The LysM-RLK CERK1 is a major chitin binding protein in Arabidopsis thaliana and subject to chitin-induced phosphorylation.

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Summary

Plants detect potential pathogens by sensing microbe associated molecular patterns via pattern recognition receptors. In the dicot model plant Arabidopsis, the Lysin Motif (LysM) containing Chitin Elicitor Receptor Kinase 1 (CERK1) has been shown to be essential for perception of the fungal cell wall component chitin and for resistance to fungal pathogens. Recent in vitro studies with CERK1 protein expressed heterologously in yeast suggested direct chitin binding activity. Here we show in an affinity purification approach that CERK1 is a major chitin binding protein of Arabidopsis cells, along with several known and putative chitinases. The ectodomain of CERK1 harbors three distinct LysM domains with potential ligand binding capacity. We demonstrate that the CERK1 ectodomain binds chitin and partially de-acetylated chitosan directly without any requirement for interacting proteins and that all three LysM domains are necessary for chitin binding. Ligand-induced phosphorylation events are a general feature of animal and plant signal transduction pathways. Our studies show that chitin, chitin oligomers and chitosan rapidly induce in vivo phosphorylation of CERK1 at multiple residues in the juxtamembrane and kinase domain. Functional analyses with a kinase dead variant provide evidence that kinase activity of CERK1 is required for its chitin-dependent in vivo phosphorylation, as well as for early defence responses and downstream signaling. Collectively, our data suggest that in Arabidopsis, CERK1 is a major chitin, chitosan and chito-oligomer binding component and that chitin signaling depends on CERK1 post-translational modification and kinase activity.

Introduction

Health and survival of all higher eukaryotic organisms depends on efficient pathogen detection and rapid activation of defence. These immediate and basic protective mechanisms are common to all multicellular organisms and are collectively referred to as innate immunity (1). In plants as well as animals, innate immunity is based on pattern recognition receptors (PRRs) which recognize so called microbe or pathogen associated molecular patterns (MAMPs/PAMPs). These are slowly evolving molecular signatures that identify whole classes of microbes but are absent from the host (2).

Despite the numerous MAMPs perceived by plants, only a handful of PRRs have been identified so far (3). The most extensively studied PRR in Arabidopsis is the leucine-rich repeat (LRR) receptor-like kinase (RLK) FLS2, which recognizes so called microbe or pathogen associated molecular patterns (MAMPs/PAMPs). These are slowly evolving molecular signatures that identify whole classes of microbes but are absent from the host (2).
chitin. Chitin is a polymer of β-1,4 linked N-acetyl-glucosamine (GlcNAc) and has long been recognized as a potent MAMP in plant-fungal interactions (5). Arabidopsis knockout mutants of the CERK1 gene entirely lack chitin induced defence responses, indicating that CERK1 is essential for chitin recognition. As a result, cerk1 mutants are more susceptible to fungal pathogens (6, 7). Recently, cerk1 mutant plants were also shown to exhibit enhanced susceptibility to the bacterial pathogen Pseudomonas syringae, suggesting that CERK1 also has a function in perception of a yet unidentified bacterial MAMP (8).

In contrast to peptide binding LRR-RLKs, CERK1 possesses three extracellular Lysin Motif (LysM) domains (6, 7). The Lysin motif is a ubiquitous protein module found in prokaryotes as well as eukaryotes. LysM proteins were first described in bacteria and shown to have binding capacity for peptidoglycan (PGN), a linear form of alternatively β-1,4 linked N-acetyl-muramic acid and GlcNAc (9). The first plant LysM proteins to be characterized were RLKs from legumes that are required for the establishment of symbiosis with rhizobial bacteria. During initiation of symbiosis, rhizobia secrete lipochitooligosaccharides known as Nod factors. The legume LysM-RLKs are involved in sensing the bacterial symbiont, likely by binding to these modified chitin oligomers (10-12). The isolation of the chitin binding protein CEBiP from plasma membranes of a rice cell culture established plant LysM proteins as important components in defense-related chitin perception (13). Similarly to Arabidopsis cerk1 knock-out mutants, rice CEBiP RNAi lines failed to mount chitin induced defence responses, indicating that CEBiP is involved in chitin perception. Interestingly, CEBiP possesses two LysM domains and a transmembrane region, but no domain that could function as a signal transduction module. In contrast to CEBiP, CERK1 contains an intracellular serine/threonine kinase domain, which makes it an excellent candidate for the Arabidopsis chitin receptor. Recent in vitro experiments with heterologously expressed CERK1 suggest that it does indeed have chitin binding capacity (14).

Despite the remarkable progress in recent years, relatively little is known about post-translational modification and downstream signalling of MAMP receptors. Phosphorylation is the most common mechanism in signal transduction and plays a key role in MAMP perception. Mutations in the kinase domain of the LRR-RLK FLS2 have been shown to render plants insensitive to flagellin, as does overexpression of kinase associated protein phosphatase KAPP (4, 15). Similarly, mutation of putative phoshorylation sites in FLS2 impairs flagellin signalling and endocytosis (16). Protein phosphorylation is also essential for the Arabidopsis chitin response. Kinase inhibitors efficiently block transcription of chitin inducible genes, whereas phosphatase inhibitors lead to induction of the same genes in the absence of chitin (17). However, the question if phosphorylation of CERK1 is required for chitin perception has not been addressed to date.

