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Craterostigma plantagineum cell wall composition is remodelled during desiccation and the glycine-rich protein CpGRP1 interacts with pectins through clustered arginines

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SUMMARY

Craterostigma plantagineum **belongs to the desiccation-tolerant angiosperm plants. Upon dehydration, leaves fold and the cells shrink which is reversed during rehydration. To understand this process changes in cell wall pectin composition, and the role of the apoplastic glycine-rich protein 1 (CpGRP1) were analysed. Cellular microstructural changes in hydrated, desiccated and rehydrated leaf sections were analysed using scanning electron microscopy. Pectin composition in different cell wall fractions was analysed with monoclonal antibodies against homogalacturonan, rhamnogalacturonan I, rhamnogalacturonan II and hemicellulose epitopes. Our data demonstrate changes in pectin composition during dehydration/rehydration which is suggested to affect cell wall properties. Homogalacturonan was less methylesterified upon desiccation and changes were also demonstrated in the detection of rhamnogalacturonan I, rhamnogalacturonan II and hemicelluloses. CpGRP1 seems to have a central role in cell adaptations to water deficit, as it interacts with pectin through a cluster of arginine residues and de-methylesterified pectin presents more binding sites for the protein–pectin interaction than to pectin from hydrated leaves. CpGRP1 can also bind phosphatidic acid (PA) and cardiolipin. The binding of CpGRP1 to pectin appears to be dependent on the pectin methylesterification status and it has a higher affinity to pectin than its binding partner CpWAK1. It is hypothesised that changes in pectin composition are sensed by the CpGRP1–CpWAK1 complex therefore leading to the activation of dehydration-related responses and leaf folding. PA might participate in the modulation of CpGRP1 activity.**

Keywords: desiccation tolerance, resurrection plant, ***Craterostigma plantagineum*****, cell wall, glycine-rich protein, pectin, leaf folding, lipid binding, phosphatidic acid.**

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INTRODUCTION

Most land plants are not able to survive prolonged periods of water shortage and even a mild water deficit can lead to irreversible damage and plant death (Zhang and Bartels, 2018). Water plays a crucial role in the maintenance of cell turgor, transport of solutes and nutrients and it mediates hydrophobic and hydrophilic interactions essential for macromolecular structures. Plants, as sessile organisms, face dehydration periods and have different strategies to reduce water loss and adapt to low water availability (Verslues and Juenger, 2011).

Desiccation tolerance is rare in vegetative tissues of vascular plants, but it is common in seeds and pollen. Only approximately 300 species of vascular plants have been reported to survive extensive dehydration during their vegetative growth phase. These plants are generally called resurrection plants (Porembski, 2000). Most angiosperm resurrection plants are native to central and southern Africa, Australia, south America and India (Gaff, 1971; Gaff and Bole, 1986). The resurrection plant *Craterostigma plantagineum* (Cp) has been extensively studied (Giarola *et al.*, 2017). *Lindernia brevidens* (Lb) and *Lindernia subracemosa* (Ls) are closely related to *C. plantagineum* (Rahmanzadeh *et al.*, 2005) and their genome sequences have recently been deciphered (VanBuren *et al.*, 2018). The interest for

comparative studies of these plants are different degrees in desiccation tolerance. *L. brevidens* is desiccation-tolerant like *C. plantagineum* (Phillips *et al.*, 2008) but *L. subracemosa* is desiccation-sensitive (Seine *et al.*, 1995).

Cell walls are the outermost structures of plant cells and essential to maintain cell and organ integrity and functionality. Mechanical stress is built up when the cell wall folds up during dehydration. When the vacuole shrinks and the cell contents are drawn inwards, tension builds up between the plasma membrane and the rigid cell wall (Levitt, 1987). The protection of the plasma membrane during dehydration is essential for the plant to survive. Iljin (1957) proposed that any plant could tolerate desiccation if the mechanical stress can be limited. In desiccation-sensitive species the cell wall loses its integrity upon desiccation. When resurrection plants encounter desiccation the leaves start to fold and the cell volume is reduced (Farrant, 2000; Farrant *et al.*, 2003; Willigen *et al.*, 2003). Previous studies showed dehydration-induced changes in cell wall architecture, cell wall composition and variations of hemicellulose polysaccharides and pectin-associated arabinans in resurrection plants (Vicré *et al.*, 1999, 2004; Moore *et al.*, 2006, 2008). It has been proposed that high levels of pectic-arabinans, arabinogalactan-proteins and arabinoxylans ensure the required cell wall plasticity upon dehydration for the resurrection plant *Myrothamnus flabellifolia* (Moore *et al.*, 2013). Changes in xyloglucan and modifications of pectin structures have been reported for *Craterostigma wilmsii* (Vicré *et al.*, 1999, 2004).

Glycine-rich proteins (GRPs) have a high-glycine-content with glycine residues arranged in $(Gly)_n$-X repeats. In addition, a cysteine-rich region, an oleosin domain, RNA-recognition motifs, a cold-shock domain or zinc-finger motifs are found in GRPs. Although several GRPs have been characterised, the function of the glycine-rich domains in these proteins is poorly understood (Czolpinska and Rurek, 2018). GRPs are classified according to the arrangement of the glycine-rich repeats and the presence of additional domains (Sachetto-Martins *et al.*, 2000*;* Fusaro *et al.*, 2001; Bocca *et al.*, 2005; Mangeon *et al.*, 2010). Class I GRPs share a high-glycine-content region with $(GGX)_n$ repeats. A C-terminal cysteine-rich region is present in the class II GRPs. The class III GRPs have a lower glycine content and may have an additional oleosin domain. Class IV GRPs are known as RNA-binding GRPs with either an RNA-recognition motif or a cold-shock domain and in some cases GRPs have additional zinc-finger motifs. Class V GRPs are similar to class I GRPs but show mixed patterns of glycine repeats. The expression patterns and the subcellular localisation of the different proteins within the GRP-superfamily are highly diverse thus suggesting that these proteins have different functions (Mangeon *et al.*, 2010). GRPs with an apoplastic signal peptide have been proposed to be an important component of cell wall structures (Condit and Meagher, 1986, 1987; Keller *et al.*, 1988). For example, the French bean PvGRP1.8 protein is part of the cell wall and plays a role in the protoxylem repair system (Ringli *et al.*, 2001). According to microarray results GRPs could also be implicated in maintaining protoxylem structures (Yokoyama and Nishitani, 2006). Glycine-rich proteins have been proposed to connect the secondary cell wall thickenings between protoxylem elements (Ryser *et al.*, 2004). The AtGRP9 protein from *Arabidopsis thaliana* interacts with a cinnamyl alcohol dehydrogenase (AtCAD5) and may be involved in lignin biosynthesis (Chen *et al.*, 2007). Besides class I GRPs also GRPs from other classes are plant cell wall components. The class V glycine-rich protein 1 (BhGRP1) from *Boea hygrometrica* was proposed to be important for cell wall integrity during dehydration, whereas the class II GRP NtCIG1 protein from tobacco was proposed to enhance callose deposition in cell walls (Ueki and Citovsky, 2002; Wang *et al.*, 2009).

The *C. plantagineum* glycine-rich protein 1 (CpGRP1) belongs to class II GRPs. CpGRP1 is highly abundant in the apoplast of desiccated leaves and interacts with the *C. plantagineum* cell wall-associated protein kinase 1 (CpWAK1) (Giarola *et al.*, 2016). WAKs contain an extracellular pectin binding domain and an intracellular serine/threonine protein kinase domain. WAKs have been suggested to link the cytoplasm to the extracellular matrix and to activate signalling pathways in response to pectin changes (He *et al.*, 1999; Anderson *et al.*, 2001; Kohorn and Kohorn, 2012). The CpGRP1−CpWAK1 complex may play a role in sensing dehydration-induced cell wall changes and thus activate dehydration-induced signalling pathways (Giarola *et al.*, 2016). A similar complex is known from *A. thaliana* where the cysteine-rich region of AtGRP3 interacts with the cell wall-associated kinase 1 (AtWAK1). This complex has been proposed to be involved in pathogen defence mechanisms (Park *et al.*, 2001).

Phosphatidic acid (PA) belongs to the group of phospholipids and consists of a glycerol backbone, a saturated fatty acid, an unsaturated fatty acid and a phosphate group and has different roles in plants (Hou *et al.*, 2016). Phospholipase C and phospholipase D are involved in the synthesis of PA from phosphatidylinositol-(4,5)-bisphosphate (PIP2), phosphatidylcholine and phosphatidyl-ethanolamine. These reactions are crucial for the maintenance of the PA pool, which serves as a source for the biosynthesis of other phospholipids (Munnik, 2001; Ufer *et al.*, 2017). PA can also function as signalling molecule in response to environmental cues. PA was shown to bind to other proteins and thus modify the activity of proteins (Hou *et al.*, 2016).

Changes in the *C. plantagineum* cell wall during a desiccation/rehydration cycle were investigated with a set of monoclonal antibodies that recognise different epitopes of homogalacturonan (HG), rhamnogalacturonan I (RG-I), rhamnogalacturonan II (RG-II) and hemicelluloses. Our

findings suggest that *C. plantagineum* cell walls undergo reversible remodelling of pectic polymers upon desiccation. A cluster of arginine residues within the CpGRP1 protein enables its binding to pectin and PA. HG methylesterification is reduced upon dehydration thus promoting CpGRP1 binding to pectin. Changes in the pectin organisation provide plasticity to the cell wall which is required for the extensive folding and expansion of cell walls during desiccation/rehydration.

