Dissecting Arabidopsis phospholipid signaling using reverse genetics

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DGK5 is required for SA responsiveness and disease resistance in Arabidopsis

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Abstract

Upon elicitation by pathogen-derived elicitors, phosphatidic acid (PA) is produced which is proposed to function as a lipid second messenger. PA is generated via either the phospholipase C (PLC)/diacylglycerol kinase (DGK) or the phospholipase D (PLD) pathway. Here, we provide genetic evidence that DGK5 is required for resistance to the virulent pathogens *Pseudomonas syringae pv maculicola (Psm)* and *Hyaloperonospora parasitica Waco9*, using an Arabidopsis DGK5 knock-out mutant. After Psm infection, the DGK5 gene is elicited within 6 hours, reaching induction levels of 5-fold. Expression analysis of the SA signaling mutants *pad4, sid2*, and *npr1* show that the induction of DGK5 by Psm is not affected in these mutants. However, the resistance defect of dgk5 is correlated with a strongly reduced expression of the *PR1* gene after Psm infection. Treatment with SA did not induce *PR1* in dgk5, suggesting that DGK5 functions downstream of or in parallel with SA. SA treatment of dgk5 led to wild-type levels of PR-2 induction, indicating that a subgroup of SA-regulated genes is affected in dgk5.
Introduction

Plants are constantly challenged by infectious pathogens; however, successful infections are relatively rare, with only a few pathogens being able to damage the plant. This is explained in part by the observation that plants are equipped with a complex network of defense strategies that are induced upon encountering microbes. Fundamental for this inducible defense system to operate is the ability of the plant to recognize potential pathogens. The plant has developed specific pattern recognition receptors that recognize pathogen-associated molecular patterns (PAMPs), such as bacterial flagellin and lipopolysaccharides, which are present in most bacteria, regardless of their pathogenic potential in plants (reviewed in [1]). This recognition triggers an array of defense reactions that limits the pathogen growth. The sum of these plant defense responses may be classified as PAMP-triggered immunity (PTI; [2]). For a pathogen to succeed in infection, it needs to weaken or delay the plant’s defense. Virulent pathogens have evolved strategies to suppress resistance by secreting effector proteins, toxins, or other virulence factors into the plant cell to alter resistance signaling that in turn cause plant disease (effector-triggered susceptibility (ETS; [2-4]). Specialized resistance (R) proteins are present in plants that monitor the integrity of host cellular targets of pathogen effectors, and as a consequence their defense is activated, referred to as gene-for-gene resistance or effector-triggered immunity (ETI; [2]).

There are many overlapping principles between PTI and ETI, as exemplified by qualitatively similar changes in global gene expression profiles of Arabidopsis plants treated with flagellin or infected with either virulent, avirulent, nonhost or hrcC mutant (deficient in secreted pathogen effectors) strains of Pseudomonas syringae bacterial pathogens [5-7]. Moreover, the induced responses require the same activated signaling pathway [5, 7, 8]. These P. syringae-related inducers all elicit the production of the molecule salicylic acid (SA) and moreover, exogenous
application of SA partly activates the same genes [7-11], indicating that the SA signaling pathway has a central role in these different resistance mechanisms. This supposition is enforced by the finding that all known mutations that interfere with SA signaling cause partly similar changes in transcriptional activation of genes and is accompanied by (enhanced) susceptibility to *P. syringae* strains [5, 7, 12].

NPR1 (nonexpressor of *PR* genes 1) is a key regulatory protein that plays a critical role in the SA-dependent signaling pathway. Mutations in the *NPR1* gene render the plant largely unresponsive to pathogen-induced SA production, thereby blocking the induction of SA-dependent *PR* (pathogenesis-related) genes [13-15]. In an uninduced state, NPR1 forms oligomers, but the redox change that is induced by SA reduces the intermolecular disulfide bonds that hold the NPR1 oligomer together so that monomeric NPR1 is released and is then translocated into the nucleus [16]. In the nucleus, NPR1 interacts with different proteins, such as TGA transcription factors and NIMINs (NIM(=NPR1)-interacting), modulating the expression of downstream genes, such as *PR1* [17-20], but also genes involved in protein folding, modification and secretion [21]. Expression of these proteins ensures a proper processing of *PR* transcripts and secretion of PR proteins, which contributes to SA-based resistance.

Whereas SA is predominantly effective against (hemi)biotrophic pathogens like *P. syringae* and the oomycete *Hyaloperonospora parasitica* [12], jasmonic acid (JA) is mostly active against necrotrophic pathogens, like *Alternaria brassicicola*, and insects [12, 22]. In addition, numerous other molecules are being induced in the plant upon pathogen recognition, including reactive oxygen species, nitric oxide, cytosolic calcium and the phospholipid phosphatidic acid [23-25].