In this study, we provide evidence that CERK1 is a major chitin binding protein in Arabidopsis cells and that its ectodomain is directly involved in chitin binding. We show that CERK1 is phosphorylated in vivo in response to chitin treatment and that this posttranslational modification is required for chitin signaling in plant innate immunity.

**Experimental Procedures**

**Preparation of soluble and microsomal protein from cultured Arabidopsis cells** 8-day old, dark grown Arabidopsis cell culture was harvested using a Buechner funnel and a vacuum pump. The cells were ground to a fine powder with mortar, pestle and sand in liquid nitrogen. Then homogenization buffer (250 mM sucrose, 50 mM HEPES-KOH pH 7.5, 5% glycerol, 50 mM Na4P2O7, 1 mM Na2MoO4, 25 mM NaF, 4 mM DTT, 1% PVPP, Roche Complete protease inhibitor) was added at 1.5 ml/g plant material. The sample was centrifuged at 5000 g and 4°C for 15 min. After ultracentrifugation at 100 000 g and 4°C for 1 h, the supernatant (soluble fraction) and microsomal pellet were separated. The pellet was washed with homogenization buffer (without PVPP), and re-centrifuged at 100 000 g and 4°C for 45 min. The microsomal pellet was solubilized in homogenization buffer supplemented with 0.5% Triton X-100 (0.8 ml/g plant material) and centrifuged again at 100 000 g and 4°C for 1 h. The supernatant from this step was used further (microsomal fraction) and the pellet discarded.
Both the soluble and microsomal fractions were passed through a 40 μm filter, followed by a 20 μm filter, to remove any particles.

**Chitin pre-clearing** Crab shell chitin (Sigma) was ground with mortar and pestle to a very fine powder and a 100 mg/ml stock slurry was prepared with water. The soluble and microsomal extracts were divided into two halves and one part was incubated 3x with 5 mg/ml chitin on a shaker at 4°C for 45 min. In between the incubations steps, the chitin was removed by centrifuging at 5000 g and 4°C for 15min.

**Chitin affinity enrichment** Chitin magnetic beads (New England Biolabs) were washed twice with wash buffer (50 mM HEPES-KOH pH 7.5, 5% glycerol, 50 mM Na₂PO₄, 1 mM Na₂MoO₄, 25 mM NaF, 4 mM DTT, 0.5% Triton X-100, Roche Complete protease inhibitor). 20 μl chitin magnetic beads (50% slurry) were added per ml of protein extract and the samples were incubated at 4°C on a rotator for 1 h. Then the samples were washed 3 x with wash buffer containing 500 mM NaCl and 3x with wash buffer without NaCl. Proteins were recovered from the beads either by boiling with 1x SDS loading buffer (50 mM TRIS-HCl pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol, 0.025% bromophenol blue) at 90°C for 5 min, or by elution with 2 mg/ml chitohexaose (Seikagaku) in wash buffer. 50 μl elution solution were added per 20 μl of beads and incubated on a shaker at room temperature for 45 min. The eluate was then concentrated 6-fold using Vivaspin 500 (MWCO 10000 Da) columns.

Affinity enrichment experiments with polysaccharides were performed as described above, but instead of chitin beads, powdered chitin, chitosan (Sigma), or PGN from different sources (Invivogen) was added to protein preparations at 5 mg/ml. Binding protein was recovered by boiling with SDS loading buffer.

**Expression and purification of CERK1 fragments in N. benthamiana** The ectodomain of CERK1 was amplified using primers EP73 and EP74 (Suppl. table 5). The resulting PCR product was cloned into vector pJL48 (pTRBO) (18) via its VspI and NotI sites. cDNA versions of CERK1 with one or two deleted LysM domains were synthesized by GenScript (Piscataway, NJ, USA). The ectodomains of these CERK1 versions were also amplified with primers EP73 and EP74 and cloned into pTRBO as described above. The resulting plasmids were transformed into A. tumefaciens GV3101-pSoup. Two-day old Agrobacterium cultures were pelleted and resuspended in infiltration buffer (10 mM MgCl₂, 10 mM MES, 150 μM acetylsringone) at OD₆₀₀=0.3 and infiltrated into N. benthamiana leaves. The transformed leaves were harvested after 4 days and stored at -80°C.

For protein purification, the plant material was ground to a fine powder with mortar and pestle under liquid nitrogen and buffer GTEN (150 mM TRIS-HCl pH 7.5, 10 mM EDTA, 150 mM NaCl, 10% glycerol, 10 mM DTT, 2% PVPP, Sigma plant protease inhibitor cocktail) was added (3 ml/g plant material). Then the sample was centrifuged for 10 min at 5000 g and 4°C. The supernatant was passed through a 60 μm and a 40 μm filter to remove particles. antiFLAG M2 affinity gel (Sigma) was washed with buffer GTEN and was added to the filtered plant extract (20 μl bed volume per ml extract). The samples were then incubated at 4°C on a roller for 1 h. Subsequently the FLAG affinity gel was washed 6x with buffer GTEN containing 250 mM NaCl and 2x with GTEN containing 150 mM NaCl. FLAG-tagged protein was eluted with GTEN containing 150 mM NaCl, 250 μg/ml FLAG-peptide and 1 mM DTT (2x bedvolume of beads). The eluate was concentrated 6-fold using Vivaspin 6 (MWCO 5000 Da) columns. The protein was further purified by gel filtration using a GE Healthcare HiLoad 16/60 Superdex 200 pg column. The running buffer was TBS (50 mM TRIS-HCl pH 7.5, 150 mM NaCl). Fractions containing pure FLAG-tagged protein were concentrated again with Vivaspin 6 (MWCO 5000 Da) columns. Purity and quantity was tested by SDS-PAGE and colloidal Coomassie staining. The purity was also verified by mass spectrometry analysis.

