

# LYK4, a Lysin Motif Receptor-Like Kinase, Is Important for Chitin Signaling and Plant Innate Immunity in Arabidopsis<sup>1[C][W][OA]</sup>

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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* (*Arabidopsis thaliana*), the LYK1/CERK1 (for LysM-containing receptor-like kinase1/chitin elicitor receptor kinase1) was shown to be essential for chitin recognition, whereas in rice (*Oryza sativa*), the LysM-containing protein, CEBiP (for 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 LYK2, -3, -4, or -5 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 as well as 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.

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 nonself microbe-associated molecular patterns (MAMPs; synonymously termed “pathogen-associated molecular patterns”), 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 GlcNAc 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, lysin motif (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 (*Oryza sativa*), CEBiP (for chitin elicitor-binding protein) 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 (for LysM-containing receptor-like kinase1/chitin elicitor receptor kinase1) as the primary PRR for chitin recognition in *Arabidopsis* (*Arabidopsis thaliana*; Miya

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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 Ser/Thr kinase domain. This protein family appears to be found exclusively in plants (Zhang et al., 2007, 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 the 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: LYK1/CERK1 and 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 reactive oxygen species, and the activation of mitogen-activated protein kinases (MPKs), and enhanced susceptibility to fungal (Miya et al., 2007; Wan et al., 2008a) and bacterial (Gimenez-Ibanez et al., 2009a, 2009b) pathogens. 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-like1, -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 the perception of the lipochitoooligosaccharide 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  $\beta$ -1,4-linked aminosugars, such as chitin, peptidoglycan, and NFs. 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 cochaperone Hop/Sti1 interacted with OsCERK1 in the endoplasmic reticulum and were critical for the efficient transport of OsCERK1 from the endoplasmic

reticulum 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 that 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, the induction of genes involved in pathogen defense (Wan et al., 2004, 2008a; Libault et al., 2007). 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, 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-like1) 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-like1, -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,

*CEBiP-like1*, *-2*, and *-3*, and also generated the triple mutant for all three genes through crossing. We then used reverse transcription (RT)-PCR to investigate whether the expression levels of CRGs were 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.

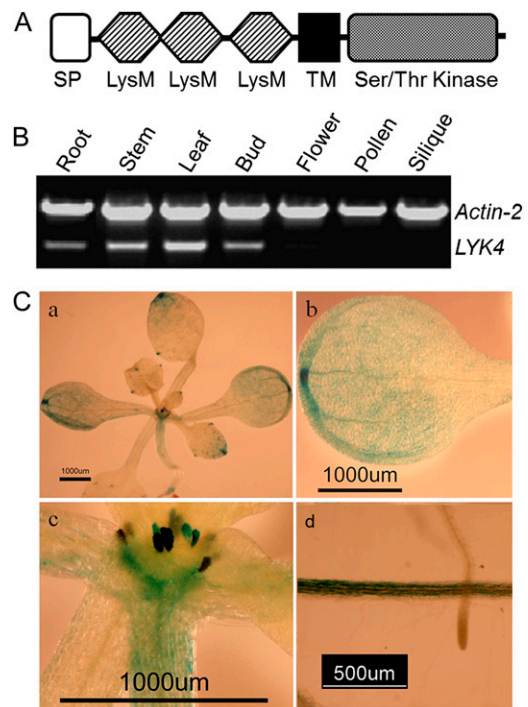
### The Expression Pattern of *LYK4*

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 (see Fig. 5 below), 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 1,839 bp long and lacks introns. The coding sequence is annotated to encode a protein of 612 amino acids, with a signal peptide, an extracellular LysM domain (containing three LysMs), a transmembrane domain, and an intracellular Ser/Thr 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 *LYK4*, we first analyzed the mRNA level in various tissues using RT-PCR. These data showed measurable expression of *LYK4* in root, stem, leaf, and flower bud, with relatively higher expression in leaf, stem, and root. Little or no *LYK4* expression was detected in older flowers, pollen, or siliques (Fig. 1B).

