Role of LysM receptors in chitin-triggered plant innate immunity

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Recent research findings clearly indicate that lysin motif (LysM)-containing cell surface receptors are involved in the recognition of specific oligosaccharide elicitors (chitin and peptidoglycan), which trigger an innate immunity response in plants. These receptors are either LysM-containing receptor-like kinases (LYKs) or LysM-containing receptor proteins (LYPs). In Arabidopsis, five LYKs (AtCERK1/AtLYK1 and AtLYK2–5) and three LYPs (AtLYP1–3) are likely expressed on the plasma membrane. In this review, we summarize recent research results on the role of these receptors in plant innate immunity, including the recent structural characterization of AtCERK1 and composition of the various receptor complexes in Arabidopsis.

Multicellular organisms activate immune systems upon recognition of microbe-derived non-self components, known as microbe elicitors or microbe-associated molecular patterns (MAMPs; synonymously termed pathogen-associated molecular patterns, PAMPs), which are invariant structures originating from microbial components and not present in the host. MAMPs are usually recognized by pattern recognition receptors (PRRs) on the cell surface triggering plant innate immunity responses.[1-3]

Oligosaccharide MAMPs, mostly microbial cell envelope components, are represented by bacterial lipopolysaccharide (LPS), peptidoglycan (PGN) and fungal chitin.[4] LPS is an outer membrane glycoconjugate from Gram-negative bacteria that is composed of a lipid and a polysaccharide joined by a covalent bond. Plant cells likely sense the sugar and lipid components of LPS separately.[5] However, the plant LPS receptor(s) has not been identified. PGN is an essential cell wall component in both Gram-positive and Gram-negative bacteria. The structure of PGN is similar to chitin being composed of alternating residues of β-1,4-linked N-acetyl-glucosamine (GlcNAc) and N-acetylmuramic acid, with a short peptide chain attached. PGNs from both Gram-positive and Gram-negative bacteria can elicit defense responses in plants.[6-8] Chitin is a homopolymer of β-1,4-linked GlcNAc (chitooligosaccharides), the major structural component of fungal cell walls, and is a potent elicitor on plants.[9,10] During plant-pathogen interaction, the fungal cell wall is degraded by chitinases releasing the chitooligosaccharide MAMP elicitors.[4] The PRRs for both PGN and chitin have been identified as lysin motif (LysM)-containing proteins as described below.

The first chitin receptor was identified in rice, the chitin elicitor-binding protein (CEBiP), encoding a LysM receptor protein, which lacks an intracellular kinase domain.[11] Subsequently, in Arabidopsis, the primary chitin receptor was identified as the chitin elicitor receptor kinase 1 (CERK1), which encodes a protein with extracellular LysM-domains, a transmembrane domain and an active, intracellular kinase domain.[12,13] Both in rice and in Arabidopsis, these respective receptors were found to be essential for chitin-triggered innate immunity. For example, mutants in these receptors are compromised in their defense against fungal pathogens, indicating that perception of chitin fragments plays a critical role in pathogen resistance.[11-13] All the chitin and PGN receptors identified to date contain one or more extracellular LysM domains.[14,15] The LysM domain was first identified in bacterial enzymes involved in remodeling peptidoglycan structure.[16-18] Consistent with a functional role in binding PGN, LysM-containing proteins (LYPs) were identified as plant PGN receptors that directly bind to PGNs.[19,20] Mutations in these PGN receptor genes completely blocked the plant response to PGN elicitation.

AtCERK1/AtLYK1 is a Major Chitin Receptor, Which is Also Required for PGN Recognition

In Arabidopsis, the cell surface receptor AtCERK1 (also termed LysM-containing receptor-like kinase1, LYK1) is an essential PRR for sensing fungal-derived chitooligosaccharides and for immunity to fungal infection.[12,13] AtCERK1 binds chitooligosaccharides by its extracellular LysM domains and presumably initiates intracellular signal transduction through activation of its cytoplasmic protein kinase domain.[21,22] Interestingly, the AtCERK1 receptor is the first PRR that lacks a non-arginine-aspartate (non-RD) signaling domain; it has a typical RD signaling domain in its catalytic loop (**Fig. 1**) and possesses autophosphorylation activity.[22]

