Plants induce immune responses against fungal pathogens by recognition of chitin, which is a component of the fungal cell wall. Recent studies have revealed that LysM receptor-like kinase 1/chitin elicitor receptor kinase 1 (LysM RLK1/CERK1) is a critical component for the immune responses to chitin in Arabidopsis thaliana. However, the molecular mechanism of the chitin recognition by LysM RLK1 still remains unknown. Here, we present the first evidence for direct binding of LysM RLK1 to chitin. We expressed LysM RLK1 fused with yeast-enhanced green fluorescent protein (LysM RLK1-yEGFP) in yeast cells. Binding studies using the solubilized LysM RLK1-yEGFP and several insoluble polysaccharides having similar structures showed that LysM RLK1-yEGFP specifically binds to chitin. Subsequently, the fluorescence microscopic observation of the solubilized LysM RLK1-yEGFP binding to chitin beads revealed that the binding was saturable and had a high affinity, with a $K_d$ of ~82 nM. This binding was competed by the addition of soluble glycol chitin or high concentration of chitin oligosaccharides having 4–8 residues of N-acetyl glucosamine. However, the competition of these chitin oligosaccharides is weaker than that of glycol chitin. These data suggest that LysM RLK1 has a higher affinity for chitin having a longer residue of N-acetyl glucosamine. We also found that LysM RLK1-yEGFP was autophosphorylated in vitro and that chitin does not affect the phosphorylation of LysM RLK1-yEGFP. Our results provide a new dimension to chitin elicitor perception in plants.

When a plant is attacked by pathogens, it rapidly induces immune responses to stop the infection. A key step of the rapid induction of immune responses is a prompt and efficient detection of microbial invaders. In plants, this is achieved by pattern recognition receptors that recognize the conserved structures of the microbial pathogens. These conserved microbial structures are called pathogen-associated molecular patterns.

The pathogen-associated molecular patterns recognized by plants include lipopolysaccharides, peptidoglycan (PGN), flagellin, and bacterial elongation factor-Tu, which are derived from the bacteria (1, 2). Plants also recognize fungal pathogen-associated molecular patterns, such as chitin and ergosterol, which are components of the fungal cell wall and plasma membrane, respectively (1, 3). Recent studies have discovered the plant pattern recognition receptors participating in the perception of chitin elicitor (4–6).

A LysM motif-containing plasma membrane protein, CEBiP (chitin oligosaccharide elicitor-binding protein), participates in the perception of chitin oligosaccharides in rice (4). The CEBiP has two LysM motifs in the extracellular domain and lacks the intracellular kinase domain that is required for signal transduction. It has been demonstrated that CEBiP directly binds to chitin oligosaccharides and plays an essential role in the perception of chitin and the induction of immunity in rice (4).

On the other hand, more recently, two groups have independently reported that a LysM receptor-like kinase (RLK) plays a key role in chitin perception in Arabidopsis thaliana (5, 6). This receptor, LysM receptor-like kinase 1/chitin elicitor receptor kinase 1 (LysM RLK1/CERK1), is localized in the plasma membrane and has three LysM motifs in the extracellular domain as well as the intracellular kinase domain. Although these studies demonstrated that LysM RLK1 plays a crucial role in the immune response against fungal pathogens, the molecular mechanism of interactions of LysM RLK1 and chitin is still unknown.

The LysM motifs are known as GlcNAc-binding motifs, which occur frequently in bacterial lysins and bind to PGN (7). In plants, this motif is also present in a chitinase and directly binds to chitin oligosaccharides (8, 9).

Although LysM RLK1 has three LysM motifs in the extracellular domain, it remains unclear whether LysM RLK1 directly binds to chitin or acts via cooperation with another protein. An affinity-labeling experiment was unable to detect any specific binding protein to chitin in a membrane preparation from A. thaliana, although the same technique could detect CEBiP in plasma membrane of rice (5). Therefore, we investigated whether LysM RLK1 directly binds to chitin.

To exclude the potential effects of other plant components, we used the recombinant LysM RLK1 protein expressed in the yeast, Saccharomyces cerevisiae. Yeast is superior to Escherichia...
coli for the expression of recombinant membrane proteins because it has a highly regulated protein quality control system in the endoplasmic reticulum (10). In this study, we demonstrated the specific and direct binding of LysM RLK1 to chitin in vitro by binding assays using recombinant LysM RLK1 fused with yeast-enhanced green fluorescence protein (LysM RLK1-yEGFP). We also found that the full length of LysM RLK1-yEGFP was autophosphorylated without chitin and that chitin did not affect the phosphorylation of LysM RLK1-yEGFP in vitro.

