Chitin-induced systemic disease resistance in rice requires both OsCERK1 and OsCEBiP and is mediated via perturbation of cell-wall biogenesis in leaves

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Summary

- Chitin is a well-known elicitor of disease resistance whose recognition by plants is crucial to perceive fungal infections. Chitin can induce both a local immune response and a systemic disease resistance when provided as a supplement in soils. Unlike local immune responses, how chitin-induced systemic disease resistance is deployed has not been studied in detail.
- In this study, we evaluated systemic disease resistance against the fungal pathogen *Bipolaris oryzae* by performing a transcriptome analysis and monitoring cell-wall composition in rice plants grown in chitin-supplemented soils. We also examined the local immune response to chitin by measuring the production of reactive oxygen species in leaves.
- Chitins induced both local immune response and systemic disease resistance with differing requirements for the receptors OsCERK1 and OsCEBiP. Transcriptome analysis suggested that a perturbation in cell-wall biogenesis is involved in the induction of systemic disease resistance, an idea which was supported by the induction of disease resistance by treatment with a cellulose biosynthesis inhibitor and alterations of cell-wall composition.
- These findings suggest that chitin-induced systemic disease resistance in rice is caused by a perturbation of cell-wall biogenesis in leaves through long-distance signalling after recognition of chitins by OsCERK1 and OsCEBiP.

(199 words)

Keywords

Rice (*Oryza sativa*), chitin, systemic disease resistance, local immune response, *Bipolaris*

63 *oryzae*, CERK1, CEBiP, cell-wall biogenesis

64 (8 words)

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Introduction

Plants have developed two types of defence mechanisms, local immune response and systemic resistance, which are used to counteract threats from pathogens (Sun & Zhang, 2021). A local immune response is first induced upon pathogen approach and infection. Plants recognize microbe- or pathogen-associated molecular patterns (MAMPs/PAMPs) via a suite of pattern recognition receptors (PRRs) that induce pattern-triggered immunity (PTI), which causes the production of reactive oxygen species (ROS) and activates the expression of *Pathogenesis-related* (*PR*) genes to defend against pathogen invasion (Bittel & Robatzek, 2007; Zipfel, 2008). However, pathogens counteract this initial defence barrier by secreting effector proteins into plant cells that disrupt PTI and allow infection to progress. In response, plants have evolved nucleotide-binding/leucine-rich repeat receptors (NLRs) to recognize pathogen effectors, which induce a robust defence response often accompanied by a localised hypersensitive response (HR) leading to cell death. This form of immunity is called effector-triggered immunity (ETI) (Jones & Dangl, 2006; Cui *et al.*, 2015).

Local pathogen infection triggers systemic acquired resistance (SAR) that spreads to distant non-infected cells and is associated with salicylic acid (SA)–dependent gene expression and the biosynthesis of secondary metabolites (Hartmann & Zeier, 2019). For instance, the synthetic SA-analogue benzothiadiazole (BTH), a chemical activator of SAR, can induce systemic resistance in tobacco (*Nicotiana tabacum*), wheat (*Triticum aestivum*), Arabidopsis (*Arabidopsis thaliana*), and rice (*Oryza sativa*) (Görlach *et al.*, 1996; Friedrich *et al.*, 1996; Lawton *et al.*, 1996; Shimono *et al.*, 2007). However, not all microbes negatively affect plant growth, as chemical treatments or beneficial microbes in the root microbiome collectively called plant growth–promoting rhizobacteria (PGPR)

and fungi (PGPF) can trigger induced systemic resistance (ISR), which is mediated by long-distance signalling (Pieterse *et al.*, 2014). Unlike the SA-dependent SAR pathway, ISR results in systemic resistance via multiple signalling pathways involving the phytohormones SA, jasmonic acid (JA), and ethylene (ET) (Pieterse *et al.*, 1998, 2014). SAR and ISR engage different mechanisms but are both considered to elicit defence priming (Pieterse *et al.*, 2014; Mauch-Mani *et al.*, 2017).

Chitin, a polymer of β-1,4-linked *N*-acetylglucosamine, is a component of the fungal cell wall and arthropod exoskeletons (Pillai *et al.*, 2009; Sharp, 2013). Plants have PRRs that recognize chitin as a MAMP/PAMP and initiate PTI (Gong *et al.*, 2020). In rice, OsCERK1 (chitin elicitor receptor kinase 1) and OsCEBiP (chitin elicitor-binding protein) are members of the protein families lysin motif (LysM)-containing receptor-like kinase (RLK) and receptor-like protein (RLP) without a kinase domain, respectively; they form a heterodimeric chitin receptor complex (Kaku *et al.*, 2006; Shimizu *et al.*, 2010; Hayafune *et al.*, 2014). OsCEBiP is the major chitin-binding protein in rice cultured cells (Kouzai *et al.*, 2014b), with two OsCEBiP molecules binding to one chitin oligomer (CO) longer than a hexamer (Hayafune *et al.*, 2014). By contrast, OsCERK1 does not directly bind to CO (Shinya *et al.*, 2012) but mediates chitin-induced PTI by binding to and phosphorylating downstream factors (Kawasaki *et al.*, 2017). OsCERK1 forms a heterodimer with the LysM-RLK OsMYR1 (Myc factor receptor 1), which perceives short-chain COs secreted by arbuscular mycorrhizal (AM) fungi and competitively inhibits OsCEBiP-dependent immune signalling (He *et al.*, 2019; Zhang *et al.*, 2021). In Arabidopsis, the LysM-RLK AtCERK1 is required for CO perception (Miya *et al.*, 2007; Wan *et al.*, 2008) by forming a receptor complex with AtLYK4 (LysM-containing receptor-like kinase 4) and AtLYK5 in the chitin signalling pathways (Cao *et al.*, 2014).

Since natural polymeric chitin is difficult to use due to its intractability and insolubility (Pillai *et al.*, 2009), water-soluble chitin forms such as COs have mainly been used in studies of plant immunity. We developed a method to produce chitin nanofiber (CNF) from original chitin polymers by simple physical treatment of crustacean exoskeletons (Ifuku & Saimoto, 2012). CNF can homogeneously disperse even in water and can be used as a solution of polymeric chitin. We previously reported that CNF, as well as a mixture of COs, elicits ROS production in Arabidopsis and rice and that spraying leaves with either COs or CNF enhances disease resistance against both the fungal pathogen *Alternaria brassicicola* and the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 in Arabidopsis (Egusa *et al.*, 2015). Moreover, CNF supplementation of soils induced systemic disease resistance in Arabidopsis, cabbage (*Brassica oleracea* var. *capitata*), and strawberry (*Fragaria* sp.) (Parada *et al.*, 2018). In addition, treatment of rice roots with a CO solution induced systemic disease resistance for a day (Tanabe *et al.*, 2006). The induction of ISR by the ectomycorrhizal fungus *Laccaria bicolor* on the nonmycorrhizal plant Arabidopsis was dependent on JA signalling and SA biosynthesis and signalling, and AtCERK1 was necessary for the effect of systemic resistance (Vishwanathan *et al.*, 2020). Thus, although ISR induced by chitin or via chitin recognition has been studied, our knowledge about the molecular basis underlying the induction of systemic disease resistance by chitin is lacking compared to our understanding of local immune responses to chitins.

In this study, we examined the local immune response and systemic disease resistance against *Bipolaris oryzae*, the causal agent of rice brown spot disease, by performing a transcriptome analysis of rice plants treated with chitins. We exposed plants to both oligomeric chitin COs and polymeric chitin CNF to test the possibility of

differential effects on chitin-induced disease resistance. Both chitins elicited ROS production and induced systemic disease resistance in leaves. Transcriptome analysis demonstrated that cell-wall biogenesis- and cytokinin-related genes are downregulated as a systemic response induced by chitins. We validated these results with a cellulose biosynthesis inhibitor, by monitoring cell-wall composition and quantifying phytohormone levels. Knockout mutants for *OsCERK1* and *OsCEBiP* revealed that both LysM receptors are required for chitin-induced systemic disease resistance in response to *B. oryzae* but not for the local immune response in leaves.

Materials and Methods

Plant growth conditions

Unless otherwise stated, the Nipponbare cultivar of *Oryza sativa* L. (*japonica* group) was used as wild-type rice. *OsCEBiP* or *OsCERK1* transformant lines were generated in the *Oryza sativa* L. *japonica* 'Nipponbare Kanto BL number 2' background, which was previously described (Kouzai *et al.*, 2014b,a). The knockout mutant and segregating wild-type siblings of *oscebip* line 169 and *oscerk1* lines 19 and 53 were used in this study. Rice seeds were soaked in distilled water (DW) for germination at 28°C for 3 or 4 days in the dark, and the germinated seeds were transplanted into sterilized culture soil (Bestmix No. 3; Nippon Rockwool, Japan) mixed with 0.1 or 0.01% (w/v) CO solution and CNF dispersion in DW at 1% (w/v) in magenta boxes (GA-7; Sigma-Aldrich, USA). Plants were grown in a growth cabinet (BiOTRON; NK-systems, Japan) under controlled conditions (28°C 14-h-light/25°C 10-h-dark cycles) and fertilised once a week with a 1:1000 HYPONeX (6-10-5; HYPONeX, Japan) solution. The COs (the mixture of DP

163 [degree of polymerization] 2-6 chitin oligomers; NA-COS-Y; Yaizu Suisankagaku
164 Industry, Japan) solution and CNF dispersion in water were prepared as previously
165 reported (Kaminaka *et al*., 2020). The cell-wall biosynthesis inhibitor isoxaben (ISX;
166 Santa Cruz Biotechnology, Germany) was resolved in dimethyl sulfoxide (DMSO), and
167 a dilution in DW was sprayed onto leaves 5 h before sampling.
168
169 **ROS measurements**
170 Fourth leaves from 3-week-old rice seedlings grown on soil without chitin
171 supplementation were excised into nine leaf discs 0.5 mm in size and floated overnight at
172 22°C in a well filled with sterilized DW (sDW). COs or CNF elicitation solutions were
173 prepared by suspending COs or CNF in sDW to a final concentration of 0.01% (w/v).
174 Peroxidase from a horseradish root (HRP: Oriental Yeast, Japan) stock solution
175 (500×HRP) and luminol L-012 (L-012; Wako, Tokyo, Japan) stock solution (20 mM)
176 were prepared as previously described (Parada *et al*., 2018). Before elicitation, the sDW
177 was carefully removed from each well without tissue damage or desiccation. The
178 elicitation solution was immediately added to each well after removing sDW, and
179 chemiluminescence was measured with a microplate reader (ARVO X3; PerkinElmer
180 Japan, Japan) for 40 min.
181
182 **Pathogen inoculation test**
183 *Bipolaris oryzae* D6 (Kihara & Kumagai, 1994) was cultured on potato dextrose agar
184 plates for 1 week at 25°C in the dark. The conidial suspension was prepared to a titre of
185 1×10^5 spores/mL in 0.25% (v/v) Tween 20. Fourth leaves from 3-week-old rice seedlings
186 grown on normal or chitin-supplemented soils were detached and inoculated with a drop

(5 μL) of spores on the leaf sheaths and incubated in the dark for 1 day and then in the light for 1 day at 25°C. Images of inoculated leaves were taken using a GT-S640 Scanner (EPSON, Japan), and each lesion diameter was measured by ImageJ (ver.1.53a).