Phosphatidic acid (PA) is a membrane-localized signaling molecule, implicated in responses to various biotic and abiotic stress factors, including PAMPs but also
wounding, drought, salinity, and cold [26, 27]. PA is present in minute amounts, yet its levels rapidly and transiently increase upon stimulation. PA is proposed to function as a signaling molecule by acting as a docking site to which specific proteins are recruited, or it may change the protein’s conformation, affecting its enzymatic activity [26, 28]. A known target of PA in plants is the protein kinase AtPDK1 [29]. PA stimulates PDK1 activity which results in phosphorylation of OXI1/AGC2-1 [30], a kinase involved in oxidative stress and disease resistance signaling [31]. OXI1 acts upstream of protein kinase PTI1-2 and the mitogen-activated protein kinases MPK3 and MPK6, which are also implicated in defense signaling [32-36]. PA has also been reported to bind to the protein phosphatase ABI1, a negative regulator of ABA responses [37]. ABA has been shown to be important for fine-tuning of resistance responses [38]. Recently, PA was reported to bind CTR1 and inhibit its activity. As CTR1 is a negative regulator of ethylene responses [39, 40], PA could be positively affecting ethylene signaling [41].

Stress induced PA formation is catalyzed by either the phospholipase D (PLD) or the phospholipase C/diacylglycerol kinase (PLC/DGK) pathway. PLD directly generates PA by hydrolysis of structural phospholipids such as phosphatidylcholine or phosphatidylethanolamine. PLC hydrolyses the phosphatidylinositol 4,5-bisphosphate into DAG, which can be phosphorylated by DGK to form PA.

In total, there are 9 PLC [42] and 7 DGK [43] genes in Arabidopsis. Different pathogen elicitors, like flagellin and xylanase have been reported to induce PA formation within minutes in cell cultures of tomato [44]. Similarly, the avirulence factor AVR4 of the fungus Cladosporium fulvum triggered a PA response in transgenic tobacco cells expressing the tomato R gene Cf-4 [45]. Production of the PA signal in these systems appeared to be derived mainly from the PLC/DGK pathway.
A recent paper showed that conditional expression of either one of the avirulence factors AvrRpm1 or AvrRpt2 of *P. syringae* results in PA formation in Arabidopsis leaf tissue [46]. Both PLC/DGK and PLD pathways appeared to contribute to the induced PA formation. Although genetic evidence is still lacking, these data point to a role for PA in plant defense.

In this study, we aimed to obtain plants affected in PLC or DGK genes to test their contribution to disease resistance. A mutant knocked out in DGK5 was identified that is impaired in resistance to the virulent pathogens *P. syringae* and *H. parasitica*. Interestingly, expression of the *PR1* gene was strongly reduced, upon either pathogen infection or SA treatment. This finding points to a role for DGK5 in disease resistance signaling, downstream of SA.

**Results**

*Knock-out mutant dgk5 is compromised in basal resistance to virulent P. syringae and H. parasitica*

To examine the role of PA in the defense response, we obtained homozygous Arabidopsis T-DNA insertion lines with mutations in the PA-generating enzymes, PLC and DGK. We obtained 20 lines with T-DNA insertions in all of the 9 PLC genes and 16 lines with mutations in all of the 7 DGK genes (Supplemental table 1). All lines were subjected to bioassays with virulent *P. syringae* pv. *maculicola* (*Psm*), avirulent *P. syringae* pv. *tomato* carrying *avrRpt2* (*Pto avrRpt2*), and the fungal necrotroph *A. brassicicola*. None of the lines displayed reduced resistance to *Alternaria* or an altered hypersensitive response to *Pto avrRpt2* (Supplemental table 1). Enhanced susceptibility to virulent *Psm* was found for three lines with T-DNA insertions in or close to PLC7, DGK2 and DGK5 (Supplemental table 1). The PLC7 T-DNA insertion line was found to over-express PLC7 and was not studied, as we
DGK5 is required for disease resistance

(a) Symptoms caused by infiltration of leaves with *Psm* 3 days post inoculation (dpi). (b) Growth of *Psm* in inoculated leaves. Data points are means (cfu/cm²) with standard deviations (represented by error bars) from 8 sets of 2 randomly selected leaf discs of 12 plants per genotype. The data presented are from a representative experiment that was repeated three times with similar results. (c) Distribution of disease severity classes of plants infected with *H. parasitica* Waco9. Classes represent the percentage of leaves with: I, no symptoms; II, trailing necrosis; III, <50% of leaf area covered with sporangia; IV, >50% of leaf area covered with sporangia, with additional chlorosis and leaf collapse. Data represent 280 leaves of 40 plants per genotype. Asterisk indicates statistical significant different frequency distribution of the disease severity classes (*χ²* test; *α* = 0.05). This experiment was repeated with similar results.

