

# A LysM Receptor-Like Kinase Plays a Critical Role in Chitin Signaling and Fungal Resistance in *Arabidopsis* <sup>W|OA</sup>

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**Chitin, a polymer of *N*-acetyl-D-glucosamine, is found in fungal cell walls but not in plants. Plant cells can perceive chitin fragments (chitooligosaccharides) leading to gene induction and defense responses. We identified a LysM receptor-like protein (LysM RLK1) required for chitin signaling in *Arabidopsis thaliana*. The mutation in this gene blocked the induction of almost all chitooligosaccharide-responsive genes and led to more susceptibility to fungal pathogens but had no effect on infection by a bacterial pathogen. Additionally, exogenously applied chitooligosaccharides enhanced resistance against both fungal and bacterial pathogens in the wild-type plants but not in the mutant. Together, our data indicate that LysM RLK1 is essential for chitin signaling in plants (likely as part of the receptor complex) and is involved in chitin-mediated plant innate immunity. The LysM RLK1-mediated chitin signaling pathway is unique, but it may share a conserved downstream pathway with the FLS2/flagellin- and EFR/EF-Tu-mediated signaling pathways. Additionally, our work suggests a possible evolutionary relationship between the chitin and Nod factor perception mechanisms due to the similarities between their potential receptors and between the signal molecules perceived by them.**

## INTRODUCTION

Chitin is a polymer of *N*-acetyl-D-glucosamine found in fungal cell walls, insect exoskeletons, and crustacean shells but not in plants. Plants do not have chitin, but they do have chitin-degrading enzymes. It was hypothesized that plant chitinases can degrade chitin in the fungal cell walls to directly affect the viability of the invading fungal pathogen and to release short chitin fragments (chitooligosaccharides) that can act as a general elicitor of plant innate immunity (Boller, 1995; Stacey and Shibuya, 1997; Shibuya and Minami, 2001; Passarinho and de Vries, 2002). This hypothesis is indirectly supported by the following observations: a number of chitinase genes are induced by fungal infection (Majeau et al., 1990; Roby et al., 1990), and chitinases accumulate at infection sites in planta (Wubben et al., 1992). Chitinases were also shown to degrade fungal cell walls and inhibit fungal growth in vitro, especially in combination with  $\beta$ -1,3-glucanases (Schlumbaum et al., 1986; Mauch et al., 1988; Arlorio et al., 1992; van den Burg et al., 2006). Transgenic plants overexpressing chitinase genes were more resistant to fungal pathogens (Brogue et al., 1991; Jach et al., 1995). In addition, a fungal chitin binding protein (Avr4) was found to specifically bind

chitin in fungal cell walls to prevent chitin degradation by plant chitinases (van den Burg et al., 2006).

In agreement with the above hypothesis, exogenously applied chitooligosaccharides enhanced plant resistance against fungal pathogens (Tanabe et al., 2006). Purified chitooligosaccharides were able to induce various defense responses in plants or cultured cells, such as induction of defense-related genes, synthesis of phytoalexin, production of reactive oxygen species, and protein phosphorylation (Felix et al., 1993; Baier et al., 1999; Peck et al., 2001; Shibuya and Minami, 2001; Ramonell et al., 2005). Gene expression profiling studies also demonstrated that chitooligosaccharides can induce a large number of genes (including many defense-related genes) in different plant species (Day et al., 2002; Ramonell et al., 2002, 2005). Mutations in selected chitooligosaccharide-responsive genes (CRGs) led to more susceptibility to a fungal pathogen (Ramonell et al., 2005). Various studies suggested that plants possess a specific system to perceive chitooligosaccharides leading to gene induction and defense (Day et al., 2001; Zhang et al., 2002; Wan et al., 2004; Libault et al., 2007).

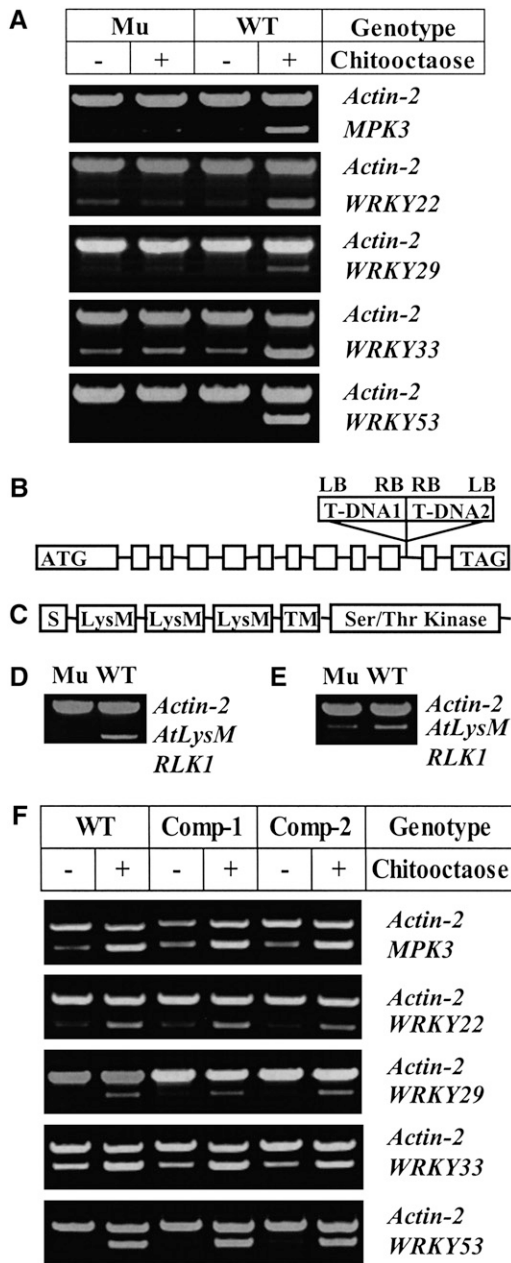
Chitin binding sites or proteins were previously identified in membrane preparations from a variety of plants (Baureithel et al., 1994; Ito et al., 1997; Day et al., 2001; Okada et al., 2002). Recently, a LysM domain-containing protein (Chitin Elicitor Binding Protein [CEBiP]) was shown to be involved in the binding and perception of chitooligosaccharides in rice (*Oryza sativa*; Kaku et al., 2006). The LysM motif was originally identified in enzymes that degrade the bacterial cell wall component peptidoglycan, which is structurally similar to chitin (Joris et al., 1992). Since CEBiP lacks a significant intracellular domain, it likely is only a part of the chitin receptor complex in rice (Kaku et al.,

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**Figure 1.** The Knockout of the *LysM RLK1* Gene Blocked the Induction of the Selected CRGs by Chitooctaose.

