The *Craterostigma plantagineum* protein kinase CpWAK1 interacts with pectin and integrates different environmental signals in the cell wall

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Abstract

Main conclusion  The cell wall protein CpWAK1 interacts with pectin, participates in decoding cell wall signals, and induces different downstream responses.

Abstract  Cell wall-associated protein kinases (WAKs) are transmembrane receptor kinases. In the desiccation-tolerant resurrection plant *Craterostigma plantagineum*, CpWAK1 has been shown to be involved in stress responses and cell expansion by forming a complex with the *C. plantagineum* glycine-rich protein1 (CpGRP1). This prompted us to extend the studies of WAK genes in *C. plantagineum*. The phylogenetic analyses of WAKs from *C. plantagineum* and from other species suggest that these genes have been duplicated after species divergence. Expression profiles indicate that CpWAKs are involved in various biological processes, including dehydration-induced responses and SA- and JA-related reactions to pathogens and wounding. CpWAK1 shows a high affinity for “egg-box” pectin structures. ELISA assays revealed that the binding of CpWAKs to pectins is modulated by CpGRP1 and it depends on the apoplastic pH. The formation of CpWAK multimers is the prerequisite for the CpWAK–pectin binding. Different pectin extracts lead to opposite trends of CpWAK–pectin binding in the presence of Ca²⁺ at pH 8. These observations demonstrate that CpWAKs can potentially discriminate and integrate cell wall signals generated by diverse stimuli, in concert with other elements, such as CpGRP1, pHapo, Ca²⁺[apo], and via the formation of CpWAK multimers.

Keywords  Abiotic stress · Biotic stimuli · Cell wall proteins · Desiccation tolerance · Resurrection plants

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Abbreviations

At  *Arabidopsis thaliana*

Cp  *Craterostigma plantagineum*

CDTA  1, 2-Cyclohexanediaminetetraacetic acid

EGF  Epidermal growth factor

GRP  Glycine-rich protein

Lbr  *Lindernia brevidens*

Lsu  *Lindernia subracemosa*

MeJA  Methyl jasmonic acid

OG  Oligo-galacturonides

SA  Salicylic acid

WAK  Wall-associated protein kinase

WAKL  WAK-like kinase

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Introduction

The plant cell wall is a highly organized macromolecular gel-like structure mainly constituted of water, polysaccharides and proteins (Vorwerk et al. 2004). Proteins in cell walls have not only structural and physiological functions but are also essential in signal transduction mediating the transmission of external stimuli to internal response systems (Cosgrove 1997; Caffall and Mohnen 2009; Chen et al. 2020).

Cell wall-associated protein kinases (WAKs), as a class of cell wall proteins, are a subset of the WAK-like (WAKL) superfamily. In Arabidopsis thaliana, five WAK and twenty-two WAK-like (WAKL) genes have been identified (He et al. 1999; Verica and He 2002). Analysis of the A. thaliana genome sequence revealed that the expansion of the ArWAK/WAKL gene family occurred via tandem duplications, segmental duplications and retrotransposon activity (Verica and He 2002). WAK/WAKL genes have been described in other plant species (Zhang et al. 2005; Liu et al. 2006; Kaur et al. 2013; Rosli et al. 2013; Hurni et al. 2015; Giarola et al. 2016; Zuo et al. 2019). In rice and apple, the number of WAKL genes was expanded up to 125 (Zhang et al. 2005) and 44 (Zuo et al. 2019), respectively. The expansion of the rice WAKL genes probably resulted from localized gene duplications (Shiu et al. 2004; Zhang et al. 2005).

The structure of WAK proteins is characterized by a conserved cytoplasmic Ser/Thr kinase domain and by a variable extracellular domain containing EGF (epidermal growth factor) repeats (Kohorn and Kohorn 2012; Kohorn 2015). Calcium is predicted to mediate the dimerization of WAKs involving asparagine residues in the EGF domains (Anderson et al. 2001; Verica et al. 2003). Conserved cysteine residues are involved in the formation of disulfide-bridged complexes (Anderson et al. 2001; Verica and He 2002). Amino acid identities between the extracellular domain of WAKs and extracellular matrix proteins like collagens, tenascins and neurexins suggest that WAK proteins may function in a carbohydrate-rich environment interacting with other proteins or forming oligomers (He et al. 1999). It was demonstrated that the extracellular domains of WAKs bind to pectins via both covalent and ionic bonds (Wagner and Kohorn 2001; Decreux and Messiaen 2005; Decreux et al. 2006; June et al. 2019). The extracellular domain of the A. thaliana WAK1 has a high affinity for oligo-galacturonides (OGs) in the “egg-box” conformation, which is formed with Ca\(^ {2+} \) as bridge linking acidic polysaccharides (Grant et al. 1973; Decreux and Messiaen 2005; Cabrera et al. 2008). The galacturonic acid appears to be the key element for WAK-pectin interaction, regardless of the chemical modification of the reducing ends of the OGs (Decreux and Messiaen 2005; Cabrera et al. 2008; Kohorn et al. 2009; Kohorn and Kohorn 2012). AtWAK2 is required for OG-mediated responses of some pectin-regulated genes (Kohorn et al. 2009). In protoplasts, the pectin-activated transcription of vacuolar invertase was triggered by WAK2 and the pectin-induced regulation of mitogen-activated protein kinases was also affected in the wak2 mutant (Kohorn et al. 2009). The proof that WAKs are the receptors for OGs comes from in vivo domain swap experiments (Brutus et al. 2010; Kohorn and Kohorn 2012). These results demonstrate the interaction between WAKs and pectins/OGs and that OGs are key elements in mediating the activation of downstream signaling pathways upon WAK–OGs interaction. Besides pectins, cell wall glycine-rich proteins (GRPs) were identified as an interaction partner of WAKs (Park et al. 2001; Kohorn and Kohorn 2012; Giarola et al. 2016). Giamegna et al. (2016) discovered that OGs, flg22 and wound treatments prolonged the expression of defense genes, increased H\(_2\)O\(_2\) accumulation, and enhanced callose deposition in both the over-expressing AtWAK1 and grp-3 loss-of-function mutants. The grp-3 mutants showed wild-type responses to OGs/flg22/wound treatments when complemented with GRP3 over-expressing plants (Giamegna et al. 2016). Taken together, these observations indicate a positive function (activation) of AtWAK1 and a negative function (repression) of AtGRP3 in the OG/flg22/wound-triggered defense responses (Giamegna et al. 2016). It is not known how OGs and AtGRP3 interact with WAK to initiate the defense responses.

