Running head

LysM RLK4 in Chitin Signaling and Defense

To whom all correspondence should be sent

Gary Stacey
Divisions of Plant Sciences and Biochemistry, National Center for Soybean Biotechnology, 271e C.S. Bond Life Sciences Center, University of Missouri-Columbia, Columbia, MO 65211, USA
Telephone number: 573-884-4752
E-mail address: staceyg@missouri.edu

Research category

Plants interacting with other organisms
LYK4, a LysM receptor-like kinase, is important for chitin signaling and plant innate immunity in Arabidopsis

Authors

Jinrong Wan², Kiwamu Tanaka², Xue-Cheng Zhang³, Geon Hui Son, Laurent Brechenmacher, Tran Hong Nha Nguyen, and Gary Stacey∗

Affiliations

Divisions of Plant Sciences, National Center for Soybean Biotechnology, C.S. Bond Life Sciences Center, University of Missouri-Columbia, Columbia, MO 65211, USA (J.W., K.T., X.C.Z., G.H.S., L.B., T.H.N.N., G.S.); Division of Biochemistry, University of Missouri, Columbia, MO 65211, USA (G.S.)
Footnotes

1The work was funded by a grant from the US Department of Energy (DE-FG02-08ER15309) to G.S.
2These authors contributed equally to this work
3Present address: Department of Molecular Biology, Massachusetts General Hospital; Department of Genetics, Harvard Medical School, Boston, MA 02114, USA.
*Corresponding author: Gary Stacey, staceyg@missouri.edu.
ABSTRACT

Chitin is commonly found in fungal cell walls and is one of the well-studied microbe/pathogen associated molecular patterns. Previous studies showed that lysin motif (LysM)-containing proteins are essential for plant recognition of chitin, leading to the activation of plant innate immunity. In Arabidopsis, the LysM-containing receptor-like kinase 1 (LYK1)/chitin elicitor receptor kinase 1 (CERK1) was shown to be essential for chitin recognition, whereas in rice the LysM-containing protein, CEBiP (chitin elicitor binding protein), was shown to be involved in chitin recognition. Unlike LYK1/CERK1, CEBiP lacks an intracellular kinase domain. Arabidopsis possesses three CEBiP-like genes. Our data show that mutations in these genes, either singly or in combination, did not compromise the response to chitin treatment. Arabidopsis also contains five LYK genes. Analysis of mutations in LysM RLK2, 3, 4 or 5 (LYK2, 3, 4, or 5 for abbreviation) showed that LYK4 is also involved in chitin signaling. The lyk4 mutants showed reduced induction of chitin-responsive genes and diminished chitin-induced cytosolic calcium elevation, and also enhanced susceptibility to both the bacterial pathogen Pseudomonas syringae pv. tomato DC3000 and the fungal pathogen Alternaria brassicicola, although these phenotypes were not as dramatic as that seen in the lyk1/cerk1 mutants. Similar to LYK1/CERK1, the LYK4 protein was also localized to the plasma membrane. Therefore, LYK4 may play a role in the chitin recognition receptor complex to assist chitin signal transduction and plant innate immunity.
INTRODUCTION

In nature, plants are confronted with a great variety of fungal pathogens, as well as other types of pathogens. In response, plants have evolved various defense mechanisms to thwart or limit these infections. One such mechanism involves the recognition of non-self microbe-associated molecular patterns (MAMPs; synonymously termed ‘pathogen-associated molecular patterns’, PAMPs), such as chitin (Bent and Mackey, 2007; Boller and Felix, 2009), by pattern recognition receptors (PRRs) to activate so called MAMP-triggered immunity (Jones and Dangl, 2006). Chitin is a linear polymer of \( \beta-1, 4 \)-linked N-acetyl-glucosamine units and a major component of fungal cell walls. The integrity of fungal cell walls is important for pathogenesis since fungal pathogens with less chitin deposition are less virulent (Madrid et al., 2003; Michielse et al., 2009; Lenardon et al., 2010). A number of publications have documented that plants activate a variety of defense responses when challenged with chitin, including the production of reactive oxygen species, the activation of defense genes, and the accumulation of phytoalexins (Shibuya and Minami, 2001).

Recently, LysM-containing proteins were shown to be involved in plant chitin recognition. The LysM domain was initially identified in bacterial enzymes involved in binding and degrading the bacterial cell wall component peptidoglycan (Joris et al., 1992; Steen et al., 2003; Buist et al., 2008), which is structurally similar to chitin. In rice, CEBiP was shown to be important in the activation of plant innate immunity upon chitin addition (Kaku et al., 2006). This protein has an extracellular domain containing two LysMs and a single transmembrane domain. The analysis of mutants identified LYK1/CERK1, as the primary PRR for chitin recognition in Arabidopsis (Miya et al., 2007; Wan et al., 2008a; Shimizu et al., 2010). The LYK proteins have an extracellular domain containing one to three LysMs, a single transmembrane domain, and an intracellular serine/threonine kinase domain. This protein family appears to be found exclusively in plants (Zhang et al., 2007; Zhang et al., 2009). In addition to these plant receptors, fungal pathogens appear to employ secreted LysM domain-containing proteins to either compete for binding chitin with the plant chitin receptors or to coat the fungal
cell wall to prevent release of elicitor active chitin fragments by plant chitinases (van den Burg et al., 2006; de Jonge and Thomma, 2009; Stergiopoulos and de Wit, 2009; de Jonge et al., 2010). Collectively, these plant and fungal studies point to a central role for LysM proteins in chitin recognition and modulating plant innate immunity in response to fungal infection.