**(A)** Analysis of the expression of the selected CRGs in both the mutant and wild-type plants using RT-PCR. Both the mutant (Mu) and wild-type plants were treated with purified chitooctaose or water (as a control) for 30 min, and gene expression of the selected CRGs was then detected using RT-PCR. *Actin-2* was used as an internal control, and the amplification of both *actin-2* and a CRG was conducted in the same PCR reaction tube with 25 cycles. The experiment was repeated at least three times with similar results.

**(B)** The gene structure of *LysM RLK1* (At3g21630) (not drawn to scale). Square boxes are exons. Solid lines between them are introns. The start codon (ATG) and stop codon (TAG) were included in the first and last exon, respectively. The two T-DNA insertions (T-DNA1 and 2) inserted in

2006). An obvious partner for CEBiP would be a membrane-associated receptor-like kinase.

LysM domain-containing receptor-like kinases (LysM RLKs; e.g., Nod Factor Receptor1 [NFR1] and NFR5) in legumes were shown to be critical for the perception of modified chitoooligosaccharides (Nod factors) in the legume–rhizobial symbiotic interaction (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Similar *LysM RLK* genes are also present in nonleguminous plants (Zhang et al., 2007). For example, there are five such *LysM RLK* genes in *Arabidopsis thaliana*. LysM domain-containing proteins are also found in animals, but LysM RLKs appear to be unique to plants (Zhang et al., 2007). Due to the similarity between the *Arabidopsis* LysM RLKs and legume NFR1 and NFR5 and the similarity between chitoooligosaccharides and Nod factors, it is logical to hypothesize that nonleguminous LysM RLKs may be involved in the perception of Nod factor-like molecules (i.e., chitoooligosaccharides).

We tested this hypothesis in this work and revealed that one *Arabidopsis* LysM RLK indeed plays a critical role in chitin signaling.

## RESULTS

### Identification of a Mutant Blocking the Induction of the Selected CRGs by Chitoooligosaccharides

To study the potential role of the *Arabidopsis* *LysM RLK* genes in chitin signaling, insertion mutants were obtained for all five *LysM RLK* genes (i.e., At1g51940, At2g23770, At2g33580, At3g01840, and At3g21630). Homozygous mutants were treated with a purified chitoooligosaccharide (chitooctaose), and the expression of known CRGs, *MPK3*, *WRKY22*, *WRKY33*, and *WRKY53* (Wan et al., 2004), was examined. Only one mutant, corresponding to At3g21630 (*LysM RLK1*), appeared to totally block the induction of the selected CRGs (Figure 1A), while mutations in the other four *LysM RLK* genes had no apparent effect on CRG expression

the 10th intron in the *LysM RLK1* mutant were indicated above the gene. LB, left border; RB, right border.

**(C)** The predicted domain structure of LysM RLK1. S, signal peptide; LysM, LysM domain; TM, transmembrane domain; WT, wild-type Col-0; Mu, *LysM RLK1* mutant.

**(D)** Analysis of the *LysM RLK1* transcript in both the mutant and wild-type plants using RT-PCR. The gene-specific primers were derived from the exons on each side of the insertions.

**(E)** Analysis of the *LysM RLK1* transcript in both the mutant and wild-type plants using RT-PCR. The gene-specific primer pairs were derived from the exons before the insertions. WT, wild-type Col-0; Mu, *LysM RLK1* mutant. *Actin-2* was used as an internal control.

**(F)** Restoration of CRG induction in the *LysM RLK1* mutant by the ectopic expression of the *LysM RLK1* cDNA. The *LysM RLK1* cDNA driven by a cauliflower mosaic virus 35S promoter was introduced into the homozygous *LysM RLK1* mutant using a modified pCambia1200 binary vector. The complemented plants (homozygous T3) were treated with chitooctaose at a final concentration of 1  $\mu$ M or water (as a control) for 30 min. RT-PCR analysis showed that the selected CRGs were induced to a similar level in the selected complemented plants (Com-1 and Com-2) to that in the wild type. Com-1 and Com-2, two independent complemented lines; WT, wild-type Col-0. *Actin-2* is used as an internal control.

(see Supplemental Figure 1 online). Therefore, the data suggest a critical role for LysM RLK1 in chitin signaling.

The *LysM RLK1* gene is 2988 nucleotides long, with 11 introns (Figure 1B) and a coding sequence of 1854 nucleotides. It encodes a LysM RLK of 617 amino acids, with an extracellular domain containing three predicted LysM motifs, a transmembrane domain, and an intracellular Ser/Thr kinase domain (Figure 1C). Interestingly, LysM RLK1 was recently shown to be phylogenetically related to the Nod factor receptor NFR1 (Zhang et al., 2007).

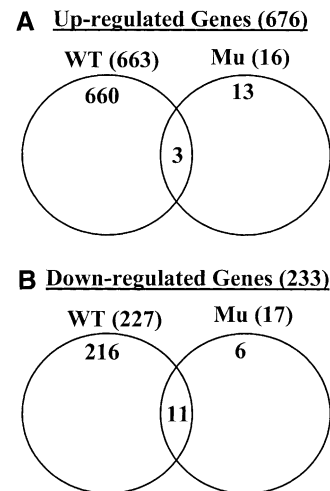
Two T-DNA insertions arranged tail-to-tail in the same location were identified in the *LysM RLK1* mutant in the 10th intron (Figure 1B). RT-PCR analysis using the primers corresponding to the exon regions on the side of the 10th intron failed to detect mRNA expression in the *LysM RLK1* mutant (Figure 1D). However, a truncated transcript derived from the gene sequence before the intron was detected by RT-PCR using the gene-specific primer pairs derived from the exons before the insertions (Figure 1E), suggesting that the T-DNA insertions in the intron blocked full-length transcription of the gene.

To confirm that the observed change in CRG expression was caused by the mutation in the *LysM RLK1* gene, the mutant was complemented with the full-length *LysM RLK1* cDNA driven by the constitutive cauliflower mosaic virus 35S promoter. As expected, complementation restored induction of CRGs (Figure 1F), confirming that it was the insertions in the *LysM RLK1* gene that caused the observed change in gene expression. The complementation data also ruled out the possibility that a truncated protein translated from the observed truncated transcript may have affected the expression of the selected CRGs.

To investigate the expression pattern of this gene, RNA from different tissues was analyzed using RT-PCR. Our data showed that the *LysM RLK1* gene was expressed ubiquitously in the whole plant, with the weakest expression in pollen (see Supplemental Figure 2 online). This expression pattern is similar to that revealed by DNA microarray analysis (<http://www.weigelworld.org/resources/microarray/AtGenExpress> and <https://www.genevestigator.ethz.ch/at/index.php?page=home>).