WAKs/WAKLs expression suggests that these genes are implicated in different aspects of the plant life cycle. In A. thaliana, AtWAKs are mainly expressed in the vegetative organs, except for AtWAK4 which is primarily detected in siliques (He et al. 1998; 1999). Of the 22 ArWAKs, ArWAKL1, ArWAKL3 and ArWAKL5 are expressed mainly in roots and flowers but not in vegetative organs (Verica et al. 2003). WAKs from other species also show tissue-specific and developmentally regulated expression patterns (Shiu et al. 2004; Kohorn et al. 2009; Kohorn 2015). WAKLs is also affected by a range of environmental stimuli. ArWAK1 is induced by pathogens, exogenous salicylic acid (SA) or its analog 2, 6-dichloroisonicotinic acid (INA) in a NPR (Nonexpressor of pathogenesis-related genes)-dependent manner (He et al. 1998). The SA-inducible ArWAKs/ArWAKLs are additionally responsive to wounding (Wagner and Kohorn 2001; Verica et al. 2003). Increasing numbers of WAKs have been identified as SA-induced or pathogen-related genes in various species (Liu et al. 2006; Li et al. 2009; Meier et al. 2010; Hu et al. 2014; Hurni et al. 2015).
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expansion to stress response, but how WAKs distinguish the 
signals and activate signaling pathways remain unknown.
Here, it was shown that CpWAK interacts with pectin and that this interaction modulated by different factors such as pH, Ca²⁺ or other cell wall proteins. The different CpWAK complexes may be required for distinguishing signal trans-
ductions in response to different cell wall signals.

Materials and methods

Plant material and treatments

Craterostigma plantagineum Hochst. (Scrophulariaceae) was grown according to Bartels et al. (1990). Plant relative water content was calculated according to Bernacchia et al. (1996) and used to monitor the dehydration status during dehydration experiments. Mature C. plantagineum plants were subjected to dehydration by withholding water until the RWC of plants was around 2% (desiccated). Desiccated plants were rehydrated for two days (RWC=80%). Leaves were collected at different time points during the dehydration and rehydration treatments. For other treatments, detached C. plantagineum leaves were incubated in water, 1 mM salicylic acid (SA) or 100 µM methyl jasmonic acid (MeJA) for 1, 3, 6, 24 and 48 h. After treatments, samples were frozen in liquid nitrogen and stored at −80 °C for further analyses.

Extraction of genomic DNA

Plant tissues (50–200 mg) were ground to a fine powder in liquid nitrogen. Then, the powder was homogenized in 300 µl 2× lysis buffer (0.6 M NaCl; 0.1 M Tris–HCl, pH 8.0; 40 mM EDTA, pH 8.0; 4% (w/v) sarcosyl; 1% (w/v) SDS), 300 µl 2 M urea and 600 µl of phenol/chloroform/ isoamyl alcohol (25/24/1). The mixture was centrifuged for 10 min (14,000g, RT) and the aqueous supernatant was collected. DNA was precipitated by mixing the supernatant with 0.7 volume of isopropanol and subsequent centrifugation for 15–20 min (14000g, 4 °C). After washing the pellet twice with 70% (v/v) ethanol, the air-dried DNA pellet was dissolved in TE buffer (10 mM Tris–HCl; 1 mM EDTA; pH 8.0) containing 20 µg/ml RNase A. RNAs in the DNA samples were removed after 5 min of incubation at 37 °C.

Transcript analysis

Total plant RNA was extracted according to Valenzuela-Avendaño et al. (2005). First-strand cDNA synthesis was performed as described by Hou and Bartels (2015). The first-strand cDNA was used directly for PCR or stored at −20 °C until use. All primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany) and are listed in Table S1. The PCR was performed as follows: 95 °C 3–5 min for initial denaturation; 95 °C 30 s for cycling denaturation; 50–65 °C 45 s for primer annealing; 72 °C for extension using Taq polymerase (1 min/kb); recycle from step 2 for 21–35 times; 72 °C 5–10 min for final extension; 4 °C for holding the samples until they were collected; The annealing temperature and cycle numbers were determined empirically for each PCR.

Gene cloning, protein expression and purification

The CpWAK genomic sequences were obtained by PCR from genomic DNA isolated from C. plantagineum leaves. The primers used are shown in Table S1. The PCR products were cloned into pJET 1.2 vectors using the CloneJET PCR Cloning Kit (Thermo Fisher Scientific, St LeonRot, Germany) and the PCR fragments were sequenced.