**EXPERIMENTAL PROCEDURES**

Chitin and Chitosan Derivatives—chitin beads were purchased from New England Biolabs. Chitin oligosaccharides (degrees of polymerization = 2–6) and a chitosan oligosaccharide (GlcNAc)9 were purchased from Seikagaku Kogyo. Colloidal chitin and colloidal chitosan were prepared as reported previously (11, 12). Glycol chitin was prepared from chitin described previously (18). For the removal of imidazole, the French press, and yEGFP was purified by a nickel column as described previously (16).

Preparation of [14C]Glycol Chitin—Glycol chitin was acetylated with [1-14C]acetic anhydride (American Radiolabeled Chemicals, Inc.) as follows. Three hundred μl of 0.04 mM Na2CO3, 250 μl of methanol, and 3 μl of [1-14C]acetic anhydride (5 mCi/mmol) were added to 2 mg of glycol chitosan. After a 24-h incubation at 25 °C, the sample was passed through the Dowex 50 column. The elution evaporated three times with methanol to remove the unreacted [1-14C]acetic acid. After the evaporation, the [14C]glycol chitin was dissolved in water.

Expression and Purification of yEGFP—yEGFP was expressed in E. coli BL21 Star (DE3) (Invitrogen). First, yEGFP was cloned into pET43a(+) (Novagen, Madison, WI). To fuse yEGFP with an octahistidine tag at the C terminus and to remove an N-terminal Nus tag, the yeast recombinational cloning was carried out as reported previously (17), except that, instead of pSU0, pYES2/CT (Invitrogen) was used as a helper plasmid for the expression of yEGFP and to remove an N-terminal octahistidine tag at the C terminus. First, the cDNA corresponding to LysM RLK1 was created by reverse transcription-PCR with SuperScript II reverse transcriptase (Invitrogen) using a gene-specific primer (5'-ATATAAATTTTCCCC-CCGGCCGACATAAGACTGA-3') from 0.5 μg of the total RNA of A. thaliana (19). The open reading frame of LysM RLK1 was obtained after two rounds of PCR using PrimeSTAR GXL DNA Polymerase (TAKARA BIO, Ohtsu, Shiga, Japan). The first round of PCR amplified the open reading frame with the gene-specific primers 5'-ACTAGTGATCCCCCAT- GAAGCTAAGATTCTCT-3' and 5'-ATATAAATTTTCCCAGGCGGACATAAGACTGA-3', whereas the second round of PCR amplified it with the primers 5'-TCGACGGAT-TCTAGAACTAGTGATCCCCC-3' and 5'-AAATTGACCTTGAAGATAATATTTCCCT-3' for providing this fragment with the ends homologous to pDDGFP2. Subsequently, the LysM RLK1 gene was subcloned into pDDGFP2 by the yeast recombinational cloning (10). The plasmid obtained from the yeast cells was cloned and propagated in E. coli DH5α (TOYOBO, Osaka, Japan) as described previously (17). The sequence of insert was confirmed by a DNA sequencer (Applied Biosystems, Foster City, CA).

Expression and Purification of LysM RLK1-yEGFP Protein—The pDDGFP2 harboring LysM RLK1 (pDDGFP2-LysM RLK1) was retransformed into the S. cerevisiae strain BY2777 (MATa prb1-1122 proc1-107 pep4-3 ura3-52 leu2 trp1). Subsequently, for the expression of LysM RLK1-yEGFP, the transformants were grown in SC-Ura liquid medium containing 2% glucose, and the protein expression was induced as reported previously (10). The cells were harvested by centrifugation at 3000 × g for 5 min at 4 °C and were suspended in the lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.6 M sorbitol, 1 mM EDTA, and protease inhibitor mixture, complete EDTA-free (Roche Applied Science). After the addition of the same volume of chilled acid-washed glass beads, the cells were broken by three rounds of a Fast Prep cell disruptor (BIO 101, Vista, CA) at 5.5 ms−1 for 20 s with 2-min intervals on ice. After lysis, the lysate was centrifuged at 3000 × g for 10 min. The supernatant was further centrifuged at 21,040 × g for 1 h at 4 °C to pellet the membranes. The crude membranes were solubilized, and LysM RLK1 proteins were purified as reported previously (10); except that a detergent, lauryl dimethylamine N-oxide (LDAO), was used in solubilization and purification instead of N-dodecyl-β-D-maltoside. To remove imidazole, the eluted protein was dialyzed against 300 volumes of the binding buffer containing 50 mM Tris-HCl (pH 7.0), 0.05% LDAO, 150 mM NaCl, 5 mM MgCl₂, 0.1 mM phenylmethylsulfonyl fluoride for 4 h at 4 °C with five buffer changes. The concentration of LysM RLK1-yEGFP protein was determined by comparing it with a yEGFP standard curve. The standard curve was generated by using known amounts of recombinant yEGFP purified from E. coli. 100 μl of the samples were transferred to a 96-well plate, and the yEGFP fluorescence in each well was read using a multimode plate reader (model DTX880, Beckman (Krefeld, Germany)), and the following settings were used: fluorescence intensity bottom method, 0.1-s integration time, 485-nm excitation filter, and 535-nm emission filter.

