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

3

4 Running head: 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.

36

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 [³³P]-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

58

59

60 INTRODUCTION

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

68 Phosphatidic acid (PA) is the most basic phosphoglycerolipids, being composed of a
69 diacylglycerol with a phosphoryl group esterified on the *sn*-3 hydroxyl of the glycerol
70 backbone. Besides being an intermediate of phospholipid synthesis (Petroustos et al. 2014),
71 PA also transduces stress signals in eukaryotic cells. In serum-withdrawn fibroblast cultures
72 for example, reverse genetic approaches have established a marked correlation between
73 elevated endogenous PA levels and apoptosis inhibition; the latter probably being partially
74 responsible for cancer establishment in tissues (Bruntz et al. 2014). In plants, upon pathogen
75 attack and situations mimicking infection by the addition of elicitors, as well as under many
76 abiotic (including drought, cold, osmotic) stresses, PA accumulation was either inferred from
77 pharmacological strategies or directly shown by biochemical methods (Pinosa et al. 2013,
78 Ruelland et al. 2015). In Eukaryotic cells, the synthesis of PA as a signalling molecule relies
79 on two distinct enzymatic systems. Phospholipases D (PLD) can hydrolyse structural
80 phospholipids within membranes, directly releasing PA (Wang et al. 2012) while,
81 diacylglycerol kinases (DGK) can catalyse the phosphorylation of diacylglycerol (DAG) to
82 produce PA (Arisz et al. 2009). DAG can be provided to DGK either by phosphoinositide-
83 dependent phospholipases C (PI-PLCs) that act on phosphorylated phosphoinositides or by
84 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* PLD δ 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 PLDB1 (Bargmann et al.
112 2006) whereas full establishment of ETI required the PI-PLC-encoding gene *sIPLC4* (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, Rustérucchi 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

139 MATERIALS & METHODS

140 Culture conditions and cryptogein elicitation of tobacco cells

141 Bright Yellow-2 (*Nicotiana tabacum*) cells were grown in modified liquid Murashige and
142 Skoog (MS, M0221, Duchefa-Kalys, Bernin, France) medium at pH 5.6 (M0221, Duchefa-
143 Kalys, Bernin, France) supplemented with 1 mg.L⁻¹ thiamine-HCl, 0.2 mg.L⁻¹ 2,4
144 dichlorophenylacetic acid, 100 mg.L⁻¹ myo-inositol, 30 g.L⁻¹ sucrose, 200 mg.L⁻¹ KH₂PO₄ and
145 2 g.L⁻¹ MES. Cells were maintained under continuous light (200 μE m⁻² s⁻¹) on a rotary shaker
146 (140 rpm) and sub-cultured to fresh medium (4:80, (v/v)) on a weekly basis.

147 For radiolabeling experiments, ROS quantification and pharmacological treatments, cells
148 were harvested from 7-day-old suspensions using a vacuum-system and resuspended at
149 0.1g/mL in either equilibration medium I2 (2 mM MES, 175 mM mannitol, 0.5 mM CaCl₂,
150 0.5 mM K₂SO₄, pH 5.9) or I20 (20 mM MES, 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM
151 K₂SO₄, pH 5.9). After a 3 h incubation period under mild shaking at 24°C, cells were elicited
152 by adding 50 nM cryptogein (final concentration) from a solution prepared according to
153 (Ricci et al. 1989).

154 Radiolabelling experiments

155 BY-2 cells were aliquoted (7 mL) in wide neck flasks (capacity 50 mL) for equilibration.
156 Cells were then labelled by the addition of 53 MBq.L⁻¹ [³³P]-orthophosphate. Total lipids
157 were extracted according to the procedure previously described by (Krinke et al. 2009). Lipids
158 were separated by thin layer chromatography (TLC) and developed either in an acidic solvent
159 system composed of chloroform:acetone:acetic acid:methanol:water (10:4:2:2:1, (v/v),
160 Lepage 1967) or in a solvent system composed of an upper phase of ethyl
161 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 μ L) 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 μ L of the assay
174 buffer (50 mM MES, 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, 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 H₂O₂ equivalents by recording cell
179 luminescence upon the addition of increasing H₂O₂ concentrations during the assay. Results
180 were normalized to BY-2-cell weight and expressed as nmoles H₂O₂ 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 1 α* (EF-1 α , 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 AAGGGTGTGTTGTTGTCCTCAATCTT-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'

195 **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.

