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LYS12 LysM receptor decelerates *Phytophthora palmivora* disease progression in *Lotus japonicus*

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SUMMARY

Phytophthora palmivora **is a devastating oomycete plant pathogen. We found that *P. palmivora* induces disease in *Lotus japonicus* and used this interaction to identify cellular and molecular events in response to this oomycete, which has a broad host range. Transcript quantification revealed that *Lys12* was highly and rapidly induced during *P. palmivora* infection. Mutants of *Lys12* displayed accelerated disease progression, earlier plant death and a lower level of defence gene expression than the wild type, while the defence program after chitin, laminarin, oligogalacturonide or flg22 treatment and the root symbioses with nitrogen-fixing rhizobia and arbuscular mycorrhiza were similar to the wild type. On the microbial side, we found that *P. palmivora* encodes an active chitin synthase-like protein, and mycelial growth is impaired after treatment with a chitin-synthase inhibitor. However, wheat germ agglutinin-detectable *N*-acetyl-glucosamine (GlcNAc) epitopes were not identified when the oomycete was grown *in vitro* or while infecting the roots. This indicates that conventional GlcNAc-mers are unlikely to be produced and/or accumulate in *P. palmivora* cell walls and that LYS12 might perceive an unknown carbohydrate. The impact of *Lys12* on progression of root rot disease, together with the finding that similar genes are present in other *P. palmivora* hosts, suggests that LYS12 might mediate a common early response to this pathogen.**

Keywords: ***Lotus japonicus*****,** ***Phytophthora palmivora*****, LysM receptor, disease, signalling.**

INTRODUCTION

Plant roots are constantly exposed to a wide range of soil microbes. Some are beneficial, like the arbuscular mycorrhizal (AM) fungi or nitrogen-fixing rhizobia, which colonize plant roots and provide the host with mineral nutrients such as phosphorus or nitrogen, respectively (Oldroyd, 2013). On the other hand, pathogens fully exploit the capacity of plants to produce photosynthetic products for their own growth and proliferation. In this category, oomycetes are recognized as some of the most devastating microbes for land plants (Drenth and Guest, 2004; Kroon *et al.*, 2012). Many species in this group are hemibiotrophs; they require living host tissues for initial infection (biotrophy), while during the later necrotrophic stage the plant cells are killed, resulting in important crop diseases. Despite their similar strategy for infection, the host range of various oomycetes varies widely. *Phytophthora sojae* is considered to be a soybean pathogen, *Phytophthora infestans* mainly infects shoots of tomato, potato and some wild tobacco species, *Aphanomyces euteiches* infects legumes in general, while *Phytophthora palmivora* is a cosmopolitan pathogen with a wide host spectrum infecting roots and shoots of cash crops such as oil palm, cacao, durian and rubber (Drenth and Guest, 2004). The interaction between *P. infestans* and potato, causing late blight disease, is currently the best-studied plant–oomycete association at molecular and cellular level (Haldar *et al.*, 2006; Kamoun *et al.*, 2015; Whisson *et al.*, 2016). However, recent studies based on analyses of the model legume *Medicago truncatula* pinpointed host genes that contribute to the disease induced by *A. euteiches* and *P. palmivora*, setting the scene for identification of host components involved in sensing infection by these oomycete

pathogens (Nars *et al.*, 2013; Rey *et al.*, 2013; Bonhomme *et al.*, 2014).

Receptor-mediated recognition of microbial carbohydrates is one of the central mechanisms controlling host–microbe interactions, and activation of this surveillance system is, in many interactions, decisive for the subsequent plant response (Radutoiu *et al.*, 2003; Miya *et al.*, 2007). The importance of lysin motif (LysM) carbohydrate receptors in plants has emerged from genetic and biochemical studies of their involvement in perception of *N*-acetyl-glucosamine (GlcNAc)-type signalling molecules (Miya *et al.*, 2007; Petutschnig *et al.*, 2010; Shimizu *et al.*, 2010; Liu *et al.*, 2012). Demonstrating the diversity of this surveillance system, legume LysM receptors distinguish chitin oligomers (COs) from their acylated derivatives, lipochitooligosaccharides (LCOs or Nod factors; Broghammer *et al.*, 2012; Madsen *et al.*, 2003; Radutoiu *et al.*, 2003) and exopolysaccharides (Kawaharada *et al.*, 2015), the symbiotic rhizobial signals that induce a signalling cascade leading to nodule organogenesis and bacterial infection. Similarly, LysM receptor kinases and LysM proteins have been shown in Arabidopsis and rice to perceive chitin and peptidoglycan, both of which are carbohydrates originating from pathogens. This ability of LysM receptors to perceive beneficial or pathogenic chitin-related microbial signals suggests that a general, ancient carbohydrate recognition mechanism is present in plants, and is one of the components of elaborate mechanisms involved in the perception of microbe-associated molecular patterns (MAMPs). The increased susceptibility of Nod-factor receptor mutants *nfp* and/or *hcl* to infection by *P. palmivora* or *A. euteiches*, respectively, indicates that LysM receptors may also play an active role in plant–oomycete interaction (Rey *et al.*, 2013) even though oomycete cell walls primarily consist of β-glucan(s) (Melida *et al.*, 2013). Oomycete species vary greatly in the GlcNAc content in their cell walls, and the detailed characterisations performed so far of *in vitro* grown pathogens revealed a suite of GlcNAc molecules in *A. euteiches* (Badreddine *et al.*, 2008) while none were detected in *P. infestans* and *P. parasitica*.

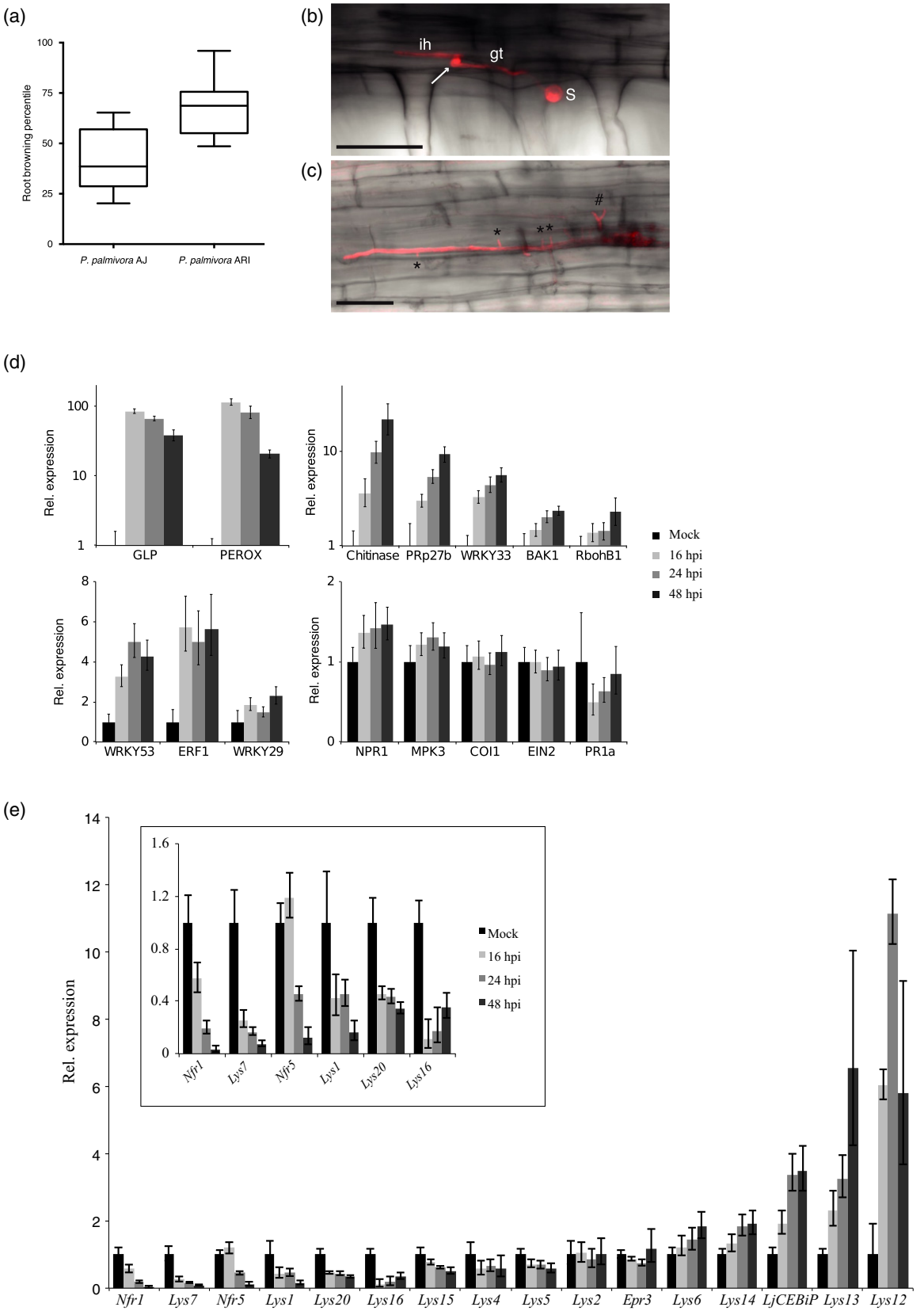
Here we show that *P. palmivora* has an active chitin synthase-like protein, but WGA (wheat-germ agglutinin)-detectable GlcNAc-mers were not observed in the hyphal/filamentous structures produced during *in vitro* growth or plant infection. We found that *P. palmivora* is a compatible pathogen for *Lotus japonicus*, and that LYS12, a LysM receptor protein of the NFR5-type, allows the legume host to keep the pace of disease progression under control.

