1 **Title of the article:**

2 Two structurally different oomycete MAMPs induce distinctive plant immune responses

3
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26

27 **Abstract**

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

50
 51 **Keywords:** Ceramide, EPA, MAMPs, Oomycete, Phytoalexins, *Phytophthora infestans*,
 52 ROS.

53

54 **Introduction**

55 During the course of their evolution, plants were always faced with the challenges of
 56 environmental microorganisms but have survived by developing physical barriers or
 57 inducible resistance strategies against pathogens. The first step of inducible plant
 58 defense against potential pathogens is the recognition of molecular patterns of microbes,
 59 referred to as MAMPs or PAMPs (microbe- or pathogen-associated molecular patterns).
 60 Plant cells recognize molecular patterns unique to a certain group of microbes as non-
 61 self molecules via pattern recognition receptors (PRRs) (Ranf, 2017). In Arabidopsis,
 62 for instance, chitin, a major component of the fungal cell wall, is recognized by a PRR
 63 complex containing LysM-RLK and co-receptor CERK1, while a conserved sequence
 64 of bacterial flagellin (flg22) and an elongation factor EF-Tu (elf18) induce plant defense
 65 via PRRs, FLS2 and EFR, respectively, in conjuncture with co-receptor BAK1 (Ranf,
 66 2017). While a variety of MAMPs of bacterial and fungal origin, as well as the
 67 mechanisms for their recognition in plants have been studied, reports for MAMPs of
 68 oomycete pathogens are relatively limited.

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

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

86 Of the various oomycete genera, *Phytophthora*, *Pythium* and *Peronospora*
87 */Hyaloperonospora* (downy mildew) are particularly noteworthy, as they include many
88 species that are problematic plant pathogens (Kamoun et al., 2015). These plant
89 pathogenic oomycete genera are predicted to have evolved from a common
90 phytopathogenic ancestor species (Thines and Kamoun, 2010). Undoubtedly the most
91 infamous representative of pathogenic oomycetes is *Phytophthora infestans*, the causal
92 agent of potato late blight, which is responsible for the Great Famine in Ireland of the
93 1840s. This pathogen is an ongoing problem even today, and the total costs for control
94 efforts and yield losses by *P. infestans* are estimated in the range of multi-billion dollars
95 annually (Garelik, 2002; Fry, 2008). Problems caused by *P. infestans* are particularly
96 serious in developing countries where fungicides are used as the primary solution for
97 disease management. Although potato cultivars with introduced resistance (*R*) gene(s)
98 have been employed, due to selection pressure on these effectors in the pathogen

99 population, *R*-gene dependent resistances have been lost in many varieties (Forbes,
100 2012).

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

111

112 **RESULTS AND DISCUSSION**

113 **Purification of *Phytophthora infestans* elicitors which induce ROS production of** 114 **potato suspension-cultured cells**

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

123 fractions were selected using their ROS inducing activity in cultured potato cells
 124 (Supplemental Figure S1). Only fractions with clear elicitor activity were used for
 125 further purification processes. Six fractions with significant ROS-producing activity
 126 were further purified (Supplemental Figure S1) and the chemical structures of these
 127 elicitors were analyzed by NMR spectroscopy (Supplemental Figures S2, 3, 5, 6, 7 and
 128 9). To compare the structural difference between elicitors and inactive related
 129 substances, two fractions with no significant elicitor activity were also collected, and
 130 the structures of substances in these fractions were analyzed (Supplemental Figures S1,
 131 4 and 8). Further structural analyses (Supplemental Figures S10-16 and Supplemental
 132 document) showed that these eight substances could be divided into two groups,
 133 ceramide (Cer) and ceramide-phosphoethanolamine (CerPE). We designated the four
 134 ceramide compounds Pi-Cer A-D (*P. infestans* Ceramide) and the remaining four
 135 compounds Pi-CerPE A-D respectively. Except for the additional phosphoethanolamine
 136 in Pi-CerPE, compounds that share the same alphabetical suffix are otherwise
 137 structurally identical (Figure 2 and Supplemental Figure S17A). Pi-Cer A, B and D
 138 were able to induce ROS production, whereas Pi-Cer C had only a marginal ROS
 139 inducing activity (Figure 2 and Supplemental Figure S1). Similarly, Pi-CerPE A, B and
 140 D were active, whereas Pi-CerPE C was inactive (Supplemental Figures S1 and S17).
 141 For both Pi-Cers and Pi-CerPEs, D is the most active, followed by B and A in that order
 142 (Figure 2 and Supplemental Figure S17).

