Diacylglycerol kinases activate tobacco NADPH oxidase-dependent oxidative burst in response to cryptogein

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4 <u>Running head:</u> Control of RBOHD activity by DGK

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27   Summary statement

28   Cryptogein is a protein secreted by the oomycete *Phytophthora cryptogea* that activates

29   defence mechanisms in tobacco. We show here in BY-2 tobacco suspension cells that

30   phosphatidic acid rapidly accumulates in response to cryptogein due to the coordinated onset

31   of phosphoinositide-dependent phospholipase C and diacylglycerol kinase activities. Both

32   enzyme specific inhibitors and silencing of the phylogenetic cluster III of the tobacco DGK

33   family were found to reduce PA production upon elicitation and to strongly decrease the

34   RBOHD-mediated oxidative burst. This establishes that phosphatidic acid production by

35   diacylglycerol kinases is upstream of the oxidative burst in response to cryptogein.

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37

38 **ABSTRACT**

39 Cryptogein is a 10 kDa-protein secreted by the oomycete *Phytophthora cryptogea* that

40 activates defence mechanisms in tobacco plants. Among early signalling events triggered by

41 this microbial-associated molecular pattern is a transient apoplastic oxidative burst which is

42 dependent on the NADPH oxidase activity of the RESPIRATORY BURST OXIDASE

43 HOMOLOG (RBOH) isoform D. Using radioactive [^{33}P]-orthophosphate labelling of tobacco

44 Bright Yellow-2 suspension cells, we here provide *in vivo* evidence for a rapid accumulation

45 of phosphatidic acid (PA) in response to cryptogein due to the coordinated onset of

46 phosphoinositide-dependent phospholipase C and diacylglycerol kinase (DGK) activities.

47 Both enzyme specific inhibitors and silencing of the phylogenetic cluster III of the tobacco

48 DGK family were found to reduce PA production upon elicitation and to strongly decrease the

49 RBOHD-mediated oxidative burst. Therefore, it appears that PA originating from DGK

50 controls NADPH-oxidase activity. Amongst cluster III DGKs, the expression of *DGK5-like*

51 was up-regulated in response to cryptogein. Besides *DGK5-like* is likely to be the main cluster

52 III DGK isoform silenced in one of our mutant line, making it a strong candidate for the

53 observed response to cryptogein. The relevance of these results is discussed with regard to

54 early signalling lipid-mediated events in plant immunity.

55

56 **KEYWORDS:** cryptogein, microbial-associated molecular pattern, diacylglycerol kinase;

57 phospholipase C, phosphatidic acid, reactive oxygen species, RBOHD, NADPH oxidase

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INTRODUCTION

Many studies have suggested that membrane lipids act as reservoirs for signalling molecules in response to developmental and environmental cues in both animal and plant cells. Indeed, under biotic or abiotic stresses, a regulated production of phospholipid-derived molecules was reported to occur, and to launch intracellular signalling cascades. Phospholipases, lipid kinases and lipid-phosphate phosphatases are considered as the main activities involved in such early signalling events (Janda et al. 2013, Sheng et al. 2015, Hawkins & Stephens 2015, Ruelland et al. 2015).

Phosphatidic acid (PA) is the most basic phosphoglycerolipids, being composed of a diacylglycerol with a phosphoryl group esterified on the s*n-3* hydroxyl of the glycerol backbone. Besides being an intermediate of phospholipid synthesis (Petroutsos et al. 2014), PA also transduces stress signals in eukaryotic cells. In serum-withdrawn fibroblast cultures for example, reverse genetic approaches have established a marked correlation between elevated endogenous PA levels and apoptosis inhibition; the latter probably being partially responsible for cancer establishment in tissues (Bruntz et al. 2014). In plants, upon pathogen attack and situations mimicking infection by the addition of elicitors, as well as under many abiotic (including drought, cold, osmotic) stresses, PA accumulation was either inferred from pharmacological strategies or directly shown by biochemical methods (Pinosa et al. 2013, Ruelland et al. 2015). In Eukaryotic cells, the synthesis of PA as a signalling molecule relies on two distinct enzymatic systems. Phospholipases D (PLD) can hydrolyse structural phospholipids within membranes, directly releasing PA (Wang et al. 2012) while, diacylglycerol kinases (DGK) can catalyse the phosphorylation of diacylglycerol (DAG) to produce PA (Arisz et al. 2009). DAG can be provided to DGK either by phosphoinositide-dependent phospholipases C (PI-PLCs) that act on phosphorylated phosphoinositides or by non-specific phospholipases C (NPCs) that use structural phospholipids (Pokotylo et al. 2013,

85   2014). In contrast to animal systems where both DAG and PA have been shown to act as

86   secondary messengers, it is commonly assumed that PA plays the central role in plant cells.

87   Indeed, no plant proteins orthologous to mammalian protein kinases C (which are activated

88   through a direct interaction with DAG) have been identified from plant genomes (Dong et al.

89   2012), even though some proteins bearing the C1 domain responsible for DAG binding to

90   PKCs have been found (Janda et al. 2013). On the other hand, up to 35 plant proteins targeted

91   by PA have been identified on the basis of their direct interaction with the lipid (Hou et al.

92   2015). However, no consensus sequence to define a canonical PA binding domain was

93   identified from these proteins.

94   Upon pathogen attack, two lines of defence can successively occur. The first relies on the

95   recognition of common pathogen motifs, named *microbial-associated molecular patterns*

96   (MAMPs), at the plasma membrane and this can result in basal resistance to a broad range of

97   pathogens. The second depends on the specific perception of pathogen effectors within host

98   cells by resistance proteins (Dangl & Jones 2001, Jones & Dangl 2006, Stotz et al. 2014,

99   Bigeard et al. 2015). This latter defence response often leads to a rapid and highly localized

100   hypersensitive cell death that prevents biotrophic pathogens from propagating (Cacas 2015).

101   Interestingly, PA has been shown to accumulate in response to many MAMPs (xylanase,

102   flagellin, N-acetylchitooligosaccharide, chitosan) in several independent plant models (van

103   der Luit et al. 2000, den Hartog 2003, Bargmann et al. 2006). PLD-, PI-PLC-, NPC- and

104   DGK-encoding genes and/or corresponding enzymatic activities were also reported to be

105   rapidly up-regulated in numerous studies dealing with either MAMP- or Effector-Triggered

106   Immunity (MTI or ETI, respectively), suggesting the involvement of these lipid-modifying

107   enzymes in early defence events (Zhang & Xiao 2015, Ruelland et al. 2015). With the notable

108   exception of DGK, this assumption was corroborated by several works using loss-of-function

109   mutants. While Arabidopsis PLDδ isoform was found to be involved in MTI, multiple PLD

110   isoforms were proposed to act redundantly in ETI (Pinosa et al. 2013, Johansson et al. 2014).

111   In tomato (*Solanum lycopersicum*) cell suspensions, MTI involved PLDß1 (Bargmann et al.

112   2006) whereas full establishment of ETI required the PI-PLC-encoding gene *slPLC4* (Vossen

113   et al. 2010).

114   The small, basic and hydrophilic 10-kDa protein named cryptogein is a MAMP secreted by

115   the oomycete *Phytophthora cryptogea* (Ricci et al. 1989). More than two decades after its

116   discovery, extensive investigations have provided a broad picture of cryptogein-induced

117   physiological responses. Although this protein exhibits both cell death-promoting and

118   systemic acquired resistance-inducing activities in tobacco (*Nicotiana tabacum*) plants (Keller

119   et al. 1996, Rustérucci et al. 1999, Cacas et al. 2005), it is also able to activate many

120   characteristic hallmarks of MTI. The interaction of cryptogein with a yet-to-be-identified

121   protein receptor at the plasma membrane results in calcium influx (Tavernier et al. 1995,

122   Pugin et al. 1997), potassium and chloride effluxes associated with apoplasm alkalization

123   (Blein et al. 1991), NADPH oxidase-dependent oxidative burst (Simon-Plas et al. 2002),

124   modification of plasma membrane order (Gerbeau-Pissot et al. 2014), activation of a MAPK

125   cascade (Dahan et al. 2009) and clathrin-dependent endocytosis (Leborgne-Castel et al. 2008).

126   Reactive oxygen species (ROS) are assumed to be the main downstream actors to PA in

127   pathogen-induced signalling cascades (Zhang & Xiao 2015). Although it was demonstrated

128   that PLD-produced PA directly regulates the NADPH oxidase-dependent oxidative burst

129   during a drought stress (Zhang *et al.* 2009), this link has not been clearly established with

130   respect to pathogen-response. Firstly, is PA production a common signature of MTI,

131   irrespective of the MAMP and the cell model? Secondly, can PA produced by DGK activities

132   act upstream of the NADPH oxidase during plant-pathogen interactions? In this present work,

133   we report on the regulatory function of DGK in controlling the cryptogein-induced oxidative

134   burst in Bright Yellow-2 (BY-2) cell suspensions. Using biochemical, pharmacological and

135   reverse genetic approaches, we show that PA, originating from a DGK coupled to PI-PLC, is

136   an upstream positive regulator of the plasma membrane-localized NADPH oxidase isoform

137   RBOHD in response to cryptogein.

138

MATERIALS & METHODS

Culture conditions and cryptogein elicitation of tobacco cells

Bright Yellow-2 (*Nicotiana tabacum*) cells were grown in modified liquid Murashige and Skoog (MS, M0221, Duchefa-Kalys, Bernin, France) medium at pH 5.6 (M0221, Duchefa-Kalys, Bernin, France) supplemented with $1\,mg.L^{-1}$ thiamine-HCl, $0.2\,mg.L^{-1}$ 2,4 dichlorophenylacetic acid, $100\,mg.L^{-1}$ myo-inositol, $30\,g.L^{-1}$ sucrose, $200\,mg.L^{-1}$ KH_2PO_4 and $2\,g.L^{-1}$ MES. Cells were maintained under continuous light ($200\,\mu E\,m^{-2}\,s^{-1}$) on a rotary shaker (140 rpm) and sub-cultured to fresh medium (4:80, (v/v)) on a weekly basis.