Phytohormone measurements

Approximately 500 mg of randomly selected leaves was excised from at least three individual 3-week-old rice seedlings grown on normal or chitin-supplemented soils. Samples were prepared with five technical replicates for each treatment. The leaves were placed in tubes and frozen in liquid nitrogen. The contents of each phytohormone were quantified using liquid chromatography–tandem mass spectrometry (LC-MS/MS) as previously described (Kanno *et al.*, 2016).

Fourier-transform infrared (FT-IR) spectroscopy

Alcohol-insoluble residue (AIR) was prepared from excised third or fourth leaves of 3-week-old rice seedlings grown on normal or chitin-supplemented soils, according to Bacete *et al*. (2017). AIR fractions were subjected to FT-IR spectroscopy using an FT-IR spectrophotometer equipped with an attenuated total reflectance accessory (Spectrum 65; PerkinElmer Japan, Japan). The FT-IR spectra were collected in the wavenumber range from 600 to 4,000 cm^{-1} with 16 scans, and the average values of three AIR fractions obtained from independent plants were used.

Transcriptome deep sequencing (RNA-seq) and data analysis

Rice plants were grown as mentioned above except for the growth conditions (28°C 14-h-light/16°C 10-h-dark cycles). About 100 mg of randomly selected leaves or roots was

211 excised from at least three individual 3-week-old rice seedlings grown on normal or
212 chitin-supplemented soils. Samples were prepared from three technical replicates for each
213 treatment. The leaves or roots were placed inside tubes with 5-mm stainless beads, frozen
214 in liquid nitrogen, and pulverised for 30 s using ShakeMan 6 (Bio Medical Science,
215 Japan). LBB solution [1 M LiCl, 100 mM Tris-HCl (pH 7.5), 1% SDS, 10 mM EDTA,
216 0.015% Antifoam A, 5 mM DTT, and 71.5 mM 2-ME, DNase/RNase-free water] was
217 added to the samples and completely dissolved by vortexing. All samples were incubated
218 for at least 5 min at room temperature with occasional inverting and mixing. After
219 centrifugation at 20,630 *g* for 10 min at room temperature, the supernatant was transferred
220 to new tubes and stored at –80°C. Sequencing libraries were produced according to the
221 BrAD-seq protocol (Ichihashi *et al.*, 2018). Sequencing was performed on a HiseqX
222 instrument (Illumina) by Macrogen Japan. Raw reads were checked for quality, and
223 adaptor sequences were trimmed using fastp (Chen *et al.*, 2018). The resulting clean reads
224 were mapped to the reference rice genome (MSU Rice Genome Annotation Project ver.
225 7.0; http://rice.plantbiology.msu.edu/) using STAR (Dobin *et al.*, 2013), and reads were
226 counted by featureCounts with the package Subread (Liao *et al.*, 2014). Results of data
227 analysis are summarised in Table **S1**. The expression profiles were obtained by comparing
228 control and chitin-treated plants using EdgeR in the R package (Robinson *et al.*, 2010)
229 with a trimmed mean of M values for normalisation. The list of differentially expressed
230 genes (DEGs) was based on a false discovery rate (FDR) < 0.05. Venn diagrams and
231 heatmaps were prepared at the Bioinformatics and Evolutionary Genomics webpage
232 (http://bioinformatics.psb.ugent.be/webtools/Venn/) and ComplexHeatmap in R package
233 (Gu *et al.*, 2016), respectively. Gene Ontology (GO) enrichment analysis was conducted
234 with the PANTHER (Mi *et al.*, 2021) and REVIGO (Supek *et al.*, 2011) tools, according

235 to Bonnot *et al.* (2019). Co-expression analysis was performed using the ShinyGO
236 (ver.0.61) website (http://bioinformatics.sdstate.edu/go/; Ge *et al*., 2020).
237
238
239 **Results**
240 **Both oligomeric and polymeric forms of chitin induce a local immune response in**
241 **rice leaves**
242 COs induce ROS production, a typical response of PTI, in Arabidopsis and rice (Kaku *et*
243 *al.*, 2006; Miya *et al.*, 2007). Furthermore, polymeric chitin in the form of CNF induces
244 ROS production in Arabidopsis seedlings, rice cultured cells, and cabbage and strawberry
245 leaf discs (Egusa *et al.*, 2015; Parada *et al.*, 2018). We confirmed that both COs and CNF
246 induce ROS production in rice leaf discs (Fig. **1a**). We previously showed that CNF
247 induces a more pronounced ROS production than COs (of DP2-6 and DP6) when treating
248 Arabidopsis seedlings and rice cultured cells (Egusa *et al.*, 2015); however, we observed
249 the opposite result in rice leaves (Fig. **1a**). These results nevertheless indicated that both
250 COs and CNF can induce a local immune response in rice leaves.
251
252 **Chitin supplementation of soils induces systemic disease resistance in rice leaves**
253 We examined the level of systemic disease resistance in chitin-treated rice plants using
254 the rice brown spot fungus *B. oryzae*. Supplementation of soils with COs and CNF
255 solution/dispersion induced disease resistance compared to untreated control plants, as
256 determined by the size of lesions on leaves; both chitin forms had comparable effects (Fig.
257 **1b**). Mounting an immune response is often accompanied by growth inhibition, a trade-
258 off between immunity and growth (Huot *et al.*, 2014). Supplementation of soils with 0.1%

(w/v) CNF hinders the development of cabbage and strawberry plants (Parada *et al*., 2018). However, 0.1% CNF added to soils did not affect leaf or stem growth in rice seedlings (Fig. **S1**). These results revealed that both COs and CNF can systemically induce disease resistance without compromising growth in rice.

Transcriptome analysis of rice plants grown in soils supplemented with chitins

To explore the molecular mechanisms underlying the induction of systemic disease resistance by chitins, we performed an RNA-seq analysis of rice leaves and roots grown in soils mixed with COs or CNF. We identified 81 and 230 DEGs in CO- and CNF-treated rice leaves, respectively (FDR < 0.05; numbered in both MSU-DB and RAP-DB) compared to control leaves (Fig. **S2**; Tables **S2**, **S3**). Of these 297 non-redundant DEGs, only 14 genes were shared between CO and CNF treatments (Fig. **S2**). The 297 DEGs consisted of 157 upregulated (LogFC > 0) and 140 downregulated (LogFC < 0) genes by chitin treatments and showed similar trends in their expression patterns in CO- and CNF-treated leaves (Fig. **2a**). A GO enrichment analysis of DEGs indicated an enrichment for categories “regulation of protein serine/threonine phosphatase activity (GO:0080163)”, “glutathione metabolic process (GO:0006749)”, and “cellular modified amino acid metabolic process (GO:0006575)” among upregulated genes (Fig. **2b**), while downregulated genes were associated with “sulfate assimilation (GO:0000103)”, “response to cytokinin (GO:0009735)”, “cytokinesis (GO: 0000910)”, and “cell wall biogenesis (GO:0042546)” (Fig. **2c**). A co-expression analysis conducted using ShinyGO (Ge *et al.*, 2020) determined that the expression of 41 genes upregulated by chitins was strongly and significantly (2.18×10^{-48}) correlated with BTH-induced genes (Fig. **S3**; Table **1**; Shimono *et al*., 2007).

We conducted a similar analysis on root samples (Fig. **S4**; Tables **S4**, **S5**). Roots exhibited a much smaller number of DEGs compared to that of leaves upon chitin treatment (Figs. **S2, S4**). GO enrichment analysis revealed that upregulated genes in response to COs and CNFs are involved in "cellular response to nitrate (GO:0071249)", "nitrogen cycle metabolic process (GO:0071941)", and "nitrate assimilation (GO:0042128)" (Fig. **S4c**). These results demonstrated that both COs and CNF induce the expression of genes involved in cytokinin signalling, cell-wall biogenesis, and defence priming in leaves, while the influence of chitin supplementation in soils was more limited in roots.

Chitin supplementation of soils affects endogenous cytokinin levels and cell-wall composition in rice leaves

Phytohormones plays important roles in ISR (Pieterse *et al.*, 2014; Hartmann & Zeier, 2019). We thus measured endogenous levels of phytohormones (auxin [IAA], gibberellins [GA_1], abscisic acid [ABA], JA, jasmonyl isoleucine [JA-Ile], *trans*-zeatin [tZ], isopentyladenine [iP], and SA) in the leaves of rice seedlings grown on soils supplemented with chitins (Table **2**). Of all phytohormones tested, only the contents for the active cytokinin tZ significantly (9.71×10^{-4}) decreased in CNF-treated samples compared to control seedlings. This finding was congruent with our RNA-seq analysis showing that the GO term "response to cytokinin" was enriched in chitin-suppressed genes in leaves (Fig. **S5**).