We further examined the expression pattern of the *LYK4* gene by analyzing the expression of a *LYK4* promoter-GUS fusion in transgenic plants. The results showed that *LYK4* is predominantly expressed in leaf (predominantly in hydathodes), stem, and root, roughly in agreement with the RT-PCR results (Fig. 1C), suggesting that *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 *LYK4*



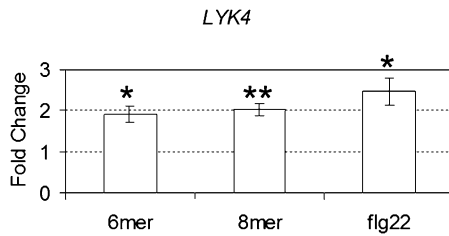
**Figure 1.** Expression patterns of *LYK4*. A, Schematic representation of the *LYK4* protein (not drawn to scale). SP, Signal peptide; TM, transmembrane domain. B, Semicquantitative RT-PCR analysis of *LYK4* gene expression in different tissues. *Actin2* was included as an internal control. C, *LYK4* promoter-GUS transgenic plants: a, whole seedling; b, leaf; c, apical meristem and stipules; d, root.

expression in different tissues and, perhaps, a role for *LYK4* in growth and development. However, *lyk4* mutants showed no obvious growth defects.

In addition, we examined the expression of *LYK4* in response to chitin and flagellin. As shown in Figure 2, *LYK4* was moderately induced by the purified chitin oligomers chitohexaose (6-mer) and chitooctaose (8-mer). A similar induction pattern was seen with *LYK1* and *LYK5*, but not *LYK2* or *LYK3*, in response to chitooctaose (Ramonell et al., 2005; Wan et al., 2008a). Interestingly, *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 *LYK1* (Zipfel et al., 2004), suggesting that the expression of both may respond to more than one MAMP.

### Subcellular Localization of *LYK4*

To investigate the subcellular localization of *LYK4*, we generated transgenic Arabidopsis plants expressing a translational fusion of *LYK4* with GFP under the control of the strong cauliflower mosaic virus (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 with that from the GFP alone, also driven by the CaMV 35S



**Figure 2.** *LYK4* expression in response to chitin and flagellin. Plants were treated with chitohexaose (6-mer), chitooctaose (8-mer), and the flagellin-derived peptide flg22 at a final concentration of 1  $\mu\text{M}$  for 30 min. The relative fold change  $\pm$  SE of *LYK4* gene expression was obtained using quantitative RT-PCR from a comparison between the chitin- or flg22-treated plants and the mock-treated plants after normalization with the reference gene *SAND*. Average results from three independent experiments are reported. Asterisks indicate statistically significant differences with respect to the wild type: \* $P < 0.05$ , \*\* $P < 0.01$ .

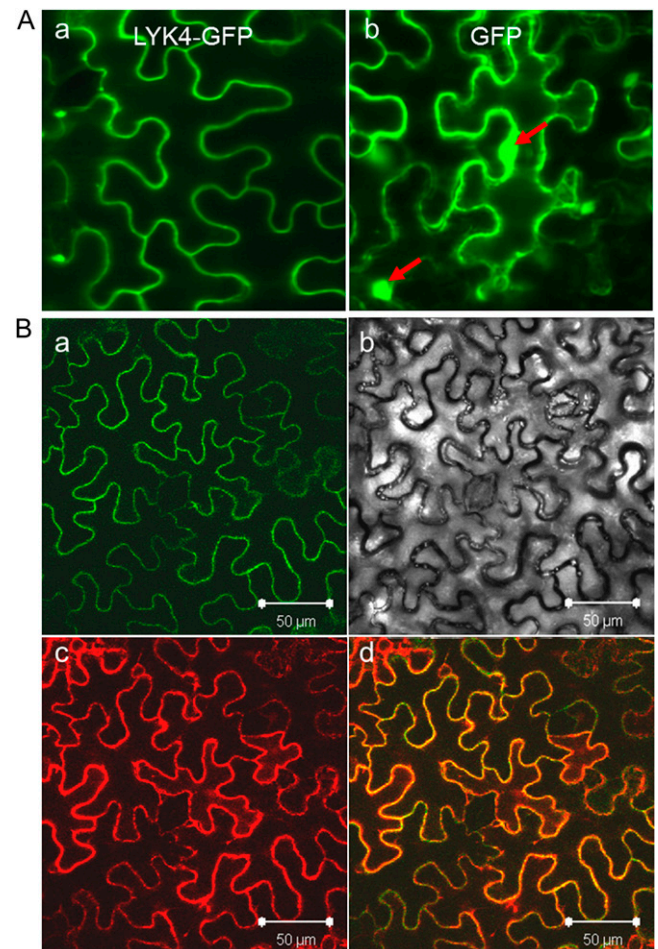
promoter. The signal was still weak even after chitin or flg22 treatment. We then tested the transient expression of the same *LYK4*-GFP construct in tobacco (*Nicotiana benthamiana*) using the pressure-mediated *Agrobacterium tumefaciens* infiltration approach (Kapila et al., 1997). In this system, 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 coinfiltrated 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 colocalize (Fig. 3B), supporting that *LYK4* is located at the PM, in agreement with the prediction that *LYK4* is primarily located at the PM using the program WoLF PSORT developed by Horton et al. (2007).