Analysis of the Proteins by In-gel Fluorescence—Protein samples were mixed with SDS sample buffer and then incubated at 45 °C for 3 min. After incubation, the samples were analyzed by SDS-PAGE. The GFP fluorescence was measured using a
Typhoon 9200 fluorescence imager (GE Healthcare). The SDS-polyacrylamide gel was scanned using the green laser (532 nm) excitation source, and the resulting fluorescence emitted (526 nm, short pass filter) was recorded using a PMT (a value for sensitivity) of 800 V at a resolution of 100 μm. The in-gel fluorescence analysis was followed by CBB staining, using CBB G-250.

**Ligand Specificity Assay**—The binding specificity of LysM RLK1 protein for various ligands was examined by a polysaccharide binding assay, which was performed as reported previously (20, 21) with minor modifications. Thirty pmol of purified LysM RLK1-yEGFP or yEGFP (negative control) was mixed with 0.25, 0.5, 2.5, 5, 12.5, and 25 μg/ml [14C]glycol chitin, 1 mm dithiothreitol, 50 μM unlabeled ATP, and 370 kBq of [γ-32P]ATP (111 TBq/mmol; PerkinElmer Life Sciences). The reactions were terminated by adding SDS sample buffer and boiled for 3 min, and the samples were separated by SDS-PAGE. The phosphorylated proteins were visualized by autoradiography after the gels were dried under the vacuum.

**RESULTS**

**Expression and Purification of LysM RLK1-yEGFP**—LysM RLK1 was expressed fused with yEGFP and octahistidine tag at the C terminus in *S. cerevisiae*. The use of yEGFP allows easy and specific detection of the target protein without antibodies and makes optimization of the protein expression and purification easy. We expressed the LysM RLK1-yEGFP fusion protein in *S. cerevisiae* strain, BY2777, following the procedure described previously (10). After the induction, we could detect the yEGFP fluorescence on the plasma membrane of the cells by a fluorescence microscope (data not shown). The membrane proteins were then analyzed by the in-gel fluorescence after SDS-PAGE, and an ~94 kDa protein band corresponding to the LysM RLK1-yEGFP was detected in the membrane fraction (Fig. 1, left, lane 1).

Subsequently, to select an adequate detergent for solubilization of LysM RLK1-yEGFP, several kinds of detergents were examined including LDAO, cholic acid, deoxycholic acid, CHAPS, and Triton X-100 at 1% (w/v). The amounts of solubilized LysM RLK1 were determined by measuring the fluorescence of the solubilized proteins with a plate reader as described previously (10). We found that the LDAO was the most effective detergent for solubilizing LysM RLK1-yEGFP from the membranes (data not shown).

The membrane proteins were then solubilized by incubating with 1% LDAO, and LysM RLK1-yEGFP protein was purified by the nickel column chromatography. The samples were analyzed by in-gel fluorescence and CBB-stained after the electrophoresis (Fig. 1). We had observed a slight shift in band size of LysM RLK1-yEGFP in the crude membrane fraction on SDS-PAGE (Fig. 1, left, lane 1). Not only the LysM RLK1-yEGFP...
protein but also other proteins in the crude membrane fraction migrated faster than that in the LDAO-solubilized fraction (Fig. 1, right, lane 1). Membrane contaminant might affect the band patterns because we could detect the non-shifted band of LysM RLK1-yEGFP after the removal of the LDAO-insoluble fraction by ultracentrifuge (Fig. 1, lane 2). The amount of LysM RLK1-yEGFP protein was determined by comparison with the yEGFP standard curve, because the CBB staining is poor and inconsistent for many membrane protein samples (10). We obtained about 0.3–0.4 mg of LysM RLK1-yEGFP protein per liter of culture after the purification.

**Ligand Specificity Assay**—To study the biochemical interactions between LysM RLK1 and chitin, we first examined the binding of LysM RLK1-yEGFP to insoluble chitin, such as chitin beads and colloidal chitin, and other insoluble polysaccharides, colloidal chitosan, and PGN, containing a common GlcNAc backbone. These insoluble polysaccharides were incubated with the same amount of LysM RLK1-yEGFP protein (Fig. 2A, Apply lane). Subsequently, the proteins bound to the polysaccharides were analyzed by in-gel fluorescence after SDS-PAGE. The LysM RLK1-yEGFP was largely specific for chitin. It strongly bound the chitin beads and colloidal chitin (Fig. 2A, B).