preferred loss-of function mutants. The phenotype of the *DGK2* T-DNA insertion line was not linked to the T-DNA insertion and was not pursued either. Instead, we choose *dgk5* for further analysis. Fig. 1a shows that *Psm* infection of *dgk5* leaves caused more water-soaked lesions and chlorosis than infection of wild-type leaves. This was accompanied by an enhanced growth rate of the pathogen in the leaves, reaching a 10-fold difference in bacterial titer between *dgk5* and wild-type at 3 days after inoculation (Fig. 1b). Under normal growth conditions, *dgk5* did not display any morphological phenotypes. Although resistance to *Pto avrRpt2* and *A. brassicicola* was still intact, infection with the virulent oomycete *H. parasitica* Waco9 resulted in significantly more disease symptoms in *dgk5* compared to wild-type (Fig. 1c), indicating that DGK5 is required for basal resistance against different virulent pathogens.
**Fig. 2.** dgk5 is a knock-out mutant that can be complemented by DGK5 cDNA
(a) Schematic representation of the DGK5 gene. Exons are shown as boxes, the T-DNA insertion point is indicated by a triangle and the region amplified by RT-PCR is indicated by a line above the gene structure. (b) Mutant dgk5 does not express the DGK5 gene. Q-RT-PCR analysis of transcript levels of DGK5 and UBI in wild-type and dgk5. C_T reflects the number of PCR amplification cycles required to reach a critical SYBR Green fluorescent value. The critical value for DGK5 in the dgk5 sample was not reached after 40 cycles (C_T expected to be infinite), indicating that there is no or an extremely low transcript level. This experiment was repeated 10 times with similar results. (c) Western blot analysis of ectopically expressed DGK5:GFP. Transgenic lines #7 and #20 express DGK5:GFP under control of the 35S promoter in dgk5 mutant background. A line expressing GFP only was included as a control. The Western blot was probed with an anti-GFP antibody (top) and stained with Ponceau to confirm equal loading (bottom). Arrows indicate the ~80 kDa DGK5:GFP fusion protein and the ~30 kDa GFP protein. (d) Restoration of resistance in dgk5 by complementation with DGK5 cDNA. Titer of Psm in leaves at 3 dpi is depicted. Lines described in the legend to Fig 1C were used. Different letters indicate statistically significant differences between genotypes (Tukey HSD test; α = 0.05, n = 8). For details see the legend to Fig 1B. The data presented are from a representative experiment that was repeated two times with similar results.

The T-DNA insertion site in dgk5 was verified by amplification of the genomic DNA, flanking the T-DNA insertion using a DGK5-specific forward primer and a T-DNA left border primer. Subsequent sequence analysis of the amplicon confirmed the presence of the T-DNA insertion 2621 bp downstream of the DGK5 translation start codon (Fig. 2a). Knockout of DGK5 gene expression was determined by RT-PCR with DGK5-specific primers (Fig. 2a). DGK5 transcript
levels were undetectable by quantitative PCR in \textit{dgk5} (Fig. 2b), confirming that \textit{dgk5} can be considered a loss-of-function allele.

No other T-DNA insertion line available from different publicly-available mutant collections with a predicted mutation in or around the \textit{DGK5} gene turned out to be a \textit{dgk5} mutant allele (data not shown). To obtain additional evidence that the observed enhanced disease susceptibility phenotype of \textit{dgk5} was caused by the mutation in \textit{DGK5} we set out to test complementation of the \textit{dgk5} mutant line with \textit{DGK5} cDNA. Western blot analysis shows that transgenic lines \#7 and \#20, which are \textit{dgk5} mutants carrying a construct of \textit{DGK5:GFP} under control of the 35S promoter, express the full length fusion protein (Fig. 2c). Infection of these transgenic lines with \textit{Psm} resulted in a disease level that was statistically significant lower than in the \textit{dgk5} mutant and comparable to that in wild-type plants (Fig. 2d). These complementation data confirm that a functional \textit{DGK5} gene is required for basal resistance against \textit{Psm}.