RESULTS

Lotus japonicus is a compatible host for *P. palmivora*

Phytophthora palmivora is a cosmopolitan pathogen which also infects leguminous plants (Rey *et al.*, 2013). To determine if the model legume *Lotus japonicus* is a compatible host for this oomycete, wild-type Gifu plants were inoculated with two different *P. palmivora* strains, ARI (P3914, Arizona) and AJ (P6390, South Sulawesi). Both were able to induce clear disease symptoms, with ARI being the more aggressive. Root browning and plant death were observed 1 day after inoculation with ARI, while AJ induced root browning after 2 days and plant death after 7 days (Figure 1a). In order to gain a better insight into the progression of *P. palmivora* infection and disease in *L. japonicus* we took advantage of the fluorescently labelled *P. palmivora* strain AJ-td (Rey *et al.*, 2013) to follow the interaction from germinating spores to fully developed infection structures inside the root (Figure 1b and Figure S1 in the Supporting Information). Previous studies performed in *M. truncatula* revealed that a high inoculum load (mycelia plug, or 10^4 spores per two plants) leads to multiple infections on the whole root system limiting the possibility of identifying subtle phenotypes in the presence of this very aggressive pathogen (Huisman *et al.*, 2015). Consequently, a low infection pressure (50 spores per plant) was used to better capture the invasion and colonization events. Under these conditions the first appressoria and root penetration were observed at 16 h post-infection (hpi) while at 24 hpi hyphae growing longitudinally along root cortical cells and forming numerous haustoria were observed (Figure 1c). At these time points no visible cellular changes were detected on the root (Figure S1). After 48 h the pathogen was found actively growing with longitudinal and transverse hyphae throughout the root, and this was followed by visible root browning after 3 days. These cellular and microscopic observations were

Figure 1. *Phytophthora palmivora* infects *Lotus japonicus* roots effectively.
(a) Root browning response of *L. japonicus* roots 3 days post-infection with *P. palmivora* AJ and ARI isolates.
(b) *Phytophthora palmivora* forms appressoria and haustoria during root infection of *L. japonicus*. Germinating tubes (gt) induced by *P. palmivora* AJ spores (S) form the appressorium (arrow) at the site of root penetration, and intercellular hyphae (ih) colonize the root interior. Scale bar = 50 μm.
(c) Haustoria (*) and occasional split haustoria (#) are formed by the intercellular hyphae. Scale bar = 50 μm.
(d) Four clusters of expression patterns are detected for *L. japonicus* defence marker genes at 16, 24 or 48 h after infection with *P. palmivora*. Note the logarithmic scales for the top two clusters.
(e) Relative expression level of *L. japonicus* LysM receptors in roots during *P. palmivora* infection. The inset shows a close-up image of the down-regulated LysM receptors. *Lys11* and *Lys21* had expression levels under the detection limit and are not shown.
In (d) and (e) relative transcript levels in three biological and three technical replicates, normalized to three housekeeping genes (ATPase, UBC, PP2A) are presented. Error bars show the 95% confidence interval. [Colour figure can be viewed at wileyonlinelibrary.com]



complemented by quantification of the pathogen biomass present in *L. japonicus* roots using a qPCR assay based on expression of *P. palmivora* genes. Transcript levels of pathogen-specific genes were significantly increased at 48 hpi, indicating proliferation of *P. palmivora* inside *Lotus* roots (Figure S2).

Next, we investigated the molecular response of the host to *P. palmivora* infection. For this we used qPCR analysis to determine the expression pattern of a diverse panel of genes regulated during the general plant immune response, defence response to oomycete infection or hormone pathways linked to plant defence. The target transcripts chosen for analysis of induced immune responses were the transcription factors *Mpk3* (Wan *et al.*, 2004), *Bak1* (Heese *et al.*, 2007), *Wrky22*, *Wrky29*, *Wrky33* and *Wrky53* (Wan *et al.*, 2004; Heese *et al.*, 2007; Nicaise *et al.*, 2009), *Chitinase* (CHIchr5; Nakagawa *et al.*, 2011), *Pathogen-response gene* (*PRp27b*; Nakagawa *et al.*, 2011) and *Respiratory burst oxidase homolog B1* (*RbohB1*; Nakagawa *et al.*, 2011). The oomycete-specific responses were analysed from the expression levels of *Peroxidase* (*Perox*) and the *Germin-like protein* (*Glp*)-encoding genes, previously shown to be upregulated during *A. euteiches* and *P. palmivora* infection in *M. truncatula* (Rey *et al.*, 2013). Finally, we tested the regulation of *Coi1* (Yan *et al.*, 2009) as a component of the jasmonic acid pathway, *Npr1* (Cao *et al.*, 1997) and *PR1* (of the salicylic acid pathway), *Ein2* (of the ethylene pathway), and the ethylene response factor 1 (*Erf1*) which integrates signals from the ethylene and jasmonate pathway (Lorenzo *et al.*, 2003). Analysis of the transcript levels for these target genes revealed four different clusters of expression patterns (Figure 1d). Two genes, *Perox* and *Glp*, were highly and rapidly induced in the first stages of infection, whereas five other genes, *chitinase*, *PRp27b*, *WRKY33*, *BAK1* and *RbohB1*, displayed a constant increase over time, with the highest expression at 48 hpi. *Erf1*, *Wrky53* and *Wrky29* were significantly induced at all time points, while *Npr1*, *Mpk3*, *Coi1*, *Ein2* and *PR1a* were not significantly induced during infection with *P. palmivora*.