In rice, Arabidopsis, and most plants, maximal activation of innate immunity requires longer-chain chitin oligomers [degree of polymerization (dp) = 7–8 GlcNAc residues].[22-24] However,
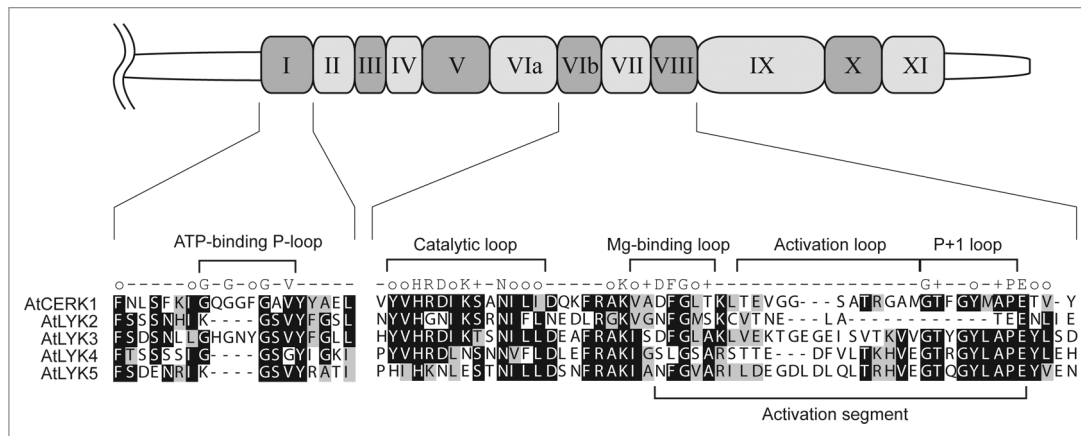
Figure 1. Sequence comparison of the kinase domain of Arabidopsis LYK proteins. Schematic representation shows the kinase domain of AtCERK1 (drawn to scale). Kinase subdomain I and VIb to VIII are selectively highlighted below the drawing to highlight the five AtLYK proteins. Identical and similar residues throughout the alignment are shown in black and gray, respectively. The consensus line among the eukaryotic protein kinase super-family[47] is given according to the following code: uppercase letters, invariant residues; o, conserved nonpolar residues; +, conserved small residues with near neutral polarity. Note that AtCERK1 and AtLYK3 have an intact intracellular kinase domain.

shorter-chain oligomers (dp < 6) do bind to AtCERK1.[21,22] Very recently, Liu et al.[25] elucidated the X-ray crystal structure of the extracellular domain of AtCERK1, as well as characterized the chitooligosaccharide-binding activity of this protein using a variety of methods (isothermal calorimetric analysis, etc.). These results indicate that the chitotetraose binds exclusively to the second LysM domain, bracketed by the two additional LysM domains found in the AtCERK1 protein. The binding affinities of AtCERK1 for chitin oligomers were found to be in the low µM range, which is inconsistent with the very high affinities (nM range) suggested by physiological experiments that measure the plant response to chitin elicitation. Also inconsistent with physiological experiments, the affinity for short-chain chitin oligomers (dp = 5) was roughly similar to that of the long-chain oligomers (dp = 8). Currently, there is no explanation for the differences seen in the affinity of chitooligosaccharide binding to the purified AtCERK1 and the apparent high affinity suggested by measuring the plant response to chitin elicitation.

The structural studies do provide an explanation as to why only the longer chain oligomers are strong inducers of innate immunity. Liu et al.[25] showed that, while AtCERK1 binds to the short-chain chitin oligomers, only the long-chain chitin oligomers (dp = 7 or 8) induce homodimerization of the receptor, which was shown to be essential for activation of downstream signaling. In contrast to the situation in Arabidopsis, the functional role of OsCERK1 appears to be quite different. In rice, OsCERK1 does not bind to chitooligosaccharides. Instead, the co-receptor, OsCEBiP is essential for chitin binding, which leads to heterodimerization of the receptor complex and subsequently activation of innate immunity.[26] Unlike AtCERK1 that contains three LysM domains, the OsCERK1 contains only one conserved LysM domain.[26] Given the fact that the requirement of three LysM domains is essential for chitin binding,[22] the differences in the chitin perception systems between rice and Arabidopsis may be explained by the structural differences between AtCERK1 and OsCERK1, although these two proteins do appear to be

orthologous based on sequence comparisons.[26] The rice model may also apply to other grass species[27] since, for example, a barley homolog of OsCEBiP, HvCEBiP, was recently shown to contribute to fungal resistance.[28]