\textit{DGK5 is plasma membrane localized}

To determine the subcellular localization of the DGK5 protein, \textit{dgk5} plants, complemented by 35S::DGK5:GFP, were subjected to confocal imaging. In the two independent lines tested, GFP was localized at the cell periphery, suggesting that DGK5:GFP is localized to the plasma membrane (Fig. 3a,b). This was confirmed by a plasmolysis control (Fig. 3c,d), consistent with enriched DGK activity in the plasma membrane fraction of various plant species [47].
Induction of DGK5 gene expression by Psm is independent of SA signaling

To determine whether the expression of the DGK5 gene is regulated by pathogen infection, we performed quantitative RT-PCR on Psm-inoculated plants. DGK5 transcript levels increased from 6 hrs after inoculation and increased up to 5-fold after 24 hrs (Fig. 4a). To investigate whether the SA signaling pathway is required for this induction, Arabidopsis mutants pad4, sid2, and npr1, with defects in SA production or responsiveness, were examined for DGK5 induction by Psm.
DGK5 is required for disease resistance

Fig. 4. Psm-induced DGK5 expression in wild-type and SA-related mutants (a) Expression of DGK5 in wild-type leaves at different times after inoculation with Psm. Transcript levels were determined by quantitative RT-PCR and the ratio of DGK5/UBI was depicted relative to that at 0 hpi (just before inoculation). In this experiment, ΔC_{T} DGK5-UBI at 0 hpi was 10.9. The experiment was repeated 5 times with similar results. (b) Expression of DGK5 in mutants pad4-1, sid2-1, and npr1-1 compared to wild-type at 0 and 24 hpi in Psm-challenged leaves. The ratio of DGK5/UBI was relative to that at 0 hpi in wild-type (just before inoculation). In this experiment, ΔC_{T} DGK5-UBI at 0 hpi was 6.93. This experiment was repeated twice with similar results.

All mutants showed DGK5 expression levels that were comparable to those in wild-type, either before or after inoculation with Psm (Fig. 4b), suggesting that SA signaling does not affect the induced expression of DGK5 by Psm. Treatment with SA did not induce DGK5 expression (data not shown), supporting this hypothesis.

gdk5 is affected in SA signaling downstream of SA

The gdk5 mutant is affected in resistance against SA-controlled pathogens like Psm and Hpp. To determine whether gdk5 has a defect in the SA-dependent signal transduction pathway, we assessed the induction of PR1, which is a marker gene for SA-triggered resistance. Fig. 5a shows that infection of wild-type with Psm greatly induced PR1 gene expression. In the gdk5 mutant, the induction level was more
Fig. 5. PR-1 and PR-2 expression and disease resistance in dgk5 and npr1
(a) Expression of PR-1 at different times after inoculation with Psm as determined by quantitative RT-PCR. The ratio of PR-1/UBI was depicted relative to that at 0 hpi in wild-type. \( \Delta C_T \) PR-1/UBI at 0 hpi was 15.57. The experiment was repeated twice with similar results. (b) Expression of PR-1 at different times after treatment with 5 mM of SA. The ratio of PR-1/UBI was depicted relative to that at 2 hpt (hours post treatment) of mock-treated wild-type. \( \Delta C_T \) PR-1/UBI at 2 hpt mock was 7.88. This experiment was repeated twice with similar results. (c) Titer of Psm in leaves 3 dpi. Different letters indicate statistically significant differences between genotypes (Tukey HSD test; \( \alpha = 0.05 \)). For details see the legend of Fig. 1b. The data presented are from a representative experiment that was repeated four times with similar results. (d) Expression of PR-2 at 24 hours after treatment with 1 mM of SA. The ratio of PR-2/UBI was depicted relative to that at 0 hpt of wild-type. \( \Delta C_T \) PR-2/UBI at 0 hpt mock was 4.41.
than 20-fold lower than found in wild-type and comparable to the established SA signaling mutant \textit{npr1}. Likewise, treatment of plants with SA resulted in a strong induction of \textit{PR1} gene expression in wild-type plants, but this induction level was much lower in \textit{dgk5} mutants, comparable to that in SA-treated \textit{npr1} mutants (Fig. 5b). These data indicate that the \textit{dgk5} mutant is impaired in SA signaling and that DGK5 has a role downstream of SA.

To verify whether the \textit{dgk5} mutation affects disease resistance signaling as severely as \textit{npr1} does, we compared the basal resistance levels of both mutants. Fig. 5c shows that \textit{in planta} growth of \textit{Psm} was higher in both mutants. However, \textit{Psm} multiplied to higher levels in \textit{npr1} compared to \textit{dgk5}, suggesting that parts of the disease resistance response other than SA-induced \textit{PR1} are intact or less affected in \textit{dgk5} compared to \textit{npr1}. This possibility was tested by determining the expression of another SA-regulated gene, \textit{PR-2}. Fig. 5d shows that SA treatment resulted in induction of \textit{PR-2} in \textit{dgk5} to the same extent as in wild-type, indicating that a subset of the SA responses is still intact in the \textit{dgk5} mutant.