Based on the microscopic visualization of hyphal development inside the root, the induced root cellular changes and the induction of defence genes we conclude that *P. palmivora* is inducing a defence response in *L. japonicus.*

Genes encoding LysM receptor proteins are transcriptionally regulated during *P. palmivora* infection

Single-pass transmembrane plant receptors containing LysM domains in their extracellular region are known to be involved in perception of microbial carbohydrate molecules (Radutoiu *et al.*, 2003; Willmann *et al.*, 2011; Broghammer *et al.*, 2012; Liu *et al.*, 2012), and specific members of this family were transcriptionally regulated after chitin treatment of *L. japonicus* roots and shoots (Lohmann *et al.*, 2010). Previous studies of *M. truncatula* infection by oomycetes identified mutants impaired in LysM receptors to be more susceptible to infection (Rey *et al.*, 2015). In *Lotus* we have investigated the transcriptional regulation of LysM receptors during the infection of roots with *P. palmivora* AJ-td using quantitative RT-PCR. We found that 11 genes coding for LysM receptors were transcriptionally regulated in roots at different time points after spore inoculation (Figure 1e). Transcription of *Lys12* and *Lys13* was mostly affected by *P. palmivora* infection. *Lys12* showed a rapid increase in transcript level by 16 hpi, when the first hyphae penetrate the root, and was upregulated 11-fold at 24 hpi. The *Lys13* transcript steadily increased throughout the 16–48 h period of analysis. In addition, three other receptors, *Lys6*, *Lys14* and the likely orthologue of *OsCEBiP* in *Lotus* (*LjCEBiP*), were found to be significantly upregulated. Interestingly, the group of genes that were significantly downregulated after infection, especially at later time points, contains genes that are known to play a role in root nodule symbiosis, such as the Nod-factor receptors *Nfr1* and *Nfr5* (Madsen *et al.*, 2003; Radutoiu *et al.*, 2003). Finally, seven *Lys* genes, among them the exopolysaccharide (EPS) receptor *Epr3*, did not show a differential response to *P. palmivora* infection, indicating that *L. japonicus* mounts a specific regulation of LysM genes during association with the *P. palmivora* pathogen, with genes involved in chitin signalling being actively upregulated (Bozsoki *et al.*, 2017).

Phytophthora palmivora encodes an active chitin synthase-like protein but no WGA-detectable GlcNAc residues were identified in the hyphal walls

Recent biochemical studies of the extracellular carbohydrates produced by different *in vitro* grown oomycete species revealed the presence of chitin and GlcNAc-type molecules in the cell wall of some, but not all, tested species (Melida *et al.*, 2013). However, GlcNAc compounds were not found in the extracts of *P. infestans* and *P. parasitica* (Melida *et al.*, 2013). This agrees with previous observations showing the lack of *P. parasitica* labeling by WGA, while this was clearly observed for *A. euteiches* which contains more than 10% GlcNAc in its cell wall (Badreddine *et al.*, 2008; Melida *et al.*, 2013). The composition of the *P. palmivora* cell wall is currently unknown, and nor is its carbohydrate elicitor. The specific upregulation of LysM genes involved in chitin perception during *P. palmivora* infection of *L. japonicus* roots prompted us to investigate whether this pathogen has the capacity to produce GlcNAc-based molecules and whether these would be deposited in its cell wall. First, we used the sequence of the *P. infestans* putative chitin synthase gene and identified the presence of a chitin synthase-like gene in *P. palmivora* encoding a protein which is highly similar to the corresponding one in *P. infestans* (94% identity) (Figure S3). This gene was found

to be transcriptionally active in *P. palmivora* spores and during intraradical hyphal growth (Figure S4). Next, we used an established pharmacological approach (Guerriero *et al*., 2010) to investigate if the activity of this putative chitin synthase is important for *in vitro* growth of *P. palmivora*. We observed that in the presence of nikkomycin Z, a potent inhibitor of chitin synthase (Guerriero *et al*., 2010), a clear decrease in the growth of mycelia occurs with increasing drug concentrations (Figures 2a and S5), indicating that the activity of chitin synthase-like proteins is important for microbial proliferation.

Next, we investigated whether GlcNAc-based molecules are produced by *P. palmivora* during *in vitro* or *in planta* growth. Incubation of plate-grown mycelia or infected *Lotus* roots with Alexa Fluor 488 tagged WGA revealed only a faint residual staining of *P. palmivora* septa (Figures 2b, c and S6). Further detailed investigations were performed using transmission electron microscopy of *P. palmivora*- or AM-infected *L. japonicus* root sections wherein the immunolocalization of GlcNAc on microbial structures was monitored using gold-labelled WGA (Balestrini *et al*., 1996). We observed that GlcNAc epitopes were detected in the AM fungal wall (Figure 2d–f), but not in *P. palmivora* while infecting *Lotus* roots (Figure 2g, h). Together, our results indicate that an active chitin synthase-like protein is necessary for *P. palmivora* growth, but WGA-detectable GlcNAc residues could not be identified in the cell wall of this pathogen during *in vitro* or *in planta* growth.

Lys12 mutation leads to higher susceptibility to *P. palmivora* disease development

Our transcriptional study identified a clear upregulation of *Lys* genes involved in chitin signalling during the *Lotus*–*P. palmivora* interaction. On the other hand, no typical WGA-detectable GlcNAc residues were found in *P. palmivora*. This conundrum prompted us to investigate whether *Lys12*, the LysM receptor most responsive to *P. palmivora* infection, has a role in the development of root rot disease in *L. japonicus.* Phylogenetically, *Lys12* belongs to the same clade as the *Nfr5* Nod factor receptor, but genes encoding highly similar proteins have been identified in both monocotyledonous and dicotyledonous species (Figure S7). We took advantage of the LORE1 (Lotus retrotransposon 1) mutant collection (Malolepszy *et al*., 2016), and identified two lines with independent retro-element insertions in the *Lys12* coding region (Figure 3a). Homozygous insertion mutants were identified and their response after *P. palmivora* inoculation was characterized. First, we monitored their ability to survive *P. palmivora* infection, and an LD_{50} value for wild-type and *lys12* mutants was calculated based on the number of dead plants over time. On average, *lys12* mutants died after 50 dpi, significantly faster than Gifu, which survived on average 4 days longer (Figure 3b). Then we assessed the extent of the development of early disease symptoms by monitoring the percentage of plants that displayed the characteristic root browning. Plants were inspected for 15 days, and the first signs of root browning were detected 3 dpi. We found that *lys12* mutants had significantly larger proportions of their roots with browning symptoms compared with wild-type roots at early (7 dpi) and later (15 dpi) stages of *P. palmivora* infection (Figures 3c and S8). These results show that *lys12* mutants differ significantly from the wild-type plants, with increased and faster development of disease symptoms after *P. palmivora* infection, indicating a clear role of this LysM receptor in the development of root rot disease.

Lys12 mutants have a normal pattern-triggered immune response to known elicitors, but defective gene regulation during *P. palmivora* infection

Analyses based on *lys12* mutant responses to *P. palmivora* infection revealed an involvement of *LYS12* in root rot disease in *Lotus*. These findings raised the question of whether the increased susceptibility of *lys12* mutants results from a generally deficient/decreased immune response after elicitation by known MAMPs. Production of reactive oxygen species (ROS) in wild-type and *lys12* mutant roots was therefore monitored after chitin (CO8), laminarin (mix of 1,3 and 1,6 β-glucan), oligogalacturonide or flg22 treatment. mitogen-activated protein kinase (MAPK) phosphorylation was assayed after CO8 and laminarin treatment, and transcriptional activation of defence genes in roots treated with CO8 was measured. ROS production and MAPK phosphorylation were detected, and induction of defence genes was observed in all genotypes irrespective of the elicitor treatment, indicating that *Lys12* is not required for perception of these pathogen-associated molecular patterns (PAMPs) (Figures 3d and S9).

