probed with anti-GFP antibodies (w/o IP).

Confocal microscopy was used to get further evidence on co-localization of CWLP and aquaporins. *N. benthamiana* leaves transiently co expressing CWLP-GFP with another of the identified aquaporins, PIP2;1, fused to mCherry, was studied using confocal microscopy (Fig. 11 C). Clear areas of co-localization are visualized in the PM. Taken together, the above data indicate that within the PM of plant cells, likely in the lipid rafts, PIP2;7 and to lesser extent other aquaporins interact with CWLP.

1.13. Putative regulatory linkage between CWLP, aquaporins and PP2A β

Water transport of plasma membrane aquaporin in tulip petals is regulated by the phosphatase PP2A (Azad *et al.*, 2004; Azad *et al.*, 2008). Site-directed mutagenesis experiments of aquaporin *TgPIP2;2* together with water channel activity study showed that PP2A is involved in the regulation of the water channel activity by dephosphorylation of Ser35, Ser116 and Ser274 of *TgPIP2;2* (Azad *et al.*, 2008). Furthermore, the regulatory subunits were suggested to be required for low temperature-dependent dephosphorylation of the PM aquaporin in tulip petals (Azad *et al.*, 2004).

Figure 11. *CWLP* interacts with aquaporins.

A Split-Ubiquitin Yeast two hybrid assay using CWLP as bait. Full sized CWLP was cloned into pBT3-SUC vector downstream to the yeast SUC2 promoter, and used as bait in NMY51 strain cotransformed with the commercially acquired Arabidopsis cDNA library NubG-X (Dual systems) cloned in the prey vector pPR3-N. Interaction on X-gal as substrate.

B Co-IP of CWLP-HA and GFP-PIP2;7. Membrane proteins of N. benthamiana leaves, transiently co-expressing the tagged CWLP and PIP2;7, were isolated and the GFP-PIP2;7-interacting fraction was immunoprecipitated (IP) using anti-GFP polyclonal antibodies irreversibly cross-linked to protein A-agarose beads. In the followed western analysis the membrane was probed with anti-HA or anti-GFP monoclonal antibodies. A sample of a detergent resistant membrane (DRM) fraction, from leaves transiently co-expressing tagged CWLP and PIP2;7, was used as control for the localization of the two tagged proteins.

(C) Co-localization by Confocal microscopy of CWLP-GFP and PIP2;1-mCherry transiently over expressed in N. benthamiana leaves

The above connection between aquaporin and PP2A β'' led us to speculate that perhaps CWLP is involved in recruiting PP2A phosphatase to the vicinity of PIP2;7 or PIP2;1, enabling the interaction between the aquaporin and phosphatase. To investigate this hypothesis, we transiently coexpressed all three (fluorescently-labeled) proteins in *N. benthamiana* leaves. Figure 12 A shows the confocal results of co-injection of CWLP-CFP, PIP2;1-mCherry and PP2A β'' -YFP. Since PP2A is a cytosolic protein it is expected to interact transiently only at certain locations with the two PM proteins, already shown to have mutual interaction. When merging the three pictures, we can detect overlapped co expression, which supports the hypothesis of the interaction among the three components of the complex.

Co-IP analysis of PIP2;1-mCherry and PP2A β'' -YFP from extracts of *N. benthamiana* leaves confirmed interaction between the two proteins. IP was performed using specific anti-PIP2;7 polyclonal antibodies. The ~87 kD PP2A β'' -YFP was immunoprecipitated together with the various oligomeric forms of PIP2;1-mCherry (Fig. 12 B, left panel and right panel, respectively). Probing the western blot with

Figure 12. Co-IP of PP2Aβ'' and PIP2;1, their co-localization with CWLP and a postulated model for the interaction of CWLP, aquaporins and PP2A.

A Confocal microscopy analysis of leaves transiently overexpressing CWLP-GFP, PP2Aβ''-YFP and PIP2;1-mCherry.

