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LYM2-dependent chitin perception limits molecular flux via plasmodesmata

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Abstract

Chitin acts as a pathogen-associated molecular pattern from fungal pathogens whose perception triggers a range of defense responses. We show that LYM2, the Arabidopsis homologue of a rice chitin receptor-like protein, mediates a reduction in molecular flux via plasmodesmata in the presence of chitin. For this response, *lym2-1* mutants are insensitive to the presence of chitin but not to the flagellin derivative flg22. Surprisingly, the chitin-recognition receptor CERK1 is not required for chitin-induced changes to PD flux suggesting that there are at least two chitin-activated response pathways in Arabidopsis, and LYM2 is not required for CERK1-mediated chitin-triggered defense responses indicating that these pathways are independent. In accordance with a role in the regulation of intercellular flux, LYM2 is resident at the plasma membrane and enriched at plasmodesmata. Chitin-triggered regulation of molecular flux between cells is required for defense responses against the fungal pathogen *Botrytis cinerea* and thus we conclude that the regulation of symplastic continuity and molecular flux between cells is a vital component of chitin-triggered immunity in Arabidopsis.

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Introduction

Plant defense responses comprise a matrix of events that define disease susceptibility. Primary defense responses involve the perception of pathogen- or microbe-associated molecular patterns (PAMPs/MAMPs) by pattern-recognition receptors (PRRs) exposed on the surface of the cell. For bacterial pathogens, the Arabidopsis receptor-like kinases FLS2 and EFR recognize the PAMPs flagellin and elongation factor Tu respectively, and the cell wall component chitin is detected by cell surface receptors during the recognition of fungal pathogens. PAMP-triggered responses are known to include calcium ion influx into the cytoplasm, a rapid increase in reactive oxygen species (ROS; known as the oxidative burst), activation of mitogen-activated protein kinases (MAPK) and callose deposition (1). These responses serve to alter gene transcription, produce anti-microbial metabolites and strengthen the cell wall, which all reduce the pathogen's ability to invade host cells and tissues.

The LysM domain-containing protein Chitin Elicitor Binding Protein (CEBiP) was identified as a chitin PRR in rice (2) and interacts with the LysM receptor-like kinase OsCERK1 for both chitin perception and the transmission of chitin-triggered signals (3). Arabidopsis homologues for CEBiP and OsCERK1 are LYM2 (also known as AtCEBiP) and CERK1 respectively (4, 5). CERK1 was identified as a component of the chitin perception machinery in Arabidopsis - its ectodomain is capable of binding chitin (6-8), and the intracellular kinase domain is required for the induction of chitin-triggered defense responses such as oxidative burst and MAPK activation (4). The observation that CERK1 is a target for the bacterial effector AvrPtoB (9) and functions as part of a peptidoglycan receptor system (10) indicates

that it also plays a role in responses triggered by pathogens that do not contain chitin. The receptor-like proteins LYM1 and LYM3 are close relatives of LYM2 and have been identified as additional components of the peptidoglycan receptor system in Arabidopsis (10) but, unlike CERK1, they do not bind chitin (11). LYM2 was identified in chitin pull-down assays suggesting that it either binds chitin itself or is a component of a chitin-binding protein complex (7, 11). Despite this affinity for chitin, there has been no direct evidence that LYM2 functions in chitin perception.

Many plant defense responses are considered as cell-autonomous processes (12, 13). However, this conflicts with the presence of cytoplasmic connections (plasmodesmata; PD) between neighboring cells and the production of diverse and potentially mobile small molecules such as ROS, calcium ions, nitric oxide (NO), and a range of defense related secondary metabolites (e.g. salicylic acid, jasmonic acid and ethylene) as part of the defense reaction. To ensure cell-specific (or non-cell autonomous) activity of these small molecules a mechanism by which cell-to-cell communication via PD is controlled following pathogen perception could be invoked. Whilst it is well established that viruses move through PD to facilitate invasion of host cells and tissues (14, 15), little is known about the role PD play in infections by other biotic pathogens. The PD-located protein PDLP5 was recently found to be required for resistance against *Pseudomonas maculicola* and associated with the deposition of callose at PD in this context (16). Also, in the interaction between rice cells and the blast fungus *Magnaporthe oryzae* invasion hyphae seek out PD as sites to cross the cell wall (17) and the effector PWL2 moves from cell-to-cell ahead of the infection front (18). Thus, cell-to-cell movement via PD appears to play a role in determining host susceptibility and pathogen virulence for non-viral biotic pathogens. However, our understanding of this role and the contribution it makes to resistance or susceptibility is yet to be defined.