Next, we investigated if the enhanced susceptibility of *lys12* mutants is caused by a defective defence response mounted during *P. palmivora* infection. For this we analysed the expression level of *Lotus* defence marker genes that were upregulated by *P. palmivora* in the wild type at 48 h (Figure 1d). We found that genes strongly induced in the wild type during *P. palmivora* infection (*Perox*, *Glp*, *Chitinase* and *PRp27b*) were significantly reduced (30–50% less) in *lys12* mutants (Figure 3e). Defence genes that were regulated to a lower level by *P. palmivora* in the wild type (*Wrky33*, *Wrky53*, *Bak1*, *RbohB1* and *Erf1*) did not show a regulatory difference between wild-type and *lys12* mutant roots (Figure 3f). This reduced activation of defence genes in *lys12* mutants was not accompanied by a consistent differential hyphal biomass. *Phytophthora palmivora* housekeeping genes had similar levels in the wild type and *lys12* mutants with an increased level at 48 h in *lys12-2* (Figure S8). This indicates that accumulation of *P. palmivora* in the root tissue is not directly affected by a lack of *Lys12*.

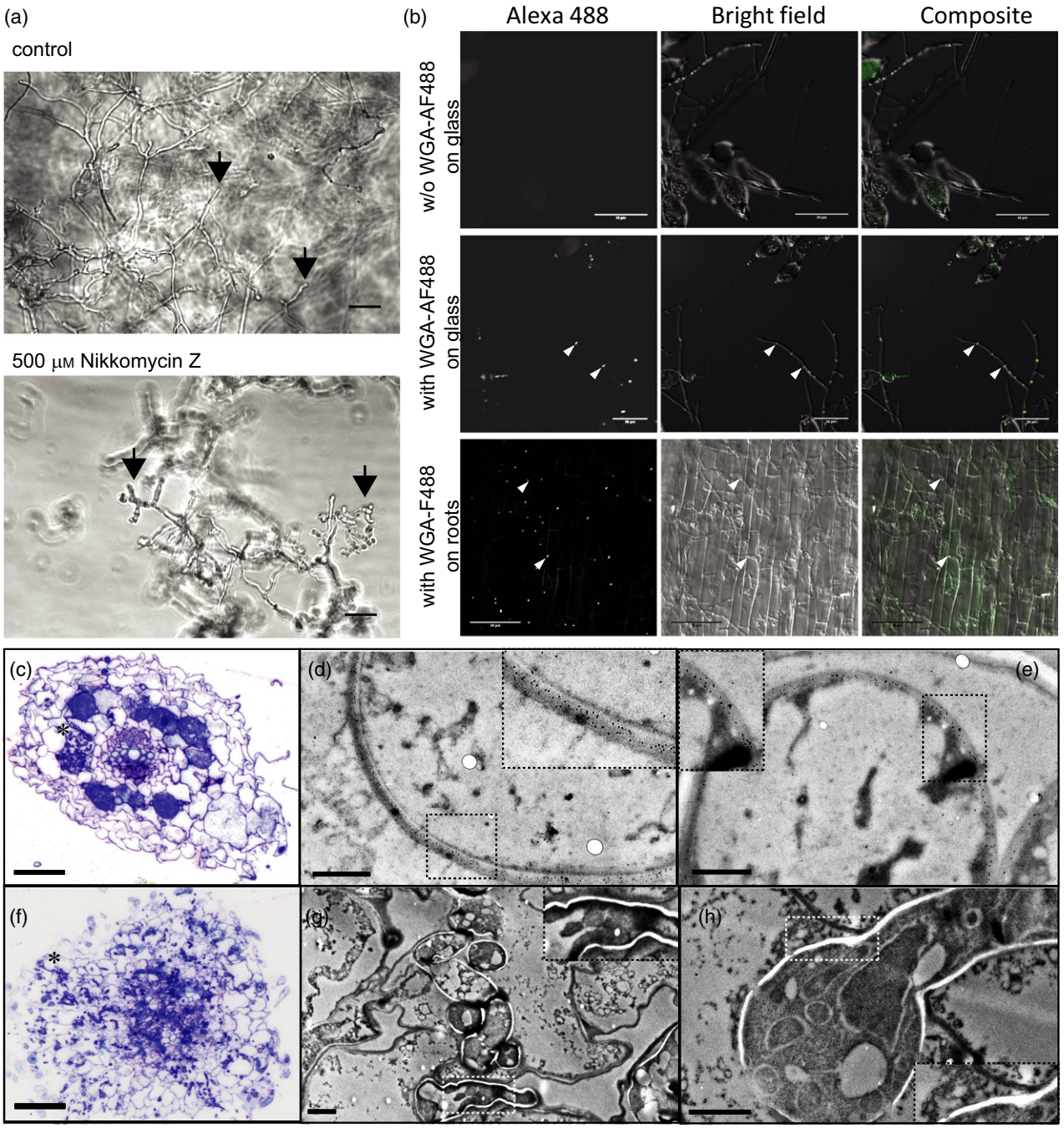


Figure 2. *Phytophthora palmivora* contains an active chitin synthase-like protein but no *N*-acetyl-glucosamine (GlcNAc)-type molecules were detected in the cell wall.

(a) Severe growth impairment of *in vitro* grown *P. palmivora* in the presence of Nikkomycin Z compared with the control treatment. Notably, hyphae develop bulbous tips and a high branching behaviour in the presence of Nikkomycin Z (arrows), and the overall number and density of hyphae is much lower than in control conditions. (b) Confocal images of *P. palmivora* structures when grown *in vitro* (top two rows) and *in planta* (bottom row, on *L. japonicus* roots) in the absence (top panels) or presence of wheat-germ agglutinin (WGA-F488). Note that only septae (arrowheads) of germ tubes can be stained with WGA, while microbial structures associated with intraradical hyphae or appressoria show no WGA-derived fluorescence. (c)–(e) *Lotus japonicus* roots infected by *Rhizophagus irregularis*. (c) Light microscopy of transverse root section showing inner cortical cells infected by the arbuscular mychorrizal (AM) fungi (*). Transmission electron micrographs of AM-infected cells from (c) where the GlcNAc epitopes are detected by gold-labelled WGA (black dots) on the hyphal wall (d) and on the fungal wall of arbuscules (e). (f)–(h) *Lotus japonicus* roots infected by *P. palmivora*. (f) Light microscopy of a transverse root section showing root cells infected by *P. palmivora* (*). Transmission electron micrographs of *P. palmivora*-infected cells from (f) where the GlcNAc epitopes are not detected by gold-labeled WGA on the hyphal wall (g) or on the haustorial wall (h). The insets in (d), (e), (g) and (h) show close-up images of the regions marked with a dotted line. Scale bars: 100 mm (a), 50 μm (c, f), 1 μm (d, e, h), 2 μm (g). [Colour figure can be viewed at wileyonlinelibrary.com]