To determine whether the binding was saturable, as would be expected for a receptor-ligand interaction, the chitin beads

![FIGURE 2. LysM RLK1 specifically bound to chitin but not to chitosan and PGN. A, binding of LysM RLK1-yEGFP or yEGFP to various polysaccharides was analyzed by in-gel fluorescence. 30 pmol of purified LysM RLK1-yEGFP (top) or yEGFP (bottom) were incubated with 200 μg of chitin beads, colloidal chitin, colloidal chitosan, and PGN. After the polysaccharides were washed with the buffer, the proteins bound to polysaccharides were eluted and electrophoresed on a 6% SDS gel for LysM RLK1 and a 12% gel for yEGFP. B, the amounts of the proteins bound to polysaccharides were analyzed by ImageJ. The experiment was repeated three times. The percentage of binding protein rate was calculated from the ratio of band fluorescence of the proteins eluted from polysaccharides and the applied proteins.](https://example.com/figure2)

![FIGURE 3. Fluorescence microscopic visualization of the interaction of LysM RLK1 and chitin beads. A, 500 nM LysM RLK1-yEGFP or yEGFP was incubated with chitin beads, and then the interactions of LysM RLK1-yEGFP or yEGFP with chitin beads were observed by the fluorescence microscope. After 20-min incubations, the strong yEGFP fluorescence was observed on the chitin beads incubated with LysM RLK1-yEGFP, but no fluorescence was detected on the beads incubated with yEGFP. B, the time course of the interaction of LysM RLK1-yEGFP and chitin beads was examined. The samples were observed after 1, 5, 10, 20, and 30 min of the incubation. The amount of the proteins bound to chitin beads was analyzed by ImageJ. C and D, the various concentrations of LysM RLK1-yEGFP (3.9, 7.8, 15.6, 31, 63, 125, 250, or 500 nM) were incubated with chitin beads. Only the beads that were incubated without or with 250, 125, 63, 31, or 7.8 nM LysM RLK1-yEGFP are shown (C). The amount of the proteins bound to chitin beads was analyzed by ImageJ (D). ■, LysM RLK1-yEGFP; ▲, yEGFP. These experiments were performed twice with similar results.](https://example.com/figure3)
LysM RLK1 Directly Binds to Chitin in Vitro

![Graph and images]

were incubated with various concentrations of LysM RLK1-yEGFP (Fig. 3C). We found that the binding was saturable, and the dissociation constant \( (K_d) \) of the proteins was \(-82 \, \text{nm} \) (Fig. 3, C and D). Furthermore, fluorescence of chitin beads was completely diminished by the addition of an excess of glycol chitin but not glycol chitosan (400 \, \mu g/\text{ml}), which are known as water-soluble chitin and chitosan derivatives (13), respectively (Fig. 4, A and B). The competition by glycol chitin and glycol chitosan was assessed in experiments in which increasing amounts of them competed with LysM RLK1-yEGFP for binding to chitin beads. Glycol chitin competed for the binding to chitin beads with a half-maximal inhibitory concentration \( (IC_{50}) \) of 4 \, \mu g/\text{ml} (Fig. 4C). These data show that LysM RLK1 specifically and directly binds to chitin.

**Competition of LysM RLK1 Binding to Chitin Oligosaccharides**—It is known that the biological activity of the soluble chitin oligosaccharides depends on their degree of polymerization. Therefore, we tested the chitin oligosaccharides of different lengths for their efficiency to compete for the binding of LysM RLK1 to chitin beads. After 250 \, \mu M concentrations of the various lengths of chitin oligosaccharides were incubated with 250 \, nm LysM RLK1-yEGFP, the chitin beads were added to this solution, and the binding of LysM RLK1-yEGFP to chitin beads was analyzed by the fluorescence microscopy. We found that the competition of chitin oligosaccharides occurred in a size-dependent manner. \( r(GlCN)_{9} \), an analog of \( (GlCN)_{9} \), \( (GlCN)_{6} \), and \( (GlCN)_{5} \) strongly competed with LysM RLK1-yEGFP for >75% of the binding to the chitin beads (Fig. 5, A and B). Although \( (GlCN)_{9} \) strongly competed the binding, \( (GlCN)_{6} \) did not compete for the binding. This finding also indicates that the LysM RLK1 specifically binds chitin oligosaccharides. \( (GlCN)_{4} \) also competed with it for about 60% of the binding (Fig. 5, A and B). However, \( (GlCN)_{3} \) and \( (GlCN)_{2} \) did not show noticeable competition with regard to the binding of LysM RLK1-yEGFP to the chitin beads (Fig. 5, A and B).