RESULTS

Morphological characterisation of leaf structures

Microscopic views of the *C. plantagineum* leaf surfaces and transverse sections during a desiccation/rehydration cycle are shown in Figure 1(a,b). Untreated and rehydrated leaf tissues are almost identical suggesting that leaves can fully recover from desiccation-induced cellular changes after 48 h of rehydration. In contrast to the hydrated and rehydrated samples, the epidermis of the desiccated leaf is extensively folded. In this compact structure leaf glands are trapped in the epidermal folds. Leaf folding mainly occurs during late dehydration when the relative water content is below 60%. Cell walls in desiccated tissues were slightly thicker than cell walls in untreated and 48 h-rehydrated tissues as shown by cell wall staining (Figure 1c,d). These findings suggest that changes in pectin composition between the different samples might be involved in cell wall adaptations to water-stress conditions.

Pectin and hemicellulose profiles determined in a desiccation/rehydration cycle

Panels of monoclonal antibodies allow the monitoring of changes in cell wall polysaccharides (Table 1). In enzyme-linked immunosorbent assays (ELISAs), JIM5, JIM7, LM20 and LM19 were used to analyse differences in the methylesterification pattern of *C. plantagineum* HG fractions upon desiccation and rehydration (Table 2). The abundance of the pectin and hemicellulose epitopes is correlated with colour intensity. JIM5, JIM7 and LM20 displayed a stronger binding to the untreated and rehydrated (1 and 2) samples than to the desiccated sample. LM19, which detects fully de-methylesterified HG, displayed a stronger signal to desiccated samples than to untreated samples. These results indicate changes in the HG methylesterification status during the desiccation/rehydration cycle and suggest homogalacturonan synthesis in the recovery process. Changes in the abundance of RG-II were analysed using the 42-6 antibody which binds RG-II monomers, crosslinked RG-II and an unknown pectic component (Table 1). The antibody bound more strongly to the untreated and rehydrated samples than to the desiccated samples (Table 2).

LM25, LM15, LM11, LM6 and LM5 monoclonal antibodies were used to analyse changes in the hemicelluloses and the rhamnogalacturonan I (Tables 1 and 2). LM25 and LM15 indicate strong signals for xyloglucan in desiccated leaves which is reversed during rehydration. LM11, which detects xylan, bound stronger to the desiccated samples, whereas LM6, which detects (1→5)-α-arabinan, displayed a slightly weaker binding to pectins of desiccated leaves than to pectins of untreated leaves. No significant changes were observed in the binding of the LM5 antibody which detects (1→4)-β-galactan. Results obtained with RG-I, RG-II and hemicellulose antibodies suggest changes in the *C. plantagineum* cell wall architecture during the desiccation/rehydration cycle.

Interaction between CpGRP1 and pectin

The glycine-rich cell wall protein CpGRP1 interacts with the CpWAK1 kinase protein in the apoplast and it has been hypothesised that CpGRP1 binds to pectin (Giarola *et al.*, 2016). The glycine-rich domain of CpGRP1 contains a positively charged arginine cluster through which it may interact with cell wall polysaccharides, such as pectins (Figure 2a,b). Three different approaches have been used to analyse a possible interaction of CpGRP1 and pectins: Blue-native page gel-shift assays, ELISA and dot-blot assays using either commercial pectin (from citrus peel; Sigma-Aldrich, USA) or the 1,2-cyclohexanediaminetetraacetic acid (CDTA)-pectin fraction isolated from *C. plantagineum* or closely related species. For binding experiments the full-length recombinant CpGRP1 protein or fragments of the CpGRP1 protein corresponding to N- or C-terminal domains or to the mutated N-terminal domain (two arginines mutated to glycine, a352g_c358g) were used (Figure 2). When the CpGRP1 full-length protein was incubated with commercial pectin and calcium, the electrophoretic mobility was retarded compared to the CpGRP1 protein in a native page (Figure 3). A similar mobility shift was observed for the N-terminal fragment, but not for the C-terminal fragment. The mutated N-terminal fragment did also show a mobility shift in the presence of pectin and calcium but in contrast to the non-mutated fragment, two protein bands were detected. No mobility shift for any of the proteins was observed in the absence of calcium. The interaction between CpGRP1 and pectins was further investigated using pectins extracted from *C. plantagineum* leaves (Figure 4a). Microtiter plate wells were coated with CDTA-soluble pectins or KOH-soluble cell wall fractions and then incubated with the CpGRP1 full-length protein, the N-terminal or the mutated N-terminal polypeptide. No complex was observed between CpGRP1 and the KOH cell wall fractions. The strongest interaction was observed with the N-terminal polypeptide, followed by the mutated N-terminal polypeptide and the full-length protein. Each of the tested proteins showed the highest value for the CDTA-pectin fraction isolated from desiccated *C. plantagineum* leaves. No differences were observed in

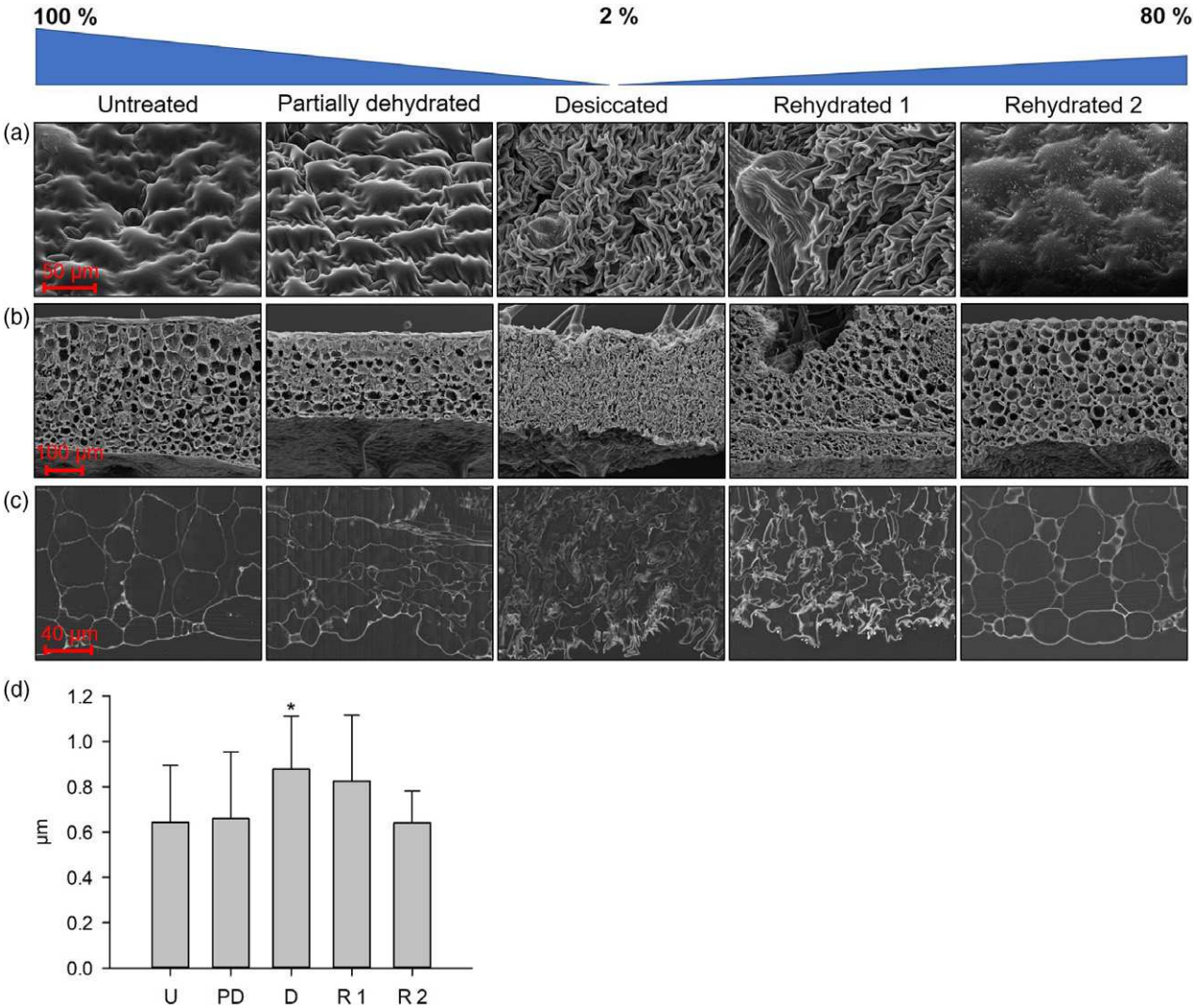


Figure 1. Morphological characterisation of *Craterostigma plantagineum* leaf structures using scanning electron microscopy. Surface images (a), transverse section images (b) and images of cell walls stained with silver nitrate (c) are shown. Micrographs were taken in a desiccation/rehydration cycle. From left to right: Untreated (RWC = 100%), Partially dehydrated (RWC = 50%), Desiccated (RWC = 2%), 24 h after rehydration (Rehydrated 1) and 48 h after rehydration (Rehydrated 2). Representative micrographs are shown. Three different samples were used for the evaluation. (d) Thickness of *C. plantagineum* cell walls in a desiccation/rehydration cycle. Images from (c) were used for the evaluation. Cell wall thickness increases slightly during dehydration and the starting point (untreated samples) is restored after 48 h of rehydration. U = Untreated, PD = Partially dehydrated, D = Desiccated, R 1 = Rehydrated 1, R 2 = Rehydrated 2. The asterisk indicates the levels of significance in comparison with the untreated sample (one-way ANOVA, Holm–Sidak method): $*P < 0.05$.

the presence or absence of calcium. In addition, pectin fractions from the two closely related species differing in desiccation tolerance, *L. brevidens* and *L. subracemosa,* were prepared and the CpGRP1 binding to those fractions was analysed (Figure 4b). The CpGRP1 full-length protein and the N-terminal polypeptide showed the strongest interaction with *C. plantagineum* pectin, followed by pectin from *L. brevidens* and *L. subracemosa*. To confirm that these differences are due to the pectin composition and not due to the amount of isolated HG the galacturonic acid (GA) content was determined for all different fractions. No significant differences in the GA content were detected which supports comparable HG contents (Table 3). In a second step the JIM5, JIM7, LM20 and LM19 antibodies were used to analyse the *L. brevidens* and *L. subracemosa* HG fractions. The methylesterification profile of HG for the desiccation-tolerant plant *L. brevidens* was similar to the one of *C. plantagineum* (Table 4). For *L. subracemosa* the signal intensity detected with the antibodies in the rehydrated samples was lower than the signal in the untreated sample suggesting that this plant is not able to restore cell wall architecture during rehydration which is consistent with the phenotype (Table 4).