The cDNA fragments encoding CpWAK1EX (amino acids 31–315), CpWAK2EX (amino acids 37–333), R-1 (amino acids 31–160), R-2 (amino acids 161–315) and R-3 (amino acids 31–220) (Fig. 3a) were amplified from a CpWAK1 cDNA clone (Giarola et al. 2016) using 1 3
primers to add an XhoI site at the 3’ end (CpWAK1_XhoI_R, CpWAK2_XhoI_R, R-1, R-2-rev, R-3, Table S1). An NcoI site is already present in the sequences of CpWAK1, CpWk2, R-1 and R-3. An NcoI site was added at the 5’ end of the R-2 sequence with the primer, R-2-for (Table S1). The NcoI/XhoI fragments were cloned into the expression vector pET28a(+) (Novagen, Darmstadt, Germany) and transformed into BL21 (DE3) E. coli cells (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The fusion constructs, pET28_CpGRP1His and pET28 CpGRP1_N-terminal His, were provided by Giarola et al. (2016) and Jung et al. (2019), respectively. Over-expression and purification of the fusion proteins were performed as described by Jung et al. (2019).

**Cell wall protein extraction and protein analysis**

Cell wall protein extraction was carried out as described by Printz et al. (2015) in protocol 3 with minor modifications. After concentration using Amicon® Ultra centrifugal filter, the CaCl$_2$ and LiCl fractions of cell wall proteins were precipitated with 4 volumes of cold acetone at −20 °C for at least 30 min or overnight. Cell wall proteins were collected by centrifugation at 15000g at 4 °C for 15 min and then the pellet was dried under a hood to eliminate acetone residues. Cell wall protein pellets were dissolved in a minimal volume of 1 × Laemmli buffer (Laemmli 1970). The protein concentration was determined according to Bradford (1976). Equal amounts of cell wall proteins from different samples were separated by 12% (w/v) SDS–polyacrylamide gel electrophoresis (PAGE) (Laemmli 1970). The separated proteins were visualized by Coomassie blue G-250 (Zehr et al. 1989) or silver staining (Mortz et al. 2001). Western blot analysis was performed according to Towbin et al. (1979). The polyclonal antibodies were diluted 1:5000 for the detection of CpWAKs and CpGRP1 (Giarola et al. 2016).

**Pectin extraction and estimation**

Pectin was extracted using CDTA (1, 2-cyclohexanediaminetetraacetic acid) according to Cornuault et al. (2014). The galacturonic acid content of the CDTA fraction was determined as described in Verma et al. (2014) with a galacturonic acid standard curve obtained with commercial polygalacturonic acid (Sigma 81325) (Fig. S1).

**Enzyme-linked immune sorbent assay (ELISA)**

The ELISA binding assay was performed according to Decreux and Messiaen (2005) with modifications. Nunc Maxisorp flat-bottom plates (Invitrogen, CA, USA) were coated with pectin solution (25 μg well$^{-1}$) at 4 °C overnight. Non-specific binding sites were blocked for 2 h at RT with 100 μL of blocking solution (3% (w/v) low fat dried milk, 20 mM Tris–HCl, 150 mM NaCl). The wells were incubated for 2 h at RT with 50 μL of purified His-tagged recombinant protein in binding buffer (1% (w/v) low fat dried milk, 20 mM Tris–HCl, 150 mM NaCl, with or without 2 mM CaCl$_2$) after removing the blocking solution. The wells were washed four times with wash buffer (20 mM Tris–HCl, 150 mM NaCl) and incubated with 50 μL of anti-His-tag antibody (1:10,000) (Invitrogen) or anti-WAK antibody (1:2500) or anti-GRP antibody (1:5000) (Giarola et al. 2016; Jung et al. 2019) in incubation buffer (1% (w/v) low-fat dried milk, 20 mM Tris–HCl, 150 mM NaCl) for 1 h at RT. After washing the wells four times, 50 μL of goat anti-rabbit IgG peroxidase antiserum (1:10,000) (Sigma, A9169) prepared in incubation buffer was added and incubated for 1 h at RT. After washing the plates six times, the bound recombinant protein was visualized in the presence of the substrate TMB (3,3′,5,5′-tetramethylbenzidine) (Sigma, T2885). The absorbance was measured at 450 nm after sufficient colour development in the dark and the reaction was stopped by adding 50 μL of 10% (v/v) phosphoric acid. The pH of all the solutions and buffers used are 4, 5, 6, 7 or 8. In the competitive ELISA binding assay, the recombinant proteins were pre-mixed with each other for 1 h at RT. The mixture was loaded on the pectin-coated and blocked wells and incubated for 2 h at RT. After washing the wells, the plate was incubated with anti-WAK or anti-GRP antiserum. The immobilized recombinant protein was detected after being incubated with goat anti-rabbit IgG peroxidase antibody and visualized with TMB as described above.

**DNA sequence, phylogenetic and gene structure analyses**

DNA sequencing was performed by GATC Biotech (https://www.gatc-biotech.com/en/index.html). Protein sequence alignments were done using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). Protein domains were identified using NCBI CD-Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) (Marchler-Bauer et al. 2011), SMART tool (http://smart.embl-heidelberg.de/)(Letunic et al. 2017) and TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

The WAK homologs (Table 1) for the phylogenetic analysis were retrieved by BLASTP from the NCBI database or by TBLASTN from L. brevidens and L. subracemosa transcriptomic databanks (VanBuren et al. 2018) with the CpWAK1 protein sequence as query (E < 10$^{-10}$). The top two hits of selected species were used for further analysis. The phylogenetic tree was constructed using the neighbor-joining method with 1000 bootstrap replications in MEGA 5.1 (Tamura et al. 2011). The genomic sequences for the gene structure analysis were obtained from NCBI database or identified using BLAST in L. brevidens and L. subracemosa.
The gene structures of all selected WAK genes are displayed using Gene Structure Display Server 2.0 (http://gsds.cbi.pku.edu.cn/) (Hu et al. 2015).