#### Mutation in the *LYK4* Gene Shows Reduced Induction of Chitin-Responsive Gene Expression in Response to Chitin

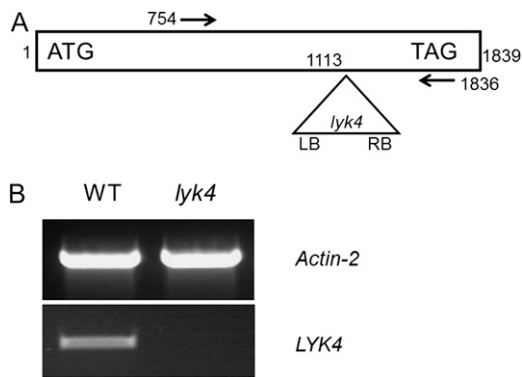
A knockout mutant of the *LYK4* gene, as shown in Figure 4A, was used to examine the role of this gene in chitin signaling (see below). The mutant WiscD-sLox297300\_01C (*lyk4*) has a transferred DNA (T-DNA) insertion after the 1,113th nucleotide downstream from the A in the ATG start codon. RT-PCR analysis using primers flanking the insertion (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 expression (Supplemental Fig. S2). However, as shown in Figure 5, the induction of the CRG genes by chitin oligomers (chitohexaose and chitooctaose) appeared to be moderately reduced in the *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



**Figure 3.** Subcellular localization of *LYK4*. A, Epifluorescence 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, Epifluorescence images of tobacco leaf cells coinfiltrated with *Agrobacterium* harboring the *LYK4*-GFP fusion construct and the PM marker-mCherry construct: a, green channel image; b, bright-field image; c, red channel image; d, merged image of the green and red channels. Bars = 50  $\mu\text{m}$ .



**Figure 4.** The *lyk4* mutant. A, Schematic representation of the *lyk4* mutant (not drawn to scale). Numbers indicate nucleotide positions. ATG, Start codon; LB, T-DNA left border; RB, T-DNA right border; TAG, stop codon. The arrows indicate the primers used in B. B, Semiquantitative RT-PCR analysis of *LYK4* gene expression in the *lyk4* mutant and wild-type (WT) plants. *Actin2* was included as an internal control.

Supplemental Figure S4, 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 genes *WRKY53*, *MPK3*, and *ZAT12* in response to the treatment with flg22 or elf26. As shown in Supplemental Figure 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

