‘Slipped Sandwich’ Model for Chitin and Chitosan Perception in *Arabidopsis*

Ekaterina Gubaeva,¹ Airat Gubaev,² Rebecca L. J. Melcher,¹ Stefan Cord-Landwehr,¹ Ratna Singh,¹ Nour Eddine El Gueddari,¹ and Bruno M. Moerschbacher^{1,†}

¹Institute for Biology and Biotechnology of Plants, University of Münster, Schlossplatz 8, 48143 Münster, Germany; and

²Institute for Physical Chemistry, University of Münster, Corrensstraße 30, 48149 Münster, Germany

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Chitin, a linear polymer of *N*-acetyl-D-glucosamine, and chitosans, fully or partially deacetylated derivatives of chitin, are known to elicit defense reactions in higher plants. We compared the ability of chitin and chitosan oligomers and polymers (chitin oligomers with degree of polymerization [DP] 3 to 8; chitosan oligomers with degree of acetylation [DA] 0 to 35% and DP 3 to 15; chitosan polymers with DA 1 to 60% and DP approximately 1,300) to elicit an oxidative burst indicative of induced defense reactions in *Arabidopsis thaliana* seedlings. Fully deacetylated chitosans were not able to trigger a response; elicitor activity increased with increasing DA of chitosan polymers. Partially acetylated chitosan oligomers required a minimum DP of 6 and at least four *N*-acetyl groups to trigger a response. Invariably, elicitation of an oxidative burst required the presence of the chitin receptor AtCERK1. Our results as well as previously published studies on chitin and chitosan perception in plants are best explained by a new general model of LysM-containing receptor complexes in which two partners form a long but off-set chitin-binding groove and are, thus, dimerized by one chitin or chitosan molecule, sharing a central GlcNAc unit with which both LysM domains interact. To verify this model and to distinguish it from earlier models, we assayed elicitor and inhibitor activities of selected partially acetylated chitosan oligomers with fully defined structures. In contrast to the initial ‘continuous groove’, the original ‘sandwich’, or the current ‘sliding mode’ models for the chitin/chitosan receptor, the here-proposed ‘slipped sandwich’ model—which builds on these earlier models and represents a consensus combination of these—is in agreement with all experimental observations.

Chitin is an evolutionary, ancient, phylogenetically widespread biopolymer present throughout most realms of eukaryotes, with the notable exception of higher plants and vertebrates, in both of which it, consequently, acts as a convenient trigger for immune responses upon nonself recognition (Boller and Felix 2009;

Boutrot and Zipfel 2017; Fong et al. 2017; Lee et al. 2011; Ranf 2017; Sánchez-Vallet et al. 2015; Tang et al. 2017; Zipfel and Oldroyd 2017). While it is widely accepted that chitin receptors in plants are multicomponent transmembrane complexes the components of which are related but differ in detail between species, and while crystal structures have been solved and models have been built, a number of questions are still debated controversially. Which components make up the receptors? How do these components bind the chitin ligands? How does chitin binding lead to receptor complex formation?

Chitin perception is best studied in *Arabidopsis* and rice (*Oryza sativa*), in which similar but not identical chitin receptor complexes have been described (Fig. 1) (Cao et al. 2014; Hayafune et al. 2014; Liu et al. 2012, 2016) and reviewed (Shinya et al. 2015; Kaku and Shibuya 2016). AtCERK1, a transmembrane protein with three extracellular chitin-binding LysM domains and an intracellular protein kinase domain, is known as the central component of the chitin receptor in *Arabidopsis* (Fig. 1A) (Liu et al. 2012), possibly in conjunction with one or both AtLYK5 and AtLYK4 (Fig. 1B) (Cao et al. 2014). In contrast, OsCERK1 in *O. sativa* does not bind chitin but, rather, interacts with OsCEBiP, which recognizes chitin, a related protein with three LysM domains but lacking the protein kinase domain (Fig. 1C) (Hayafune et al. 2014; Kaku et al. 2006; Liu et al. 2016; Shimizu et al. 2010). In both *Arabidopsis* and rice, interaction with chitin octamer appears to lead to dimerization and activation of the receptor. Two alternative hypotheses have been put forward in *Arabidopsis*. Either binding of the chitin octamer directly dimerizes AtCERK1 (Fig. 1A) (Liu et al. 2012) or it may lead to the formation of a hetero-tetramer of a preformed AtLYK5 dimer and two AtCERK1 moieties (Fig. 1B) (Cao et al. 2014). In rice, a preformed hetero-dimer of OsCERK1 and OsCEBiP is believed to dimerize upon chitin octamer binding, which also brings together two OsCERK1 proteins, resulting in downstream signaling (Fig. 1C) (Hayafune et al. 2014; Liu et al. 2016).

There is also controversy concerning how the receptor binds chitin. The crystal structure of the LysM domains of AtCERK1 revealed four ligand binding subsites on subunit LysM2 (Liu et al. 2012), three of them directly interacting with GlcNAc residues. Recently, the crystal structure (Liu et al. 2016) of OsCEBiP revealed that chitin is bound in a similar way as seen in the AtCERK1-chitin complex. In both cases, the chitin oligomer appears to bind to LysM2 only, the other two LysM domains are not involved in ligand interaction. An alternative mode of chitin binding has been shown for Ecp6, the LysM containing effector of the fungal tomato pathogen *Cladosporium fulvum*, which binds chitin by intramolecular LysM domain dimerization involving LysM1 and LysM3, i.e., by

[†]Corresponding author: Bruno M. Moerschbacher;
E-mail: moersch@uni-muenster.de

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chitin-mediated intrachain noncovalent crosslinking of these two domains (Sánchez-Vallet et al. 2013). Interestingly, the model build for chitin binding to AtLYK4 and AtLYK5 in *Arabidopsis* was based on the Ecp6 model, suggesting a different conformation and a different mode of ligand interaction compared with AtCERK1 (Cao et al. 2014).