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

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

153 The most evident structural difference between active Pi-Cers and Pi-Cer C is the
154 8*E*,10*E*-double bonds and methyl-branching at C-9 in the sphingoid base, which are
155 found in Pi-Cer A, B and D, but not in Pi-Cer C (Figure 2). Kato et al. (2021) showed
156 that that 9-methyl branching and 4*E*-double bond structure of sphingoid base are
157 important for its elicitor activity in Arabidopsis. Previously, cerebroside were identified
158 as elicitor molecules from rice blast pathogen, *Pyricularia oryzae* (Koga et al., 1998).
159 Cerebroside elicitors isolated from *P. oryzae* also contain 9-methyl-branching and a 4*E*-
160 double bond in the sphingoid base. Given that 9-methyl-branching in the sphingoid base
161 is essentially not found in plants (Sperling and Heinz, 2003), this structure appears to be
162 a molecular pattern that can be leveraged by the plant to recognize potentially
163 pathogenic microorganisms. By mutagenesis of the Arabidopsis pWRKY33-LUC
164 marker line and screening of mutants insensitive to Pi-Cer D, Kato et al. (2021)
165 revealed a lectin receptor kinase is required for the recognition of Pi-Cer D (9-methyl
166 sphingoid base) in Arabidopsis.

167

168 **Pi-Cer D and Pi-CerPE D are elicitors of ROS production in dicot and monocot** 169 **plants**

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

183

184 **Treatment of Pi-Cer D and Pi-CerPE D enhances the resistance of potato against *P.*** 185 ***infestans***

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

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

201

202 **Isolation of Pi-DAGs as elicitors for the induction of phytoalexins production in** 203 **potato tuber**

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

214 in potato tubers. Substantial elicitor activity was only observed in the first fraction
 215 (RM-I-130-1) and it was therefore further purified (Supplemental Figure S21). Among
 216 the five fractions obtained from the following flash column chromatography, the third
 217 (ST-I-8-3) and fourth (ST-I-8-4) fraction showed elicitor activity that resulted in
 218 phytoalexin production. These fractions were later found to contain mainly 1,3-DAG
 219 (diacylglycerol) and 1,2-DAG, respectively. Both ST-I-8-3 and -4 were further
 220 separated by HPLC using an ODS column and at least 5 out of 9 resultant fractions
 221 showed elicitor activity (Supplemental Figure S21). These results indicated that several
 222 1,3- and 1,2-DAGs derived from *P. infestans* are elicitors that can induce the
 223 phytoalexin production of potato tubers. Two fractions with relatively higher elicitor
 224 activity, yield and purity, ST-I-14-4 and ST-I-23-4, were selected for further structural
 225 analyses. Two additional fractions, which showed almost no elicitor activity (ST-I-14-8
 226 and ST-I-23-8), were also subjected for structural analyses in order to narrow down the
 227 molecular patterns that may be critical for the elicitor activity. Further structural
 228 analyses (Supplemental Figures S22-25) revealed that active substances from ST-I-14-4
 229 and ST-I-23-4 were 1,2- and 1,3-DAG which both contained an eicosapentaenoic acid
 230 (EPA) and linoleic acid fatty acid chain each, and were designated as Pi-DAG A and B,
 231 respectively (Figure 5 and Supplemental Figure S21). The inactive substances in ST-I-
 232 14-8 and ST-I-23-8 were found to be 1,2- and 1,3-DAG containing palmitic acid and
 233 linoleic acid as fatty acid chains, and were designated as Pi-DAG C and D, respectively.
 234 Pi-DAG A and B, as well as Pi-DAG C and D, are also contained in *Py.*
 235 *aphanidermatum*, suggesting that Pi-DAGs are molecules commonly found in oomycete
 236 plant pathogens (Supplemental Figure S26).

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

247

248 **RNAseq analysis of Arabidopsis treated with EPA, Pi-Cer D or a mixture of EPA** 249 **and Pi-Cer D**

250 To compare the elicitor activity of EPA and Pi-Cer D in Arabidopsis, an Arabidopsis
 251 transformant containing a *LUC* transgene under the control of *WRKY33* (AT2G38470)
 252 promoter (pWRKY33-LUC) (Kato et al., 2021) was treated with EPA and Pi-Cer D.
 253 While Pi-Cer D can induce the activation of the *WRKY33* promoter, only marginal
 254 induction of *WRKY33* promoter was detected by the treatment with EPA (Figure 6A).
 255 RNAseq analysis was performed for Arabidopsis seedlings 24 h after the treatment with
 256 20 µg/ml EPA, Pi-Cer D or a mixture of 20 µg/ml EPA and 20 µg/ml Pi-Cer D.
 257 Clustering analysis of significantly up- or down-regulated genes revealed that treatment
 258 with EPA or Pi-Cer D leads to distinct gene expression patterns. While some genes
 259 respond similarly to either treatment, others genes are induced exclusively by only one