The plant cell wall offers a passive physical defence barrier to prevent pathogen access to plant cells; in agreement, alteration of cell-wall composition is associated with disease resistance (Bacete *et al.*, 2018). Modification of cell-wall composition caused by

307 genetic inactivation or overexpression of cell-wall-related genes in Arabidopsis resulted
308 in enhanced disease resistance or susceptibility against various pathogens (Bacete *et al.*,
309 2018; Molina *et al.*, 2021). Since the GO enrichment analysis suggested an alteration of
310 cell-wall composition in leaves by chitin supplementation of soils (Fig. **2c**), we purified
311 the cell-wall fraction from leaves of control and chitin-treated rice seedlings and
312 measured its absorbance using FT-IR spectroscopy. We selected the wavenumber range
313 of 800–1700 cm^{-1} as in Molina *et al*. (2021), which can be assigned to main cell-wall
314 components (Alonso-Simón *et al.*, 2011). As shown in Fig. **3a**, we observed different FT-
315 IR spectra in seedlings grown on chitin-supplemented soils, indicating that chitin
316 supplementation of soils results in an alteration of cell-wall composition in rice leaves.
317
318 **Cellulose biosynthesis inhibition induces disease resistance in rice leaves**
319 Defects in cellulose biosynthesis are associated with disease resistance against
320 *Plectosphaerella cucumerina*, *Botrytis cinerea*, and *Ralstonia solanacearum* in
321 Arabidopsis (Hernández-Blanco *et al.*, 2007). However, an effect of cellulose
322 biosynthesis inhibition on disease resistance has not been reported in rice. Accordingly,
323 we examined disease resistance against *B. oryzae* in rice leaves treated with the cellulose
324 biosynthesis inhibitor ISX (Heim *et al*., 1990; Tateno *et al*., 2016). ISX treatment
325 significantly enhanced disease resistance against *B. oryzae*, compared to control and
326 DMSO-treated seedlings ($p < 0.005$), indicating that, as in Arabidopsis, inhibition of
327 cellulose biosynthesis increases resistance against pathogens in rice (Fig. **3b**).
328
329 **Both LysM receptors, OsCERK1 and OsCEBiP, are required to induce systemic**
330 **disease resistance by chitins in rice leaves**

To assess whether OsCERK1 or OsCEBiP contributes to the local immune response in chitin-treated rice seedlings, we quantified ROS production in their respective knockout mutants and corresponding wild-type segregants (Kouzai *et al.*, 2014b,a), which we used as wild-type plants in the following experiments. ROS production by chitins was compromised in the *oscerk1* mutant compared to its wild-type siblings (Fig. **4a**). However, ROS production was comparable between the *oscebip* mutant and its wild-type siblings (Fig. **4b**). We also tested chitin-induced systemic disease resistance in all genotypes and observed that both wild-type siblings and wild-type plants exhibited a systemic induction of disease resistance against *B. oryzae* upon chitin treatments, whereas neither knockout mutant did (Fig. **5**). Taken together, these findings indicate that both OsCERK1 and OsCEBiP are required for chitin-induced systemic disease resistance in rice, but OsCEBiP did not appear to be essential for a local immune response in leaves.

To investigate the effects of the *oscerk1* or *oscebip* mutants on chitin-induced gene expression, we performed an RNA-seq analysis on the leaves of *oscerk1* and *oscebip* mutants grown on soils mixed with chitins. Using the 297 DEGs in the wild type in response to chitin treatment as reference (Fig. **2a**), we established that the expression patterns in the *oscebip* mutant background were drastically different from those observed in the wild type and the *oscerk1* mutant (Fig. **6a**; Tables **S6–S9**). Next, we determined DEGs specific to the knockout mutants by comparing expression levels between control and chitin-treated seedlings and identified 1744 DEGs in *oscebip* and 1495 DEGs in *oscerk1*, of which 535 genes were common to both receptor mutants with both chitin treatments (Fig. **6b**). In fact, only 32 of the 297 chitin-induced DEGs in the wild type were differentially expressed in both knockout mutants, and 162 genes were specifically induced by chitin treatment in the wild type (Fig. **6b**). A GO enrichment analysis of these

355 162 genes identified the terms “mitotic cytokinesis (GO:0000281)” and “plant-type cell
356 wall organization or biogenesis (GO:0071669)” as enriched (Fig. **6c**), which
357 corresponded to the GO terms obtained in the DEGs downregulated by chitin
358 supplementation (Fig. **2c**).
359
360
361 **Discussion**
362 This study aimed to elucidate the molecular mechanism underlying the systemic
363 resistance induced by chitins in rice. To this end, we used two types of chitins, COs (DP2-
364 6) and polymeric chitin CNF, and determined their effects on systemic disease resistance,
365 ROS production, and the transcriptome using knockout mutants of the well-characterized
366 LysM-containing chitin receptors, OsCERK1 and OsCEBiP. Both COs and CNF induced
367 ROS production, and we showed that OsCERK1, but not OsCEBiP, is required to elicit
368 ROS production by chitins in rice leaves (Figs. **1a**, **4a, 4b**). Supplementation of soils with
369 COs or CNF significantly induced systemic disease resistance against *B. oryzae* in rice
370 leaves of wild-type plants and wild-type siblings of the knockout mutants (Figs. **1b**, **5**),
371 while both *oscerk1* and *oscebip* mutants compromised chitin-induced systemic disease
372 resistance (Fig. **5**). These results indicated that OsCERK1 and OsCEBiP regulate chitin-
373 induced systemic disease resistance, although a chitin-induced local immune response in
374 leaves likely requires another chitin-binding protein(s). When chitins are supplemented
375 in soils, chitin perception would be expected to take place in roots and then initiate a long-
376 distance signalling from roots to shoots to induce systemic disease resistance in leaves.
377 Since both OsCERK1 and OsCEBiP are required for chitin-induced systemic disease
378 resistance, these LysM receptors should function as chitin receptors in roots, but OsCEBiP

379 is not required for ROS production in leaves (Fig. **4b**). However, the *oscebip* mutant also
380 compromises elicitor activity in rice suspension cultured cells (Kouzai *et al.*, 2014b). This
381 discrepancy may be explained by the different materials used for analysis and ROS
382 measurements: photosynthetic (leaves) versus non-photosynthetic (cell suspensions).

383 In rice leaves, COs induced ROS production more strongly than CNF (Fig. **1a**).
384 By contrast, CNF supplementation of soils resulted in more DEGs than CO
385 supplementation (Fig. **2**), as was previously observed with the transcriptomes of soybean
386 (*Glycine max*) roots grown in soils mixed with COs or CNF (Kaminaka *et al*., 2020). In
387 addition, the RNA-seq analysis of the chitin receptor mutants demonstrated that the
388 expression patterns of DEGs in rice leaves are similar between soils supplemented with
389 COs and CNF, with some differences as well (Fig. **6**). CNF can be degraded into
390 oligomeric chitins by chitinase more rapidly than non-nanofibrillated chitin (Egusa *et al*.,
391 2015). Thus, CNF treatment may be caused by these degraded forms of oligomeric chitins
392 rather than directly by CNF. However, previous reports indicated that AtCERK1 also
393 binds to polymeric chitin, which plays an essential role in chitin signalling (Petutschnig
394 *et al*., 2010; Wan *et al*., 2012). Therefore, oligomeric and polymeric chitins may have
395 specific roles in local immune responses and systemic disease resistance.

396 RNA-seq analysis of the leaves of rice seedlings grown on soils supplemented
397 with chitins revealed the involvement of cell-wall biogenesis, cytokinin signalling, and
398 regulation of phosphorylation in the systemic response induced by chitins (Fig. **2**). Cell-
399 wall biogenesis may play a key role in chitin-induced systemic response, as this function
400 would require the chitin receptors OsCERK1 and OsCEBiP (Fig. **6b**, **c**). This finding was
401 also supported by the evidence that chitin supplementation of soils disturbs cell-wall
402 composition, as evidenced by FT-IR spectrometry (Fig. **3a**). The leaves of chitin-treated

403 rice seedlings showed spectra quite similar to those of the cellulose-deficient mutant
404 *procuste 1-8* (*prc1-8*) and the pectin-deficient mutant *quasimodo 1-1* (*qua1-1*) of
405 Arabidopsis (Mouille *et al*., 2003). We observed the same lower absorbance from 1170
406 to 1050 cm^{-1} in chitin-treated rice seedlings that was attributed to cellulose and
407 xyloglucans observed in the Arabidopsis *powdery mildew resistant 5* (*pmr5*) and *pmr6*
408 mutants, which exhibit enhanced resistance to powdery mildew (Vogel *et al*., 2004). In
409 addition, the cellulose biosynthesis inhibitor ISX significantly induced disease resistance
410 in leaves (Fig. **3b**). ISX targets cellulose synthase (CESA) subunits in Arabidopsis
411 (Desprez *et al*., 2002; Scheible *et al*., 2001), which is in line with the strong reduction in
412 the expression of genes encoding CESA or cellulose synthase-like (CSLA) among CNF-
413 induced DEGs compared to control seedlings (Fig. **S5**). Cell-wall-derived
414 oligosaccharides released from hemicellulose activate the immune response via
415 OsCERK1 during infection by the fungal pathogen *Magnaporthe oryzae* in rice (Yang *et*
416 *al*., 2021). Thus, damage-associated molecular pattern (DAMP)-triggered immunity
417 caused by cell-wall-derived molecules, particularly cellulose, might be involved in chitin-
418 induced systemic resistance in rice.

419 We measured a significant reduction in cytokinin levels in leaves of rice
420 seedlings grown on CNF-supplemented soils (Table **2**). ISX treatment reduces the
421 contents of the active cytokinin tZ as well as iP types in Arabidopsis (Gigli-Bisceglia *et*
422 *al*., 2018). The expression levels of several genes encoding type-A response regulators,
423 which regulate cytokinin signalling, were lower upon supplementation of soils with both
424 chitins in rice leaves (Fig. **S5**). Loss of function of type-A ARR6 (Arabidopsis Response
425 Regulator 6) induces disease resistance to *P. cucumerina* BMM and *Hyaloperonospora*
426 *parasitica* Noco2 and is accompanied by an alteration in cell-wall composition (Bacete

et al., 2020). These findings suggest that downregulation of genes involved in cytokinin signalling, which is associated with alterations of cell-wall components, participates in chitin-induced systemic disease resistance.

LysM-containing receptors perceive ligands for both immune responses and when establishing symbiosis. In rice, OsCERK1 is involved in recognizing both immune and symbiotic signals. For chitin-triggered immunity, OsCERK1 forms a receptor complex with OsCEBiP that binds to long-chain COs such as chitooctaose (CO8) (Hayafune *et al*., 2014; Shimizu *et al*., 2010). OsMYR1/OsLYK2, which directly binds to short-chain COs like chitotetraose (CO4) released by beneficial symbiont AM fungi, forms a heteromer with OsCERK1 to establish AM symbiosis (He *et al*., 2019). Disruption of OsCERK1 decreases the colonisation of AM fungi and the production of calcium spikes, whereas the *oscebip* mutant does not have any effect on symbiosis (Carotenuto *et al*., 2017; Miyata *et al*., 2014). OsMYR1 depletes OsCERK1 for OsCERK1-OsCEBiP formation and prevents immune signalling induced by CO8, while OsCEBiP inhibits OsCERK1-OsMYR1 binding in a CO8-dependent manner (Zhang *et al*., 2021). This competition between OsCERK1-OsCEBiP and OsCERK1-OsMYR1 might balance immunity and symbiosis (Zhang *et al*., 2021). Since the systemic induction of disease resistance by chitins appears similar to what takes place during ISR caused by beneficial fungi, chitin-induced systemic disease resistance may employ the recognition mechanism for chitins involved in local immune response via OsCERK1-OsCEBiP but not the OsCERK1-OsMYR1 receptor complex participating in AM symbiosis. In addition, the expression of DEGs upregulated by chitins in leaves displayed a strong positive correlation with the genes induced by BTH, an SA analogue that induces defence priming (Shimono *et al*., 2007) (Table **1**). Our previous study reported that CNF supplementation

of soils induces defence priming in cabbage and strawberry (Parada *et al*., 2018). Taken together with the evidence that fungal ISR caused by *L. bicolor* in Arabidopsis occurs via AtCERK1 (Vishwanathan *et al*., 2020), chitin-induced systemic disease resistance may mimic ISR induced by plant growth–promoting fungi.