Similarly, different models have been proposed for the mechanism of chitin-induced receptor dimerization. The observation of four GlcNAc binding sites in AtCERK1 suggested a simple, ‘continuous groove’ model in which a chitin octamer, which is considered the most elicitor-active chitin oligomer, would span and bridge two receptor molecules, dimerizing them (Liu et al. 2012). In rice, however, based on sophisticated saturation transfer difference nuclear magnetic resonance studies, it was first suggested that chitin is ‘sandwiched’ between the two OsCEBiP receptor units, connecting them via the acetyl groups oriented alternately to both sides (Fig. 1C) (Hayafune et al. 2014), but solving of the crystal structure (Supplementary Fig. S1) (Liu et al. 2016) led to the ‘sliding mode’ model for receptor dimerization, based on an alignment to the LysM domain in the chitin hexamer-mediated NlpC/P60-LysM homo-dimer formation of an endopeptidase (Wong et al. 2015), in which three GlcNAc residues are bound by the first OsCEBiP molecule and the next three GlcNAc residues are bound by the second. This sliding mode model, thus, is a combination of the earlier continuous groove and sandwich models.

Clearly, the AtCERK1 and OsCEBiP binding sites accommodate only four GlcNAc residues, of which three interact directly with the LysM domain (Liu et al. 2012, 2016), which seems at odds with the necessity of a chitin octamer for optimal elicitor activity (Liu et al. 2012; Okada et al. 2002; Shibuya and Minami 2001; Shimizu et al. 2010), if the sandwich model (Hayafune et al. 2014) applies. Similarly, the sliding mode model (Liu et al.

2016) does not explain why chitin octamer is a much better elicitor than chitin hexamer (Liu et al. 2012; Okada et al. 2002; Shibuya and Minami 2001; Shimizu et al. 2010). In contrast, the continuous groove model (Liu et al. 2012) does not explain why a chitosan octamer consisting of alternating GlcN and GlcNAc residues acts as an inhibitor of chitin octamer-induced elicitation rather than as an elicitor (Hayafune et al. 2014).

To address these questions, we tested a larger set of chitosan oligomers and polymers, differing in their degree of polymerization (DP) and degree of acetylation (DA), for their elicitor activities, both in wild-type *Arabidopsis* seedlings and in knockout mutants lacking the AtCERK1 chitin receptor. Plants are known to recognize chitosans, i.e., partially de-N-acetylated chitin, but the mechanism of recognition is unknown (Cabrera et al. 2006; Vander et al. 1998). It has been speculated that electrostatic interactions may be involved (Kauss et al. 1989) or plants may contain chitosan-specific receptors (Liéart et al. 1991; Senstad and Mattiasson 1989). Alternatively, it has been suggested that the chitin receptor may also recognize chitosan, provided its DA is high enough (Petutschnig et al. 2010). Here, we show that AtCERK1 is, indeed, required for the elicitation of an oxidative defense reaction in *Arabidopsis* by a broad range of chitosan polymers and oligomers, and, based on these experimental results, we propose an improved model for chitin- and chitosan-induced dimerization of the chitin receptor in *Arabidopsis* that should also be applicable for chitin and chitosan perception in rice.

RESULTS AND DISCUSSION

Wild-type *Arabidopsis thaliana* seedlings treated with chitin oligosaccharides clearly showed a size- (DP) and dose-dependent induction of H₂O₂ (Fig. 2A), as expected (Liu et al. 2012; Okada et al. 2002; Shibuya and Minami 2001). Starting

Fig. 1. Models of chitin receptors and their activation. **A**, Chitin-induced AtCERK1 dimerization in *Arabidopsis*. Direct binding of a chitin oligomer to the AtCERK1 LysM2 domain leads to homo-dimer formation and AtCERK1 activation. **B**, Chitin-induced hetero-tetramer receptor complex in *Arabidopsis*. Two AtLYK5 forming a homo-dimer bind two chitin oligomers by all three LysM domains and recruit two AtCERK1 to build the active receptor complex. **C**, Chitin-induced hetero-tetramer receptor complex in rice. OsCERK1 and OsCEBiP form a hetero-dimer; a chitin oligomer either ‘sandwiches’ between two OsCEBiP proteins leading to the formation of the active receptor complex or, alternatively, the chitin oligomer induces homo-dimerization of two OsCEBiP molecules in the ‘sliding mode’. In both cases, elevated levels of association of OsCEBiP with OsCERK1 in the presence of chitin lead to OsCERK1 dimerization and activation.

from a DP of 6, we observed an increase in reactive oxygen species (ROS) generation with increasing DP at the concentration of 1 $\mu\text{g/ml}$; the maximum of eliciting activity was observed for chitin oligomers of DP 7 and DP 8, confirming published results (Cao et al. 2014; Liu et al. 2012). Also, as expected, the *Atcerk1* mutant seedlings did not show any response to chitin even at 10 $\mu\text{g/ml}$, thus corroborating that AtCERK1 is involved in recognition of chitin oligomers (Iizasa et al. 2010; Miya et al. 2007; Petutschnig et al. 2010; Wan et al. 2008) and validating the assay. Fully deacetylated chitosan oligomers (DA 0%, DP 3 to 8) did not trigger an immune response in *A. thaliana* seedlings (Fig. 2B).

