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Two structurally different oomycete MAMPs induce distinctive plant immune responses

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Abstract

Plants recognize a variety of external signals and induce appropriate mechanisms to increase their tolerance to biotic and abiotic stresses. Precise recognition of attacking pathogens and induction of effective resistance mechanisms are critical functions for plant survival. Some molecular patterns unique to a certain group of microbes (MAMPs, microbe-associated molecular patterns) are sensed by plant cells as non-self molecules via pattern recognition receptors. While a variety of MAMPs of bacterial and fungal origin have been identified, reports on MAMPs of oomycete pathogens are relatively limited. This study aimed to identify unique MAMP elicitors from the oomycete pathogen *Phytophthora infestans*, the causal agent of potato late blight. Using reactive oxygen species (ROS) production and phytoalexin production in potato as markers for the purification of oomycete elicitors, we identified two structurally different groups of elicitors, namely ceramides and diacylglycerols. *P. infestans* ceramide (Pi-Cer) elicitors induced ROS production, while diacylglycerol (Pi-DAG) elicitors, containing eicosapentaenoic acid (EPA) as a substructure, induced the formation of phytoalexins in potato. Pi-Cer and Pi-DAG are also contained in the mycelia of another oomycete pathogen *Pythium aphanidermatum*, indicating that they are MAMPs of oomycetes. When Arabidopsis was treated with Pi-Cer and EPA, partially overlapping but different sets of genes were induced. Furthermore, simultaneous treatment with Pi-Cer and EPA did not have a cumulative effect on induced genes, but rather the expression of some genes induced by EPA was attenuated by the co-treatment with Pi-Cer. These results indicate that plants may combine the signals from simultaneously recognized MAMP elicitors to specifically adapt the defense response to a particular pathogen.

Keywords: Ceramide, EPA, MAMPs, Oomycete, Phytoalexins, *Phytophthora infestans*, ROS.

Introduction

During the course of their evolution, plants were always faced with the challenges of environmental microorganisms but have survived by developing physical barriers or inducible resistance strategies against pathogens. The first step of inducible plant defense against potential pathogens is the recognition of molecular patterns of microbes, referred to as MAMPs or PAMPs (microbe- or pathogen-associated molecular patterns). Plant cells recognize molecular patterns unique to a certain group of microbes as non-self molecules via pattern recognition receptors (PRRs) (Ranf, 2017). In Arabidopsis, for instance, chitin, a major component of the fungal cell wall, is recognized by a PRR complex containing LysM-RLK and co-receptor CERK1, while a conserved sequence of bacterial flagellin (flg22) and an elongation factor EF-Tu (elf18) induce plant defense via PRRs, FLS2 and EFR, respectively, in conjuncture with co-receptor BAK1 (Ranf, 2017). While a variety of MAMPs of bacterial and fungal origin, as well as the mechanisms for their recognition in plants have been studied, reports for MAMPs of oomycete pathogens are relatively limited.

Oomycete plant pathogens resemble fungal pathogens in various aspects. Besides morphological similarities, such as filamentous hyphae, they are also able to form appressoria and haustoria during infection. These similarities are however merely the result of convergent evolution, since oomycetes are phylogenetically closely related to diatoms and brown algae (Stramenopiles), and only distantly related to fungi (Opisthokonta). Therefore, the natures of oomycetes and true fungi are rather different. For example, the somatic thallus of oomycetes is diploid, while it is haploid (or dikaryotic) for true fungi, and oomycetes have mitochondria with tubular cristae

whereas true fungi have flattened ones. More importantly in terms of interaction with plants, the composition of the cell wall and cell membrane of oomycete and fungi is distinctive. Although it varies from species to species, the fungal cell wall mainly contains β-1,3-glucan and chitin (β-1,4-N-acetylglucosamine) (Free, 2013), whereas the oomycete cell wall consists mainly of cellulose (β-1,4-glucan) and β-1,3-glucan (Mélida et al., 2013). The plasma membrane of oomycetes contains fucosterol as the end sterol, but ergosterol is the major end sterol for fungi (Gaulin et al., 2010). Consequently, plants may have distinct mechanisms to recognize oomycete and fungal pathogens to activate appropriate defense reactions.

Of the various oomycete genera, *Phytophthora*, *Pythium* and *Peronospora* /*Hyaloperonospora* (downy mildew) are particularly noteworthy, as they include many species that are problematic plant pathogens (Kamoun et al., 2015). These plant pathogenic oomycete genera are predicted to have evolved from a common phytopathogenic ancestor species (Thines and Kamoun, 2010). Undoubtedly the most infamous representative of pathogenic oomycetes is *Phytophthora infestans*, the causal agent of potato late blight, which is responsible for the Great Famine in Ireland of the 1840s. This pathogen is an ongoing problem even today, and the total costs for control efforts and yield losses by *P. infestans* are estimated in the range of multi-billion dollars annually (Garelik, 2002; Fry, 2008). Problems caused by *P. infestans* are particularly serious in developing countries where fungicides are used as the primary solution for disease management. Although potato cultivars with introduced resistance (*R*) gene(s) have been employed, due to selection pressure on these effectors in the pathogen

population, *R*-gene dependent resistances have been lost in many varieties (Forbes, 2012).

In this study, we purified two structurally distinctive elicitors from *P. infestans*. Using induction of reactive oxygen species (ROS) production or phytoalexin production in potato as markers for the purification of elicitors, we identified two structurally different groups of elicitors, namely ceramides and diacylglycerols. *P. infestans* ceramide (Pi-Cer) elicitors induced ROS production, while diacylglycerol (Pi-DAG) elicitors, containing eicosapentaenoic acid (EPA) as a substructure, induced the formation of phytoalexins in potato tubers. RNAseq analysis was performed in Arabidopsis treated with Pi-Cer, EPA, or the mixture of both elicitors, to investigate the difference in the activity of the two elicitors on the induction of defense genes, and the effect of simultaneous recognition of two MAMP elicitors.

RESULTS AND DISCUSSION

Purification of *Phytophthora infestans* elicitors which induce ROS production of potato suspension-cultured cells

Previously, we have reported that crude elicitor derived from the mycelia of *P. infestans* extracted by methanol (Pi-MEM) can induce the resistance reactions in potato (Monjil et al., 2015). Treatment with Pi-MEM can induce the ROS production in potato suspension cells and leaves, and production of sesquiterpenoid phytoalexins (rishitin, lubimin and oxylubimin) in potato tubers (Figure 1). To purify the elicitor molecules from the mycelia of *P. infestans* and determine their structure, Pi-MEM was first fractionated by its solubility in water and butanol. In this study, the lipophilic (butanol soluble) fraction was further separated by column chromatography, and the elicitor

fractions were selected using their ROS inducing activity in cultured potato cells (Supplemental Figure S1). Only fractions with clear elicitor activity were used for further purification processes. Six fractions with significant ROS-producing activity were further purified (Supplemental Figure S1) and the chemical structures of these elicitors were analyzed by NMR spectroscopy (Supplemental Figures S2, 3, 5, 6, 7 and 9). To compare the structural difference between elicitors and inactive related substances, two fractions with no significant elicitor activity were also collected, and the structures of substances in these fractions were analyzed (Supplemental Figures S1, 4 and 8). Further structural analyses (Supplemental Figures S10-16 and Supplemental document) showed that these eight substances could be divided into two groups, ceramide (Cer) and ceramide-phosphoethanolamine (CerPE). We designated the four ceramide compounds Pi-Cer A-D (*P. infestans* Ceramide) and the remaining four compounds Pi-CerPE A-D respectively. Except for the additional phosphoethanolamine in Pi-CerPE, compounds that share the same alphabetical suffix are otherwise structurally identical (Figure 2 and Supplemental Figure S17A). Pi-Cer A, B and D were able to induce ROS production, whereas Pi-Cer C had only a marginal ROS inducing activity (Figure 2 and Supplemental Figure S1). Similarly, Pi-CerPE A, B and D were active, whereas Pi-CerPE C was inactive (Supplemental Figures S1 and S17). For both Pi-Cers and Pi-CerPEs, D is the most active, followed by B and A in that order (Figure 2 and Supplemental Figure S17).