B Co-IP of PP2Aβ''-YFP and PIP2;1-mCherry.

Membrane proteins of *N. bentamiana* leaves, transiently co-expressing PP2Aβ''-YFP and PIP2;1-mCherry, were isolated and PP2Aβ''-YFP interacting proteins were immunoprecipitated using anti-PIP2;7 polyclonal antibodies irreversibly cross-linked to protein A - agarose beads. The proteins that were immunoprecipitated together with PIP2;1-mCherry were detected in Western blot by probing with anti-GFP antibody. Input – total membrane proteins prior to the IP. PIP2;1-mCherry was detected either by anti-mCherry or anti-PIP2;7 antibodies.

C Postulated model for the interaction of CWLP, aquaporins and PP2A.

anti-GFP antibody again showed a smaller form of phosphatase, that might be an alternatively spliced form. The different aquaporin oligomers were visualized by using either anti-mCherry or anti-PIP2;7 antibodies.

The above results indicate interaction between CWLP, aquaporins (PIP2;7 and PIP2;1) and the β'' regulatory subunit of PP2A. Based on this observation we suggest a putative model of interaction between these three proteins (Fig. 12 C). This model suggest the recruiting of PP2A to the aquaporin part of the PM, that protrudes to the cytosol, by the interaction of PP2A β'' with CWLP and aquaporin. The recruited phosphatase dephosphorylates the aquaporin leading to the closure of the water channel.

1.14. Identification of additional proteins, including endocytotic proteins, that interact with over expressed CWLP in planta

Upon observing that proper assembly of the CWLP or CWLP-GFP in the PM requires appropriate functioning of the actin-cytoskeleton components, a general approach was undertaken to identify proteins that interact CWLP *in planta* by affinity purification and Omicrosequencing.

The following strategy was undertaken, in which all proteins were extracted from three-day-old WT and CWLP-GFP-over expressing etiolated seedlings that were frozen in liquid nitrogen and ground using a mortar and a pestle. An extraction buffer of 50 mM Tris HCl, pH 8.0, containing 150 mM NaCl and 1% Triton X-100 was added to the powder and incubated at 4°C for 1 hour. After centrifugation at 100,000g, the supernatant was filtered through a 0.45- μ m Millex HV filter (Millipore). CWLP-GFP protein was affinity-purified/enriched from the filtered supernatant using anti-GFP antibody-conjugated magnetic beads according to the manufacturer's instructions (Miltentyi Biotec). Purified proteins were analyzed by 7.5% SDS-PAGE gels. Gels were stained with silver nitrate or transferred to polyvinylidene fluoride membranes (Millipore) for further immunoblotting analysis. CWLP-GFP was detected by mouse monoclonal anti-GFP antibodies (Covance and Antibodies, Inc.) and dynamins were detected by anti-DRP1A/ADL1 or anti-DRP1C/ADL1C antibodies (Kang *et al.*, 2003a; Kang *et al.*, 2003b). The anti- α -tubulin DM1A monoclonal antibody (Sigma) and an anti-actin

monoclonal antibody (Andersland *et al.*, 1994a; Andersland *et al.*, 1994b) were also used in the immunoblotting experiment.

For microsequencing, polypeptides that were co-purified with CWLP-GFP, were separated by SDS-PAGE and stained with GelCode™ Blue Safe Protein Stain (Thermo Scientific). Specific bands were excised and digested with trypsin followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). In order to identify specific peptides, microsequencing was performed and the results were analyzed using X! Tandem (www.thegpm.org) and Scaffold 3.0 (Proteome Software Inc.). The cutoff settings are: minimum 2 unique peptides, protein identification probability >95%, and peptide identification probability >80%.