The Arabidopsis genome encodes five LYKs, including LYK1/CERK1 and the others are named LYK2 to LYK5 (Zhang et al., 2007; Wan et al., 2008a). As shown previously, a knockout mutation in lyk1/cerk1 blocked the induction of virtually all chitin-responsive genes (CRGs), the production of ROS, and the activation of MAP kinases (MPKs), and enhanced susceptibility to fungal (Miya et al., 2007; Wan et al., 2008a) and bacterial pathogens (Gimenez-Ibanez et al., 2009; Gimenez-Ibanez et al., 2009). Recent biochemical studies confirmed that both CEBiP and LYK1/CERK1 proteins can directly bind chitin (Iizasa et al., 2010; Petutschnig et al., 2010). A protein similar to LYK1/CERK1, called OsCERK1, was also shown to be involved in chitin signaling in rice and was proposed to function together with CEBiP (Kaku et al., 2006; Shimizu et al., 2010). Proteins similar to rice CEBiP, i.e., CEBiP-like 1, 2, and 3, are also encoded by the Arabidopsis genome, but so far their role in chitin signaling has not been established.

Interestingly, in legumes, LYK proteins were shown to be the essential receptors for perception of the lipo-chitooligosaccharide nodulation factors (NFs), produced by rhizobia and essential for establishment of the nitrogen fixing symbiosis (Limpens et al., 2003; Madsen et al., 2003; Mulder et al., 2006; Radutoiu et al., 2007; Smit et al., 2007). Therefore, the LysM domain appears to be capable of recognizing/binding a sugar backbone of β1-4 linked aminosugars, such as chitin, peptidoglycan, and Nod factors. Surprisingly, the different LysM domains found in the various LYKs were shown to specifically recognize only their cognate signal, which, in the case of NF recognition, was mapped to only a few, critical amino acid residues (Radutoiu et al., 2007).

Although the chitin receptors have been identified from both Arabidopsis and rice, relatively little is known about the downstream signaling pathway, especially how the
Chitin signal is transduced into intracellular responses leading to plant innate immunity. In rice, Chen et al. (2010) showed that Hsp90 and its co-chaperone Hop/Sti1 interacted with OsCERK1 in the endoplasmic reticulum (ER) and were critical for efficient transport of OsCERK1 from the ER to the plasma membrane (PM). Furthermore, Hop/Sti1 and Hsp90 were also localized in a complex at the PM with the plant-specific Rho-type GTPase OsRac1, and Hop/Sti1 were required for chitin-triggered immunity and resistance to rice blast fungus. These data suggest that in rice the Hop/Sti1-Hsp90 chaperone complex may play an important role in transducing the perceived chitin signal to downstream components in the chitin signaling pathway (Chen et al., 2010).

In Arabidopsis, a simple pathway has been proposed, which starts with the perception of chitin by LYK1/CERK1, followed by activation of the MPK pathway, leading to the activation of a variety of transcription factors and ultimately induction of genes involved in pathogen defense (Wan et al., 2004; Libault et al., 2007; Wan et al., 2008a). An analysis of the genes induced by various MAMPs, including chitin, flagellin and EF-Tu, showed that, although each is recognized by a unique PRR, these pathways converge on a common set of genes (Wan et al., 2008a; Wan et al., 2008b).

In this work, we examined the question of whether other LysM proteins in Arabidopsis, in addition to LYK1/CERK1, are involved in chitin perception. Additionally, some of these genes were also induced by chitin, for instance, *LYK4* and *LYK5*, as well as *LYK1* (Miya et al., 2007; Wan et al., 2008a), indicating their potential involvement in chitin signaling. Furthermore, recently, Petutschnig et al. (2010) found that several LysM proteins (i.e., *LYK1/CERK1*, *LYK4*, *LYK5*, and CEBiP-like 1 protein) can be pulled down from an Arabidopsis cellular extract by chitin magnetic beads, supporting the potential involvement of other LysM proteins in chitin perception.

In order to test the role of the three CEBiP-like proteins (i.e., CEBiP-like 1, 2, and 3), we identified mutations in each of the encoding genes and also generated a triple-mutant totally lacking in CEBiP expression. However, unlike the case in rice, mutations in one or all of the genes encoding CEBiP-like proteins showed no significant effects on the plant
response to chitin addition.

We also obtained mutants for the other four LYK genes. Only the mutations in the *LYK4* gene (At2g23770) resulted in a significant reduction in the plant chitin response, including reducing the induction of CRGs and chitin-induced calcium signaling, and enhancing susceptibility to both fungal and bacterial pathogens. Similar to LYK1/CERK1, the LYK4 protein is also localized to the PM. Therefore, LYK4 is partially, but clearly involved in chitin signaling, likely functioning in a chitin recognition receptor complex.
RESULTS

Analysis of CEBiP-like genes

To investigate whether CEBiP-like genes are involved in chitin signaling in Arabidopsis, we obtained homozygous mutants for each of the three genes, i.e., CEBiP-like 1, 2, and 3, and also generated the triple mutant for all three genes through crossing. We then used qRT-PCR (quantitative reverse transcription-polymerase chain reaction) to investigated whether the expression levels of CRGs was changed in these mutants after treatment with chitin. The results showed that the mutants responded in a similar fashion to the wild-type (Supplemental Fig. S1) suggesting that, contrary to the case of the rice OsCEBiP (Kaku et al., 2006), these genes are not involved in chitin signaling in Arabidopsis. Consistent with this, expression of the CEBiP-like genes was not induced by chitin treatment as shown by our previous microarray experiments (Ramonell et al., 2005; Wan et al., 2008a). Therefore, although OsCEBiP and OsCERK1 appear to function as part of a chitin receptor complex in rice, it is unlikely that CEBiP-like proteins play a similar role in Arabidopsis.