Here we have identified LYM2 as a chitin PRR that mediates a decrease in molecular flux between cells in the presence of chitin. The work identifies a reduction in cell-to-cell connectivity via PD as a novel PAMP-triggered response that, for chitin, occurs independently of the known intracellular signaling pathways employed in PAMP-triggered immunity (PTI). Importantly, *lym2* mutant plants show altered susceptibility to a fungal pathogen indicating that this pathway is a key component of PTI. This study significantly advances our understanding of the role of symplastic cell-to-cell communication during pathogen perception and its potential for regulating disease outcomes.

Results

The PAMPs chitin and flg22 trigger a reduction in PD flux

LYM2 was identified in the PD proteome (19) and therefore we hypothesized that it plays a specific role in the regulation of cell-to-cell connectivity. However, the dynamics of molecular flux through PD in the presence of chitin and other PAMPs had not been addressed. Therefore, we examined the effect of chitin and the flagellin-derived peptide flg22 on cell-to-cell movement of a constitutively expressed cytosolic marker by microprojectile bombardment of leaf tissue. This assay monitors the expression of fluorescent proteins such as GFP and mRFP in individual transformed cells, from where they can diffuse freely through PD and thus provide a measure of conductivity through the pore (20) (Figure S1). Co-bombardment of cDNAs for a cell-restricted protein (ER-localised mRFP; $mRFP_{ER}$) with a cytosolic protein (GFP) confirmed our identification of the transformed cell within a patch of fluorescent cells (Figure S1). Western blot analysis following PAMP treatment (Figure S1) demonstrated that 35S expression is not affected by chitin or flg22, allowing for comparison

of untreated and treated tissue. To determine the effect of PAMPs on intercellular flux we bombarded leaves with *mRFP* and then treated tissue with either chitin or flg22. Both chitin and flg22 caused a significant reduction in cell-to-cell diffusion of mRFP relative to untreated tissue, demonstrating that molecular flux through PD in wild-type Arabidopsis plants is altered in the presence of both these PAMPs (Figure 1).

LYM2, and FLS2, mediate PAMP-triggered reduction in PD flux

The receptor-like kinases CERK1 and FLS2 are Arabidopsis PRRs for chitin and flagellin respectively (6, 21). To critically test the influence of these PRRs and LYM2 on PD function, knock-out lines in *LYM2* were obtained in Col-0 (*lym2-1*) and No-0 (*lym2-2*) backgrounds and compared to *cerk1-2* and *fls2* mutants. As observed in wild-type Col-0 plants, *cerk1-2* mutant plants showed a reduction in mRFP diffusion following chitin treatment (Figure 1), indicating that CERK1 is not necessary for chitin-triggered changes to molecular flux via PD. By contrast, diffusion of mRFP from bombardment sites was unchanged following chitin treatment in *lym2-1* leaves (Figure 1). Diffusion of mRFP in plants carrying the independent mutant allele *lym2-2* also remained unchanged following chitin treatment, different to the response in No-0 wild type plants (Figure 1). While flg22-treated *lym2-1* leaves showed reduced diffusion of mRFP relative to untreated leaves (Figure 1), *fls2* leaves showed no change in mRFP diffusion in the presence or absence of flg22. These data implicate LYM2 and FLS2 independently in PD-regulation following PAMP treatment.

LYM2 and CERK1 act in independent, chitin-responsive signaling pathways

The receptor-like kinase CERK1 is required for chitin-triggered oxidative burst and MAPK activation (4, 7). To assess whether loss of LYM2 affects the chitin binding capacity of CERK1, chitin pull-down assays using chitin beads were performed on extracts from Col-0

and *lym2-1* plants. Antibody detection of CERK1 in the extracts, combined with elution with chitin oligomers, showed that binding of chitin by CERK1 is independent of LYM2 (Figure 2A). Hence, CERK1 was captured and released by chitin equally for both extracts. Nevertheless, it remained possible that the pathway following activation by chitin may be affected. To address this we monitored CERK1 phosphorylation, the chitin-induced oxidative burst and MAPK activation in wild-type and *lym2-1* plants. Phosphorylation of CERK1 is induced by the binding of chitin and, when monitored by changes in electrophoretic migration in gel-shift assays, was comparable in *lym2-1* mutants and Col-0 (Figure 2B). Thus CERK1 phosphorylation does not require LYM2. Luminol-based detection of ROS in Col-0 leaves demonstrated a 10-fold increase in ROS following chitin treatment. A slightly smaller increase (6-7- fold) was observed in *lym2-1* but this was not significantly different from Col-0. By contrast, chitin treatment of *cerk1-2* showed no change in luminescence output over the untreated control (Figure 2C). Chitin-triggered MAPK activation of MPK6 is dependent upon CERK1 and was absent in *cerk1-2* chitin-treated tissues. *lym2-1* mutants showed phosphorylation of MPK6 similar to Col-0 at 0, 10 and 30 minutes after chitin treatment (Figure 2D). In combination with the bombardment assays, these results indicate that LYM2 is not necessary for CERK1 activation and CERK1-mediated responses and *vice versa*, demonstrating that these two proteins respond to chitin independently.