For radiolabeling experiments, ROS quantification and pharmacological treatments, cells were harvested from 7-day-old suspensions using a vacuum-system and resuspended at 0.1g/mL in either equilibration medium I2 (2 mM MES, 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, pH 5.9) or I20 (20 mM MES, 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, pH 5.9). After a 3 h incubation period under mild shaking at 24°C, cells were elicited by adding 50 nM cryptogein (final concentration) from a solution prepared according to (Ricci et al. 1989).

Radiolabelling experiments

BY-2 cells were aliquoted (7 mL) in wide neck flasks (capacity 50 mL) for equilibration. Cells were then labelled by the addition of 53 $MBq.L^{-1}$ [^{33}P]-orthophosphate. Total lipids were extracted according to the procedure previously described by (Krinke et al. 2009). Lipids were separated by thin layer chromatography (TLC) and developed either in an acidic solvent system composed of chloroform:acetone:acetic acid:methanol:water (10:4:2:2:1, (v/v), Lepage 1967) or in a solvent system composed of an upper phase of ethyl acetate:isooctane:acetic acid: water (12:2:3:10 (v/v), Munnik et al. 1995). Radiolabelled spots

162   were quantified by autoradiography using a Storm phosphorimager (Amersham Biosciences,

163   Buckinghamshire, UK). Separated phospholipids were identified by co-migration with

164   authentic non-labelled standards visualized by primuline staining (under UV light) or by

165   phosphate staining.

166   **Quantification of reactive oxygen species**

167   Reactive oxygen species were measured using a luminol-based method as described

168   previously (Simon-Plas et al. 2002) but with slight modifications. Briefly, after a 3 h-

169   equilibration period in I20 medium, cells were treated with cryptogein. Before elicitation,

170   samples corresponding to zero time were harvested and the luminescence background of the

171   assay was measured. Samples (250 µL) were regularly harvested over the first 90 min for

172   ROS determination using a luminometer (Lumat LB9507, Berthold Technologies, Bad

173   Wilbad, Germany). Fifty microliters of a 0.3 mM luminol solution and 300 µL of the assay

174   buffer (50 mM MES, 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, pH 6.5) were

175   automatically added to cell aliquots before luminescence was measured (delay in injection of

176   1.2 s, data acquisition time of 10 s). For each biological and technical replicates, 2 h after

177   elicitation (when the oxidative burst was over), a calibration curve was made in order to

178   convert relative luminescence units (RLU) into H_2O_2 equivalents by recording cell

179   luminescence upon the addition of increasing H_2O_2 concentrations during the assay. Results

180   were normalized to BY-2-cell weight and expressed as nmoles H_2O_2 equivalents per gram of

181   cells.

182   **Reverse transcription-quantitative polymerase chain reaction**

183   RNA extraction, reverse transcription and quantitative measurement of gene expression by Q-

184   PCR were carried out as described by (Anca et al. 2014). Two reference genes were used for

185   expression normalization: *ELONGATION FACTOR 1α* (EF-1α, AF120093), forward, 5'-

186 TGAGATGCACCACGAAGCTC-3', and reverse, 5'-CCAACATTGTCACCAGGAAGTG-

187 3'; and *L25* gene coding for a ribosomal subunit (L18908), forward, 5'-

188 CCCCTCACCACAGAGTCTGC-3', and reverse, 5'-

189 AAGGGTGTTGTTGTCCTCAATCTT-3'. Primer sequences for *NtDGK5-like* gene are as

190 follows: *NtDGK5-like* specific couple: forward (F85sq), 5'-

191 AGTCCGAGCTCAATGACAACA-3'; and reverse (R85sq2), 5'-

192 GTACGAAGAAGTTCTCCTCCAAGTT-3'; Cluster III gene-targeting couple: forward

193 (ClusterIII-F), 5'-TTCAGCATGGGGATGGATGCA-3'; and reverse (ClusterIII-R), 5'-

194 AACCATCCTTGYGTACATCC-3'

Cloning of *NtDGK5*-like coding sequence

196 *NtDGK5-like* coding sequence was amplified by PCR from cDNA obtained from untreated 7-

197 day-old BY-2 cells using high fidelity DNA polymerase (Phire Hot Start, ThermoFisher

198 SCIENTIFIC) and specific primers: forward (F1c85), 5'-ATGGCGAATTCTGAGTCCGA-

199 3'; and reverse (R1c85), 5'-CTAACTGAGACGAGAAACATCGA-3'. Primers were

200 designed according to the sequence of SGN-U439985 (Solanacea Genomic Network

201 database). Resulting PCR products were cloned into pSC-B Amp/Kan vector using the

202 topoisomerase-based StrataClone Blunt PCR cloning kit (Agilent). Seven clones were

203 sequenced among which one was selected for further work. The sequence was deposited in

204 GenBank (accession number KU934207). This cDNA was named *NtDGK5-like* because its

205 closest *Arabidopsis thaliana* ortholog is AtDGK5.

Production of overexpressing *NtDGK5-like* transgenic lines

207 The *NtDGK5-like* coding sequence was first amplified by PCR using high fidelity DNA

208 polymerase from *pSC-B::DGK5-like* using the following primer pairs: couple 1, forward

209 (CACC-F85), 5'-CACCATGGCGAATTCTGAGTCCGA-3'; and the reverse R1c85; couple

210 2:   the   forward   (CACC-F85)   and   reverse   (R85-c-myc),   5'-

211 CTAGAGGTCTTCTTCGCTGATGAGCTTTTGTTCACTGAGACGAGAAACATCGAC-

212 3'. Two independent intermediate constructs to express a C-terminally c-myc tagged protein

213 and an untagged version were generated by cloning the PCR products into pENTR/D-TOPO

214 (ThermoFisher SCIENTIFIC). Sequencing of both inserts revealed no mutations. Using

215 Gateway LR Clonase II enzyme mix (ThermoFisher SCIENTIFIC), MluI-digested

216 *pENTR/D::DGK5-like-c-myc* and MluI-digested *pENTR/D::DGK5-like* were then

217 independently recombined with pMDC32 (Karimi et al. 2007) to generate plant-expressing

218 vectors that were checked for correct recombination by restriction digestions before being

219 used to transform WT BY-2 cell suspensions following a previously described procedure

220 (Simon-Plas *et al.* 2002). Among the 6 hygromycin-resistant calli isolated, only one

221 overexpressed the untagged version of *Nt*DGK5-like (named *line OE.NtDGK5-A*) while five

222 out of twenty hygromycin-resistant calli overexpressed the C-terminally tagged version of

223 *Nt*DGK5-like. The two lines, named *OE.NtDGK5myc-A* and *OE.NtDGK5myc-B*, which

224 exhibited the strongest levels of transgene expression, were selected for further

225 characterization.

Generation of cluster III DGK-silenced cell lines

227 A conserved region of cluster III DGKs of the *NtDGK5-like* coding sequence, from

228 nucleotides 511 to 692 (see supporting information Fig. S2) was chosen to be inserted into the

229 post-transcriptional gene silencing vector (pH7GWIWG2(II), Karimi et al. 2007). This

230 fragment was PCR-amplified using high fidelity DNA polymerase and the following specific

231 primers: forward (Fc-ClusterIII), 5'-<u>CACC</u>TCATTTTTGAAGCAAGTAATGAATGCA-3',

232 and reverse (Rc-ClusterIII), 5'-TGGAAACCTTCCACATTCAGTTCA-3'. The resulting PCR

233 product was directionally cloned into pENTR/D-TOPO and the *pENTR::DGK5-like* fragment

234 was sequenced before the plasmid was recombined with pH7GWIWG2(II). The final

235   construct was verified by restriction digestion and the fragment-intron loop-fragment was also

236   sequenced. To check that the construct was functional, a transient tobacco expression system

237   was designed (explained hereafter). Wild-type (WT) BY-2 cells were transformed as

238   previously described (Simon-Plas et al. 2002). Among the 30 hygromycin-resistant calli

239   isolated, 5 were screened and 2 independent transgenic lines (named *hp-clusterIII-A* and *hp-*

240   *clusterIII-B* lines) silenced for *NtDGK5-like* were identified and selected for further

241   characterization.