We also assessed the competition by the serial diluted chitin oligosaccharides. Even larger chitin oligosaccharides could weakly compete for the binding of LysM RLK1-yEGFP to chitin beads with an \( IC_{50} \) of about 100 \, \mu M (Fig. 5C).

**Binding of \( ^{14}C \)-Labeled Glycol Chitin to LysM RLK1-yEGFP**—\( ^{14}C \)-Labeled glycol chitin was used to confirm the binding of chitin to LysM RLK1. The purified LysM RLK1-yEGFP was bound to nickel-chelating Sepharose and suspended in 100 \, \mu l of binding buffer containing the various concentrations of \( ^{14}C \)-glycol chitin with or without a 100-fold excess of unlabeled glycol chitin. The binding of \( ^{14}C \)-glycol chitin to LysM RLK1-yEGFP was saturable, and a \( K_d \) value was 3.5 \, \mu g/\text{ml} (Fig. 6A). We also performed the competition assay with the serial diluted glycol chitin, glycol chitosan, and oligosaccharides of various lengths. Glycol chitin but glycol chitosan competed for the binding of \( ^{14}C \)-glycol chitin to LysM RLK1-yEGFP with an \( IC_{50} \) of 10 \, \mu g/\text{ml} (Fig. 6B). However, even larger chitin oligosaccharides could weakly compete for the binding of it at a higher concentration, such as 500 \, \mu M (Fig. 6C).

**Phosphorylation of LysM RLK1**—Many animal receptor kinases exhibit ligand-dependent autophosphorylation (24). To determine whether the early events in chitin perception of plants share this mechanism, we carried out an in vitro kinase assay. Various concentrations of LysM RLK1-yEGFP and yEGFP were assayed for autophosphorylation. The samples were electrophoresed and analyzed by autoradiography. The bands corresponding to LysM RLK1-yEGFP were detected in a dose-dependent manner without chitin, but those of yEGFP were not detected (Fig. 7A). Furthermore, to examine whether its ligand, chitin, affects the phosphorylation state of LysM RLK1, LysM RLK1-yEGFP was incubated with 500 \, \mu M r(GlCN)_{9} or 25 \, \mu g/\text{ml} glycol chitin and assayed for autophosphorylation. However, neither of them affected the phosphorylation state of LysM RLK1-yEGFP (Fig. 7, A and B). These results demonstrate that the LysM RLK1 was autophosphorylated without chitin and that the addition of chitin does not affect the phosphorylation state of it in vitro.
DISCUSSION

Recent studies have identified a plant receptor, LysM RLK1, playing an essential role in defense responses to chitin in *A. thaliana* (5, 6). Although these studies revealed that the mutations in LysM RLK1 abolished the defense responses against chitin, the molecular mechanisms of the perception of chitin by LysM RLK1 were not described. In this study, we showed that LysM RLK1 specifically and directly binds to chitin.

We first examined whether LysM RLK1 binds specifically and directly to chitin, using various polysaccharides, chitin, chitosan, and PGN, which has a common backbone, GlcNAc. Interestingly, although these polysaccharides have similar structures, we could detect the strong binding of LysM RLK-yEGFP only to chitin, chitin beads, and colloidal chitin and not colloidal chitosan and PGN, at least under our experimental conditions (Fig. 2). This result demonstrates that LysM RLK1 may recognize the acetyl group of *N*-acetylglucosamine residues of chitin, and it may be inhibited by the bulky peptide group cross-linked to *N*-acetylmuramic acid residues of PGN.

The PGN is a pathogen-associated molecular pattern derived from bacteria. Therefore, this result is consistent with a previous study showing that the mutations in the LysM RLK1 gene did not affect the defense response against bacteria (6). Moreover, a previous study also suggested that the recognition site of chitin is different from that of PGN in the leaf of *A. thaliana* (2). The LysM motifs are known as the PGN binding motif, and it is known that there are four other putative LysM RLKs in *A. thaliana* (25). Hence, we speculate that some of them may be involved in PGN perception in *A. thaliana*.

To examine the binding property of LysM RLK1, we next directly visualized the binding of LysM RLK1 to chitin beads. The binding studies on many chitin-binding proteins were performed using powdered chitin or colloidal chitin (26, 27). However, these methods required a large amount of protein, and it was difficult to detect a small amount of proteins bound to chitin because the binding proteins were quantified by measuring the UV absorbance at 280 nm. However, the direct observation by fluorescence microscopy revealed the binding of LysM RLK-yEGFP to chitin beads in only 50 μl of the sample with high sensitivity. Moreover, fluorescence of bound proteins could also be monitored without the removal of unbound proteins. Therefore, this method is considered to be better than the conventional methods.