To investigate whether the substances corresponding to Pi-Cers are contained in another phytopathogenic oomycetes, a modified purification process was applied to partially purify the Pi-Cers from *Pythium aphanidermatum*. *Py. aphanidermatum* is an

oomycete plant pathogen with a wide host range, causing seedling damping-off, root and stem rots and blights of a wide range of important crops (e.g. tomato, soybean, cucumber and cotton). Although the composition of Pi-Cers was different from that in *P. infestans*, Pi-Cer A, B, C and D were detected in *Py. aphanidermatum*. On the other hand, Pi-Cers were not detected in several tested fungal plant pathogens, such as *Botrytis cinerea*, *Fusarium oxysporum* and *Colletotrichum orbiculare* (Supplemental Figure S18). These results suggest that the Pi-Cers are molecules specific to oomycetes.

The most evident structural difference between active Pi-Cers and Pi-Cer C is the 8*E*,10*E*-double bonds and methyl-branching at C-9 in the sphingoid base, which are found in Pi-Cer A, B and D, but not in Pi-Cer C (Figure 2). Kato et al. (2021) showed that that 9-methyl branching and 4*E*-double bond structure of sphingoid base are important for its elicitor activity in Arabidopsis. Previously, cerebrosides were identified as elicitor molecules from rice blast pathogen, *Pyricularia oryzae* (Koga et al., 1998). Cerebroside elicitors isolated from *P. oryzae* also contain 9-methyl-branching and a 4*E*-double bond in the sphingoid base. Given that 9-methyl-branching in the sphingoid base is essentially not found in plants (Sperling and Heinz, 2003), this structure appears to be a molecular pattern that can be leveraged by the plant to recognize potentially pathogenic microorganisms. By mutagenesis of the Arabidopsis pWRKY33-LUC marker line and screening of mutants insensitive to Pi-Cer D, Kato et al. (2021) revealed a lectin receptor kinase is required for the recognition of Pi-Cer D (9-methyl sphingoid base) in Arabidopsis.

Pi-Cer D and Pi-CerPE D are elicitors of ROS production in dicot and monocot plants

Because Pi-Cer D and Pi-CerPE D had the highest elicitor activity among Pi-Cers and Pi-CerPEs, respectively, and were also more abundantly purified than the A and B type substances (Supplemental Figure S1), Pi-Cer D and Pi-CerPE D were mainly used in the following experiments. In potato suspension-cultured cells, ROS production induced by Pi-MEM peaks approx. 3 h after treatment. The same pattern was observed after Pi-Cer D and Pi-CerPE D treatment (Supplemental Figure S19), suggesting that Pi-Cers and Pi-CerPEs could be the major ROS inducing substances in Pi-MEM. Potato leaves were treated with Pi-Cer D, Pi-CerPE D or Pi-MEM by syringe infiltration and induction of ROS production was detected. ROS production was induced by these elicitors with a peak at approx. 12 h after the treatment. Similarly, ROS production was detected in leaves of *A. thaliana* and rice treated with Pi-Cer D or Pi-CerPE D 12 h after the treatment (Figure 3). These results indicated that Pi-Cer D and Pi-CerPE D are recognized by both dicot (potato, Arabidopsis) and monocot (rice) plants as MAMPs.

Treatment of Pi-Cer D and Pi-CerPE D enhances the resistance of potato against *P. infestans*

The effect of pre-treatment with ceramide elicitors on the resistance of potato against *P. infestans* was investigated (Figure 4). Potato leaves were treated with 10 μg/ml Pi-Cer D, Pi-CerPE D or 100 μg/ml Pi-MEM, and inoculated with a zoospore suspension of *P. infestans* at 24 h after the elicitor treatment. Within 3 days after the inoculation, leaves of the control plant showed water-soaked disease symptoms, and the lesions on

most leaves extended over the entire leaves within 7 days. In contrast, fewer and smaller spots were developed in leaves treated with Pi-Cer D, Pi-CerPE D or Pi-MEM. Evaluation of disease symptoms up to 7 days after the inoculation indicated that pretreatment with Pi-Cer D and Pi-CerPE D can enhance the resistance of potato leaves against *P. infestans* (Figure 4). Inoculated leaves were stained with aniline blue to visualize the penetration of *P. infestans* as fluorescent spots of deposited callose beneath the penetration sites at 24 h after the inoculation. Compared with control leaves, fewer fluorescence spots were detected in leaves pretreated with Pi-Cer D, Pi-CerPE D or Pi-MEM elicitors (Figure 4D), which indicates that Pi-Cer D and Pi-CerPE D can enhance the penetration resistance of potato leaves against *P. infestans*.

Isolation of Pi-DAGs as elicitors for the induction of phytoalexins production in potato tuber

In potato tubers, accumulation of phytoalexins (rishitin, lubimin and oxylubimin) was induced by the treatment with the Pi-MEM (Figure 1). However, treatment of Pi-Cers or Pi-CerPEs did not induce the production of phytoalexins in potato tubers (Supplemental Figure S20), which suggested that Pi-MEM includes one or more additional elicitors that can induce phytoalexin production. The initial step of our purification protocol consisted of fractioning the butanol-soluble compounds via silica gel flash column chromatography, yielding eight fractions. Of these eight fractions, the third (RM-I-130-3) and sixth (RM-I-130-6) fraction had shown ROS inducing activity and were used for the isolation of Pi-Cers and Pi-CerPEs (Supplemental Figures S1 and 21). The same eight fractions were now used to evaluate their ability to induce phytoalexin production

in potato tubers. Substantial elicitor activity was only observed in the first fraction (RM-I-130-1) and it was therefore further purified (Supplemental Figure S21). Among the five fractions obtained from the following flash column chromatography, the third (ST-I-8-3) and fourth (ST-I-8-4) fraction showed elicitor activity that resulted in phytoalexin production. These fractions were later found to contain mainly 1,3-DAG (diacylglycerol) and 1,2-DAG, respectively. Both ST-I-8-3 and -4 were further separated by HPLC using an ODS column and at least 5 out of 9 resultant fractions showed elicitor activity (Supplemental Figure S21). These results indicated that several 1,3- and 1,2-DAGs derived from *P. infestans* are elicitors that can induce the phytoalexin production of potato tubers. Two fractions with relatively higher elicitor activity, yield and purity, ST-I-14-4 and ST-I-23-4, were selected for further structural analyses. Two additional fractions, which showed almost no elicitor activity (ST-I-14-8 and ST-I-23-8), were also subjected for structural analyses in order to narrow down the molecular patterns that may be critical for the elicitor activity. Further structural analyses (Supplemental Figures S22-25) revealed that active substances from ST-I-14-4 and ST-I-23-4 were 1,2- and 1,3-DAG which both contained an eicosapentaenoic acid (EPA) and linoleic acid fatty acid chain each, and were designated as Pi-DAG A and B, respectively (Figure 5 and Supplemental Figure S21). The inactive substances in ST-I-14-8 and ST-I-23-8 were found to be 1,2- and 1,3-DAG containing palmitic acid and linoleic acid as fatty acid chains, and were designated as Pi-DAG C and D, respectively. Pi-DAG A and B, as well as Pi-DAG C and D, are also contained in *Py. aphanidermatum*, suggesting that Pi-DAGs are molecules commonly found in oomycete plant pathogens (Supplemental Figure S26).