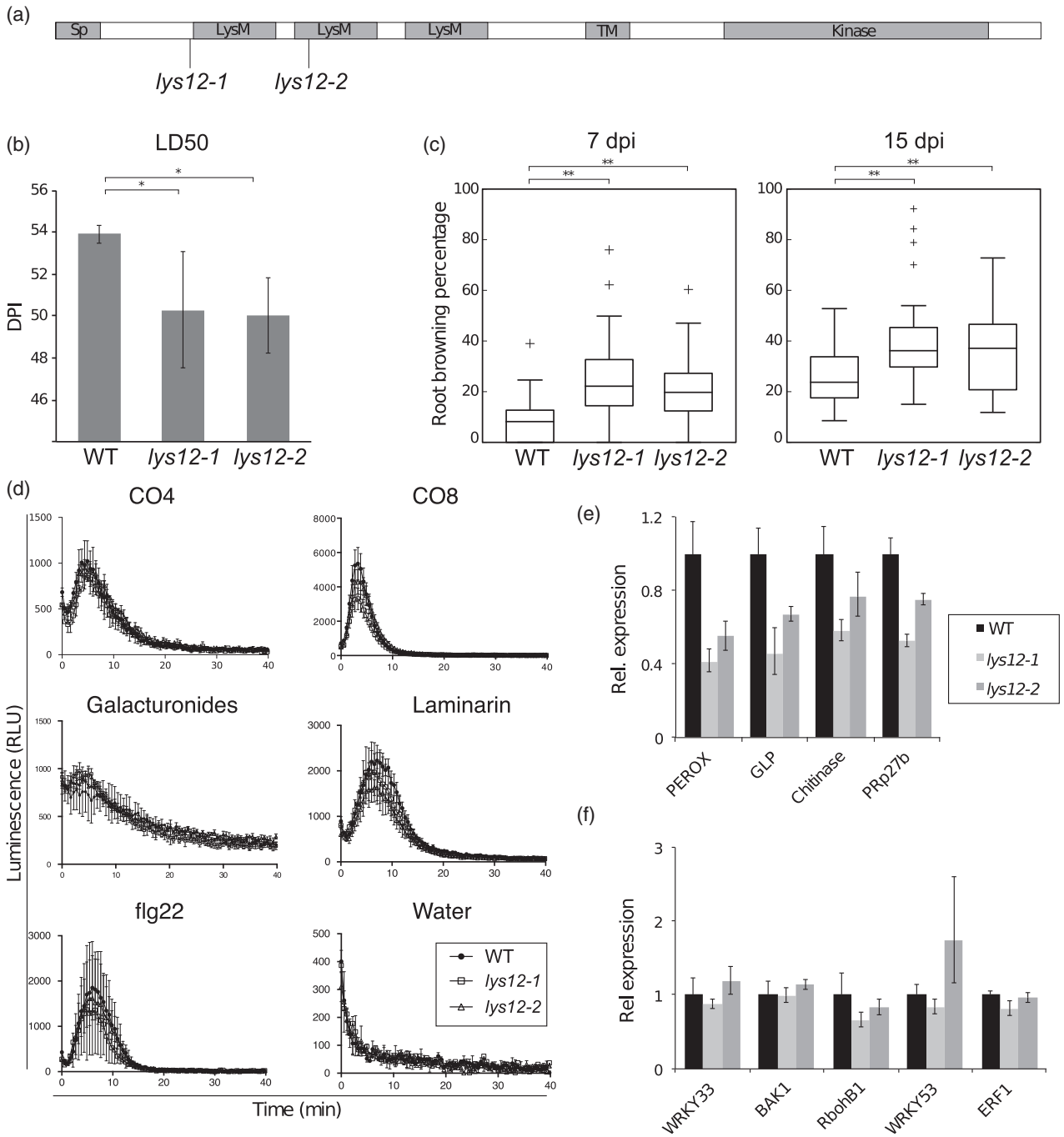


Figure 3. *Lotus japonicus lys12* mutants have a specific defective phenotype during *Phytophthora palmivora* infection.
(a) LYS12 domain organization and localization of the two LORE1 insertions in the *lys12* mutants. Lore1 insertion in *lys12-1* is at position C180 and in *lys12-2* at position C606 from the starting A in the coding region. Sp, signal peptide; LysM, LysM domain; TM, transmembrane region. (b) LD50 (lethal dose 50%) shows the average time point (dpi, days post-infection) when 50% of wild-type ($n = 47$), *lys12-1* ($n = 55$) and *lys12-2* ($n = 53$) seedlings from individual plates were dead. The error bars show the 95% confidence interval. $*P < 0.02$ and $**P < 0.001$ according to the non-parametric Kolmogorov–Smirnov test. (c) Root browning was evaluated as the percentage of the root displaying browning disease symptoms at 7 and 15 dpi. The boxplots show average and quartiles for wild-type (WT), *lys12-1* and *lys12-2* plants. (d) Wild-type plants and *lys12* mutants produce reactive oxygen species (ROS) after treatment with CO4 (10^{-6} M), CO8 (10^{-6} M), oligogalacturonides (2.5 mg ml^{-1}), laminarin (3.3 mg ml^{-1}), flagellin (flg22, 5×10^{-7} M) but not water (H_2O). Values are plotted as relative light units (RLU) and error bars represent the SEM. (e), (f) Transcriptional regulation of *L. japonicus* defence markers in wild-type, *lys12-1* and *lys12-2* mutant roots 48 h after infection with *P. palmivora*. Relative transcript levels in three biological and three technical replicates normalized to three housekeeping genes (*ATPase*, *UBC*, *TIP41*) are presented. Error bars show the 95% confidence interval. In (e) transcription levels of *Peroxidase* (*Perox*), *Germin-like protein* (*Glp*), *Chitinase* and *PRp27b* were reduced in *lys12* mutants compared with the wild type. (f) Transcription levels of *Wrky33*, *Bak1*, *RbohB1*, *Wrky53* and *Erf1* were similar in *lys12* mutants and wild-type plants.

Taken together, our results from the phenotypic response to treatment with known elicitors, and from marker gene regulation during *P. palmivora* infection, show that *lys12* mutants display an impaired ability to mount a *P. palmivora*-specific defence program and, consequently, display an increased sensitivity to this pathogen.

LYS12 is a plasma membrane receptor expressed in *P. palmivora*-infected root zones

Lys12 encodes for a receptor protein predicted to have three LysM domains in the extracellular region, followed by a single transmembrane domain and an intracellular kinase that, like NFR5, lacks the ATP-binding domain (Lohmann *et al.*, 2010). The domain structure, together with the presence of a signal peptide in front of the LysM domain, predicted plasma membrane localization of LYS12. This prompted us to analyse the cellular localization of LYS12 before and during pathogen infection. For this we used *Nicotiana benthamiana* leaves which were transformed with the 35S:*Lys12:YFP* construct prior to infection with *P. palmivora*. We observed that LYS12 localized to the plasma membrane both in the absence of the pathogen (Figure S10) and during infection with *P. palmivora*. LYS12 was not detected at the extrahaustorial membrane (Figure 4a, b), indicating that it is not part of the intracellular structure (host membranes) induced by the pathogen.

The rapid and sharp increase in the overall transcript level of *Lys12* in roots inoculated with *P. palmivora* spores prompted us to perform a more detailed analysis of the spatial expression of this gene before and during infection. The activity of a *pLys12*:GUS transcriptional reporter (1.54-kb promoter) was followed in the transformed roots. In the uninfected roots we found a high level of promoter activity at sites of lateral root emergence from primordia and throughout its out-growth from the main root. Furthermore, reduced and rather scattered expression was occasionally detected in the epidermis and root hairs (Figure S11a, b) and very rarely at the root tip. Strong *Lys12* promoter activity was found at the sites coinciding with *P. palmivora* infection, which included the root tips or the epidermal sectors covering large areas of the root (Figures 4c and S11c, d). Occasional expression was also detected in the inner cortex, and these areas coincided with the presence of *P. palmivora* (Figure 4d, e). Taken together, these results obtained from promoter analyses corroborate those obtained from transcript-level measurements on whole roots and demonstrate a specific regulation of *Lys12* which is associated with *P. palmivora* infection.