**Elicitor treatment of plant material** Chitin, chitosan (Sigma), chitin oligomers (IsoSep), chitosan oligomers (Seikagaku) as well as PGN from different source organisms (Invivogen) and LPS (Sigma) were used at a concentration of 1-100 μg/ml. flg22 and elf18 were used at a final concentration of 100 nM. Leaves of soil-grown plants were treated by vacuum infiltration. To treat cultured cells, the MAMPs were added to the medium.
Total protein extraction and Western blot analysis
Total protein was extracted by grinding leaves in homogenization buffer containing 0.5% Triton X-100 and subsequent centrifugation at 10,000 g for 15 min. Proteins were separated by 8% or 10% SDS-PAGE and blotted to polyvinylidene difluoride membranes (Millipore). Membranes were probed with anti-CERK1 (8) or anti-FLAG M2 (Sigma) antibodies. Goat-anti-rabbit or goat-anti-mouse alkaline phosphatase conjugates (Sigma) were used as secondary antibodies. The reaction was detected using ImmunStar AP substrate (BioRad).

Lambda phosphatase treatment
Microsomal extracts from untreated and chitin-treated Arabidopsis cell culture were prepared. CERK1 protein was pulled down from the extracts with chitin magnetic beads or powdered chitin. The lambda phosphatase (Sigma) treatment was performed on the beads/chitin powder according to the manufacturer’s instructions.

MAP kinase assays
The plant material used was leaves of soil grown plants. To avoid MAPK activation by infiltration, the upper and lower surface of detached leaves was cut with a razor blade. Cut leaves were incubated in water for 5 h to allow the wounding response to diminish. Elicitors were added to the water and the samples were incubated for 12 min. Immunocomplex MAP kinase assays were performed as described previously (19). Antibodies against AtMPK6 and AtMPK4 were purchased from Sigma.

ROS burst assays
The production of ROS was measured using a luminol based chemiluminescent assay. Leaf discs (4 mm diameter) were floated on water overnight. Then the water was replaced with luminol solution (100 µl per leaf disc; 10 mM TRIS pH 9.5, 100 µM luminol, 10 µg/ml horse radish peroxidase). ROS production was induced with 100 µg/ml chitin, chitosan or chitin/chitosan oligomers, or 100 nM flg22. The equivalent volume of water was added to control samples. Luminescence was measured using a Tecan infinite M200 plate reader over time and data were recorded at the maximum intensity of the response.

Results
Several proteins from Arabidopsis cell extracts, including LysM-RLK CERK1, bind polymeric chitin. In order to identify proteins with potential function in chitin-induced plant innate immunity, we developed an affinity purification method to enrich for plant chitin binding proteins (Suppl. Fig. 1A). Proteins from Arabidopsis thaliana cell culture were separated into soluble and microsomal fractions and the resulting protein extracts were incubated with chitin magnetic beads. To discriminate between chitin binding proteins and proteins that bind non-specifically, we incubated half of the extracts with powdered chitin before binding to chitin beads. This pre-clearing step did not visibly alter the overall composition of extracts (Suppl. Fig. 1B). After incubation, the beads were washed with a high salt buffer (0.5 M NaCl). Bound proteins were recovered by boiling in SDS buffer and subsequently separated by SDS-PAGE (Fig. 1). Protein bands that were reproducibly depleted from the extracts by pre-clearing were analysed by LC-MS/MS. In the membrane fraction, a 70 kDa band was identified as CERK1, suggesting that CERK1 isolated from Arabidopsis cells indeed has chitin binding activity (Table 1). In the soluble fraction several differential bands were detected which corresponded to chitinases and glycosyl hydrolases, including the pathogenesis related chitin binding proteins PR3 and PR4 (20, 21) (Table 1). To identify less abundant chitin binding proteins, complete gel lanes were divided into slices and analysed by mass spectrometry. This approach revealed several additional chitinases, the LysM protein At2g17120 (closest Arabidopsis homolog of the rice chitin binding protein CEBiP) (13) and LysM-RLK At2g23770 as potential chitin binding proteins (Suppl. Table 1). The closely related LysM-RLK At2g33580 was found in a similar experiment (Suppl. Table 3C).

CERK1 was also detected in protein samples that were specifically eluted from chitin beads with chitoheaxose, corroborating CERK1 chitin binding activity and suggesting that CERK1 can also bind to chitin oligomers (Suppl. Table 2). Mascot and X!Tandem scores of all proteins identified in this study are presented in Suppl. Table 3.