The expression pattern of LYK4

The Arabidopsis genome encodes five LYK proteins and one of them, LYK1/CERK1, was previously shown to be involved in chitin perception (Miya et al., 2007; Wan et al., 2008a). To examine the potential involvement of the remaining four LYKs in chitin recognition, we obtained knockout mutants for each of these four genes, LYK2 to LYK5. Interestingly, only mutations in LYK4 affected the chitin response (Fig. 5), whereas mutants of LYK2, LYK3 or LYK5 (Zhang et al., 2007; Wan et al., 2008a), as well as the triple mutant, showed no significant effect on chitin signaling based on the induction of CRGs, e.g., WRKY53 (Supplemental Fig. S2). Hence, we focused on further characterization of LYK4 as described below.

The LYK4 gene is 1839 base pairs long and lacks intron. The coding sequence is
annotated to encode a protein of 612 amino acids, with a signal peptide, an extracellular LysM domain (containing 3 LysMs), a transmembrane domain, and an intracellular serine/threonine kinase domain (Fig. 1A). This protein appears to be evolutionarily related to NF receptors and the chitin receptor LYK1/CERK1 (Zhang et al., 2007).

To examine the expression pattern of \textit{LYK4}, we first analyzed the mRNA level in various tissues using RT-PCR. These data showed measurable expression of \textit{LYK4} in root, stem, leaf, and flower bud, with relatively higher expression in leaf, stem, and root. Little or no \textit{LYK4} expression was detected in older flowers, pollen, or siliques (Fig. 1B).

We further examined the expression pattern the \textit{LYK4} gene by analyzing the expression of an \textit{LYK4} promoter-GUS fusion in transgenic plants. The results showed that \textit{LYK4} is predominantly expressed in leaf (predominantly in hydathodes), stem and root, roughly in agreement with the RT-PCR results (Fig. 1C), suggesting that \textit{LYK4} may function in these tissues. Interestingly, a higher expression level was found in the apical meristem and stipule (Fig. 1C), suggesting a possible spatial regulation of \textit{LYK4} expression in different tissues and, perhaps, a role for \textit{LYK4} in growth and development. However, \textit{lyk4} mutants showed no obvious growth defects.

In addition, we examined the expression of \textit{LYK4} in response to chitin and flagellin. As shown in Fig. 2, \textit{LYK4} was moderately induced by the purified chitin oligomers chitohexaose (6mer) and chitooctaose (8mer). A similar induction pattern was seen with \textit{LYK1} and \textit{LYK5}, but not \textit{LYK2} or \textit{LYK3}, in response to chitooctaose (Ramonell et al., 2005; Wan et al., 2008a). Interestingly, \textit{LYK4} was also moderately induced by the addition of flg22, an elicitor-active epitope of 22 amino acids derived from flagellin (Felix et al., 1999) (Fig. 2), as was seen with \textit{LYK1} (Zipfel et al., 2004), suggesting that expression of both may respond to more than one MAMP.

**Subcellular Localization of LYK4**

To investigate the subcellular localization of \textit{LYK4}, we generated transgenic Arabidopsis
plants expressing a translational fusion of the LYK4 with GFP, under the control of the strong CaMV 35S promoter (Benfey and Chua, 1990). However, the signal from the LYK4-GFP fusion was very weak in both seedling leaves and roots compared to that from the GFP alone, also driven by the CaMV 35S promoter. The signal was still weak even after chitin or fig22 treatment. We then tested the transient expression of the same LYK4-GFP construct in tobacco *Nicotiana benthamiana* using the pressure-mediated *Agrobacterium* infiltration approach (Kapila et al., 1997). In this system, the expression of LYK4-GFP appeared to be localized at the PM of leaf cells, although the GFP alone appeared to be present in both the cytoplasm and nucleus (Fig. 3A). To further support this localization, the LYK4-GFP construct was co-infiltrated with a specific PM marker, the Arabidopsis PM aquaporin AtPIP2A fused with the red fluorescent protein mCherry (Nelson et al., 2007). After merging the images obtained from the green and red channels, both LYK4-GFP and AtPIP2A-mCherry appeared to co-localize (Fig. 3B), supporting that LYK4 is located at the PM, in agreement with the prediction that LYK4 is primarily located at PM using the program WoLF PSORT developed by Horton et al. (Horton et al., 2007).

**Mutation in the **LYK4** gene shows reduced induction of chitin-responsive gene expression in response to chitin**

A knockout mutant of the *LYK4* gene, as shown in Fig. 4A, was used to examine the role of this gene in chitin signaling (see below). The mutant WiscDsLox297300_01C (*lyk4*), has a T-DNA insertion after the 1113th nucleotide downstream from the A in the ATG start codon. RT-PCR analysis using primers flanking the insertion (shown as the solid arrows in Fig. 4A) failed to detect *LYK4* mRNA expression in the mutant, which was easily detected in the wild-type plant (Fig. 4B).