206 **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 CTAGAGGTCTTCTTCGCTGATGAGCTTTTGTTCCTGAGACGAGAAACATCGAC-
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 *NtDGK5-like* (named *line OE.NtDGK5-A*) while five
222 out of twenty hygromycin-resistant calli overexpressed the C-terminally tagged version of
223 *NtDGK5-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.

226 **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'-CACCTCATTTTTGAAGCAAGTAATGAATGCA-3',
232 and reverse (Rc-ClusterIII), 5'-TGGAACCTTCCACATTCAGTTCA-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 µg.mL⁻¹
246 rifampicin, 50 µg.mL⁻¹ gentamycin and 50 µg.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 GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTggaaac-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 GGATGCGATCATAACCAGCACT-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 [³³Pi]-orthophosphate for 20 min
296 before lipid extraction and [³³P]-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 μ M 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 μ M wortmannin resulted in a 46% inhibition of cryptogein-induced PA
322 accumulation whereas 1 μ M 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 *NtDGK5-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 probably 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 cryptogin elicitation. The *DGK*-silenced lines were shown to no longer
430 over-accumulate PA, 10 min after cryptogin 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).

440 DISCUSSION

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

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 *NtDGK5-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 PLD α 1 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, Pinoso 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

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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
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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 isoctane 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 \pm 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 \pm 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 H₂O₂ 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

834 **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 *Lycopersicon 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

856 **Figure 5: Characterization of *NtDGK5-like* overexpressing transgenic lines.** (a)
857 Expression levels of *NtDGK5-like* overexpressing lines. Gene expression was determined by
858 Q-PCR using primers that amplify both the endogenous and transgene-encoding transcripts.
859 Data are means and SD for 4 independent experiments. These experiments were carried out
860 on material obtained from untreated 7-day-old cells. BY-2, WT cells; the empty vector-
861 containing line is a BY-2 line transformed with an empty pMDC32; *OE.NtDGK5-A*, line
862 overexpressing an untagged version of NtDGK5-like protein; *OE.NtDGK5myc-A* and
863 *OE.NtDGK5myc-B* are independent lines overexpressing a C-terminally tagged version of
864 NtDGK5-like protein. (b) Radioactivity associated with PA in cryptogein-elicited
865 overexpressors. Cells were radiolabeled for 20 min before lipid extraction. Lipids were
866 extracted 10 min after cryptogein addition. TLC plates were developed in an acid solvent
867 system. The radioactivity associated with PA was normalized to that of PE and PC. The data
868 are expressed as % of the value in the empty vector control line elicited with cryptogein. Data
869 are means and SD, $n=3$. (c) Total ROS levels produced during a 90 min period (same
870 experimental design as that described in the legend of figure 4). Data are expressed as percent
871 compared to the ROS level of BY-2 cells being set to 100 (corresponding to 500 nmoles H_2O_2
872 eq./g of cells). Means and SD for 3 independent experiments. Statistical differences for the
873 total ROS level was assessed using a one-way ANOVA, with a Tukey honestly significant
874 difference (HSD) multiple mean comparison *post hoc* test. Different letters indicate a
875 significant difference, Tukey HSD, $P<0.05$. (d) Cell density of untreated transgenic lines.
876 Packed cell volume (PCV) and dry weight (DW), were expressed in percent. Presented data
877 are means and SD for 6 independent experiments ($n=6$ samples for each cell line). Control
878 (empty vector) and overexpressors were compared to WT BY-2 suspensions using a one-way
879 ANOVA, with a Tukey honestly significant difference (HSD) multiple mean comparison *post*
880 *hoc* test. For PCV, different letters indicate a significant difference, Tukey HSD, $P<0.05$. No