LYM2 is a plasma membrane-located protein with PD association

LYM2 is one of a three member protein family and was identified in protein extracts from purified PD (19). To determine their subcellular localization, and considering that LYM1 and LYM2 are predicted GPI-anchored proteins (5), *LYM1*, *LYM2* and *LYM3* were cloned as translational fusions expressing the homologous proteins fused internally to fluorescent mCitrine in Arabidopsis. Fluorescence microscopy of transgenic lines revealed LYM1-mCit,

LYM2-mCit and LYM3-mCit are located at the plasma membrane (Figure 3); LYM3 was also observed in the endoplasmic reticulum. In contrast to LYM1-mCit and LYM3-mCit, LYM2-mCit was distributed unevenly in the plasma membrane. Patches of increased LYM2-mCit fluorescence corresponded with aniline blue-stained spots of PD-associated callose (Figure 3). This was also observed when the construct was transiently expressed in *N. benthamina* leaves (Figure S2). Following our observation that FLS2 mediates flg22-triggered closure of PD we carefully examined leaves of transgenic lines expressing FLS2-GFP (22) and found unevenly distributed signals in the plasma membrane. Similar to LYM2-mCit, bright domains of fluorescence corresponded with aniline blue-stained PD-associated callose (Figure S2).

LYM2 is required for resistance to *Botrytis cinerea*

To ascertain whether or not chitin-induced regulation of cell-to-cell connectivity contributes to the development of infection we assayed for increased resistance or susceptibility to two fungal pathogens and one bacterial pathogen in the *lym2-1* line. Pathogenicity assays with *Botrytis cinerea* demonstrated that relative to Col-0, *lym2-1* developed larger disease lesions 3 dpi (Figure 4A, D). Trypan blue staining of inoculated leaves indicated that at the early stages of infection (24 and 32 hpi) there was no difference between Col-0 and *lym2*-1 infection sites. At 24 hpi epidermal cells beneath penetrating hyphae stained blue, indicating cell death. At 32 hpi, mesophyll cells beneath the infection site (defined microscopically by the presence of fungal hyphae on the leaf surface) showed evidence of cell death. By contrast, at 48 hpi mesophyll cell death in *lym2-1* leaves had spread beyond the infection site, but was restricted beneath the infection site in Col-0 leaves (Figure 4E).

Colletotrichum higginsianum employs a similar infection mechanism to *M. oryzae*; invasion hyphae cross between cells at PD (23). To examine the impact of a chitin-responsive PD regulator on this mode of intercellular spread we performed pathogenicity assays with *C. higginsianum*. In contrast to the difference in susceptibility to *B. cinerea*, lesions (5 dpi) that developed following drop inoculation on *lym2-1* mutant leaves were similar in size and appearance to those that developed on Col-0 leaves (Figure 4B, D). Lesions on leaves of *cerk1-2* mutants also showed no difference to lesions on Col-0 (Figure S3).

As our bombardment assays indicate that LYM2 responds to chitin but not to flg22 we hypothesised that LYM2 is not required for PTI against a pathogen that does not display chitin. To test this we assayed for pathogenicity of *Pseudomonas syringae* pv. *tomato* DC3000 (*Pto* DC3000). Colony counts showed that there was no difference in bacterial growth on Col-0 and *lym2-1* plants at 2 dpi (Figure 4C).

Discussion

It is well established that the earliest defense responses to bacterial and fungal pathogens are triggered by the activity of different PRRs upon binding to specific PAMPs. In rice, chitin perception employs the receptor-like protein OsCEBiP but a similar function for its Arabidopsis homologue, LYM2, has been elusive in spite of evidence that LYM2 is a chitin-binding protein (7, 11). This study has identified that LYM2 has a specific role that mediates a reduction in molecular flux through PD in the presence of chitin. This implicates changes to cell-to-cell connectivity as an integral component of plant defenses against fungal pathogens.

Both chitin and flg22 elicit changes to intercellular flux. LYM2 mediates the chitin-triggered response but the flg22-triggered response is mediated through its cognate PRR FLS2, demonstrating ligand specificity (Figure 1). PAMP-triggered changes to molecular flux infer that PAMP responses include changes to symplastic domains and the movement of molecules between cells. Significantly, the chitin-induced reduction in PD flux observed in this study is independent of other chitin-triggered responses. *lym2-1* mutant plants are incapable of chitin-triggered PD flux decreases, but exhibit wild-type chitin-triggered MAPK activation, oxidative burst, CERK1 chitin-binding and chitin-triggered CERK1 phosphorylation (Figure 2). These results support previous work that demonstrated that chitin-triggered, CERK1-dependent increases in gene expression are normal in a *lym1,2,3* triple knockout (11). Chitin-induced PD flux reduction occurs in *cerk1-2* mutant plants indicating that, as LYM2 is not required for CERK1-mediated responses, neither is CERK1 required for LYM2-mediated responses (Figure 1). These two proteins function in independent chitin-response pathways.