lys12 mutants form normal endosymbioses with rhizobia and AM fungi

Previous studies identified that the LYS12 orthologue from *M. truncatula*, LYR3, has the capacity to bind and specifically recognize different LCO molecules when expressed and purified from a heterologous system (Fliegmann *et al.*, 2013; Malkov *et al.*, 2016). Therefore, in order to gain further insight into the types of molecules that are recognized by LYS12 we investigated whether LYS12 could be involved in LCO perception in *L. japonicus*. Two beneficial types of microbe, AM fungi and nitrogen-fixing rhizobia, produce LCO molecules, and both can be recognized by *L. japonicus*. Thus, we analysed the capacity of *lys12* mutants to form efficient symbioses with *Rhizophagus irregularis* mycorrhizal fungi and *Mesorhizobium loti*, the nitrogen-fixing bacterial symbiont of *Lotus*. To assess the symbiotic association with AM fungi we exposed the *lys12-1* mutant as well as wild-type plants to AM spores for 4 weeks and assessed the proportion of roots infected by the fungus or containing symbiotic fungal structures (arbuscules). Quantitative and qualitative analysis of AM-infected roots revealed that mutant and wild-type plants were equally well colonized by the symbiotic fungus (Figure S12a, b). In order to test the ability of *lys12* mutants to form symbiosis with *M. loti* the number of root hair infection threads at 9 and 14 dpi, and the number of nodules at 5 weeks post-inoculation were quantified. We found that both *lys12* alleles resemble wild-type plants for the onset and development of infection threads and nitrogen-fixing nodules (Figure S12c, d). We took our analysis one step further and tested whether LYS12 could replace NFR5 for perception of LCOs produced by *M. loti*. The *Lys12* gene was expressed from a strong ubiquitin promoter in the nodulation-defective *nfr5-2* mutant and the complementation was assayed by scoring root nodule development. This analysis revealed that LYS12, unlike NFR5 or LYS11 (Madsen *et al.*, 2003; Rasmussen *et al.*, 2016), did not rescue the nodulation-defective phenotype of *nfr5-2* mutants, and the plants remained nitrogen starved (Figure S13). Finally, the expression of the *pLys12*:GUS reporter was analysed in transgenic roots after inoculation with *M. loti* or AM fungi. No change in *Lys12* promoter activity associated with symbiotic bacterial or fungal infection was observed (Figure S14).

Our results from phenotypic analyses of endosymbiotic development in *lys12* mutants and the lack of genetic complementation of *nfr5-2* mutants demonstrate that *lys12* has a normal response to these symbiotic microbes that produce LCOs; therefore, we conclude that LYS12 alone is not required for perception of these symbiotic forms of chitin-derived carbohydrates in *L. japonicus*.

DISCUSSION

We have found that *L. japonicus* is susceptible to *P. palmivora*, and that LYS12 regulates the progression of the disease. We have shown that *Lys12* is induced rapidly and specifically during *Lotus–P. palmivora* association, and that mutant plants have an increased and accelerated disease

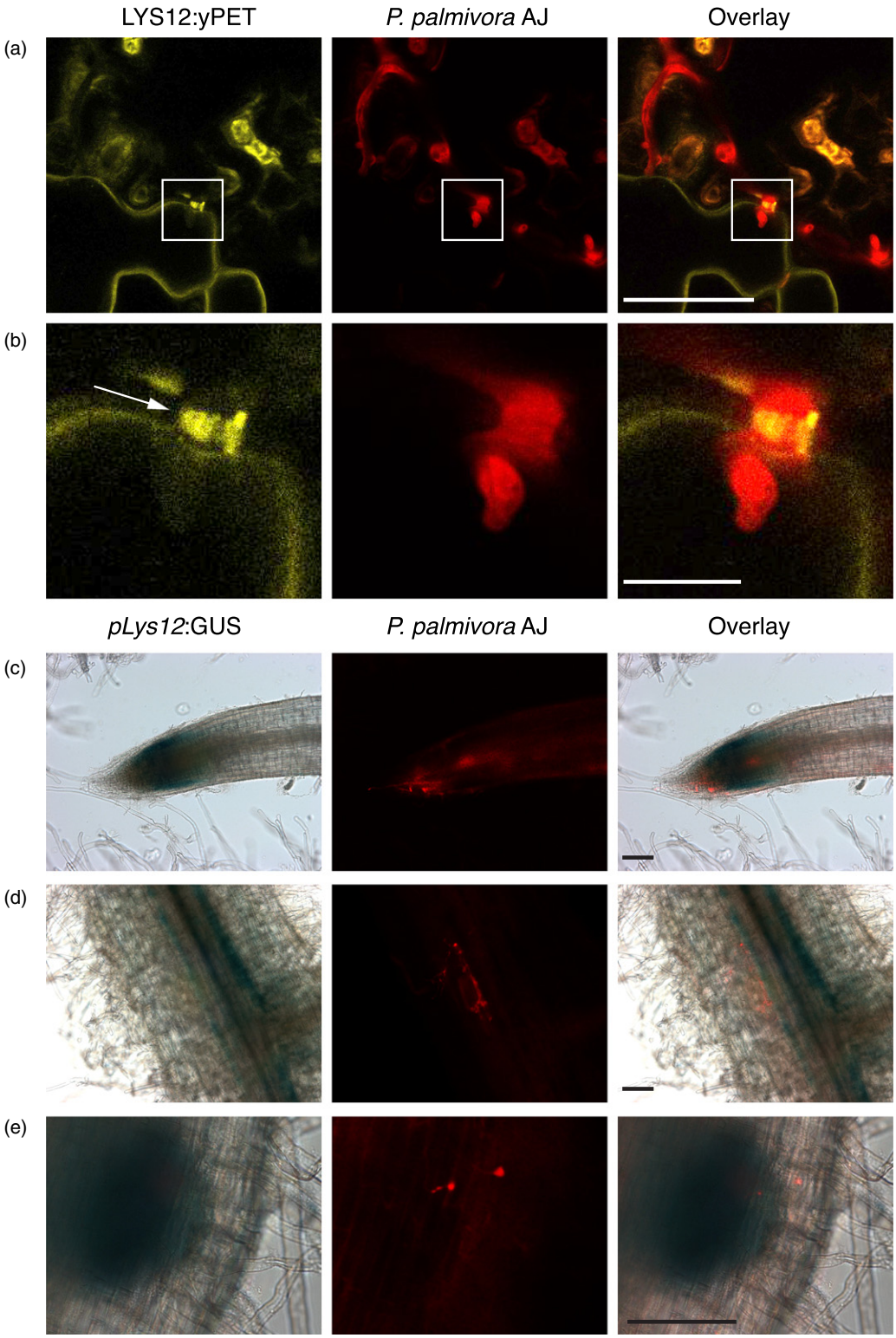


Figure 4. LYS12 is a plasma membrane receptor expressed in *Phytophthora palmivora*-infected root zones.
(a) LYS12:yPET translational fusion localizes to the plasma membrane of *Nicotiana benthamiana* leaf cells during infection and haustoria formation by *P. palmivora* AJ-red strain. Top panels show LYS12:yPET localization (left), the location of *P. palmivora* (middle) and the overlay (right).
(b) Close-up images of the insets in panels (a) showing the haustoria formed by *P. palmivora* AJ-red, and the plasma membrane-localized LYS12.
pLys12:GUS activity induced in the root tip (c) or the inner cortex (d, e) coincides with the *P. palmivora*-infected root zones.
Scale bars represent 50 μm (a), 10 μm (b) and 100 μm (c)–(e). [Colour figure can be viewed at wileyonlinelibrary.com]

progression, while maintaining a normal, wild-type response during symbiotic associations with nitrogen-fixing rhizobia or AM fungi.

LYS12 belongs to the family of NFR5-type LysM receptor kinases that lack the ATP-binding loop and the typical DFG motif in subdomain VII (Lohmann *et al.*, 2010), indicating that LYS12 may be a pseudo-kinase that interacts with other LysM receptors having an active kinase (Madsen *et al.*, 2011). Genes encoding proteins with high similarity to LYS12 were found in both monocots and dicots, including *Arabidopsis thaliana* (Figure S7), indicating a conserved function across land plants. The closest receptor to LYS12 in Arabidopsis is LYK4, which was previously shown to assist LYK5 in controlling chitin signalling and innate immune responses (Cao *et al.*, 2014). In *Lotus*, we found that *lys12* mutants respond to short (CO4) and long (CO8) chitin oligomers, 1,3 and 1,6 β-glucan (laminarin) or flg22 treatment when ROS and MAPK phosphorylation were measured or CO8-induced genes were quantified. This indicates that, in *Lotus*, LYS12 alone is not strictly required for perception and early downstream signalling defence responses associated with these elicitors. On the other hand, reports based on biochemical characterization of the most likely orthologue of LYS12 in *M. truncatula*, LYR3, showed high-affinity binding to LCOs but not to COs when expressed in a heterologous system (Fliegmann *et al.*, 2013). The functional role of *Lyr3* in *M. truncatula* is currently unknown. Our analyses based on *lys12* mutant phenotypes in the presence of the LCO-producing microbes, *M. loti* and AM fungi, together with the lack of functional complementation of *nfr5-2* in the presence of *M. loti*, demonstrate that *Lys12* alone is not required for these symbiotic associations, and is likely not essential for LCO perception. *Lys12* was, however, found to contribute to defence responses after infection with *P. palmivora*. Phenotypic analyses of *lys12* mutants revealed increased root browning, accelerated plant death and reduced transcriptional activation of host marker genes associated with oomycete infection (Rey *et al.*, 2013, 2015). Transcriptional analyses identified *Lys12* as having the fastest and highest increase in transcript level (6- and 11-fold increase at 16 and 24 hpi, respectively) among *Lotus Lys* genes after *P. palmivora* infection, while the expression pattern monitored by the *pLys12*:GUS reporter revealed clear promoter activity in the root zones that overlapped with the outer and inner root zones where the pathogen was present.