242 Transient expression system in tobacco plants

243   To test for functional miRNA constructs, *Agrobacterium tumefaciens* (GV3101) were

244   transformed with the Cluster III-targeting vector and selected clones were grown overnight

245   under mild shaking at 28°C in regular Luria-Bertani medium containing 50 $\mu g.mL^{-1}$

246   rifampicin, 50 $\mu g.mL^{-1}$ gentamycin and 50 $\mu g.mL^{-1}$ spectinomycin. Bacteria were collected

247   the following day by centrifugation and the pellets were resuspended in induction buffer (20

248   mM MES pH 5.5, 10 mM MgSO4, 200 μM acetosyringone) so that the $OD_{600\ nm}$ was 0.5-0.6.

249   After incubation at room temperature for 3 h, the bacterial suspension was infiltrated into the

250   abaxial side of detached tobacco leaves (*Nicotiana tabaccum*, 4 to 6 week-old plants) using a

251   needleless syringe. Samples for RT-PCR were harvested 2 days-post inoculations. Each

252   experiment included non-infiltrated and infiltrated leaf material with bacterial clones

253   containing the empty vector as controls. Stem-loop reverse transcription reactions to show

254   specific pri-miRNA was performed on 2 independent biological replicates according to (Chen

255   2005) using the following primer: 5'-

256   GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGAC<u>tggaaac</u>-3'; the

257   underscored sequence corresponding to the gene specific part of the primer whereas the other

258   part corresponds to the stem-loop backbone of the microRNA *miR172* (Martin et al. 2009).

259   Stem-loop RT controls were carried out using a 5S-rRNA-specific oligonucleotide 5'-

260   GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACgcaaca-3'.   The

261   generated cDNA matrix was amplified by regular PCR with a gene specific-forward primer

262   and a *miR172* reverse primer (5'-GGGTCGACATCAAGTCATCAATTTGCCA-3'). For the

263   5S-rRNA   template,   the   forward   primer   was   as   follows: 5S-rRNA-F,   5'-

264   GGATGCGATCATACCAGCACT-3'; for the Cluster III-targeting miRNA, the primer used

265   for generating the construct (Fc-ClusterIII, described above) was used. PCR products were

266   then sequenced and proved to correspond to Cluster III pri-miRNA.

267   **Western blot analysis**

268   Seven-day old cells from the *OE.NtDGK5myc-A* and *OE.NtDGK5myc-B* lines and the

269   corresponding empty vector line were harvested by filtration and protein extracts (cytosol,

270   microsomes and plasma membrane) were prepared as described by (Mongrand et al. 2004).

271   Proteins were solubilized in a buffer containing 40 mM Tris-HCl pH 6.8, 5% β-

272   mercaptoethanol, 1.5% SDS, 1 mM EDTA, 2M urea, 1M thiourea, 1% n-octyl-glucoside,

273   10% glycerol and bromophenol blue for 2h at room temperature, separated by SDS-PAGE

274   (8% (w/v) acrylamide-bisacrylamide) and transferred onto nitrocellulose membrane (Hybond

275   ECL; GE Healthcare, Chalfont St Giles, UK). The membrane was blocked overnight in a 5%

276   (w/v) milk solution and probed with an anti-c-myc antibody directly coupled to horseradish

277   peroxidase (GenScript, Piscataway, New Jersey, USA). Specific signals were revealed using

278   an ECL kit (Perkin Elmer, Waltham, MA, USA) according to the manufacturer's instructions.

279   **Assessment of cell suspension density**

280   The packed cell volume (PCV) and dry weight (DW) were used as proxies of cell density.

281   PCV was determined upon centrifugation of 15mL cell suspension samples (700 x g, 3 min).

282   It represents the ratio of pelleted cell volume to the total sample volume, expressed in percent

283   (Cacas et al. 2005). Dry weight was measured upon drying 12mL cell suspension aliquots at

284   55°C for 3 days; a condition that allows the culture medium to fully evaporate. It is expressed

285   in percent with reference to WT BY-2 conditions set to 100.

286   **Phylogenetic analysis**

287   Sequence alignment and phylogenetic tree building were carried out as described previously

288   (Cacas et al. 2011).

289

290   **RESULTS**

291   **The MAMP cryptogein triggers rapid accumulation of phosphatidic acid in tobacco BY-**

292   **2 cell suspensions**

293   The potential production of PA following elicitation of BY-2 tobacco cell suspensions with

294   cryptogein was investigated by treating cells with the peptide and extracting lipids for up to

295   one hour after elicitor addition. Cells were incubated with [^{33}Pi]-orthophosphate for 20 min

296   before lipid extraction and [^{33}P]-labelled PA levels were measured after lipid separation by

297   thin layer chromatography (TLC). Marked changes in labelled PA levels were observed after

298   the addition of cryptogein (Fig. 1a) and quantification of the signal showed that after a 3 min

299   lag, radiolabelled PA increased and reached a maximum within the first 10 min and then

300   plateaued (Fig. 1b).

301   **PLC and DGK inhibitors reduce the accumulation of phosphatidic acid in response to**

302   **cryptogein**

303   Under our experimental conditions, i.e. short-time radioisotopic labelling, the main pool of

304   radiolabel is incorporated into ATP whereas structural phospholipids are only weakly

305   labelled. This experimental design is commonly used to detect *in vivo* activity of lipid kinases,

306   including DGKs, because these enzymes use the γ-phosphate group of ATP as their substrate.

307   Hence, it could be postulated from our data that, in response to cryptogein, a phospholipase C

308   activity produced DAG that was then processed by DGK into PA. These two hypotheses were

309   tested by a pharmacological approach relying on the use of edelfosine, an inhibitor of

310   phosphoinositide-dependent PLC (PI-PLC; Wong *et al.* 2007; Kelm *et al.* 2010; Djafi *et al.*

311   2013) and R59022, an inhibitor of DGK (Gómez-Merino et al. 2004). Wild-type cell

312   suspensions were preincubated with inhibitors and radioactivity incorporated into PA was

313   quantified 10 min after cryptogein addition, i.e. when PA levels are maximal. Edelfosine was

314   found to significantly alter cryptogein-induced PA accumulation in a dose-dependent manner,

315   leading to a maximum inhibition of radioactive PA production of around 70% (Fig. 2a).

316   Furthermore, 75 µM R59022 provoked a similar decrease of radioactive PA accumulation

317   (Fig. 2b).

318   It was shown in *Arabidopsis thaliana* that phosphoinositides, substrates of PI-PLCs, are

319   provided by type III-phosphatidylinositol-4 kinases (PI4K) that are sensitive to micromolar

320   concentrations of wortmannin (Delage et al. 2012, Djafi et al. 2013). Pre-treatment of BY-2

321   cells with 30 µM wortmannin resulted in a 46% inhibition of cryptogein-induced PA

322   accumulation whereas 1 µM of the inhibitor, a concentration that represses

323   phosphatidylinositol-3 kinase activity (Krinke et al. 2007) but not that of PI4K, did not exhibit

324   any effect (Fig. 2c). Furthermore, since PI-PLC activity is highly dependent on calcium (Hunt

325   et al. 2004, Pokotylo et al. 2014), lanthanides that can act as inhibitors of plasma membrane

326   calcium channels (Tester 1990, Knight et al. 1997) were tested for their ability to impact PA

327   production upon elicitation. As expected, increasing concentrations of La^{3+} led to decreasing

328   PA accumulation in response to cryptogein (Fig. 2d).

329   Taken together, these findings clearly point to a role for a PI-PLC/DGK pathway in

330   cryptogein-induced PA production in BY-2 tobacco cell cultures.

331   **PLC and DGK inhibitors diminish RBOHD-mediated oxidative burst intensity in**

332   **response to cryptogein**

333   Among early signalling events triggered by cryptogein in BY-2 cells is the RBOHD-mediated

334   oxidative burst which takes place within the first 10-15 min following elicitation and is,

335   therefore, concomitant to the onset of PA production. To examine the hypothesis that a PI-

336   PLC/DGK pathway could control the oxidative burst, a pharmacological approach was

337   undertaken. The exogenous application of the PI-PLC inhibitor edelfosine was found to

338   drastically alter the cryptogein-induced oxidative burst (Fig. 3a), resulting in a 70% decrease

339   of the total amount of ROS when compared to untreated WT cells (Fig. 3b). Similarly,

340   treatment with the DGK inhibitor R59022 also promoted a significant decrease in ROS levels

341   (Fig. 3c,d). Interestingly, neither PI-PLC nor DGK inhibitors led to the inhibition of

342   extracellular pH alkalization, another early signalling event associated with cryptogein

343   elicitation (Supporting information Fig. S3). As the results of both PA production and ROS

344   burst supported a working scenario where a PI-PLC/DGK pathway controls the cryptogein-

345   induced signalling cascade, a reverse genetic strategy was developed to identify DGK

346   candidates involved in the regulation of RBOHD.