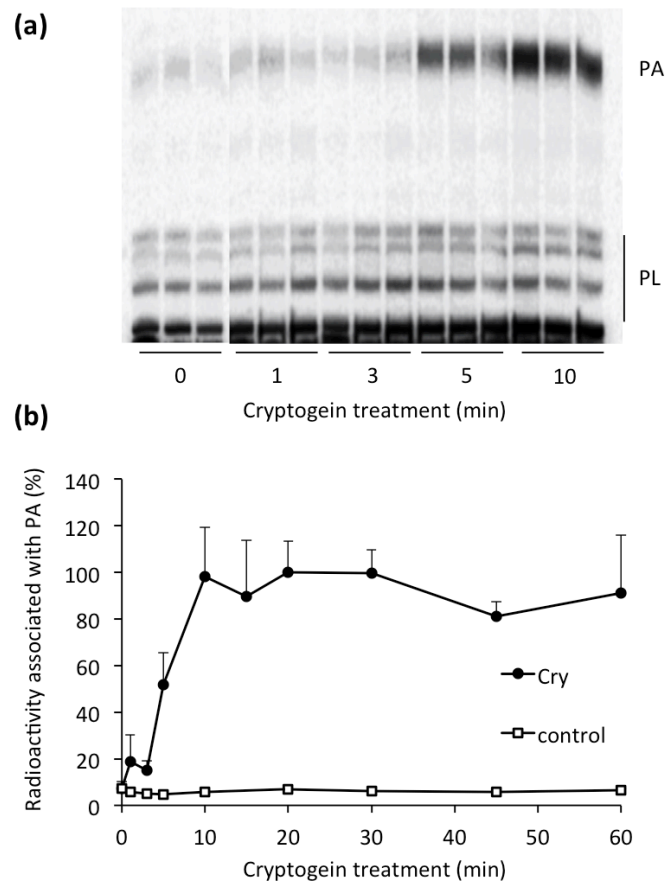
881 significant difference could be detected for DW (Tukey HSD, $P < 0.05$.) significant differences
882 (Tukey HSD, $P < 0.01$) in PCV and DW between genotypes were detected. Letters marked
883 with an apostrophe (') are used for DW data.

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

905 **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 \pm 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