The rice chitin receptor complex, being composed of OsCERK1 and OsCEBiP, is more similar to the proposed PGN receptor complex in Arabidopsis. In this latter case, PGN recognition requires two, non-redundant LYPs, AtLYP2/LYM1 and AtLYP3/LYM3, but also AtCERK1.[20] However, direct interaction between these proteins has yet to be shown experimentally. The rice PGN receptor complex is likely similar to that in Arabidopsis since recent work showed that OsLYP4 and OsLYP6, the closest homologs of AtLYP2 and AtLYP3 in rice, are essential for perception of PGN and chitin.[19] OsLYP4 and OsLYP6 are distinct proteins from OsCEBiP (OsLYP1). A role of OsCERK1 in PGN recognition in rice has not been reported.

Five AtLYK and Three AtLYP Proteins

Published data clearly show that AtCERK1 is the major chitin receptor in Arabidopsis and essential for the induction of innate immunity upon chitin elicitation. However, Arabidopsis also has other LysM receptor proteins and, therefore, what is their function, relative to chitin or PGN recognition and innate immunity? We here designate the Arabidopsis LYKs as AtCERK1/AtLYK1 and AtLYK2–5, and LYPs as AtLYP1–3 to avoid any confusion (**Table 1** shows a summary); a nomenclature based on our earlier publication.[14,15]

As mentioned above, AtLYP2 and AtLYP3 are involved in PGN recognition and play indispensable roles for immunity to bacterial infection. Two independent studies demonstrated that neither of these proteins, in contrast to the rice LYPs, binds to chitin oligosaccharides.[20,29] Therefore, AtLYP2 and AtLYP3 appear to specifically respond to bacterial PGN elicitors.

Three different labs reported that, in addition to AtCERK1, other LysM-containing receptors, AtLYK4, AtLYK5 and AtLYP1

are able to bind to chitin molecules.[22,29,30] AtLYP1, an OsCEBiP homolog, showed high-affinity binding as measured using biotinylated chitooligosaccharides.[29] The binding characteristics of AtLYP1 were very similar to that of OsCEBiP. However, mutants disrupted in the expression of AtLYP1, AtLYP2 and AtLYP3, either singly or in combination, showed no difference from the wild-type in their response to chitin elicitation.[29,30] Therefore, it would appear that none of the Arabidopsis OsCEBiP homologs are necessary for chitin recognition. Similar mutant studies demonstrated that AtLYK2, AtLYK3 and AtLYK5 do not appear to be involved in chitin recognition.[30]

AtLYK4 is Important for Chitin-Mediated Innate Immunity

Wan et al.[30] recently reported that a knockout mutation of AtLYK4 reduced, but did not eliminate, the response to chitin elicitation, e.g., induction of chitin-responsive gene expression and elevation of cytosolic calcium levels. In contrast, a knockout mutant of AtCERK1 shows essentially no response to chitin elicitation. The data suggest that, while not essential for the chitin response, AtLYK4 is involved in chitin recognition, perhaps as a co-receptor to increase AtCERK1 affinity or activity. As would be expected, AtLYK4 mutant plants showed enhanced susceptibility to both the fungal pathogen *Alternaria brassicicola* and the bacterial pathogen *Pseudomonas syringae* pv tomato DC3000. These data raise the possibility that AtLYK4 may also be involved in responding to bacterially produced MAMPs (e.g., PGN). However, this hypothesis remains to be tested.

An analysis of Arabidopsis plants expressing a *AtLYK4* promoter-GUS construct showed that this gene is expressed in most tissues (except for flowers, pollens and siliques).[30] Interestingly, in leaves, *AtLYK4* is predominantly expressed in hydathodes.[30] Hydathodes are open pores located on the margin of leaves, which permit the discharge of excess water from the plant, but lack structural barriers against pathogens. A number of pathogens likely enter leaves through hydathodes in addition to leaf stomatal openings.[31] In fact, chitinases and other genes related to the defense response are highly expressing in hydathodes, presumably to position them to respond to the invading pathogens.[32,33] The restricted expression of the *AtLYK4* gene in the hydathodes contrasts with that of *AtCERK1*, which is expressed throughout the leaf tissue. This difference suggests that the rather moderate phenotype of *lyk4* mutant plants, relatively to *cerk1* mutants, could be due to the restricted expression of AtLYK4. If true, this might suggest that other LysM receptor proteins could substitute for the role of AtLYK4 depending on their specific pattern of tissue expression. Such functional redundancy among LysM receptors would also explain why mutations in the various receptor genes result in no observable phenotypic changes in the plants relative to the chitin response.