1.14.1. Enrichment of CWLP-GFP interacting proteins from seedling extracts

Using the wild-type (WT) seedling extracts as the negative control, we purified/enriched the CWLP-GFP fusion protein from transgenic seedling expressing the protein. A number of specific polypeptides were purified (Figure 13). In order to detect the bait protein – CWLP-GFP, two anti-GFP antibodies were used to detect the fusion protein purified *in vivo*. A band near the 85-kDa marker position was detected by both antibodies, likely representing the intact CWLP-GFP polypeptide which appeared in a diffuse wide band on an SDS-PAGE gel (Figure 13, arrows). The results confirm our previous results (Fig. 3E) that CWLP has post-translational modifications such as glycosylation *in vivo*. In addition to the intact fusion protein, two major degradation products were detected during the purification.

We hypothesized that CWLP might interact with the cytoskeleton *in vivo*. In order to test this possibility, the purified proteins were probed using anti-actin and α -tubulin antibodies. Our results showed that neither tubulin nor actin was detected in the preparation (Figure 13), confirming the confocal observations (Figure 6 A, B) that CWLP does not interact directly with these cytoskeletal proteins.

1.14.2. Identification of CWLP-interacting proteins

In order to identify proteins co-purified with CWLP-GFP, co-IP polypeptides excised from gels, were subjected to mass spectrometric analysis. Four major specific bands were

identified together with the CWLP-GFP band (Figure 14 A). Bands 1-4 contained proteins in two categories. Band 1 was identified as the BiP1 protein encoded by the At5g28540 locus with a predicted molecular mass of 73.6 kDa. Band 4 corresponded to the BAG7 (Bcl-2-associated athanogene) protein encoded by At5g62390 with a predicted molecular mass of 51.6-kDa. These proteins are associated with the endoplasmic reticulum and may interact with each other, and are known to play critical roles in unfolded protein response (UPR) in the ER (Noh *et al.*, 2003; Williams *et al.*, 2010).

Figure 13. Purification of CWLP-GFP.

Proteins purified from transgenic plants expressing CWLP-GFP and the wild-type (WT) plants were separated by SDS-PAGE and analyzed by silver staining and immunoblotting. The CWLP-GFP fusion protein is indicated by arrows as detected by two different anti-GFP antibodies. Two degradation products are marked by #. Tubulin and actin are not detected by immunoblotting. Molecular weight markers in kDa are shown on the left.

Bands 2 and 3 were identified as the dynamin-related proteins DRP1C and DRP1A encoded by At1g14830 and At5g42080, respectively. The predicted 68.2-kDa DRP1A had 74% peptide coverage, and the 68.7-kDa DRP1C had 78% coverage (Figure 14 B and Table 1). Both DRP isoforms appear at cell cortex and play critical roles in endocytic membrane trafficking and cell expansion (Konopka and Bednarek, 2008; Konopka *et al.*, 2008). Thus, we further verified the interaction by immunoblotting using antibodies specifically recognizing DRP1A and DRP1C, respectively (Kang *et al.*, 2003a; Kang *et al.*, 2003b). Using the wild-type as a negative control, again, the CWLP-GFP fusion

protein was specifically purified by anti-GFP affinity chromatography as detected by the anti-GFP antibody (Figure 15). Both DRP1A and DRP1C were detected in the CWLP-GFP-purified fraction at positions close to the 70-kDa marker (Figure 15). The apparent molecular weights of these two proteins shown here are similar to what has been reported (Kang *et al.*, 2003b). The DRP1C band had a slightly higher apparent molecular mass than DRP1A. This result further supports the notion that CWLP interacts with at least two DRP1 family proteins *in vivo*.

A

B

Gene ID	Protein	# peptides	# spectra	coverage
At5g42080	DRP1A	60	454	74%
At1g14830	DRP1C	49	335	78%

Figure 14. Analysis of polypeptides co-purified with CWLP-GFP. (A) Together with the CWLP-GFP band, four specific bands (1-4) were excised and subjected to mass spectrometry-based analysis followed by peptide identification. Molecular weight markers in kDa are shown on the left. (B) Identification of DRP1C (band 2) and DRP1A (band 3). Gene ID, genetic locus identified by TAIR; # peptides, number of unique peptides detected; # spectra, number of total spectra detected; coverage, percent of protein sequence covered.