921 Figure 1

922

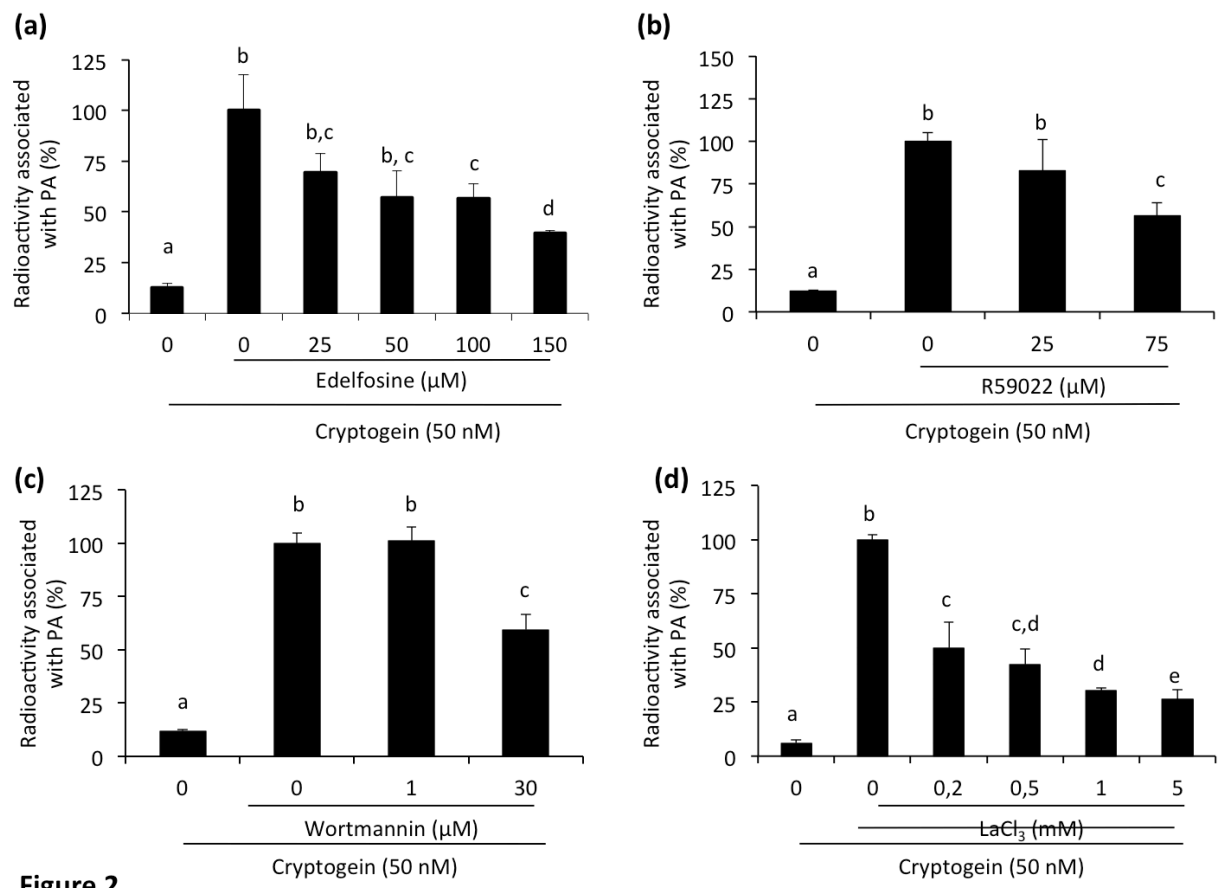
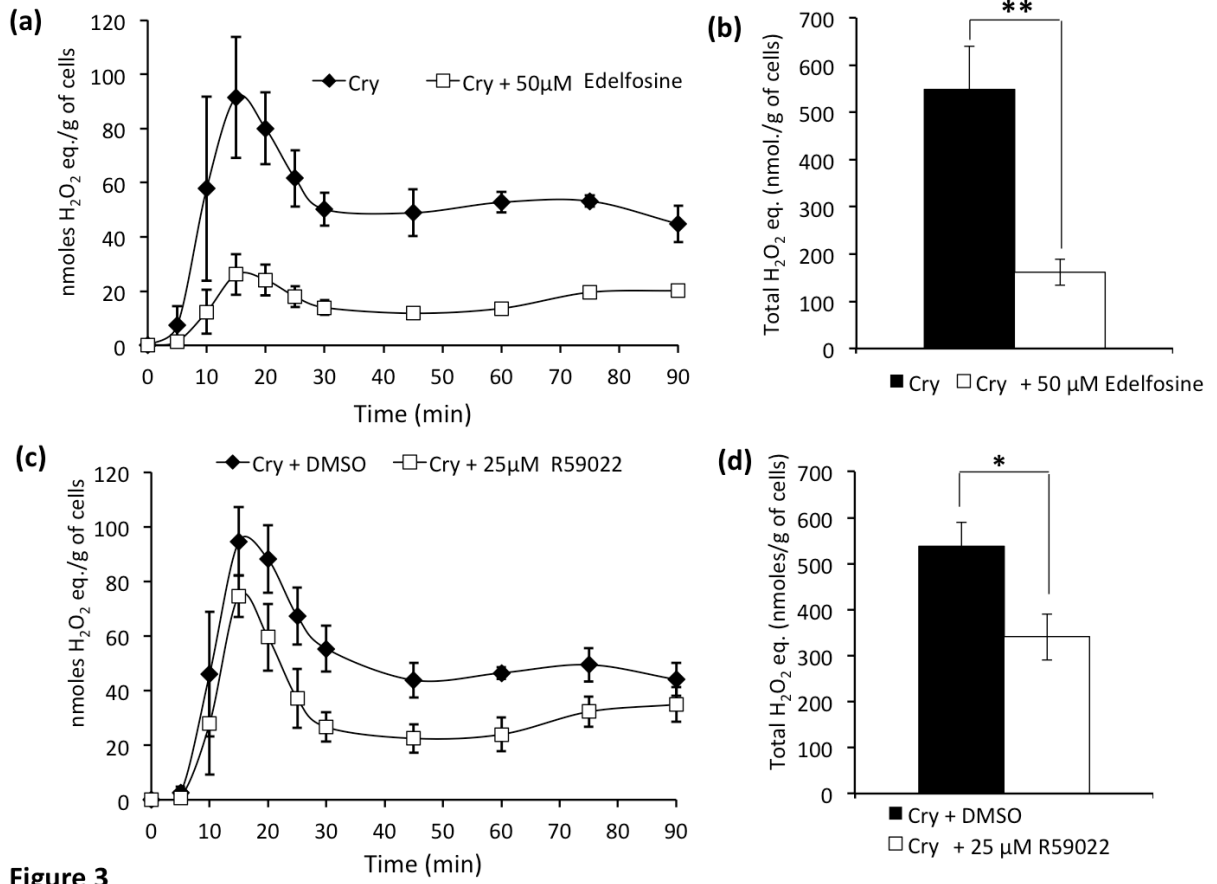