Based on the structural difference of active and inactive Pi-DAGs (Figure 5A and B), EPA was expected to be the essential structure for the elicitor activity of Pi-DAG A and B. Consistently, EPA from *P. infestans* has previously been identified as an elicitor molecule (Bostock et al., 1981). EPA can be considered as a MAMP of oomycete, as EPA is not found in higher plants and higher fungi, but an essential component of the plasma membrane of oomycete cells (Bostock et al., 2011). We confirmed that EPA, but not Pi-Cer D, can induce the production of phytoalexins in potato tubers (Figure 5C and D). On the contrary, Pi-Cer D, but not EPA, induced ROS production in potato leaves (Supplemental Figure S27), thus indicating that the structurally different oomycete MAMPs, EPA and Pi-Cer D, induce distinctive plant immune responses.

RNAseq analysis of Arabidopsis treated with EPA, Pi-Cer D or a mixture of EPA and Pi-Cer D

To compare the elicitor activity of EPA and Pi-Cer D in Arabidopsis, an Arabidopsis transformant containing a *LUC* transgene under the control of *WRKY33* (AT2G38470) promoter (pWRKY33-LUC) (Kato et al., 2021) was treated with EPA and Pi-Cer D. While Pi-Cer D can induce the activation of the *WRKY33* promoter, only marginal induction of *WRKY33* promoter was detected by the treatment with EPA (Figure 6A). RNAseq analysis was performed for Arabidopsis seedlings 24 h after the treatment with 20 µg/ml EPA, Pi-Cer D or a mixture of 20 µg/ml EPA and 20 µg/ml Pi-Cer D. Clustering analysis of significantly up- or down-regulated genes revealed that treatment with EPA or Pi-Cer D leads to distinct gene expression patterns. While some genes respond similarly to either treatment, others genes are induced exclusively by only one

of the two treatments (Figure 6B). Furthermore, simultaneous treatment with Pi-Cer D and EPA did not result in an expression pattern that would be expected by the mere combination of either treatment. Instead, the expression of some genes induced by EPA was attenuated by the co-treatment with Pi-Cer D, resulting in an expression pattern different from either single treatment (Figure 6B). These results indicate that plants may combine the signals from simultaneously recognized MAMP elicitors to specifically adapt the defense response to a particular pathogen. Insight into how these elicitor signals are processed, as well as how the response pathways of both elicitors affect one another, will require further analysis. RNAseq analyses of potato and Arabidopsis treated either with single, or simultaneous treatments of EPA and Pi-Cer D at different time points should allow a better understanding of how these elicitors function.

MATERIALS AND METHODS

Biological materials, growth conditions and inoculation

Potato suspension-cultured cells were prepared from calluses induced from potato tuber discs (cv. Sayaka carrying the *R1* and *R3* genes) in callus-inducing medium [1 ml/l B5-vitamin (100 g/l Myo-inositol, 2.5 g/l glycine, 500 mg/l thiamine-HCl, 500 mg/l pyridoxine-HCl, 5 g/l nicotinic acid, 50 mg/l biotin, 50 mg/l folic acid), 2 mg/l NAA, 0.5 mg/l kinetin, 3% sucrose, 0.2% phytagel and Murashige-Skoog basal medium], under dark condition. Suspension-cultured potato cells were grown by agitation at 130 rpm, 23˚C in 95 ml of MS medium supplemented with 30 mg/ml sucrose, 1 mg/l thiamine, 100 mg/l myo-inositol, 200 mg/l KH_2PO_4, and 0.2 mg/l 2,4-dichlorophenoxyacetic acid. Cells were sub-cultured every week and used for

experiments 4-5 days after the subculturing. Potato, rice and Arabidopsis (Col-0) plants were grown at 23-25˚C under a 16 h photoperiod and an 8 h dark period in an environmentally controlled growth room. Tubers of potato (cv. Rishiri) carrying the *R1* gene were stored at 4˚C until use. *P. infestans* isolate 08YD1 and PI1234-1 (Shibata et al., 2011) was maintained on rye-media at 10˚C. Inoculation of potato leaves with a zoospore suspension of *P. infestans* was performed as described previously (Shibata et al., 2010). *Pythium aphanidermatum* strain Pyaph isolated from cucumber, *Botrytis cinerea* strain AI18 isolated from strawberry leaves in Mie Prefecture in 2018, *Fusarium oxysporum* f. sp. *melonis* strain Mel02010 (Namiki et al., 1998) and *Colletotrichum orbiculare* strain 104-T (Ishida and Akai 1969) were grown on potato dextrose agar (PDA) at 25˚C and maintained at -80˚C in 20% glycerol.

Preparation of methanol extract of *Phytophthora infestans* mycelia (Pi-MEM)

The *Phytophthora infestans* isolates PI1234-1 were grown on rye-seed extract agar medium in test tubes at 18°C in dark condition for 2 weeks. Parts of the growing mycelia were placed in 100 ml flasks containing 20 ml of rye liquid nutrient medium of rye seed-extract (60 g rye seed), 20 g of sucrose and 2 g of yeast extract per 1 liter and incubated in the dark for 2 weeks at 18°C to allow the growth of the mycelia. The mycelial mats grown in the liquid medium were washed thoroughly with water. To remove excess water, water was filtered through filter paper (Toyoroshi No. 2) under reduced pressure and the tissue then frozen at -20°C. The collected mycelia of *P. infestans* were ground in liquid nitrogen by mortar and pestle. The ground mycelia were transferred to a 50 ml tube containing methanol (10 ml/g mycelia). The mycelia

306 suspension was finely grounded using a polytron type homogenizer (HG30, Hitachi
307 Koki, Japan) for 2 min. After centrifugation at 4°C, 3000 × g for 30 min, the supernatant
308 was collected and dried using an evaporator and used as Pi-MEM elicitor. Further
309 procedures for the purification of Pi-Cer and Pi-DAG from Pi-MEM are described in
310 the supplemental document. Mycelia of *Pythium aphanidermatum*, *Botrytis cinerea*,
311 *Fusarium oxysporum* f. sp. *melonis* and *Colletotrichum orbiculare* were grown in 50 ml
312 of potato dextrose broth (PDB) in 100 ml flasks at 25˚C with gentle shaking.
313
314 **Measurement of ROS production in potato suspension-cultured cells**
315 The relative intensity of ROS generation in potato suspension-cultured cells was
316 measured by counting photons from L-012-mediated chemiluminescence. The potato
317 suspension-cultured cells (50 mg/ml) were washed with the assay buffer (175 mM
318 mannitol, 50 mM MES-KOH, 0.5 mM $CaCl_2$ and 0.5 mM K_2SO_4, pH 5.7) twice for
319 removal of the liquid culture medium. For the detection of ROS produced in cultured
320 cells, the cells were resuspended in assay buffer, equilibrated for 1 h at 100 rpm at 23°C.
321 Cells were then treated with elicitors and incubated under the same condition for 3 h.
322 After the incubation, chemiluminescence was measured using 20 mM L-012 (Wako
323 Pure Chemical, Osaka, Japan) in a multimode microplate reader Mithras LB940
324 (Berthold Technologies, Bad Wildbad, Germany).
325
326 **Measurement of ROS production in plant leaves**
327 The relative intensity of ROS production was determined by counting photons from
328 L-012-mediated chemiluminescence. To detect the ROS production in potato,