The nucleotide sequences of the *C. plantagineum* genes described in this study are deposited in the NCBI GenBank database under the following accession numbers: KT893872 (CpWAK1), KT893873 (CpWAK2), MW580911 (CpWAK3), and KT893871 (CpGRP1).

### Statistical analysis

All the experiments were conducted using three biological replicates. The mean and the standard error of mean (SEM) values shown in the ELISA binding assays were calculated from three biological replicates including three technical replicates each \((n = 9)\). Statistical significance was determined by t-test in Fig. 3b, e and Fig. 4a–d and by one-way ANOVA with Bonferroni’s post-test in Figs. 3d and Fig. 4e. All statistical analyses were performed with Excel and Graphpad prism 5.0 (San Diego, CA; https://www.graphpad.com/).

### Results

#### Phylogenetic analysis of CpWAK genes

This study is focused on three very closely related *CpWAK* genes isolated from the desiccation-tolerant plant *C. plantagineum*: *CpWAK1*, 2 and 3. The deduced amino acid sequences of the three *CpWAK* genes show high sequence conservation of the three predicted proteins (Fig. S2). All three *CpWAK* proteins display the features of wall-associated kinases: the extracellular galacturonide-binding domain, EGF repeats, a transmembrane domain and a cytoplasmic protein kinase domain (Fig. S2).

To investigate the evolutionary relationships of the *CpWAK* genes with other species, a phylogenetic analysis was carried out of WAK genes from *C. plantagineum*, the moss *Physcomitrella patens* and selected vascular plants including two Linderniaceae species closely related to *C. plantagineum* (Fig. 1). The WAK homologs used for the phylogenetic analysis are reported in Table 1. As shown in Fig. 1 homologs are divided into four clusters which reflect the genus classification (group I: moss, group II: monocots, group III: dicots, group IV: Linderniaceae family). Only the WAK genes in group IV have no introns or fewer introns than the other genes (Fig. 1). The protein structures of all WAK homologs are highly similar, especially in vascular plants. All WAK proteins from vascular plants contain the conserved kinase domain, the EGF-like domain and the extracellular galacturonan-binding domain, while a MATE-like domain (cd13132) is present in RXH_67760.1 (*Malus domestica* (apple)) (Fig. 1). The MATE-like domain is related to iron homeostasis under osmotic stress. No transmembrane domain is predicted in NP_00132332.1 (*Arabidopsis thaliana*). The predicted WAK proteins of *P. patens* show similarity only within the conserved kinase domain and contain a cupredoxin motif (cl19115) located in the extracellular domain.

#### Expression analyses of CpWAKs

It was previously shown that *CpWAK1* and 2 genes are mainly expressed in well-watered leaves of *C. plantagineum*.

**Table 1 Selected homologs of CpWAK1**

| Accession number | Organism          | Query coverage | Percent identity | E value |
|------------------|-------------------|----------------|------------------|---------|
| Lbr_010788       | Lindernia brevidens | 99%            | 69.73%           | 0.0     |
| Lbr_015766       | Lindernia brevidens | 88%            | 53.20%           | 0.0     |
| Lsu_027987       | Lindernia subracemosa | 97%          | 69.60%           | 0.0     |
| Lsu_004124       | Lindernia subracemosa | 96%            | 61.30%           | 0.0     |
| RXH67760.1       | Malus domestica (apple) | 94%         | 44.85%           | 0.0     |
| XP_008380329.1   | Malus domestica (apple) | 95%            | 44.44%           | 0.0     |
| NP_00132332.1    | Arabidopsis thaliana | 95%            | 43.20%           | 6e-174  |
| NP_001185009.1   | Arabidopsis thaliana | 95%            | 43.06%           | 3e-175  |
| EA221467.1       | Oryza sativa (rice) | 91%            | 38.14%           | 1e-134  |
| XP_015627146.1   | Oryza sativa (rice) | 94%            | 37.73%           | 2e-135  |
| XP_024376490.1   | Physcomitrella patens | 51%            | 43.34%           | 2e-84   |
| XP_024376488.1   | Physcomitrella patens | 51%            | 43.34%           | 3e-84   |

Putative CpWAK homologs identified by BLASTP from the non-redundant protein sequences NCBI database (nr) or by TBLASTN from *L. brevidens* and *L. subracemosa* transcriptomic databanks (VanBuren et al. 2018). The first two hits of selected species were used for further phylogenetic analysis.
and repressed by dehydration (Giarola et al. 2016). This suggests that expression of these two genes is associated with the rehydration process. To extend our understanding of the processes involving *CpWAK* genes we analyzed the expression of the three *CpWAK* genes using gene specific probes and a polyclonal antiserum. A polyclonal antiserum was raised against a recombinant fragment of *CpWAK1*. However, the antiserum detects probably also *CpWAK2* and *CpWAK3* proteins as well as other *CpWAKs* with conserved extracellular domains due to the high conservation of the amino acid sequences. The protein expression during dehydration/rehydration of *CpWAKs* is illustrated in Fig. 2a.

The two protein bands on the blot may represent not only the *CpWAK1* protein but other *CpWAK* proteins, or some posttranslationally modified WAKs.