### The Mutation in the *LysM RLK1* Gene Blocked the Induction of Virtually All CRGs by Chitooligosaccharides

To investigate the effect of the mutation on the genome-wide gene expression in response to chitin, the Affymetrix *Arabidopsis* whole-genome array ATH1 (with ~22,000 genes) was used to compare the response of the *LysM RLK1* mutant and wild-type plants to purified chitooctose. Microarray data analysis showed that a total of 890 genes responded >1.5-fold ( $P < 0.05$ ) to chitooctose elicitation in the wild-type plants 30 min after the treatment, with 663 upregulated and 227 downregulated (Figure 2; see Supplemental Table 1 online). Out of these 890 genes, 876 (660 upregulated + 216 downregulated) were responsive to chitooctose in the wild type but not in the mutant. By contrast, only 33 genes were responsive to chitooctose in the mutant, with 16 upregulated and 17 downregulated (Figure 2; see Supplemental Table 2 online). Among these 33 genes, 14 (three upregulated + 11 downregulated) were also similarly regulated in the wild-type plants, leaving only 19 genes that were specifically regulated by chitooctose in the mutant. The regulation of this small number of genes by



**Figure 2.** The Mutation in the *LysM RLK1* Gene Blocked the Induction of Virtually All CRGs.

**(A)** Upregulated genes in the wild-type and mutant (Mu) plants in response to chitooctose, revealed by the Affymetrix *Arabidopsis* Whole Genome Array ATH1. Upregulated genes: 1.5-fold and  $P < 0.05$ .

**(B)** Downregulated genes in the wild-type and mutant (Mu) plants in response to chitooctose. Downregulated genes: 1.5-fold and  $P < 0.05$ .

chitooctose in the mutant may be due to some redundant function provided by one of the other four LysM RLKs and/or due to possible experimental variation, since these genes were only weakly to moderately regulated (–1.7- to 2.7-fold). Therefore, the mutation in the *LysM RLK1* gene appeared to block the induction of virtually all CRGs by chitooligosaccharides, suggesting a critical role for LysM RLK1 in chitin signaling.

The CRGs revealed in the above microarray analysis included many defense-related genes and signal transduction-related genes (see Supplemental Table 1 online), suggesting a potential relationship between gene induction and plant defense mediated by chitooligosaccharides.

To examine whether the mutation in the *LysM RLK1* gene causes any changes in gene expression in the absence of chitooligosaccharides, the mutant sample treated with water was compared with the similarly treated wild-type sample. Interestingly, the comparison revealed a significant expression change in 316 genes, with 100 upregulated and 216 downregulated in the mutant (see Supplemental Table 3 online). Out of these 316 genes, 94 overlapped with the above CRGs listed in Supplemental Table 1 online. This finding suggests that LysM RLK1 may also be involved in other events in addition to chitin signaling.

### The Mutation in the *LysM RLK1* Gene Led to More Susceptibility to Fungal Pathogens but Had No Effect on Bacterial Infection

If chitooligosaccharide recognition is an integral part of the response pathway by which plants defend against fungal pathogens, then mutations in the *LysM RLK1* gene should affect plant resistance to fungal pathogens. To test this, we inoculated 3-week-old mutant and wild-type plants with the biotrophic powdery mildew fungal pathogen *Erysiphe cichoracearum*. Ten

days later, the mutant plants appeared to support more fungal growth than the wild-type plants. The susceptibility appeared to be less than that observed in NahG plants, which express a bacterial salicylate hydrolase, preventing the accumulation of salicylic acid (SA), making them very susceptible to the fungal pathogen (Figure 3A). Trypan blue staining of the infected leaves also showed that the *LysM RLK1* mutant supported more hyphal growth and production of conidiophores earlier than the wild-type plants (Figure 3B). To measure the difference quantitatively, the number of conidiophores (stalks bearing asexual spores) per colony were counted 6 d after inoculation. Many more conidiophores per colony were produced on the *LysM RLK1* mutant (and also on the NahG plants) than on the wild-type plants (Figure 3C). Therefore, the *LysM RLK1* mutant was more susceptible to the pathogen than the wild-type plants. Additionally, we inoculated 4-week-old plants with the necrotrophic fungus *Alternaria brassicicola*. Three days after inoculation, the mutant developed slightly bigger lesions than the wild-type plants (Figures 4A and 4B). In agreement with this, the mutant plants also produced more spores per lesion than the wild-type plants (Figure 4C).

To examine how CRGs responded to *A. brassicicola* in both the wild-type and mutant plants, *MPK3* and *WRKY53* were monitored over time. Interestingly, both genes were still induced by the

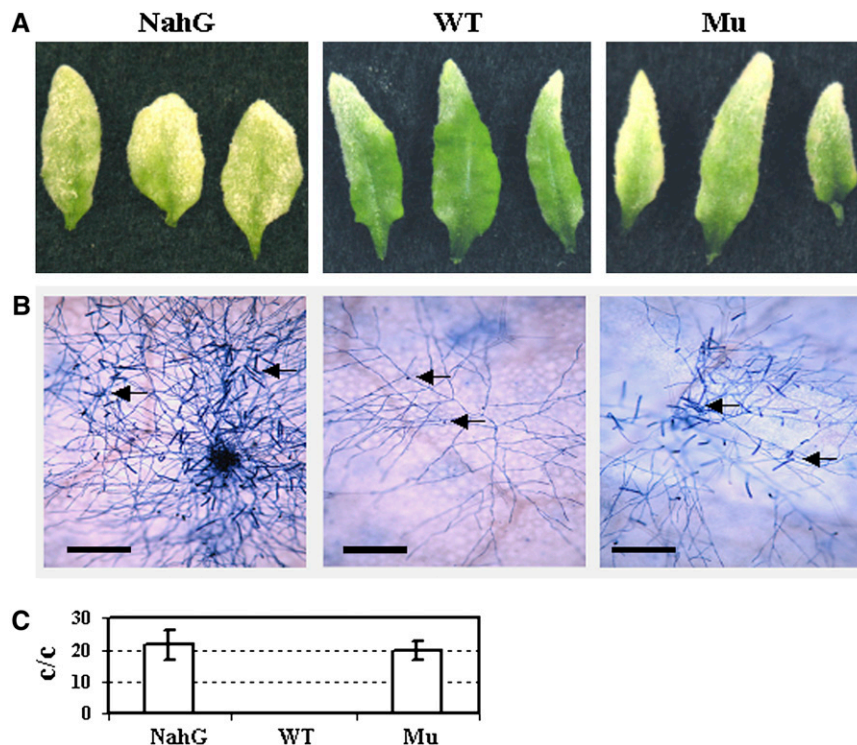
pathogen in the mutant, although to a lower level than that in the wild-type plants (see Supplemental Figure 3 online). As would be expected, these results show that blocking recognition of the chitin response pathway does not totally eliminate the ability of the plant to respond to pathogen invasion. This may explain why the mutant was only moderately susceptible to the fungal pathogens.