Arabidopsis and rice leaves, 0.5 mM L-012 in 10 mM MOPS-KOH (pH 7.4) was allowed to infiltrate to the intercellular space of leaves. For potato and Arabidopsis, a syringe without a needle was used for leaf infiltration, whereas vacuum infiltration was used for rice. Chemiluminescence was monitored using a photon image processor equipped with a sensitive CCD camera in the dark chamber at 20°C (Aquacosmos 2.5; Hamamatsu Photonics, Shizuoka, Japan) and quantified using the U7501 program (Hamamatsu Photonics). For the data shown in Supplemental Fig. S27, chemiluminescence was monitored using Lumino Graph II EM (ATTO, Tokyo, Japan).

Detection of potato phytoalexins by thin-layer chromatography (TLC)

Potato phytoalexins, exuded from potato tissue, were extracted with ethyl acetate as described previously (Noritake et al., 1996). The extract was separated on TLC plates (TLC aluminum sheet of silica gel 60, Merck, Whitehouse Station, NJ, USA), which were developed with cyclohexane:ethyl acetate (1:1, v/v) and visualized by spraying with sulfuric acid containing 0.5% vanillin followed by heating at 120°C.

Detection of potato phytoalexins by liquid chromatography/mass spectrometry (LC/MS)

Potato tuber discs (2 cm in diameter and approx. 4 mm thick) were prepared and incubated in a humidified chamber in dark at 23˚C for 24 h before treatment with elicitors. The upper side of incubated potato tubers was then treated with 100 µl of elicitor solution and further incubated at 23°C in the dark for 48 h before the extraction of phytoalexins. Four tuber disks/sample were immersed in 5 ml of ethyl acetate and

shaken for 1 h, then the organic solvent was collected and evaporated. The residual material was redissolved in 100 µl of 50% (v/v) acetonitrile and 2 µl was injected for the analysis with LC/MS (Accurate-Mass Q-TOF LC/MS 6520, Agilent Technologies, Santa Clara, CA, USA).

Measuring the activation of *AtWRKY33* promoter and RNA-seq analysis

Seeds of Arabidopsis containing *Luciferase* marker gene under the control of *AtWRKY33* (AT2G38470) promoter (Kato et al., 2021) were surface sterilized with 3% hydrogen peroxide and 50 % ethanol for 1 min with gentle shaking, washed with sterilized water, and then individual sterilized seeds were placed in separate wells of a 96 microwell plate containing 150 µl of Murashige and Skoog (MS) liquid medium (1/2 MS salts, 0.05% [w/v] MES, 0.5% [w/v] Sucrose, adjusted to pH 5.8 with NaOH) with 50 µM D-Luciferin potassium salt (Biosynth Carbosynth, Compton, UK), covered with a clear plastic cover. Plates containing Arabidopsis seeds were then incubated in a growth chamber for approx. 12 days at 23ºC with 24 h light. After the treatment of elicitors, chemiluminescence intensity derived from expressed luciferase was measured using Mithras LB940 (Berthold) for 12 h. Total RNA was extracted from 6 seedlings/sample of 10-days old Arabidopsis 24 h after the treatment with elicitors using the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Libraries were constructed using KAPA mRNA Capture Kit (Roche, Basel, Switzerland) and MGIEasy RNA Directional Library Prep Set (MGI, Shenzhen, China), and sequenced on DNBSEQ-G400RS (MGI) with 150 bp paired-end protocol. The RNA-seq reads were filtered using trim-galore v.0.6.6 (Martin, 2011, bioinformatics.babraham.ac.uk) and mapped to the

Arabidopsis genome (TAIR 10.1; RefSeq: GCF_000001735.4) using HISAT2 v.2.2.1 (Kim et al., 2019) and assembled via StringTie v.2.1.7 (Kovaka et al., 2019). Significant differential expression was determined using DESeq2 v.1.32.0 (Love et al., 2014). All software used during RNA-seq analysis was run with default settings. The expression profile was calculated from the log2-fold expressions using the clustermap function from seaborn v. 0.11.1 (Waskom, 2021).

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SUPPLEMENTAL DATA

Supplemental Figure S1. Purification procedure of *Phytophthora infestans* ceramide (Pi-Cer) and ceramide phosphoethanolamine (Pi-CerPE) elicitors.

Supplemental Figure S2. NMR spectra of Pi-Cer A ($CDCl_3$, 400 MHz for ^1H, 100 MHz for ^{13}C).

Supplemental Figure S3. NMR spectra of Pi-Cer B ($CDCl_3$, 600 MHz for ^1H, 150 MHz for ^{13}C).

Supplemental Figure S4. NMR spectra of Pi-Cer C ($CDCl_3$, 400 MHz for ^1H, 100 MHz for ^{13}C).

Supplemental Figure S5. NMR spectra of Pi-Cer D ($CDCl_3$, 600 MHz for ^1H, 150 MHz for ^{13}C).

Supplemental Figure S6. NMR spectra of Pi-CerPE A ($CDCl_3$-CD_3OD 4:1, 400 MHz for ^1H, 100 MHz for ^{13}C).

Supplemental Figure S7. NMR spectra of Pi-CerPE B ($CDCl_3$-CD_3OD 4:1, 600 MHz for ^1H, 150 MHz for ^{13}C).

Supplemental Figure S8. NMR spectra of Pi-CerPE D ($CDCl_3$-CD_3OD 4:1, 600 MHz for ^1H, 150 MHz for ^{13}C).

Supplemental Figure S9. NMR spectra of Pi-CerPE D ($CDCl_3$-CD_3OD 4:1, 600 MHz for ^1H, 150 MHz for ^{13}C).

Supplemental Figure S10. MS/MS fragmentation of Pi-Cer A (precursor ion: $[M + Na]^+$).

Supplemental Figure S11. Two-dimensional NMR correlations of Pi-Cer B (thick bonds: DQF-COSY, curved arrows: HMBC, l + m =15).

Supplemental Figure S12. MS/MS fragmentation of Pi-Cer B (precursor ion: $[M + Na]^+$).

Supplemental Figure S13. Two-dimensional NMR correlations of Pi-Cer B (thick bonds: DQF-COSY, curved arrows: HMBC, l + m + n = 19).

Supplemental Figure S14. DQF-COSY (thick bonds), HMBC (curved arrows), and NOESY (dashed curves) correlations of Pi-Cer D.

Supplemental Figure S15. Linked-scan FAB MS/MS (negative ion mode) of the fatty acid derived from Pi-Cer D by acid hydrolysis.

Supplemental Figure S16. DQF-COSY (thick bonds) and HMBC (curved arrows) correlations of Pi-CerPE D.