To confirm the ELISA analyses a series of dot-blot experiments was carried out. The interaction of the CpGRP1 protein and pectin was either detected using a 6x His-tag

Table 1 Monoclonal antibodies used in this study for pectin and hemicellulose characterization

	Antibody	Specificity	Reference
HG[a]	JIM5	Partially or de-methylesterified HG	Knox *et al.* (1990)
	JIM7	Partially methylesterified HG	Knox *et al.* (1990)
	LM20	Methylesterified HG	Verhertbruggen *et al.* (2009)
	LM19	Fully de-methylesterified HG	Verhertbruggen *et al.* (2009)
RG-II[b]	42-6	B-RG-II, RG-II monomers, unknown pectic fragment	Zhou *et al.* (2018)
RG-I[b]	LM6	(1→5)-α-arabinan	Willats *et al.* (1998)
	LM5	(1→4)-β-galactan	Jones *et al.* (1997)
Hemicelluloses	LM25	Xyloglucan (XXLG, XLLG)	Pedersen *et al.* (2012)
	LM15	Xyloglucan (XXXG)	Marcus *et al.* (2008)
	LM11	(1→4)-β-xylan	McCartney *et al.* (2005)

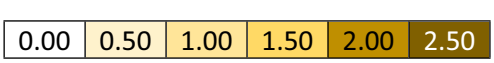
[a]Homogalacturonan.
[b]Rhamnogalacturonan.

antibody or the JIM5 antibody (Figure 5a). The dot-blot analyses confirmed the interaction between the CpGRP1 full-length protein and the two N-terminal protein fragments with pectins. BSA (Carl Roth, Karlsruhe, Art.-Nr. 8076.2) and the LEA-like 11-24His recombinant protein (Petersen *et al.*, 2012) were used as negative controls and did not show any interaction. Figure 5(b) presents the species-specific interactions between the CpGRP1 full-length protein, the N-terminal fragment and the mutated N-terminal fragment with the CDTA fractions of *C. plantagineum*, *L. brevidens* and *L. subracemosa*. Mutation of arginines in the N-terminal fragment led again to weaker interactions with pectins. Quantification of signal intensities of the dot blots fully supports the ELISA results (Figure 5c). The binding of CpGRP1 and CpGRP1 protein fragments to pectins from the desiccated samples was stronger than the binding to pectins from the untreated or rehydrated samples in all experiments. The highest signal intensity was obtained for pectin isolated from *C. plantagineum*. To investigate and compare the CpGRP1–pectin interaction, the pectin binding of two other apoplastic proteins, CpWAK1 and the *C. plantagineum* germin-like protein 1 (CpGLP1), was investigated. Figure 6 demonstrates that the CpGRP1 binds much stronger to pectin than CpWAK1 or CpGLP1.

Pre-treatment of pectin with CAPS-buffered solutions (pH 7–11) or with 0.1 M sodium carbonate reduced the extent of HG methylesterification. CpGRP1 full-length protein bound more strongly to de-methylesterified than to methylesterified pectin from *C. plantagineum* (Figure 7a).

Table 2 Analysis of changes in the cell wall composition of *Craterostigma plantagineum* leaves in a desiccation/rehydration cycle

		Ab	Untreated	Desiccated	Rehydrated 1	Rehydrated 2
C. plantagineum	HG[a]	JIM5	0.85 ± 0.14^{BCD}	0.37 ± 0.09^{Ad}	0.33 ± 0.06^{AD}	0.52 ± 0.10^{AbC}
		JIM7	1.22 ± 0.18^{BCD}	0.70 ± 0.08^{AcD}	0.90 ± 0.11^{Ab}	0.93 ± 0.07^{AB}
		LM20	2.10 ± 0.21^{BCd}	0.65 ± 0.11^{ACD}	1.70 ± 0.14^{AB}	1.83 ± 0.16^{aB}
		LM19	0.77 ± 0.12^{BCD}	1.32 ± 0.17^{ACD}	0.46 ± 0.05^{AB}	0.41 ± 0.06^{AB}
	RG-II[b]	42-6	3.49 ± 0.27^{BCD}	0.41 ± 0.11^{ACD}	1.63 ± 0.26^{ABD}	2.55 ± 0.23^{ABC}
	RG-I[b]	LM6	1.66 ± 0.31	1.33 ± 0.25	1.50 ± 0.22	1.45 ± 0.19
		LM5	0.28 ± 0.05	0.29 ± 0.08^{d}	0.27 ± 0.03	0.22 ± 0.03^{b}
	Hemicelluloses	LM25	1.30 ± 0.10^{b}	1.50 ± 0.19^{a}	1.35 ± 0.11	1.34 ± 0.14
		LM15	0.16 ± 0.02^{B}	0.31 ± 0.04^{ACD}	0.18 ± 0.03^{B}	0.19 ± 0.02^{B}
		LM11	0.15 ± 0.07^{BCd}	0.65 ± 0.11^{ACD}	0.42 ± 0.09^{ABD}	0.26 ± 0.08^{aBC}



JIM5, JIM7 and LM20 detect varying levels of methylesterification in HG. LM19 detects fully de-methylesterified HG. The 42-6 antibody detects RG-II, crosslinked RG-II and an unknown pectic epitope. LM6 and LM5 detect pectic arabinan and pectic galactan, epitopes present in the RG-I, respectively. LM25 and LM15 detect xyloglucan and LM11 detects xylan. For specificity of different antibodies see Table 1. The colour scale in relation to absorbance values is shown bottom left. Results for HG were generated analysing the CDTA fractions in 1:5 dilutions and results for RG-I and RG-II were generated analysing the KOH fractions in 1:5 dilutions. Values shown are means of three biological replicates ± SD. The letters indicate the levels of significance (one-way ANOVA, Holm–Sidak method): a,b,c,d $P < 0.05$; ABCD $P < 0.01$; aA = significantly different from 'Untreated' sample, bB = significantly different from 'Desiccated' sample, cC = significantly different from 'Rehydrated 1' (24 h) sample, dD = significantly different from 'Rehydrated 2' (48 h) sample.
[a]Homogalacturonan.
[b]Rhamnogalacturonan.

We hypothesize that the de-methylesterification of HG provides more binding sites for the CpGRP1 protein. Highly methylesterified commercial apple pectin (Apple Pectin Powder; Solgar, USA) was used to confirm the effectiveness of the de-methylesterification procedure as indicated by the specificities of the LM19 and LM20 probes. Figure 7(b) shows that untreated apple pectin is recognised by the LM20 antibody but not by LM19, whereas the sodium carbonate-treated and hence de-methylesterified apple pectin is recognised by the LM19 antibody and not by LM20.

As lipids are a major component of cell membranes and are modified during desiccation/rehydration in *C. plantagineum* (Gasulla *et al.*, 2013) it was tested whether CpGRP1 may also bind to lipids. CpGRP1 binding to different lipids was analysed in protein-lipid overlay assays (Figure 8a). The LEA-like 11–24 protein was used as a positive control and it was used with or without an additional His-tag to account for possible His-tag background binding (Petersen *et al.*, 2012). The CpGRP1 full-length recombinant protein showed strong binding to PA and cardiolipin (CL), but not to any other lipid tested (Figure 8b). The N-terminal fragment of CpGRP1 and the full-length CpGRP1 showed similar binding to PA, whereas the binding of the C-terminal fragment of CpGRP1 to PA was very weak. These results indicate that the binding of CpGRP1 to PA is mainly mediated by the N-terminal region of the protein. Mutations of two arginines in this region led to weaker binding, suggesting the involvement of positively charged arginines in the CpGRP1–PA interaction.

DISCUSSION

Pectin fractions are remodelled upon dehydration

Folding of cell walls and changing of texture and chemical composition is an adaptation to desiccation in resurrection plants (Bartels and Hussain, 2011). Intensive cell wall folding of desiccated cells is also in place in *C. plantagineum* as shown by scanning electron microscopy (SEM) (Figure 1). Similar observations have been made for *L. brevidens* and *L. subracemosa*, which are closely related to *C. plantagineum* and *C. wilmsii* (Farrant, 2000; Phillips *et al.*, 2008). Although the leaves of the desiccation-sensitive plant *L. subracemosa* are folded upon dehydration, they became brown and necrotic and did not recover after rewatering, which points to alterations in the composition of the cell wall of *C. plantagineum*. To address this question pectins and hemicelluloses associated with cell wall flexibility were analysed.