The ectodomain of CERK1 binds chitin and chitin derivatives directly. The affinity purification experiments were performed under very stringent conditions to enrich for proteins that bind chitin directly rather than via protein-protein
interactions. To confirm direct chitin binding activity of the CERK1 ectodomain, we transiently expressed the FLAG-tagged N-terminus of CERK1 (aa 1 to 229) in N. benthamiana, using the recently described pTRBO overexpression system (18). The CERK1 ectodomain-FLAG protein was then purified by FLAG-affinity chromatography and gel filtration. Subsequently, we determined that the purified protein binds to chitin beads (Fig. 2A), indicating that the CERK1 ectodomain alone has chitin binding activity and interacting proteins are not required for efficient chitin binding.

Chitosan, a partially de-acetylated derivative of chitin, has been described as a potent elicitor of defence responses in plants (22, 23). Similarly, plants recognize the bacterial cell wall component peptidoglycan (PGN), which is chemically similar to chitin (24, 25). Therefore, we examined CERK1 binding to chitosan and PGN. We found that CERK1 binds strongly to chitin and more weakly to chitosan, whereas binding to PGN from either a gram-positive or a gram-negative bacterium could not be observed (Fig. 2B). CERK1 binding to chitosan appears to be physiologically relevant as cerk1-2 knockout mutants (6) show neither a reactive oxygen burst nor MAP kinase activation in response to chitosan (Suppl. Fig. 2A, B). Collectively, these data demonstrate that CERK1 is not only required for chitin, but also for chitosan perception, whereas it is unlikely to play a role in PGN recognition.

Not only polymeric chitin, but also chitin oligomers induce defence responses in plants (5). Elution of CERK1 from chitin beads with chitohexaose suggested that CERK1 also binds chitin oligomers. To address this question in more detail, we performed binding of CERK1 to chitin beads in the presence of chitin and chitosan oligomers of different length. Chitin oligomers with a polymerization degree of 5 and longer decreased the binding of CERK1 to chitin beads, indicating that CERK1 can bind these chitin fragments. In contrast, shorter chitin oligomers or chitosan oligomers had no effect (Fig 2C).

All three LysM domains of CERK1 are required for chitin binding. The CERK1 extracellular domain comprises three distinct LysM domains. In order to investigate which of the LysM domain(s) is/are necessary or sufficient for chitin binding, we generated a comprehensive set of FLAG-tagged CERK1-ectodomain versions with one or two LysM domains deleted (Fig 3A). Transient expression in N. benthamiana yielded proteins that corresponded well to their expected molecular weights. (Fig. 3B, left panel). Next we tested binding of the respective proteins to chitin beads. We found that only the full length CERK1 ectodomain could be pulled down with chitin beads, indicating that all three LysM domains are required for chitin binding (Fig. 3B, right panel).

Chitin and chitin derivatives induce transient phosphorylation of CERK1. Western blotting experiments with CERK1 from Arabidopsis leaves that were vacuum infiltrated with chitin revealed that chitin treatment induces a transient bandshift of the CERK1 protein (Fig 4A). To study the band shift kinetics in more detail we next used Arabidopsis cell culture which allows for a more simultaneous chitin application to all cells (Fig 4B). These experiments showed that the bandshift is very rapid, appears within a few minutes after chitin treatment and lasts up to a few hours.

To test the specificity of the bandshift response, we treated Arabidopsis cell culture with a range of known polysaccharide and peptide PAMPs (Fig. 4C). We found that in addition to chitin, the structurally closely related polysaccharide chitosan induced a partial CERK1 bandshift, corroborating the idea that CERK1 is involved in chitosan-induced plant defence activation. In contrast to chitin, chitosan can be solubilised in weak acids, which increases its availability and thereby the bandshift-inducing capacity. Peptidoglycan (PGN) from different source organisms, lipopolysaccharides (LPS) or peptide PAMPs did not induce a bandshift.

Next, we compared the bandshift induced by polymeric chitin and chitosan to the effect of different chitin and chitosan oligomers (Fig. 4D). These experiments showed that the ability of chitin oligomers to induce a bandshift of CERK1 is length-dependent: Monomeric GlcNAc and chitin dimers did not cause a CERK1 protein shift, tri- and tetramers induced a weak shift, whereas chitin pentamers and longer oligomers led to a bandshift comparable to that induced by polymeric chitin. Polymeric chitosan induced a weak bandshift, whereas chitosan oligomers did not. This is likely due to the fact that polymeric chitosan contains a significant proportion of acetylated glucosamine residues, whereas the chitosan oligomers used here are completely de-
acetylated (Fig 4D). The chitin oligomers that induced a clear band shift of CERK1 were also able to induce a ROS burst and activate MAP kinases in wild type Arabidopsis, but not in the knock-out mutant cerk1-2 (Suppl. Fig. 3A, B). These findings confirm that chitin oligomers (dp \geq\ 5) are recognized as PAMPs in Arabidopsis and that their perception is dependent on CERK1. MAP kinase assays are highly sensitive and long chitin oligomers induced phosphorylation activity already at low concentrations (1 \mu g/ml) (Suppl. Fig. 3 B). At higher concentrations (\geq\ 10 \mu g/ml) also chitin tri- and tetramers activated MAPKs, suggesting that they function as PAMPs in Arabidopsis, albeit with low efficiency (data not shown).