347   **Phylogenetic clustering of the tobacco diacylglycerol kinase family**

348   Tobacco DGK nucleotide sequences were retrieved by both a keywords and a sequence

349   homology search with coding sequences of the seven identified *Arabidopsis thaliana* DGK

350   genes and those published for rice (*Oryza sativa*) and tomato (*Solanum lycopersicum*)

351   (Snedden & Blumwald 2000, Zhang et al. 2008) using the BLAST algorithm of the *Solanacea*

352   *Genomics Network* (SGN) server. No unigene could be identified, but 16 partial contigs were

353   isolated (Supporting information Fig. S4). Manual EST and contig assembly led to the

354   identification of two potential full-length mRNAs corresponding to the accessions SGN-

355   U440091 and SGN-U439985. A phylogenetic tree was built using the longest *in silico*

356   translated tobacco primary amino acid sequences, including SGN-U440091, SGN-U439985

357   and **7** additional partial coding sequences, in addition to Arabidopsis, rice and tomato

358   sequences (Fig. 4a).

359   DGKs are bipartite proteins composed of one catalytic domain and one accessory domain,

360   assumed to be necessary for the kinase activity. While the animal DGK family can be

361   subdivided into 5 classes (Goto & Kondo 2004), plant DGKs only form three phylogenetic

362 clusters characterized by distinct protein structures (Fig. 4a). Cluster I encompasses the

363 closest sequence orthologous of metazoan enzymes with proteins that display in their N-

364 terminus one or two DAG-binding domain(s) (noted C1) and either one or no transmembrane

365 helix (Vaultier et al. 2008). Contrary to Cluster II DGKs that have a minimal DGK structure,

366 cluster III DGKs can exhibit a C-terminal calmodulin binding region (referred to as CBD)

367 depending on splicing variants (Arisz et al. 2009). SGN-U440091 and SGN-U439985 appear

368 to belong to clusters II and III, respectively. Furthermore, amongst the 7 partial tobacco DGK

369 coding sequences that were retrieved, four are present in cluster I, one in cluster II and two in

370 cluster III.

371 **Overexpressing a member of the phylogenetic cluster III, *NtDGK5-like*, does not result**

372 **in a gain-of-function**

373 Among tobacco cluster III members, the full-length cDNA corresponding to the unigene

374 SGN-U439985 (hereafter referred to as *NtDGK5-like* after its closest *Arabidopsis thaliana*

375 ortholog) was cloned and deposited in GenBank (Accession number KU934207). The isolated

376 *NtDGK5-like* cDNA corresponds to a 1482 bp-long coding sequence that encodes a 493-

377 amino acid protein displaying a molecular weight of 55.24 kDa and a theoretical pI of 6.75,

378 with no predicted signal peptide. The *NtDGK5-like* gene is constitutively expressed in BY-2

379 cells but its expression is significantly up-regulated in response to cryptogein (Fig. 4b). Using

380 transgenic lines overexpressing a C-terminal c-myc-tagged NtDGK5-like protein (named

381 *OE.NtDGK5myc-A* and *OE.NtDGK5myc-B* lines), a cell fractioning approach was developed

382 to identify where the protein was located. Cytosolic, microsomal and plasma membrane

383 fractions were isolated and their proteins analysed by western blotting. The tagged protein

384 was found in both the endomembrane and plasma membrane fractions (Fig. 4c).

385 To study the role of *NtDGK5-like*, the two *OE.NtDGK5myc-A* and B lines were analysed

386 further along with a third line (named *OE.NtDGK5-A*) allowing the expression of an untagged

387 *Nt*DGK5-like protein under the control of the CaMV35S promoter. Despite a constitutive 10-

388 to-15-fold increase in steady-state levels of *NtDGK5-like* transcripts with respect to

389 endogenous gene expression (Fig. 5a), all three overexpressor lines accumulated PA levels

390 comparable to those of WT BY-2 and an empty vector-containing cell line in response to

391 cryptogein (Fig. 5b). Accordingly, no differences in ROS production could be seen between

392 overexpressing lines and WT or empty vector-control genotypes (Fig. 5c), with the notable

393 exception of the *OE.NtDGK5myc-B* line. This line displays a significant difference with the

394 WT line, but not with the empty vector control line. Because cryptogein-induced oxidative

395 burst intensity was assessed on 7-day-old cell suspensions in our experimental system, the

396 impact of overexpressing *NtDGK5-like* on cell density was estimated at this time-point.

397 Packed cell volume (PCV) and dry weight (DW) of overexpressing lines were measured as

398 two independent proxies of biomass and compared to those of WT cell culture. Only the

399 *OE.NtDGK5myc-B* line had a slight defect in cell size (Fig. 5d) and this could explain the

400 small but significant decrease in ROS levels produced upon elicitation.

401 **Silencing the phylogenetic cluster III DGK family of tobacco strongly inhibits**

402 **cryptogein-induced oxidative burst**

403 In an attempt to specifically silence *NtDGK5-like* gene expression, a miRNA transgenic cell

404 line strategy was developed but it was unsuccessful due to the early death of transformed

405 calli. However, to get insights into the function of cluster III DGKs, a construct targeting a

406 conserved DNA region of cluster III members was engineered. A 180 bp-long portion of the

407 *NtDGK5-like* coding sequence was cloned into the hairpin vector pH7GWIWIG2(II) (Fig. 6a).

408 Using an *Agrobacterium*-mediated transient expression system, this construct was shown to

409 produce DGK specific miRNA *in planta* (Fig. 6b) and it was therefore used to generate stable

410 transgenic cell lines in the WT BY-2 genetic background. Amongst the 30 transformed clones

411 obtained, two independent lines (named *hp-clusterIII-A* and *hp-clusterIII-B* lines) were

412 identified and selected on the basis of their reduced constitutive expression of *NtDGK5-like*.

413 While an empty vector-containing line displayed slightly higher levels of expression

414 compared to the WT cell line, miRNA cell lines exhibited similar lower steady state-levels of

415 *NtDGK5-like* transcripts, averaging 30 to 40% of that of WT cells (Fig. 6c). To test whether

416 the expression of other cluster III DGK genes were affected in the two transgenic lines, Q-

417 PCR was carried out using primers that allowed to simultaneously amplify the coding

418 sequences of each cluster III genes. In this way, it was found that the bulk expression of

419 cluster III genes was not altered in *hp-clusterIII-A* line whereas it was silenced approximately

420 3-fold in line *hp-clusterIII-B* line (Fig. 6c). A 3-fold down-regulation of *NtDGK5-like* is

421 propbably not enough to induce changes in the bulk expression of cluster III genes (see *hp-*

422 *clusterIII-A* cell line). This implies that that *NtDGK5-like* is not the only cluster III isoform to

423 be silenced in the *hp-clusterIII-B* line. On the contrary, this gene is likely to be the main

424 cluster III-DGK isoform to be silenced in the *hp-clusterIII-A* cell line *NtDGK5-like*.

425 The impact of silencing DGK cluster III on cell density was estimated. No evidence for

426 statistical differences between the four analysed genotypes (BY-2, empty vector line, *hp-*

427 *clusterIII-A* and *hp-clusterIII-B* lines) on PCV and DW at day 7 after sub-culturing was found

428 (Fig. 6d). Hence, potential effects of cluster III silencing on both PA and ROS production was

429 determined upon cryptogein elicitation. The *DGK*-silenced lines were shown to no longer

430 over-accumulate PA, 10 min after cryptogein addition (Fig. 7a). Remarkably, the absence of

431 early PA accumulation in these elicited transgenics could be correlated with a marked

432 decrease in oxidative burst intensity. Wild-type and empty-vector lines showed a similar

433 stereotyped behaviour characterized by a rapid and transient peak of ROS, being maximum

434 within 10-15 min post-treatment before decreasing and stabilizing for the last 45min, whereas

435   ROS profiles of the two *hp-clusterIII-* lines were devoid of a well-defined peak, exhibiting

436   rather weak and stable ROS levels over 90 min (Fig. 7b). Indeed, total levels of extracellular

437   ROS generated by the miRNA lines accounted for approximately one third to one half of

438   those recorded in WT and empty vector-containing lines upon cryptogein application (Fig.

439   7c).

DISCUSSION

In the present work, radio-labelling experiments clearly showed that *in vivo* levels of PA rapidly increased when BY-2 cell suspensions were challenged with cryptogein, reaching a maximum after 10 min and then staying stable during 1 h. Under our experimental conditions, [^{33}Pi]-orthophosphate was added to cell cultures 20 min before lipid extraction. In contrast to long time periods of isotope incubation that lead to labelled structural phospholipids, short time labelling results in the incorporation of isotopic phosphorus into ATP. Hence, this setup is optimized to detect products of lipid kinases using ATP as substrate (Vaultier et al. 2006) and being active within the duration of labelling. These kinase activities include DGK and others, such as PI4-kinase and PI4P-5-kinase. Indeed, the radioactivity incorporated into PI4P is typically 4- to 5-fold that of PI, in control cells, although PIP (not labelled) is much less abundant than PI (Furt et al. 2010). However, this experimental design is not optimal for detecting the possible occurrence of PLD activity. The radioactivity associated with PA upon cryptogein treatment represented 3- to 4-fold of that monitored in phosphatidylcholine, phosphatidylethanolamine and phosphatidylglycerol, putative substrates of PLDs. This strongly supports the idea that radiolabelled PA found after a 20 min labelling cannot arise from PLD action. Yet, this does not mean a PLD is not activated upon cryptogein addition. A long time labelling with [^{33}Pi]-orthophosphate (16 hours) designed to reveal PLD activity was also carried out. In the presence of primary alcohol *n*-butanol, PLD catalyses a trans-phosphatidylation reaction transferring the phosphatidyl moiety from structural lipids onto the hydroxyl group of the primary alcohol. In our hands, no radiolabeled phosphatidylbutanol accumulated in response to cryptogein (data not shown). However, since the signal-to-noise ratio was low in these experiments, we cannot rule out the possible activation of PLD by cryptogein.