To test the specificity of *LysM RLK1* in fungal disease resistance, the response of the mutant to the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 was also examined. Both the mutant and wild-type plants supported a similar bacterial growth in planta 1 and 3 d after infiltration with the pathogen (Figure 5), indicating that bacterial multiplication was not affected.

Together, the above data suggest a direct connection between *LysM RLK1*-mediated chitin signaling and fungal resistance.

### Exogenously Applied Chitooligosaccharides Enhanced Plant Resistance to Both Fungal and Bacterial Pathogens

To further test the role of chitin signaling and *LysM RLK1* in plant defense, both the wild-type and mutant plants were pretreated with chitooligosaccharides and then tested for their response to different pathogens. Wild-type plants pretreated with a crab shell chitin (CSC) mixture had reduced disease symptoms upon

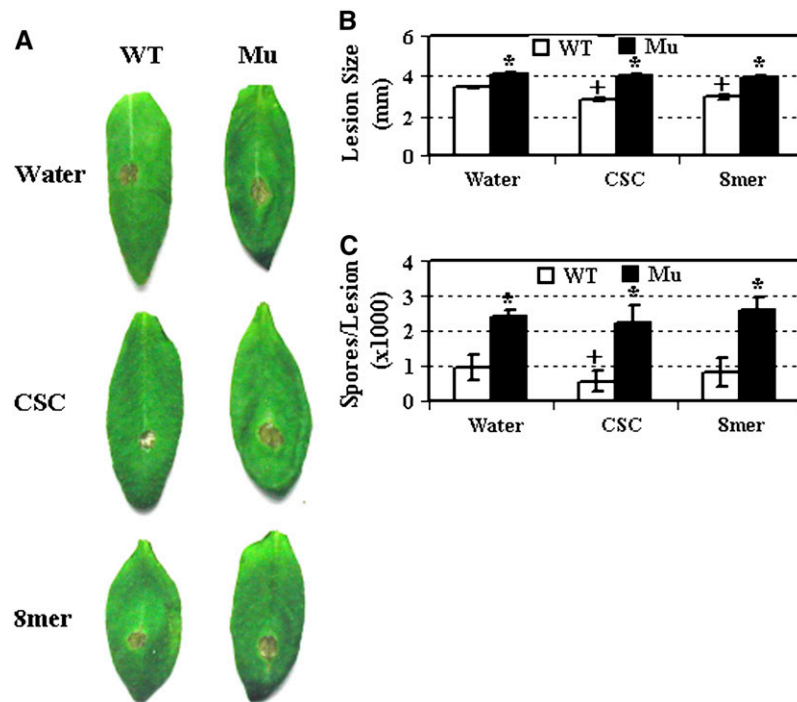


**Figure 3.** The *LysM RLK1* Mutant Was More Susceptible to a Biotrophic Fungal Pathogen Than Wild-Type Plants.

(A) Three-week-old plants were inoculated with *E. cichoracearum*. Pictures were taken 10 d after inoculation.

(B) Trypan blue staining showing fungal hyphae and conidiophores of *E. cichoracearum* on *Arabidopsis* leaves. Arrows indicate sites where conidiophores were forming. Pictures were taken 6 d after inoculation. Only one colony was shown in the pictures. Bars = 0.1 mm.

(C) The quantification of the number of conidiophores (stalks bearing asexual spores) per colony (c/c). Conidiophores were counted 6 d after inoculation. The average value and SE were based on at least 15 replications. WT, wild-type Col-0; Mu, *LysM RLK1* mutant; NahG, transgenic plants expressing a bacterial salicylate hydrolase.



**Figure 4.** The *LysM RLK1* Mutant Was More Susceptible to a Necrotrophic Fungal Pathogen Than Wild-Type Plants, and Exogenously Applied Chitooligosaccharides Enhanced Resistance in the Wild-Type Plants.

**(A)** Four-week-old plants were inoculated with *A. brassicicola*. Plants were pretreated twice (24 and 4 h before pathogen inoculation) with CSC (200  $\mu$ g/mL), purified chitooctaose (8mer, 5  $\mu$ M), or water. Pictures were taken 3 d after inoculation.

**(B)** The average diameter of the lesions caused by *A. brassicicola* on both the mutant and wild-type *Arabidopsis* leaves. The diameters of lesions from 30 leaves were measured 3 d after inoculation.

**(C)** The average number of spores of *A. brassicicola* produced per lesion by both the mutant and wild-type plants 6 d after inoculation. The number of spores per lesion was measured from 30 leaves. WT, wild-type Col-0; Mu, *LysM RLK1* mutant; CSC, crab shell chitin; 8mer, chitooctaose; water, distilled water. The asterisk indicates a significant difference between the mutant and wild-type plants under the same treatments based on a Student's *t* test ( $P < 0.05$ ). The plus symbol indicates a significance difference between the chitin-treated plants and control (water-treated) plants of the same genotype based on a Student's *t* test ( $P < 0.05$ ). Error bars indicate SE. Each experiment was repeated at least twice with similar results.

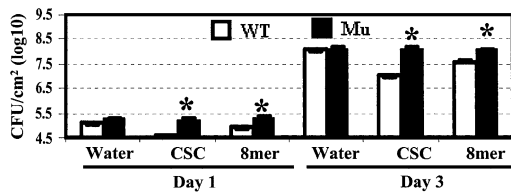
inoculation with the fungal pathogen *A. brassicicola*, as reflected by the reduced lesion size and spore production (Figures 4A to 4C). However, the protective effect from the purified chitooctaose was not as effective as from CSC. Interestingly, pretreatment also inhibited the growth of the bacterial pathogen *P. syringae* pv *tomato* DC3000 inside plants (Figure 5), reflecting a general induction of plant innate immunity by chitooligosaccharides. By contrast, similar pretreatment of the *LysM RLK1* mutant plants did not enhance resistance. These data further support the critical role of *LysM RLK1* in chitin signaling and plant innate immunity. Similar enhanced fungal resistance upon chitooligosaccharide treatment was also recently observed in rice (Tanabe et al., 2006).