WAK proteins are associated with cell wall compartments (Giarola et al. 2016) and were not detected in total protein extracts. Therefore, cell wall protein fractions were prepared according to the protocol 3 of Printz et al. (2015) and two different fractions (CaCl$_2$ and LiCl fractions) were used for protein detection in immunoblots (Fig. 2a). In both fractions the *CpWAK* proteins are present in extracts of well-watered and rehydrated leaves and decreased during dehydration (Fig. 2a). Thus, the protein accumulation profile is similar...
to what was previously observed for transcripts (Giarola et al. 2016) suggesting that WAK gene expression is mainly regulated on the transcriptional level during dehydration/rehydration.

WAK proteins interact with GRP proteins in *C. plantagineum* and *A. thaliana* (Park et al. 2001; Giarola et al. 2016). In *Arabidopsis*, both WAK and GRP are up-regulated upon SA or the SA analog 2, 6-dichloroisonicotinic acid (INA) treatment (He et al. 1999; Park et al. 2001; Verica et al. 2003), which suggests that the two proteins are involved in defense-response pathways. Thus, the transcript and protein expression of the CpWAK genes and CpGRP1 were analyzed in *C. plantagineum* upon SA and MeJA treatments (Fig. 2b, c). *CpWAK1, 2 and 3* show a faster response than *CpGRP1* and they accumulate after 1 h of SA treatment, while *CpGRP1* accumulated after 3 h of SA and MeJA treatments (Fig. 2b). *CpWAK1* and *CpWAK3* decrease in response to MeJA, while *CpWAK2* increases (Fig. 2b). The accumulation of all transcripts is transient and the levels decrease quickly after reaching the peak. No significant difference was observed in the expression of CpWAKs and CpGRP1 proteins after 1 h of SA and MeJA treatments, but CpWAKs and CpGRP1 proteins accumulate after 24 h of treatments (Fig. 2c). The expression of CpWAKs and CpGRP1 proteins is still detectable in the water-treated samples even up to 24 h, which is in line with the transcript expression pattern (Fig. 2b, c). The transcript and protein expression patterns point to a different regulation in response to SA or MeJA than in response to dehydration. The expression analysis suggests that CpWAK and CpGRP1 proteins are involved in defense-response pathways possibly triggered in the cell wall.
CpWAKs form aggregates and have a high affinity to the pectins in “egg-box” conformations

To better understand how WAK proteins can mediate signalling we overexpressed different CpWAK protein fragments in bacteria and used these fragments for binding studies (Fig. 3a). Here, CpWAK1EX and CpWAK2EX are used to represent the extracellular domains of CpWAK proteins without signal peptides; whereas, R-1, R-2 and R-3 are truncated fragments of the CpWAK1 protein, containing the amino acids 31–160, 161–315 and 31–220, respectively (Fig. 3a, Fig. S3). The putative molecular weight and pI of the His-tagged recombinant proteins are reported in Table 2. Western-blot analyses of the CpWAK recombinant proteins confirmed that all proteins can be immunologically detected with His-tag antibody (1:10,000). All the mean absorbance values were calculated from three biological of which each included three technical repetitions. Error bars indicate SEM and Mock indicates only buffer without pectin (ns means no significant, ***P < 0.0001, **P < 0.01, t test compared to Mock in b and S-/Ca2+- in c, respectively). The letters in d show the significance determined by one-way ANOVA with Bonferroni’s post-test (a–d P < 0.01)
SDS-PAGE gels in the samples containing CpWAK1 extracellular protein fragments supplemented with 2 mM Ca$^{2+}$ (Fig. S4).

The extracellular domain of the AtWAK1 binds to pectins (Decreux and Messiaen 2005); therefore, the five CpWAK protein fragments were tested for interaction with either commercial pectin (poly-D-galacturonic acid methyl ester and galacturonic acid content ≥ 74%, Sigma Aldrich, P9135) from citrus peel or pectin isolated from *C. plantagineum*. CpWAK1EX–pectin binding was observed with the pectin extracted from *C. plantagineum* leaves, but not with commercial pectins or polygalacturonic acid (PGA, Sigma.

| Recombinant proteins | Amino acids covered | Molecular weight (kDa) | Isoelectric point (pI) |
|----------------------|---------------------|------------------------|-----------------------|
| CpWAK1EX             | 31–315              | 32.2                   | 4.71                  |
| CpWAK2EX             | 37–333              | 34.1                   | 5.31                  |
| R-1                  | 31–160              | 14.9                   | 7.41                  |
| R-2                  | 161–315             | 18.6                   | 4.31                  |
| R-3                  | 31–220              | 21.8                   | 6.22                  |
| CpGRP1               | 22–156              | 14.18                  | 8.35                  |