In summary, chitins supplemented into soils systemically induce disease resistance against the fungal pathogen *B. oryzae* via recognition of chitins by the LysM receptors OsCERK1 and OsCEBiP in rice. Cell-wall biogenesis and cytokinin signalling are perturbed as a systemic response in leaves, and defence priming-related genes and phosphorylation-related genes are upregulated. These effects, together with another unknown function, eventually induce disease resistance (Fig. **7**). This study uncovers the molecular basis underlying chitin-induced systemic disease resistance. These findings may also contribute to elucidating the molecular basis of ISR, which is not well understood, and provides support for the application of chitins as a promising material in agriculture to confer disease resistance. However, it remains unknown how plants systemically induce disease resistance in response to chitins. In addition, both oligomeric and polymeric chitins caused similar effects on chitin-induced systemic disease resistance but differently affected local immune responses and global gene expression in leaves. Thus, it will be essential to expand our knowledge regarding chitin-induced disease resistance in rice, for example, by identifying the molecules involved in long-distance signalling and those derived from cell walls and by confirming the direct perception of CNF by LysM receptors.

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Author Contributions

YN, SI, AM, and HK conceived and designed the experiments; TM, KH, KN, SM, ME, YK, MS, and AM performed the experiments; TM, KH, and AM analysed the sequencing data; TM, ME, YN, SI, AM and HK wrote the manuscript. All authors approved the final manuscript.

Data availability

The raw read data for RNA-seq were deposited in the DNA Data Bank of Japan under the accession number DRA012267.

References

Alonso-Simón A, García-Angulo P, Mélida H, Encina A, Álvarez JM, Acebes JL. **2011**. The use of FTIR spectroscopy to monitor modifications in plant cell wall architecture caused by cellulose biosynthesis inhibitors. *Plant signaling & behavior* **6**: 1104–1110.

Bacete L, Mélida H, López G, Dabos P, Tremousaygue D, Denancé N, Miedes E, Bulone V, Goffner D, Molina A. **2020**. *Arabidopsis* response regulator 6 (ARR6)

499 modulates plant cell-wall composition and disease resistance. *Molecular Plant-*
500 *Microbe Interactions* **33**: 767–780.

501 **Bacete L, Mélida H, Miedes E, Molina A**. **2018**. Plant cell wall-mediated immunity:
502 cell wall changes trigger disease resistance responses. *The Plant Journal* **93**: 614–
503 636.

504 **Bacete L, Mélida H, Pattathil S, Hahn MG, Molina A, Miedes E**. **2017**.
505 Characterization of Plant Cell Wall Damage-Associated Molecular Patterns
506 Regulating Immune Responses. *Methods in molecular biology (Clifton, N.J.)*
507 **1578**: 13–23.

508 **Bittel P, Robatzek S**. **2007**. Microbe-associated molecular patterns (MAMPs) probe
509 plant immunity. *Current Opinion in Plant Biology* **10**: 335–341.

510 **Bonnot T, Gillard M, Nagel D**. **2019**. *A Simple Protocol for Informative Visualization*
511 *of Enriched Gene Ontology Terms*.

512 **Cao Y, Liang Y, Tanaka K, Nguyen CT, Jedrzejczak RP, Joachimiak A, Stacey G**.
513 **2014**. The kinase LYK5 is a major chitin receptor in *Arabidopsis* and forms a
514 chitin-induced complex with related kinase CERK1. *eLife* **3**: 1–19.

515 **Carotenuto G, Chabaud M, Miyata K, Capozzi M, Takeda N, Kaku H, Shibuya N,**
516 **Nakagawa T, Barker DG, Genre A**. **2017**. The rice LysM receptor-like kinase
517 OsCERK1 is required for the perception of short-chain chitin oligomers in
518 arbuscular mycorrhizal signaling. *New Phytologist* **214**: 1440–1446.

519 **Chen S, Zhou Y, Chen Y, Gu J**. **2018**. fastp: an ultra-fast all-in-one FASTQ
520 preprocessor. *Bioinformatics* **34**: i884–i890.

Cui H, Tsuda K, Parker JE. **2015**. Effector-Triggered Immunity: From Pathogen Perception to Robust Defense. *Annual Review of Plant Biology* **66**: 487–511.

Desprez T, Vernhettes S, Fagard M, Refrégier G, Desnos T, Aletti E, Py N, Pelletier S, Höfte H. **2002**. Resistance against herbicide isoxaben and cellulose deficiency caused by distinct mutations in same cellulose synthase isoform CESA6. *Plant Physiology* **128**: 482–490.

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR. **2013**. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics* **29**: 15–21.

Egusa M, Matsui H, Urakami T, Okuda S, Ifuku S, Nakagami H, Kaminaka H. **2015**. Chitin nanofiber elucidates the elicitor activity of polymeric chitin in plants. *Frontiers in Plant Science* **6**: 1–7.

Friedrich L, Lawton K, Ruess W, Masner P, Specker N, Rella MG, Meier B, Dincher S, Staub T, Uknes S, *et al.* 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *The Plant Journal* **10**: 61–70.

Ge SX, Jung D, Yao R, Jung D, Yao R. **2020**. ShinyGO: A graphical gene-set enrichment tool for animals and plants. *Bioinformatics* **36**: 2628–2629.

Gigli-Bisceglia N, Engelsdorf T, Strnad M, Vaahtera L, Khan GA, Jamoune A, Alipanah L, Novaḱ O, Persson S, Hejatko J, *et al.* 2018. Cell wall integrity modulates arabidopsis thaliana cell cycle gene expression in a cytokinin-and nitrate reductase-dependent manner. *Development (Cambridge)* **145**: 1–14.

Gong B-Q, Wang F-Z, Li J-F. **2020**. Hide-and-Seek: Chitin-Triggered Plant Immunity

and Fungal Counterstrategies. *Trends in plant science* **25**: 805–816.

Görlach J, Volrath S, Knauf-Beiter G, Hengy G, Beckhove U, Kogel KH, Oostendorp M, Staub T, Ward E, Kessmann H, *et al.* 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *The Plant Cell* **8**: 629–643.

Gu Z, Eils R, Schlesner M. 2016. Complex heatmaps reveal patterns and correlations in multidimensional genomic data. *Bioinformatics* **32**: 2847–2849.

Hartmann M, Zeier J. 2019. *N*-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance. *Current opinion in plant biology* **50**: 44–57.

Hayafune M, Berisio R, Marchetti R, Silipo A, Kayama M, Desaki Y, Arima S, Squeglia F, Ruggiero A, Tokuyasu K, *et al.* 2014. Chitin-induced activation of immune signaling by the rice receptor CEBiP relies on a unique sandwich-type dimerization. *Proceedings of the National Academy of Sciences of the United States of America* **111**.

He J, Zhang C, Dai H, Liu H, Zhang X, Yang J, Chen X, Zhu Y, Wang D, Qi X, *et al.* 2019. A LysM Receptor Heteromer Mediates Perception of Arbuscular Mycorrhizal Symbiotic Signal in Rice. *Molecular Plant* **12**: 1561–1576.

Heim DR, Skomp JR, Tschabold EE, Larrinua IM. 1990. Isoxaben Inhibits the Synthesis of Acid Insoluble Cell Wall Materials In *Arabidopsis thaliana*. *Plant Physiology* **93**: 695–700.

Hernández-Blanco C, Feng DX, Hu J, Sánchez-Vallet A, Deslandes L, Llorente F, Berrocal-Lobo M, Keller H, Barlet X, Sánchez-Rodríguez C, *et al.* 2007.

565 Impairment of cellulose synthases required for *Arabidopsis* secondary cell wall
566 formation enhances disease resistance. *Plant Cell* **19**: 890–903.

567 **Huot B, Yao J, Montgomery BL, He SY**. **2014**. Growth-Defense Tradeoffs in Plants: A
568 Balancing Act to Optimize Fitness. *Molecular Plant* **7**: 1267–1287.

569 **Ichihashi Y, Fukushima A, Shibata A, Shirasu K**. **2018**. High Impact Gene Discovery:
570 Simple Strand-Specific mRNA Library Construction and Differential Regulatory
571 Analysis Based on Gene Co-Expression Network BT - Plant Transcription
572 Factors: Methods and Protocols. In: Yamaguchi N, ed. New York, NY: Springer
573 New York, 163–189.

574 **Ifuku S, Saimoto H**. **2012**. Chitin nanofibers: preparations, modifications, and
575 applications. *Nanoscale* **4**: 3308–3318.

576 **Jones JDG, Dangl JL**. **2006**. The plant immune system. *Nature* **444**: 323–329.

577 **Kaku H, Nishizawa Y, Ishii-Minami N, Akimoto-Tomiyama C, Dohmae N, Takio K,**
578 **Minami E, Shibuya N**. **2006**. Plant cells recognize chitin fragments for defense
579 signaling through a plasma membrane receptor. *Proceedings of the National*
580 *Academy of Sciences of the United States of America* **103**: 11086–11091.

581 **Kaminaka H, Miura C, Isowa Y, Tominaga T, Gonnami M, Egusa M, Ifuku S**. **2020**.
582 Nanofibrillation is an Effective Method to Produce Chitin Derivatives for
583 Induction of Plant Responses in Soybean. *Plants* **9**.

584 **Kanno Y, Oikawa T, Chiba Y, Ishimaru Y, Shimizu T, Sano N, Koshiba T, Kamiya**
585 **Y, Ueda M, Seo M**. **2016**. AtSWEET13 and AtSWEET14 regulate gibberellin-
586 mediated physiological processes. *Nature Communications* **7**: 13245.

Kawasaki T, Yamada K, Yoshimura S, Yamaguchi K. **2017**. Chitin receptor-mediated activation of MAP kinases and ROS production in rice and Arabidopsis. *Plant Signaling & Behavior* **12**: e1361076.

Kihara J, Kumagai T. **1994**. Ecotypes of the fungus *Bipolaris oryzae* with various responses of the mycochrome system. *Physiologia Plantarum* **92**: 689–695.

Kouzai Y, Mochizuki S, Nakajima K, Desaki Y, Hayafune M, Miyazaki H, Yokotani N, Ozawa K, Minami E, Kaku H, *et al.* 2014a. Targeted gene disruption of *OsCERK1* reveals its indispensable role in chitin perception and involvement in the peptidoglycan response and immunity in rice. *Molecular Plant-Microbe Interactions* **27**: 975–982.

Kouzai Y, Nakajima K, Hayafune M, Ozawa K, Kaku H, Shibuya N, Minami E, Nishizawa Y. **2014b**. CEBiP is the major chitin oligomer-binding protein in rice and plays a main role in the perception of chitin oligomers. *Plant Molecular Biology* **84**: 519–528.