Unlike AtCERK1, in vitro assays failed to demonstrate kinase activity in purified preparations of AtLYK4.[30] These results are supported by an analysis of the AtLYK4 protein sequence that shows the lack of critical residues necessary for kinase catalytic activity (**Fig. 1**). For example, AtLYK4 differs significantly from AtCERK1 in the ATP-binding P-loop (GxGxF/YG) and Mg-binding loop (DFG). If AtCERK1 and AtLYK4 do indeed form a receptor complex then this would resemble the receptor complex involved in recognition of lipo-chitooligosaccharides in the legume *Lotus japonicus*.[34] Recognition of the lipo-chitooligosaccharide Nod factor, produced by the legume bacterial symbiont, is essential for formation of the nitrogen-fixing symbiosis. The Nod factor receptor complex is composed of LjNFR1, an active kinase homolog of AtCERK1, and LjNFR5, an inactive kinase homolog of AtLYK4. Therefore, LjNFR5 and AtLYK4 appear to be (lipo-) chitooligosaccharide-binding receptor-like kinases with a pseudo kinase domain (categorized as LysM-RLK II or LYR; see **Table 1**).[35,36] While genetic data suggest that AtLYK4 may partner with AtCERK1 to compose the chitin receptor, another possible partner could be AtLYK3, which also possesses a functional, intracellular kinase domain (**Figs. 1 and 3**). However, AtLYK3 has no known function since mutations do not appear to affect plant susceptibility to either fungal or bacterial pathogens.[30]

As mentioned above, independent laboratories showed that AtLYK4 can bind to chitin.[22,29,30] Therefore, utilizing the published X-ray crystal structure of AtCERK1, we modeled the structure of the AtLYK4 extracellular domain (**Fig. 2A**). The computer modeling was performed as described previously.[37] This model revealed that AtLYK4 uses fewer and different amino acid residues than AtCERK1 to interact with chitotetraose at the second LysM domain (**Fig. 2C and D**), suggesting that AtLYK4 likely has lower binding affinity to chitotetraose than AtCERK1. Indeed, the AutoDock program calculates a binding affinity of -8.6 kcal/mol for AtLYK4 and -9.2 kcal/mol for AtCERK1, respectively. However, the predicted model shows that the second LysM domain has a very similar structure between AtLYK4 and AtCERK1 (the root-mean-square deviation values are 0.648 Å, for second LysM doamin and 0.893 Å, for whole extracellular domain, respectively) (**Fig. 2B**). This prediction of a lower affinity for AtLYK4 is consistent with the previous reports that this protein showed a weaker interaction with a chitin bead column than AtCERK1.[22,30]

Perspectives

Recent research findings have provided a significant amount of additional detail regarding the role of LysM receptors (especially AtCERK1, AtLYK4, AtLYP2 and AtLYP3) in chitin and PGN recognition, as well as their role in plant innate immunity. However, there are still many unanswered questions regarding the exact composition of the respective receptor complexes, other auxiliary proteins, the mechanism of signaling and other components of the signaling cascade leading ultimately to enhanced disease resistance.