Figure 15. Specific interaction between CWLP and DRP1. Proteins recovered from anti-GFP affinity purifications using the CWLP-GFP plants (lanes 2, 4, 6, 8) and the negative control of wild-type (lanes 1, 3, 5, 7). Total proteins are shown by silver staining. The CWLP-GFP fusion protein is detected by the anti-GFP antibody, and DRP1A and DRP1C are detected by respective specific antibodies. Molecular weight markers in kDa are shown in the left.

Sample#	Gene ID	Gene Name	# peptides	Peptide sequence
CWLP-GFP IP	At5g42080	Dynamin 1A	60	AAQSEIDAVAWSK ACTALGDHGDSSALPTLWDSLPAAVVGQSSGK AGGEKVYVWFNDQLPAALKR DDGTREYAEFLHLPK EREYFSNTTEYR EVDPSGDRTFGLTK LYAEFLHLPK EYFSNTTEYR FTDFAAVR FTDFAAVRK GGNPTHISIFDR GGNPTHISIFORYNDSYLR GTDAVEILEGR GYQPHIAPEQGYR IDDGTREYAEFLHLPK IDLMDKGTDAVEILEGR ILAISPANQDLATSDAIK IPGQSLINK KFTDFAAVR KFTDFAAVR KLVTADGYQPHIAPEQGYR KLVTADGYQPHIAPEQGYRR LFDQIFKEHLDGVR LGKPIAADAGGK LIESIVSIK LKYPWVGIVNR LPGLTKVAVDQSSQSIK LQFDKQLAMDNIR LQFDKQLAMDNIRK LSSLNEDPAIMER LVTEADGYQPHIAPEQGYR LVTEADGYQPHIAPEQGYRR LYSIMEIKR MENUSLVNKK MENUSLVNKKR NKNVDMIAAR QDVEKGGNPTHISIFDR QLAMDNIR QLAMDNIRK QLAMDNIRKLVTADGYQPHIAPEQGYR RLSSLNEDPAIMER RPLVLQLQK SIVYQVVR SLLDHFAELGTMDMKR SQADINKVDMIAAR SRIPGQSLINK SSVLESIVGR SSVLESIVGRDFLPR SVNETVELK SVNETVELKQYPALR SYNEKPNCILAIISPANQDLATSDAIK TVLELETELK VAVDQSSQSIK VAVDQSSQSIKDIENMVR VEVTNAAIESLDK VEVTNAAIESLDKMR VEVTNAAIESLDKMRGSK VYVWFNDQLPAALK VYVWFNDQLPAALKR YPPWVGIVNR
CWLP-GFP IP	At1g14830	Dynamin 1C	49	ACTVLGDHGGEGMSLWEALPTVAVVGQSSGK AVVYQVVR DDIDAVAWK EIEDETDRITGK EKEKLGAMLDEDPQLMER EKLGLAMLDEDPQLMER EQEYFETSPYGHLSR ESIVQDIENMVR FADFAAVR FADFAAVRK FPTLASDIAAAANEALR GPAEATVDVAVHVLK IGRPVAVDSGAQLVTLLECR IGSNVSAYINMVCOTLR IPSIVALINK IYGVFDHQLPAALK KEIETEDRITGK KLHLEPEKEKPNR KSISETEELK KSISETEELKRFPPTLASDIAAAANEALR LDIMDKGTDCDLEGR LGAMLDEDPQLMER LGAMLDEDPQLMERR LIDGSISYFK LLSQHLETIVR LQHPWVGIVNR LVDMESSYLTVEFFR MGSEYLA NAPAPNADPYSDNHR NAPAPNADPYSDNHRK QARDIDAVAWK QKIPSIVALINK RKEQYFETSPYGHLSR RVDMAAR SIDEINAELOK SISETEELK SISETEELKRFPPTLASDIAAAANEALR SLGLINK SLNFFYAQVGR SSVLESIVGR SSVLESIVGRDFLPR SYVEKPNCILAIISPANQDIATSDAIK TEDGTTTEYAEFLHAPK TFGVATK TLIDLPGLTKVAVDQSPESIVQDIENMVR VAVDQSPESIVQDIENMVR VDMIAAR VWSEADGYQPHIAPEQGYR VWSEADGYQPHIAPEQGYRR