The rapid inducibility of the bandshift suggested a post-translational modification of the CERK1 receptor kinase. To analyze the nature of the bandshift in more detail, we first tested shifted and non-shifted CERK1 purified from cell culture with chitin beads, for ubiquitination. However, immunodetection experiments with an ubiquitin-specific antibody yielded negative results (data not shown). We next investigated phosphorylation, because it plays an essential role in signal transduction and is a very common post-translational modification of protein kinases. Supporting evidence came from inhibitor studies: The broad specificity kinase inhibitors staurosporine and K252a partially blocked the band shift, whereas the phosphatase inhibitor okadaic acid induced a slight band shift in absence of chitin (Suppl. Fig. 4). To test if CERK1 is indeed phosphorylated, we treated CERK1 protein pulled down from Arabidopsis cell culture with lambda phosphatase. Subsequent immunoblots revealed that dephosphorylation with lambda phosphatase completely reversed the bandshift, whereas control treatments without the enzyme had no effect, indicating that phosphorylation is the cause of the bandshift (Fig. 5).

CERK1 is phosphorylated on multiple residues after chitin treatment. We then analysed shifted and non-shifted CERK1 from Arabidopsis cells by mass spectrometry in order to identify chitin induced phosphorylation sites. Several phosphorylated residues were identified within a serine/threonine rich region in the juxtamembrane domain and one (T519) in the kinase domain (Table 2, Suppl. Fig 5). The juxta-membrane phosphorylation sites were all identified from the same, serine-rich peptide. Therefore we examined the respective spectra manually and with the assistance of PhosCalc (26). There was clear support for phosphorylation on residues S266, S268 and S274, whereas S270 represents a potential phosphorylation site.

Dynamic changes in phosphorylation can be assessed by comparing the peak intensities of phosphorylated and non-phosphorylated peptides in extracted ion chromatograms (27-31). The peptide isoforms corresponding to the independent phosphorylation sites discussed above appeared as well separated peaks in LC ion chromatograms (Suppl. Fig. 6 and data not shown). This allowed us to investigate chitin induced changes in phosphorylation for all identified residues except S270. One phosphorylated residue (S266) was identified in unchallenged as well as chitin treated samples (Table 2), and the quantitative analysis suggested that the corresponding peak was not significantly different upon chitin treatment. In contrast, the other two residues in the juxtamembrane domain (S268, S274) and the residue in the kinase domain (T519) were clearly more phosphorylated in chitin induced samples. (Suppl. Table 4). These results demonstrate that chitin induces phosphorylation of CERK1 on multiple residues. The size of the bandshift observed in our experiments suggests that there might be additional chitin-induced phosphorylation sites in CERK1.

CERK1 kinase activity is necessary for CERK1 phosphorylation and chitin signaling. CERK1 kinase domain expressed in E. coli has been shown to autophosphorylate and to phosphorylate the artificial substrate myelin basic protein (MBP) (6). To test if kinase activity of CERK1 is required for its chitin-induced phosphorylation, we generated a loss of function (kinase dead) version of CERK1 (cerk1-LOF) with a mutated ATP binding site (K350N). To confirm that this mutation abolishes kinase activity, we expressed His-tagged versions of the mutant and wild type CERK1 kinase domains in E.coli. In in-vitro phosphorylation assays, wild type kinase domain showed autophosphorylation and was also able to phosphorylate the artificial substrate MBP, whereas neither activity was detected with cerk1-LOF kinase domain (Suppl. Fig. 7).
To assess the effect of the kinase dead (LOF) mutation in planta, we generated stable transgenic plants expressing wild type (WT) CERK1 or cerk1-LOF in the knock-out mutant cerk1-2 (6) under the control of native CERK1 promoter. Lines were selected that expressed CERK1 at similar levels to the wild type. Western blotting experiments revealed that cerk1-LOF did not shift in response to chitin, whereas control plants transformed with wild type CERK1 displayed a normal bandshift (Fig. 6A). These data show that the kinase activity of CERK1 is essential for phosphorylation after chitin treatment.

CERK1 is required for chitin-induced generation of reactive oxygen species (ROS) and activation of MAP kinases (6). Therefore, we tested the chitin responsiveness of our transgenic plants in ROS-burst and MAP kinase assays (Fig. 6B, C). In these experiments, cerk1-2 plants transformed with wild type CERK1 showed normal ROS-burst generation and activation of MPK4 and MPK6 in response to chitin, suggesting full complementation capacity. In marked contrast, cerk1-LOF expression could not rescue the chitin insensitive phenotype of cerk1-2 in either of the two assays. These findings clearly demonstrate that CERK1 kinase activity and phosphorylation of the CERK1 protein are essential for chitin signalling in Arabidopsis.