C. plantagineum showed a lower degree of methylesterification in HG upon dehydration, which is reversed during rehydration (Table 2). *L. brevidens* did show a similar methylesterification status as *C. plantagineum*, which is in agreement with the fact that this plant is also desiccation tolerant. In contrast, no significant changes in the HG fraction were detected for the desiccation-sensitive plant *L. subracemosa* upon rehydration (Table 4). The degree of methylesterification may be of importance for desiccation tolerance, at least among the Linderniaceae. HG is thought to be synthesised in the Golgi apparatus in a highly methylesterified form and is de-methylesterified in the cell

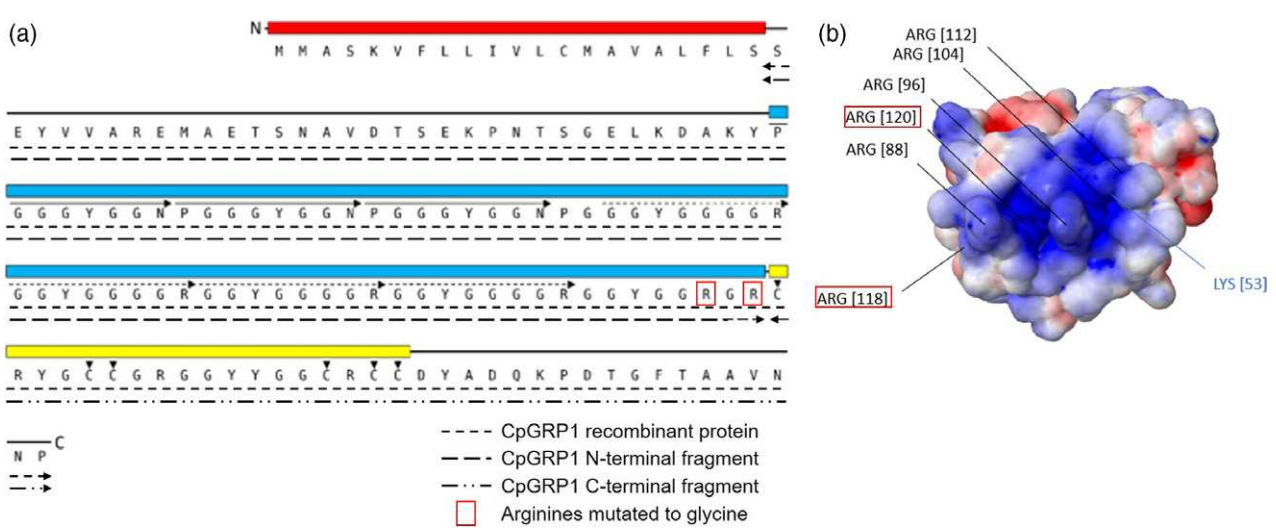


Figure 2. (a) *Craterostigma plantagineum* CpGRP1 amino acid sequence and protein domains. The different protein domains of CpGRP1 are indicated by coloured boxes above the sequence. Red: predicted signal peptide; blue: Semi-repetitive glycine-rich region; the two different amino acid tandem repeat motifs are shown with solid or dashed arrows respectively. Yellow: Cysteine-rich region; the six cysteine residues are marked by black triangles. Different protein fragments are marked by dotted lines. Additionally, the full-length CpGRP1 recombinant protein and the other polypeptides carried six histidine residues. (b) Electrostatic surface model of the recombinant CpGRP1 protein. Clustered arginines in the glycine-rich domain of the protein build up a negative cluster. Arginines at positions 118 and 120 were mutated to glycine (red boxes). The image in (a) was modified from Giarola et al. (2016).

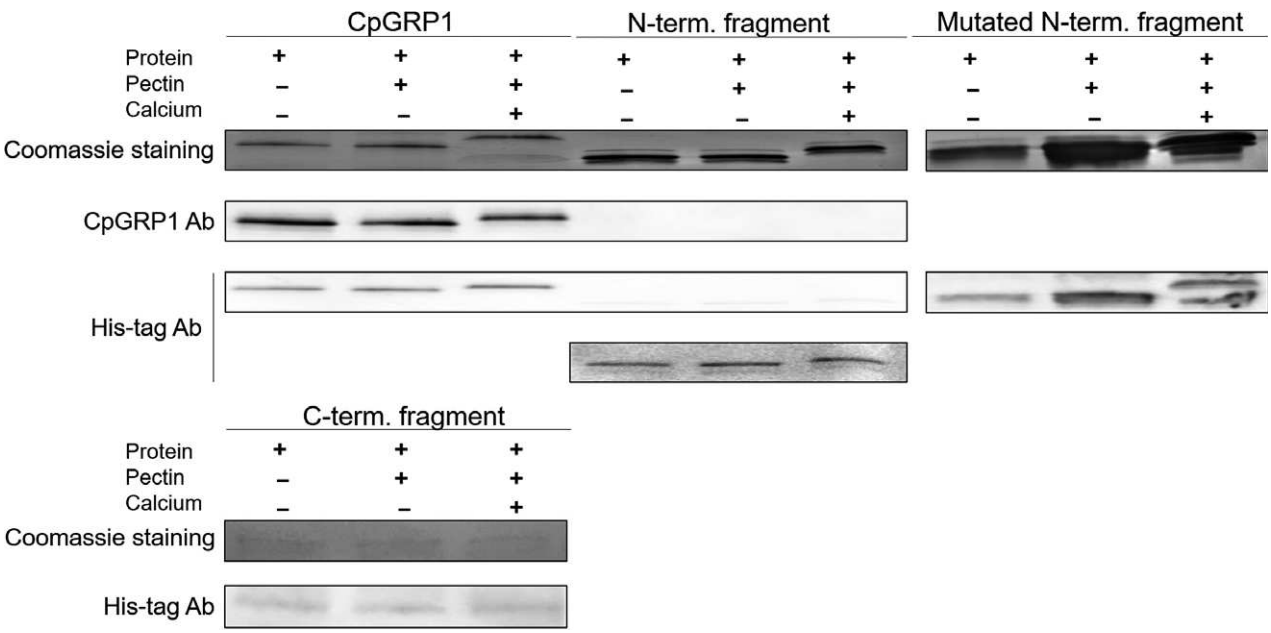


Figure 3. Analysis of CpGRP1–pectin interaction by Blue-native page gel-shift assay. The full-length recombinant CpGRP1 protein, a CpGRP1 N-terminal protein fragment and the mutated N-terminal protein fragment were used for this analysis. The proteins were detected with CpGRP1 or His-tag antibodies and SDS-gels were stained using Coomassie brilliant blue.

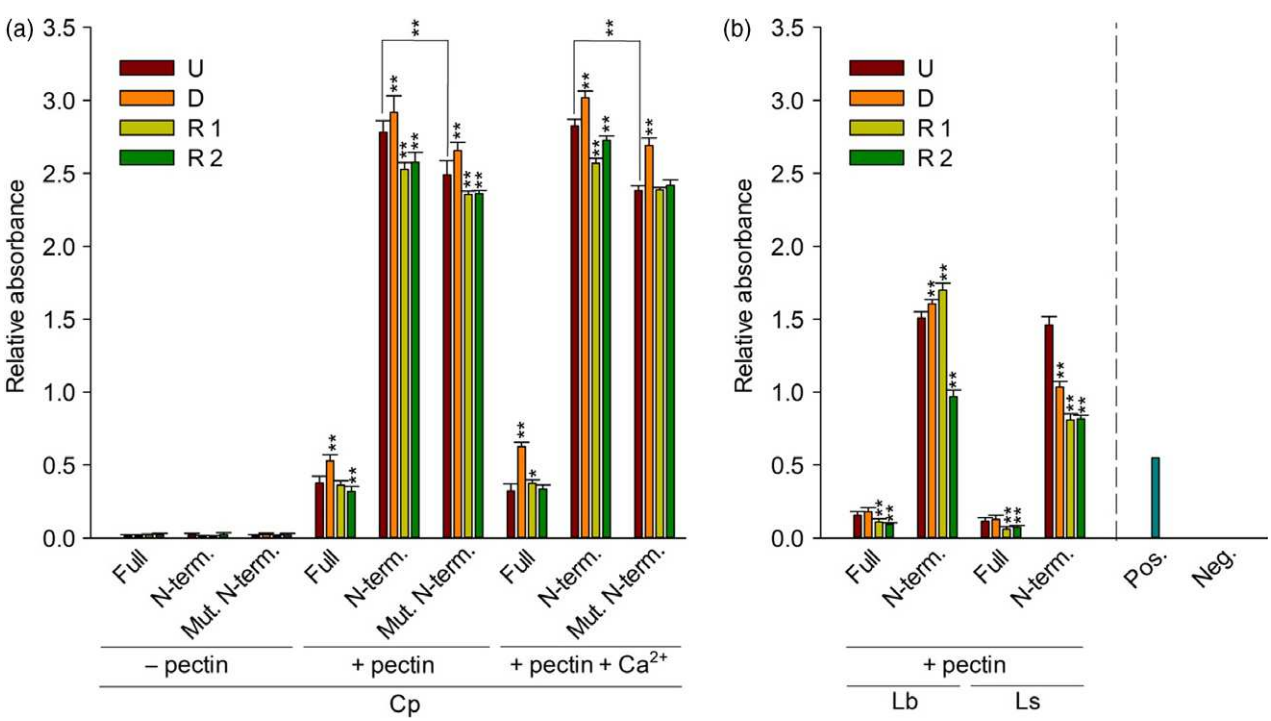


Figure 4. Quantification of the protein–pectin interaction using the CpGRP1 full-length protein, the N-term. fragment and the mutated N-term. fragment and pectin isolated from (a) *Craterostigma plantagineum*, (b) *Lindernia brevidens* and *Lindernia subracemosa* during dehydration and rehydration. All signals shown were detected using 1:5 dilutions of CDTA fractions. No signals were detected in KOH fractions. The LEA-like 11–24 protein was incubated with commercial pectin and 2 mM Ca^{2+} and used as a negative control (Neg.). The CpGRP1 full-length protein was incubated with commercial pectin and Ca^{2+} and used as a positive control (Pos.). U = Untreated, D = Desiccated, R 1 = Rehydrated 1 (24 h), R 2 = Rehydrated 2 (48 h). Values shown are means of three biological replicates ± SD. The star indicates the levels of significance in comparison with the untreated sample (one-way ANOVA, Holm–Sidak method): $*P < 0.05$; $**P < 0.01$.