\textit{PA treatment protects \textit{dgk5} against \textit{Psm} but not by restoring \textit{PR1} expression}

The reduced resistance of \textit{dgk5} to \textit{Psm} is likely due to a reduced increase in PA levels. We wanted to verify whether PA functions either as a signaling molecule mediating activation of defense responses or exerts a direct toxic effect on the pathogen. An \textit{in vitro} \textit{Psm} growth assay in the presence of PA was performed to determine the effect of PA on \textit{Psm} growth. The bacteria were cultured in King’s medium B (KB) without MgSO\textsubscript{4}, because PA precipitates in the presence of Mg\textsuperscript{2+}. KB without MgSO\textsubscript{4} allowed bacterial proliferation, albeit to a lesser extent (Fig. 6a). Addition of 0.1 mM of PA did not significantly affect the growth of \textit{Psm}, while 1 mM of PA was even found to stimulate growth (Fig. 6a). A direct inhibitory effect of PA on \textit{Psm} is therefore very unlikely.
Fig. 6. Study of the effect of PA treatment on disease resistance
(a) In vitro growth of Psm in KB medium with or without MgSO_4 (Mg) and supplemented with either 1 mM or 0.1 mM of PA. Bacterial titer was determined at the times indicated. (b) 1 mM of PA was pressure infiltrated in leaves at 20 hours before challenge inoculation with Psm of the same leaves. Control (Ctrl) plants were not pre-infiltrated and mock plants were pre-infiltrated with water. Growth of Psm is depicted as means (cfu/cm²) with standard deviations from 8 sets of 2 randomly selected leaf discs of 12 plants per genotype at 3 days after inoculation. The experiment was repeated with similar results. (c) For treatments, see legend to Fig. 5a. PR-1 expression was determined by quantitative RT-PCR and the ratio of PR-1/UBI was depicted relative to that at 0 dpi in wild-type. ΔCt PR-1/UBI at 0 hpi was 8.27. (d) Uptake of NBD-PA by wildtype mesophyll cells. Confocal images were taken 1 day after pressure infiltration of the leaf with liposomes containing 5 μM NBD-PA:95 μM PA (16:0 18:1).
Next, we tested whether PA could restore the resistance defect of *dgk5* by pressure infiltration of PA in the leaves. As expected, untreated *dgk5* allowed more growth of *Psm* than wild-type plants (Fig. 6b). The mock treatment, consisting of pressure infiltration of water in leaves 20 hrs before challenging them with *Psm*, caused a small decrease in *Psm* growth in both wild-type and *dgk5*. Treatment with PA led to an additional reduction of *Psm* growth in the *dgk5* mutant, reaching levels comparable to those in mock-infiltrated wild-type plants. The PA treatment did not affect *Psm* growth in the wild-type plants (Fig. 6b). Thus, application of PA protected *dgk5* mutants to *Psm* infection, suggesting that it supplemented for the lost increase in PA production due to the *dgk5* mutation.

To verify whether the PA treatment complemented the defect in DGK5-mediated defense signaling in *dgk5*, we studied the expression behavior of the *PR1* gene in *dgk5* after PA treatment. Fig. 6c shows that PA treatment alone did not induce *PR1* in wild-type plants, which is in line with our bioassay findings that no resistance is induced by PA in wild-type. *PR1* is also not induced in *dgk5* by PA treatment, which is to be expected as in wild-type also no *PR1* is induced. Infection with *Psm* led to *PR1* induction, but the expression level was not influenced by pretreatment with PA, neither in wild-type nor in *dgk5* (Fig. 6c). This indicates that the PA treatment did not complement the defect signaling pathway in *dgk5*, and that consequently, the protection to *Psm* in *dgk5* accomplished by PA treatment is unlikely due to a restoration of the DGK5-mediated signaling pathway.

To gain more insight in the mechanisms responsible for the protection accomplished by PA treatment, the fate of the infiltrated PA in the leaves was examined. PA labeled with the fluorophore nitrobenzoxadiazole (NBD) allowed us to visualize the PA. Liposomes consisting of PA-NBD mixed with non-labeled PA with or without the carrier (PC; phosphatidylcholine) were taken up by the cells within minutes after infiltration, as was seen by fluorescent cytoplasmic strands (not
shown). After 20 hrs, the PA-NBD was present at the edges of the cells, possibly the plasma membrane or cell wall (Fig. 6d). Remarkably, it seemed trapped at this location, because upon photobleaching of a section of a cell, which renders this part non-fluorescent, no recovery of the signal was observed, implying that PA-NBD had not moved from fluorescent parts of the tissue to the bleached section. These data support our finding that exogenous PA application unlikely functions as DGK5-generated PA.