**Discussion**

It has recently been shown that the LysM-RLK CERK1 is indispensable for chitin signalling in Arabidopsis (6, 7) and that CERK1 protein expressed heterologously in yeast has chitin binding activity (14). However, previous attempts to demonstrate chitin binding of endogenous Arabidopsis CERK1 by affinity labeling failed (6). Here we describe an affinity purification method based on chitin magnetic beads, which allowed us to isolate from Arabidopsis cells a number of proteins with chitin binding activity, including CERK1. Indeed, CERK1 was among the most abundant chitin binding proteins that we identified in our experiments. This enzyme has confirmed polygalacturonase (pectinase) activity and has been shown to play a role in dehiscence of floral organs and siliques (32, 33). Commercially available pectinases have been used to hydrolyze partially acetylated chitosan as well as chitin (34, 35). Thus, it will be interesting to test if PGAZAT/ADPG2 can act as a chitinase and if loss of its activity has any effect on plant-microbe interactions.

Our affinity purification approach also retrieved a number of less abundant, potential chitin binding proteins. Among these were several additional chitinases as well as the LysM protein At2g17120 (LYM2), the closest Arabidopsis homolog of the rice chitin binding protein CEBiP (13) and the LysM-RLKs At2g23770 and At2g33580. The low levels detected of these proteins suggest that they may have a lower affinity to chitin than CERK1, or are less abundant in Arabidopsis cultured cells. Knock-out lines of At2g23770 and At2g33580 did not show any altered response to chitin (7, 6). Therefore, these two closely related LysM-RLKs may either be only minor players in chitin signaling, may function redundantly, or may serve a completely different function.

It has previously been speculated that CERK1 may form a complex with the Arabidopsis ortholog of rice CEBiP, in which the CEBiP-like protein would provide the chitin binding site and CERK1 would function as signaling module (6, 3). However, our chitin binding experiments with purified CERK1-ectodomain showed that CERK1 binds chitin directly in the absence of any other proteins, which argues against this model. Also, recent studies on receptor maturation processes in rice indicate that OsCERK1 is required for chitin perception besides CEBiP (36). Chitin binding activity of OsCERK1 has not been demonstrated, but we identified the CEBiP homolog LYM2 as a potential chitin binding protein in our affinity chromatography experiments. Therefore it seems conceivable that CERK1/OsCERK1 may act in concert with LYM2/CEBiP and both proteins may be involved in binding the chitin ligand.

We have shown that the CERK1-ectodomain also binds to chitosan, a partially de-acetylated derivative of chitin. Moreover, we demonstrated by ROS-burst and MAP kinase assays that the chitin insensitive knockout-mutant cerk1-2 is also
unresponsive to partially de-acetylated chitosan. Chitosan derived from crab or shrimp shells is used in crop protection. In addition to its direct antimicrobial activity it elicits various defence pathways in crop plants (23). Our data suggest that these defence responses may be mediated by CERK1-like proteins. Iizasa and colleagues (14) reported very weak binding of heterologously expressed CERK1 to chitosan. However, the authors do not provide any information on the degree of acetylation, which is a major determinant of chitosan properties (22, 37) and may account for the observed difference. CERK1 did not bind to peptidoglycan from gram-positive or gram-negative bacteria, which is consistent with previous findings that cerk1 knock-out mutants are not impaired in peptidoglycan perception (38).

Since LysM domains from bacteria are peptidoglycan binding modules (9) and peptidoglycan is a major bacterial MAMP in Arabidopsis (24, 25) it is tempting to speculate that one of the other four Arabidopsis LysM-RLKs might be required for peptidoglycan perception.

We demonstrated that endogenous Arabidopsis CERK1 can be eluted from chitin beads with chitohexaose, indicating that CERK1 can bind to chitoooligomers. However, the yield of chitoooligomer elution was low compared to recovery with SDS buffer. This suggests that affinity of Arabidopsis CERK1 to chitin oligomers is much weaker than to polymeric chitin, which is in accordance with studies on heterologously expressed CERK1(14).

So far, research on plant chitin signaling has focused on chitoooligomers (6, 7, 13). It has been assumed that insoluble polymeric chitin is not an effective elicitor in plants and that chitin signaling requires the activity of apoplastic chitinases which cleave the polymer into biologically more active oligomers (5). However, our data suggest that CERK1 binds to polymeric chitin more strongly than to oligomers. Thus polymeric chitin is potentially an active molecule in chitin signaling and generation of short chitoooligomers by apoplastic chitinases might not be an absolute prerequisite for chitin recognition in Arabidopsis.

By binding competition assays with chitin and chitosan oligomers of various lengths, we could show that CERK1 binds to chitin penta- to octamers, which is not observed with chitosan oligomers of the same length. The fact that fully de-acetylated chitoooligomers are not effective in competition assays implies that acetylation of GlcNAc residues is required for CERK1 binding. This notion is substantiated by GlcNAc being the general constituent in all binding substrates of LysM proteins from bacteria as well as eukaryotes, suggesting that GlcNAc is the common sugar bound by LysM domains (39). Furthermore, N-acetylation of Nod factors is vital for their biological activity (40), highlighting the importance of N-acetylation for perception by LysM-RLKs. The polymeric chitosan used in this study is not completely de-acetylated and therefore able to bind to CERK1 and to induce CERK1-dependent defense responses.