The binding of LysM RLK1 to chitin beads was completed within 20 min of the start of incubation and the half-maximal binding is about 3 min. This is unexpectedly slow for simple ligand binding. This result shows that the interaction of LysM RLK1 and chitin is not a simple ligand binding reaction, and this interaction may be accompanied with a conformational change of receptor protein as often seen in other receptor-ligand interactions (28, 29). Therefore, it takes several minutes to reach the equilibrium.

This binding was saturable, and the dissociation constant ($K_d$) was $\sim 82$ nM (Fig. 3, C and D). These values might be dif-
LysM RLK1 Directly Binds to Chitin in Vitro

A

![Graph A](image1)

B

![Graph B](image2)

C

![Graph C](image3)

**FIGURE 6.** Binding of [14C]glycol chitin to LysM RLK1-yEGFP. A, 40 pmol of LysM RLK1-yEGFP bound to nickel-chelating Sepharose was incubated with 0.025, 0.5, 2.5, 12.5, and 25 μg/ml at room temperature for 30 min in the absence of (•, total binding) or presence of (▲, nonspecific binding) 100-fold unlabeled glycol chitin. To determine the specific binding (●), nonspecific binding was subtracted from total binding. B, competition of the binding of [14C]glycol chitin to LysM RLK1-yEGFP by the unlabeled glycol chitin. 40 pmol of LysM RLK1-yEGFP bound to nickel-chelating Sepharose was incubated with various concentrations of glycol chitin and glycol chitosan (0.125, 1.25, 12.5, 125, and 1250 μg/ml) and then incubated with 12.5 μg/ml [14C]glycol chitin at room temperature for 30 min. C, competition of the binding of [14C]glycol chitin to LysM RLK1-yEGFP by chitin oligosaccharides. 40 pmol of LysM RLK1-yEGFP bound to nickel-chelating Sepharose was incubated with various concentrations of chitin oligosaccharides (0.05, 0.5, 5, 50, and 500 μM) and then incubated with 12.5 μg/ml [14C]glycol chitin at room temperature for 30 min. The relative percentage binding was calculated by comparison with the dpm in the absence of competitors (100% binding). The experiments were repeated three times (A) or twice (B and C), and the averages of the results are shown.

Different from that of those in vivo, because we used soluble LysM RLK1-yEGFP that was not anchored. A previous study reported that the dimerization of RLK or its anchoring is important for high affinity interaction of the receptor and its ligand (30). However, the $K_d$ value of LysM RLK1 for chitin beads was lower than that of other chitin-binding proteins to chitin beads (21), and this binding was eliminated by the excess amount of 400 μg/ml glycol chitin but glycol chitosan. Therefore, we conclude that LysM RLK1 directly and specifically binds to chitin.

We also examine the direct binding of [14C]glycol chitin with LysM RLK1-yEGFP. The binding of [14C]glycol chitin to LysM RLK1-yEGFP was saturable, and the $K_d$ value was 3.5 μg/ml (Fig. 6A). Previous research studied elicitor activity of $A. thaliana$ by using glycol chitin in a concentration of about 0.05% (500 μg/ml) (31, 32). Moreover, chitin was previously shown to induce expression of the gene, such as lectin-like protein or zinc finger protein, in $A. thaliana$ (33). Its half-maximal effective concentration ($EC_{50}$) was more than 1 μg/ml (33). Therefore, the $K_d$ value may be in the physiological range.

Subsequently, we tested the chitin oligosaccharides of different lengths for their efficiency to compete with the binding of LysM RLK1 to chitin beads. Two hundred fifty μM of the $r(GlcNAc)_9$ ($GlcNAc)_9$, and $(GlcNAc)_5$ strongly competed for the binding of LysM RLK1-yEGFP to chitin beads (Fig. 5, A and B). On the other hand, $(GlcNAc)_3$ and $(GlcNAc)_2$ did not show noticeable competition with regard to the binding of LysM RLK1-yEGFP to the chitin beads (Fig. 5, A and B). The biological activity of the chitin oligomers was observed to depend on their degree of polymerization. A previous study showed that the larger chitin oligosaccharides (degree of polymerization = 6–8) had the most effective activity in $A. thaliana$ (33). However, smaller chitin oligosaccharides (degree of polymerization = 2–5) had a little biological activity (33). The competition efficiency of the various lengths of chitin oligosaccharides beads was consistent with the previous data, except that the $(GlcNAc)_5$ had a relatively higher competition activity in our experiment. This might be due to the difference in the efficiency of the length of the chitin oligosaccharides on the binding and biological activity.