#### The Mutation in the *LysM RLK1* Gene Did Not Affect Other Defense-Related Pathways

To test whether the mutation in the *LysM RLK1* gene affected the SA- and jasmonic acid/ethylene-responsive pathways, the *LysM RLK1* mutant and wild-type plants were treated with SA, methyl jasmonic acid (MeJA), and 1-aminocyclopropane-1-carboxylic acid (ACC), and expression of the pathway hallmark genes, *PR-1*

(in the SA pathway) (Ryals et al., 1996) and *PDF1.2* (in the jasmonic acid/ethylene pathway) (Penninckx et al., 1996) was examined. Quantitative RT-PCR data demonstrated that both the mutant and wild-type plants showed similar induction of *PR-1* by SA and of *PDF1.2* by MeJA or ACC (see Supplemental Figure 4 online), suggesting that the mutation did not affect these defense pathways.

To examine whether the mutation in the *LysM RLK1* gene affected the flagellin/FLS2-mediated signaling pathway, both the mutant and wild-type plants were challenged with the flagellin-derived peptide flg22 (Gomez-Gomez and Boller, 2000). The RT-PCR analysis showed that the selected flagellin-responsive genes (*MPK3*, *WRKY22*, *WRKY29*, and *WRKY53*) were similarly induced by flg22 in the mutant as in the wild-type plants (Figure 6), indicating that the flagellin signaling pathway was not affected in the mutant. Therefore, chitin signaling and flagellin signaling pathways appear to be independent, at least at the initial signal perception stages. However, increasing data suggest that different pathogen-associated molecular patterns (PAMPs) may activate a common downstream pathway to induce pathogen resistance (Asai et al., 2002; Wan et al., 2004; Zipfel et al., 2006).



**Figure 5.** The Mutation in the *LysM RLK1* Gene Did Not Affect Growth of a Bacterial Pathogen.

Four-week-old plants were pretreated twice (24 and 4 h before pathogen inoculation) with CSC (200  $\mu$ g/mL), purified chitooctase (5  $\mu$ M), or water before being infiltrated with *P. syringae* pv *tomato* DC3000. Leaf discs were collected 1 and 3 d after inoculation to determine bacterial growth. WT, wild-type Col-0; Mu, *LysM RLK1* mutant; 8mer, chitooctase; water, distilled water. Asterisks indicate that the mutant was statistically significantly different from the wild type based on a Student's *t* test ( $P < 0.05$ ). Error bars indicate SE. Each experiment was repeated at least twice with similar results.

To get a better view of this possible downstream convergence, we compared the genes differentially regulated by chitin (chitooctase, 8mer), flagellin (flg22), and EF-Tu (elf26) in *Arabidopsis* (Zipfel et al., 2004, 2006; this work). As expected, a large number of genes (441 genes) were commonly upregulated by all three stimuli (Figure 7; see Supplemental Table 4 online). This overlap supports that different pathways mediated by different PAMPs converge at a downstream step leading to induction of common downstream genes. However, only a small overlap (15 genes) was observed among the downregulated genes (Figure 7). Interestingly, chitooctase appeared to downregulate many more genes than flg22 and elf26 (Figure 7; see Supplemental Table 4 online), suggesting that the plant chitin response may have unique features, compared with the response to protein-derived PAMPs.

#### Mutations in the Potential Nod Factor Receptors Did Not Affect Chitin Signaling in the Legume *Lotus japonicus*

To test whether the recently identified legume Nod factor receptors NFR1 and NFR5 are also involved in chitin signaling, the *L. japonicus* mutants in the these two genes (*nfr1* and *nfr5*) (Limpens et al., 2003; Radutoiu et al., 2003) were challenged with chitin (chitooctase) and the expression of the selected corresponding CRGs, corresponding to the *Arabidopsis* CRGs (Wan et al., 2004), was examined using RT-PCR. As expected, the selected CRGs (Lj *MPK3*, Lj *WRKY22*, Lj *WRKY33*, and Lj *WRKY53*) were similarly induced in the mutants as in the wild-type plants (see Supplemental Figure 5 online), indicating that chitin signaling was not affected by these mutations. Therefore, our data suggest that the Nod factor receptors have evolved to such a level that they can only recognize and perceive the modified lipochitooligosaccharides, Nod factors (Zhang et al., 2007).

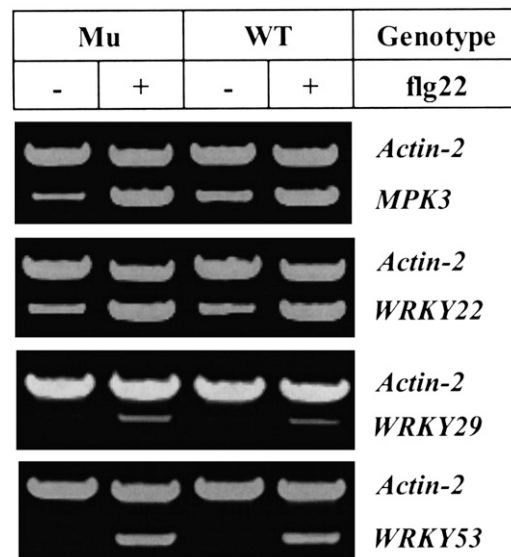
## DISCUSSION

### LysM RLK1 Is Critical for Chitin Signaling

Since the mutation in the *LysM RLK1* gene blocked the induction of virtually all CRGs, LysM RLK1 appears to be a critical component in chitin signaling. Considering its structural features (an

extracellular LysM motif-containing domain + a transmembrane domain + an intracellular kinase domain), LysM RLK1 likely plays a critical role in chitin perception. The LysM (or lysin motif) domain is a protein module that binds peptidoglycan (Joris et al., 1992; Bateman and Bycroft, 2000), which is structurally similar to chitin. Legume LysM RLKs NFR1 and NFR5 were shown to be the putative receptors of Nod factors (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). However, LysM RLK1 may not function alone in chitin perception. Like many other receptor kinases (Goring and Walker, 2004), LysM RLK1 may also require a partner protein. Indeed, recently, a rice LysM domain-containing protein CEBiP (without an obvious intracellular domain) was shown to bind chitin (Kaku et al., 2006). Therefore, LysM RLK1 may act in concert, either with another LysM RLK or a protein similar to the rice CEBiP. Since mutations in the other four *LysM RLK* genes had no obvious effect on the expression of selected CRGs, it seems unlikely that the products of these genes are essential for chitin perception. However, there are three CEBiP-like proteins in *Arabidopsis* encoded by genes At1g21880, At1g77630, and At2g17120. These CEBiP-like proteins lack an obvious intracellular domain to transduce the received chitin signal. Therefore, they may function in chitin perception by interaction with LysM RLK1. We are currently examining this possibility in *Arabidopsis*.