**Discussion**

Here, we provide genetic evidence that DGK5 is required for resistance of Arabidopsis against the virulent pathogens *Psm* and *H. parasitica* (Fig. 1). None of the tested lines carrying mutations in other DGKs or in PLCs displayed an altered resistance level to these pathogens due to reduced expression of the PLC or DGK genes. This suggests that either none of these enzymes is involved in resistance to these pathogens, or that (several of) the PA-generating enzymes function redundantly in the activation of defense signaling.

Expression of the *DGK5* gene was induced upon infection with *Psm*, which was unaffected in *pad4, sid2* and *npr1*, indicating that *DGK5* induction is independent of SA signaling (Fig. 4). *Psm*-induced *PRI* expression was strongly affected in *dgk5* (Fig. 5a). The levels of *PRI* expression in *dgk5* were comparable to those found in the SA signaling mutants *npr1* (Fig. 5a), *pad4* and *sid2* (data not shown), placing DGK5 firmly in the SA signaling pathway. Unlike *pad4* and *sid2*, the *dgk5* phenotype could not be rescued by exogenous application of SA, suggesting that DGK5 acts downstream of this important signaling molecule (Fig. 5b).

*NPR1* responds to SA by translocating to the nucleus where it activates gene expression amongst which *PRI* [48]. We are currently crossing the *dgk5* mutation
into the transgenic line 35S::NPR1:GFP to determine whether DGK5 affects the nuclear localization of NPR1 upon activation of the defense. It is unlikely that loss of DGK5 fully undermines the function of NPR1, because bacterial proliferated to lower levels in dgk5 compared to npr1 (Fig. 5c) and the defense gene PR-2 is induced in to wild-type levels by SA in dgk5 (Fig. 5d). This indicates that only a subset of NPR1-dependent defense responses are affected in dgk5. Besides PR genes, also genes involved in protein secretion have been reported to be regulated upon nuclear translocation of NPR1 monomers [21]. Evidence was provided that NPR1 regulates the expression of secretion-related and PR genes through different transcription factors and cis-elements. DGK5 may be involved in PRI gene-regulating activity, while other responses downstream of NPR1 that contribute to resistance are still intact. An example of such a protein is the whirlly transcription factor AtWHY1 that has been shown to bind to promoters of PR genes in a SA-dependent and NPR1-independent manner [49, 50]. Mutations of AtWHY1 in its DNA-binding domain were found to affect SA-induced PRI expression and resistance to H. parasitica [50]. Together, these results suggest that AtWHY1 works in conjunction with NPR1 to activate SA-dependent defenses [49]. It may be possible that DGK5 affects AtWHY1 function, which would render the plant unresponsive to SA with regard to PRI gene expression and resistance.

Confocal microscopy studies showed that GFP-labeled DGK5 is localized at the plasma membrane (Fig. 3). Since ectopic expression of DGK5:GFP functionally complemented the dgk5 mutant (Fig. 2), it is likely that the observed plasma-membrane localization of DGK5:GFP reflects the true localization of DGK5. In accordance, DGK activity is typically enriched in plasma membrane preparations [51, 52]. It is unclear what targets DGK5 to the plasma membrane, as no obvious membrane targeting domains were identified [43].
Our study indicates that DGK5 acts downstream of SA in plant defense signaling. SA is synthesized in the chloroplast and exported to the cytoplasm where it induces a change in redox and consequently induces monomerization of NPR1, leading to its translocation into the nucleus [16]. It is unclear how plasma membrane-localized DGK5 can influence SA responsiveness. A candidate downstream response of DGK5 is the PA-mediated activation of the protein kinase cascade composed of PDK1 and OXI1, which has been shown to function in disease resistance (29-31).

In yeast, PA binds to the transcription factor Opi1p. Only when PA levels decrease, Opi1p is released and subsequently enters the nucleus where it represses target genes [53]. Although no such mechanism has been described for plants, it could explain how DGK5 influences gene expression downstream of SA. Identification of additional PA binding proteins will increase our understanding of how PA functions in disease resistance.

Several pathogen elicitors have been reported to induce PA formation in plant cell cultures [54-56]. Effort was taken to measure PA formation induced by live Psm, Psm cell extract and flagellin, but none of the treatments reproducibly induced PA in wild-type leaf discs or seedlings (data not shown). This may be due to very localized and transient increases in PA content. To solve this problem, a PA biosensor should be developed, as has been described for PI3P [57] and PI(4,5)P2 [58]. These biosensors are fusions of GFP with specific lipid binding domain which have been expressed in cell cultures and plants. Currently different PA-binding motifs are constructed and tested (J.E.M. Vermeer, R. van Wijk, C. Testerink and T. Munnik, unpublished data).