**Table 2** Basic characteristics of the His-tag recombinant proteins

Fig. 4 CpGRP1–CpWAK1–pectin interaction and the factors involved in CpWAK1–pectin binding. a The interaction of the pectin extracts of *C. plantagineum* leaves with CpGRP1 is much stronger than with CpWAKs. b Heatmaps showing the contribution of CpGRP1 to the binding activity of CpWAK1EX to pectins. c CpWAK1EX–CpGRP1 complex binding affinity for pectin extracts is affected by pH values. Each heatmap depicts the ELISA absorbance values with anti-CpWAK1 (orange, 1:2500) and anti-CpGRP1 (purple, 1:5000) antibody, respectively. Values shown in the heatmaps are means of three biological replicates which included three technical replicates ± SD. The lines above the heatmap in b are the combinations for t test. Different protein combinations or different pH values are visualized longitudinally, and the ratios of the amounts of CpWAKs (W) and CpGRPs (G) are shown horizontally. The proteins were pre-mixed for 1 h before incubating with the pectin-immobilized ELISA plates. d The effect of Ca$^{2+}$ on the CpWAK1EX–pectins binding is related to pH values and the source of pectin extracts. Pectins were extracted from untreated *C. plantagineum* leaves (Fresh) and *C. plantagineum* leaves incubated in water (H2O). The treated leaves were soaked for 48 h. Asterisks in the heatmaps and above bars represent statistically significant differences compared to control samples (Mock in a, W:G = 1:0 or W:G = 1:1 in b, pH 5 in c, Ca$^{2+}$ – in d) (t-test, ns means no significant, *P < 0.05, **P < 0.01, ***P < 0.0001). e CpWAK1EX showed different binding capacity to the pectin extracts prepared from detached *C. plantagineum* leaves exposed to water, SA or MeJA for 48 h. Statistical analysis was performed using one-way ANOVA with Bonferroni’s post-test (a, b, P < 0.01); n = 9. Error bars indicate SEM.
81325) (Fig. 3b). Binding of CpWAK1EX to commercial pectin was achieved after saponification with and without 
Ca\(^{2+}\) (Fig. 3c). Saponification and Ca\(^{2+}\) facilitate the formation of “egg-box” structures of pectins (Grant et al. 1973; 
Cabrera et al. 2008). CpWAK1EX showed a higher preference to the pectins in “egg-box” conformation (supple-
mented with Ca\(^{2+}\)) than those only saponificated but without Ca\(^{2+}\) (Fig. 3b). Like CpWAK1EX, CpWAK2EX also showed
affinity for pectins from C. plantagineum and Ca\(^{2+}\) facilitates the binding of the two extracellular domain of the CpWAKs
to pectin extracts (Fig. 4a, S5a). Next, we tested the affinity of R-1, R-2, and R-3 fragments to pectins. R-1 includes
the galacturonan-binding domain, R-2 contains the EGF-like domains and R-3 corresponds to R-1 but includes alkaline
amino acids more towards the 3’ end without the EGF-like domains (Fig. 3a). Among the three fragments, R-1 showed
the strongest binding capacity to C. plantagineum pectin extracts, closely followed by R-3. R-2, containing no galac-
turonic binding domain, had less affinity than other fragments (Fig. 3d).

The CpWAK1–pectin–Cgrp1 complex

CpWAK1 does not only interact with pectins but also with the glycine-rich cell wall protein Cgrp1 (Giarola et al. 2016). 
Cgrp1 has a stronger affinity to pectins than CpWAKs (Jung et al. 2019) (Fig. 4a). Therefore, the interaction of 
CpWAK1 and pectin was further tested including the Cgrp1 protein using competitive ELISA assays. Equal
molar amounts of CpWAK1EX and Cgrp1 proteins were premixed before incubating them with pectin. The heatmaps
in Fig. 4b show that full-size Cgrp1 contributes to significantly more binding of CpWAK1EX and R-2 to pectin
extracts. However, the presence of CpWAK1 decreases the number of Cgrp1 molecules binding to pectin extracts,
especially in experiments with the R-2 fragment, which might contain the site for the interaction with Cgrp1.
The N-terminal fragment of Cgrp1 without the pectin-binding domain (Jung et al. 2019) significantly decreases
CpWAK1EX–pectin binding when the ratio of WAK/GRP is 1:2 (Fig. 4b).

The apoplastic pH is normally around five and oscil-
lates between 4 and 7, which is observed in specific develop-
mental stage or under stress conditions (Geilfus 2017). 
Thus, we investigated the effect of the apoplastic pH on
the CpWAK1/Cgrp1/pectin interaction varying the pH of
ELISA binding assays (Fig. 4c). The binding between
CpWAK1EX and pectins was not significantly affected over
the pH range of 4–7, but with increasing pH, a decrease
was observed. However, Cgrp1 showed stronger binding
capacity at pH 4, 6 and 7. When Cgrp1 and CpWAK1EX
proteins were mixed and the pectin binding was tested at
different pH values, the same trend was seen in all assays
as for Cgrp1 alone despite the insignificant difference in
pH 7. Ca\(^{2+}\) affects CpWAK1EX–pectin binding depending
on the pH and the source of the pectin, only when the pH
value increased to 8.0 (Fig. 4d). The binding was strengthen-
e when the pectin was extracted from untreated leaves, 
while weakened when pectin was prepared from water-
soaked leaves. The SA and MeJA treatments also affected
the CpWAK1EX–pectin binding, with less CpWAK1EX
being immobilized by pectin extracts from detached leaves
subjected to SA and MeJA treatments (Fig. 4e). However,
CpWAK1EX did not show significantly different affinity for
the pectin extracted from untreated, partially dried, desic-
cated and rehydrated C. plantagineum leaves (Fig. S5b).