All partially acetylated chitosan polymers tested, spanning a wide range of DA from 1 to 60%, elicited an oxidative burst; elicitor activity increased gradually with increasing DA (Fig. 3A). In all cases, the elicitor activity of the chitosans was dependent on the presence of AtCERK1, arguing against disturbance of plant membranes through electrostatic interactions as a perception mechanism for chitosan polymers, which had previously been proposed (Kauss et al. 1989).

A minimum DP of 9 was required when chitosan oligomers of DA 35% were used as elicitors, and a maximum response was reached with DP 14 (Fig. 3C). In contrast, chitosan oligomers of DA 10% were elicitor-inactive in the DP range investigated (from 4 to 17) (Supplementary Fig. S2). Again, the *Atcerk1* mutant seedlings did not show any response when treated with the chitosan oligomers, arguing against chitin receptor-independent perception mechanisms for chitosans, which also had been previously proposed (Kauss et al. 1989). Dose-response curves showed that elicitor activity increased with increasing DA and increasing DP. At higher concentrations, elicitor activities tended to decrease, indicating either a toxic effect of the chitosans to the seedlings or a lack of ligand-induced receptor dimerization at superoptimal ligand concentrations. Interestingly, chitosan oligomers of DA 35% and DP 13 had slightly higher elicitor activities at low concentrations than fully acetylated chitin oligomers of DP 7 (Fig. 3B), indicating that longer molecules may be more efficient in activating the receptor complex. Presumably, such molecules

require a minimum number, and possibly distribution, of acetyl groups for efficient binding and a minimum number of free amino groups for solubility.

Chitosan oligomers with DA 10% and DP 17, which on average contain less than two acetyl groups ($17 \times 10\% = 1.7$) per oligomer, did not induce ROS generation, although they fulfill the minimum length requirement for chitin oligomer perception (DP ≥ 6) (Fig. 2A). In contrast, chitosan oligomers with DA 35% and DP 9, which on average contain more than three ($9 \times 35\% = 3.2$) acetyl groups per molecule, did induce a ROS response (Fig. 3C). Therefore, to induce ROS generation, we assume that a minimum of four acetyl groups would be required in a chitosan hexamer to show elicitor activity.

In an attempt to explain our experimental results on the basis of the models proposed for chitin perception in plants, we took a closer look at the crystal structure of AtCERK1 in complex with chitin pentamer (Liu et al. 2012). When we tried to model a dimer consisting of two AtCERK1 molecules with a chitin tetramer bound, we were surprised to find that this is possible without steric clashes between the two receptors if we superimpose the GlcNAc residue at the reducing end (NAG-4) of one tetramer and the GlcNAc residue at the nonreducing end (NAG-1) of the other tetramer (Fig. 4). Thus, two chitin tetramers are aligned and form one chitin heptamer. This model for chitin-induced AtCERK1 dimerization which we termed the ‘slipped sandwich’ model—in analogy to the slipped sandwich of unsymmetric metallocenes in organometallic chemistry—combines the original sandwich model (Hayafune et al. 2014) and the recently described sliding mode model (Liu et al. 2016) for chitin-induced OsCEBiP dimerization. It differs from the sandwich model by offsetting the two receptor molecules by three rather than just one glycosyl residue, leading to just one instead of three shared residues binding to both receptor molecules. It differs from the sliding mode model by taking into account four instead of three binding subsites per receptor molecule, leading to dual binding of the central GlcNAc unit that is not seen in the sliding mode model. In contrast to the sliding mode model, the slipped sandwich model explains why chitin heptamer is a much better elicitor than chitin hexamer. In contrast

Fig. 2. Validation of the assay method to quantify chitin-induced reactive oxygen species (ROS) production (H_2O_2) in *Arabidopsis thaliana*. **A**, ROS production by wild-type (Col-0) and *Atcerk1* (CERK1) seedlings in response to the addition of chitin oligomers with different degrees of polymerization (DP) (from 3 to 8). **A**, Starting from DP 6, chitin oligomers induced ROS formation that was strongest for DP 7 and DP 8, at concentrations as low as 1 $\mu\text{g/ml}$. ROS formation increased with increasing concentrations of chitin oligomers. The *Atcerk1* mutant did not show any detectable ROS response caused by addition of chitin oligomers. **B**, Fully deacetylated chitosan oligomers with different DP (from 3 to 8) did not elicit ROS production in wild-type (Col-0) or *Atcerk1* (CERK1) seedlings; ROS formation in the presence of chitin oligomer with DP 8 served as positive control. Water addition to the seedlings served as negative control. Data given are means $\pm$ standard deviation of four replicates from one, representative of three independent experiments.

to the continuous groove model, it also explains why chitin heptamer and chitin octamer have equally strong elicitor activities. Of course, the situation may be different in *Arabidopsis* and rice, but the slipped sandwich model seems to be consistent with all experimental observations made in both systems and, thus, might apply to chitin perception in both species.

The model would also allow partially acetylated chitosans and chitosan oligomers to bind and dimerize the receptor as long as their pattern of acetylation (PA) is such that enough acetyl groups are facing the binding site on the receptors while free amino groups are oriented away from the receptors. In fact, when analyzing the ligand-bound AtCERK1 and the OsCEBiP structures, we realized that, of the four GlcNAc subunits interacting directly or indirectly with the LysM domains, the acetyl group of the third

GlcNAc from the nonreducing end points away from the receptor and, thus, makes no interaction with it. Consequently, the monodeacetylated chitosan tetramer GlcNAc-GlcNAc-GlcN-GlcNAc should bind equally well as the fully acetylated chitin tetramer. Consequently, experiments using partially acetylated chitosan oligomers with fully defined structure in terms of DP, DA, and PA might help to critically assess the different models for ligand binding and receptor dimerization.