LYM2 is predicted to be a GPI-anchored receptor-like protein with no intracellular domains. Therefore, LYM2 activity must be restricted to the extracellular space or be mediated by another protein(s) that activates an intracellular signalling pathway leading to a reduction in PD aperture. While not exclusively localized to PD, LYM2 does show increased association with the plasma membrane in the vicinity of PD. In combination with its extraction from purified PD (19), this indicates it is resident in PD membranes. Whether or not LYM2 has an alternate function in the non-PD plasma membrane remains to be determined. The finding here, that FLS2 mediates flg22-triggered changes in cell-to-cell flux, suggests that there are also unconsidered facets to FLS2 activity. Like LYM2, FLS2 was observed in the vicinity of PD, and its regulation of PD flux suggests either a site-specific function for FLS2 or a downstream signaling component.

It would be expected that LYM2 is required for defense responses against fungal, but not bacterial pathogens. Indeed, LYM2 is not required for defence against *Pto* DC3000. Our data indicates that while LYM2 is required for defence against the fungal pathogen *B. cinerea*, it does not contribute to interactions with *C. higginsianum*. It is possible that this difference arises from differences in lifestyle (*B. cinerea* is a necrotroph and *C. higginsianum* is a hemi-bitotroph) and infection mechanism, but we observed that *cerk1-2* mutants are also equally susceptible to *C. higginisianum* when compared with Col-0. This suggests that a general failure of chitin perception is responsible for the absence of a difference in susceptibility between Col-0 and *lym2-1* mutants to *C. higginsianum*. It is possible that, like *Cladosporum fulvum* and *M. oryzae, C. higginsianum* is capable of evading chitin detection (24, 25).

Trypan blue staining of *B. cinerea* infections revealed that prior to the appearance of measurable necrotic lesions, mesophyll cell death had spread beyond the site of infection in *lym2-1* mutants but remained restricted in Col-0. A similar difference in the spread of mesophyll cell death was observed in *wrky33* mutants (26), which have enhanced expression of genes involved in the salicylic acid signaling pathway. Increased spread of cell death in *lym2-1* may arise as a direct result of the inability of *lym2-1* cells to regulate the flux of defense-associated signals that trigger cell-death such as salicylic acid.

We have determined that intercellular flux is reduced by the PAMPs chitin and flg22, and that this is required for the deployment of a full suite of defense responses against a fungal pathogen. The corollary of this finding is that signals must be transmitted between cells to trigger or suppress cellular defence responses. Given the plethora of small, defence-associated molecules involved in plant defence it must be determined which of these have a

role in intercellular signaling. With respect to the different mechanisms of chitin perception between rice and Arabidopsis it remains to investigate chitin-triggered PD closure in rice and whether this is mediated by the CEBiP/OsCERK1 complex or by another member of the Lysin Motif-Containing Protein family (27). The purpose served by a reduction in cell-to-cell communication during defense responses remains to be explained but through its identification and its protein mediator it we can now actively pursue deeper questions relating to PD function during plant-pathogen interactions.

Materials and Methods

Plant Material

lym2-1 is SAIL_343_B03 in the Columbia (Col-0) background and *lym2-2* is 11-4398-1 (Riken) in the Nossen (No-0) background. *cerk1-2* is GABI_096F09 (GABI line, (4)) in the Col-0 background. *fls2* is SAIL_691_C04 in the Col-0 background. Plants were grown in short day conditions (10 h light, 14 h dark) for all experiments.

DNA Constructs and Transgenic Plants

LYM1-mCit, LYM2-mCit and LYM3-mCit were generated by insertion of mCitrine downstream of the predicted signal peptide at positions 78 bp, 72 bp and 75bp respectively. Gene fusions were generated by overlap PCR and then cloned by Gateway cloning into the pB7WG2.0 expression vector. These constructs were used to generate stably expressing Arabidopsis by floral dipping. *mRFP* was cloned into pB7WG2.0 for bombardment assays.

Chemicals

Chitin oligosaccharides were purchased from Yaizu Suisankagaku, chitin magnetic beads from New England Biolabs and chitin pentamers and hexamers from IsoPep or Seikagaku. flg22 peptides were obtained from Peptron.