Our data show that exogenous application of PA led to an enhanced protection of the \textit{dgk5} mutant to \textit{Psm} infection but this was not accompanied by an enhanced expression of \textit{PR1} after \textit{Psm} challenge (Fig. 6b,c). Therefore, it is unlikely that PA
treatment complemented the defense signaling defect of *dgk5*. More likely, PA exerted its protective effect to *Psm* through blocking the accessibility of the plant cells to the pathogen, as we saw that the NBD-labeled PA got stuck at the outer layer of the cells (Fig. 6d). Somehow, PA treatment rigidified the cell structure. Others have reported that leaf discs floating in PA solutions exhibited chlorosis and cell death [59, 60]. The expression of *PR1* observed in these symptomatic leaf discs [59] does not necessarily has to be a direct effect of PA and could be explained by a last attempt of self defense by the macerated tissue. We never observed the cell death effects of PA treatment in infiltrated leaves or floating leaf discs, also not after careful studying of trypan blue-stained tissue (data not shown). This discrepancy may be explained by the fact that we apply PA in the more natural form of liposomes generated by an extruder instead of solutions that were sonicated only.

**Materials and methods**

*Arabidopsis genotypes and growth conditions*

*Arabidopsis thaliana* ecotype Col-0 was used throughout the study. Mutants *npr1* was *npr1-1* [61], *pad4* was *pad4-1* [62], and *sid2* was *sid2-5* [63]. The *dgk5* mutant was isolated from the Syngenta Arabidopsis Insertion Library [64]. Mutant *dgk5* was line SAIL_1212_E10 (see Fig. 2a and the legend to it for the structure of the *DGK5* gene and the localization of the mutation). The wild-type *DGK5* locus was identified by PCR amplification using *DGK5*-specific primers F (5'-GAC ATT GCC TGA GTG CTA CAT-3') and R (5'-CCT GAT GGC GGG GTG TTG A-3') that flank the insertion. The mutant *dgk5* locus was identified using primer F and a T-DNA specific primer (5'-GCT TCC TAT TAT ATC TTC CCA AAT TAC CAA TAC A-3'). Plants were grown in soil and assayed in a controlled-environment chamber at 21°C, 70% relative humidity, and 150 μM·m⁻²·sec⁻¹ of cool white fluorescent illumination with a 13-h light/11-h dark cycle.
Bioassays

*Psm* and *Pto avrRpt2* were cultured in King’s B medium (KB) as described [63]. Leaves were inoculated with the bacteria by pressure infiltration using a syringe. Bacterial titers were determined in cut leaf discs that were homogenized and plated on KB agar containing 25 mg·L⁻¹ streptomycin as described [63]. Culturing of *A. brassicicola* and bioassays were performed as described [65]. For bioassays with *H. parasitica* Waco9, three-week-old plants were misted with a spore suspension containing 5x10⁴ sporangiospores/mL [66]. Disease symptoms were scored at 9 days after inoculation for about 280 leaves per genotype.

Construction of transgenic plants

*DGK5* cDNA was PCR-amplified with Gateway-compatible primers 5’- AA AAA GCA GGC TCA ATG GAG AAA TAC AAC AGT T-3’ and 5’ A GAA AGC TGG GTT ACA GAG CAC ATG TGA CCA TG –3’, followed by PCR-amplification with Gateway-adapter primers. The resulting DNA fragment was recombined into pDONR yielding pENTR-DGK5. The DGK5 cDNA fragment was subsequently recombined into the binary vector pGWB5 yielding pGWB5-DGK5. pGWB5-DGK5 harbors *DGK5:GFP* under control of the 35S promoter. pGWB5-DGK5 was transformed to *dgk5* by floral dipping. Primary transformants were selected on 20 μg/ml hygromycin and allowed to self. T₂ progeny plants that displayed fluorescence were selected and bred to homozygousity. The first two homozygous T₃ families isolated were tested for complementation.

Protein extraction and western blot

Protein extraction buffer (9.5 M Urea, 0.1M Tris-HCl pH 6.8, 2% (w/v) SDS and 2% (v/v) β-mercapto-ethanol) was added to an equal volume of ground leaf tissue, mixed and centrifuged in an eppendorf centrifuge for 10 min at maximal speed. Sample buffer was added to the supernatant and samples were loaded on a 10% SDS-PAGE gel, blotted on nitrocellulose and incubated overnight in PBST with 5%
(w/v) powdered milk and a monoclonal anti-GFP antibody (mouse ascites fluid, Sigma). The blot was washed three times in PBST and incubated for 1 hour with an appropriate peroxidase-conjugated secondary antibody. The peroxidase activity was detected by enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Equal loading was confirmed by staining the blot with Ponceau S.