Chitin binding assays with fragments of the CERK1 ectodomain revealed that all three LysM domains are necessary for full chitin binding activity. Many LysM proteins contain two or more LysM domains, including the chitin binding protein CEBiP from rice (13) and chitinase PrChi-A from the fern Pteris ryukyuensis (41, 42). Also, legume LysM-RLKs, which are closely related to CERK1 and implicated in Nod-factor perception, contain more than one LysM motif (10, 11). The requirement of several LysM domains for optimum substrate binding has been demonstrated for bacterial peptidoglycan hydrolases (39). Isothermal titration calorimetry of LysM domains from a fern chitinase suggests that each LysM motif can bind one chitin oligomer (41).

Although the function of the CxC motifs is not known, it is tempting to speculate that intramolecular cysteine disulfide bridge formation might provide structural cues for spatial distribution of LysM domains and thus for CERK1 ligand binding capacity. Loss of any LysM domain (or CxC motif) might therefore impede overall ligand binding activity as observed with our deletion constructs.

Our analyses revealed that chitin induces CERK1 phosphorylation in vivo on several residues in the juxtamembrane and kinase
domains. Phosphorylation was specific to chitin and partially de-acetylated chitosan. Chitin oligomers with a polymerization degree of 5 or higher induced CERK1 phosphorylation effectively, while only a weak effect could be observed with shorter oligomers. Fully de-acetylated chitosan oligomers did not induce CERK1 phosphorylation. These findings correlate well with our binding competition assays, suggesting that CERK1 phosphorylation is a direct consequence of chitin binding. Chitin oligomers that induced a band shift were also triggers of MAPK activation and ROS burst. Thus, these data confirm the general observation that longer chitin oligomers are more effective inducers of plant defense than shorter ones (37).

Experiments with transgenic plants expressing a kinase dead version of CERK1 demonstrated that CERK1 kinase activity is required for chitin induced phosphorylation of CERK1. Moreover, we showed that downstream signaling events, such as activation of MAP kinases and generation of ROS, depend on CERK1 kinase activity. In analogy to mechanisms known from mammalian growth factor receptor tyrosine kinases (43), chitin-binding induced homodimerization and autophosphorylation of CERK1 represents an attractive mechanistic working hypothesis. However, heterologously expressed CERK1 fails to autophosphorylate in response to chitin in vitro (14) suggesting that adequate in planta subcellular localization or plant-specific receptor complex partners may be necessary for chitin induced CERK1 phosphorylation. In this context it is important to note that the Arabidopsis brassinosteroid receptor kinase BR11 is phosphorylated after brassinosteroind treatment and, like in CERK1, phosphorylation is abolished by a mutation that renders BR11 kinase dead (44). BR11 forms homodimers as well as heterodimers with the LRR-RLK BAK1/SERK3 and both interactions are required for full phosphorylation and brassinosteroid signaling (45, 46, 47). The LysM-RLKs At2g23770 and At2g33580, which we detected in our chitin bead affinity purification, represent good candidates for CERK1 coreceptors. However, these LysM-RLKs lack the first protein kinase subdomain. Therefore, they might not be functional kinases and thus incapable of CERK1 trans-phosphorylation.

Based on our data, we propose a model where CERK1 binds polymeric chitin, chitosan and chitin oligomers. Subsequently, ligand binding leads to phosphorylation of CERK1 in the juxtamembrane and kinase domain, either via autophosphorylation or transphosphorylation by yet unknown kinases. The phosphorylation status of CERK1 may in turn control its affinity and phosphorylation activity towards different substrates or govern interactions with other regulatory proteins such as inhibitors or scaffold proteins required for chitin signal transduction in plant innate immunity.

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References

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Figure and Table Legends

FIGURE 1. Differential chitin affinity chromatography allows identification of potential chitin binding proteins from *Arabidopsis* cell cultures. Soluble and microsomal protein extracts from *Arabidopsis* cell culture were bound to chitin beads and chitin binding proteins were recovered by boiling with SDS buffer. Half of each extract was pre-cleared with powdered chitin prior to chitin bead incubation. Differential bands that were clearly and reproducibly depleted by chitin pre-clearing are marked with numbers. A 70 kDa band in the microsomal fraction (#8) was identified as CERK1 by mass spectrometric analysis (Table 1). The upper panel shows a 10 % gel, the lower panel a 15 % gel.

FIGURE 2. Purified CERK1-ectodomain protein directly binds polymeric chitin and chitosan. A FLAG-tagged version of the CERK1 ectodomain (CeF) was expressed in *N. benthamiana* and purified by FLAG affinity chromatography followed by gel filtration. For pull-down experiments, purified CeF (25 ng/µl) was diluted 1:100. A, 200 µl diluted CeF were incubated with chitin beads. After stringent washing, bound protein was recovered by boiling with SDS buffer (beads). The flow through was loaded as control. Western blots were probed with αFLAG and specific αCERK1 antibodies. B, 200 µl diluted CeF were incubated with 1 mg of the following polysaccharides: chitin, chitosan, peptidoglycans (PGN) from *Staphylococcus aureus* and from *Escherichia coli*. After incubation the polysaccharides were washed and bound proteins recovered by boiling in SDS buffer. Western blotting was performed with αCERK1 antibody. C, 200 µl diluted CeF were incubated with chitin beads in the presence of 1 mM chitin or chitosan oligomers as indicated. Washing, recovery and detection were performed as described above.