Supplemental Figure S17. Ceramide phosphoethanolamine (Pi-CerPE) elicitors purified from methanol extract of *P. infestans* mycelia (Pi-MEM), which can induce the production of reactive oxygen species (ROS) in potato suspension-cultured cells.

Supplemental Figure S18. Pi-Cer contents in mycelia of oomycete and fungal plant pathogens.

Supplemental Figure S19. Induction of reactive oxygen species (ROS) production by Pi-Cer D and Pi-CerPE D treatment in potato suspension-culture cells.

Supplemental Figure S20. Production of phytoalexins in potato tuber is not induced by Pi-Cers and Pi-CerPEs.

Supplemental Figure S21. Purification procedure of *Phytophthora infestans* diacylglycerol (Pi-DAG) elicitors.

Supplemental Figure S22. ESI-TOF MS of Pi-DAG A. NMR spectra of Pi-DAG A ($CDCl_3$, 400 MHz for ^1H and 100 MHz for ^{13}C). Two-dimensional NMR of Pi-DAG A ($CDCl_3$, 400 MHz). Determination of fatty acids of Pi-DAG A by MS/MS.

Supplemental Figure S23. ^1H NMR spectrum of Pi-DAG B ($CDCl_3$, 400 MHz). Determination of fatty acids in Pi-DAG B by negative ion FAB MS

Supplemental Figure S24. NMR spectra of Pi-DAG C ($CDCl_3$, 400 MHz for ^1H, 100 MHz for ^{13}C). Determination of fatty acids in Pi-DAG C by positive ion ESI MS/MS. Confirmation of fatty acid linkage in Pi-DAG C.

Supplemental Figure S25. NMR spectra of Pi-DAG D (MeOD, 400 MHz for ^1H, 100 MHz for ^{13}C). Determination of fatty acids in Pi-DAG D by hydrolysis followed by negative ion FAB MS and MS/MS analysis of two fatty acid products.

Supplemental Figure S26. Pi-DAG contents in mycelia of oomycete plant pathogens.

Supplemental Figure S27. Eicosapentaenoic acid (EPA) does not induce the production of reactive oxygen species (ROS) in potato leaves.

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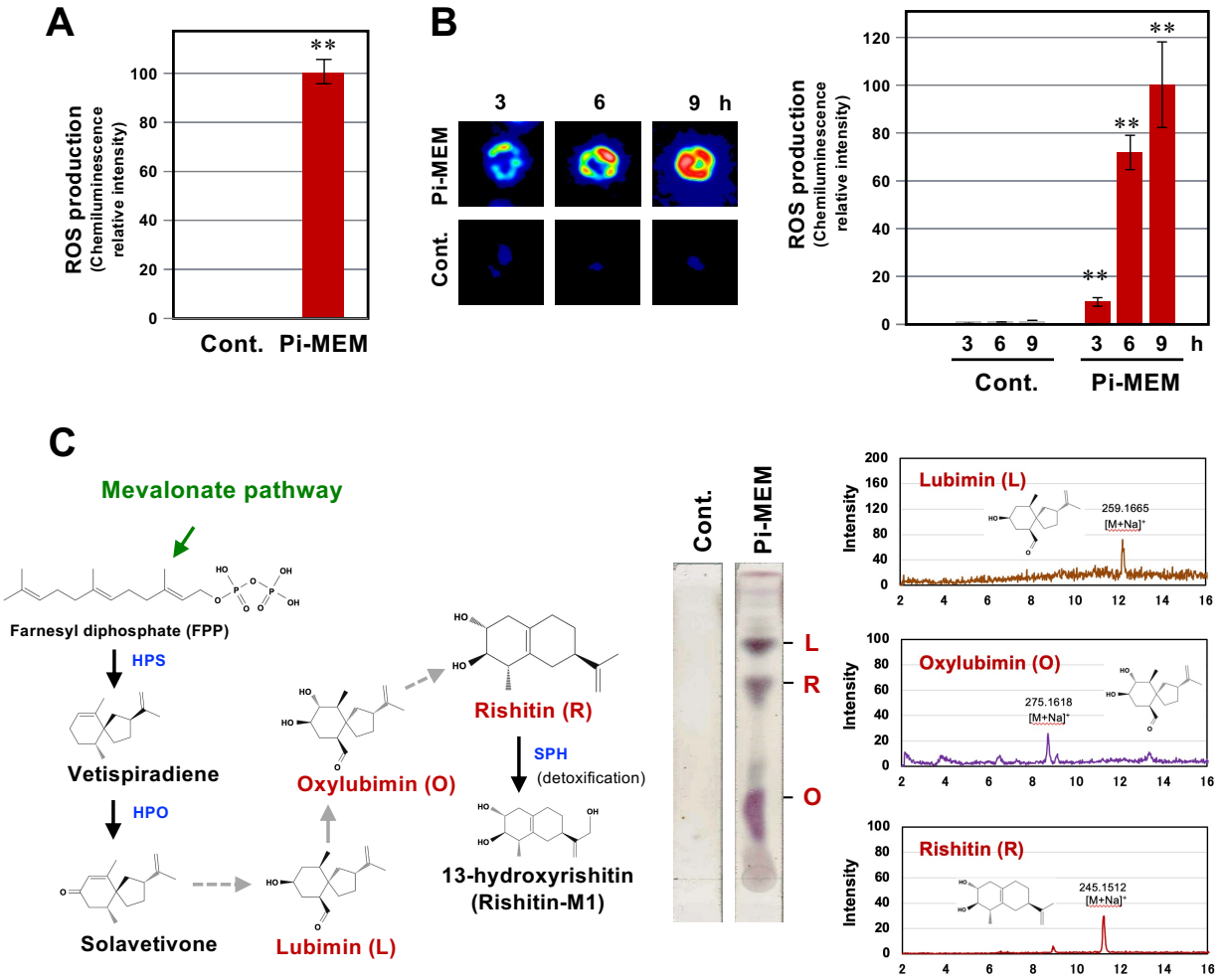

Figure 1. Elicitor activity of methanol extract of *Phytophthora infestans* mycelia (Pi-MEM) in potato.
(A) Potato suspension cultured cells were treated with 0.3% DMSO (Cont.) or 30 µg/ml Pi-MEM and production of reactive oxygen species (ROS) was detected as L-012-mediated chemiluminescence 3 h after the treatment. Data are means ± SE (n = 3). **(B)** Potato leaves were treated with 1 % DMSO (Cont.) or 100 µg/ml Pi-MEM and production of ROS was detected as L-012-mediated chemiluminescence. Data are means ± SE (n = 3). Data marked with asterisks are significantly different from control as assessed by two-tailed Student's *t* tests: **P < 0.01. **(C)** Left, putative biosynthetic pathway of potato phytoalexins, lubimin, oxylubimin and rishitin. HPS, *Hyoscyamus muticus* premnaspirodiene synthase; HPO *H. muticus* premnaspirodiene oxygenase; SPH, sesquiterpenoid phytoalexins hydroxylase (Camagna et al., 2020). Potato tubers were treated with 3 % DMSO (Cont.) or 1 mg/ml Pi-MEM and phytoalexins were extracted 48 h after treatment. Produced phytoalexins were detected by thin-layer chromatography (middle) or LC/MS (right).