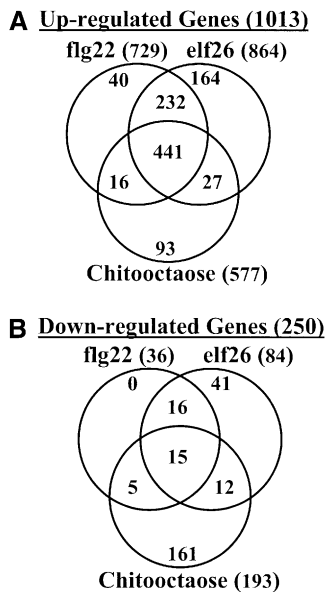
During the review of this manuscript, a report by Miya et al. (2007) appeared that described similar results also showing a critical role for LysM RLK1 (termed Chitin-Elicitor Receptor Kinase



**Figure 6.** The *LysM RLK1* Mutation Did Not Block the Induction of Flagellin-Responsive Genes.

Fourteen-day-old, hydroponically grown seedlings were treated with the flagellin-derived flg22 peptide (dissolved in DMSO) at a final concentration of 10  $\mu$ M or with an equivalent amount of DMSO (as a control). The selected flagellin-responsive genes were detected using RT-PCR. *Actin-2* was used as an internal control, and the amplification of both *actin-2* and a flagellin-responsive gene was conducted in the same PCR reaction tube with 25 cycles. The experiment was repeated twice with similar results.





**Figure 7.** Comparison of the Genes Regulated by flg22, elf26, and Chitooctaose.

(A) Venn diagram showing the overlap of the upregulated genes ( $\geq 2$ -fold) by flg22, elf26, and chitooctaose.

(B) Venn diagram showing the overlap of the downregulated genes ( $\geq 2$ -fold) by flg22, elf26, and chitooctaose. The genes regulated by flg22 and elf26 were obtained from publications (Zipfel et al., 2004, 2006). The elf26 data (60 min after treatment) were chosen, instead of elf18 data, for comparison due to the following reasons: at 60 min, elf26 induced a comparable number of genes to that by flg22 at 30 min; at 30 min, elf26 only induced approximately half of the genes by flg22; no microarray data were available from the wild-type plants after treated with elf18; elf18 data were obtained using the *fls2* mutant instead of the wild type. To be consistent with the twofold cutoff used for flg22 and elf26-regulated genes, only CRGs with a change  $\geq 2$ -fold were included in the comparison.

[CERK1] in chitin signaling. For example, Miya et al. (2007) also found that mutations in LysM RLK1 (CERK1) totally blocked chitin elicitation of CRG expression and conferred greater sensitivity to fungal infection. These authors were also unable to detect specific chitoooligosaccharide binding proteins in various membrane preparations from wild-type *Arabidopsis* plants, although the same technique was able to detect a specific binding protein in the membrane preparation from suspension-cultured rice cells (Kaku et al., 2006). Hence, the question of whether LysM RLK1 (CERK1) directly binds chitin or acts via cooperation with another protein remains to be resolved. We prefer the LysM RLK1 nomenclature since it conforms to that previously used for the legume Nod factor receptors (Limpens et al., 2003) and also conforms to the recent nomenclature suggestions for naming all members of the LysM RLK family (Zhang et al., 2007).

#### LysM RLK1 Is Involved in Basal Resistance or Plant Innate Immunity

Since the *LysM RLK1* mutation led to only moderate susceptibility to fungal pathogens and LysM RLK1 was required for the

enhanced resistance to both fungal and bacterial pathogens caused by exogenously applied chitin, LysM RLK1 appears to play a role in basal resistance to pathogens through mediating chitin signaling. This result is not surprising since it is well documented that fungal pathogens produce multiple elicitors that can be recognized by other receptors in plant cells to trigger plant innate immunity (Nürnberg et al., 2004; Zipfel and Felix, 2005). Thus, blocking recognition of the chitin response pathway would not be expected to completely block the plant defense responses. This is supported by our observation that CRGs (e.g., *MPK3* and *WRKY53*) were still induced by a fungal pathogen in the mutant, although to a lower level than that in the wild-type plants (see Supplemental Figure 3 online). This may explain why blocking chitin signaling in the *LysM RLK1* mutant only led to moderate susceptibility to fungal pathogens.

#### The Chitin Signaling Pathway Mediated by LysM RLK1 Is Unique but May Share a Common Downstream Pathway with the Flagellin/FLS2- and EF-Tu/EFR-Mediated Pathways

This work and a previous study (Zhang et al., 2002) demonstrated that the chitin signaling pathway is independent of the SA, jasmonic acid, and ethylene pathways. Chitin perception and flagellin perception also appear to be two independent processes, since the LysM RLK1 mutant was fully responsive to the flagellin-derived peptide flg22. However, similar downstream signaling components are found in these two pathways, such as the MAPK cascade and WRKY transcription factors (Asai et al., 2002; Wan et al., 2004), suggesting that a common downstream pathway may be activated by these two different PAMPs to induce pathogen resistance. A similar overlap was also revealed between the flagellin and EF-Tu (another bacterial PAMP) signaling pathways (Zipfel et al., 2006).

The comparison of the genes differentially regulated by chitooctaose (8mer), flagellin (flg22), and EF-Tu (elf26) in *Arabidopsis* (Zipfel et al., 2004, 2006; this work) revealed a large number of genes (441 genes) that were commonly upregulated by all three stimuli (Figure 7A; see Supplemental Table 4 online). These data suggest that different pathways mediated by different PAMPs converge at a downstream step leading to induction of common downstream genes.

#### LysM RLK Family Members Are Critical for Both Nod Factor and Chitin Signaling, Suggesting a Possible Evolutionary Relationship between Two Pathways

Recently, specific legume LysM RLKs were proposed to act as the receptors for Nod factors, which are critical for rhizobial bacteria to invade and induce nodulation on their legume hosts (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). Nod factors are lipochitoooligosaccharides, usually composed of 3-6 *N*-acetyl-D-glucosamine residues (Stacey et al., 2006). This study and the work from Miya et al. (2007) have now demonstrated that another LysM RLK (LysM RLK1/ CERK1) is essential for chitin signaling (likely as a part of the receptor complex) and the induction of plant innate immunity, further supporting that LysM receptors can recognize both friend and foe (Knogge and Scheel, 2006). Collectively, these results suggest that Nod factor and chitin signaling pathways are evolutionarily related,

especially at the initial perception stages. However, Nod factor recognition has become highly specific, since mutations in either of the *L. japonicus* *NFR1* and *NFR5* genes did not block the induction of the selected CRGs by chitin in this plant (see Supplemental Figure 5 online). Since chitin elicitation is widespread in plants, while Nod factor recognition is largely limited to legumes, it appears that the latter was derived from the former within the legumes. This hypothesis is also supported by phylogenetic analyses, which shows a possible evolutionary relationship between the *LysM* RLK1 and the Nod factor receptors (Zhang et al., 2007).