In order to test whether the *lyk4* mutant is compromised in chitin signaling, we treated seedlings with chitin oligomers (i.e., chitohexaose and chitooctaose) and then examined the expression of selected CRG genes (Wan et al., 2004; Libault et al., 2007). As mentioned above, mutations in *LYK2, 3* and *5* showed no effect on CRG expressions
(Supplemental Fig. S2). However, as shown in Fig. 5, the induction of the CRG genes by chitin oligomers (chitohexaose and chitooctaose) appeared to be moderately reduced in lyk4 mutant, when compared with wild-type plants. As expected, the induction of these genes was totally blocked in the mutant lyk1 (Fig. 5), as reported previously (Miya et al., 2007; Wan et al., 2008a). A similar pattern of gene expression was found when mRNA levels in aerial tissues (mostly containing leaves) and root tissues were examined separately (Supplemental Fig. S3). Additionally, we generated complementation lines in which the LYK4 gene was expressed under the control of the CaMV 35S promoter in the lyk4 mutant background. As shown in Supplemental Fig. 4, the induction of the CRG genes appeared to be normal in the complementation lines of the lyk4 mutant. Taken together, the attenuation of CRG expression caused in the lyk4 line suggests that LYK4 may play an auxiliary role in chitin signaling.

To see whether LYK4 is possibly involved in other signaling pathways, we examined the effect of the mutation in LYK4 on the induction of the following genes: WRKY53, MPK3 and ZAT12, in response to the treatment with flg22 or elf26. As shown in Supplemental Fig. S5, the induction of these genes was normal in response to flg22 and elf26 in both the lyk4 and lyk1 mutants, suggesting that LYK4 functions specifically in chitin signaling.

**The LYK4 mutant shows a reduced cytosolic calcium response to chitin treatment**

The elevation of cytosolic free calcium ion concentration ([Ca\(^{2+}\)\(_{\text{cys}}\)) is one of the plant cell responses to chitin elicitation (Kwaaitaal et al., 2011). To assess the chitin-induced [Ca\(^{2+}\)\(_{\text{cys}}\) changes in the lyk4 mutant, we generated a mutant line in the aequorin transgenic background by cross-pollination (see “Materials and Methods”). We also generated an aequorin-expressing lyk1 mutant line as a control. The individual mutants were treated with chitin to measure the chitin-induced [Ca\(^{2+}\)\(_{\text{cys}}\) response. As shown in Fig. 6, the [Ca\(^{2+}\)\(_{\text{cys}}\) in the wild type was significantly elevated 0-150 sec after addition of the chitin mixture or at 300-500 sec after addition of the purified chitin oligomers. In contrast, the chitin-induced [Ca\(^{2+}\)\(_{\text{cys}}\) responses in the lyk4 mutant were delayed and moderately
reduced in comparison to that in the wild type, while the responses were completely blocked in the \textit{lyk1} mutant (Fig. 6). The reduced chitin-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ responses were recovered in the complementation lines of the \textit{lyk4} mutant (Supplemental Fig. S6). Collectively, the results show that the mutation in the \textit{LYK4} gene significantly reduces the chitin-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ response but not to the same degree as found in the \textit{LYK1} mutant line.

\textbf{Mutation in \textit{LYK4} causes enhanced susceptibility to fungal and bacterial pathogens}

To investigate the potential role of LYK4 in plant defense, we performed disease assays with the necrotrophic fungal pathogen \textit{Alternaria brassicicola}. The results clearly showed that the \textit{lyk4} plants were more susceptible to this fungal pathogen than wild-type plants, as exhibited by more severe symptoms and higher spore production per lesion (Fig. 7). These data suggest that LYK4 is involved in the plant defense pathway to fungal infection, likely mediated by chitin recognition.

We also tested the \textit{lyk4} mutant to discern the susceptibility to infection with the hemibiotrophic bacterial pathogen \textit{Pseudomonas syringae pv. tomato} DC3000. As shown in Fig. 8, both \textit{lyk4} and \textit{lyk1} mutants appeared to be significantly more susceptible to this bacterial pathogen than wild-type plants, as indicated by a higher bacterial growth rate (colony-forming units per square centimeter, cfu/cm$^2$). These data suggest that LYK4 may also be involved in plant defense against bacterial pathogens.
DISCUSSION

The LysM domain, first identified in enzymes involved in degrading peptidoglycan in the bacterial cell wall (Joris et al., 1992), has the capacity to recognize and bind sugar polymers composed of β-1, 4-linked aminosugars, such as chitin, peptidoglycan, and the lipo-chitooligosaccharide NFs (Limpens et al., 2003; Madsen et al., 2003; Mulder et al., 2006; Miya et al., 2007; Radutoiu et al., 2007; Smit et al., 2007; Wan et al., 2008a). All sequenced plant genomes appear to have multiple LYK genes, with the possibility that each protein plays a different role in perceiving slightly different signals.

The Arabidopsis genome encodes three LysM domain-containing proteins (i.e., CEBiP-like 1, 2, and 3), similar to OsCEBiP, which was shown to be critical for chitin perception in rice (Kaku et al., 2006). Interestingly, analyses of mutations in each or in combination of these genes indicate that they are not critical for chitin signaling in Arabidopsis. This difference suggests that different plants may employ different LysM proteins to perceive chitin; rice employs OsCERK1/OsCEBiP, and we suggest that Arabidopsis employs LYK1/LYK4 (see below).

The Arabidopsis genome encodes five LYKs (Zhang et al., 2007; Zhang et al., 2009), of which LYK1/CERK1 appears to be the essential PRR involved in chitin perception and plant innate immunity (Miya et al., 2007; Wan et al., 2008a). In the current work, we showed that LYK4 also plays an important role in chitin signaling, since a mutation in LYK4 led to diminished induction of CRGs and \([\text{Ca}^{2+}]_{\text{cyt}}\) levels, and enhanced susceptibility to both fungal and bacterial pathogens. Since mutations in LYK1 blocked every known response elicited by chitin, and LYK1 was shown to directly bind to chitin without apparent need of accessory proteins, this protein is clearly the major and essential PRR for chitin recognition. Given these findings, what is the role of LYK4?