Discussion

Evolution of CpWAKs

The genome-wide analyses of WAKs in rice, Arabidopsis
and apple indicated that tandem duplication and segment-
ral duplications contribute to the expansion of WAK gene
families (Shiu et al. 2004; Zhang et al. 2005; Zuo et al.
2019). The high sequence similarity among the CpWAKs
(Fig. S2) suggests also duplications for the CpWAK genes
in C. plantagineum. The phylogenetic analysis shows that
the WAK homologs from different species are clustered in
distinct species-specific groups (Fig. 1), which is consist-
ent with that in rice, Arabidopsis and apple (Zhang et al.
2005; Zuo et al. 2019). This suggests that gene expansion/
gen duplicate takes place after species divergence. It is
remarkable that a non-intron gene structure is only seen in
the Linderniaceae family (group IV) among the selected
WAK gene homologs (Fig. 1). The amino acid sequences
of the selected WAKs are conserved within the cytoplasmic
kinase domain, the EGF-like domain and the galacturonan-
binding domains, but they are variable within the extracel-
ular domains. This variability in the extracellular domain
may be connected with a differential response of the WAK
genes to environmental stimuli (Fig. 1). The extracellular
domains exert special functions in some biological pro-
cesses, such as the copper-binding-like domain (Cupredoxin
domain) in XP_024376490.1 and XP_024376488.1
(Fig. 1). The conserved kinase domains of plant WAKs have
also evolved into two classes: WAK-RD and WAK-non-RD
after the monocot–dicot separation (de Oliveira et al. 2014).
The classification of RD and non-RD classes depends on
the presence of a conserved arginine (R) residue before the
catalytic motif DxxxxN. All the three CpWAKs possess the
RDxxxxN motif (Fig. S2) and, thus, belong to the WAK-RD
class. The non-RD WAKs only occur in monocots. The dif-
ferent catalytic domains in the two WAK classes may lead to
different signaling pathways (Kohorn 2015). The variability
within the extracellular domain and the kinase domain may determine the specificity of WAKs in different biological processes.

**The expression patterns of CpWAKs**

WAK gene expression is modulated by diverse stimuli and differential expression patterns have been observed on the transcript and protein level in *C. plantagineum*. For example, the transcript expression of *CpWAKs* was only slightly up-regulated after 1 h of SA treatment and reduced after 24 h treatment (Fig. 2b). The transcripts of *CpWAK1* and *CpWAK3* were suppressed under MeJA treatment while *CpWAK2* accumulated after 1 h MeJA treatment and then sharply declined (Fig. 2b). CpWAK proteins accumulated after 6 h and 24 h of SA and MeJA treatments while no significant up-regulated expression patterns were observed on the transcript level (Fig. 2b,c). This means that the expression of CpWAKs under SA and MeJA are mainly post transcriptionally controlled. In contrast to the modulation of CpWAK expression under SA and MeJA treatment, the expression of *CpWAK* genes is regulated on the transcriptional level in response to dehydration, because the expression of CpWAKs showed the same trend on the transcript and protein level (Giarola et al. 2016) (Fig. 2a). Therefore, CpWAK expression may be controlled depending on the stimulus.

**CpWAKs form multimers**

Western blot analyses of purified recombinant proteins reveal two or more bands with His-tag or CpWAK1 antisera (Fig. S3). The multiple bands suggest that CpWAKs can form dimers or multimers. The formation of multimers is mainly due to the presence of a protein domain within the R-1 fragment because more bands are seen when only the R-1 fragment was run on the gel. It was noticed that except for protein fragment R-2 the proteins of truncated fragments of CpWAKs do not migrate into the SDS-PAGE gels without DTT (Fig. S6). There are several cysteine residues localized in the galacturonan-binding domains, in the EGF-like domains and other parts of the CpWAK proteins (Fig. S2). These cysteine residues, especially those in the R-1 segment, are presumably responsible for the formation of CpWAK dimers or multimers via intermolecular disulfide bonds (Fig. 5). Besides the cysteine-rich domains, the EGF repeats can also lead to the dimerization of proteins mediated by calcium (Anderson et al. 2001; Verica et al. 2003).

The recombinant protein corresponding to the extracellular region of CpWAK1 precipitates in the presence of Ca$^{2+}$ (Fig. S4), which may result from the calcium-mediated protein dimerization of the EGF repeats.

**CpWAKs bind to pectins**

It had been suggested that CpWAK1 is involved in the reversible folding of the cell wall during dehydration and rehydration in *C. plantagineum* (Giarola et al. 2016). Therefore, the underlying mechanisms of CpWAK-mediated cell wall folding were analyzed in detail.

Decreux and Messiaen (2005) first identified the WAK–pectin binding in vitro. WAK–pectin binding was also observed using the recombinant proteins of CpWAKs and the pectin extracts from untreated *C. plantagineum* (Fig. 3b) (Jung et al. 2019). According to Decreux and Messiaen (2005), AtWAK1 shows higher affinity for the pectins in the “egg-box” model. This is consistent with the observation for *C. plantagineum* (Fig. 3c, S5a). CpWAK1EX did not bind to polygalacturonic acid (PGA, Sigma) or commercial pectin (pectin from citrus peel, Sigma) (Fig. 3b), but CpWAK1EX–pectin binding was observed after saponification of commercial pectin in the presence of Ca$^{2+}$ (Fig. 3c). Saponification breaks ester bonds and then the pectic chains can form the “egg-box” structure in a calcium environment (Sedan et al. 2007). CpWAK1EX showed a weak binding to saponificated pectins in buffer without Ca$^{2+}$ (Fig. 3c), which indicates that CpWAK1 can bind to the negatively charged pectins. Fully de-methylesterified homogalacturonan was detected in the pectin extracts (CDTA fraction) from untreated *C. plantagineum* leaves (Jung et al. 2019). This is the prerequisite for the formation of “egg-box” structures in a calcium environment, which also explains the stronger binding between CpWAK1EX and *C. plantagineum* pectin. The binding assays using different fragments of the CpWAK1 proteins showed that both R-1 and R-3 fragments containing the galacturonan-binding domain bound more strongly to pectin than the R-2 fragment containing only EGF repeats (Fig. 3a, d). This demonstrates the importance of the galacturonan-binding domain in the CpWAK–pectin interaction. Protein band shift assays with CpWAK protein and pectin did not show a band shift on PAGE gels in the presence of DTT (Fig. S7). Therefore, the cleavage of the disulfide bonds with DTT not only disrupts the formation of multimers but also prevents the binding of CpWAKs to pectins. This suggests that CpWAKs bind to pectins as dimers or multimers via disulfide bonds (Fig. 5), consistent with the proposal that the extracellular domain of WAK proteins may function in a carbohydrate-rich environment involving protein interactions or oligomerization (He et al. 1999).