Table 1

Peptides defined by microsequencing derived from bands isolated from the gel shown in Figure 14.

1.15. Conclusions of CWLP study

1.15.1 CWLP function

Our results indicate that within the PM of plant cells, likely in lipid rafts, PIP3 and to a lesser extent other aquaporins, form complexes with CWLP, which are involved in transport of water, and perhaps other small molecules. In nature, *Arabidopsis* plants are mainly facing cold stress rather than drought, being grown in well water-supplied

environment. Therefore, the natural increase in CWLP content is observed during cold stress (Figure 7). However, when an excess of CWLP molecules is provided by constitutive expression of ectopic CWLP, those molecules form complexes with aquaporins in the PM lipid rafts, stabilizing these structures by forming CW-PM-cytosol continuum and increase coping capacity with other environmentally imposed water shortage conditions (Figure 8). CWLP molecules recruit PP2A to the aquaporin complex at the PM inner side and thereby promote dephosphorylation of two Ser residues responsible for the closing of the aquaporin water channel (Figure 12). Hence, the assembly of CWLP molecules, capable of forming continuum between the cell wall and cytosol through the PM as well as concomitantly interact with membrane aquaporins, generates supporting scaffold structures in the PM lipid rafts. These structures contribute to the PM maintenance, especially when stress effects cause changes in water potential and membrane fluidity. Our findings unravel the function of CWLP in mediating cellular water loss (plasmolysis) drought and freezing conditions and demonstrate the mechanism responsible for improved tolerance to water shortage. Although this mechanism has been evolved in *Arabidopsis* to cope with freezing effects, here we show its utilization for dehydration withstanding.

1.15.2. CWLP, dynamin-related proteins and endocytosis

The interaction with DRP1A/C is in line with the membrane-associated localization of CWLP-GFP at the cell cortex. Both DRP1 proteins have been proven to play non-redundant critical roles in the maintenance of the plasma membrane in rapidly growing cells (Konopka and Bednarek, 2008). It has been shown that at least DRP1C co localizes with the clathrin light chain CLC in dynamic foci at the cell cortex (Konopka et al., 2008). Therefore, it likely participates in clathrin-mediated membrane remodeling during endocytosis. Similarly, DRP1A is also involved in clathrin-mediated endocytosis and is probably involved in rapid internalization of auxin efflux carriers *via* endocytosis (Mravec et al., 2011). Two possible implications arise from the co-purification of CWLP and DRP1. CWLP may assist the endocytic machinery for its function in membrane internalization at the cell cortex. It has been reported that the internalization of the PIP2 aquaporin and likely plasma membrane is induced by drought or salt stress (Lee *et*

- A Amino acid sequence and domain organization. The 8CM domain with the conserved Cys residue organization is shown.*
- B Transsient expression of GPRP-GFP driven by the CaMV 35S promoter in epidermis cells of *N. benthamiana*. The protein is organized in clusters in the PM.*
- C Expression of GPRG gene in *Arabidopsis* during development.*