One possibility is that LYK4 may function differentially in different parts of the plant, such as leaf and root. However, analysis of mRNA levels showed that LYK4 is expressed in both leaf and root, as well as in other tissues (Fig. 1), and mutations in LYK4 had a
similar effect on CRG expression in both leaf and root (Supplemental Fig. S3). Another possibility is that LYK4 may differ in its specificity for specific chitin oligomers, perhaps expanding the range of chitin oligomers that can be recognized by LYK1, which can recognize all known elicitor-active chitin oligomers (i.e., chitohexaose, chitoheptaose, and chitooctaose) (Zhang et al., 2002; Miya et al., 2007; Wan et al., 2008a). However, our data indicate that lyk4 mutant plants were similarly defective in their response to both chitohexaose and chitooctaose in terms of gene regulation (Fig. 5). Similar results were seen in the calcium responses (Fig. 6). Therefore, LYK4 does not appear to be able to distinguish, at least, between chitohexaose and chitooctaose. Oligomers smaller than chitohexaose have only weak elicitor activity in wild-type, lyk4 and lyk1 mutant plants. Additionally, we cannot rule out the possibility of recognition of other, unknown chitin-like molecules by LYK4.

Another possibility is that LYK4 may be a part of the receptor complex in which it may interact with LYK1 to mediate chitin perception and signaling. In the case of rice, data suggest that OsCEBiP likely mediates chitin recognition via interaction with the OsCERK1 protein (Shimizu et al., 2010). These proteins likely act as part of a protein complex that may also involve the Hop/Sti1, Hsp90, and OsRAC proteins (Chen et al., 2010). In the case of legumes, at least two LysM proteins appear to be involved in NF recognition. For example, in *Lotus japonicus*, two LYKs, LjNFR1 and LjNFR5, are postulated to interact and both are required for recognition of the lipo-chitooligosaccharide NF (Madsen et al., 2003; Radutoiu et al., 2003). Therefore, it is possible that LYK4 may interact directly or indirectly with LYK1 to aid in chitin recognition. However, direct evidence for this interaction is currently lacking. Recently, Petutschnig et al. (2010) showed that LYK4, LYK5, and CEBiP-like 1 protein were pulled down together with LYK1 by chitin magnetic beads and were further eluted by chitohexaose from the beads (Petutschnig et al., 2010). We repeated this experiment and also found that LYK1, LYK4, LYK5 and CEBiP-like 1 protein were pulled down by chitin magnetic beads and eluted by chitooctaose (Supplemental Table S1). Therefore, it remains a possibility that these proteins may compose a chitin-recognition complex. However, only LYK1 appears to be essential for chitin recognition and, hence, at best
LYK4 is serving to enhance the chitin response.

In the case of rice, it is presumed that OsCERK1 provides the necessary kinase function, since OsCEBiP does not have a kinase domain (Kaku et al., 2006; Miya et al., 2007; Chen et al., 2010; Shimizu et al., 2010; Shinya et al., 2010). In the case of LjNFR1 and LjNFR5, while both are LysM RLKs, only the LjNFR1 protein appears to have an active kinase (Radutoiu et al., 2003; Madsen et al., 2011). Hence, it is possible that chitin recognition requires two, interacting LysM proteins with at least one possessing the necessary kinase activity to initiate the downstream chitin signaling pathway.

Interestingly, similar to LjNFR5, LYK4 also appears to be an inactive kinase, since the comparison of the LYK4 kinase domain with other typical kinases revealed that certain key residues are missing (Supplemental Fig. S6), especially those residues involved in aligning ATP for $\gamma$-phosphate group transfer in domain VII (Hanrs and Hunter, 2000). Additionally, kinase assays using recombinant LYK4 protein isolated after expression in E. coli failed to show phosphorylation of the common kinase substrate MBP (myelin basic protein), although a kinase domain derived from a similar LYK was capable of phosphorylating the same substrate (Supplemental Fig. S7). Therefore, we speculate that like LjNFR1 and LjNFR5 or OsCERK1 and OsCEBiP, Arabidopsis LYK1 and LYK4 may interact either directly or indirectly to form a chitin-receptor complex in which a single kinase domain, provided by LYK1, is the key step in downstream chitin signaling.

Our current work also suggests that LYK4 may be involved in plant defense against bacterial pathogens. These results are consistent with the observation that lyk1 mutant plants were more susceptible to DC3000 and that LYK1 is targeted by the bacterial effector protein (AvrPtoB) for degradation (Gimenez-Ibanez et al., 2009; Gimenez-Ibanez et al., 2009). Moreover, the mutations in LYK1 and LYK4 have a negligible impact on fls22 or elf26 signaling based on the gene expression analysis (Supplemental Fig. S5). The findings that LYK1/CERK1 and LYK4 are involved in defense against both fungal and bacterial pathogens suggest that, in addition to chitin, they may be able to recognize and bind another unidentified elicitor from bacterial pathogens. Consistent with this hypothesis, a recent study demonstrated that the flagellin receptor FLS2 also...
mediates the perception of *Xanthomonas* AX21 secreted peptides to lead to defense against this pathogen (Danna et al., 2011). A likely candidate would be the cell wall peptidoglycan of pathogenic bacteria as recently reported (Willmann et al., 2011), since the LysM domain has the potential to recognize and bind peptidoglycan (Buist et al., 2008).