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

271

272 **MATERIALS AND METHODS**

273 **Biological materials, growth conditions and inoculation**

274 Potato suspension-cultured cells were prepared from calluses induced from potato
275 tuber discs (cv. Sayaka carrying the *R1* and *R3* genes) in callus-inducing medium [1 ml/l
276 B5-vitamin (100 g/l Myo-inositol, 2.5 g/l glycine, 500 mg/l thiamine-HCl, 500 mg/l
277 pyridoxine-HCl, 5 g/l nicotinic acid, 50 mg/l biotin, 50 mg/l folic acid), 2 mg/l NAA,
278 0.5 mg/l kinetin, 3% sucrose, 0.2% phytigel and Murashige-Skoog basal medium],
279 under dark condition. Suspension-cultured potato cells were grown by agitation at 130
280 rpm, 23°C in 95 ml of MS medium supplemented with 30 mg/ml sucrose, 1 mg/l
281 thiamine, 100 mg/l myo-inositol, 200 mg/l KH_2PO_4 , and 0.2 mg/l 2,4-
282 dichlorophenoxyacetic acid. Cells were sub-cultured every week and used for

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

294

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

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

suspension was finely grounded using a polytron type homogenizer (HG30, Hitachi Koki, Japan) for 2 min. After centrifugation at 4°C, 3000 × g for 30 min, the supernatant was collected and dried using an evaporator and used as Pi-MEM elicitor. Further procedures for the purification of Pi-Cer and Pi-DAG from Pi-MEM are described in the supplemental document. Mycelia of *Pythium aphanidermatum*, *Botrytis cinerea*, *Fusarium oxysporum* f. sp. *melonis* and *Colletotrichum orbiculare* were grown in 50 ml 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 CaCl₂ and 0.5 mM K₂SO₄, 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,

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

337

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

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

344

345 **Detection of potato phytoalexins by liquid chromatography/mass spectrometry** 346 **(LC/MS)**

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

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

356

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

358 Seeds of Arabidopsis containing *Luciferase* marker gene under the control of
359 *AtWRKY33* (AT2G38470) promoter (Kato et al., 2021) were surface sterilized with 3%
360 hydrogen peroxide and 50 % ethanol for 1 min with gentle shaking, washed with
361 sterilized water, and then individual sterilized seeds were placed in separate wells of a
362 96 microwell plate containing 150 µl of Murashige and Skoog (MS) liquid medium (1/2
363 MS salts, 0.05% [w/v] MES, 0.5% [w/v] Sucrose, adjusted to pH 5.8 with NaOH) with
364 50 µM D-Luciferin potassium salt (Biosynth Carbosynth, Compton, UK), covered with
365 a clear plastic cover. Plates containing Arabidopsis seeds were then incubated in a
366 growth chamber for approx. 12 days at 23°C with 24 h light. After the treatment of
367 elicitors, chemiluminescence intensity derived from expressed luciferase was measured
368 using Mithras LB940 (Berthold) for 12 h. Total RNA was extracted from 6
369 seedlings/sample of 10-days old Arabidopsis 24 h after the treatment with elicitors using
370 the RNeasy Plant Mini Kit (QIAGEN, Hilden, Germany). Libraries were constructed
371 using KAPA mRNA Capture Kit (Roche, Basel, Switzerland) and MGIEasy RNA
372 Directional Library Prep Set (MGI, Shenzhen, China), and sequenced on DNBSEQ-
373 G400RS (MGI) with 150 bp paired-end protocol. The RNA-seq reads were filtered using
374 trim-galore v.0.6.6 (Martin, 2011, bioinformatics.babraham.ac.uk) and mapped to the