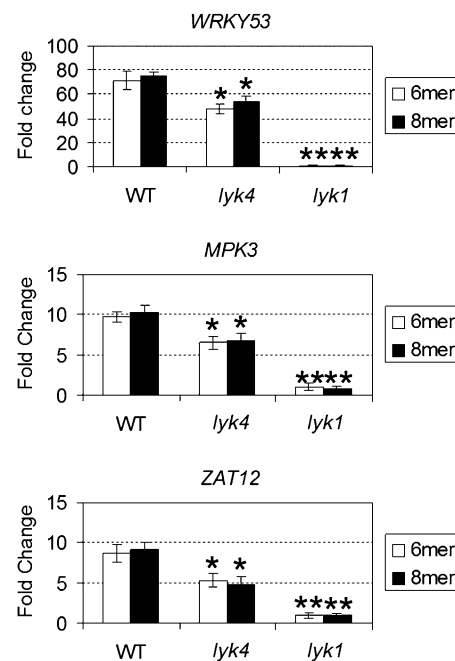
The elevation of cytosolic free calcium ion concentration ( $[Ca^{2+}]_{cyt}$ ) is one of the plant cell responses to chitin elicitation (Kwaaitaal et al., 2011). To assess the chitin-induced  $[Ca^{2+}]_{cyt}$  changes in the *lyk4* mutant, we generated a mutant line in the aequorin transgenic background 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  $[Ca^{2+}]_{cyt}$  response. As shown in Figure 6, the  $[Ca^{2+}]_{cyt}$  in the wild type was significantly elevated 0 to 150 s after addition of the chitin mixture or at 300 to 500 s after addition of the purified chitin oligomers. In contrast, the chitin-induced  $[Ca^{2+}]_{cyt}$  responses in the *lyk4* mutant were delayed and moderately reduced in comparison with that in the wild type, while the responses were completely blocked in the *lyk1* mutant (Fig. 6). The reduced chitin-induced  $[Ca^{2+}]_{cyt}$  responses were recovered

in the complementation lines of the *lyk4* mutant (Supplemental Fig. S6). Collectively, the results show that the mutation in the *LYK4* gene significantly reduces the chitin-induced  $[Ca^{2+}]_{cyt}$  response but not to the same degree as found in the *LYK1* mutant line.

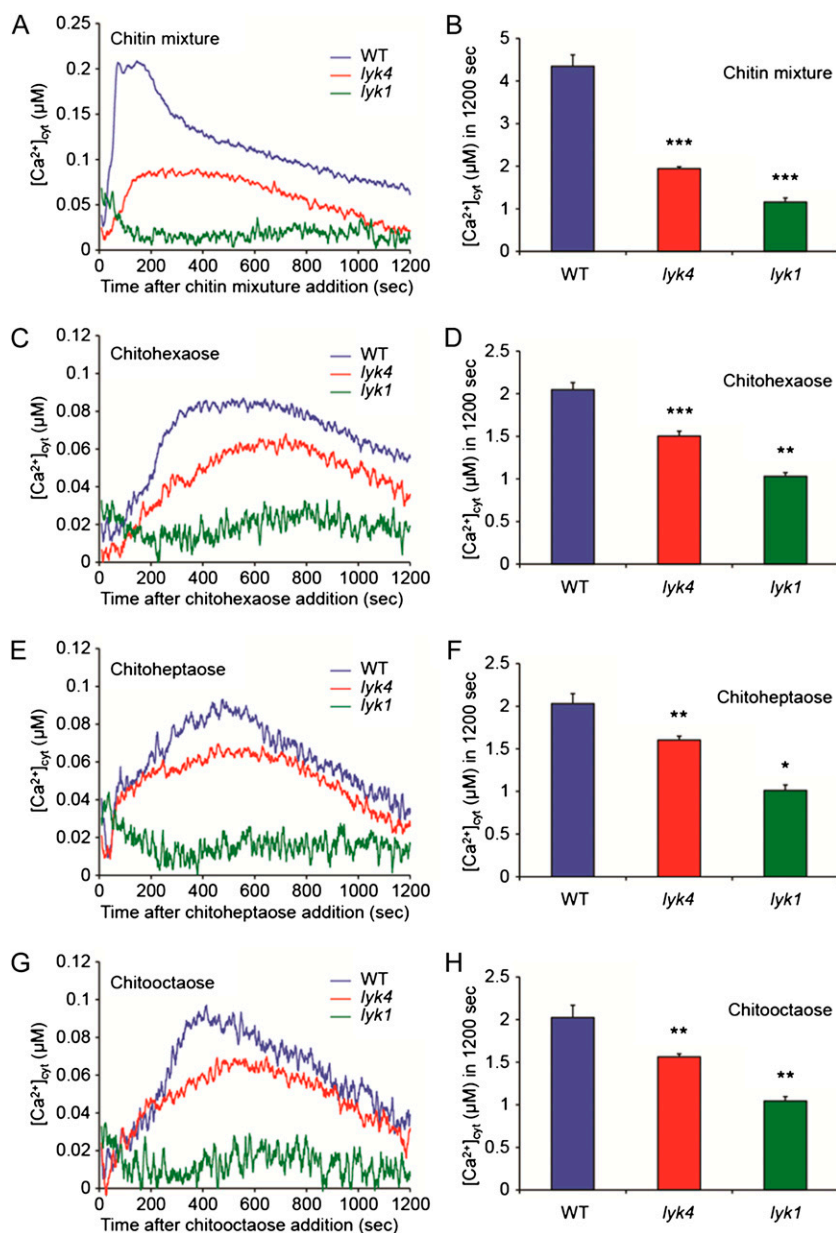
#### Mutation in *LYK4* Causes Enhanced Susceptibility to Fungal and Bacterial Pathogens