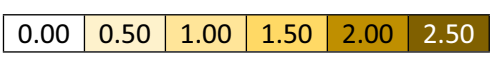
Table 3 Quantification of galacturonic acid content in the different CDTA-pectin fractions in μg μl^{-1}

	Untreated	Desiccated	Rehydrated 1	Rehydrated 2
Craterostigma plantagineum	2.01 ± 0.31	2.12 ± 0.22	2.00 ± 0.24	1.62 ± 0.41
Lindernia brevidens	1.81 ± 0.24	1.55 ± 0.26	1.58 ± 0.27	1.60 ± 0.21
Lindernia subracemosa	2.05 ± 0.25	1.92 ± 0.21	1.65 ± 0.27*	2.02 ± 0.20

Values shown are means of three biological replicates ± standard deviation (SD). The star indicates the levels of significance in comparison to the untreated sample (one-way ANOVA, Holm–Sidak method): *$P < 0.05$.

Table 4 Analysis of changes in cell wall methylesterification of *Lindernia brevidens* (Lb) and *Lindernia subracemosa* (Ls) leaves in a desiccation/rehydration cycle

		Ab	Untreated	Desiccated	Rehydrated 1	Rehydrated 2
HG[a]	Lb	JIM5	0.77 ± 0.14^{BCd}	0.31 ± 0.08^{AcD}	0.45 ± 0.11^{Abd}	0.62 ± 0.15^{aBc}
		JIM7	1.42 ± 0.21^{BCd}	0.67 ± 0.17^{AcD}	1.02 ± 0.22^{Ab}	1.14 ± 0.19^{aB}
		LM20	2.05 ± 0.28^{BC}	0.87 ± 0.18^{ACD}	1.52 ± 0.20^{ABd}	1.89 ± 0.16^{Bc}
		LM19	0.58 ± 0.09^{BCd}	1.52 ± 0.15^{ACD}	0.36 ± 0.07^{AB}	0.45 ± 0.06^{aB}
	Ls	JIM5	0.67 ± 0.18^{BCD}	0.22 ± 0.02^{A}	0.31 ± 0.06^{A}	0.24 ± 0.04^{A}
		JIM7	1.36 ± 0.16^{BCD}	0.88 ± 0.18^{A}	0.74 ± 0.11^{A}	0.81 ± 0.14^{A}
		LM20	2.41 ± 0.33^{BCD}	0.99 ± 0.25^{A}	0.84 ± 0.17^{A}	0.88 ± 0.18^{A}
		LM19	0.61 ± 0.09^{BCD}	1.44 ± 0.17^{ACD}	1.02 ± 0.22^{AB}	0.97 ± 0.21^{AB}



JIM5, JIM7 and LM20 detect varying levels of methylesterification in HG. LM19 detects fully de-methylesterified HG. For specificity of different antibodies see Table 1. The colour scale in relation to absorbance values is shown bottom left. Results were generated analysing the CDTA fractions in 1:5 dilutions. Values shown are means of three biological replicates ± standard deviation (SD). The letters indicate the levels of significance (one-way ANOVA, Holm–Sidak method): a,b,c,d $P < 0.05$; ABCD $P < 0.01$; aA = significantly different from 'Untreated' sample, bB = significantly different from 'Desiccated' sample, cC = significantly different from 'Rehydrated 1' (24 h) sample, dD = significantly different from 'Rehydrated 2' (48 h) sample.
[a]Homogalacturonan.

wall (Zhang and Staehelin, 1992; Staehelin and Moore, 1995; Sterling, 2001). Thus, the de-methylesterification of HG in the cell wall is a one-way process and there is no evidence of methylesterification of pectin in cell walls. The increasing binding of antibodies detecting methylesterified HG in rehydrated samples suggests that pectin is synthesised *de novo* in *C. plantagineum* and *L. brevidens* during the recovery process (Tables 2 and 4).

Pectin de-methylesterification is catalysed by a large enzyme family of pectin methylesterases (PMEs) (Fleischer *et al.*, 1999; Le Gall *et al.*, 2015). Little information is known about the activities of PMEs in *C. plantagineum*. PMEs are thought to be crucial in influencing cell wall properties and to be important for the modulation of apoplastic Ca^{2+} concentrations (Micheli, 2001; Wu and Jinn, 2010; Wu *et al.*, 2010, 2018). Ca^{2+} mediates the interaction between de-methylesterified pectin chains leading to the formation of "egg-box" pectin structures (Grant *et al.*, 1973; Jarvis, 1984; Moore *et al.*, 1986; Lloyd, 1991). An increase in apoplastic Ca^{2+} was observed in *C. wilmsii* upon dehydration suggesting enhanced cell wall strength in *Craterostigma* upon dehydration (Vicré *et al.*, 1999).

RG-II is crosslinked by borate (Kobayashi *et al.*, 1996). The interaction between RG-II, borate and HG is important for the physical and biochemical properties of the cell wall (O'Neill *et al.*, 2001). The antibody 42-6 detects RG-II monomers, borate-crosslinked RG-II and an unknown pectic component (Zhou *et al.*, 2018). We found significant changes in RG-II during desiccation/rehydration (Table 2). The decrease in the 42-6 signals in response to dehydration suggests pectin remodelling which could affect cell wall rigidity.

Xyloglucan connects cellulose fibrils and contributes to cell wall extensibility (Moore *et al.*, 1986; Fry, 1989). Higher abundance of xyloglucan and de-methylesterified pectins are both known to strengthen the cell wall. This fits well with the increase in xyloglucan and de-methylesterified pectins in desiccated *C. plantagineum* leaves (Table 2) and *C. wilmsii* (Vicré *et al.*, 1999). Increased detection of xylan, mostly present in secondary cell walls, was observed upon dehydration in *C. plantagineum*. Higher xylan contents may contribute to cell wall strength, as xylan together with xyloglucan connects cellulose fibrils. *A. thaliana* plants with reduced xylan contents have weakened cell walls

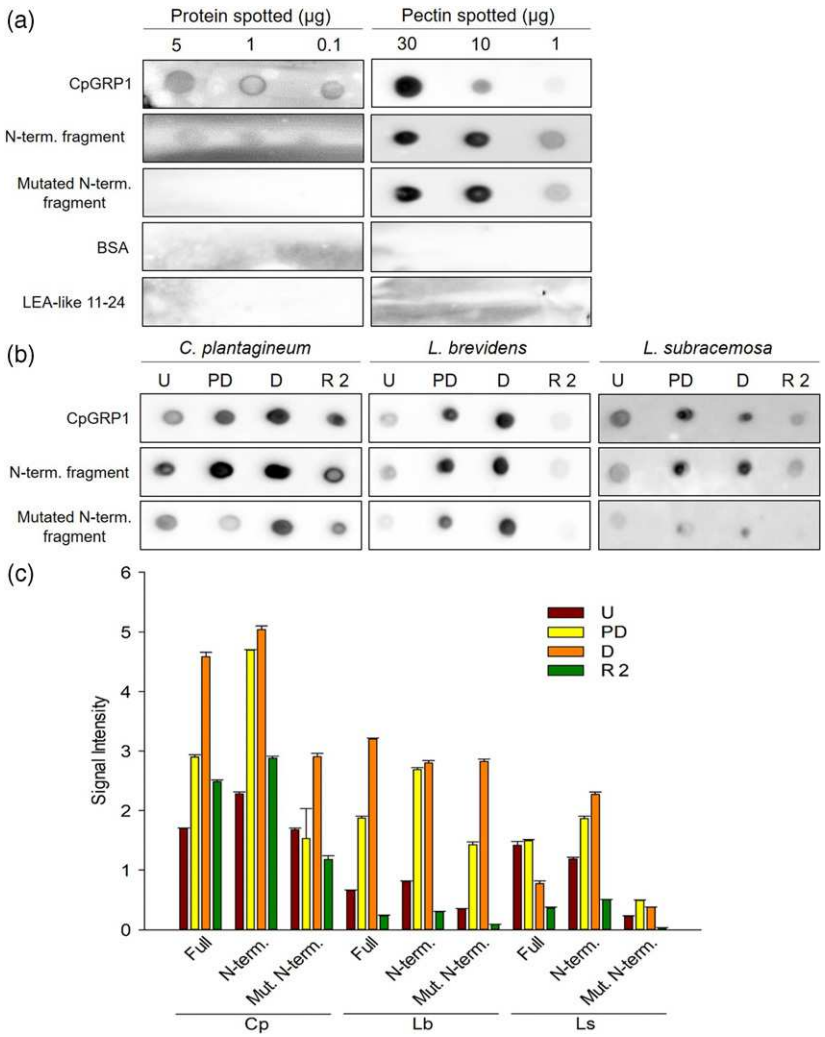


Figure 5. Evaluation of CpGRP1 interaction with *Craterostigma plantagineum*, *Lindernia brevidens* and *Lindernia subracemosa* pectin fractions during dehydration and rehydration by dot-blot analyses. (a) Binding of CpGRP1 full-length, N-terminal fragment and mutated N-terminal fragment to commercial pectin. BSA and the LEA-like 11-24 protein were used as negative controls. In the left-hand column the protein was spotted on the membrane first and then incubated with pectin, the right-hand column represents the opposite experiment, the pectin was spotted first and then the protein was added. (b) Interaction of CpGRP1 full-length, N-terminal fragment. and mutated N-terminal fragment with CDTA fractions. From each fraction 1 µg of pectin was spotted. Quantification of signals from (b) is shown in (c). U = Untreated, PD = Partially dehydrated, D = Desiccated, R 2 = Rehydrated 2 (48 h), Cp = *Craterostigma plantagineum*, Lb = *Lindernia brevidens,* Ls = *Lindernia subracemosa*. Values shown are means of three technical replicates ± SD.