**CONCLUSION**

Our current work demonstrated that the mutation in LYK4 reduced the induction of CRGs in Arabidopsis and enhanced susceptibility to both a fungal and a bacterial pathogen. LYK4 was further shown to be induced by chitin and located at PM. Therefore, LYK4 likely plays an auxiliary role in the chitin receptor complex to assist chitin signal transduction and plant innate immunity.
MATERIALS AND METHODS

Plants

Ds insertion mutants pst15072 and pst17581 were obtained for CEBiP-like 1 gene (At2g17120) and CEBiP-like 2 gene (At1g21880) from RIKEN BioResource Center, Japan, respectively. One T-DNA insertion mutant SALK_111212 was obtained for CEBiP-like 3 gene (At1g77630) from ABRC (Arabidopsis Biological Resource Center, Ohio, USA). All the mutants were genotyped and raised to homozygosity. Additionally, crossing was made to generate a homozygous triple mutant for all three genes.

The lyk4 mutant, WiscDsLox297300_01C (also named CS850683 and CS863985 in ABRC, with CS863985 derived from the CS850683) was obtained from the Genome Center of Wisconsin (University of Wisconsin-Madison, Madison, Wisconsin, USA). Genomic DNA was isolated according to the protocol described by Edwards et al. (1991). The homozygous plants were identified by genotyping using the following gene-specific primers: 5’-CCACAATCGGTTTCTCCTCATTGTC-3’ and 5’-GTACGACGATTCTTCCCAGTTCTGCGTAG-3’, together with the T-DNA left border primer: 5’-AACGTCCGCAATGTGTTATTAAGTTGTC-3’. These two primers were also used to detect whether the expression of the LYK4 gene was blocked in the mutant plants by RT-PCR (see below).

The mutant lines, SALK_152226 for LYK2 (At3g01840) and SALK_140374 for LYK3 (At1g51940) were obtained from ABRC, and CSHL_GT7089 for LYK5 (At2g33580) was obtained from Dr. Rob Martienssen. From these single mutants, a homozygous triple mutant was also made for all the three genes through crossing.

Mutants of LYK4 and LYK1 in the Aequorin transgenic background and [Ca^{2+}]_{cyt} measurement

The lyk1 (a Gabi-Kat line: 096F09; Wan et al., 2008a) and lyk4 mutants in the aequorin
transgenic background were screened from F2 generation pools after cross-pollination between each mutant and the aequorin transgenic Arabidopsis line (kindly provided by Dr. Marc R. Knight). Homozygous T-DNA insertions were detected by PCR using the gene-specific primers and the T-DNA left-border-specific primer (described above). The presence of the aequorin transgene was confirmed in the F2 generation by detection of bioluminescence in its dissected cotyledon using reconstitution buffer and discharging solution (Tanaka et al., 2010); homozygosity was then determined in the F3 generation by the same method. The [Ca\(^{2+}\)]\(_{cyt}\) measurement was performed as described previously (Tanaka et al., 2010).

**Growth of seedlings and treatment with chitin and flg22**

Arabidopsis seedlings were grown in the ½ MS liquid medium as previously described (Zhang et al., 2002). Approximately 10-d-old seedlings were treated with either chitohexaose or chitooctaose (Sigma, St. Louis, Missouri, USA) at a final concentration of 1 µM, or the flagellin-derived flg22 peptide, or elf26 peptide (GeneScript) at a concentration of 1 µM for 30 min. As a negative control, plants were similarly treated with an equivalent amount of solvents used to solubilize the elicitors. After treatment, samples were collected and frozen in liquid nitrogen for RNA isolation.

**RNA isolation and RT-PCR**

Total RNA was isolated using the Trizol Reagent according to the manufacturer’s instructions (Invitrogen, Carlsbad, California, USA). The isolated RNA was further treated with Turbo DNase (Ambion, Austin, Texas, USA) according to the manufacturer’s instructions to remove potential DNA contamination. cDNA was synthesized using M-MLV reverse transcriptase according to the manufacturer’s instructions (Promega, Madison, Wisconsin, USA).

To analyze the expression of *LYK4* in different tissues using RT-PCR, the following *LYK4*-specific primer pair was used: 5’-ATGATCTCGTTTTCATTTTCATCTCCTC-3’
and 5'-GATACTTCACGCCATCTTCGTTGATC-3', together with an internal control actin-2 (At3g18780) primer pair as follows: 5'-GACTAAGAGAGAAAGTAAGAGATAATCCAG-3' and 5'-CAGCCTTTGATTTCAATTTGCATGTAAGAG-3'. RT-PCR reaction was performed with both the LYK4-specific primer pair and the actin-2 primer pair under the following PCR conditions: 94°C, 3 min; 94°C, 30 sec; 55°C, 30 sec; 72°C, 1.5 min; 25 cycles; and 72°C, 3 min. The resultant PCR products were resolved on a 1% agarose gel for comparison. In the case of detecting the LYK4 gene expression for comparison between wild type and the lyk4 mutant, the cycles were increased from 25 to 39.

For qRT-PCR, primer sequences for the following genes we tested are described previously (Libault et al., 2007): WRKY53 (At4g23810), MPK3 (At3g45640), ZAT12 (At5g59820), and a SAND gene (At2g28390). The SAND gene was used as an internal control to normalize gene expression across different samples. The reactions were conducted on a 7500 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using the SYBR®Green Master Mix (Applied Biosystems). The relative fold change of the target gene, normalized by the expression level of the SAND gene and relative to the gene expression in the control sample, was calculated as described before (Libault et al., 2007).