Although it is clear that some LysM receptor proteins have affinity for chitin-like molecules, the biological/biochemical function of the other Arabidopsis LysM receptor proteins remains enigmatic (i.e., AtLYK2, AtLYK3, AtLYK5 and AtLYP1). In these cases, mutant studies have not suggested a functional role for these proteins. However, sequence analysis and biochemical assays indicate that AtLYK5 and AtLYP1 likely recognize chitin molecules and AtLYK3 likely possesses a functional, intracellular kinase domain. All of these proteins are presumably located

Table 1. LysM-containing receptors in Arabidopsis

Gene name (other names)	Locus	LysM domain arrangement[a]	Ligand	Receptor Type[f]	Functional kinase? (kinase type)	Mutant phenotype to chitin treatment[i]	Note
AtCERK1	At3g21630	I + II + IV	Chitin[b]	LysM-RLK-I	Yes[g]	Insensitive	Also involved in PGN perception[b]
(AtLYK1, LysM RLK1)				LYK	(RD kinase)		LjNFR1 paralog[a]
AtLYK2	At3g01840	* + * + V	Unknown	LysM-RLK-II	No[h]	Normal	LjNFR5 paralog II[a]
				LYR	(Pseudo kinase)		
AtLYK3	At1g51940	* + VII + *	Unknown	LysM-RLK-I	Yes[h]	Normal	
				LYK	(RD kinase)		
AtLYK4	At2g23770	I + II + III	Chitin[c]	LysM-RLK-II	No[g]	Moderately insensitive	LjNFR5 paralog I[a]
				LYR	(Pseudo kinase)		
AtLYK5	At2g33580	I + II + III	Chitin[c]	LysM-RLK-II	No[h]	Normal	
				LYR	(Pseudo kinase)		
AtLYP1	At2g17120	* + VI + VII	Chitin[c, d]	LYP	-	Normal	Contains a C-terminal GPI anchor signal
(CEBiP-like1, LYM2)							Ortholog of OsCEBiP
AtLYP2	At1g21880	* + VI + VIII	PGN[e]	LYP	-	Normal	Contains a C-terminal GPI anchor signal
(CEBiP-like2, LYM1)							Ortholog of OsLYP4 and OsLYP6
AtLYP3	At1g77630	* + VI + VIII	PGN[e]	LYP	-	Normal	Contains a C-terminal GPI anchor signal
(CEBiP-like3, LYM3)							Ortholog of OsLYP4 and OsLYP6

[a]Classified by Zhang et al.[14,15] Putative LysM domains with less sequence conservation are represented by asterisk (*). [b]Based on the direct-binding assay.[21,25] Shown involvement of AtCERK1 in PGN perception, but unable to bind directly to PGNs.[20] Also shown to weakly bind to deacetylated chitosan by affinity column-binding assay.[22] [c]Detected in chitin affinity column from plant crude extracts.[22,30] [d]Based on the chitin-binding assay using tobacco BY-2 cells.[29] [e]Shown by directly bind assay to PGNs.[20] [f]Categorized based on kinase domain features.[35,36] [g]Confirmed by in vitro kinase assay.[12,13] [h]Predicted based on protein sequence alignment analysis (see **Fig. 1**). [i]Based on the gene expression result of chitin-responsive genes.[30] Abbreviations: LYK, LysM receptor-like kinase; LYR, LYK-related; CERK, Chitin elicitor receptor kinase; CEBiP, Chitin elicitor binding protein; LYP, LysM receptor-like protein; LYM, LysM domain-containing GPI-anchored protein; PGN, peptidoglycan.

in the plasma membrane due to the presence of an N-terminal signal peptide and a transmembrane segment or a GPI anchor signal sequence for membrane attachment (**Table 1** and **Fig. 3**). However, this prediction needs to be confirmed by experimentation. Unlike AtCERK1, AtLYK2 and AtLYK3 have only one conserved LysM domain in their extracellular domain (**Table 1** and **Fig. 3**). Whether this would disrupt interaction with an extracellular ligand is not clear. AtLYK2 and AtLYK5 likely lack an active, intracellular kinase domain (**Fig. 1**), suggesting that they may function in conjunction with a co-receptor. It may be that one or more of these proteins do function in MAMP signaling, which is being masked in mutant studies due to functional redundancy. It is still possible that these receptors are functioning in a spatial or temporal manner,[38] which would make the identification of a mutant phenotype difficult. It is possible that one or more of these LysM-domain proteins could bind MAMPs but not induce downstream signaling, for example, as a decoy receptor. Such an example has been found for plant recognition of the fungal elicitor xylanase where only one receptor (Eix2) mediates defense signaling while the other (Eix1) acts as a decoy receptor to attenuate the MAMP response.[39,40] Decoy receptors are well-known in mammals,[41-43] in which a decoy receptor manipulates the signaling of a cognate receptor by competing for ligand binding resulting in inhibition of downstream signaling to regulate tumor proliferation and cell death. If acting as a decoy, the LysM receptor protein could attenuate the MAMP response by titrating the elicitor in the extracellular matrix. Alternatively, it is still possible that these LYKs or LYPs recognize different size chitin oligomers, since it is possible that different oligomers could have distinct functions,[44,45] as shown for chitin recognition by mammalian macrophages.[46] Further studies are required to disclose the nature of molecular functions of all LysM receptors in plant innate immunity.