(Brown *et al.*, 2007; Wu *et al.*, 2009). (1–>4)-β-galactan, the most flexible component of the cell wall, decreases the ability of pectin molecules to crosslink and also (1–>5)-*a*-arabinan side chains are motile (Ha *et al.*, 1996; Jones *et al.*, 1997). These components do not change or even decrease upon dehydration in *C. plantagineum* (Table 2).

Our results demonstrate major changes in cell wall components. The flexible cell wall components are not changing or are even decreasing upon drying. Conversely, components associated with stiffer and stronger cell walls increase. These findings are in agreement with the results published by Vicré *et al.* (1999) but are different to the results by Jones and McQueen-Mason (2004). They found an increase in cell wall flexibility and a higher activity of expansins. We propose that the *C. plantagineum* cell wall folds in a distinct way and this process is tightly influenced by different factors. Some connections are strengthened, while more flexibility is added to others. Different mechanisms need to be in place to protect the cells from being damaged by the mechanical tension during the dehydration process.

CpGRP1 binds to de-methylesterified pectin through clustered arginines

Glycine-rich proteins have been connected to cell wall properties of plants (Condit and Meagher, 1986, 1987;

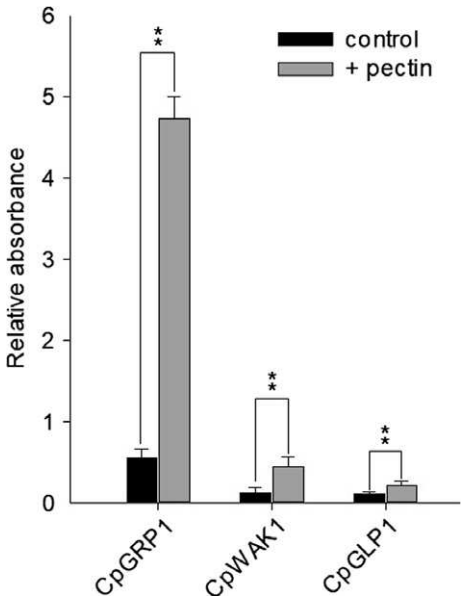


Figure 6. Quantification of protein–pectin interaction using the CpGRP1 full-length recombinant protein, the CpWAK1 extracellular recombinant protein fragment and the CpGLP1 full-length recombinant protein together with CDTA-pectin extracts from *C. plantagineum* leaves. Values shown are means of three biological replicates ± SD. The asterisk indicates the levels of significance in comparison to the control sample (one-way ANOVA, Holm–Sidak method): $^{**}P < 0.01$.

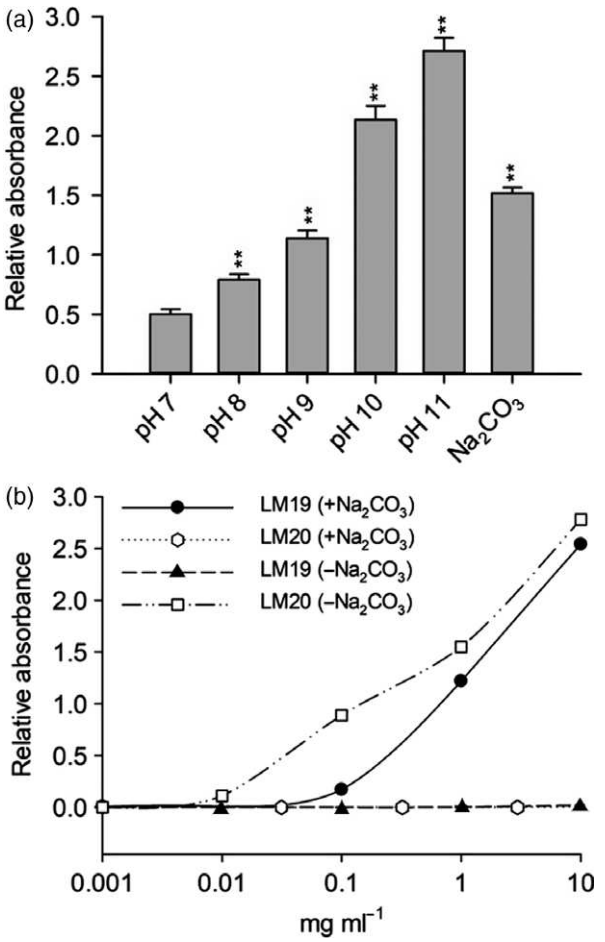


Figure 7. Quantification of CpGRP1–pectin interaction after pectin demethylesterification. (a) ELISA to quantify GRP1–pectin interaction after pre-treatment of coated homogalacturonan with CAPS-buffers (pH 7– 11) or with sodium carbonate. (b) Binding of LM19 and LM20 to different concentrations of apple pectin with and without sodium carbonate pre-treatment. Values shown are means of three biological replicates ± SD. The asterisk indicates the levels of significance in comparison with the untreated sample (one-way ANOVA, Holm–Sidak method): $^{**}P < 0.01$.

Keller *et al.*, 1988). In *C. plantagineum* the class II glycine-rich protein CpGRP1 accumulates in the apoplast of dehydrated leaf tissues (Giarola *et al.*, 2015). The glycine-rich protein 1 from the resurrection plant *Boea hygrometrica* (BhGRP1) was proposed to be correlated with cell wall flexibility (Wang *et al.*, 2009). The *A. thaliana* glycine-rich protein 3 (AtGRP-3), structurally similar to CpGRP1, interacts with a cell wall-associated protein kinase 1 (AtWAK1). This interaction is involved in plant-pathogen defence mechanisms and requires the C-terminal cysteine-rich domain (Park *et al.*, 2001). Giarola *et al.* (2016) showed that CpGRP1 binds to a WAK protein of *C. plantagineum*, CpWAK1, and proposed that the CpGRP1-CpWAK1 complex could be involved in dehydration-induced mechanisms. WAKs can bind to cell wall pectins (Kohorn and Kohorn, 2012) but a GRP–pectin interaction has not been characterised so far. Here we showed that the CpGRP1 protein binds to pectins which requires the glycine-rich domain of CpGRP1 (Figure 3). The substitution of two arginines within the glycine-rich domain by glycines was sufficient to reduce the protein–pectin interaction (Figures 3, 4 and 5). The involvement of an arginine cluster in pectin binding was also shown for *Phaseolus vulgaris* polygalacturonase inhibitor proteins (Spadoni *et al.*, 2006). The glycine-rich domain of the CpGRP1 protein is important for pectin binding. The repetitive character of this domain leads to multiple arginines with a distinct spacing. This spacing could be important for the spatial organisation of a positive amino acid cluster, which plays a crucial role in pectin binding. Similarly, the binding to PA seems to be mediated through the N-terminal domain of the protein and the arginine cluster. Multiple binding capacities of CpGRP1 suggests that this protein may coordinate different ligands involved in cell wall/membrane modifications during the folding process.

The role of Ca^{2+} in the protein–pectin binding is not clear. The gel-shift assay suggests Ca^{2+} to be important for the interaction but the ELISAs showed that Ca^{2+} was not essential for the interaction between CpGRP1 and pectin (Figures 3 and 4). One explanation could be that gel shift assays are less sensitive than ELISAs and therefore in gel

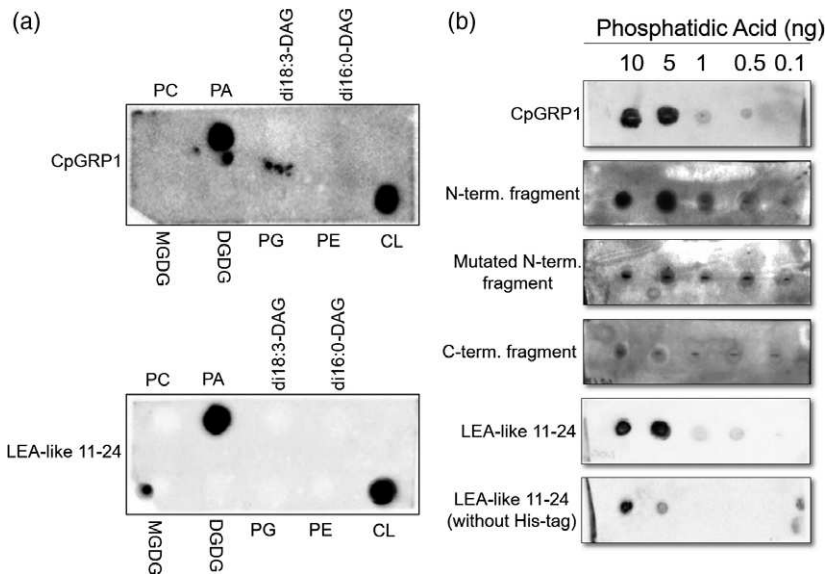


Figure 8. Protein–lipid overlay assays using the recombinant CpGRP1 full-length protein, the N. term. polypeptide, the mutated N-term. polypeptide and the C-term. polypeptide. Anti-His antibody was used to detect the different protein fragments. The LEA-like 11-24 protein (with and without His-tag) served as a positive control and was detected with an antibody specific to LEA-like 11-24. (a) Investigation of CpGRP1 binding properties to different lipids: phospatidylcholine (PC), phosphatidic acid (PA), diacylglycerol 18:3 (di18:3-DAG), diacylglycerol 16:0 (di16:0-DAG), monogalactosyldiacylglyceride (MGDG), digalactosyldiacylglyceride (DGDG), phosphatidylglycerin (PG), phosphatidylethanolamine (PE) and cardiolipin (CL). (b) Evaluation of differences in PA binding strength of protein fragments using filters containing 10, 5, 1, 0.5 and 0.1 ng of PA.

shift assays Ca^{2+} might be required to crosslink pectins and therefore increases the strength of the interaction. In *A. thaliana* a Ca^{2+}-induced modification of the pectin structure supports the interaction with AtWAK1 (Decreux and Messiaen, 2005). Ca^{2+} seems not to be necessary for CpGRP1–pectin binding *in vitro*, but a role of Ca^{2+} *in planta* cannot be excluded.