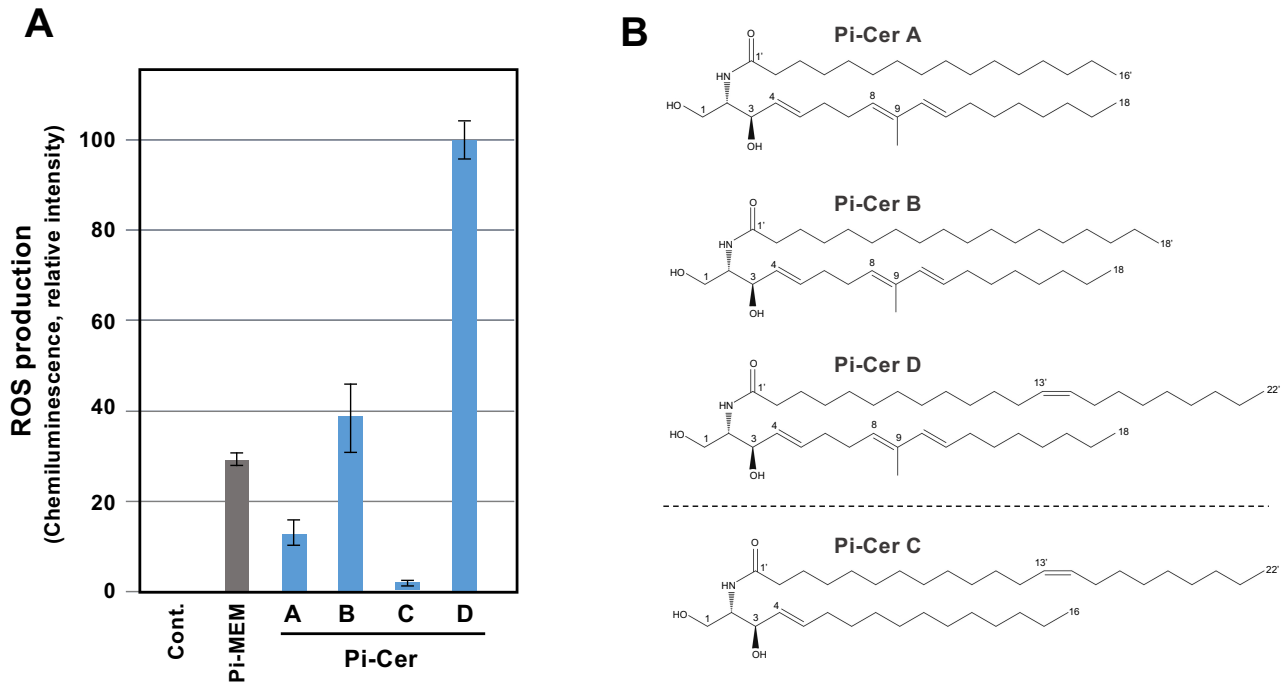

Figure 2. *Phytophthora infestans* ceramide elicitors (Pi-Cer) can induce the production of reactive oxygen species (ROS) in potato suspension cultured cells. **(A)** Potato suspension cultured cells were treated with 0.3% DMSO (Cont.), 30 µg/ml methanol extract of *P. infestans* mycelia (PiMEM), 3 µg/ml Pi-Cer A, B, C or D and production of ROS was detected as L-012 mediated chemiluminescence 3 h after the treatment. **(B)** Structures of Pi-Cer A, B, C and D. See Supplemental Figure S1 for the procedures of purification of elicitors and Supplemental Figures S2-5, S10-15 and supplemental document for details of their structural analysis.

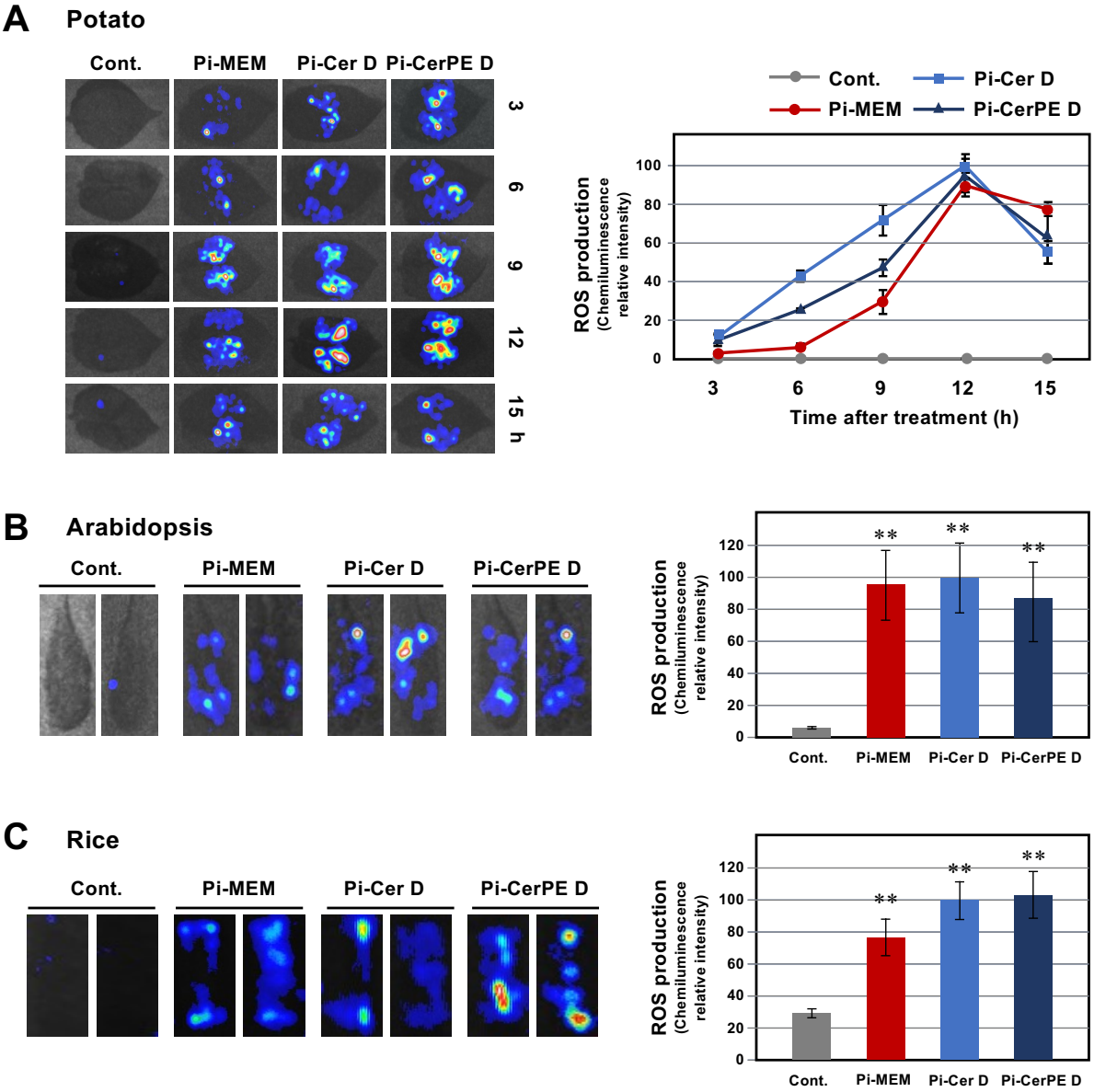

Figure 3. Pi-Cer D and Pi-CerPE D can induce the production of reactive oxygen species (ROS) in different plant species.