Cell Biology and Microscopy

Confocal microscopy was performed on a Leica SP5 or a Zeiss LSM 510 Meta confocal microscope with a 25x water dipping lens (HCX IRAPO 25.0x0.95 water), a 40x oil immersion lens (HCX PLAPO CS 40.0x1.25 OIL) or a 63x oil immersion lens (Plan-APOCHROMAT 63x/1.4 OIL). mCitrine was excited with a 488nm or 514nm argon laser and collected at 525-560nm. mRFP was excited with a DPSS laser and collected at 580-610 nm. For callose staining aniline blue (0.1mg/mL) was infiltrated into 4-5 week old leaves up to two hours before imaging. The aniline blue fluorochrome was excited with a 405nm laser and collected at 440-490nm. Dual labeling with aniline blue and mCitrine was imaged by sequential scanning. For trypan blue staining, infected leaf material was boiled in trypan blue in lactophenol and cleared with chloral hydrate (28). Material was imaged on a Leica DM6000 microscope.

Microprojectile Bombardment

Microprojectile bombardment assays were performed as described (20). 4-6 week old expanded leaves of relevant Arabidopsis lines were bombarded with gold particles coated with pB7WG2.0.mRFP using a Bio-Rad Biolostic® PDS-1000/He Particle Delivery System. Bombardment sites were imaged 24 h post-bombardment by confocal microscopy. For PAMP treatment 500 μg/mL chitin oligosaccharides or 100 nM flg22 was infiltrated into bombarded leaves 4 h post-bombardment. For each treatment data was collected from at least 3 independent bombardment events, each of which consisted of leaves from at least two

individual plants. Statistical nonparametric Mann-Whitney analysis was performed using GraphPad InStat software.

Pull down and gel-shift assays

Total protein was extracted from Arabidopsis leaves as described previously (7). For each sample 1 mg of total protein was bound to chitin magnetic beads. Chitin bound protein was eluted with SDS-loading buffer, 1 mM chitin hexamers or water. Eluted proteins were probed with anti-CERK1 antibodies (9) or anti-GFP antibodies (Chromotek) by Western blot analysis. For gel-shift analysis leaves were pre-treated with 100 μg/mL chitin pentamers prior to protein extraction.

MAPK Activation

MAPK activation assays were performed as described (29). Briefly, seeds were germinated on MS and 7 day old seedlings were transferred to liquid culture. At 14 days plants were treated with 500 μg/mL chitin oligosaccharides and harvested at 0, 10 and 30 minutes after chitin treatment. MAPK activation was determined by Western blot analysis with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) rabbit monoclonal antibodies (Cell Signaling). Equal loading was verified by Coomassie blue staining.

Oxidative Burst

ROS were elicited by treatment of leaf discs taken from 5 week old Arabidopsis plants with 100 μg/mL chitin or water and detected as described (9). Luminescence was detected for 40 min using an ICCD photon counting camera (Photek).

Pathogen Infection

Botrytis cinerea spores (2.5 x 10^5 spores/mL) were drop inoculated on expanded leaves of 5 week old Arabidopsis plants and developing disease lesions were measured at 3 days post inoculation. 6 leaves per plant were inoculated to provide 6 measurements per plant, and 3 replicate experiments, each containing 20 individuals, were performed.

Colletotrichum higginsianum spores (2 x 10^6 spores/mL) were drop inoculated on 4-5 week old Arabidopsis plants and the diameter of necrotic lesions was measured 5 days post inoculation. 6 leaves per plant were inoculated to provide 6 measurements per plant, and 3 replicate experiments, each containing 15 individuals, were performed.

Pseudomonas syringae pv. *tomato* DC3000 was spray inoculated on to 5 week old Arabidopsis plants. Whole plants were harvested 2 days post-inoculation. Two individual plants were combined for a single measurement and 10 measurements were taken per replicate experiment. A total of 3 replicate experiments were performed.

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Figure Legends

Figure 1: PAMP-triggered reduction in molecular flux through PD. (A) Wild type (Col-0 and No-0) plants and mutants lines (*cerk1-2, fls2, lym2-1,* and *lym2-2*) were bombarded with cDNA constructs capable of producing mRFP protein. Diffusion of mRFP to surrounding cells provided a measure of molecular flux through PD. Numbers of individual expression foci (n) are indicated on the bars. The *lym2-2* mutant has No-0 as its parental control; all others were compared with Col-0. Error bars indicate SE. Asterisks indicate statistical significance: * $p<0.05$, ** $p<0.01$, *** $p<0.001$. (B) Representative bombardment sites from Col-0 and *lym2-1* leaves in untreated, chitin treated and flg22 treated tissue. Bombarded cells are indicated with an asterisk, scale bars are 20 μm.