23

464   The fact that the increase in radiolabelled PA in our experimental design was due to DGK was

465   indicated by the pharmacological approach where DGK inhibitors led to an inhibition of

466   radiolabelled PA accumulation. Moreover, the inhibiting effects of wortmannin and

467   edelfosine strongly suggest that the DAG substrate of DGK was produced by a PI-PLC

468   activity, the substrate of which is provided by a type III PI4K. In *Arabidopsis thaliana*, it has

469   been shown that PI-PLC substrates are provided by such PI4Ks, either for basal PI-PLC

470   activity (i.e. active in control cell, Djafi et al. 2013) or during PI-PLC activation by chilling

471   (Delage et al. 2012). Therefore it seems that the role of type III PI4Ks to supply substrates to

472   PI-PLC is likely to be the case for other plant species, including tobacco. Besides, PI-PLCs

473   are strictly dependent on calcium and therefore it was not surprising that a blocker of

474   plasmalemma calcium channels, lanthanides, inhibited radioactive PA accumulation.

475   Since inhibitors of different chemical nature acting on different enzymes (PI4K, PI-PLC,

476   DGK) of a given pathway led to a similar inhibition of cryptogein-induced accumulation of

477   radiolabelled PA, it is unlikely that this is due to hypothetical side effects of each inhibitor

478   (Djafi et al. 2013, Ruelland et al. 2014). Moreover, silencing the phylogenetic cluster III of

479   tobacco DGKs resulted in the loss of the cryptogein-induced PA increase thus demonstrating

480   unambiguously the involvement of such enzymes in this response to cryptogein. Our

481   knowledge concerning plant DGKs is still limited. Prokaryotic DGKs exhibit a peculiar

482   multimeric membrane-embedded structure with three transmembrane spanning-regions per

483   subunit (Van Horn & Sanders 2012). Metazoan counterparts display a conserved bidomain

484   architecture associated with catalytic activity and decorated by a huge diversity of additional

485   functional domains that are responsible for membrane or protein interactions and subcellular

486   localization (Goto & Kondo 2004). Predictions of plant DGK structures using the Conserved

487   Domain Architecture Retrieval Tool (Geer et al. 2002) reveal a simpler organization that falls

488   into three evolutionary phyla (Vaultier et al. 2008). In this work, we focused our efforts on

489   DGKs of phylogenetic cluster III. Two members of this cluster have been either

490   biochemically or functionally characterized. The rice DGK1 was stably expressed in tobacco

491   plants and found to confer enhanced disease resistance to tobacco mosaic virus (TMV) and to

492   the oomycete *Phytophthora infestans* (Zhang et al. 2008). Two distinct spliced mRNA

493   variants were reported for the tomato LeDGK1/CBK1-encoded gene and this transcriptional

494   regulatory mechanism was demonstrated to regulate protein docking at the plasma membrane

495   (Snedden & Blumwald 2000) In addition, the *AtDGK5* gene expression was found to be

496   upregulated in response to flagellin 22, a bacterial MAMP, as well as several avirulent

497   pathogens (GENEVESTIGATOR database, Hruz et al. 2008).

498   In this work, one member of cluster III was cloned and named *NtDGK5-like* after its closest

499   Arabidopsis ortholog. Independent transgenic cell lines were generated that allowed

500   constitutive overexpression of either native *Nt*DGK5-like (*OE.NtDGK5-A*) or a C-terminally

501   c-myc tagged version of the protein (*OE.NtDGK5myc-A* and *OE.NtDGK5myc-B*). Despite the

502   absence of any predicted signal peptide and transmembrane domain, western-blotting

503   established that the protein was localized to the plasma membrane, as already reported for a

504   *LeDGK1/CBK1* variant from tomato (Snedden & Blumwald 2000). In all *NtDGK5-like*

505   overexpressing lines the accumulation of radioactive PA after 10 min of cryptogein addition

506   was similar to that of control and WT lines. Thus it is tempting to speculate that DGK is not a

507   limiting step in the coupled reaction leading to PA in response to cryptogein.

508   Previously, we had established that early cryptogein-induced ROS synthesis was fully

509   dependent on the isoform D of the RBOH family in BY-2 cell suspensions (Simon-Plas et al.

510   2002). In addition, Zhang et al. (2009) demonstrated that PA could directly interact with the

511   Arabidopsis RBOHD isoform *via* an N-terminally-localized PA-binding domain and,

512   subsequently, stimulate its activity. Alignment of the highly homologous tobacco RBOHD

513   isoform with its Arabidopsis counterpart pinpointed that the location and sequence of the PA-

514   binding domain, as well as the basic nature of the two amino acid residues involved in the

515   interaction with PA, are conserved between the two plant species (Supporting information Fig

516   S5). Both PI-PLC and DGK inhibitors reduced the RBOHD-dependent oxidative burst.

517   Furthermore, transgenic lines deficient for the tobacco cluster III DGKs displayed a

518   significantly reduced oxidative burst upon cryptogein addition. Such findings strongly point

519   to a direct regulation of RBOHD activity by PA, and place the PI-PLC/DGK pathway

520   upstream from the enzyme in a signalling cascade induced by the MAMP cryptogein. It can

521   be postulated that at least one DGK member of cluster III is responsible for the required DGK

522   activity. Even though our genetic strategy concerning *NtDGK5-like* was partly unsuccessful,

523   this gene still remains a good candidate since it was the only cluster III DGK gene suppressed

524   in the *hp-clusterIII-A* cell line.

525   Distinct regulatory mechanisms have been described for plant RBOH proteins. These include

526   post-translational modifications like phosphorylation (Kobayashi et al. 2007, Ogasawara et al.

527   2008) and S-nitrosylation (Yun et al. 2011), association with RacGTPase (Wong et al. 2007b,

528   Oda et al. 2010) as well as regulation by extracellular ATP level and calcium influx (Song et

529   al. 2006, Demidchik et al. 2009). Zhang *et al.* (2009) also elegantly demonstrated that direct

530   binding of PA activates AtRBOHD, *in fine* controlling stomatal aperture upon drought stress.

531   Contrary to our model, where a PI-PLC/DGK pathway is involved, the authors showed that

532   PLDα1 was the main source of PA under their experimental conditions. In the context of plant

533   defence, whether it is MTI or ETI, to date there is no genetic evidence for a role of DGKs in

534   the positive regulation of RBOH enzymes, even though it has been suggested previously by

535   several works (de Jong et al. 2004, Andersson et al. 2006, Vossen et al. 2010). Instead, many

536   reports indicate PLDs as the main suppliers of PA in response to MAMP/pathogen effectors,

537   irrespective of the downstream protein target (Young et al. 1996, van der Luit et al. 2000, de

538   Torres Zabela et al. 2002, Bargmann et al. 2006, Laxalt et al. 2007, Krinke et al. 2009,

539 Yamaguchi et al. 2009, Kalachova et al. 2013, Zhao et al. 2013, Pinosa et al. 2013). Thus, our

540 work sheds light on new insights into regulatory mechanisms that control early signalling

541 events associated with NADPH oxidase since PA produced by a PI-PLC/DGK pathway can

542 also control NADH oxidase activity. As underlined by (Zhang & Xiao 2015), this raises

543 interesting questions as to how the spatiotemporal regulation of PA neo-synthesis and the

544 engaged molecular species of PA could influence the specificity of downstream responses.

545

554 **AUTHOR'S CONTRIBUTION:** JLC, PGP, JF, ER, CC, DT, TK & KM carried out

555 experiments; FSP, SM, EJ, PGP, JLC & ER designed experiments and discussed results; JLC,

556 PGP & ER wrote the manuscript; SM & FSP coordinated the project and retrieved ANR

557 funds.

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798

799 **FIGURE LEGENDS**

800

801 **Figure 1: Early accumulation of phosphatidic acid in response to cryptogein.** Lipids were

802 extracted at different time points after cryptogein addition. Cells were radiolabeled for 20 min

803 before lipid extraction. (a) TLC plates were developed in an isooctane based solvent system.

804 (b) The radioactivity associated with PA was quantified relative to the radioactivity associated

805 with all others phospholipids (PL) and then expressed as % of the highest value. For each

806 point of the kinetics, 3 flasks of tobacco cells were treated independently with cryptogein.

807 Data are mean ± SD, n=3. Note that the control error bars are within the symbol.

808

809   **Figure 2: Decrease in cryptogein-induced phosphatidic acid accumulation by PLC and**

810   **DGK inhibitors.** Edelfosine (a) and R50022 (b) were used as PI-PLC and DGK inhibitors,

811   respectively. Wortmannin (c) and lanthanum (d) were used as type III-PI4K and calcium

812   channel inhibitors, respectively. Cells were radiolabeled for 10 min and then cryptogein was

813   added. Lipids were extracted at 10 min after cryptogein addition. When necessary, inhibitors

814   were added 15 min before the cryptogein. Labelling was carried out during 20 min for each

815   point. Extracted lipids were separated using TLC plates and developed in an acidic solvent

816   system. Radioactivity in PA spots was quantified relative to PE and results are expressed as %

817   of that in cryptogein treated cells. For each treatment, 3 flasks of tobacco cells were used to be

818   elicited or not with cryptogein in presence or not of inhibitors. The data are mean ± SD, $n=3$.