Disclosure of Potential Conflicts of Interest

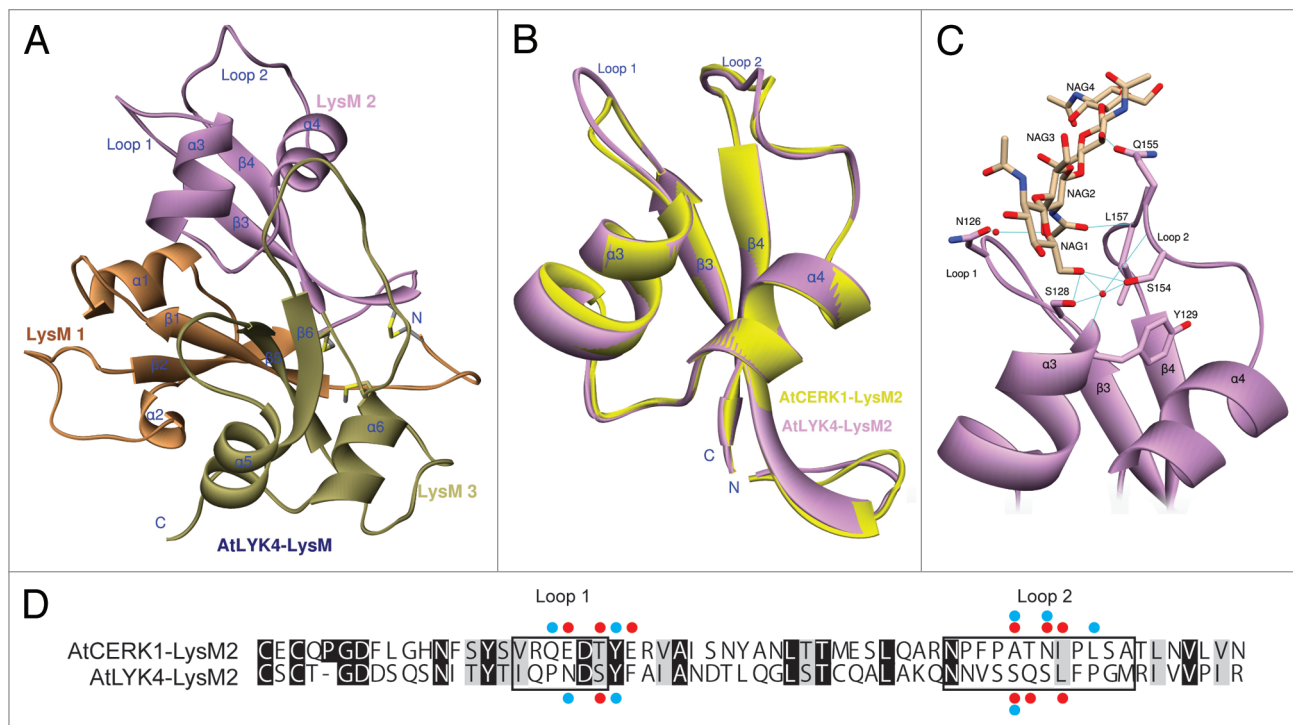No potential conflicts of interest were disclosed.

Figure 2. Three-dimensional model prediction of the structure of the AtLYK4 extracellular domain, including a ligand docking model. (**A**) The 3D model of AtLYK4 extracellular domain was built based on the crystal structure of AtCERK1 (PDB code: 4EBY). Each LysM domain is represented in a different color: first LysM (orange), second LysM (purple), and third LysM (green). (**B**) The second LysM domains of AtLYK4 (purple) and AtCERK1 (yellow) are superimposed to highlight the similarity in structure. Note that the overall folds are highly conserved between the two models, although the AtLYK4 has the longer extended Loop 1 which is a constitutive part of the cleft where the predicted chitin binding site is found. (**C**) Docking model between the second LysM domain of AtLYK4 and chitotetraose. (**D**) Pairwise sequence comparison of the second LysM domains of AtLYK4 and AtCERK1. Identical and similar residues throughout the alignment are shown in black and gray, respectively. Enclosed boxes represent Loop 1 and Loop 2. Red and blue dots indicate the residues involved in direct interactions and water molecule-mediated interactions with chitotetraose, respectively. The residues involved in van der Waals interactions were neglected here.