To investigate the potential role of LYK4 in plant defense, we performed disease assays with the necrotrophic fungal pathogen *Alternaria brassicicola*. The results clearly showed that the *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 *lyk4* mutant to discern the susceptibility to infection with the hemibiotrophic bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000. As shown in Figure 8, both *lyk4* and *lyk1* mutants appeared to be significantly more susceptible to this bacterial pathogen than wild-type plants, as indicated



**Figure 5.** Analysis of chitin-responsive genes in the *lyk4* mutant. Seedlings were treated with the purified chitin oligomers, chitohexaose (6-mer) and chitooctaose (8-mer), at a final concentration of 1  $\mu$ M for 30 min. The relative fold change  $\pm$  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 the wild type (WT): \*P < 0.05, \*\*P < 0.01, \*\*\*\*P < 0.0001.



**Figure 6.** Effects of chitin treatments on the  $[Ca^{2+}]_{cyt}$  response in the *lyk4* mutant. Five-day-old aequorin transgenic seedlings of the wild type (WT) and *lyk4* and *lyk1* mutants were treated with  $10 \mu\text{g mL}^{-1}$  chitin mixture (A and B) or  $1 \mu\text{M}$  chitoheptaose (E and F), or chitooctaose (G and H). Line graphs show kinetic differences in chitin-induced  $[Ca^{2+}]_{cyt}$  responses. Histograms represent integrated  $[Ca^{2+}]_{cyt}$  values over 1,200 s after chitin treatments. Each value shows a mean of 12 seedlings  $\pm$  SE. Asterisks indicate statistically significant differences compared with the wild-type control: \* $P < 0.05$ , \*\* $0.001 < P < 0.01$ , \*\*\* $P < 0.001$ .

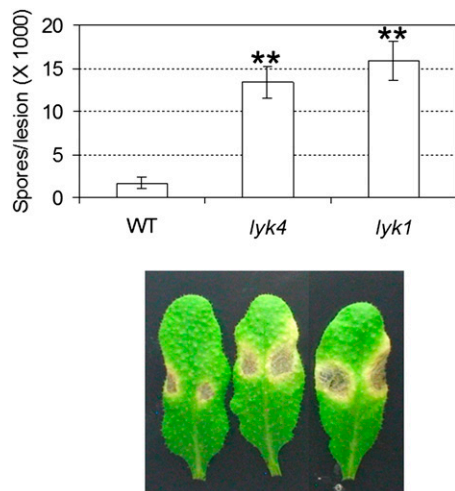
by a higher bacterial growth rate (colony-forming units  $\text{cm}^{-2}$ ). These data suggest that LYK4 may also be involved in plant defense against bacterial pathogens.

## 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  $\beta$ -1,4-linked aminosugars, such as chitin, peptidoglycan, and the lipochitooligosaccharide 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-like1, -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 gene 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).



**Figure 7.** Response of the *lyk4* mutant to a fungal pathogen. The fungal pathogen *A. brassicicola* was spot inoculated onto different plants at a concentration of  $5 \times 10^5$  spores  $\text{mL}^{-1}$ . Asterisks indicate statistically significant differences with respect to the wild type (WT) :  $**P < 0.01$ . The experiment was repeated three times with similar results. [See online article for color version of this figure.]