Confocal microscopy
For confocal microscopy, Arabidopsis seedlings expressing AtDGK5:GFP were grown for 5 days at 21 °C and transferred to object slides containing a fixed coverslide and ½ MS supplemented with 1% (w/v) sucrose. Microscopy was performed on a Zeiss LSM 510 CLSM (Confocal laser scanning microscope) (Carl Zeiss GMBH, Jena, Germany). Confocal configurations were as described before [67].

Preparation of PA suspensions
Synthetic PA 16:0 18:1 (Avanti Polar Lipids, Alabaster, AL, USA) was used in all experiments. PA that was suspended in chloroform was dried in the speedvac and sonicated in deionized water. This was used for the in vitro growth assays of Psm and the Psm bioassays. For the confocal imaging of PA-NBD (Avanti Polar Lipids, ask Christa) liposomes of PA-NBD mixed with non-labeled PA or PC (dioleoyl phosphatidylcholine; Avanti Polar Lipids) were used (5 μM PA-NBD:95 μM PA:400 μM PC). For this purpose, the lipids were mixed in the right molar ratios before drying in the speed vac. After sonification in 1 mM of MES buffer, unilamellar vesicles were produced using a lipid extruder (0.2 μm filters; Avanti Polar Lipids) according to the manufacturer's instructions.

RNA extraction and quantitative RT-PCR
Total RNA was extracted as described previously [68]. Gene expression was analyzed by quantitative RT-PCR. Five μg of RNA was digested with Turbo DNA-
free (Ambion, Huntingdon, United Kingdom) according to the manufacturer’s instructions. To check for contamination with genomic DNA a PCR was performed on the DNase-treated RNA. DNA-free RNA was converted to cDNA using oligo-dT18 primers, dNTPs, and SuperScript III Reverse Transcriptase (Invitrogen, Breda, The Netherlands) according to the manufacturer’s instructions. Quantitative PCR was performed on the cDNA using SYBR Green Supermix reagent (Invitrogen) in a final volume of 15 μL, following the manufacturer’s protocol, using the Applied Biosystems 7500 real time PCR machine. Gene-specific primers were designed for DGK5 and PRI: DGK5 F (5'- GAC TCA GTG GCT GAA GGC G-3'), DGK5 R 5'- ACT GTG TTC TCC CTC ATC AGG AA 3' (over intron), PRI F (5'-CTC GGA GCT ACG CAG AAC T-3'), and PRI R (5'-TTG CGA CAC ATG TTC A-3'). Primers for the reference gene UBI10 (At4g05320) were UBI10 F (5'-GGC CTT GTA TAA TCC CTG ATG AAT AAG-3') and UBI10 R (5'-AAA GAG ATA ACA GGA ACG GAA ACA TAG T-3'). Ct values were normalized to Ct of UBI10, after which the fold-differences in transcript levels were calculated.

Acknowledgments

We would like to thank Dorus Gadella and Erik Manders of the Centre for Advanced Microscopy for using their microscopes. Dieuwertje van der Does and Hana Návarová are acknowledged for excellent technical assistance.

References

5. Tao, Y., Z. Xie, W. Chen, J. Glazebrook, H.-S. Chang, B. Han, T. Zhu, G.Z. Zou and F. Katagiri 2003 Quantitative nature of Arabidopsis responses during compatible and

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incompatible interactions with the bacterial pathogen *Pseudomonas syringae*. Plant Cell. 15: 317-330.


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**Supplemental table 1**

1. Insertion site based on sequence read using the left border primer for the insertion as obtained from the website [http://signal.salk.edu/](http://signal.salk.edu/), depicted as base number relative to the ATG start site. > and < indicate that the insertion site is further away or closer to the predicted site, respectively (depending on the orientation of the insertion).

2. Bioassays were performed as described in materials and methods. For *Psm* assays, disease was quantified by determining bacterial titer at 3 days after inoculation. For *Pto avrRpt2* and *Alternaria* assays, the amount of necrosis of the inoculated tissue was scored by eye. x, no difference in disease compared to wild type; eds, enhanced disease susceptible.

3. Susceptible phenotype was not genetically linked to the mutation in *DGK2*.

4. This line constitutively expressed high levels of the *PLC7* gene due to the insertion upstream of the coding sequence.

5. No plants carrying a homozygous mutation in the particular PLC genes were identified, only heterozygous plants, which were not tested in the bioassays.

6. These lines were kindly provided by J.E. Gray (University of Sheffield, UK).

7. Enhancer Trap line from Jack collection ([http://www.dartmouth.edu/~tjack/](http://www.dartmouth.edu/~tjack/)) and SLAT line from Jones collection ([http://arabidopsis.info/info/slat_info1.htm](http://arabidopsis.info/info/slat_info1.htm))
Supplemental table 1. Resistance to different pathogens of Arabidopsis T-DNA insertion lines carrying mutations in DGK or PLC.

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DGK5 is required for disease resistance.