LysM receptor proteins have been shown to bind GlcNAc-derived and EPS microbial signals (Broghammer *et al.*, 2012; Liu *et al.*, 2012; Kawaharada *et al.*, 2015) and to activate specific downstream signalling cascades leading to defence or mutualistic association (Radutoiu *et al.*, 2003; Kaku *et al.*, 2006; Willmann *et al.*, 2011; Zhang *et al.*, 2015). Hence, a role for LYS12 in monitoring carbohydrate signals produced by the oomycete pathogen (PAMPs) or by the plant during *P. palmivora* infection (damage-associated molecular patterns, DAMPs) seems plausible. Genome inspections of a wide range of oomycete species, including *P. infestans*, identified the presence of at least one chitin synthase-like gene in all tested genomes (Bulone *et al.*, 1992; Werner *et al.*, 2002; Kamoun, 2003). True enzymatic activity was, however, only demonstrated for *Saprolegnia monoica* chitin synthase CHS2, and not for CHS1 (Guerriero *et al.*, 2010). We found that *P. palmivora* encodes for a chitin synthase-like protein that shares a higher degree of similarity to CHS1 (45%) than to CHS2 (33%), indicating that, like CHS1, it may lack a typical chitin synthase activity. The *P. palmivora* gene was found expressed in spores and during intraradical stages of infection (24 and 48 hpi), while it had a reduced transcriptional activity in the early stages (16 hpi) of root infection. This indicates that the pathogen modulates the activity of this gene at the transcriptional level in a specific, stage-wise manner. Even though most oomycete species have been shown to contain a chitin synthase gene, they were originally found to contain only minute amounts of chitin, and their cell walls were considered to be primarily made of cellulose. Later developments in carbohydrate analytical methodology identified a large diversity among oomycetes in terms of their cell wall composition (Melida *et al.*, 2013), and atypical chitooligosaccharides linked to β-glucans were isolated from the cell wall of *A. euteiches* (Nars *et al.*, 2013). However, the content of GlcNAc-based molecules was found to vary from none in *P. infestans* and *P. parasitica* to 10% in *A. euteiches* (Melida *et al.*, 2013). These recent results, based on extractions and detailed biochemical characterization from *in vitro* grown mycelia, correlate with previous reports showing the selective ability of *A. euteiches*, a member of Saprolegniales, and the two *Phytophthora* species belonging to Peronosporales, to be labelled and visualized after WGA staining (Badreddine *et al.*, 2008). So far, details of *P. palmivora* cell wall composition are not available, and herein we demonstrate that WGA-detectable residues could not be observed by confocal microscopy or by immunogold labelling and transmission electron microscopy (TEM) of infected roots. Clearly future work is needed to define the function and product of *P. palmivora* chitin synthase. Our results therefore provide key molecular, and cellular findings on *P. palmivora* supporting future biochemical and genetic analyses targeting identification and characterization of its specific carbohydrate elicitor.

On the host side, we found that *Lotus* has a fast response to the presence of the pathogen by activating the transcription of several defence marker genes, including *Peroxidase* and *Glp*, which were previously identified as transcripts induced by *A. euteiches* and *P. palmivora* in *M. truncatula* (Rey *et al.*, 2015). Furthermore, we found that LYS12 contributes to plant defence, ensuring an optimal

expression of defence genes associated with oomycete infection. Nonetheless, *P. palmivora* is an aggressive pathogen that employs various molecular cues to ensure successful infection of plants (Kamoun, 2003), therefore wild-type *Lotus* plants, regardless of the presence of an active *Lys12*, become infected and eventually die. Interestingly, LysM receptors involved in chitin perception and signalling in *Lotus* (*Lys6*, *Lys13* and *Lys14*) (Bozsoki *et al.*, 2017) were induced during *P. palmivora* infection, especially at the later stages of infection (48 hpi) when the transcription of *P. palmivora* putative chitin synthase was high. The activation of this ample chitin-signalling response can be explained either by the need for a complex signalling system for perception of *P. palmivora*-produced carbohydrate-based signals during intraradical growth or as part of the generally increased host alertness during pathogen infection. Future studies based on biochemical characterization of *in vitro* and *in planta* grown *P. palmivora* cell wall composition and produced MAMPs or DAMPs would allow a better understanding of the protein–carbohydrate recognition code established by this cosmopolitan pathogen which allows it to infect numerous and diverse plant taxa.

EXPERIMENTAL PROCEDURES

Seed germination

Lotus Gifu wild-type, *lys12-1* and *lys12-2* seeds were scarified with sandpaper, surface sterilized with 0.5% hypochlorite for 15 min and soaked overnight in water at 4°C. The following day, seeds were moved to sterile plates with filter paper for germination at 21°C. Seedlings were then transferred to 0.8% agar (*P. palmivora* infection), ¼ B&D (Broughton and Dilworth, 1971) (*M. loti* inoculation) or ½ B5 (hairy root transformation).

Lotus japonicus–microbe interaction assays

Phytophthora palmivora infection assay. *Phytophthora palmivora* AJ-td (Rey *et al.*, 2015) was grown on V8 plates and spores were harvested by flooding the plates with sterile water at 4°C. Spores were quantified under a light microscope. Seedlings were inoculated 6 days after germination with 25–1000 *P. palmivora* AJ spores per plant depending on the experiment. The number of *P. palmivora* spores used for different analyses is as follows: disease development with AJ and ARI (Rey *et al.*, 2015), 1000 spores per plant; expression studies on transformed roots containing the *pLys12*:GUS construct, 1000 spores per plant; transcription analysis using qRT-PCR, 1000 spores per plant; disease phenotyping of *lys12*, 25 spores per plant. Inoculated plants were kept at 25°C in the light. Disease development was quantified as described previously (Rey *et al.*, 2015).

Nodulation and infection thread assay after Mesorhizobium *inoculation*. Germinated seedlings were moved to ¼ B&D medium (at 21°C, light conditions 16 h/8 h day/night), and infection threads were counted per centimetre 9 and 14 days after inoculation with 1 ml *M. loti* R7A (OD_{600} = 0.015). For the nodulation assay, plants were grown in greenhouse conditions, and nodules were counted 5 weeks after inoculation with *M. loti* NZP2235.

Arbuscular mycorrhiza assay. Seedlings at the stage of first true leaves were placed between two nitrocellulose discs, together with 100–150 *R. irregularis* spores, and the discs were placed in sand-filled magentas watered with Long Ashton medium (Gutjahr *et al.*, 2009). Plants were grown for 4 weeks. For quantification, *R. irregularis*-colonized roots were stained with 5% ink in 5% acetic acid solution (Vierheilig *et al.*, 1998). The estimation of mycorrhizal parameters was performed as described by Trouvelot *et al.* (1986) using MYCOCALC (http://www2.dijon.inra.fr/mychintec/mycocalc-prg/download.html).