Lawton KA, Friedrich L, Hunt M, Weymann K, Delaney T, Kessmann H, Staub T, Ryals J. **1996**. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. *The Plant Journal* **10**: 71–82.

Liao Y, Smyth GK, Shi W. **2014**. featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* **30**: 923–930.

Mauch-Mani B, Baccelli I, Luna E, Flors V. **2017**. Defense Priming: An Adaptive Part of Induced Resistance. *Annual Review of Plant Biology* **68**: 485–512.

Mi H, Ebert D, Muruganujan A, Mills C, Albou L-P, Mushayamaha T, Thomas PD. **2021**. PANTHER version 16: a revised family classification, tree-based classification tool, enhancer regions and extensive API. *Nucleic acids research* **49**: D394–D403.

Miya A, Albert P, Shinya T, Desaki Y, Ichimura K, Shirasu K, Narusaka Y, Kawakami N, Kaku H, Shibuya N. **2007**. CERK1, a LysM receptor kinase, is essential for chitin elicitor signaling in *Arabidopsis*. *Proceedings of the National Academy of Sciences of the United States of America* **104**: 19613–19618.

Miyata K, Kozaki T, Kouzai Y, Ozawa K, Ishii K, Asamizu E, Okabe Y, Umehara Y, Miyamoto A, Kobae Y, *et al.* 2014. The bifunctional plant receptor, OsCERK1, regulates both chitin-triggered immunity and arbuscular mycorrhizal symbiosis in rice. *Plant and Cell Physiology* **55**: 1864–1872.

Molina A, Miedes E, Bacete L, Rodríguez T, Mélida H, Denancé N, Sánchez-Vallet A, Rivière M-P, López G, Freydier A, *et al.* 2021. *Arabidopsis* cell wall composition determines disease resistance specificity and fitness. *Proceedings of the National Academy of Sciences* **118**: e2010243118.

Mouille G, Robin S, Lecomte M, Pagant S, Höfte H. **2003**. Classification and identification of Arabidopsis cell wall mutants using Fourier-Transform InfraRed (FT-IR) microspectroscopy. *The Plant Journal* **35**: 393–404.

Parada RY, Egusa M, Aklog YF, Miura C, Ifuku S, Kaminaka H. **2018**. Optimization of nanofibrillation degree of chitin for induction of plant disease resistance: Elicitor activity and systemic resistance induced by chitin nanofiber in cabbage and strawberry. *International Journal of Biological Macromolecules* **118**: 2185–

2192.

Petutschnig EK, Jones AME, Serazetdinova L, Lipka U, Lipka V. **2010**. The lysin motif receptor-like kinase (LysM-RLK) CERK1 is a major chitin-binding protein in *Arabidopsis thaliana* and subject to chitin-induced phosphorylation. *The Journal of biological chemistry* **285**: 28902–28911.

Pieterse CMJ, van Wees SCM, van Pelt JA, Knoester M, Laan R, Gerrits H, Weisbeek PJ, van Loon LC. **1998**. A Novel Signaling Pathway Controlling Induced Systemic Resistance in Arabidopsis. *The Plant Cell* **10**: 1571–1580.

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker PAHM. **2014**. Induced systemic resistance by beneficial microbes. *Annual review of phytopathology* **52**: 347–375.

Pillai CKS, Paul W, Sharma CP. **2009**. Chitin and chitosan polymers: Chemistry, solubility and fiber formation. *Progress in Polymer Science* **34**: 641–678.

Robinson MD, McCarthy DJ, Smyth GK. **2010**. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**: 139–140.

Scheible WR, Eshed R, Richmond T, Delmer D, Somerville C. **2001**. Modifications of cellulose synthase confer resistance to isoxaben and thiazolidinone herbicides in *Arabidopsis Ixr1* mutants. *Proceedings of the National Academy of Sciences of the United States of America* **98**: 10079–10084.

Sharp RG. **2013**. A Review of the Applications of Chitin and Its Derivatives in Agriculture to Modify Plant-Microbial Interactions and Improve Crop Yields.

Agronomy **3**.

Shimizu T, Nakano T, Takamizawa D, Desaki Y, Ishii-Minami N, Nishizawa Y, Minami E, Okada K, Yamane H, Kaku H, *et al.* 2010. Two LysM receptor molecules, CEBiP and OsCERK1, cooperatively regulate chitin elicitor signaling in rice. *Plant Journal* **64**: 204–214.

Shimono M, Sugano S, Nakayama A, Jiang C-J, Ono K, Toki S, Takatsuji H. 2007. Rice WRKY45 plays a crucial role in benzothiadiazole-inducible blast resistance. *The Plant cell* **19**: 2064–2076.

Shinya T, Motoyama N, Ikeda A, Wada M, Kamiya K, Hayafune M, Kaku H, Shibuya N. 2012. Functional characterization of CEBiP and CERK1 homologs in arabidopsis and rice reveals the presence of different chitin receptor systems in plants. *Plant and Cell Physiology* **53**: 1696–1706.

Sun T, Zhang Y. 2021. Short- and long-distance signaling in plant defense. *The Plant Journal* **105**: 505–517.

Supek F, Bošnjak M, Škunca N, Šmuc T. 2011. REVIGO Summarizes and Visualizes Long Lists of Gene Ontology Terms. *PLOS ONE* **6**: e21800.

Tanabe S, Okada M, Jikumaru Y, Yamane H, Kaku H, Shibuya N, Minami E. 2006. Induction of resistance against rice blast fungus in rice plants treated with a potent elicitor, *N*-acetylchitooligosaccharide. *Bioscience, Biotechnology and Biochemistry* **70**: 1599–1605.

Tateno M, Brabham C, DeBolt S. 2016. Cellulose biosynthesis inhibitors – a multifunctional toolbox. *Journal of Experimental Botany* **67**: 533–542.

Vishwanathan K, Zienkiewicz K, Liu Y, Janz D, Feussner I, Polle A, Haney CH. **2020**. Ectomycorrhizal fungi induce systemic resistance against insects on a nonmycorrhizal plant in a CERK1-dependent manner. *New Phytologist* **228**: 728–740.

Vogel JP, Raab TK, Somerville CR, Somerville SC. **2004**. Mutations in *PMR5* result in powdery mildew resistance and altered cell wall composition. *The Plant Journal* **40**: 968–978.

Wan J, Tanaka K, Zhang X-C, Son GH, Brechenmacher L, Nguyen THN, Stacey G. **2012**. LYK4, a Lysin Motif Receptor-Like Kinase, Is Important for Chitin Signaling and Plant Innate Immunity in Arabidopsis. *Plant Physiology* **160**: 396–406.

Wan J, Zhang XC, Neece D, Ramonell KM, Clough S, Kim SY, Stacey MG, Stacey G. **2008**. A LysM receptor-like kinase plays a critical role in chitin signaling and fungal resistance in *Arabidopsis*. *Plant Cell* **20**: 471–481.

Yang C, Liu R, Pang J, Ren B, Zhou H, Wang G, Wang E, Liu J. **2021**. Poaceae-specific cell wall-derived oligosaccharides activate plant immunity via OsCERK1 during *Magnaporthe oryzae* infection in rice. *Nature Communications* **12**: 2178.

Zhang C, He J, Dai H, Wang G, Zhang X, Wang C, Shi J, Chen X, Wang D, Wang E. **2021**. Discriminating symbiosis and immunity signals by receptor competition in rice. *Proceedings of the National Academy of Sciences* **118**: e2023738118.

Zipfel C. **2008**. Pattern-recognition receptors in plant innate immunity. *Current Opinion in Immunology* **20**: 10–16.

699

700 **Figure Legends**

701 **Fig. 1.** Chitins induce a local immune response and systemic disease resistance in rice.
702 (a) Reactive oxygen species (ROS) production induced by chitins in rice leaves. Leaf
703 discs were prepared from 3-week-old rice seedlings grown on soil and treated with
704 distilled water (DW; Control), 0.01% (w/v) chitin oligomers (COs), or chitin nanofiber
705 (CNF). ROS production triggered by chitin treatments was measured in relative
706 luminescence units (RLUs) in the presence of the probe L-012 for 40 min. Representative
707 data from three independent experiments are shown. Error bars, standard deviation (SD,
708 $n = 6$). (b) Induced systemic disease resistance against *Bipolaris oryzae* by chitins, as
709 measured by lesion diameter (in centimetres) of leaves 2 days after inoculation. Three-
710 week-old rice seedlings grown on soil mixed with DW (Control) and 0.1% or 0.01% (w/v)
711 COs and CNF were inoculated with *B. oryzae*. Representative results from three
712 independent experiments are shown. Error bars, SD ($n > 14$). Different letters indicate
713 significant differences by Tukey's test ($p < 0.05$).

714

715 **Fig. 2.** Transcriptome analysis of rice leaves grown on chitin-supplemented soils.
716 (a) Heatmap representation of gene expression levels of differentially expressed genes
717 (DEGs) in response to COs and CNF (left). LogFC is shown between –2 and 2, with
718 outside values indicated as 2 or –2. Red, upregulated genes; blue, downregulated genes.
719 DEGs in each treatment are indicated on the right. Red, DEG; ivory, not differentially
720 expressed. (b, c) Results of Gene Ontology (GO) enrichment analysis summarised as plot
721 data for upregulated DEGs in chitin-treated samples (b) and downregulated DEGs (c).

722

723 **Fig. 3.** Perturbation of cell-wall biogenesis upon chitin treatments and induced systemic
724 disease resistance in rice leaves.
725 (a) Supplementation of soils with chitins systematically induced alterations of cell-wall
726 composition in rice leaves. Each line represents the differential Fourier-transform infrared
727 (FT-IR) spectra between control plants and seedlings grown on CO- or CNF-containing
728 soils ($n = 3$). (b) A cellulose biosynthesis inhibitor induces disease resistance in rice leaves.
729 The leaves of 3-week-old rice seedlings grown on soil were sprayed with control (DW),
730 DMSO, or isoxaben (1 , 0.1 , and 0.01 μM, respectively) 5 h before *B. oryzae* inoculation.
731 Lesion diameter (cm) of leaves 2 days after inoculation are shown. Representative results
732 from three independent experiments are shown. Error bars, SD ($n > 14$). Different letters
733 indicate significant differences by Tukey's test ($p < 0.05$).
734
735 **Fig. 4.** Chitins induce a local immune response in LysM-receptor mutants.
736 (a, b) ROS production induced by chitins in rice LysM-receptor mutants. Leaf discs
737 prepared from 3-week-old knockout (*ko*) mutants and wild-type siblings (*ws*) of
738 *OsCERK1* (a) or *OsCEBiP* (b) grown on soil were used for ROS measurements in relative
739 luminescence units (RLUs). Representative results from three independent experiments
740 are shown. Error bars, SD ($n = 3$).
741
742 **Fig. 5.** Chitins induce systemic disease resistance in LysM-receptor mutants.
743 Lesion diameters upon *B. oryzae* inoculation in wild-type plants (WT), wild-type siblings
744 (*ws*), and knockout (*ko*) mutants of *OsCERK1* or *OsCEBiP*, conducted as in Fig. 1.
745 Representative results from three independent experiments are shown. Error bars, SD (n
746 > 9). Different letters indicate significant differences by Tukey's test ($p < 0.05$).