Figure 2

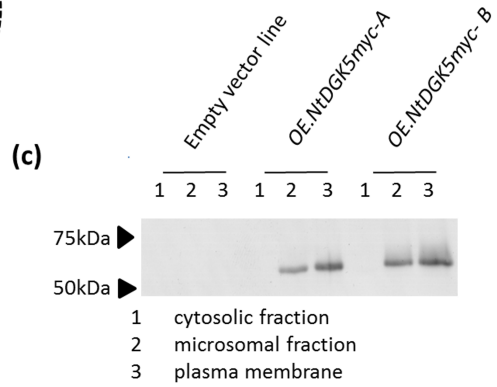
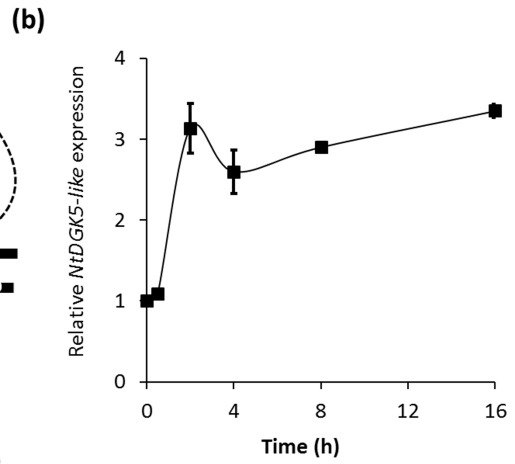
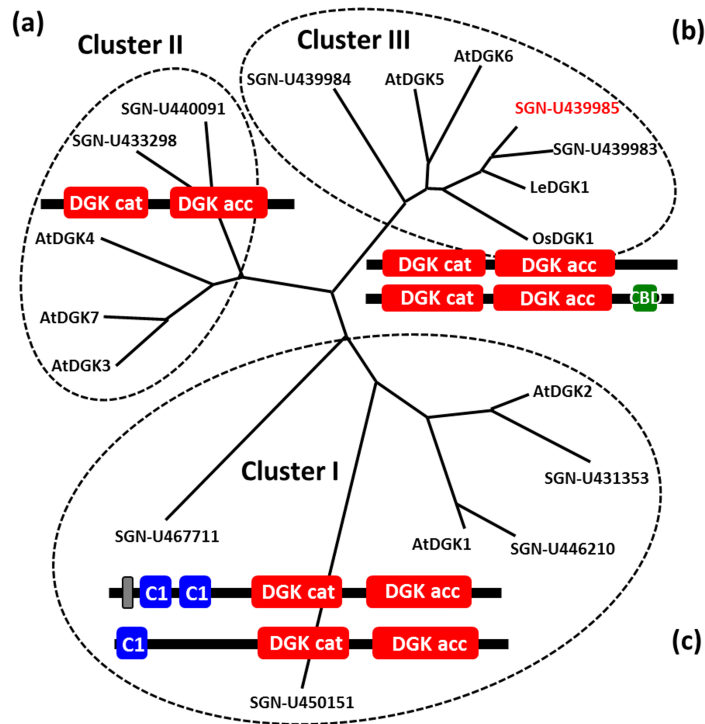