Figure 2: CERK1 chitin-binding and chitin-induced responses are normal in *lym2-1* mutants. (A) Total protein extracts (1) from Col-0 and *lym2-1* plants were applied to chitin beads and eluted with SDS-loading buffer (2), chitin hexamers (3) and water (4). Eluted proteins were probed with anti-CERK1 antibodies. (B) Col-0 and *lym2-1* plants were treated with water (1) or chitin pentamers (2) and total protein extracts were separated by SDS-PAGE. CERK1 was detected with anti-CERK1 antibodies in extracts from Col-0 and *lym2-1* plants pre-treated with chitin. *lym2-1* mutants have normal oxidative burst and MAPK activation following chitin treatment. (C) Luminescence assay for ROS measured over 40 min after treatment of Col-0, *lym2-1* and *cerk1-2* mutants with water (mock) or chitin. n=20 for all lines. Error bars

indicate SE. (D) MAPK activation was monitored by immunodetection (upper panel) of phosphorylated MPK6 0, 10 and 30 minutes following chitin treatment of Col-0 (1), *lym2-1* (2) and *cerk1-2* (3) seedlings. Loading was visualized with Coomassie Brilliant Blue (CBB).

Figure 3: LYM proteins localize to the plasma membrane. Internal fusions of LYM1 (A), LYM2 (B) and LYM3 (C) to mCitrine show that each protein is located at the plasma membrane. The fluorescence associated with LYM2 is uneven in the membrane; patches of increased fluorescence co-localise with aniline blue-stained, PD-associated callose (arrowheads). Scale bars are 20 μm.

Figure 4: *lym2-1* mutants have increased susceptibility to *B. cinera* but not to *C. higginsianum* and *Pto* DC3000. *B. cinerea* (A) or *C. higginsianum* (B) was drop inoculated on leaves of Col-0 or *lym2-1* plants. Lesion diameter measured after 3 days (*B. cinerea*, D) or 5 days (*C. higginisianum*, D). (C) Col-0 and *lym2-1* were spray inoculated with *Pto* DC3000 and bacterial growth assessed as colony counts ($\log_{10}$ CFU/g) determined 2 dpi from pooled extracts (2 plants per count). Error bars indicate SD. Asterisks indicate statistical significance: *** $p<0.001$. (E) Trypan blue stained leaves infected with *B. cinerea* showed that at 24 and 36 hpi infection sites appeared the same in Col-0 and *lym2-1* showing a similar degree of cell death in the epidermis (24 h) and mesophyll (36 h). At 48 hpi, mesophyll cell death in Col-0 leaves was restricted to the site of infection (solid line) but in *lym2-1* leaves was observed beyond this boundary (dotted line). Scale bars are 0.5 cm (D) and 100 μm (E).