**Generation of the CaMV35S-LYK4, LYK4 promoter-GUS, and LYK4-GFP transgenic plants**

In order to express the LYK4 gene, the full-length coding sequence (1839 base pairs) was amplified from the genomic DNA isolated from the Arabidopsis ecotype Col-0 using the following primer pair: 5’-GGTACCATCACGATGATCTCGTTTTC-3' (with an engineered KpnI site, underlined) and 5’-TCTAGATTAGTCGACGATTCTTCCAG-3’ (with an underlined XbaI site). The amplified sequence was cloned into the modified binary vector pCAMBIA1200-35S, under the control of the CaMV 35S promoter. The cloned LYK4 sequence was confirmed by sequencing. The final construct was electroporated into Agrobacterium tumefaciens AGL1. The resultant A. tumefaciens was
then used to transform Arabidopsis Col-0 plants via floral dipping (Clough and Bent, 1998). Transgenic plants were selected in the presence of hygromycin.

To make the **LYK4** promoter-GUS fusion transgenic plants, the 2046 base pair sequence before the **LYK4** start codon was amplified from the genomic DNA isolated from Arabidopsis Col-0 plants using the following primer pair: 5’-GTCGACGATCCGATTGTCACTTCTCTG-3’ (with an underlined *SalI* site) and 5’-CCATGGCGTGATTCTGTAAGATTG-3’ (with an underlined *NcoI* site). The amplified sequence was cloned into the binary vector pCAMBIA1391Z, and confirmed by sequencing. The final construct was then used to generate transgenic plants, using the afore-mentioned methods. The GUS staining was conducted as described (Jefferson et al., 1987).

To make the **LYK4**-GFP fusion transgenic plants, the **LYK4** coding sequence (without the stop codon) was cloned into the binary vector pMDC83 to form a translational fusion with the GFP sequence via Gateway cloning strategies (Curtis and Grossniklaus, 2003), and the final construct was then used to generate transgenic plants, using the afore-mentioned methods. The GFP fluorescence in transgenic plants was observed with a confocal microscope (a Zeiss LSM 510 META NLO two-photon point-scanning confocal system).

**Transient expression of the LYK4-GFP fusion construct in tobacco**

The **LYK4**-GFP fusion construct, as well as the GFP construct, was electroporated into *Agrobacterium tumefaciens* GV3101. The different *Agrobacterium* strains were cultured overnight, pelleted, and resuspended in 10 mM MgCl₂. After pretreatment with 40 µM acetylsyringone (Sigma, St. Louis, Missouri, USA) for approximately 2 h at room temperature, the *Agrobacterium* strains harboring either the **LYK4**-GFP or GFP construct was mixed with *A. tumefaciens* (C58C1) expressing the silencing suppressor HC-Pro (Llave et al., 2000), with each strain adjusted to a final optical density of 0.3 at 600 nm. The bacterial mixture was co-infiltrated into leaves of 4-week-old *Nicotiana benthamiana*
plants using a syringe, to examine the transient expression of LYK4-GFP or GFP. Three
days after infiltration, the infiltrated area was cut and observed using the afore-mentioned
confocal microscope.

**Disease assays**

Disease assays with *Alternaria brassicicola* were conducted as described (van Wees et
al., 2003; Veronese et al., 2006) with a spore suspension of 5 x 10^5 spores/mL by dot-
inoculating 5 µl of the spore solution onto leaves. The disease assays with *P. syringae* pv.
tomato DC3000 carrying the luxCDABE operon (DC3000-lux for abbreviation) (Fan et
al., 2008; Bartels et al., 2009) were conducted at a bacterial concentration of 5 x 10^4
colony-forming units/mL as described (Fan et al., 2008; Bartels et al., 2009) using the
Photek HRPCS4 photon detection camera system (Photek, UK).

**Expression of the LYK4 recombinant protein**

To test the LYK4 chitin binding capability, the coding sequence encoding the
extracellular part (minus the signal peptide) of LYK4 was cloned into the expression
vector pGEX-4T-3 to form the GST fusion construct. The fusion protein was purified
with the Gluthathione Sepharose 4B beads (GE Healthcare, Piscataway, New Jersey,
USA) and used in the chitin binding assay.

To test the kinase activity of LYK4, the coding sequence encoding the intracellular part
of LYK4 was cloned into pGEX-4T-3 to form the GST fusion construct. The fusion
protein was purified and used in the in-gel kinase assay.

**Chitin binding assay and pull-down of microsomal fraction proteins using chitin
magnetic beads**

In order to demonstrate protein binding to the chitin magnetic beads (New England
Biolabs, Ipswich, MA, USA), 30 µg of the purified protein was inoculated with the chitin
beads according to the method described by Petutschnig et al. (2010). The proteins pulled down by the beads were separated on a 12% SDS gel, transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA) and detected with the GST antibody from Sigma (St. Louis, MO, USA).

The pull-down of microsomal fraction proteins by chitin magnetic beads was conducted according to Petutschnig et al. (2010).

**In-gel kinase assay**

The purified recombinant proteins were input in the in-gel kinase assay as previously described (Zhang and Klessig, 1997). The phosphorylated substrate was visualized by autoradiography after being separated on a 12.5% SDS-polyacrylamide gel.

**ACKNOWLEDGMENTS**

We thank Dr. Patrick J. Krysan (University of Wisconsin-Madison) for the lyk4 mutant WiscDsLox297300_01C, Dr. Rob Martienssen (Cold Spring Harbor Laboratory) for the lyk5 mutant CSHL_GT7089, Dr. Christopher Lawrence (Virginia Bioinformatics Institute) for *Alternaria brassicicola*, Dr. Chris Lamb (John Innes Centre) for the *Pseudomonas syringae* pv. *tomato* DC3000 carrying the luxCDABE operon, Dr. Marc R. Knight (University of Oxford) for aequorin transgenic Arabidopsis, and Dr. Scott C. Peck (University of Missouri) for technical advice on using the Photek HRPCS4 photon detection camera.