FIGURE 3. CERK1 ectodomain chitin binding activity requires combined presence of all three LysM domains. FLAG-tagged versions of the CERK1 ectodomain with one or two deleted LysM domains were expressed in *N. benthamiana*. A, Overview of deletion constructs. The expected mass (kDa) without glycosylation and the number of N-glycosylation sites predicted with NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc) are shown in parentheses. SP, signal peptide. B, Protein extracts of transformed *N. benthamiana* leaves were incubated with chitin magnetic beads and after stringent washing, chitin binding proteins were recovered by boiling in SDS buffer. Protein extracts (left panel) and protein recovered from beads (right panel) were analyzed by Western blotting using an αFLAG antibody.

FIGURE 4. Chitin, chitosan and chitin oligomers induce a CERK1 protein band shift. In all experiments, protein extracts were analyzed by Western blotting using specific αCERK1 antibody (upper panels). A, Leaves of Col-0 plants were vacuum-infiltrated with 100 µg/ml chitin and protein extracts were analysed after the indicated time points. The lower panel shows Coomassie stained loading control. B, *Arabidopsis* cell culture was incubated with 100 µg/ml chitin and samples were taken at the indicated time points. The upper panel shows a Western blot and the lower panel a colloidal Coomassie stained gel of CERK1 after affinity purification from microsomal fractions. C, *Arabidopsis* cell culture was incubated for 30 min with 100 nM elf18 or flg22, or with 100 µg/ml of chitin, chitosan or PGN from different sources. Two different chitosan preparations were used: A suspension in water and a solution in 0.005 % (final
concentration) acetic acid. To control samples, an equal volume of water was added. The panels show Western blot and Coomassie stained loading control, respectively. D, Arabidopsis Col-0 leaves were infiltrated with 100 µg/ml polymeric chitin, chitosan or chitin and chitosan oligomers of different length and incubated for 30 min. Control samples were infiltrated with water. Western blot and loading control are shown.

**FIGURE 5.** Chitin induces *in vivo* CERK1 phosphorylation. CERK1 was purified from microsomal fractions of chitin treated (100 µg/ml, 15 min) and untreated Arabidopsis cell culture using chitin magnetic beads. One third of the beads were boiled immediately with SDS buffer to release CERK1 (dir). The remainder of the beads was incubated with lambda phosphatase at room temperature for 30 min (λ) or used in a control incubation without the phosphatase enzyme (ctrl). CERK1 was detected by Western blotting with specific αCERK1 antibody (upper panel) or by staining with colloidal Coomassie brilliant blue (CCBB, lower panel).

**FIGURE 6.** CERK1 kinase activity is required for chitin induced CERK1 phosphorylation and for downstream chitin signaling. In all three experiments, transgenic plants were used expressing cerk1-LOF or wild type CERK1 in a cerk1-2 background. Col-0 and cerk1-2 plants were included as positive and negative controls, respectively. A, Leaves of transgenic plants and controls were infiltrated with 100 µg/ml chitin and incubated for 30 min. Protein extracts were analyzed by Western blotting with αCERK1 antibody. B, Leaf discs were treated with 100 µg/ml chitin and ROS generation was measured using a luminol based assay. Data were recorded at time of maximum response (12 min) and are presented as mean of 8 samples ± SEM. C, Leaves of transgenic and control plants were treated with 100 µg/ml chitin for 12 min. Immunocomplex MAPK assays were performed with specific αMPK4 and αMPK6 antibodies. Upper panel shows phosphorylation of substrate MBP, lower panels show Western blots probed with αMPK4 or αMPK6.

**TABLE 1.** Arabidopsis chitin binding proteins identified by affinity purification. Protein bands that were strongly reduced by chitin pre-clearing (Fig. 1) were excised and analysed by mass spectrometry. Corresponding gel slices of pre-cleared samples were analysed in parallel. For each band normalized spectrum counts of the most abundant protein are shown and the difference between untreated and pre-cleared samples is given in %.

**TABLE 2.** CERK1 phosphorylation sites identified by mass spectrometry. CERK1 was affinity purified with chitin beads from untreated and chitin-elicited Arabidopsis cell culture. A part of the protein was dephosphorylated with lambda phosphatase. Phosphorylation sites were analyzed by LC-MS/MS. The number of spectra identifying a phosphoresidue is given for each sample type. * Peptide spectra identifying the individual phosphoresidues are shown in Suppl. Fig. 5A-E. § LC peaks corresponding to the SKGDSFSSSIPLSTK phosphopeptide isoforms are shown in Suppl.Fig. 6. NA: not applicable. * Peak #1 did not meet the quality control criteria for relative quantitation.
### TABLE 1

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* Topology and subcellular location as predicted by [http://aramemnon.botanik.uni-koeln.de](http://aramemnon.botanik.uni-koeln.de)

s = soluble, m = membrane, sec = secreted

** Chitinase At2g43610 was also detected in membrane samples likely because of its extremely high abundance
### TABLE 2

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FIGURE 1

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kDa
98  64  50  36
36  22  16  6
FIGURE 2
FIGURE 3
FIGURE 4
FIGURE 5
FIGURE 6
The LysM-RLK CERK1 is a major chitin binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation

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