A chitosan oligomer consisting of alternating GlcN and GlcNAc units has been shown to act as an inhibitor of chitin perception in rice (Hayafune et al. 2014). In the original sandwich model (Hayafune et al. 2014), this was explained by the oligomer binding to one OsCEBiP molecule but then being unable to bind a second OsCEBiP molecule (Fig. 5A). In the

Fig. 3. Elicitor activity of partially acetylated chitosan polymers and oligomers. **A**, Reactive oxygen species (ROS) production by wild-type (Col-0) and *Atcerk1* *Arabidopsis thaliana* seedlings in response to the addition of chitosan polymers with varying degree of acetylation (DA) (from 1 to 60%) and constant degree of polymerization (DP) (around 1,300). Elicitor activity increased with increasing DA and was already similar to the positive control (chitin octamer) at rather low DA. The *Atcerk1* mutant did not show any detectable ROS response caused by the addition of chitosan in comparison with the water control. **B**, Dependence of ROS formation on the concentration of chitosan oligomers and polymers (from 0 [water control] to 50 µg/ml) with different DP and DA in wild-type *A. thaliana* seedlings. ROS formation increased with increasing chitosan concentration until it reached a maximum beyond which ROS formation decreased, except for very low DA chitosan oligomers,* which did not elicit any detectable ROS production. **C**, ROS production by wild-type (Col-0) and *Atcerk1* *A. thaliana* seedlings in response to the addition of chitosan oligomers with different DP (from 6 to 15) and constant DA of 35%. Chitosan oligomers of DP 9 and larger induced AtCERK1-dependent ROS formation that increased with increasing DP. Data given are means ± standard deviation of four replicates from one, representative of three independent experiments.

continuous groove model (Liu et al. 2012), two OsCEBiP molecules would align to form a continuous binding groove with eight (2×4) binding sites, and the alternating chitosan oligomer would be expected to be able to bind, as all acetyl groups would point toward one side of the oligomer, thus dimerizing the receptor, in which case, this chitosan oligomer should act as an elicitor rather than as an inhibitor. The continuous groove model, thus, is not compatible with this experimental result, in contrast to the other three models. In case of the sliding mode model (Liu et al. 2016), the LysM2 domain of the first OsCEBiP molecule could bind to a motif within this oligomer, but the second OsCEBiP molecule could then not bind in the required close proximity to the first one to lead to dimerization. Hence, in this model, the alternating chitosan octamer would act as an inhibitor, as observed. The same would be true in our slipped sandwich model, which differs from the sliding mode model only in the presence of the shared middle GlcNAc unit of chitin as well as in four rather than three GlcNAc units bound per LysM domain, and from the original sandwich model in being staggered three instead of just one glycosyl unit.

Similarly, the experimental observation that chitin tetramer acts as an inhibitor (Iizasa et al. 2010) rather than as an elicitor (Zhang et al. 2002; Petutschnig et al. 2010) argues against the original sandwich model, while it is compatible with the other three models (Fig. 5A). Then, the experimental evidence shows that chitin heptamer is an equally strong elicitor as chitin octamer, while chitin hexamer is a weak elicitor (Fig. 2) (Petutschnig et al. 2010; Zhang et al. 2002). This observation again is not easily compatible with the continuous groove

model, which would, instead, predict lower activity for the heptamer and no activity for the hexamer. While the sandwich model as well as the sliding mode model would predict equal activities for these three oligomers, only the slipped sandwich model explains the lower elicitor activity of the hexamer. The experimental evidence gained from elicitor assays using fully acetylated chitin oligomers of different DP thus tends to exclude the continuous groove and the original sandwich model and disfavors the sliding mode model, while favoring the slipped sandwich model.

To further distinguish between the sliding mode and the slipped sandwich model, we performed experiments using two defined, partially deacetylated chitosan hexamers. Using a recombinant bacterial chitin deacetylase from *Vibrio cholerae*, VcCDA (Li et al. 2007), we specifically deacetylated the unit next to the nonreducing end of the chitin hexamer, yielding the monodeacetylated chitosan hexamer GlcNAc-GlcN-GlcNAc-GlcNAc-GlcNAc-GlcNAc (ADAAAA). An aliquot of this hexamer was then further incubated with a recombinant fungal chitin deacetylase from *Pestalotiopsis* sp., PesCDA (Cord-Landwehr et al. 2016), for a short time, yielding the double-deacetylated chitosan hexamer GlcNAc-GlcN-GlcNAc-GlcNAc-GlcN-GlcNAc (ADAADA) as main product, with small amounts of ADAAAA and A3D3. The structure and purity of both hexamers were verified using mass spectrometry (Supplementary Fig. S3). When these two chitosan hexamers were tested for elicitor activity, only the monodeacetylated hexamer was still active, though less than the fully acetylated chitin hexamer, while the double-deacetylated hexamer was elicitor-inactive (Fig. 5B). While these experimental results are

Fig. 4. Model of chitin-induced AtCERK1-AtCERK1 ectodomain homo-dimerization. The binding pocket is formed by the LysM2 domains of both AtCERK1 ectodomains. To build the chitin heptamer, shown in sticks representation, from two chitin tetramers, each positioned, as seen in the crystal structure, together with its corresponding AtCERK1 ectodomain (Liu et al. 2012), we aligned the nonreducing end GlcNAc residue of one of the tetramers to the same position (NAG4 in heptamer) as the reducing end GlcNAc unit of the second tetramer. Arrows indicate possible important N-acetyl groups for binding (NAG1, NAG2, NAG4, NAG5, NAG7).