**CpGRP1 contributes to the binding of CpWAK1 to pectins**

CpWAK1 does not only bind pectins, but it also forms a complex with the cell wall protein CpGRP1 (Giarola et al. 2016), which in turn influences the CpWAK1–pectin
interaction. The heatmap in Fig. 4b shows that more CpGRP1 molecules lead to more binding of CpWAK1EX to pectins but in contrast more CpWAK1EX molecules lead to less CpGRP1 binding. The N-terminal fragment of CpGRP1 without the pectin-binding domain competes with pectins for interacting with CpWAK1EX (Fig. 4b). These results indicate that CpGRP1 contributes to the binding of CpWAK1EX to pectins by interacting with CpWAK1EX (Fig. 5) which supports the hypothesis proposed by Giarola et al. (2016) that CpWAK1 interactions with cell wall components are modulated by additional interaction partners such as CpGRP1. GRPs interact with the extracellular domain of WAKs (Park et al. 2001; Giarola et al. 2016). The effect of CpGRP1 on the binding of CpWAK1 and pectins was also observed using the CpWAK1 fragments, R-1 and R-2 (Fig. 4b). The R-2 fragment reduces the binding of CpGRP1 to pectins, which implies that CpGRP1 mainly interacts with a protein domain present within the R-2 fragment of the CpWAK1.
Multiple factors influence CpWAK–pectin binding

CpWAK protein abundance decreases in response to dehydration (Fig. 2a). Based on the responses of CpWAKs and CpGRP1 to dehydration, Giarola et al. (2016) proposed that the CpGRP1–CpWAK1 complex is implicated in the cell wall remodelling during dehydration and rehydration. The association of WAKs with wounding and pathogenesis-related processes is partially due to the increased expression after wounding or pathogen infection (Park et al. 2001; Kohorn and Kohorn 2012; Kohorn 2015). Plant hormones SA and JA are involved in the responses of plants to pathogens and wounding (Dong 1998; Reymond and Farmer 1998). The protein expression of CpWAKs can be induced by both SA and MeJA (Fig. 2c). Treatments of C. plantagineum leaves with the two hormones also resulted in increased expression of CpGRPs (Fig. 2c). The accumulation of CpWAK and CpGRP proteins seem to follow similar kinetics, with an increase after 6 h (Fig. 2c). The simultaneous accumulation of CpWAKs and CpGRPs make it possible that CpGRP acts as a modulator in regulating the cell wall signal perception of CpWAKs after wounding or pathogen infection. CpGRPs and CpWAKs show antagonism in the OG/flg22/wound-triggered defense responses according to Gramegna et al. (2016).

All pectin binding assays described above were performed in buffered solutions with pH 8. However, the pH value of the apoplast (pHapo) is generally around 5 and can be formed in buffered solutions with pH 8. However, the pH kinetics, with an increase after 6 h (Fig. 2c). The simultaneous accumulation of CpWAKs and CpGRPs make it possible that CpGRP acts as a modulator in regulating the cell wall signal perception of CpWAKs after wounding or pathogen infection. CpGRPs and CpWAKs show antagonism in the OG/flg22/wound-triggered defense responses according to Gramegna et al. (2016).

The de-methylesterified pectins in the cell wall are necessary for the formation of “egg-box” gelatin. The pectins can be de-methylesterified by pectin methylesterases, which is inhibited by the pectin methylesterase inhibitors (Micheli 2001). Previous studies showed that SA and MeJA led to the up-regulated expression of pectin methylesterase inhibitors and, thus, gave rise to the controlled activity of pectin methylesterases and decreased de-methylesterified pectins (An et al. 2008; Meng et al. 2009). Thus, the weaker binding of CpWAK1EX to pectin extracts from SA and MeJAtreated C. plantagineum leaves (Fig. 4e) may result from fewer “egg-box” structures in the cell wall pectins resulting from lower activity of pectin methylesterases.

Although more pectins in “egg-box” conformation may be present in desiccated tissues (Vicré et al. 2004; Jung et al. 2019), CpWAK1EX did not show different affinities to pectins extracted from hydrated or dehydrated C. plantagineum leaves (Fig. S5b). These observations imply that CpGRP1 may affect CpWAK–pectin binding during the early stages of dehydration (Fig. 5). During desiccation, the low abundance of CpWAKs leads to a decrease of WAK dimers and multimers which promote CpWAK–pectin binding; therefore, abundance of CpWAKs may distinguish the signals under desiccation (Fig. 5).

WAKs as wall-associated receptor kinases should be capable of recognizing different signals with the help of ions and protein ligands. It is a big challenge to understand how WAKs distinguish the different signals from cell walls. In this work, CpGRP1, pHapo, Ca2+[apo] and the formation of CpWAK multimers or dimers are considered as potential factors involved in the orchestrated processes. Each factor has a potential to play a role in the regulation of signal perception under stress (Fig. 5). The hypothesis is that CpWAK decodes cell wall signals in concert with CpGRP1, which modulates CpWAK–pectin interaction in responses to wounding or pathogen infections, and a decrease of CpWAKs multimers may activate downstream signals during desiccation.

Author contribution statement  PC planned and designed the research, conducted the experiments and wrote the manuscript. DB and VG designed the research, supervised the work and corrected the manuscript.

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Data availability statement All data supporting the findings of this study are available within the manuscript and within its supplementary materials published online.

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