Figure 1

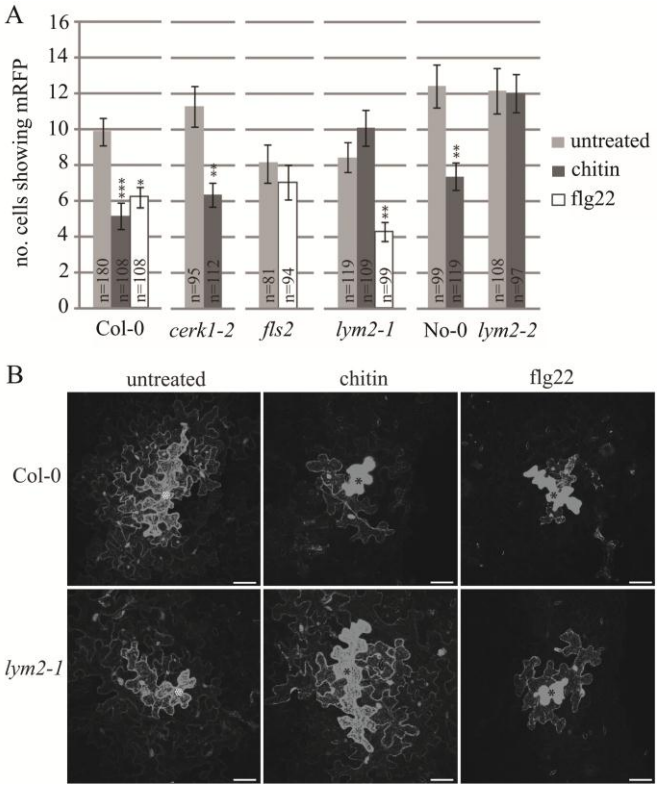


Figure 2

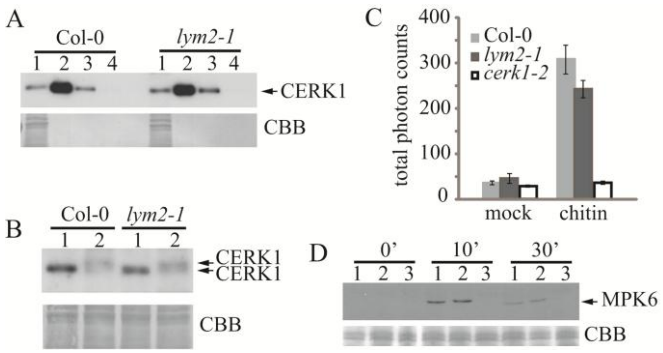


Figure 3

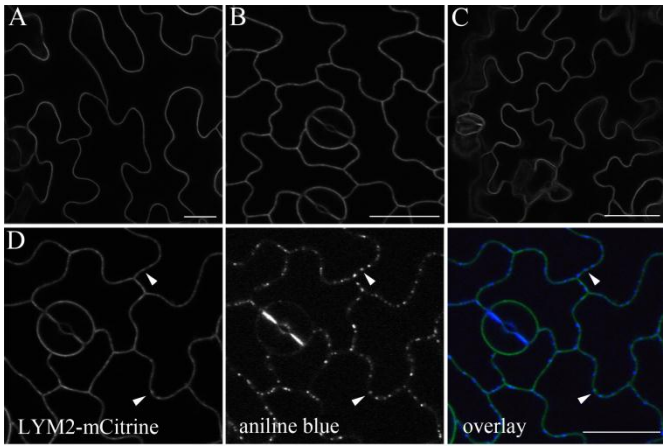
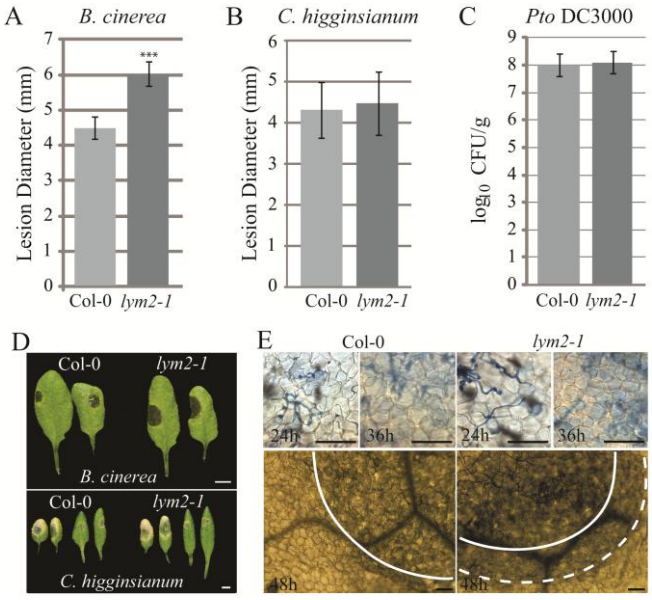


Figure 4



Supporting Materials and Methods

Microprojectile bombardments

4-6 week old Col-0 were bombarded as described with gold coated with pB7WG2.0.GFP DNA or co-bombarded with gold coated in pB7WG2.0.GFP and pB7WG2.0.mRFP$_{ER}$ (20).

35S Expression

Seedlings that stably express *35S::photoactivatableGFP* were grown as for MAPK assays. At 14 days seedlings were vacuum infiltrated with MS medium containing 500 μg/mL chitin oligosaccharides or 100 nM flg22. Seedlings were harvested after 4, 12 or 20h PAMP treatment. Crude protein extracts were analyzed by Western blot analysis with an anti-GFP antibody (Roche) to determine the expression level of photoactivatable GFP (PAGFP) following the treatments.

Transient Expression

pB7WG2.0.LYM2-mCit was transformed into *Agrobacetrium tumefaciens* GV3101 and bacteria were infiltrated into *Nicotiana benthamiana* leaves. Leaves were imaged by confocal microscopy 2 dpi.

Supporting Figure Legends

Figure S1: Microprojectile bombardment of a single fluorescent protein gene can be used to assay molecular flux of a fluorescent protein between Arabidopsis epidermal cells. (A) Microprojectile bombardment of gold particles coated with pB7WG2.0.mRFP causes transformation of single cells. Expression of *mRFP* leads to accumulation of mRFP in the transformed cell and its diffusion into surrounding cells. The extent of movement from a