The expression of GPRP is limited to the shoot meristems and developing seeds (Figure 16 C). Transgenic *Arabidopsis* plants, over expressing *GPRP* driven by the CaMV 35S promoter, were generated. Despite the presence of the protein, which was defined by western blots using anti-GFP antibodies, no GFP fluorescence was observed in any of the transgenic plants. Therefore, transient expression of GPRP was examined in *N. benthamiana* leaves. As is shown in Figure 16 B, GPRP-GFP is localized to the PM, and appears in clusters similarly to the appearance of the CWLP-GFP. Like the CWLP, also GPRP is found in lipid rafts as well as in the soluble membrane fraction. Assuming that perhaps GPRP has a function that is similar to that of CWLP, we performed drought experiments comparing WT and GPRP-over expressing *Arabidopsis* plants. A slight but significant increase in dehydration withstanding was observed (data not shown). The K/O mutant of this gene does not have any phenotype. Thus, GPRP may act similarly to CWLP, however, a broader study with this protein is required to estimate its mode of action, and whether it can support plant cells during short dehydration periods.

2.2. GPR940

This proline-rich protein, encoded by the At2g10940 gene in *Arabidopsis*, possesses a shorter proline-rich domain compared with that of the CWLP, but still maintains highly homologous 8CM domain (Figure 1). When this protein was fused to GFP and transiently expressed in *N. benthamiana* leaves, a completely different arrangement of PRP940 molecules in the periphery of the cells was observed (Figure 17 B, right panel). The PRP940 molecules appeared to be aligned in parallel to the orientation of the cortical microtubules (Figure 17 B b-c). This orientation was interrupted by the addition of oryzalin which selectively causes microtubule depolymerization.

A PRP 940: 876bp cDNA, 291aa sequence containing 73 Pro (25%), 43 Leu (14.7) and 40 Val (13.7%) residues. Nine repeats of PVPPVTVPKL.

MDSSKLSLSLCLFLICIIYLPQHSLACGSCNPRKGGKHSPKAPKLPVPPVTVPKLPVPPVTV
PKLPVPPVTVPKLPVPPVTIPKLPVPPVTVPKLPVPPVTVPKLPVPPVTVPKLPVPPVTVPK
LPVPPVTVPKLPVPPVTVPKLPVPPVTVPKLPVPPVTVPKLPVPPVTVPKLPVPPVTVPK
DCPPPPGSKPPSGGGKATCPIDTLKLGACVDLLGGLVKIGLGDPAVNKCCPLLKGLVEVE
AAACLCITTLKALDLNLYVPVALQLLTGKNPPPGYTCSIA

Figure 17. PRP940 characteristics and assembly

A PRP940 amino acid sequence, indicating various domains including the conserved 8CM domain (also indicated in Figure 1).

B Transient expression of PRP940-GFP in *N. benthamiana* epidermis cells showing parallel orientation with cortical microtubules (b, c) with no overlapping with the ER marker (c).

Transgenic plants that contain the 35S promoter –PRP940-GFP were generated. However, despite the presence of the PRP940 expression cassette, no GFP fluorescence was observed.

To examine the involvement of PRP940 in dehydration withstanding, dehydration experiments were performed, comparing WT and the K/O mutant Salk_0831138 plants. No difference was observed in several experiments. Hence, the difference in the assembled pattern of PRP940-GPR in cells, which is microtubule dependent, and the lack

of response to dehydration suggest that PRP940 has a different function, not related to water shortage and probably linked to the tubulin-cytoskeleton function.

Collaboration

Along the study a tight collaboration between the three groups has been maintained. Constant consultations and discussions about results, exchange of know-how and fluorescently labeled proteins have contributed to these innovative findings that show the possible option of regulating water channels in plant cells. This collaboration still continues and currently we are finalizing the manuscript that describes the functions of the CWLP and also characterizing the functions of PRP940.

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List of publications

1. Thesis submitted for the degree “Doctor of Philosophy” by Arik Honig (October 2009)
Characterization of the Structure and Function of *Arabidopsis thaliana* Pro-rich Cell Wall Linker Protein (*AtCWLP*)
2. A manuscript summarizing the above results and those achieved later is currently being submitted.