Fig. 5. Elicitor activity of chitin oligomers of different degree of polymerization and of chitosan oligomers with defined pattern of acetylation. **A**, Expected and observed binding and elicitor activities of chitin/chitosan oligomers to LysM-containing receptors, according to the 'continuous groove', 'sandwich', 'sliding mode', and 'slipped sandwich' models. The letter A in the LysM domain indicates a binding site where acetylation is required for binding. A letter A written inside the domain indicates that the acetyl group must be oriented to face the binding site, one written outside that the acetyl group needs to point away from the protein, as suggested by the three-dimensional structure of the binding pocket in the respective model (Liu et al. [2016] depicts a sliding mode model). For the sandwich model, the weak binding of the first subsite of each LysM (Hayafune et al. 2014), which is also corroborated by the crystal structure underlying the sliding mode model (Liu et al. 2016), is indicated by a dotted line. Only the slipped sandwich model is fully compatible with the existing experimental data. **B**, Eliciting activity of the mono- and double-deacetylated chitosan hexamers ADAAAA and ADAADA; the fully acetylated chitin tetramer served as a positive control. **C** and **D**, Inhibiting activity of the fully acetylated chitin tetramer (**C**) and the double-deacetylated chitosan hexamer ADAADA (**D**); the fully acetylated chitin hexamer served as an elicitor. Data given are means $\pm$ standard deviation of four replicates from one, representative of three independent experiments.

compatible with the slipped sandwich model, they clearly eliminate the sliding mode model that would predict full elicitor activity for both of these chitosan hexamers (Fig. 5A).

Finally, we also tested potential inhibitor activities that should be expected for oligomers that can bind to one receptor molecule but are unable to bind a second one in the required conformation to yield an active receptor complex triggering an oxidative burst. For these experiments, we chose the rather weakly elicitor-active chitin hexamer, expecting that potentially antagonistic oligomers will be more readily detectable than when working with a strong elicitor such as the chitin hepta- or octamer. As expected by all four models and as shown previously, the fully acetylated chitin tetramer acted as an inhibitor (Fig. 5C). More revealing was the clear inhibitor activity observed for the double-deacetylated chitosan hexamer ADAADA (Fig. 5D), confirming that it is bound by AtCERK1 or, possibly, by AtLYK5. As seen in Figure 5B, this hexamer would act as an elicitor in the sliding mode model while acting as an antagonistic inhibitor in the here-proposed slipped sandwich model.

Taking all together, i) unlike the continuous groove model that does not explain why the alternating chitosan octamer acts as an inhibitor rather than as an elicitor, ii) unlike the original sandwich model that is at odds with the observation that the chitin tetramer is not an elicitor, and iii) unlike the sliding mode model that does not explain why the double-deacetylated chitosan hexamer ADAADA is not an elicitor but, rather, acts as an inhibitor, our slipped sandwich model is in agreement with all the experimentally observed facts.

According to the latest described model of chitin perception in *A. thaliana*, the chitin receptor is formed as a hetero-tetrameric complex of two AtCERK1 and two AtLYK5 proteins (Fig. 1B) (Cao et al. 2014). Even though we, like others, were unable to reproduce the reported necessity of AtLYK5 for chitin or chitosan perception (data not shown), we nevertheless tried to accommodate AtLYK5 into our slipped sandwich model of the AtCERK1 dimer proposed above. To model the binding groove

structure, in contrast with Cao et al. (2014), we based our model (using SWISS-MODEL workspace) (Arnold et al. 2006) of AtLYK5 on the available AtCERK1 structure (PDB ID 4EBZ) (Liu et al. 2012) rather than on the structure of the fungal chitin-binding protein Ecp6 (Sánchez-Vallet et al. 2013).

Like AtCERK1 and AtLYK5, Ecp6 possesses three LysM domains, but its structure was found to be significantly different (Sánchez-Vallet et al. 2013). In the AtCERK1 structure, three disulfide bonds are formed between different LysM motifs (Supplementary Fig. S4), resulting in a rigid structure, while the four disulfide bonds formed in Ecp6 are intra- rather than intermotif bonds, allowing much more flexibility and, hence, formation of the ultra-high affinity binding site between LysM1 and LysM3. However, when AtLYK5 is modeled based on the Ecp6 structure (Cao et al. 2014; Sánchez-Vallet et al. 2013), none of these four intramotif disulfide bonds can be formed. In contrast, when modeling is based on the AtCERK1 structure (Liu et al. 2012), all three intermotif disulfide bonds can be formed. We therefore conclude that AtLYK5 most likely adopts the same conformation as AtCERK1, OsCEBiP, and, possibly, OsCERK1. In the original model (Cao et al. 2014), chitin binding to the interdomain binding site buried deep inside one AtLYK5 molecule, or rather, the binding of two chitin oligomers to two AtLYK5 molecules, was supposed to 'somehow' lead to the binding of two AtCERK1 molecules to the preformed AtLYK5-AtLYK5 homo-dimer, forming the active hetero-tetrameric receptor-ligand complex. Surprisingly, in this original model, chitin binding to AtCERK1 is not even required. However, assuming that AtLYK5 takes the same conformation as AtCERK1, we can easily explain how chitin binding leads to the formation of the AtCERK1-AtLYK5 hetero-dimer and subsequently, due to the preformed AtLYK5-AtLYK5 homo-dimer, to the hetero-tetrameric receptor-ligand complex. Interestingly, and finally, the same dimerization model may then apply for an AtCERK1-AtCERK1 homo-dimer (Fig. 6A), an AtCERK1-AtLYK5 heterodimer (Fig. 6B), an OsCEBiP-OsCEBiP homo-dimer, and possibly, an

Fig. 6. Alternative schemes of receptor complexes in *Arabidopsis thaliana*. **A**, Two monomers of AtCERK1 bind one molecule of chitin heptamer and form a homo-dimeric receptor complex. **B**, Two monomers of AtCERK1 bind via two chitin heptamers to a preformed AtLYK5 homo-dimer and form a hetero-tetrameric receptor complex. In both cases, dimerization of AtCERK1 within the complex will lead to phosphorylation and downstream signaling.