819   Results were analysed using a one-way ANOVA, with a Tukey honestly significant difference

820   (HSD) multiple mean comparison *post hoc* test. Different letters indicate a significant

821   difference (Tukey HSD, $P<0.05$).

822

823   **Figure 3: Inhibition of RBOHD-dependent oxidative burst by both DGK and PLC**

824   **inhibitors.** Time course of ROS production of WT BY-2 cells pretreated with either R59022,

825   an inhibitor of DGK activity (a), or edelfosine, an inhibitor of PLC activity (c). Total amounts

826   of ROS produced over a 90 min period following elicitation were calculated for cell

827   suspensions treated with R59022 (b) or edelfosine (d). Cells were preincubated in I20 medium

828   for 30 min with the inhibitor at the indicated concentrations before cryptogein was added.

829   Extracellular ROS synthesis was regularly quantified using a luminol-based method. Data are

830   expressed as nmoles of H_2O_2 equivalents per gram of cells. Means and SD for 3 independent

831   replicates. Statistical difference for the total ROS level was assessed by a Student T-test,

832   where * indicates $P<0.05$ and ** refers to $P<0.01$.

833

Figure 4: Phylogenetic clustering of tobacco DGKs and characterization of one member

835 **of cluster III.** (a) Phylogenetic cluster of plant DGKs. Nucleic acid sequences were retrieved

836 for DGKs from the *Solanacea Genomics Network* (SGN) database (https://solgenomics.net/)

837 using the BLAST algorithm and keyword search tool. Known Arabidopsis, rice and tomato

838 DGKs were used as nucleic acid sequence queries. The phylogenetic analysis was carried out

839 using identified nucleic acid sequences (indicated by their SGN references) as previously

840 described (Cacas et al. 2011). Plant DGKs were grouped into 3 phyla, each showing slightly

841 different protein domain structures. Abbreviations: C1, C1 DAG binding domain; CBD,

842 calmodulin-binding domain; DGKcat, DGK catalytic domain; DGKacc, DGK accessory

843 domain. For plant species, At refers to *Arabidopsis thaliana*, Le to *Solanum esculentum*

844 (previously *Lycopersicum esculentum*), Nt to *Nicotiana tabacum* and Os to *Oryza sativa* (b)

845 Time course of *NtDGK5-like* gene expression in response to cryptogein. Gene expression was

846 determined by Q-PCR as described in the *Material and Methods*. Data are means and SD for

847 3 independent biological replicates (n=3 samples per cell line). (c) Subcellular localization of

848 NtDGK5-like protein. Cells from overexpressing *NtDGK5-like*-c-myc lines

849 (*OE.NtDGK5myc-A* and *OE.NtDGK5myc-B*) and the corresponding empty vector-containing

850 line (*i.e.* line transformed with an empty pMDC32) were harvested 7 days after subculture.

851 Proteins were extracted from cytosolic (lanes 1), microsomal (lanes 2) and plasma membrane

852 (lanes 3) fractions. After western blotting using anti-c-myc antibodies, a specific signal was

853 only found in membrane fractions. Two independent biological replicates were carried out

854 (n=2 samples per cell line).

855

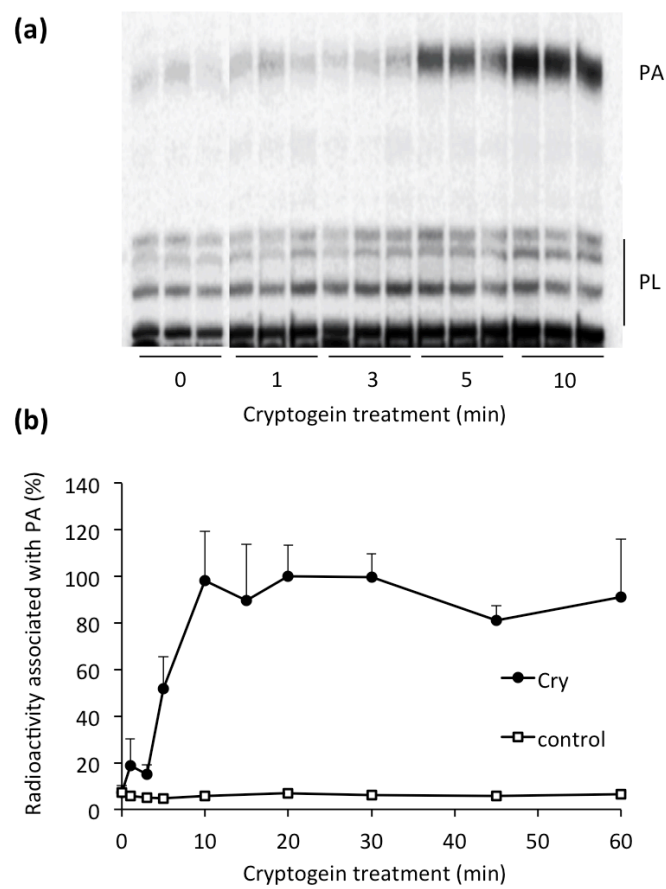**Figure 5: Characterization of *NtDGK5-like* overexpressing transgenic lines.** (a)

Expression levels of *NtDGK5-like* overexpressing lines. Gene expression was determined by

Q-PCR using primers that amplify both the endogenous and transgene-encoding transcripts.

Data are means and SD for 4 independent experiments. These experiments were carried out

on material obtained from untreated 7-day-old cells. BY-2, WT cells; the empty vector-

containing line is a BY-2 line transformed with an empty pMDC32; *OE.NtDGK5-A*, line

overexpressing an untagged version of NtDGK5-like protein; *OE.NtDGK5myc-A* and

OE.NtDGK5myc-B are independent lines overexpressing a C-terminally tagged version of

NtDGK5-like protein. (b) Radioactivity associated with PA in cryptogein-elicited

overexpressors. Cells were radiolabeled for 20 min before lipid extraction. Lipids were

extracted 10 min after cryptogein addition. TLC plates were developed in an acid solvent

system. The radioactivity associated with PA was normalized to that of PE and PC. The data

are expressed as % of the value in the empty vector control line elicited with cryptogein. Data

are means and SD, *n*= 3. (c) Total ROS levels produced during a 90 min period (same

experimental design as that described in the legend of figure 4). Data are expressed as percent

compared to the ROS level of BY-2 cells being set to 100 (corresponding to 500 nmoles H_2O_2

eq./g of cells). Means and SD for 3 independent experiments. Statistical differences for the

total ROS level was assessed using a one-way ANOVA, with a Tukey honestly significant

difference (HSD) multiple mean comparison *post hoc* test. Different letters indicate a

significant difference, Tukey HSD, $P<0.05$. (d) Cell density of untreated transgenic lines.

Packed cell volume (PCV) and dry weight (DW), were expressed in percent. Presented data

are means and SD for 6 independent experiments (n=6 samples for each cell line). Control

(empty vector) and overexpressors were compared to WT BY-2 suspensions using a one-way

ANOVA, with a Tukey honestly significant difference (HSD) multiple mean comparison *post*

hoc test. For PCV, different letters indicate a significant difference, Tukey HSD, $P<0.05$. No

881   significant difference could be detected for DW (Tukey HSD, P<0.05.) significant differences

882   (Tukey HSD, P<0.01) in PCV and DW between genotypes were detected. Letters marked

883   with an apostrophe (') are used for DW data.

884

885 **Figure 6: Characterization of DGK Cluster III-targeted miRNA transgenic cell lines.** (a)

886 Scheme of the nucleic region targeted by the miRNA construct (see also Supporting

887 information Fig. S2). (b) Evidence for miRNA production *in planta*. The cluster III-specific

888 miRNA construct was expressed in tobacco leaves using *Agrobacterium tumefaciens*

889 (GV3101). Foliar samples were harvested 2 day-post inoculation. Total RNAs were extracted,

890 the cluster III pri-miRNA was specifically reverse transcribed using a stem-loop system and

891 generated cDNA used as template for PCR amplification (expected size of PCR

892 product=181bp). Sequencing showed PCR products to be the hairpin construct. (c) Expression

893 levels of cluster III and *NtDGK5-like* genes in miRNA cell lines. Expression levels were

894 quantified by Q-PCR on samples obtained from untreated 7-day-old cell suspensions. Means

895 and SD are from 4 independent biological replicates (n=4 samples for each cell line). The

896 empty vector line corresponds to a line transformed with an empty pH7GWIWG2(II). (d) Cell

897 suspension density of 7 day-old transgenic lines. Packed cell volume (PCV) and dry weight

898 (DW), are expressed as a %. Presented data are means and SD for 6 independent biological

899 replicates (n=6 samples for each cell line). The empty vector line and the *hp-clusterIII-A* and

900 *hp-clusterIII-B* cell lines were compared to WT BY-2 suspensions using a one-way ANOVA,

901 with a Tukey honestly significant difference (HSD) multiple mean comparison *post hoc* test.