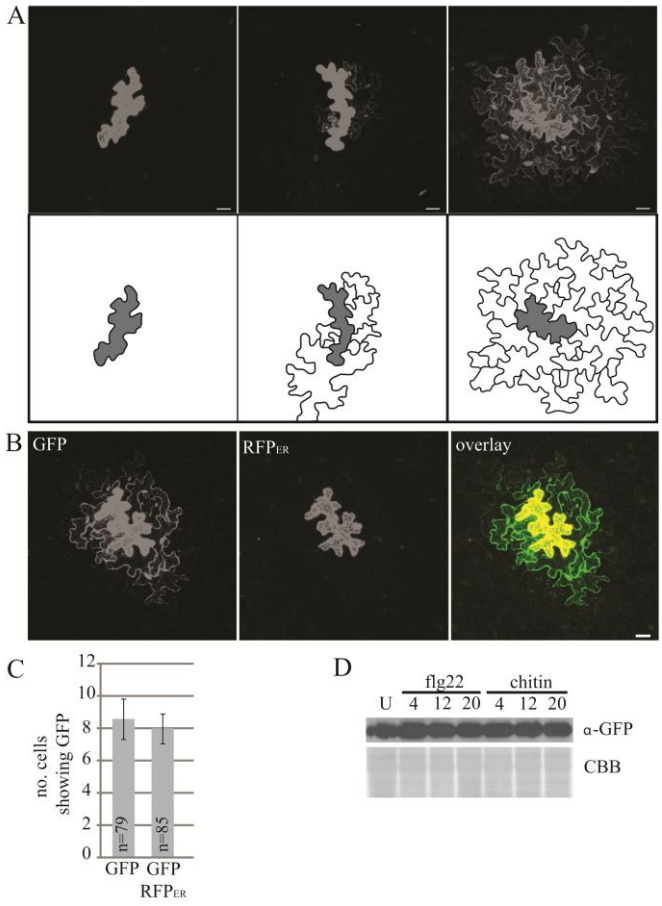
single transformed cell varies and the panels show, from left to right, bombardment events that display increasing spread of mRFP. The cartoons beneath the panels illustrate how bombardment sites were interpreted: bombarded cells are shaded grey while cells showing mRFP counted for analysis are outlined. Scale bars are 20 μm. (B) To confirm bombardment sites were correctly interpreted, bombardments were performed on Col-0 leaves with gold coated with either pB7WG2.0.GFP only or with both pB7WG2.0.GFP and pB7WG2.0.mRFP$_{ER}$. mRFP$_{ER}$ is restricted to the bombarded cell, marking the site of transformation, while GFP diffuses from this cell into surrounding cells. In the overlayed image GFP is coloured green and mRFP$_{ER}$ is coloured red. Co-localisation appears yellow. Scale bar is 20 μm. (C) Quantification of cellular spread of GFP was compared for bombardments of GFP alone with GFP and mRFP$_{ER}$ co-bombardments. Thus, the number of cells to which GFP diffused was counted for sites in which the bombarded cell was defined by an independent marker and those for which it was not. The average number of cells showing GFP was not different between bombardments indicating bombardment of a diffusible probe alone was sufficient. The number of transformation sites counted for each bombardment type (n) is indicated on the bars, error bars are SE. (D) Gene expression from the 35S promoter is not changed by treatment with flg22 or chitin. Arabidopsis plants stably expressing PAGFP from the 35S promoter were treated with flg22 or chitin for 4, 12 or 20 minutes. Soluble proteins were extracted and Western blot analysis with an anti-GFP antibody indicates that expression levels did not change between untreated (U) and treated plants. Loading was visualized with CBB.

Figure S2: (A) Transient expression of *LYM2-mCit* in *N. benthamiana* leaves shows that LYM2-mCit is unevenly distributed in the plasma membrane. Domains of bright fluorescence correspond with aniline blue-stained, PD-associated callose (arrowheads). In the overlay

image LYM2-mCit is green, aniline blue is blue and chlorophyll autofluorescence is red. (B) In Col-0 plants expressing *FLS2-GFP* from its native promoter, FLS2-GFP was similarly observed in bright patches in the plasma membrane. (C) Domains of bright fluorescence of FLS2-GFP co-localised with aniline blue stained, PD-associated callose (arrowheads). In overlay image FLS2-GFP is green, aniline blue is blue and chlorophyll autofluorescence is red. Scale bars are 20 μm.

Figure S3: *cerk1-2* is equally susceptible to *C. higginsianum* when compared with Col-0 and *lym2-1*. The diameter and appearance of necrotic lesions caused by *C. higginsianum* on Col-0, *lym2-1* and *cerk1-2* leaves were not significantly different.

Figure S1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RFPER
overlay
C
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8
6
4
2
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GFP
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D
flg22
chitin
U 4 12 20 4 12 20
α-GFP
CBB

Figure S2

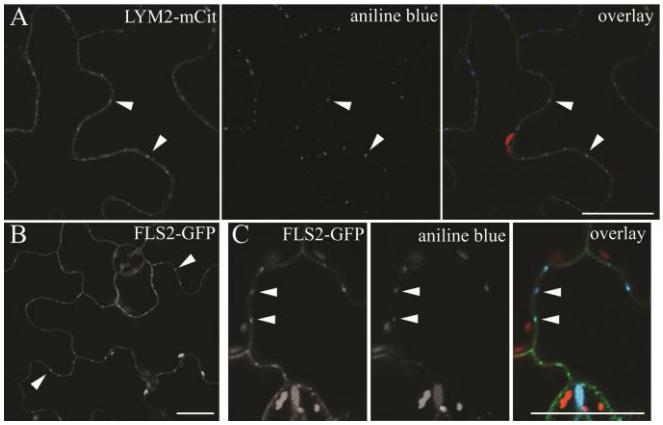

Figure S3