**SUPPLEMENTAL DATA**

The following materials are available in the online version of this article.

**Supplemental Table S1.** Microsomal fraction proteins pulled down by chitin magnetic
Supplemental Fig. S1. Analysis of chitin-responsive genes in the CEBiP-like mutants.

Supplemental Fig. S2. Induction of WRKY53 by chitin is not blocked in the mutants of the LYK2, LYK3, and LYK5 genes.

Supplemental Fig. S3. Expression levels of chitin-responsive genes in the aerial tissue or root tissue of the lyk4 mutant seedlings.

Supplemental Fig. S4. Analysis of chitin-responsive genes in complementation lines of the lyk4 mutant.

Supplemental Fig. S5. Analysis of flg22- or elf26-induced gene expression in the lyk4 and lyk1 mutants.

Supplemental Fig. S6. Effects of chitin treatments on the [Ca^{2+}]_{cyt} response in complementation lines of the lyk4 mutant.

Supplemental Fig. S7. Comparison of the LYK4 kinase domain with other kinases.

Supplemental Fig. S8. Kinase assay of the recombinant LYK4 protein.
LITERATURE CITED


essential for chitin elicitor signaling in Arabidopsis. Proc Natl Acad Sci U S A 104: 19613-19618


Figure legends

**Fig. 1.** Expression patterns of *LYK4*. A, Schematic representation of the LYK4 protein (not drawn to scale). SP, signal peptide; LysM, lysin motif; TM, transmembrane domain; Ser/Thr Kinase, serine/threonine kinase domain. B, Semi-quantitative RT-PCR analysis of the *LYK4* gene expression in different tissues. *Actin-2* was included as an internal control. C, *LYK4* promoter-GUS transgenic plants. a, whole seedling; b, leaf; c, apical meristem and stipules; d, root.

**Fig. 2.** *LYK4* expression in response to chitin and flagellin. Plants were treated with chitoheaxose (6mer), chitoctaose (8mer), and the flagellin-derived peptide flg22 at a final concentration of 1 μM for 30 minutes, respectively. The relative fold change (±SE) of the *Lyk4* gene expression was obtained using qRT-PCR from comparison between the chitin- or flg22-treated plants and the mock-treated plants after normalization with the reference gene, *SAND*. The average results from three independent experiments were reported in the figure. Asterisks indicate statistically significant differences with respect to WT (*P*<0.05, **P**<0.01).

**Fig. 3.** Subcellular localization of LYK4. A, Epi-fluorescence images of tobacco leaf cells infiltrated with *Agrobacterium* harboring the LYK4-GFP fusion construct (a) or the GFP construct (b). The red arrows indicate nuclei. B, Epi-fluorescence images of tobacco leaf cells co-infiltrated with *Agrobacterium* harboring the LYK4-GFP fusion construct and the plasma membrane marker-mCherry construct. a, Green channel Image; b, Bright field image; c, Red channel image; d, Merged image of the green and red channels. The scale bar = 50 µm.

**Fig. 4.** *LYK4* mutant. A, Schematic representations of the *LYK4* mutant (not drawn to scale). The numbers in the figure indicate nucleotide positions. ATG, start codon; TAG, stop codon, LB, T-DNA left border; RB, T-DNA right border. The solid arrows indicate the primers used in B. B, Semi-quantitative RT-PCR analysis of
the *LYK4* gene expression in the *lyk4* mutant and wild-type (WT) plants. *Actin-2* was included as an internal control.

**Fig. 5.** Analysis of chitin-responsive genes in the *LYK4* mutant. Seedlings were treated with the purified chitin oligomers, chitohexaose (6mer) and chitooctaose (8mer), at a final concentration of 1 μM for 30 minutes, respectively. The relative fold change (±SE) of the chitin-responsive genes (*WRKY53*, *MPK3*, and *ZAT12*) in a particular genotype was obtained from the comparison between the chitin-treated plants and the mock-treated plants after normalization with the reference gene, *SAND*. Asterisks indicate statistically significant differences with respect to WT (*P<0.05, **P<0.01*).

**Fig. 6.** Effects of chitin treatments on the \([Ca^{2+}]_{cyt}\) response in the *lyk4* mutant. Five-day-old aequorin transgenic seedlings of wild type, *lyk4*, and *lyk1* mutants, were treated with 10 mg mL\(^{-1}\) of chitin mixture (*A, B*), or 1 μM of chitohexaose (*C, D*), chitoheptaose (*E, F*), or chitooctaose (*G, H*). Line graph shows kinetic differences of chitin-induced \([Ca^{2+}]_{cyt}\) responses. Histogram represents integrated \([Ca^{2+}]_{cyt}\) values over 1200 sec after chitin treatments. Each value shows a mean of 12 seedlings with SE. Asterisks indicate statistically significant differences compared with wild type control (*P<0.05, **P<0.01, ***P<0.001*).

**Fig. 7.** Response of the *lyk4* mutant to a fungal pathogen. Fungal pathogen *Alternaria brassicicola* was spot-inoculated onto different plants at a concentration of 5x10^5 spores/ml. Asterisks indicate statistically significant differences with respect to WT (**P<0.01*). The experiment was repeated three times with similar results.

**Fig. 8.** Response of the *lyk4* mutant to a bacterial pathogen. *Pseudomonas syringae* pv. *tomato* DC3000. The pathogen was inoculated at a concentration of 5x10^4 cfu/ml. Asterisks indicate statistically significant differences with respect to WT (*P<0.05*). The experiment was repeated three times with similar results.