Fig. 6. Transcriptome analysis of leaves from LysM-receptor mutants grown on chitin-supplemented soils.

(a) Heatmap representation of expression levels of genes identified in the wild type (WT) as being differentially expressed in leaves upon chitin treatment and listed in Table **S2** and **S3** in the leaves of the WT and *oscebip* or *oscerk1* mutants. LogFC is shown between –2 and 2, with outside values indicated as 2 or –2. Red, upregulated; blue, downregulated. (b) Venn diagram showing the overlap between chitin-induced DEGs in the WT and genes that were DEGs in the knockout mutants (blue: in the WT, red: in *oscebip* mutants, green: in *oscerk1* mutants). (c) Results of GO enrichment analysis of the genes not differentially expressed in *oscebip* and *oscerk1* mutants defined above [162 genes; in the WT-specific group of (b)].

Fig. 7. Hypothetical model of chitin-induced systemic disease resistance in rice.

Chitins supplemented in the soil induce disease resistance in leaves against the fungal pathogen *B. oryzae*. Chitins are first recognized by the LysM receptors OsCERK1 and OsCEBiP in roots. Then, long-distance signalling initiated in the roots perturbs cell-wall biogenesis and cytokinin signalling and upregulates defence priming-related genes and phosphorylation-related genes in leaves, inducing disease resistance.

Supporting information

Fig. S1. Effects of chitin supplementation of the soil on the growth of rice seedlings.

Fig. S2. Venn diagram showing the overlap between the number of DEGs in rice leaves treated with COs or CNF.

771 **Fig. S3.** BTH-induced genes are also upregulated in response to chitin treatments.

772 **Fig. S4.** Results of RNA-seq analysis of chitin-treated rice roots.

773 **Fig. S5.** Expression levels of cell-wall- and cytokinin-related genes.

774 **Table S1.** Summary of RNA-seq analysis.

775 **Table S2.** DEGs in wild-type rice leaves induced by supplementation of soils with COs.

776 **Table S3.** DEGs in wild-type rice leaves induced by supplementation of soils with CNF.

777 **Table S4.** DEGs in wild-type rice roots induced by supplementation of soils with COs.

778 **Table S5.** DEGs in wild-type rice roots induced by supplementation of soils with CNF.

779 **Table S6.** DEGs in leaves of the *oscebip* knockout mutant induced by supplementation
780 of soils with COs.

781 **Table S7.** DEGs in leaves of the *oscebip* knockout mutant induced by supplementation
782 of soils with CNF.

783 **Table S8.** DEGs in leaves of the *oscerk1* knockout mutant induced by supplementation
784 of soils with COs.

785 **Table S9.** DEGs in leaves of the *oscerk1* knockout mutant induced by supplementation
786 of soils with CNF.

787 **Table S10.** DEGs in leaves of the wild type induced by supplementation of soils with
788 chitin that are not included in the DEGs induced by chitin treatment in the *oscebip* or
789 *oscerk1* mutants.

790

791

792 **Table 1.** Expression levels and annotation of genes upregulated by chitin treatment
793 correlated with BTH-induced genes.

Gene ID	COs LogFC	CNF LogFC	Annotation
Os01g0176000	0.632	1.487	flavonol-3-*O*-glycoside-7-*O*-glucosyltransferase 1, putative, expressed
Os01g0510200	1.921	3.168	expressed protein
Os01g0585200	0.883	1.784	expressed protein
Os01g0627800	0.904	2.160	cytochrome P450 72A1, putative, expressed
Os01g0638000	1.507	3.712	anthocyanin 3-*O*-beta-glucosyltransferase, putative, expressed
Os01g0695800	0.767	1.242	ABC transporter, ATP-binding protein, putative, expressed
Os01g0795200	2.290	3.552	OsSub8 - Putative Subtilisin homologue, expressed
Os02g0726700	1.111	2.136	helix-loop-helix DNA-binding domain containing protein, expressed
Os03g0235000	1.745	0.875	peroxidase precursor, putative, expressed
Os03g0757600	1.590	2.651	UDP-glucoronosyl and UDP-glucosyl transferase domain containing protein, expressed
Os04g0339400	2.810	4.817	oxidoreductase, aldo/keto reductase family protein, putative, expressed
Os04g0373400	0.796	1.494	MATE efflux family protein, putative, expressed
Os04g0581000	1.475	0.702	naringenin,2-oxoglutarate 3-dioxygenase, putative, expressed
Os04g0627900	1.507	2.599	expressed protein
Os05g0527000	1.658	2.435	anthocyanidin 5,3-*O*-glucosyltransferase, putative, expressed
Os06g0493100	1.310	0.905	RALFL28 - Rapid ALkalinization Factor RALF family protein precursor, expressed
Os07g0175600	1.549	0.882	LTPL78 - Protease inhibitor/seed storage/LTP family protein precursor, expressed
Os07g0442900	2.977	3.307	membrane associated DUF588 domain containing protein, putative, expressed
Os07g0605400	0.746	0.450	EGG APPARATUS-1, putative, expressed
Os07g0677200	1.227	0.694	peroxidase precursor, putative, expressed
Os08g0153900	2.204	0.956	expressed protein
Os08g0155900	1.831	3.026	expressed protein
Os08g0185900	1.146	1.798	ubiquitin family protein, putative, expressed
Os08g0412700	1.173	1.989	expressed protein
Os09g0492900	2.017	4.108	expressed protein
Os10g0109600	2.748	1.908	peroxidase precursor, putative, expressed
Os10g0527400	2.717	3.543	glutathione *S*-transferase GSTU6, putative, expressed
Os10g0527800	3.119	3.641	glutathione *S*-transferase, putative, expressed
Os10g0529500	1.311	2.201	glutathione *S*-transferase GSTU6, putative, expressed
Os10g0535800	0.945	0.954	uncharacterized Cys-rich domain containing protein, putative, expressed
Os10g0542900	2.095	1.632	CHIT14 - Chitinase family protein precursor, expressed

Os10g0558700	0.932	1.340	flavonol synthase/flavanone 3-hydroxylase, putative, expressed
Os11g0687100	2.968	1.771	von Willebrand factor type A domain containing protein, putative, expressed
Os12g0268000	1.387	2.397	cytochrome P450 71A1, putative, expressed
Os12g0555200	1.568	1.714	pathogenesis-related Bet v I family protein, putative, expressed
Os12g0555500	0.732	0.319	pathogenesis-related Bet v I family protein, putative, expressed

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796 **Table 2.** Levels of endogenous phytohormones upon induction by chitins.

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	Control	0.1% COs	0.1% CNF
IAA	68.37±13.48	84.08±43.8	72.39±22.83
GA_1	4.14±1.08	4.42±2.26	3.06±0.71
ABA	23.60±0.63	25.54±11.22	25.40±7.69
JA	55.55±48.20	29.02±18.93	16.08±8.66
JA-Ile	9.14±6.55	3.41±2.03	2.28±1.09
tZ	2.98±0.26	3.51±1.30	***1.45±0.55
iP	0.39±0.03	0.46±0.24	0.35±0.11
SA	48.53±11.00	49.19±18.64	47.55±16.04

798 ***, $p < 0.001$, $n = 5$; IAA, GA_1, ABA, JA, JA-Ile, tZ, iP, pg/mg; SA, ng/mg.

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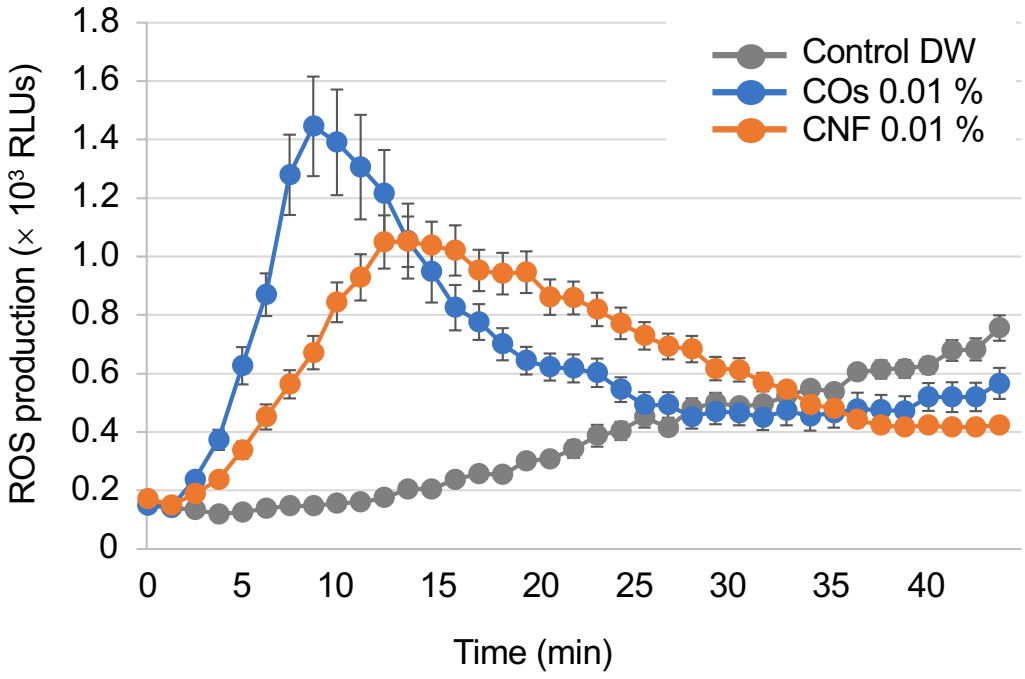
Fig. 1

(a)