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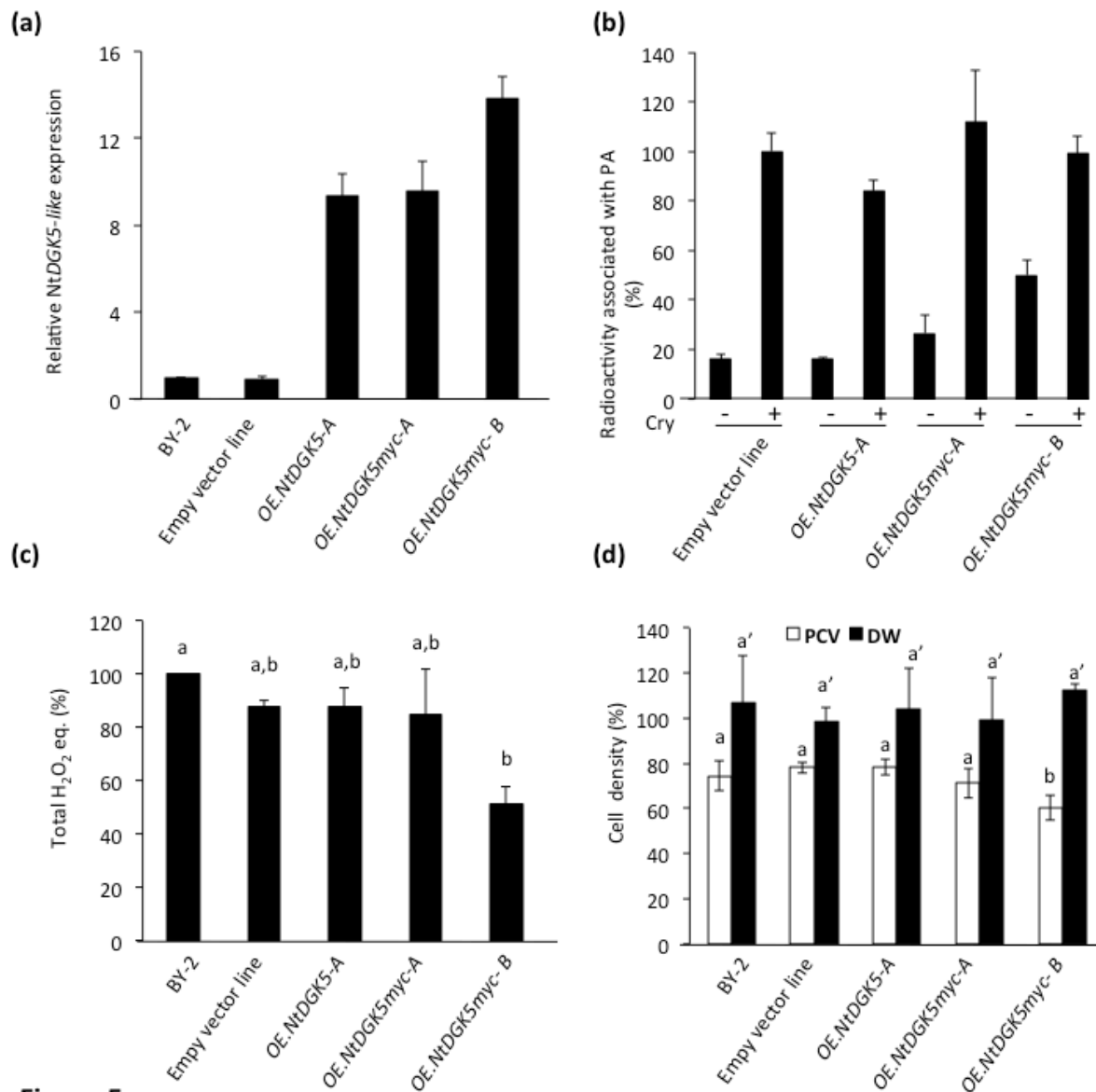
925 **Figure 3**