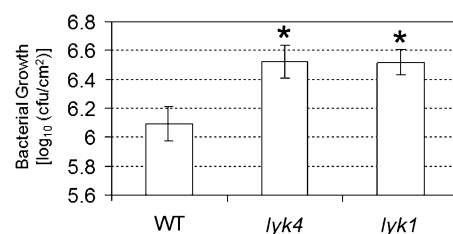
The Arabidopsis genome encodes five LYKs (Zhang et al., 2007, 2009), of which LYK1/CERK1 appears to be the essential PRR involved in chitin perception and plant innate immunity (Miya et al., 2007; Wan et al., 2008a). In this work, we showed that LYK4 also plays an important role in chitin signaling, since a mutation in *LYK4* led to diminished induction of CRGs and  $[\text{Ca}^{2+}]_{\text{cyt}}$  levels as well as 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 between chitohexaose and chitooctaose. Oligomers smaller than chitohexaose have

only weak elicitor activity in wild-type, *lyk4*, and *lyk1* 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, the 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 lipochitooligosaccharide 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-like1 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-like1 were pulled down by chitin magnetic beads and eluted by chitooctaose (Supplemental Table S1). Therefore, it remains a possibility that these proteins may constitute a chitin-recognition complex. However, only LYK1 appears to be essential for chitin recognition; 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



**Figure 8.** Response of the *lyk4* mutant to a bacterial pathogen, *P. syringae* pv *tomato* DC3000. The pathogen was inoculated at a concentration of  $5 \times 10^4$  colony-forming units (cfu)  $\text{mL}^{-1}$ . Asterisks indicate statistically significant differences with respect to the wild type (WT):  $*P < 0.05$ . The experiment was repeated three times with similar results.

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. S7), especially those residues involved in aligning ATP for  $\gamma$ -phosphate group transfer in domain VII (Hanks and Hunter, 1995). Additionally, kinase assays using recombinant LYK4 protein isolated after expression in *Escherichia coli* failed to show phosphorylation of the common kinase substrate Myelin Basic Protein, although a kinase domain derived from a similar LYK was capable of phosphorylating the same substrate (Supplemental Fig. S8). 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 work here 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 *P. syringae* pv *tomato* DC3000 and that LYK1 is targeted by the bacterial effector protein (AvrPtoB) for degradation (Gimenez-Ibanez et al., 2009a, 2009b). 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 reported recently (Willmann et al., 2011), since the LysM domain has the potential to recognize and bind peptidoglycan (Buist et al., 2008).

## CONCLUSION

This work has 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 the 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

Arabidopsis (*Arabidopsis thaliana*) Ds insertion mutants *pst15072* and *pst17581* were obtained for the *CEBiP-like1* gene (At2g17120) and the *CEBiP-*

*like2* gene (At1g21880), respectively, from the RIKEN BioResource Center. One T-DNA insertion mutant, SALK\_111212, was obtained for the *CEBiP-like3* gene (At1g77630) from the Arabidopsis Biological Resource Center (ABRC). All the mutants were genotyped and raised to homozygosity. Additionally, crossing was done to generate a homozygous triple mutant for all three genes.

The *lyk4* mutant, WiscDsLox297300\_01C (also named CS850683 and CS863985 in the ABRC, with CS863985 derived from CS850683), was obtained from the Genome Center of Wisconsin (University of Wisconsin-Madison). 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'-CCACAATCGGTTTCTCTCTCC-ATTGTC-3' and 5'-GTACGACGATTCTTCCAGTCTGCGTAG-3', together with the T-DNA left border primer 5'-AACGCCGAATGTGTTAT-TAAGTTGTC-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 the 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 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 the 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.  $[Ca^{2+}]_{cyt}$  measurement was performed as described previously (Tanaka et al., 2010).

### Growth of Seedlings and Treatment with Chitin and *flg22*

Arabidopsis seedlings were grown in one-half-strength Murashige and Skoog liquid medium as described previously (Zhang et al., 2002). Approximately 10-d-old seedlings were treated with either chitohexaose or chitoctaose (Sigma) at a final concentration of 1  $\mu$ M, or the flagellin-derived *flg22* peptide, or *elf26* peptide (GeneScript) at a concentration of 1  $\mu$ M 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). The isolated RNA was further treated with Turbo DNase (Ambion) according to the manufacturer's instructions to remove potential DNA contamination. Complementary DNA was synthesized using Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Promega).