Nikkomycin Z hyphal growth inhibition assay

Two microlitres of *P. palmivora* AJ-td zoospore solution (about 300 spores) was added to wells of a 96-well plate containing 98 μl of liquid Plich medium and 0–1000 μg Nikkomycin Z (Sigma-Aldrich, http://www.sigmaaldrich.com/). The plate was transparently sealed and incubated for 2 days at 25°C under constant illumination. Hyphal morphology was documented 24 hpi using an inverted light microscope (Nikon Eclipse TS100 equipped with a Nikon D5100 Digital Camera, http://www.nikon.com/). To quantify hyphal mass at 48 hpi, the OD_{595} of triplicates was measured using a plate reader (SpectraMax i3x, Molecular Devices, https://www.moleculardevices.com/).

Wheat germ agglutinin staining and transmission electron microscopy

Lotus japonicus roots infected with *P. palmivora* were boiled in 10% KOH at 96°C for 10 min, and subsequently washed in PBS solution. The cleared roots, or *P. palmivora* spore solution, were stained with 25 μg ml^{-1} WGA–fluorescein isothiocyanate overnight in the dark and visualized for green fluorescence under a fluorescence microscope (Zeiss LSM780, https://www.zeiss.com/). *Lotus japonicus* roots infected with *P. palmivora* or *R. irregularis* were fixed and embedded in resin according to James and Sprent (1999). Ultrathin sections (80 nm) were collected on pyroxylin-coated nickel grids and labelled using a WGA 10-nm gold complex (Biovalley, https://www.biovalley.fr/), which binds to fungal chitin (Balestrini *et al.*, 1996). The sections were observed and photographed using a JEOL 1400 JEM TEM.

Constructs for promoter analyses and protein expression

A 1540-bp fragment upstream of the start codon of *Lys12* and a 399-bp fragment after the stop were amplified from Gifu and used to create the *pLys12*:GUS:*tLys12* construct with the GoldenGate cloning system (Engler *et al.*, 2008). T located at 639 nucleotides upstream of the start codon was mutated to A in order to remove a *Bsa*I site and allow further cloning. Likewise, the *Lys12* coding sequence was amplified and used to create the *pLjUbi*:*Lys12*:35S construct for *nfr5-2* complementation studies, and the *p35S*:*Lys12*:yPET:35S for cellular localization studies in *N. benthamiana* as previously described (Madsen *et al.*, 2011).

Hairy root transformation

Transformed roots of *L. japonicus* Gifu or *nfr5-2* were obtained as described previously (Radutoiu *et al.*, 2003, 2007). Composite plants with wild-type shoots and transformed roots were inoculated with *M. loti*, AM, *P. palmivora* or Mock (water). *Mesorhizobium loti*-inoculated plants were harvested after 1, 7, 14 and 21 days for the promoter:GUS assay, and after 5 weeks for the *nfr5* complementation assay. Plant roots colonized by *R. irregularis* were harvested at 4 weeks after inoculation. *Phytophthora*

palmivora-infected roots were investigated for infection under the fluorescent microscope every day until several infection areas were observed per root.

Quantitative RT-PCR

Seedling roots inoculated with *P. palmivora* were harvested after 16, 24 or 48 h. Three biological replicates ($n = 10$) were used for each genotype and condition. Messenger RNA was extracted using Invitrogen Dynabeads (http://www.invitrogen.com/). RT-PCR using gene-specific primers (Table S1) and FastStart DNA Master SYBR green I kit (Roche Molecular Biochemicals, https://lifescience.roche.com/) was performed on the Roche LightCycler480. Relative quantification and normalization to a calibrator was performed as previously described (Lohmann *et al.*, 2010).

Measurement of ROS

Roots of 7-day old seedlings were cut into 0.5-cm pieces and placed into white 96-well flat-bottomed polystyrene plates (Greiner Bio-One, http://www.greinerbioone.com/) then incubated overnight in sterile water at room temperature (i.e. 21°C) in the dark. Measurements of ROS were conducted in a Varioskan Flash Multimode Reader (Thermo Scientific, http://www.thermofisher.com/) using the luminometric measurement mode immediately after exchanging the water for reaction mixture [20 μM luminol (Sigma), 5 μg ml^{-1} horseradish peroxidase (Sigma) and the respective elicitor] on the root pieces. The following were used as elicitors: 10^{-6} M tetra-*N*-acetyl-chitotetraose, CO4 (Megazyme, https://www.megazyme.com/), octa-*N*-acetyl-chitooctaose, CO8 (IsoSep, http://www.isosep.com/), 3.3 mg ml^{-1} laminarin (from *Laminaria digitata*, Sigma), 5×10^{-7} M flg22 (the 30–51 22-amino-acid peptide of *Pseudomonas aeruginosa* flagellin, Alpha Diagnostic, http://www.4adi.com/), 2.5 mg ml^{-1} oligogalacturonides [prepared by partial digestion of polygalacturonic acid (Sigma Aldrich) following the protocol of Spiro *et al.* (1993) or water. Six roots (10 mg of root material) were used per biological replicate. Three biological replicates were measured for every treatment and genotype. The treatments were repeated at least twice with similar results.

MAPK phosphorylation

Roots of 7-day-old seedlings were treated for 10 min with 10^{-6} M CO8, 3.3 mg ml^{-1} laminarin (Sigma) or water, and for 15 min with 5×10^{-7} M flg22. Total root protein was isolated in a buffer containing 50 mM 2-amino-2-(hydroxymethyl)-1,3-propanediol (TRIS)-HCl (pH 7.5), 100 mM NaCl, 15 mM EGTA (pH 7.5), 10 mM $MgCl_2$, 0.1% Triton X-100, 1 mM NaF, 30 mM β-glycerophosphate, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor cocktail (P9599, Sigma), 1% phosphatase inhibitor cocktail 2 (P5726, Sigma) and 1% phosphatase inhibitor cocktail 3 (P0044, Sigma). Twin gels were run, visualizing the phospho-MAPK3/6 and α/β-tubulin bands using anti phospho-p44/42 MAPK (no. 4370, Cell Signaling, https://www.cellsignal.com/) and anti α/β-tubulin (no. 2148, Cell Signaling) primary antibodies, respectively.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

WSF, TY, ZB, GA, and EKJ performed experiments and analysed the data, SS and SR conceived and coordinated analyses, SR wrote the manuscript with contribution from JS, EKJ, WSF, and SS.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *Phytophthora palmivora* infection stages in *Lotus japonicus* roots.

Figure S2. *Phytophthora palmivora* hyphal accumulation in *Lotus japonicus* roots during root infection.

Figure S3. Chitin synthase-like genes in selected oomycete species.

Figure S4. *Phytophthora palmivora* chitin synthase-like gene is expressed and upregulated during *Lotus* root infection.

Figure S5. *Phytophthora palmivora* hyphal growth is impaired by Nikkomycin Z.

Figure S6. *Phytophthora palmivora* hyphae are not stained by WGA-Alexa Fluor488.

Figure S7. Proteins with high similarity to LjLYS12 are present in monocot and dicot plant species.

Figure S8. *Lys12* mutants are more sensitive to *Phytophthora palmivora* infection.

Figure S9. Mitogen-activated protein kinase phosphorylation occurs in wild-type and *lys12* mutants after treatment with elicitors.

Figure S10. LYS12 localizes to the plasma membrane of *Nicotiana benthamiana* leaf cells.

Figure S11. Spatial expression of *Lys12* in uninoculated roots and during *Phytophthora palmivora* infection.

Figure S12. *lys12* mutants form functional symbioses with rhizobia and arbuscular mycorrhizal fungi.

Figure S13. Overexpression of *Lys12* is not sufficient to rescue the *nfr5-2* nodulation defective phenotype.

Figure S14. *Lys12* expression is not regulated during arbuscular mycorrhiza or root nodule symbioses.

Table S1. List of primers used for RT-qPCR.

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