902 No significant differences (Tukey HSD, $P<0.01$) in PCV and DW between genotypes were

903 detected. Letters marked with an apostrophe (') are used for DW data.

904

Figure 7: Silencing DGK Cluster III of tobacco abolishes PA production and strongly

906 **inhibits the oxidative burst in response to cryptogein.** (a) Levels of PA in response to

907 cryptogein. Cells were radiolabeled for 20 min before lipid extraction. Lipids were extracted

908 10 min after cryptogein addition. TLC plates were developed using an acid solvent system.

909 The radioactivity associated with PA was normalized to that of PE and PC. For each line, the

910 data are expressed as % of the value of the non-elicited cells. For each treatment, 3 flasks of

911 cells were used and the data are mean ± SD, $n=3$. Statistical difference between treated and

912 non-treated conditions was assessed by a Student T-test, where * indicates P<0.05. (b) Time-

913 course of ROS levels following elicitation with cryptogein. Data are means and SD for 8

914 independent biological replicates (n=8 samples per kinetic point for each cell line). (c) Total

915 amounts of ROS produced over a 90 min period after cryptogein addition (same data as in

916 panel b). Statistical differences was assessed using a one-way ANOVA, with a Tukey

917 honestly significant difference (HSD) multiple mean comparison *post hoc* test. Different

918 letters indicate a significant difference, Tukey HSD, P<0.01.
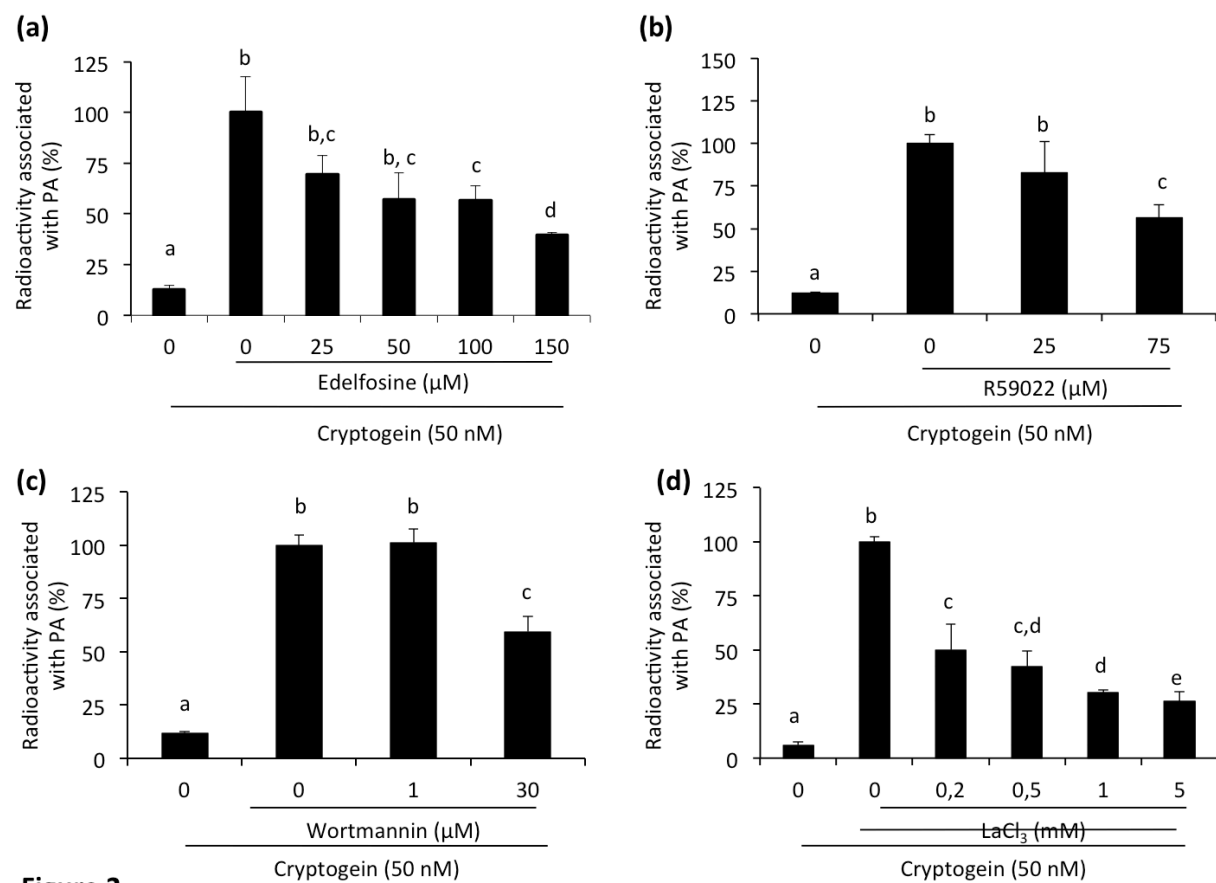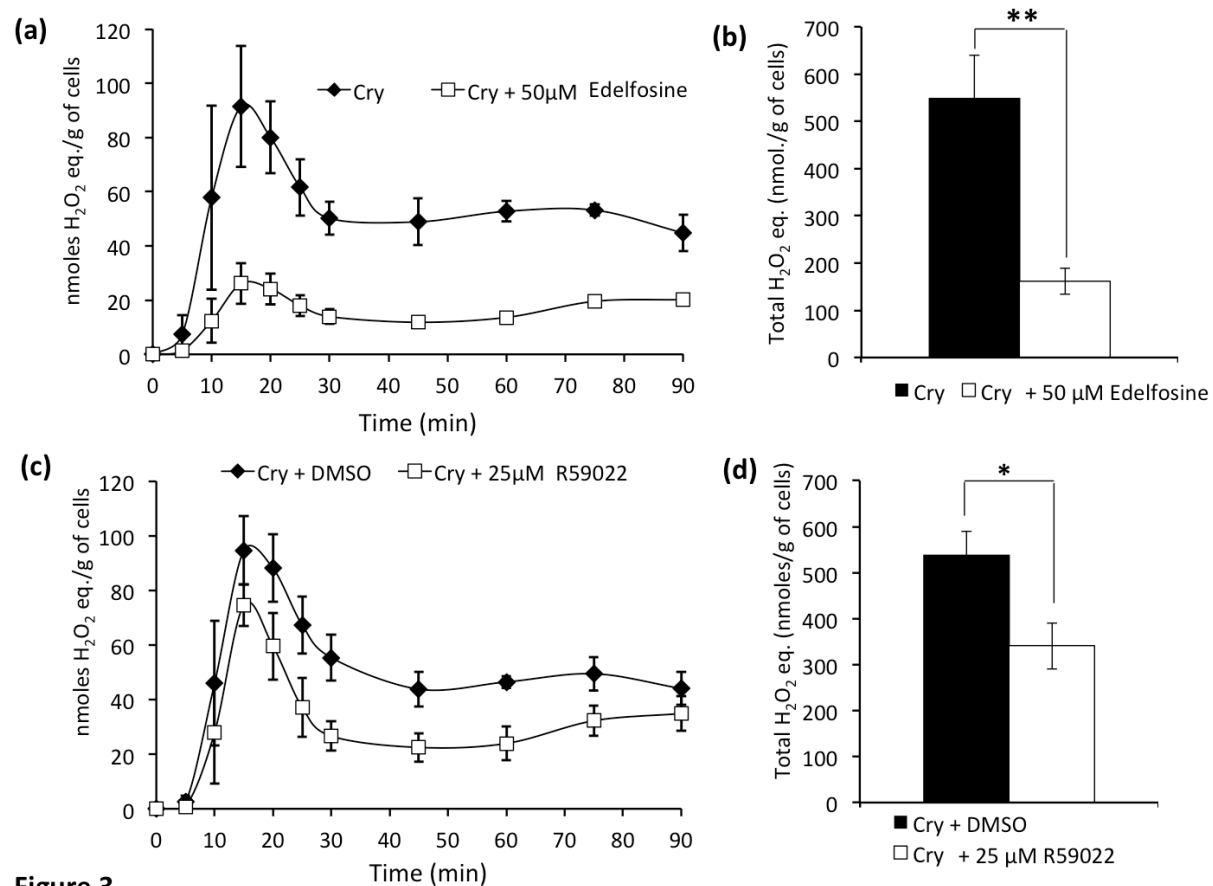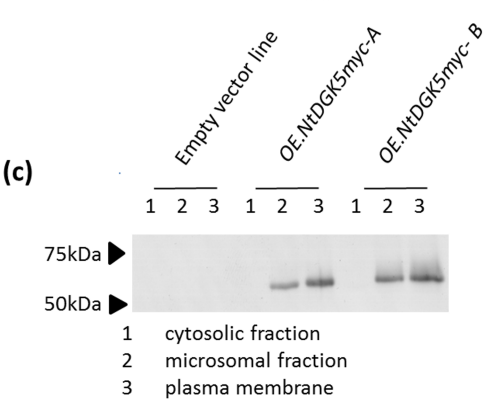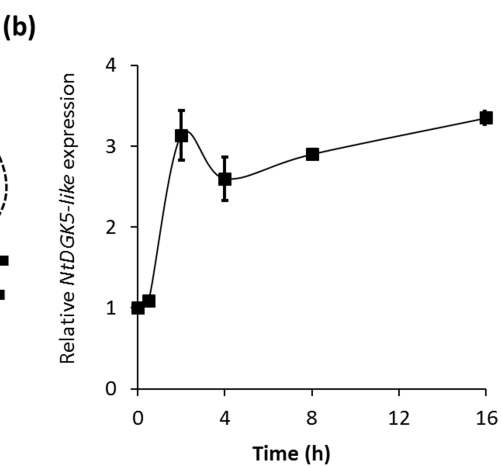
919