In the competition assay of Fig. 5, although the larger chitin oligosaccharides (degree of polymerization = 4–8) competed for the binding of LysM RLK1-yEGFP to chitin beads at their higher concentration of 250 μM (Fig. 5B), the competition of these larger chitin oligosaccharides is also weaker than that of glycol chitin. $IC_{50}$ of $r(GlcNAc)_9$ and $(GlcNAc)_9$ is about 100 μM in Fig. 5B, and that is also about 180 and 120 μg/ml, respectively, in weight per volume concentration units. This value is much higher than that of glycol chitin ($IC_{50} = 4 μg/ml$).

In the competition assay of Fig. 6, chitin oligosaccharides also weakly competed for the binding of [14C]glycol chitin to LysM RLK1-yEGFP (Fig. 6, B and C). The $IC_{50}$ values in the competition assay of Fig. 6 are different from those in competition assays in Figs. 4 and 5. The discrepancy of the $IC_{50}$ may be caused by the use of different assays. However, in both cases, the competition of chitin oligosaccharides is much weaker than that of glycol chitin. These data show that LysM RLK1 has a higher affinity for chitin polymer than chitin oligosaccharides.
LysM RLK1 Directly Binds to Chitin in Vitro

It is proposed that a single LysM motif binds 4–5 residues of N-acetyl glucosamine (9, 34). It is also known that CEBiP, which directly binds (GlcNAc)_8 in rice, has two extracellular LysM motifs (4). On the other hand, LysM RLK1 has three LysM extracellular motifs. Therefore, it may have the binding capacity for 12–15 residues of N-acetyl glucosamine. CEBiP may be a main binding protein for (GlcNAc)_8 in rice because a similarity for 12–15 residues of motifs (4). On the other hand, LysM RLK1 has three LysM motifs (35). Therefore, CEBiP may bind (GlcNAc)_8 with its three LysM motifs that LysM RLK1 very weakly binds to chitin oligosaccharides having more than 8 residues and induces the innate immunity because it is difficult to obtain enough of these large chitin oligosaccharides for the assays.

Although LysM RLK1 weakly binds chitin oligosaccharides having 8 or fewer N-acetyl glucosamine residues, previous results showed that LysM RLK1 is also essential for (GlcNAc)_8 elictor signaling (5, 6). It is known that the TLR4 (Toll-like receptor-4)-MD2 receptor complex recognizes lipopolysaccharides, and other proteins LBP and CD14 increase the lipopolysaccharide sensitivity of TLR4-MD2 (38). Therefore, we speculate that there might be other receptor molecules like CEBiP that can bind (GlcNAc)_8 also in A. thaliana, and these receptors condense the (GlcNAc)_8 molecules on the plasma membrane and allows LysM RLK1 to bind (GlcNAc)_8 to activate the chitin receptor signaling.

We also examined the kinase activity of LysM RLK1 because the kinase activity is considered to be important for signal transductions. When various concentrations of LysM RLK1-yEGFP were incubated with its substrate [γ-32P]ATP, we detected specific autophosphorylation bands corresponding to LysM RLK1-yEGFP by autoradiography (Fig. 7A). This result is consistent with the previous result, which demonstrated the kinase activity of the intracellular domain of LysM RLK1 expressed in E. coli (5). We subsequently examined whether its ligand chitin oligosaccharides or glycol chitin affected the kinase activity of LysM RLK1. Many animal receptor kinases are known to exhibit ligand-dependent autophosphorylation (24). However, neither r(GlcNAc)_8 nor glycol chitin affected the autophosphorylation activity of LysM RLK1 in vitro (Fig. 7A and B). We presume that LysM RLK1 may require other endogenous components to activate its intracellular kinase domain. A recent study reported that the brassinolide receptor BRI1 is basally phosphorylated, and its perception of brassinolide induces the phosphorylation and activation of BRI1 by a regulator component, BAK1 (39).

In legumes, it is known that other LysM RLKs, such as NFR1 and NFR5, play important roles in the perception of Nod factors, which are the lipochitin oligosaccharides secreted by symbiotic bacteria, and in the symbiotic nitrogen fixation with rhizo- (40, 41). However, it remains unclear whether these LysM RLKs directly bind to Nod factors. Interestingly, NFR1 is found to be relatively close to LysM RLK1 in the evolution of plant LysM RLKs (25). Thus, our result may be useful for examining the interaction of NFR1 and Nod factors.
LysM RLK1 Directly Binds to Chitin in Vitro

In this study, we showed that LysM RLK1 directly binds to chitin. This is the first report demonstrating that LysM RLK1 directly binds to chitin, and this result may provide a new insight into chitin elicitor perception as well as the interactions of LysM RLKs and their ligands in plants.