(A) (Left) Potato leaves were treated with 1% DMSO (Cont.), 100 µg/ml methanol extract of *P. infestans* mycelia (Pi-MEM), 10 µg/ml Pi-Cer D or Pi-CerPE D by syringe infiltration, and production of ROS was detected as L-012 mediated chemiluminescence 3-15 h after the treatment. Data are means ± SE (n = 3).

(B) (Left) *Arabidopsis thaliana* leaves were treated with 1% DMSO (Cont.), 100 µg/ml methanol extract of *P. infestans* (Pi-MEM), 10 µg/ml Pi-Cer D or Pi-CerPE D by syringe infiltration, and production of ROS was detected as L-012 mediated chemiluminescence 12 h after the treatment. Data are means ± SE (n = 3).

(C) (Left) Rice leaves were treated with 1% DMSO (Cont.), 100 µg/ml methanol extract of *P. infestans* (Pi-MEM), 10 µg/ml Pi-Cer D or Pi-CerPE D by vacuum infiltration, and production of ROS was detected as L-012 mediated chemiluminescence 12 h after the treatment. Data are means ± SE (n = 3). Data marked with asterisks are significantly different from control as assessed by two-tailed Student's *t* tests: **P < 0.01.

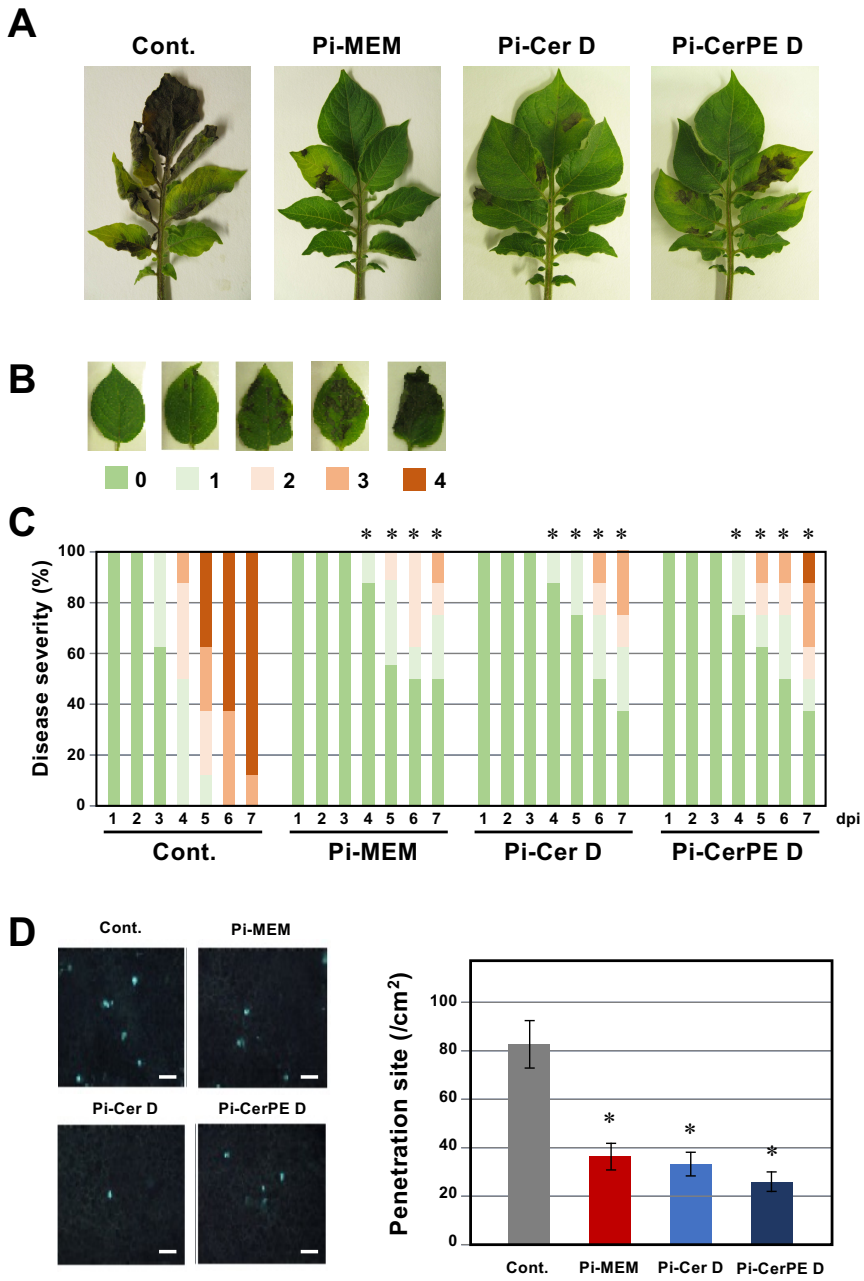

Figure 4. Pretreatment with Pi-Cer D and Pi-CerPE D enhances the resistance of potato leaves against *P. infestans.*

Potato leaves were treated with 0.5 % DMSO (control), 100 µg/ml Pi-MEM, 10 µg/ml Pi-Cer D or Pi-CerPE D and incubated for 24 hrs, and inoculated with a spore suspension of *P. infestans*. **(A)** Disease symptoms of *P. infestans* on potato leaves treated with DMSO, Pi-MEM, Pi-Cer D or Pi-CerPE D. Photographs were taken 6 days post inoculation. **(B)** Leaves representative of the disease severities used for classification. **(C)** Plots showing percentages of potato leaves with disease symptom severities using the classification depicted in (B). Leaves were pretreated with DMSO or elicitors, and disease severity of a subsequent *P. infestans* inoculation was observed from 1 - 7 days post inoculation (dpi). (n = 8). Data marked with asterisks are significantly different from control as assessed by one-tailed Mann–Whitney U-tests: *P < 0.05. **(D)** Left, Penetration sites of *P. infestans* in elicitor treated leaf-discs were detected as callose depositions by aniline blue staining 24 h after inoculation. Bars = 50 µm. Right, Number of fluorescent spots were counted in elicitor-treated leaf discs inoculated with *P. infestans* 24 hrs after inoculation. Data are means ± SE (n = 3). Data marked with asterisks are significantly different from control as assessed by two-tailed Student's *t* tests: p < 0.01.