To compare the CpGRP1–pectin interaction with other apoplastic proteins, CpWAK1 and CpGLP1 were used. CpWAK1 and CpGLP1 were chosen, because they are exposed to pectin in a similar way as CpGRP1 due to their localisation. Both proteins are able to bind pectin (Figure 6) but the interaction of CpGRP1 with pectin is much stronger. To exclude the pectin binding of CpGRP1, CpWAK1 and CpGLP1 is just due to a high electrostatic charge, two other proteins with high lysine and arginine frequencies were used as additional controls (Figure 5). The recombinant LEA-like 11-24 protein (Petersen *et al.*, 2012) has a lysine frequency of 9.1% which is more than double compared with CpGRP1, which has a lysine frequency of 3% and an arginine frequency of 7.4%. BSA (UniProtKB accession number P02769) has a lysine frequency of 9.9% and an arginine frequency of 4.3%. Despite the high lysine and arginine frequencies LEA-like 11–24 and BSA did not show any interaction to pectin, proving a high electrostatic charge alone is not sufficient for a protein–pectin interaction and a particular spatial arrangement of the amino acid residues is required. The strong pectin binding of CpGRP1 seems to be specific, as other apoplastic proteins or proteins with a high electrostatic charge did not show any or a weak protein–pectin binding.

After identifying the protein domains important for pectin binding, we investigated the pectin fractions with regards to CpGRP1 binding. Both the CpGRP1 full-length protein and the N-term. fragment showed a slightly stronger binding to pectin samples isolated from desiccated *C. plantagineum* leaves than to pectins isolated from untreated leaves (Figure 4a). This could be due to the methylesterification level of HG which decreased upon dehydration. Previous reports demonstrated the importance of de-methylesterified HG stretches for protein binding (Spadoni *et al.*, 2006; Chevalier *et al.*, 2019). Pectin with a lower degree of methylesterification provides more binding sites for proteins. The small N-term. fragment showed always a higher signal in ELISA pectin binding assays than the full-length CpGRP1 protein (Figure 4a,b). Likely, more protein molecules can bind to the de-methylesterified pectin and this explains the stronger signal. To demonstrate a link between the degree of pectin methylesterification and protein binding, the *C. plantagineum* pectin was treated with solutions of different pH values to stepwise de-methylesterify the HG. Our results demonstrate that de-methylesterification of HG provides more binding sites for CpGRP1 (Figure 7).

Reversible methylesterification of HG in the *C. plantagineum* cell wall during dehydration might be

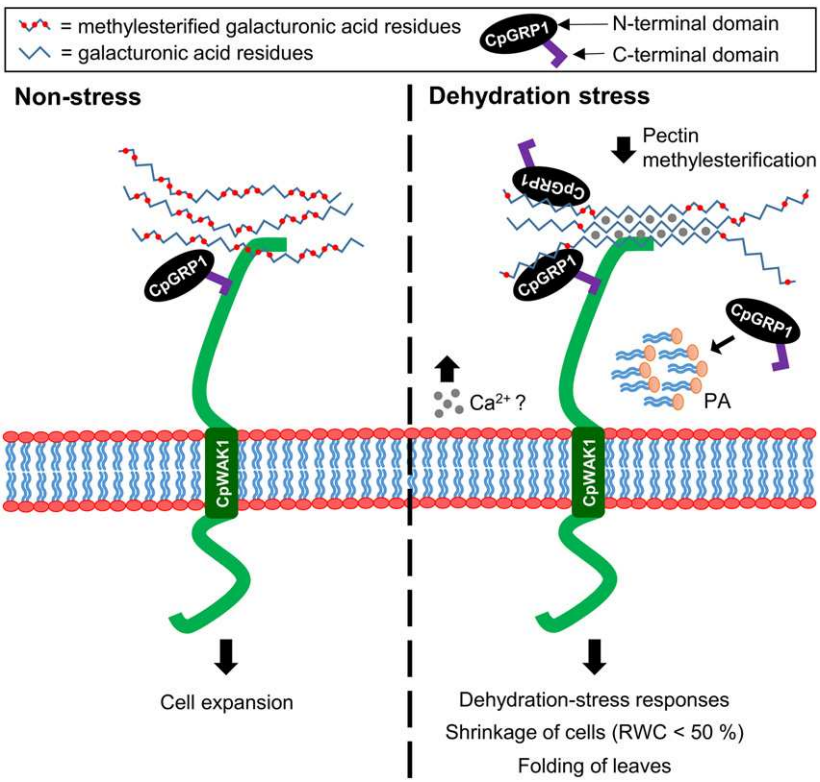


Figure 9. Model depicting the interaction between the *Craterostigma plantagineum* glycine-rich protein 1 (CpGRP1), the *C. plantagineum* cell wall associated protein kinase 1 (CpWAK1) and cell wall pectins and their role in regulating growth and dehydration-stress responses (modified from Giarola *et al.*, 2016). CpGRP1 is a part of the pectin matrix. Under non-stress conditions cell wall pectins (jagged blue lines) with a high degree of methylesterification (red dots) are bound to CpWAK1 and are involved in cell expansion. Upon dehydration, CpGRP1 is more abundant. Clustered arginines in the N-terminal domain of CpGRP1 are involved in binding PA and de-methylesterified stretches of pectin, which are more abundant upon dehydration. The binding of CpGRP1 to CpWAK1 is most likely mediated by cysteines in the C-terminal domain of CpGRP1. CpGRP1 might be either bound to PA or pectin with both competing for CpGRP1 binding. Ca^{2+} levels increase upon dehydration which may lead to the formation of egg-box structures resulting in an increase in rigidity of the cell walls.

responsible for the reduction of mechanical stress and might provide more binding sites for proteins. However, the protein-binding experiments using pectin from *L. brevidens* and *L. subracemosa* suggest that other factors in the structure of pectins also contribute to protein–pectin interactions. No significant changes were detected in the degree of methylesterification between *C. plantagineum*, *L. brevidens* and *L. subracemosa*, but the binding of the CpGRP1 protein to the pectin fractions from the three species was different (Figures 4 and 5). There seems to be a correlation between desiccation tolerance and the CpGRP1-binding capacity to pectin, as the signal is strongest for the *C. plantagineum* pectin, weaker for the *L. brevidens* pectin and even weaker for the pectin isolated from the non-tolerant *L. subracemosa*. The reasons for these differences are unknown.

Pectins and hemicelluloses of the *C. plantagineum* cell wall are modified, especially the HG and xyloglucan. These changes strengthen the cell wall. A higher proportion of de-methylesterified HG provides more binding sites for the CpGRP1 protein. CpGRP1 interacts through arginines with HG or PA and via cysteines in the C-terminal part of CpGRP1 with the extracellular domain of CpWAK1. These results are now integrated in a model how CpGRP1, CpWAK1, pectins and PA may interact and what are their possible roles in regulating cell shrinkage and expansion (Figure 9). We propose, that CpGRP1 is an essential factor in cell wall adaptations to desiccation.

EXPERIMENTAL PROCEDURES

Cultivation of plants

C. plantagineum Hochst., *L. brevidens* Skan, and *L. subracemosa* De Wild plants were grown as described in Bartels *et al.* (1990) and Phillips *et al.* (2008). Plants were subjected to desiccation and rehydration treatments and the hydration status of leaves was determined by calculating the relative water content (RWC) with the formula: RWC [%] = (Initial weight − dry weight)/(full turgor weight − dry weight) × 100 (Bernacchia *et al.*, 1996). For dehydration, fully-grown plants were dehydrated in pots until partial dehydration (PD, 50%) or desiccation (D, 2%) samples. Completely desiccated plants were rehydrated for either 24 or 48 h. Leaves from dehydrated and rehydrated plants were ground in liquid nitrogen with a mortar and a pestle and stored at −80°C.

Molecular biology techniques and DNA sequencing analysis

Molecular biology techniques were performed according to Green and Sambrook (2012). DNA sequencing was carried out by GATC Biotech (https://www.gatc-biotech.com/en/index.html) and primer synthesis by Eurofins MWG Operon (http://www.eurofinsgenomics.eu). All primers used are listed in Table S1.

Scanning electron microscopy

C. plantagineum leaves were frozen in liquid nitrogen and sputtered with palladium for 2 min using a sputter-coater (SCD 040; Balzer, http://www.oerlikon.com/balzers). Leaves were fixed on the sample holder and analysed under the electron microscope at ×100 and ×400 magnification (Cambridge Stereoscan S 200; Cambridge Instrument Company, UK).

Cell walls were stained as follows: leaf material was cut with a razor blade and immediately immersed in cold FAA (10% (v/v) formalin, 10% (v/v) acetic acid, 30% (v/v) water and 50% (v/v) ethanol) solution for at least 24 h at 4°C. After fixation samples were incubated for 30 min in 85% (v/v) ethanol, 50% (v/v) ethanol/50% (v/v) acetone and finally in 100% (v/v) acetone solutions. Samples were embedded using the Agar Low Viscosity Kit (Plano GmbH, Wetzlar, https://www.plano-em.de). Cell wall structures were stained with a 30% (w/v) silver nitrate solution for 10 min. After staining samples were incubated in 10% (v/v) HCl for 5 min before analysing the surface under the SEM (Blockface imaging).

Critical point drying (CPD 020; Balzers, http://www.oerlikon.com/balzers) was performed according to Svitkina *et al.* (1984) before analysing with the SEM.