## METHODS

### Insertion Mutant

The *LysM* *RLK1* insertion mutant (096F09) used in this work was generated in the context of the GABI-Kat program and provided by Bernd Weisshaar (Max Planck Institute for Plant Breeding Research, Cologne, Germany) (Rosso et al., 2003). The homozygous plants were identified by genotyping using the gene-specific primers 5'-AGAATATATCCACGAG-CACACGGTTCCAG-3' (forward) and 5'-GACGAAAAGAGAGTGGATA-AAGCAACCAC-3' (reverse), together with the T-DNA left border primer, 5'-CCCATTGGACGTGAATGTAGACAC-3'.

These two primers were also used to detect the expression of the *LysM* *RLK1* gene via RT-PCR. The other primers used to detect the transcript 5' of the insertion site were as follows: 5'-ATGAAGCTAAAGATTCTC-TAATCGCTC-3' and 5'-GAAATGCACCATTGGATCTCTCCAG-3'.

### Complementation

To complement the *LysM* *LRK1* mutant, the full-length cDNA was obtained from total RNA isolated from seedlings via RT-PCR (see below for RNA isolation and cDNA synthesis). After confirmation by sequencing, the cDNA was cloned into the modified binary vector pCambia1200 that contains a 35S promoter-multiple cloning site poly(A) signal downstream of the 35S promoter. The final construct was electroporated into *Agrobacterium tumefaciens* EHA105. The resultant *A. tumefaciens* was then used to transform the homozygous *LysM* *RLK1* mutant via floral dipping (Clough and Bent, 1998). Multiple transgenic lines were obtained and raised to homozygous T3 lines.

### Growth of Seedlings and Treatment with Chitoooligosaccharides or Other Chemicals

*Arabidopsis thaliana* seedlings were grown in liquid medium as described (Zhang et al., 2002). Fourteen-day-old seedlings were treated with chitooctaose (Sigma-Aldrich) at a concentration of 1  $\mu$ M or with distilled water (as a control) for 30 min. To test flagellin-responsive genes, 14-d-old seedlings were also treated with the flagellin-derived flg22 peptide (dissolved in DMSO) at a final concentration of 10  $\mu$ M or with an equivalent amount of DMSO (as a control) for 30 min. To test other defense pathways, *Arabidopsis* seedlings were also treated for 24 h with 5 mM SA, 100  $\mu$ M MeJA, and 0.5 mM ACC (all obtained from Sigma-Aldrich) and dissolved in 0.1% ethanol. The control plants were similarly treated with an equivalent amount of ethanol. After treatment, the seedlings were collected and frozen in liquid nitrogen for RNA isolation.

### RNA Isolation and cDNA Synthesis

Total RNA was isolated using the Trizol reagent according to the manufacturer's instructions (Invitrogen). The isolated RNA was further purified using Qiagen RNeasy mini columns according to the manufacturer's

instructions and treated with Turbo DNase (Ambion). cDNA was synthesized using M-MLV reverse transcriptase according to the manufacturer's instructions (Promega).

### RT-PCR

The gene-specific primer pairs (forward and reverse) for detecting the following CRGs are as follows: 5'-CTCACGGAGGACAGTTCATAAG-3' and 5'-GAGATCAGATTCTGTGCGGTGTG-3' for *MPK3* (At3g45640); 5'-GTAAGCTCATCAGCTACTACCAC-3' and 5'-ACCGCTAGATGATC-CTCAACAG-3' for *WRKY22* (At4g01250); 5'-ATGGACGAAGGAGACC-TAGAAG-3' and 5'-CCGCTTGGTGCCTACTCGTTTC-3' for *WRKY29* (At4g23550); 5'-CTCCGACCACAACACTACAAC-3' and 5'-GGCTCTC-TCACTGTCTTCTTC-3' for *WRKY33* (At2g38470); 5'-CCTACGAGA-GATCTCTTCTCTG-3' and 5'-AGATCGGAGAACTCTCCACGTG-3' for *WRKY53* (At4g23810). As an internal control, the following forward and reverse primers of *actin-2* (At3g18780) were designed: 5'-GACTAAGA-GAGAAAGTAAGAGATAATCCAG-3' and 5'-CAGCCTTTGATTTCAATTT-GCATGTAAGAG-3'.

To analyze gene expression, equivalent amounts of total RNA from both control and treated samples were input in cDNA synthesis (see above), and then equivalent amounts of cDNA were included in PCR reactions that contained both a gene-specific primer pair and the *actin-2* primer pair under the following PCR conditions: 94°C, 3 min; 94°C, 30 s; 55°C, 30 s; 72°C, 1.5 min; 25 cycles; 72°C, 3 min. The resultant PCR products were resolved on a 1.2% agarose gel for comparison. The *actin-2* band served as an internal control.

### Quantitative PCR

To quantify gene expression using quantitative PCR, the forward and reverse primers of each gene were as follows: 5'-AACACGTGCAATG-GAGTTTGTGGTCACT-3' and 5'-ACCATTGTTACACCTCACTTTGGCA-CAT-3' for *PR-1* (At2g14610); 5'-AGTGCATTAACCTGAAGGAGCCAAA-CAT-3' and 5'-AACAGATACACTTGTGTGCTGGGAAGACA-3' for *PDF1.2* (At5g44420); 5'-TGGCCATTGATCTTGTGACAGAATGTTGA-3' and 5'-TCGTGCAATTTAGCAAGGTAAGTGTGATT-3' for *MPK3* (At3g45640); 5'-TTTAGGCGCCAAATCCCAAGGAGTTATT-3' and 5'-TCTGGACTT-GTTTGTGTTGCCAACAGTTT-3' for *WRKY53* (At4g23810); 5'-GGTAT-TCTTACCTGAAGTATCCTATTG-3' and 5'-CTCATTGTAGAAAGTGTG-ATGCCAGATC-3' for *actin-2* (At3g18780). *Actin-2* 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 SYBR Green Master Mix (Applied Biosystems) with the following conditions: 95°C, 10 min; 95°C, 15 s; 60°C, 1 min; 40 cycles, followed by the dissociation curve analysis to verify the single amplicon. The fold change in the target gene, normalized to *actin-2* and relative to the gene expression in the control sample, was calculated as described (Libault et al., 2007).