OsCEBiP-OsCERK1 hetero-dimer (though no chitin-binding has been shown yet for OsCERK1).

This improved model for the interaction of LysM-containing chitin-binding proteins and chitin or chitosan oligomers in a slipped sandwich mode dimerizing two receptor moieties through their similarly folded LysM2 domains has further implications that we are only briefly mentioning here. The different binding affinities for chitin reported for AtCERK1 at different concentrations (Cao et al. 2014; Iizasa et al. 2010; Liu et al. 2012) suggest that there is a strong cooperativity for chitin binding, indicating that the second AtCERK1 protein would bind much more easily to an AtCERK1-chitin complex than the first AtCERK1 binding to chitin. Similarly, cooperativity can be expected in the AtCERK1-AtLYK5 dimer. As AtLYK5 seems to have a higher affinity for chitin oligomers than AtCERK1 (Cao et al. 2014), this would suggest that the elicitor-active (or inhibiting) ligands would first bind to the existing AtLYK5 dimer, which would then easily recruit (or be unable to recruit) two AtCERK1 proteins.

MATERIALS AND METHODS

Seedling preparation.

Arabidopsis seeds of the wild-type Col-0 and its AtCERK1 knockout mutant *cerk1-2* (Wan et al. 2008) were sterilized with 1 ml of 70% ethanol for 1 min, then, ethanol was substituted with 20% sodium hypochlorite solution containing 1% Tween 20. Seeds were washed four or five times with sterile water and were plated aseptically on 0.1% agar, containing 2.2 g of Murashige and Skoog salts per liter (Deutsche Phytoengineering Chemical Company) supplemented with 1% sucrose, at pH 5.7. Seedlings were grown in Percival AR-66 L4 growth chambers (Percival Scientific) at 22°C in a 8-h light and 16-h dark photoperiod.

Luminol chemiluminescence assay for H₂O₂ detection in 96-well plates.

To monitor H₂O₂ accumulation, we modified the method of ROS measurement after Albert et al. (2006), which quantifies luminol chemiluminescence as described previously (Klauser et al. 2013). Seven-day-old seedlings were transferred individually into single wells of a 96-well microtitre plate (Thermo Fisher Scientific Nunc A/S), containing 170 µl of nutrients solution (Naito et al. 1994) complemented with 1% sucrose per well. Using a multichannel pipette, 30 µl of Luminol L-012 (Waco Pure Chemical Industries), at the stock concentration of 100 µg/ml, was quickly added to each well. Then, the plate was incubated for 3 h at 22°C, covered with aluminum foil to protect the luminol from degradation. Directly before the measurement, elicitors were added to each well and the plate was placed in the microtitre plate reader (Luminoskan Ascent Microplate Luminometer, Thermo Scientific). For the detection of ROS released by *Arabidopsis* seedlings, every well was measured for 5 s every 10 min for 10 h. Kinetics of H₂O₂ production was determined by integration of data for every well over the reading period. Every timepoint is the mean value of four seedlings from four wells (either mock or chitosan-elicited). All experiments were repeated independently at least three times.

To assess possible inhibitory activities of chitin/chitosan oligomers, seedlings were preincubated with potential antagonists (60 µM) for 15 min in the microtiter plate, followed by the addition of chitin hexamer as an elicitor (10 µM), as described above.

Preparation and characterization of chitin and chitosan oligomers.

Purified chitin (DA 100%) and chitosan (DA 0%) oligomers with DP ranging from 3 to 8 were prepared by HCl hydrolysis

of α -chitin (Mahtani Chitosan) or DA 0% chitosan (Mahtani Chitosan), respectively, as described (Trombott et al. 2008). Separation of oligomers was achieved using size exclusion chromatography (SEC), as described (Hamer et al. 2015). Purified oligomers were analyzed using ultrahigh-pressure liquid chromatography evaporative light scattering detection electrospray ionization-mass spectrometry (ESI-MS) (Cord-Landwehr et al. 2017).

Preparation and characterization of partially N-acetylated chitosan polymers.

Polymeric chitosans (average DP 1,300) with DAs ranging from 1 to 60%, in which the GlcNAc and GlcN residues are randomly distributed along the polymer chain, were prepared and analyzed as described before (Lamarque et al. 2005).

Preparation and characterization of partially N-acetylated chitosan oligomers.

Purified partially acetylated chitosan oligomers were produced by enzymatic hydrolysis of partially re-N-acetylated chitosan with DA 10 and 35%, as described above. Purified recombinant *Bacillus licheniformis* chitinase (Neeraja et al. 2010) and *Bacillus* sp. chitosanase (Nampally et al. 2012) were used to produce chitosan oligomers with DA 10% and DA 35%, respectively (S. Basa and B. M. Moerschbacher *unpublished*). Oligomers were separated using SEC (Lamarque et al. 2005) and were analyzed using matrix-assisted laser desorption-ionization time of flight MS (Loboda et al. 2003).