Protein analyses

The sequence encoding the N-terminal fragment (aa 22–120) and the C-terminal fragment (aa 121–156) of CpGRP1 (GenBank accession number ALQ43973.1) was amplified with primers from the pET28 CpGRP1His plasmid to add a *Nco*I site and a *Xho*I site at the 5′ and 3′ ends, respectively (CpGRP1_NTERM_R/T7 promoter and CpGRP1_CTERM_F/T7 terminator, Table S1). The sequence encoding the extracellular domain of CpWAK1 (aa 31–315) was amplified with primers from a CpWAK1 cDNA clone (GenBank accession number KT893872.1; Giarola *et al.*, 2016) to add a *Xho*I site at the 3′ end (pJET1.2 fwd and CpWAK1_XhoI_R, Table S1). A *Nco*I site is already present in the CpWAK1 sequence. The sequence encoding the *C. plantagineum* germin-like protein 1 (CpGLP1, Dulitz, 2016) without the signal peptide (aa 27–226) was excised from a pAD vector using *Eco*RI and *Sal*I restriction enzymes. N- and C-terminal fragments of CpGRP1 as well as the CpWAK1 extracellular fragment were cloned between the *Nco*I and the *Xho*I sites of the pET28a expression vector (NOVAGEN, http://www.novagen.com) to create the corresponding protein-His-tag translation fusion constructs (pET28 CpGRP1_N-terminalHis, pET28 CpGRP1_C-terminalHis, and pET28 CpWAK1_extracellularHis, respectively). The CpGLP1 fragment was cloned between *Eco*RI and *Sal*I sites of the pET28a vector to generate the pET28 CpGLP1_His fusion construct. Overexpression constructs were transformed into BL21 (DE3) *Escherichia coli* cells (Amersham Pharmacia Biotech, https://www.gelifesciences.com) and protein overexpression was induced by adding 1 mM isopropyl-1-thio-β-D-galaytopyranoside (IPTG). The recombinant proteins were purified from bacteria 5 h after IPTG induction using affinity chromatography (Kirch and Röhrig, 2010). Purified protein fragments were concentrated using Amicon Ultracel-10K centrifugal concentrators (MILLIPORE, http://www.millipore.com) and desalted with PD10-columns (http://www.gelifesciences.com) before freeze-drying. Freeze-dried proteins were used for Blue-native page gel-shift assays, ELISA pectin binding and dot-blot assays.

The CpGRP1 sequence coding for two arginine residues (arginine [118] and [120]) in the CpGRP1 N-terminal fragment was mutated with primers (CpGRP1_NTERM_a352g_c358g_F and CpGRP1_NTERM_a352g_c358g_R. Table S1) using the QuikChange II site-directed mutagenesis kit according to the manufacturer's instructions (Agilent, https://www.agilent.com). Separation of proteins was performed as described in He (2011) based on the method first described by Laemmli (1970). The separation of the CpGRP1 C-terminal fragment was performed as described by Schägger and von Jagow (1987) using a peptide page. Proteins of SDS-PAGE gels were visualised with Coomassie brilliant blue R250 (0.1% (w/v) Coomassie R250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid, 40% (v/v) water). Immunoblot analyses were performed according to Towbin *et al.* (1979). CpGRP1 or 6x-His-Tag polyclonal antibodies (http://www.thermofisher.com) were used at a dilution of 1:5000. Detection of proteins was performed using the ECL Western Blotting detection kit (GE Healthcare, http://www.gehealthcare.com). Signals were visualised using the Azure Biosystems c300 chemiluminescent detection system (http://www.biozym.com).

Extraction of cell wall components

Cell wall components were obtained by following extractions as described by Cornuault *et al.* (2014). The 1,2-cyclohexanediaminetetraacetic acid (CDTA) fraction was obtained by vortexing 1 mg freeze-dried material in 1 ml 50 mM CDTA pH 7.5 for 1 h. The KOH fraction was obtained by vortexing the pellet from the CDTA extraction in 1 ml 4 M KOH, 1% (w/v) $NaBH_4$ solution for 1 h. Acetic acid (80%) was used to neutralise the pH of the KOH fraction after extraction. All samples were stored at −20°C until use.

Enzyme-linked immunosorbent assay

Isolated CDTA and KOH fractions were incubated in microtiter plates (NUNC-Immuno MicroWell 96 well solid plates, flat bottom, http://www.sigmaaldrich.com) overnight at 4°C. Plate wells were washed vigorously six times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4) and then blocked using 200 µl per well of 4% (w/v) non-fat milk powder in PBS for 2 h at room temperature. The plates were washed nine times with PBS and padded dry. Primary antibodies were added and incubated overnight at 4°C. The plates were washed 12 times with PBS and shaken dry. Then 150 µl secondary peroxidase-coupled anti-rabbit or anti-rat IgG antibodies (http://www.sigmaaldrich.com) were added at a 5000-fold dilution in 4% milk powder/PBS for 1 h at room temperature. After washing 12 times with PBS, microtitre plates were developed using 150 µl of substrate solution (0.1 M sodium acetate buffer, pH 6, 0.1% (w/v) tetramethyl benzidine, 0.006% (v/v) H_2O_2) in each well. The enzyme reaction was stopped by adding 40 µl of 2.5 M H_2SO_4 to each well, and the absorbance at 450 nm was determined for each well.

Analysis of the cell wall composition. The experiment was performed as described in Cornuault *et al.* (2014) with minor changes. Nine different rat monoclonal antibodies (mAB) were used in this study: JIM5, JIM7, LM20, LM19, LM25, LM15, LM11, LM5 and LM6 and one rabbit mAb: 42-6 (Table 1). CDTA and KOH 1:5 dilutions were used for ELISAs as they showed the most appropriate signal intensity (Figure S1). Ten-fold dilution of hybridoma cell culture supernatants in 4% (w/v) non-fat milk powder/PBS and a 1:10 000 dilution for 42-6 were used as primary antibodies (150 µl each well).

CpGRP1, CpWAK1, CpGLP1 pectin binding assay. After incubating CDTA and KOH fractions overnight, the recombinant proteins were dissolved in 4% (w/v) non-fat milk powder at a concentration of 1 µg $µl^{-1}$ and incubated in the plates overnight at 4°C. The protein was detected using a 6x-His-Tag polyclonal antibody (http://www.thermofisher.com) at a 1:5000 or 1:10 000 dilution for CpGRP1, 1:10 000 for CpWAK1 and 1:10 000 for CpGLP1. Pre-treatment of pectin was performed by incubating the plates with 50 mM CAPS-buffered solution at alkaline pH (adjusted between 7 and 11 with HCl and KOH) or with 0.1 M sodium carbonate.

Determination of GA

The GA content was determined according to Blumenkrantz and Asboe-Hansen (1973) and Verma *et al.* (2014). All CDTA and KOH fractions were analysed for their GA content by m-hydroxydiphenyl method, using GA as a standard (Figure S2).

Blue-native page gel-shift assays

For gel-shift assays 0.5 μg of CpGRP1 were incubated with 0.5 μg of citrus pectin (http://www.sigmaaldrich.com) and 2 mM $CaCl_2$ for 4 h at room temperature. The samples were mixed with 5× sample buffer (15.5 ml 1 M Tris–HCl, pH 6.8; 2.5 ml of 1% bromophenol blue solution; 7 ml of water; 25 ml of glycerol) and separated in 15% polyacrylamide gels without SDS and without stacking gel. A Tris/boric acid buffer (89 mM Tris, 89 mM boric acid; pH 9.25) was used for gel preparation and gel electrophoresis. Gels were either stained with Coomassie brilliant blue or incubated in 50 mM Tris/1% (w/v) SDS buffer prior to immunoblotting. Proteins were detected using a 6x-His-Tag polyclonal antibody (http://www.thermofisher.com) at a 1:5000 dilution.

Dot-blot pectin binding assays

Dot-blot assays were either based on proteins or pectins immobilised on a nitrocellulose membrane. Dots of 1.5 μl of either polygalacturonic acid (PGA) or of the CDTA-pectin fraction or of the CpGRP1 recombinant protein were spotted on a nitrocellulose membrane and allowed to dry. Membranes were blocked with 4% (w/v) non-fat milk powder in TBST for 2 h and were then washed with TBST three times for 5 min. The membranes were incubated with CpGRP1 recombinant protein (1 μg ml^{-1}; membranes with PGA and CDTA spots) or pectin (2 mg ml^{-1}; membranes with CpGRP1 spots) in TBST overnight at 4°C followed by three washing steps. The membranes were incubated with 6x-His-tag antibody (1:5000 dilution) or JIM5 antibody (1:10 dilution) overnight at 4°C and then washed again three times. Immunodetection was performed as described above and quantification of dot intensity was done using *ImageJ* (http://www.imagej.net).

Protein-lipid overlay assay

Protein-lipid overlay assays were performed according to Deak *et al.* 1999 and Ufer *et al.* 2017 to analyse lipid-binding properties of CpGRP1.

Electrostatic surface modelling

The *Phyre2* (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley *et al.*, 2015) web service was used to create a PDB-file from the CpGRP1 protein sequence, which was then used for electrostatic surface modelling using the website http://www.charmm-gui.org/?doc=input/pbeqsolver (Im *et al.*, 1998; Jo *et al.*, 2008a,b).

ACCESSION NUMBERS

The GenBank accession numbers used here are KT893871.1 (CpGRP1), KT893872.1 (CpWAK1) and JQ067608.1 (LEA-like 11–24). The UniProtKB accession numbers used here are A0A0S2ZYI4 (CpGRP1), A0A0S2ZYM7 (CpWAK1), P02769 (BSA) and O23764 (CpLEA-like 11-24).

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CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

NJ and PC planned and designed the research and conducted the experiments. NJ wrote the manuscript. DB, PK and VG designed the research, supervised the work and corrected the manuscript.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Standard-curve to calculate the galacturonic acid (GA) content in the different pectin fractions.

Figure S2. LM25 binding to different dilutions of the KOH fraction extracted from *C. plantagineum* to determine the most appropriate dilution for ELISA studies.

Table S1. List of primers used in this study.

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