### Microarray Experiment and Data Analysis

Double-stranded cDNA was synthesized from 8  $\mu$ g of purified total RNA and purified and turned into cRNA according to the Affymetrix GeneChip Expression Analysis Technical Manual. The purified biotin-labeled cRNA was fragmented and hybridized with microarrays according to the manufacturer's instructions (Affymetrix GeneChip Expression Analysis Technical Manual). Three biological replicates were included in the microarray experiment.

The scanned data (CEL or DAT files) were analyzed using the software DNA-Chip Analyzer (dChip) (version release September 23, 2005) (Li and Wong, 2001). The default setting were employed for normalization using the default array with median brightness (i.e., Mu-8mer-I) as the baseline. Model-based expression values were computed using the default



settings. To identify significantly regulated genes between the two different samples, the following criteria were selected: 1.5-fold, with a *t* test *P* value < 0.05, the absolute signal intensity difference between a baseline and its corresponding treatment >100, and the *P* (presence) call% in the samples involved  $\geq 20\%$ . Additionally, the false discovery rate (FDR) was also computed by applying the above comparison criteria to the sample-wise permuted data sets and recording the number of obtained genes at each permutation. After 200 permutations, the median of the 200 values was reported as the median FDR, and the 90th percentile (90th largest value) of the 200 values was reported as 90% FDR (see the dChip website for details; www.dchip.org).

The microarray data were deposited in the Gene Expression Omnibus database at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/geo/; accession number GSE8319).

### Disease Assays

Disease assay with *Erysiphe cichoracearum* isolate UCSC1 was conducted as previously described (Wilson et al., 2001). Trypan blue staining of fungal structures was conducted as described (Ramonell et al., 2005). The conidiophores (stalks bearing asexual spores) per colony were counted 6 d after inoculation. Disease assay with *Alternaria brassicicola* was conducted as described (Van Wees et al., 2003) with a spore suspension of  $5 \times 10^5$  spores/mL by dot-inoculating 5  $\mu$ L of the spore solution. The disease assays with *Pseudomonas syringae* pv *tomato* DC3000 were conducted as previously described (Zhang and Gassmann, 2003) with a bacterial concentration of  $5 \times 10^4$  colony-forming units/mL.

To test whether exogenously applied chitooligosaccharides can enhance defense, plants were pretreated twice (24 and 4 h before pathogen inoculation) by spraying with either CSC mixture at a final concentration of 200  $\mu$ g/mL supplemented with 0.01% of Silwet L-77 or purified chitoctase supplemented with 0.01% of Silwet L-77 at a final concentration of 5  $\mu$ M until runoff. Control plants were similarly treated with water supplemented with 0.01% of Silwet L-77.

### Identification and Detection of the Selected CRGs in the Legume Plant *Lotus japonicus*

The corresponding CRG genes in *L. japonicus* were identified by searching the cDNA sequences of the selected *Arabidopsis* CRGs (and also *actin-2*) used in this work against The Institute for Genomic Research *Lotus japonicus* Gene Index (<http://compbio.dfci.harvard.edu/tgi/cgi-bin/tgi/gimain.pl?gudb=Ljaponicus>). The closest hits were chosen and arbitrarily named after their *Arabidopsis* counterparts with the prefix Lj (for *L. japonicus*). The following primer pairs were designed to detect the corresponding genes: 5'-CACCTTGGCTAGAGAGTTTACTGATGTC-3' and 5'-GTTGACGAGGATATTGAGGAAGTTGTCTG-3' for Lj *MPK3* (TC8079); 5'-TCACCTTGCTGGTCTGGTTCTGGTTCTG-3' and 5'-TCT-GATAGGGGTGCAACCCCATCTTCTTC-3' for Lj *WRKY22* (AV423663); 5'-AGTTGTGGTTCAGACCACAGTGACATTG-3' and 5'-ACCCATTGAGTTTCCAAACCCTGATGAG-3' for Lj *WRKY33* (TC14849); 5'-CCC-ATCAAAAGAACCAACCACAACAAGAG-3' and 5'-ATCCGCACGCACT-TGAACCATGTATTGTG-3' for Lj *WRKY53* (TC9074); and 5'-AAG-GTTTCGTAACGATGGCTGATGCTGAG-3' and 5'-ACCTTGATCTCA-TGCTGCTAGGAGCAAG-3' for Lj *Actin-2* (TC14247). Lj *Actin-2* was used as an internal control in RT-PCR reactions.

### Accession Numbers

The Arabidopsis Genome Initiative accession numbers for the genes used in this study are as follows: *LysM RLK1*, At3g21630; *LysM RLK2*, At1g51940; *LysM RLK3*, At2g33580; *LysM RLK4*, At2g23770; *LysM RLK5*, At3g01840; *MPK3*, At3g45640; *WRKY22*, At4g01250; *WRKY29*, At4g23550; *WRKY33*, At2g38470; *WRKY53*, At4g23810; *PR-1*,

At2g14610; *PDF1.2*, At5g44420; and *actin-2*, At3g18780. The accession numbers of some corresponding genes in *L. japonicus* were as follows: Lj *MPK3*, TC8079; Lj *WRKY22*, AV423663; Lj *WRKY33*, TC14849; Lj *WRKY53*, TC9074; and Lj *Actin-2*, TC14247.

### Supplemental Data

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

**Supplemental Figure 1.** Initial Screen of the Five LysM RLK Mutants in Response to Chitin Treatment.

**Supplemental Figure 2.** Expression Analysis of the *LysM RLK1* Gene in Different Tissues or Organs.

**Supplemental Figure 3.** The Selected CRGs Were Still Induced in the *LysM RLK1* Mutant by a Fungal Pathogen but to a Reduced Level.

**Supplemental Figure 4.** The Mutation in the *LysM RLK1* Gene Did Not Affect Other Defense-Related Pathways.

**Supplemental Figure 5.** The Mutations in the Nod Factor Receptor Genes *NFR1* and *NFR5* in the Legume *Lotus japonicus* Did Not Affect the Induction of the Selected CRGs in the Plant.

**Supplemental Table 1.** The 890 Genes Regulated in the Wild Type by Chitoctase.

**Supplemental Table 2.** The 33 Genes Regulated in the Mutant by Chitoctase.

**Supplemental Table 3.** The 316 Genes Affected by the Mutation in the *LysM RLK1* Gene in Absence of Chitoctase.

**Supplemental Table 4.** The 456 Genes Commonly Regulated by flg22, elf26, and Chitoctase.

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