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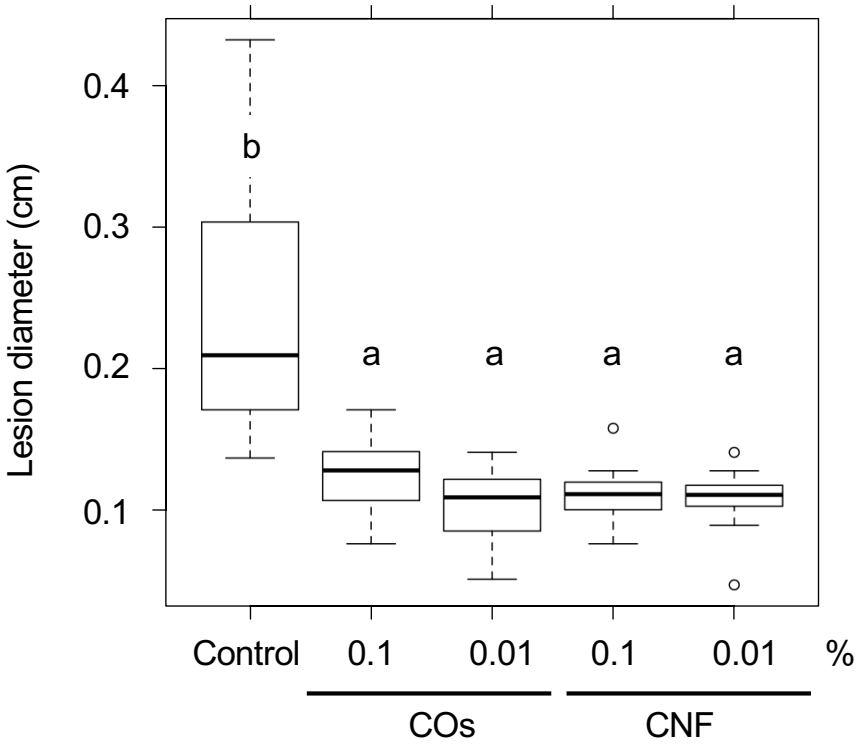