926



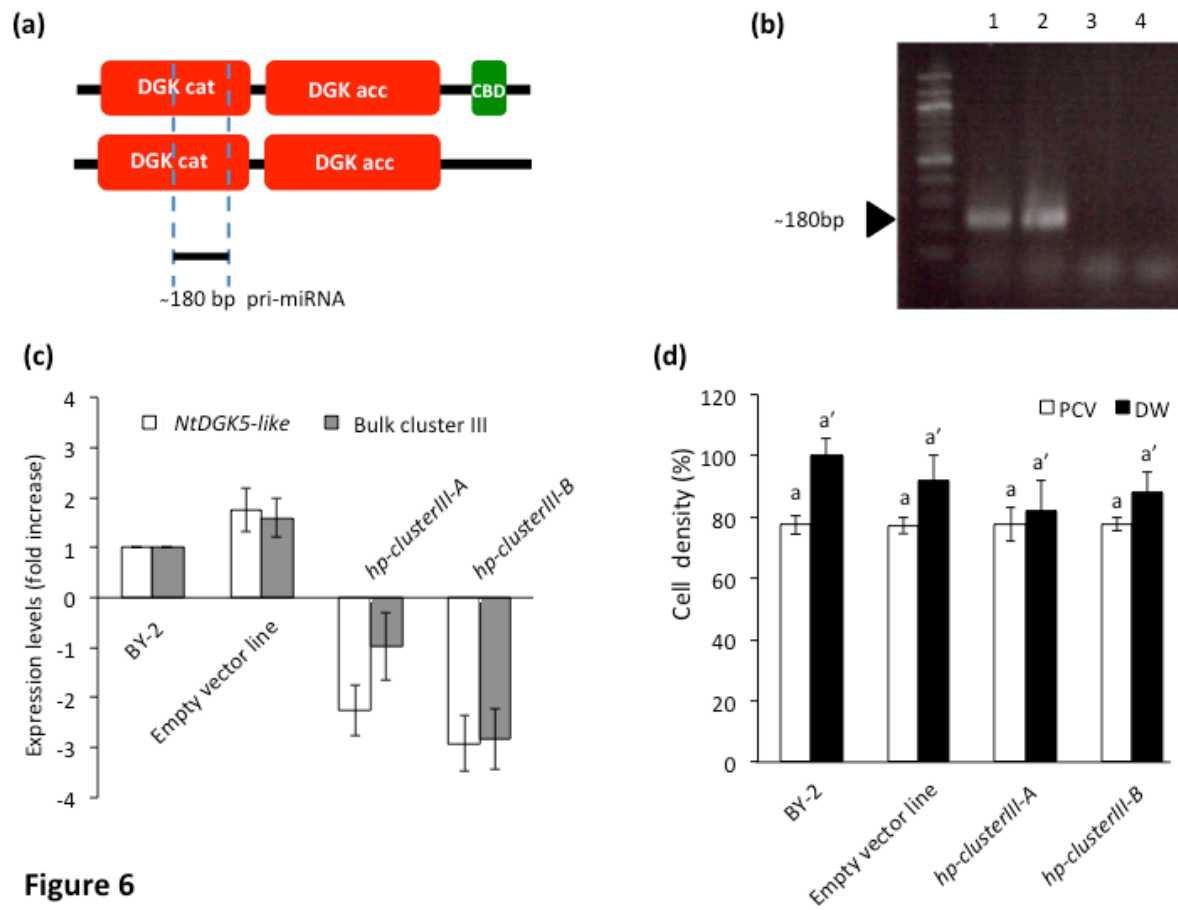
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929 **Figure 5**