Defined mono- and double-deacetylated chitosan hexamers were produced by enzymatic deacetylation of fully acetylated chitin hexamer (Megazyme). Purified recombinant chitin deacetylases VcCDA from *Vibrio cholerae* (Li et al. 2007) and PesCDA from a *Pestalotiopsis* sp. (Cord-Landwehr et al. 2016) were used to produce GlcNAc-GlcN-GlcNAc-GlcNAc-GlcNAc-GlcNAc (VcCDA only) or GlcNAc-GlcN-GlcNAc-GlcNAc-GlcN-GlcNAc (first VcCDA followed by PesCDA incubation) chitosan hexamers. The reactions were stopped by removing the enzymes with a 3K-Pes-filter (VWR) at 4°C, centrifuging at 16,000 × g for 30 min. The target hexamers were analyzed using hydrophilic interaction chromatography ESI-MS (Cord-Landwehr et al. 2017).

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LITERATURE CITED

- Albert, P., Miya, A., Hiratsuka, K., Kawakami, N., and Shibuya, N. 2006. A high-throughput evaluation system for *Arabidopsis* mutants for defense signaling. *Plant Biotechnol.* 23:459-466.
- Arnold, K., Bordoli, L., Kopp, J., and Schwede, T. 2006. The SWISS-MODEL workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* 22:195-201.
- Boller, T., and Felix, G. 2009. A renaissance of elicitors: Perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annu. Rev. Plant Biol.* 60:379-406.
- Boutrot, F., and Zipfel, C. 2017. Function, discovery, and exploitation of plant pattern recognition receptors for broad-spectrum disease resistance. *Annu. Rev. Phytopathol.* 55:257-286.
- Cabrera, J. C., Messiaen, J., Cambier, P., and Van Cutsem, P. 2006. Size, acetylation and concentration of chitooligosaccharide elicitors determine the switch from defence involving PAL activation to cell death and water peroxide production in *Arabidopsis* cell suspensions. *Physiol. Plant.* 127: 44-56.
- Cao, Y., Liang, Y., Tanaka, K., Nguyen, C. T., Jedrzejczak, R. P., Joachimiak, A., and Stacey, G. 2014. The kinase LYK5 is a major chitin receptor in *Arabidopsis* and forms a chitin-induced complex with related kinase CERK1. *eLife* 3:e03766.