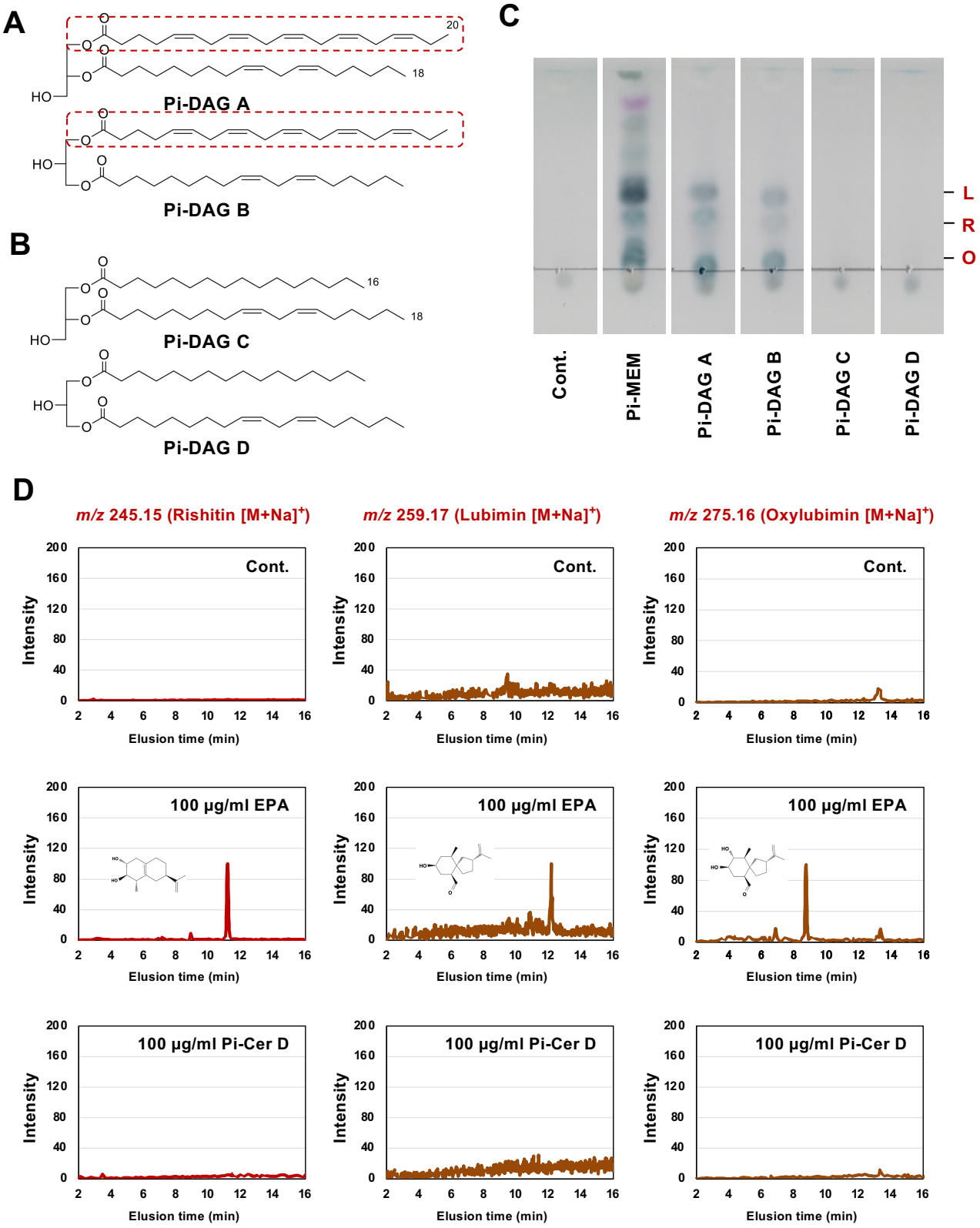

Figure 5. *Phytophthora infestans* diacylglycerol (Pi-DAG) induce the production of phytoalexins in potato tubers. **(A)** Structures of Pi-DAG A and B. Structures equivalent to eicosapentaenoic acid (EPA) are shown in red, dotted boxes. **(B)** Structures of Pi-DAG C and D which have significantly weaker elicitor activity compared with Pi-DAG A and B. See Supplemental Figure S22 for the procedures of purification of Pi-DAGs and Supplemental Figures S23-26 for their structural analysis. **(C)** Potato tubers were treated with 0.3% DMSO (Cont.), 30 µg/ml methanol extract of *P. infestans* mycelium (Pi-MEM), or 100 µg/ml Pi-DAGs, and production of phytoalexins was detected by thin-layer chromatography. L, Lubimin; R, Rishitin; O, Oxylubimin. **(D)** EPA, but not Pi-Cer D, can induce the production of phytoalexins in potato tubers. Potato tubers were treated with 0.3% DMSO (Cont.), 100 µg/ml EPA or 100 µg/ml Pi-Cer D and produced phytoalexins were detected by LC/MS.

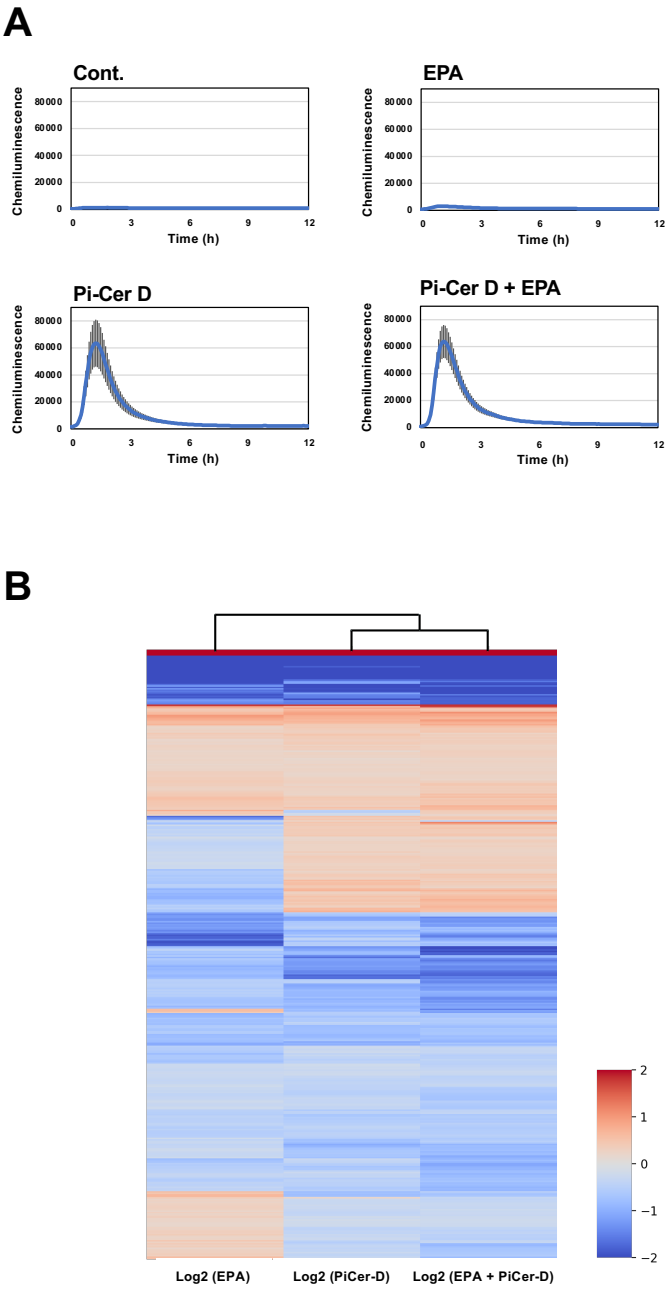

Figure. 6 Distinctive sets of Arabidopsis genes were upregulated by the treatment with EPA and Pi-Cer D. **(A)** Arabidopsis transformant pWRKY33-LUC containing *LUC* transgene under the control of *AtWRKY33* (AT2G38470.1) promoter was treated with 20 µg/ml EPA, Pi-Cer D or a mixture of 20 µg/ml EPA and Pi-Cer D. Chemiluminescence was monitored for 12 h after the treatment. (B) Expression profile of Arabidopsis genes treated with 20 µg/ml EPA, Pi-Cer D or a mixture of 20 µg/ml EPA and Pi-Cer D. RNAseq analysis was performed 24 h after the treatment.