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

381

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394

395 **SUPPLEMENTAL DATA**

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

- 398 **Supplemental Figure S2.** NMR spectra of Pi-Cer A (CDCl₃, 400 MHz for ¹H, 100
399 MHz for ¹³C).
- 400 **Supplemental Figure S3.** NMR spectra of Pi-Cer B (CDCl₃, 600 MHz for ¹H, 150
401 MHz for ¹³C).
- 402 **Supplemental Figure S4.** NMR spectra of Pi-Cer C (CDCl₃, 400 MHz for ¹H, 100
403 MHz for ¹³C).
- 404 **Supplemental Figure S5.** NMR spectra of Pi-Cer D (CDCl₃, 600 MHz for ¹H, 150
405 MHz for ¹³C).
- 406 **Supplemental Figure S6.** NMR spectra of Pi-CerPE A (CDCl₃-CD₃OD 4:1, 400 MHz
407 for ¹H, 100 MHz for ¹³C).
- 408 **Supplemental Figure S7.** NMR spectra of Pi-CerPE B (CDCl₃-CD₃OD 4:1, 600 MHz
409 for ¹H, 150 MHz for ¹³C).
- 410 **Supplemental Figure S8.** NMR spectra of Pi-CerPE D (CDCl₃-CD₃OD 4:1, 600 MHz
411 for ¹H, 150 MHz for ¹³C).
- 412 **Supplemental Figure S9.** NMR spectra of Pi-CerPE D (CDCl₃-CD₃OD 4:1, 600 MHz
413 for ¹H, 150 MHz for ¹³C).
- 414 **Supplemental Figure S10.** MS/MS fragmentation of Pi-Cer A (precursor ion: [M +
415 Na]⁺).
- 416 **Supplemental Figure S11.** Two-dimensional NMR correlations of Pi-Cer B (thick
417 bonds: DQF-COSY, curved arrows: HMBC, l + m = 15).
- 418 **Supplemental Figure S12.** MS/MS fragmentation of Pi-Cer B (precursor ion: [M +
419 Na]⁺).
- 420 **Supplemental Figure S13.** Two-dimensional NMR correlations of Pi-Cer B (thick
421 bonds: DQF-COSY, curved arrows: HMBC, l + m + n = 19).
- 422 **Supplemental Figure S14.** DQF-COSY (thick bonds), HMBC (curved arrows), and
423 NOESY (dashed curves) correlations of Pi-Cer D.
- 424 **Supplemental Figure S15.** Linked-scan FAB MS/MS (negative ion mode) of the fatty
425 acid derived from Pi-Cer D by acid hydrolysis.
- 426 **Supplemental Figure S16.** DQF-COSY (thick bonds) and HMBC (curved arrows)
427 correlations of Pi-CerPE D.

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

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

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

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

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

439 **Supplemental Figure S22.** ESI-TOF MS of Pi-DAG A. NMR spectra of Pi-DAG A
440 (CDCl₃, 400 MHz for ¹H and 100 MHz for ¹³C). Two-dimensional NMR of Pi-DAG A
441 (CDCl₃, 400 MHz). Determination of fatty acids of Pi-DAG A by MS/MS.

442 **Supplemental Figure S23.** ¹H NMR spectrum of Pi-DAG B (CDCl₃, 400 MHz).
443 Determination of fatty acids in Pi-DAG B by negative ion FAB MS

444 **Supplemental Figure S24.** NMR spectra of Pi-DAG C (CDCl₃, 400 MHz for ¹H, 100
445 MHz for ¹³C). Determination of fatty acids in Pi-DAG C by positive ion ESI MS/MS.
446 Confirmation of fatty acid linkage in Pi-DAG C.

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

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

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

453

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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* prenaspirodiene synthase; HPO *H. muticus* prenaspirodiene 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 μ g/ml methanol extract of *P. infestans* mycelia (PiMEM), 3 μ g/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.

A Potato

B Arabidopsis

C Rice

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 μ g/ml methanol extract of *P. infestans* mycelia (Pi-MEM), 10 μ g/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 $\pm$ SE (n = 3).

(B) (Left) *Arabidopsis thaliana* leaves were treated with 1% DMSO (Cont.), 100 μ g/ml methanol extract of *P. infestans* (Pi-MEM), 10 μ g/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 $\pm$ SE (n = 3).

(C) (Left) Rice leaves were treated with 1% DMSO (Cont.), 100 μ g/ml methanol extract of *P. infestans* (Pi-MEM), 10 μ g/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 $\pm$ 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.

A **B**

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 $\mu\text{g/ml}$ EPA, Pi-Cer D or a mixture of 20 $\mu\text{g/ml}$ EPA and Pi-Cer D. Chemiluminescence was monitored for 12 h after the treatment. **(B)** Expression profile of Arabidopsis genes treated with 20 $\mu\text{g/ml}$ EPA, Pi-Cer D or a mixture of 20 $\mu\text{g/ml}$ EPA and Pi-Cer D. RNAseq analysis was performed 24 h after the treatment.