FIGURES

(a)

PA

PL

0   1   3   5   10

Cryptogein treatment (min)

(b)

Figure 1

(a)

Radioactivity associated with PA (%)

Edelfosine (μM)

Cryptogein (50 nM)

(b)

Radioactivity associated with PA (%)

R59022 (μM)

Cryptogein (50 nM)

(c)

Radioactivity associated with PA (%)

Wortmannin (μM)

Cryptogein (50 nM)

(d)

Radioactivity associated with PA (%)

LaCl$_3$ (mM)

Cryptogein (50 nM)

923 **Figure 2**

924

925   **Figure 3**

926

(a) Cluster II, Cluster III, Cluster I phylogenetic tree

(b) Relative NtDGK5-like expression vs Time (h)

(c)

927

928

52

929　　**Figure 5**

930

(a)

DGK cat DGK acc CBD

DGK cat DGK acc

~180 bp pri-miRNA

(b)

1   2   3   4

~180bp

(c)

□ *NtDGK5-like* ■ Bulk cluster III

Expression levels (fold increase)

BY-2

Empty vector line

hp-clusterIII-A

hp-clusterIII-B

(d)

□PCV ■DW

Cell density (%)

BY-2   Empty vector line   *hp-clusterIII-A*   *hp-clusterIII-B*

931   **Figure 6**

932

54

Figure 7

933

934
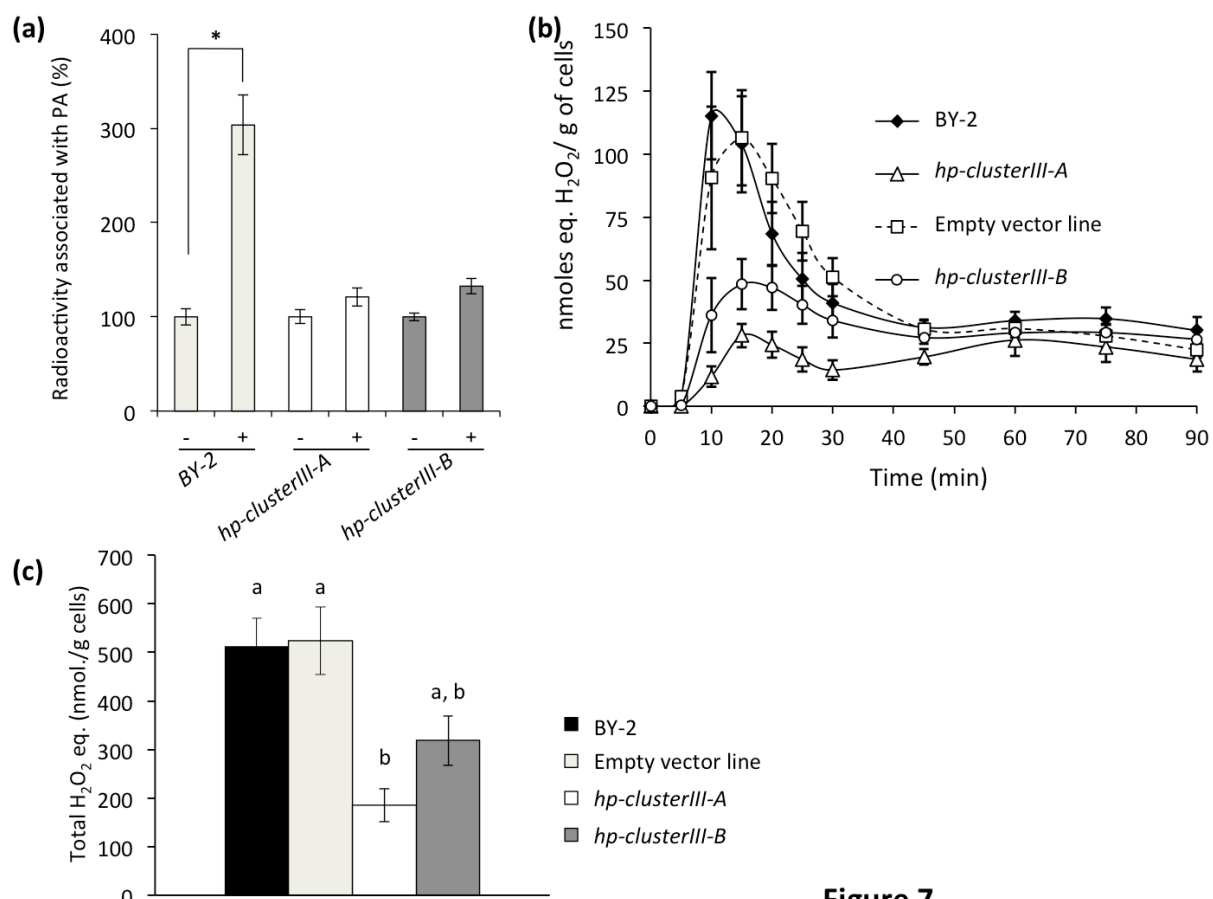
SUPPORTING INFORMATION

Supporting information figure S1: The translated coding sequence of the tobacco *NtDGK5-like* gene. The identified nucleic acid sequence was deposited in GenBank under the reference KU934207. It codes for a 493-amino acid-long protein (55.24kDa), having a predicted pI of 6.75. *In silico* translation was performed using the freeware ORF Finder available at the NCBI website (http://www.ncbi.nlm.nih.gov/gorf/gorf.html).

Supporting information figure S2: Partial alignment of plant DGK coding sequences from clusters II and III. This figure shows the nucleic acid coding region used for miRNA design (squared in red), which is highly conserved among cluster III members and divergent with cluster II counterparts. Full-length nucleic acid sequences were aligned using version 2.0.10 of Clustal X (Larkin et al. 2007) and the resulting alignment was processed online at the BoxShade server (http://www.ch.embnet.org/software/BOX_form.html). Roman numbers (II and III) on the right of the alignment indicate DGK cluster.

Supporting information figure S3: Effects of inhibitors on the alkalization triggered by cryptogein. Cells were preincubated 15 min with inhibitors (or DMSO, solvent of the inhibitors) before cryptogein (50 nM) elicitation. Final concentrations of edelfosine, R59022 and R59949 were 25 µM, 75 µM and 75 µM, respectively. R59949 is another DGK inhibitor. Extracellular pH alkalinization was measured and reported as the difference between initial and final (1 hour of cryptogein treatment) pH values. Means +/- SD.

Supporting information figure S4: Topology of the sixteen identified tobacco DGK proteins. Protein structure is depicted for the 3 phylogenetic clusters. DGK nucleic acid sequences were retrieved from the *Solanacea Genomics Network* (SGN) database (https://solgenomics.net/) using the BLAST algorithm and keyword search tool. Known Arabidopsis, rice and tomato DGKs were used as nucleic acid sequence queries. Upon *in*

959   *silico* translation using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html), the

960   resulting polypeptides were compared to their Arabidopsis counterparts and the Conserved

961   Domain Architecture Retrieval Tool (CDART, (Geer et al. 2002)) was used to predict domain

962   location. Within a panel (corresponding to one protein cluster), the closest Arabidopsis

963   relative is followed by orthologous tobacco translated sequences. The latter are referenced by

964   their SGN numbers and the identified part of the protein is presented. The length of the

965   corresponding contigs is also indicated in green. Abbreviations: AtDGK5lv, AtDGK5 long

966   splice variant; AtDGK5sv, AtDGK5 short splice variant; C1, DAG binding domain; CBD,

967   calmodulin-binding domain; DGKcat, DGK catalytic domain; DGKacc, DGK accessory

968   domain. The grey rectangle localized to the N-terminal part of AtDGK1 represents a

969   transmembrane domain.

970   **Supporting information figure S5:** Identification of the PA binding domain of NtRBOHD

971   by alignment with the cognate domain of Arabidopsis isoform D. Full length primary amino-

972   acid sequences of RBOHD from *Arabidopsis thaliana* (SwissProt:Q9FIJ0.1) and *Nicotiana*

973   *tabacum* (GenBank:ABN58915) were aligned using version 2.0.10 of Clustal X (Larkin et al.

974   2007) and the resulting alignment was then processed online at the BoxShade server

975   (http://www.ch.embnet.org/software/BOX_form.html). Stars indicate the two basic amino-

976   acid residues experimentally proven to be critical for interaction with PA (Zhang *et al.* 2009).

977   Note the conservation of these residues between the two proteins.