Fig. 2

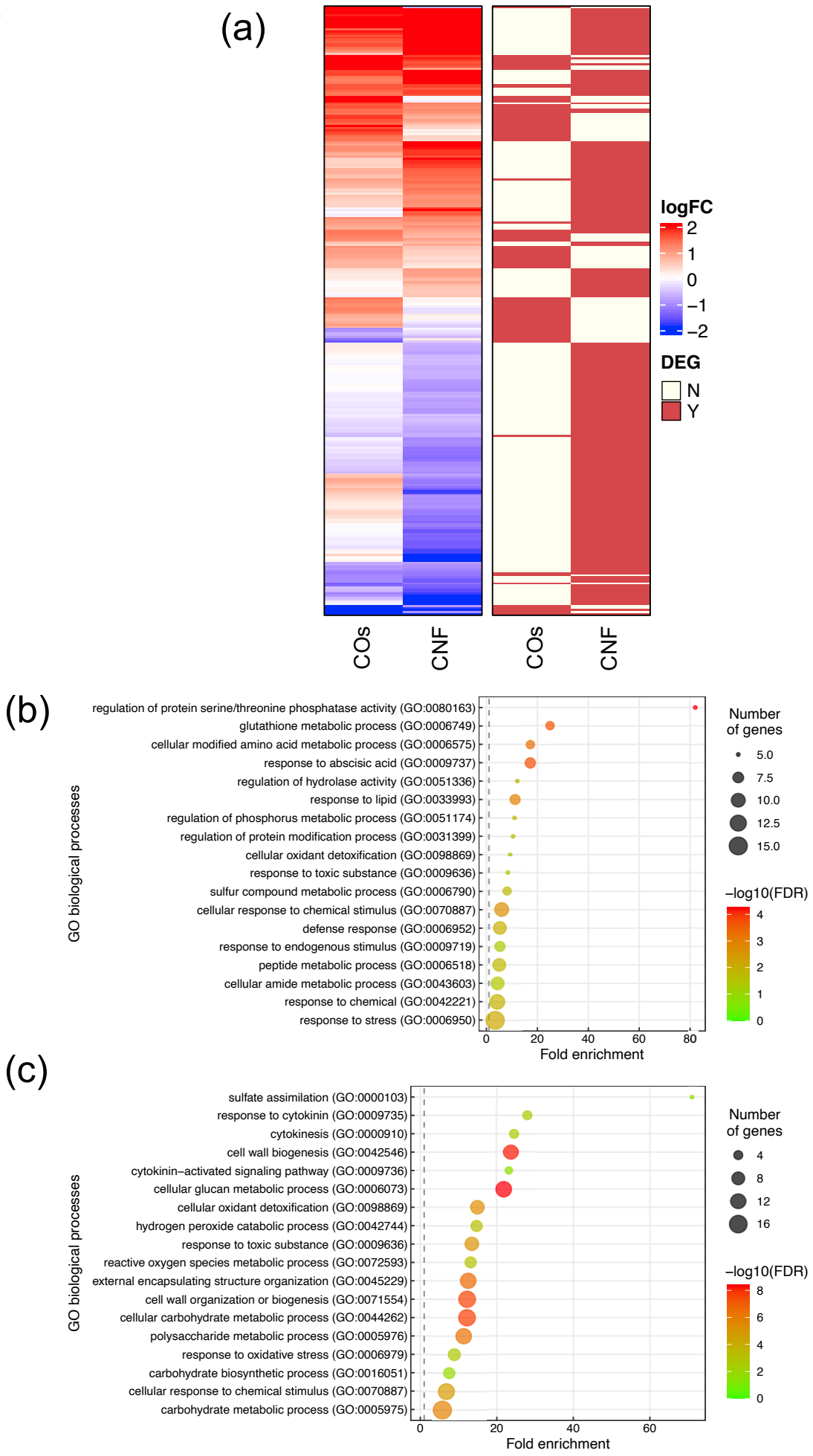


Fig. 3

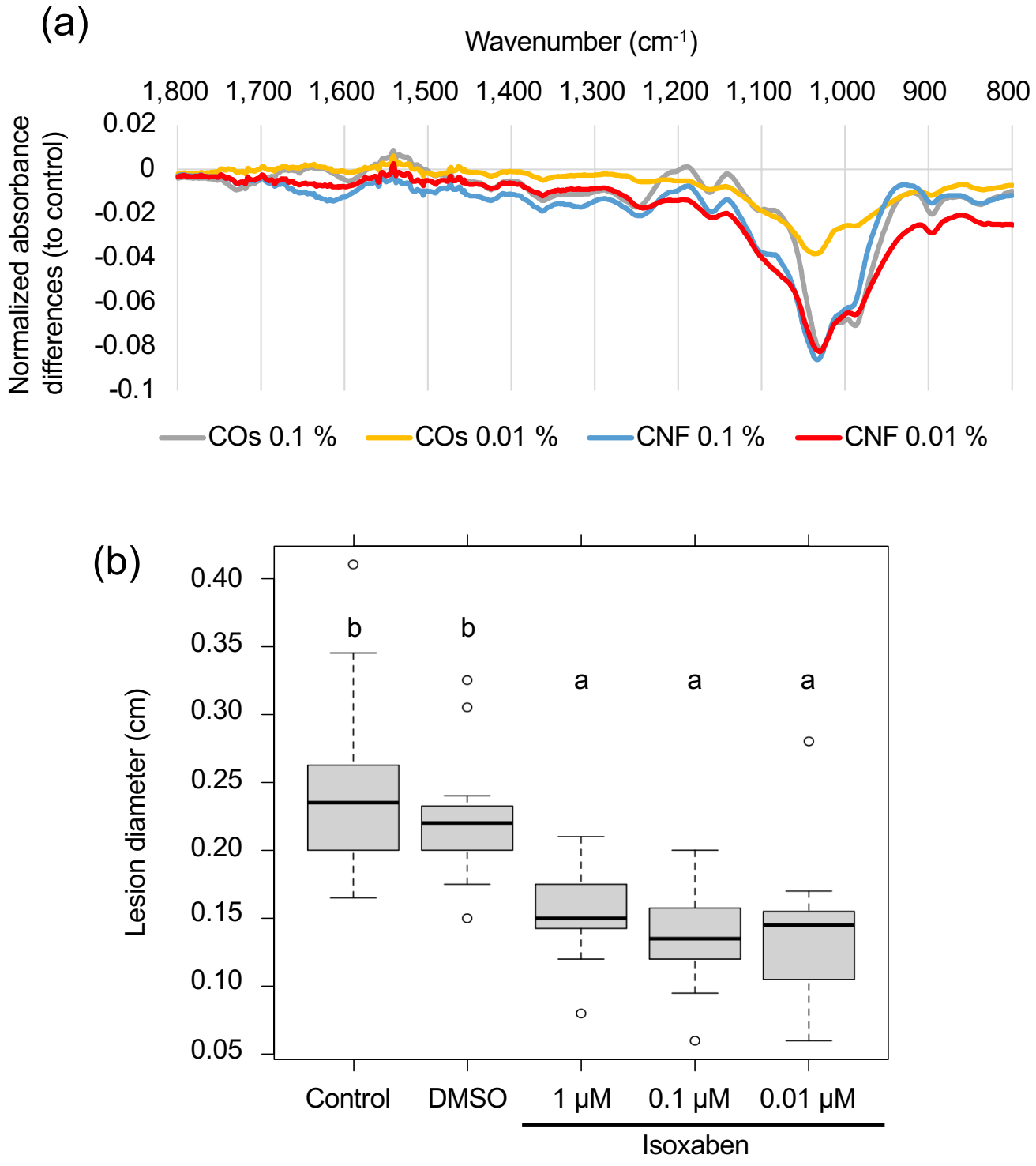


Fig. 4

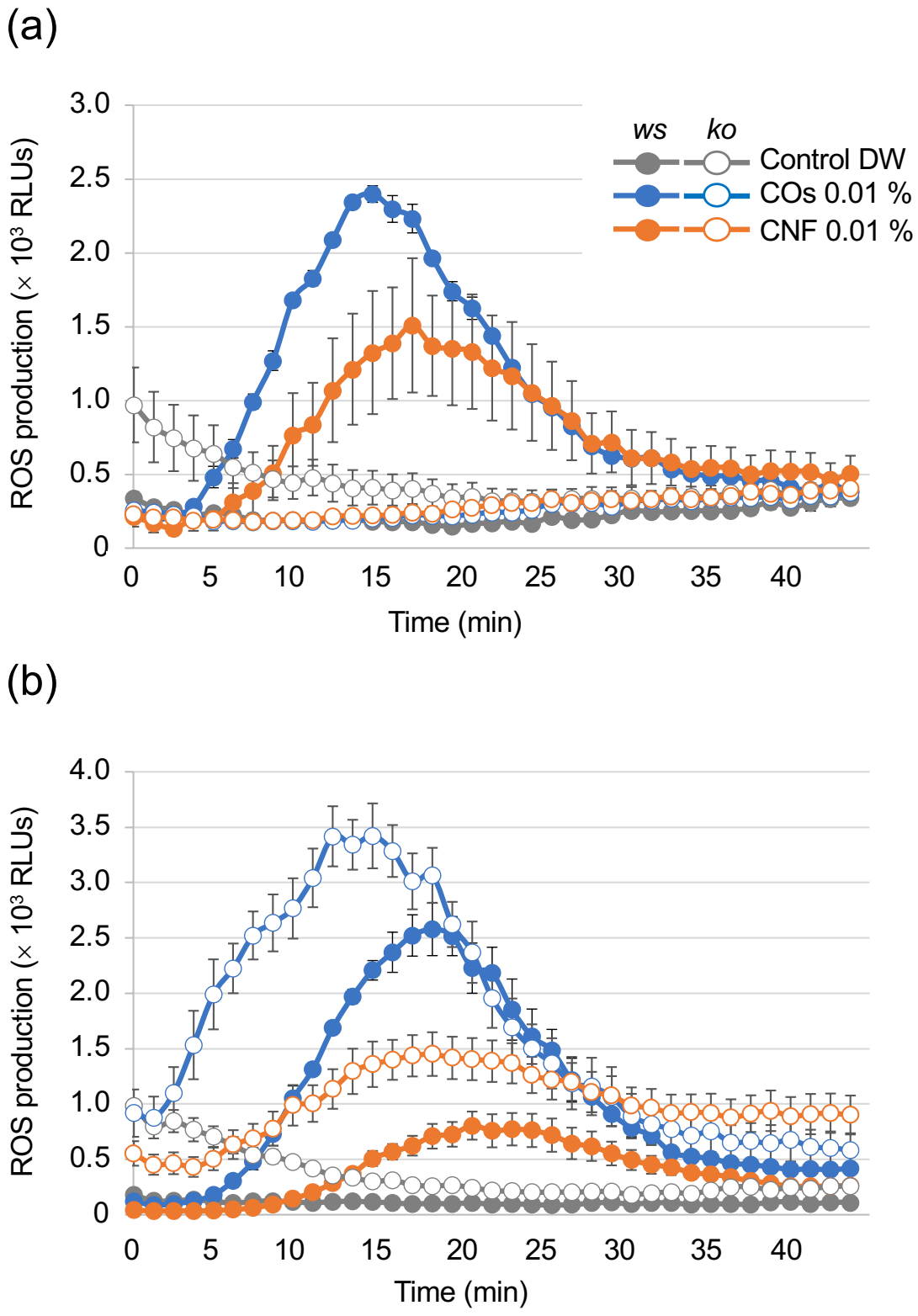


Fig. 5

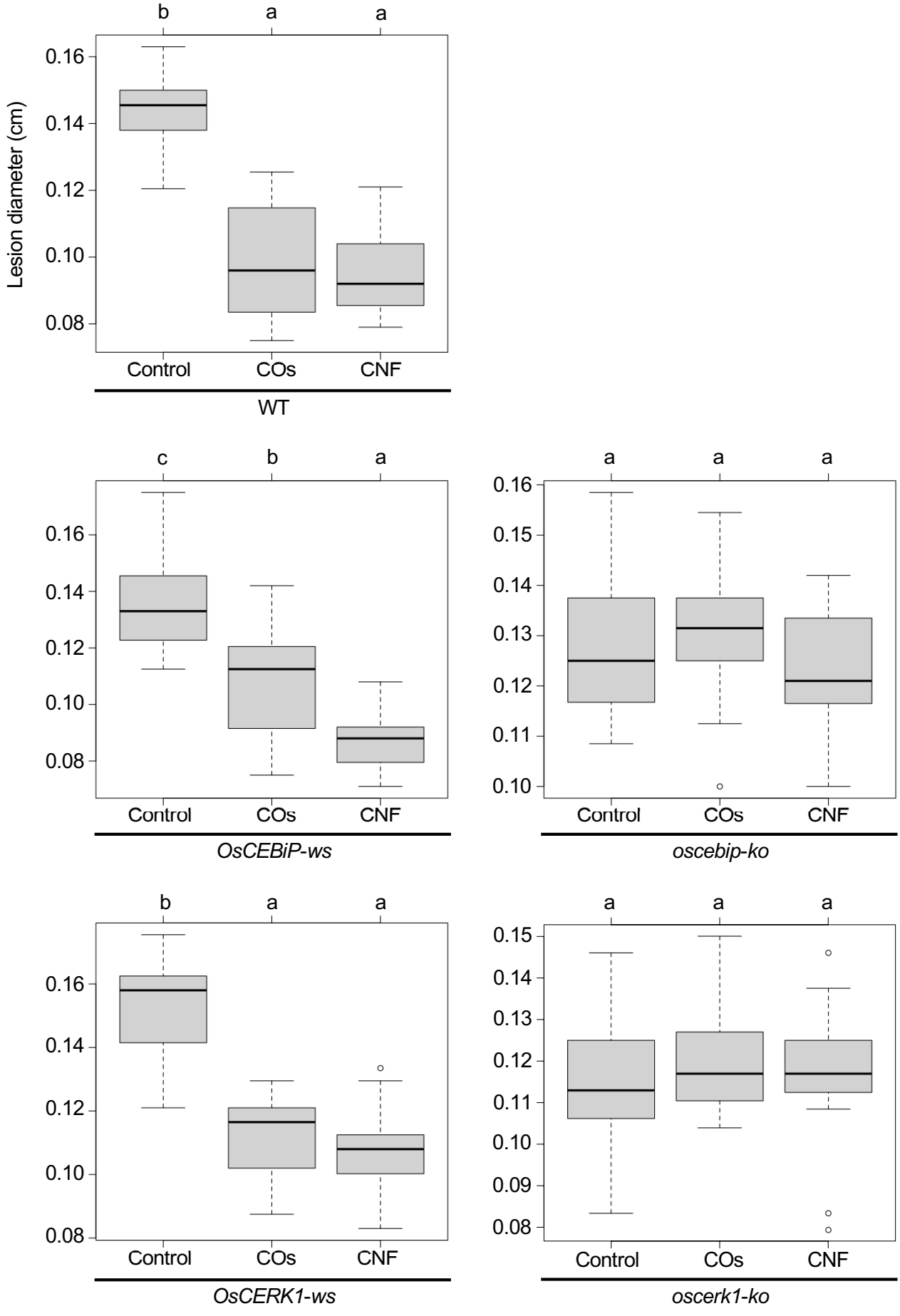
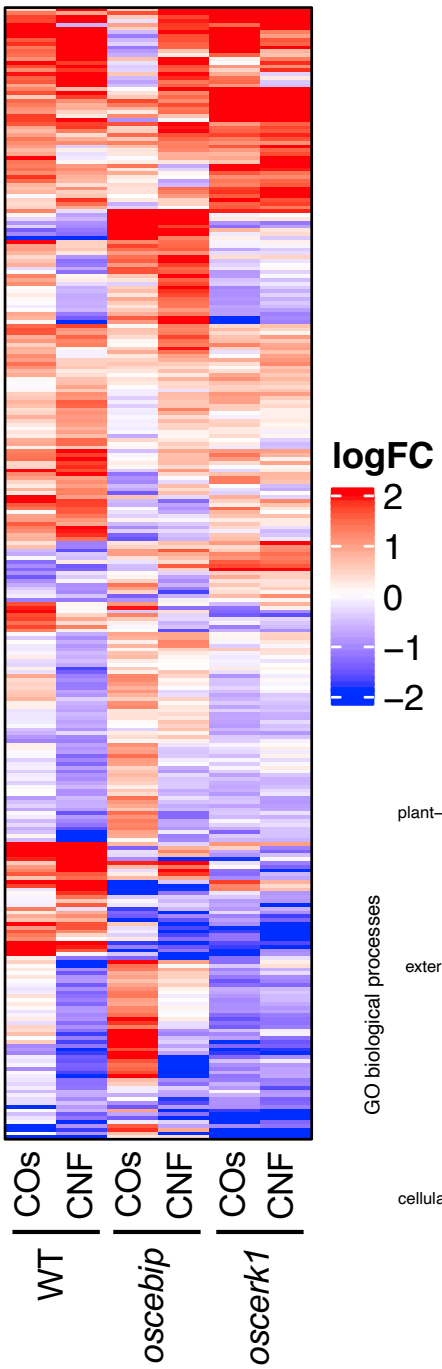
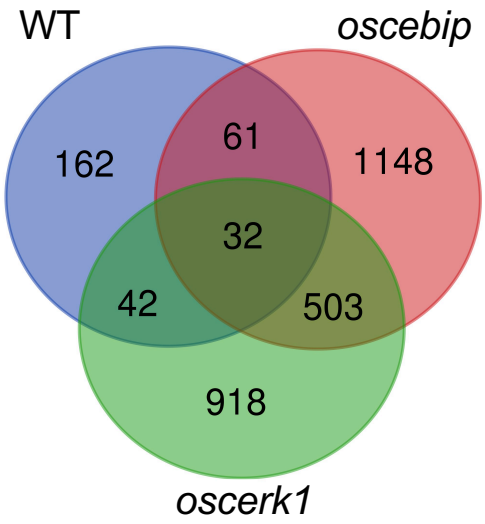


Fig. 6

(a)



(b)



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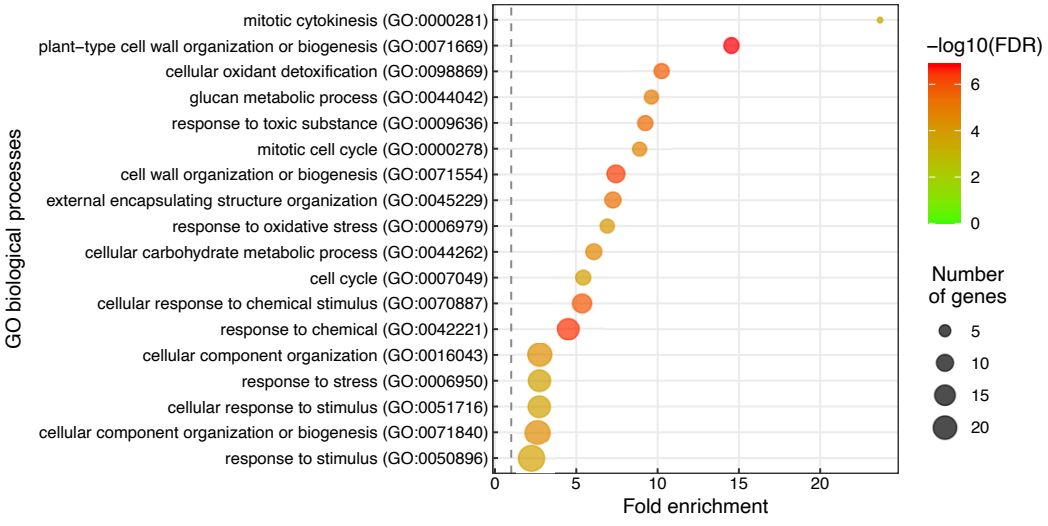

Fig. 7