Acknowledgments—We thank Dr. Y. Hayakawa and Dr. S. Agarie for the use of the fluorescence plate reader DTX880 and Typhoon 9200, respectively, and Dr. D. Drew for providing the plasmid pDDGFP2 for technical suggestions. We are grateful to Dr. K. Watanabe, Dr. F. Yagi, Dr. Y. Hagihara, and Dr. S. Katayama for helpful discussions. We also thank K. Horie for technical support in the construction of the Yeast Genetic Resource Center.

REFERENCES

1. Zipfel, C. (2009) Curr. Opin. Plant Biol. 12, 414–420
2. Gust, A. A., Biswas, R., Lenz, H. D., Rahut, T., Ranf, S., Kemmerling, B., Götz, F., Glawischnig, E., Lee, J., Felix, G., and Nürnberg, T. (2007) J. Biol. Chem. 282, 32338–32348
3. Granado, J., Felix, G., and Boller, T. (1995) Plant Physiol. 107, 485–490
4. Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., and Albert, P. (2006) Glycobiology 18, 414–423
5. Ohnuma, T., Onaga, S., Murata, K., Taira, T., and Katoh, E. (2008) J. Biol. Chem. 283, 5178–5187
6. Newstead, S., Kim, H., von Heijne, G., Iwata, S., and Drew, D. (2007) Proc. Natl. Acad. Sci. U.S.A. 104, 19613–19618
7. Miya, A., Albert, P., Shinya, T., Desaki, Y., Ichimura, K., Shirasu, K., Narusaka, Y., Kawakami, N., Kaku, H., and Shibuya, N. (2006) Proc. Natl. Acad. Sci. U.S.A. 103, 11086–11091
8. Onaga, S., and Taira, T. (2008) J. Biol. Chem. 283, 28287–28296
9. Shibuya, N., Ebisu, N., Kamada, Y., Kaku, H., Cohn, J., and Ito, Y. (1996) Plant Cell Physiol. 37, 801–809
10. Shimosato, H., Yokota, N., Shiba, H., Iwano, M., Entani, T., Che, F. S., Watanabe, M., Isogai, A., and Takayama, S. (2007) Plant Cell 19, 107–117
11. Kim, C. Y., Gal, S. W., Choe, M. S., Jeong, S. Y., Lee, S. I., Cheong, Y. H., Lee, S. H., Choi, Y. J., Han, C. D., Kang, K. Y., and Cho, M. J. (1998) Plant Mol. Biol. 37, 523–534
12. Park, H. C., Kim, M. L., Kang, Y. H., Jeon, J. M., Yoo, J. H., Kim, M. C., Park, C. Y., Jeong, J. C., Moon, B. C., Lee, J. H., Yoon, H. W., Lee, S. H., Chung, W. S., Lim, C. O., Lee, S. Y., Hong, J. C., and Cho, M. J. (2004) Plant Physiol. 135, 2150–2161
13. Zhang, B., Ramonell, K., Somerville, S., and Stacey, G. (2002) Mol. Plant Microbe Interact 15, 963–970
14. Mulder, L., Lefebvre, B., Cullimore J., and Imbert A. (2006) Glycobiology 16, 801–809
15. Shibuya, N., Ebisu, N., Kamada, Y., Kaku, H., Cohn, J., and Ito, Y. (1996) Plant Cell Physiol. 37, 894–898
16. Day, R. B., Okada, M., Ito, Y., Tsukada, K., Zaghouni, H., Shibuya, N., and Stacey, G. (2001) Plant Physiol. 126, 1162–1173
17. Albert, P., Miya, A., Hiratsuka, K., Kawakami, N., and Shibuya, N. (2006) Plant Biotechnol. 23, 459–466
18. Jeral R. (2007) Int. J. Med. Microbiol. 297, 353–363
19. Wang, X., Kota, U., He, K., Blackburn, K., Li, J., Goshe, M. B., Huber, S. C., and Clouse, S. D. (2008) Dev. Cell 15, 220–235
20. Radutoiu, S., Madsen, L. H., Madsen, E. B., Felle, H. H., Umehara, Y., Grenlund, M., Sato, S., Nakamura, Y., Tabata, S., Sandal, N., and Stougard, J. (2003) Nature 425, 585–592
21. Madsen, B. E., Madsen, L. H., Radutoiu, S., Olbryt, M., Rakwalska, M., Szczegolowski, K., Sato, S., Kaneko, T., Tabata, S., Sandal, N., and Stougard, J. (2003) Nature 425, 637–640