930



931 **Figure 6**

932

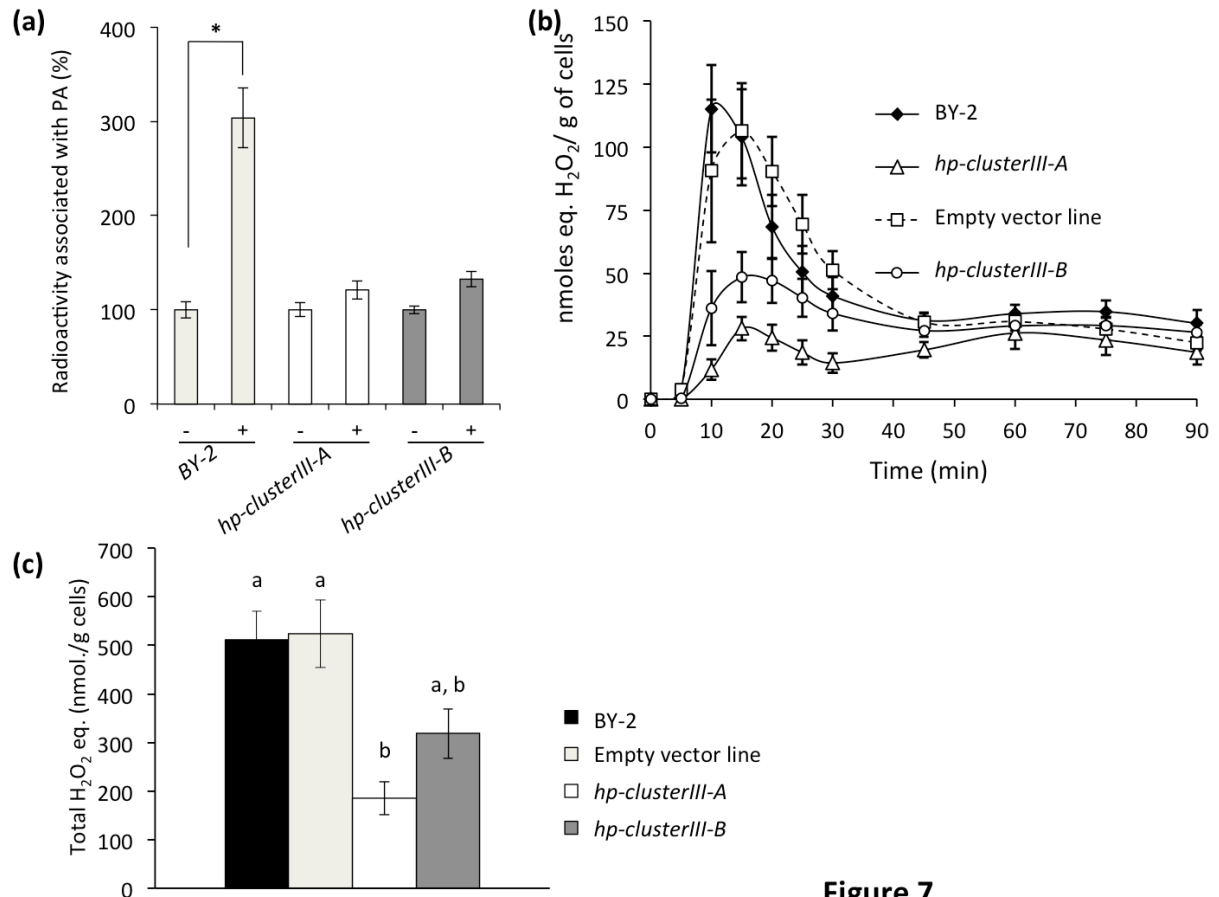


Figure 7

933

934

935 **SUPPORTING INFORMATION**

936 **Supporting information figure S1:** The translated coding sequence of the tobacco *NtDGK5-*
937 *like* gene. The identified nucleic acid sequence was deposited in GenBank under the reference
938 KU934207. It codes for a 493-amino acid-long protein (55.24kDa), having a predicted pI of
939 6.75. *In silico* translation was performed using the freeware ORF Finder available at the
940 NCBI website (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>).

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

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

954 **Supporting information figure S4:** Topology of the sixteen identified tobacco DGK
955 proteins. Protein structure is depicted for the 3 phylogenetic clusters. DGK nucleic acid
956 sequences were retrieved from the *Solanacea Genomics Network* (SGN) database
957 (<https://solgenomics.net/>) using the BLAST algorithm and keyword search tool. Known
958 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.