To analyze the expression of *LYK4* in different tissues using RT-PCR, the following *LYK4*-specific primer pair was used, 5'-ATGATCTCGTTTT-CATTTCATCTCCTC-3' and 5'-GATACTTCACGCCATCTTCGTTGATC-3', together with an internal control *Actin2* (At3g18780) primer pair as follows: 5'-GACTAAGAGAGAAAGTAAGAGATAATCCAG-3' and 5'-CAGCCTTT-GATTTCAATTTGCATGTAAGAG-3'. RT-PCR was performed with both the *LYK4*-specific primer pair and the *Actin2* primer pair under the following PCR conditions: 94°C for 3 min; 25 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1.5 min; and then 72°C for 3 min. The resultant PCR products were resolved on a 1% agarose gel for comparison. In the case of detecting *LYK4* gene expression for comparison between the wild type and the *lyk4* mutant, the cycles were increased from 25 to 39.

For quantitative RT-PCR, primer sequences for the following genes we tested were 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) using the SYBRGreen 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 CaMV 35S-LYK4, LYK4 Promoter-GUS, and LYK4-GFP Transgenic Plants

In order to express the *LYK4* gene, the full-length coding sequence (1,839 bp) was amplified from the genomic DNA isolated from Arabidopsis ecotype Columbia (Col-0) using the following primer pair: 5'-acaaaGGTACCAT-CACGATGATCTCGTTTC-3' (with an engineered *KpnI* site underlined) and 5'-acaaaTCTAGATTAGTACGACGATTCTCCAG-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 *Agrobacterium* 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 2,046-bp sequence before the *LYK4* start codon was amplified from the genomic DNA isolated from Arabidopsis Col-0 plants using the following primer pair: 5'-acaaaGTCGACGATCCGATTGCTACTCTCTG-3' (with an underlined *SaI* site) and 5'-acaaaCCATGCGGTGATTCTGTAAGATTGGT-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 previously mentioned methods. 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 previously 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, were electroporated into *Agrobacterium tumefaciens* GV3101. The different *Agrobacterium* strains were cultured overnight, pelleted, and resuspended in 10 mM MgCl<sub>2</sub>. After pretreatment with 40 μM acetosyringone (Sigma) for approximately 2 h at room temperature, the *Agrobacterium* strains harboring either the *LYK4*-GFP or GFP construct were mixed with *Agrobacterium* 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 coinfiltrated into leaves of 4-week-old tobacco (*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 previously 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 × 10<sup>5</sup> spores mL<sup>-1</sup> by dot inoculating 5 μL of the spore solution onto leaves. The disease assays with *Pseudomonas syringae* pv *tomato* DC3000 carrying the *luxCDABE* operon (Fan et al., 2008; Bartels et al., 2009) were conducted at a bacterial concentration of 5 × 10<sup>4</sup> colony-forming units mL<sup>-1</sup> as described (Fan et al., 2008; Bartels et al., 2009) using the Photech HRPCS4 photon detection camera system.

### 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 glutathione S-transferase (GST)

fusion construct. The fusion protein was purified with Glutathione Sepharose 4B beads (GE Healthcare) 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), 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 polyvinylidene difluoride membrane (Bio-Rad), and detected with the GST antibody from Sigma.

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

### In Vitro Kinase Assay

The purified recombinant proteins were input in the in vitro kinase assay as described previously (Liu et al., 2011). The phosphorylated substrate was visualized by autoradiography after being separated on a 12.5% SDS-polyacrylamide gel.

### Supplemental Data

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

**Supplemental Figure S1.** Analysis of chitin-responsive genes in the *CEBIP-like* mutants.

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

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

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

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

**Supplemental Figure S6.** Effects of chitin treatments on the [Ca<sup>2+</sup>]<sub>cyt</sub> response in complementation lines of the *lyk4* mutant.

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

**Supplemental Figure S8.** Kinase assay of the recombinant *LYK4* protein.

**Supplemental Table S1.** Microsomal fraction proteins pulled down by chitin magnetic beads.

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