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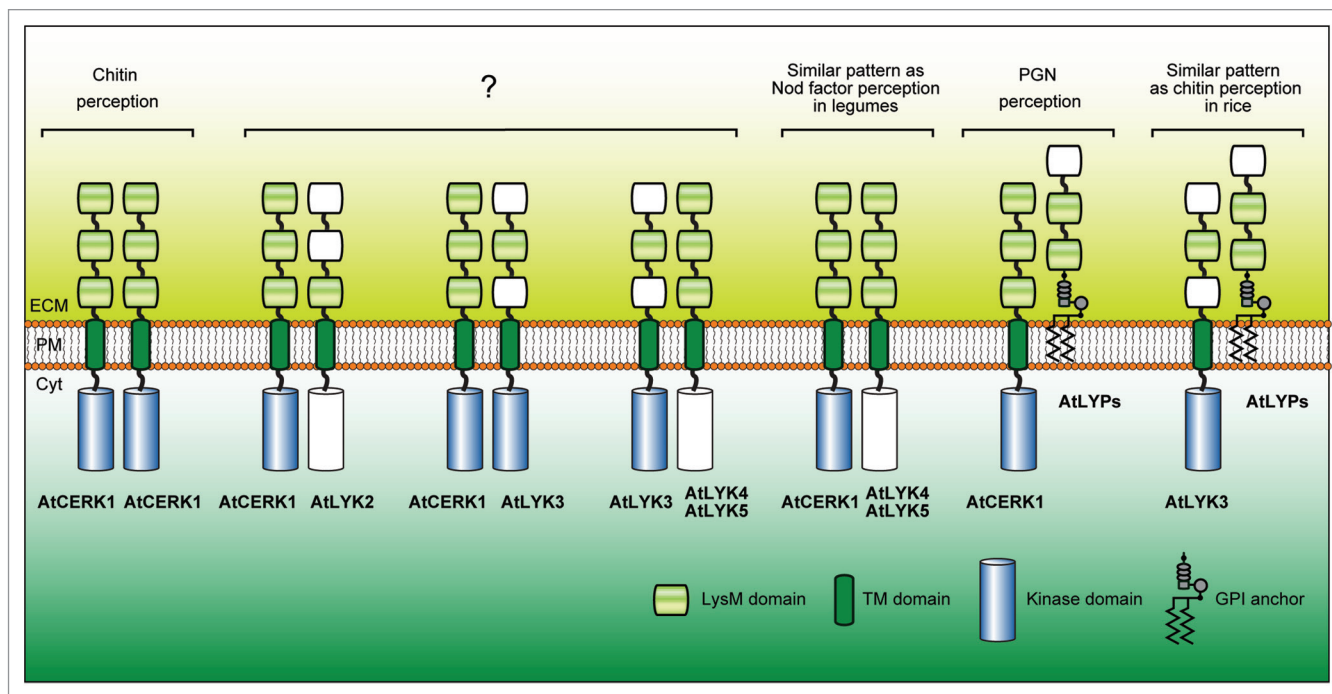
Figure 3. Possible combinations of Arabidopsis LysM receptor proteins involved in oligosaccharide MAMP recognition. The picture represents theoretical combinations of LysM receptors with a prerequisite condition that two or more LysM domains are present in the extracellular region and that at least one of the receptors possess an active, intracellular kinase domain. Putative LysM domains with less sequence conservation and pseudo kinase domains are drawn colorless. Note that AtCERK1 is a unique LysM receptor in Arabidopsis since it alone can form a homodimer given the prerequisites imposed. Abbreviations: LysM, lysin motif; TM, transmembrane; GPI, glycosylphosphatidylinositol; ECM, extracellular matrix; PM, plasma membrane; Cyt, cytoplasm.

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