- Cord-Landwehr, S., Ihmor, P., Niehues, A., Luftmann, H., Moerschbacher, B. M., and Mormann, M. 2017. Quantitative mass-spectrometric sequencing of chitosan oligomers revealing cleavage sites of chitosan hydrolases. *Anal. Chem.* 89:2893-2900.
- Cord-Landwehr, S., Melcher, R. L. J., Kolkenbrock, S., and Moerschbacher, B. M. 2016. A chitin deacetylase from the endophytic fungus *Pestalotiopsis* sp. efficiently inactivates the elicitor activity of chitin oligomers in rice cells. *Sci. Rep.* 6:38018.
- Fong, D., Grégoire-Gélinas, P., Cheng, A. P., Mezheritsky, T., Lavertu, M., Sato, S., and Hoemann, C. D. 2017. Lysosomal rupture induced by structurally distinct chitosans either promotes a type 1 IFN response or activates the inflammasome in macrophages. *Biomaterials* 129:127-138.
- Hamer, S. N., Cord-Landwehr, S., Biarnés, X., Planas, A., Waegeman, H., Moerschbacher, B. M., and Kolkenbrock, S. 2015. Enzymatic production of defined chitosan oligomers with a specific pattern of acetylation using a combination of chitin oligosaccharide deacetylases. *Sci. Rep.* 5:8716.
- Hayafune, M., Berisio, R., Marchetti, R., Silipo, A., Kayama, M., Desaki, Y., Arima, S., Squeglia, F., Ruggiero, A., Tokuyasu, K., Molinaro, A., Kaku, H., and Shibuya, N. 2014. Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. *Proc. Natl. Acad. Sci. U.S.A.* 111:E404-E413.
- Iizasa, E., Mitsutomi, M., and Nagano, Y. 2010. Direct binding of a plant LysM receptor-like kinase, LysM RLK1/CERK1, to chitin in vitro. *J. Biol. Chem.* 285:2996-3004.
- Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiya, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. 2006. Plant cells recognize chitin fragments for defense signaling through a plasma membrane receptor. *Proc. Natl. Acad. Sci. U.S.A.* 103:11086-11091.
- Kaku, H., and Shibuya, N. 2016. Molecular mechanisms of chitin recognition and immune signaling by LysM-receptors. *Physiol. Mol. Plant Pathol.* 95:60-65.
- Kauss, H., Jeblick, W., and Domard, A. 1989. The degrees of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Catharanthus roseus*. *Planta* 178:385-392.
- Klauser, D., Flury, P., Boller, T., and Bartels, S. 2013. Several MAMPs, including chitin fragments, enhance AtPep-triggered oxidative burst independently of wounding. *Plant Signal. Behav.* 8:e25346.
- Lamarque, G., Lucas, J. M., Viton, C., and Domard, A. 2005. Physicochemical behavior of homogeneous series of acetylated chitosans in aqueous solution: Role of various structural parameters. *Biomacromolecules* 6: 131-142.
- Lee, C. G., Da Silva, C. A., Dela Cruz, C. S., Ahangari, F., Ma, B., Kang, M.-J., He, C.-H., Takyar, S., and Elias, J. A. 2011. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. *Annu. Rev. Physiol.* 73:479-501.
- Li, X., Wang, L.-X., Wang, X., and Roseman, S. 2007. The chitin catabolic cascade in the marine bacterium *Vibrio cholerae*: Characterization of a unique chitin oligosaccharide deacetylase. *Glycobiology* 17:1377-1387.
- Liénart, Y., Gautier, C., and Domard, A. 1991. Isolation from *Rubus* cell-suspension cultures of a lectin specific for glucosamine oligomers. *Planta* 184:8-13.
- Liu, S., Wang, J., Han, Z., Gong, X., Zhang, H., and Chai, J. 2016. Molecular mechanism for fungal cell wall recognition by rice chitin receptor OsCEBiP. *Structure* 24:1192-1200.
- Liu, T., Liu, Z., Song, C., Hu, Y., Han, Z., She, J., Fan, F., Wang, J., Jin, C., Chang, J., Zhou, J.-M., and Chai, J. 2012. Chitin-induced dimerization activates a plant immune receptor. *Science* 336:1160-1164.
- Loboda, A. V., Ackloo, S., and Chernushevich, I. V. 2003. A high-performance matrix-assisted laser desorption/ionization orthogonal time-of-flight mass spectrometer with collisional cooling. *Rapid Commun. Mass Spectrom.* 17:2508-2516.
- Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. 2007. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 104:19613-19618.
- Naito, S., Hirai, M. Y., Chino, M., and Komeda, Y. 1994. Expression of a soybean (*Glycine max* [L.] Merr.) seed storage protein gene in transgenic *Arabidopsis thaliana* and its response to nutritional stress and to abscisic acid mutations. *Plant Physiol.* 104:497-503.
- Nampally, M., Moerschbacher, B. M., and Kolkenbrock, S. 2012. Fusion of a novel genetically engineered chitosan affinity protein and green fluorescent protein for specific detection of chitosan in vitro and in situ. *Appl. Environ. Microbiol.* 78:3114-3119.
- Neeraja, C., Moerschbacher, B., and Podile, A. R. 2010. Fusion of cellulose binding domain to the catalytic domain improves the activity and conformational stability of chitinase in *Bacilluslicheniformis* DSM13. *Bioresour. Technol.* 101:3635-3641.
- Okada, M., Matsumura, M., Ito, Y., and Shibuya, N. 2002. High-affinity binding proteins for N-acetylchitooligosaccharide elicitor in the plasma membranes from wheat, barley and carrot cells: Conserved presence and correlation with the responsiveness to the elicitor. *Plant Cell Physiol.* 43: 505-512.
- Petutschnig, E. K., Jones, A. M. E., Serazetdinova, L., Lipka, U., and Lipka, V. 2010. The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *J. Biol. Chem.* 285:28902-28911.
- Ranf, S. 2017. Sensing of molecular patterns through cell surface immune receptors. *Curr. Opin. Plant Biol.* 38:68-77.
- Sánchez-Vallet, A., Mesters, J. R., and Thomma, B. P. H. J., 2015. The battle for chitin recognition in plant-microbe interactions. *FEMS Microbiol. Rev.* 39:171-183.
- Sánchez-Vallet, A., Saleem-Batcha, R., Kombrink, A., Hansen, G., Valkenburg, D.-J., Thomma, B. P. H. J., and Mesters, J. R. 2013. Fungal effector Ecp6 outcompetes host immune receptor for chitin binding through intrachain LysM dimerization. *eLife* 2:e00790.
- Senstad, C., and Mattiasson, B. 1989. Purification of wheat germ agglutinin using affinity flocculation with chitosan and a subsequent centrifugation or flotation step. *Biotechnol. Bioeng.* 34:387-393.
- Shibuya, N., and Minami, E. 2001. Oligosaccharide signalling for defence responses in plant. *Physiol. Mol. Plant Pathol.* 59:223-233.
- Shimizu, T., Nakano, T., Takamizawa, D., Desaki, Y., Ishii-Minami, N., Nishizawa, Y., Minami, E., Okada, K., Yamane, H., Kaku, H., and Shibuya, N. 2010. Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant J.* 64:204-214.
- Shinya, T., Nakagawa, T., Kaku, H., and Shibuya, N. 2015. Chitin-mediated plant-fungal interactions: Catching, hiding and handshaking. *Curr. Opin. Plant Biol.* 26:64-71.
- Tang, D., Wang, G., and Zhou, J.-M. 2017. Receptor kinases in plant-pathogen interactions: More than pattern recognition. *Plant Cell* 29: 618-637.
- Trombotto, S., Ladavière, C., Delolme, F., and Domard, A. 2008. Chemical preparation and structural characterization of a homogeneous series of chitin/chitosan oligomers. *Biomacromolecules* 9:1731-1738.
- Vander, P., Vårum, K. M., Domard, A., El Gueddari, N. E., and Moerschbacher, B. M. 1998. Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. *Plant Physiol.* 118:1353-1359.
- Wan, J., Zhang, X.-C., Neece, D., Ramonell, K. M., Clough, S., Kim, S. Y., Stacey, M. G., and Stacey, G. 2008. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell* 20:471-481.
- Wong, J. E. M. M., Midtgaard, S. R., Gysel, K., Thygesen, M. B., Sørensen, K. K., Jensen, K. J., Stougaard, J., Thirup, S., and Blaise, M. 2015. An intermolecular binding mechanism involving multiple LysM domains mediates carbohydrate recognition by an endopeptidase. *Acta Crystallogr. D Biol. Crystallogr.* 71:592-605.
- Zhang, B., Ramonell, K., Somerville, S., and Stacey, G. 2002. Characterization of early, chitin-induced gene expression in *Arabidopsis*. *Mol. Plant-Microbe Interact.* 15:963-970.
- Zipfel, C., and Oldroyd, G. E. D. 2017. Plant signalling in symbiosis and immunity. *Nature* 543:328-336.