Nitric Oxide is Critical for Inducing Phosphatidic Acid Accumulation in Xylanase-elicited Tomato Cells

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Nitric Oxide (NO) is a second messenger related to development and (a)biotic stress responses in plants. We have studied the role of NO in signaling during plant defense responses upon xylanase elicitation. Treatment of tomato cell cultures with the fungal elicitor xylanase resulted in a rapid and dose-dependent NO accumulation. We have demonstrated that NO is required for the production of the lipid second messenger phosphatidic acid (PA) via the activation of the phospholipase C (PLC) and diacylglycerol kinase (DGK) pathway. Defense-related responses downstream of PA were studied. PA and, correspondingly, xylanase were shown to induce reactive oxygen species production. Scavenging of NO or inhibition of either the PLC or the DGK enzyme diminished xylanase-induced reactive oxygen species production. Xylanase-induced PLDβ1 and PR1 mRNA levels decreased when NO or PA production were compromised. Finally, we have shown that NO and PA are involved in the induction of cell death by xylanase. Treatment with NO scavenger cPTIO, PLC inhibitor U73122, or DGK inhibitor R59022 diminished xylanase-induced cell death. On the basis of biochemical and pharmacological experimental results, we have shown that PLC/DGK-derived PA represents a novel downstream component of NO signaling cascade during plant defense.

Plants are constantly challenged by pathogens. To resist these pathogens, they can activate a battery of responses or defense mechanisms as part of what is generally referred to as the plant defense response. The first step in the induction of the plant defense response is the recognition of certain pathogen-derived molecules known as elicitors. Downstream signal transduction cascades become activated upon recognition of these elicitors.

One of the second messengers reported to participate in plant defense responses is nitric oxide (NO). NO treatments induce plant defense-related transcript accumulation (1, 2), whereas treatments with different inhibitors of NO accumulation compromise the hypersensitive response (HR), a form of programmed cell death induced during plant defense (3). Cytotoxic, an elicitor from oomycete Phytophthora cryptogea, triggers NO production that subsequently participates in the mobilization of Ca2+ from internal stores, thereby increasing Ca2+ cytosolic concentrations (4). In animals, NO has been shown to control Ca2+ homeostasis (5). In addition, NO and reactive oxygen species (ROS) have been found to act together triggering apoptosis and executing invasive pathogens (6). In plants, a balanced production of NO and ROS was required for HR (7, 8). Nevertheless, how exactly these two second messengers are related is unknown.

The oxidative burst is a massive production of ROS that can act (i) directly on the pathogens, (ii) indirectly by strengthening the cell wall, and (iii) as a signal activating downstream responses such as the induction of pathogenesis-related (PR) proteins and HR. In plants, there is pharmacological evidence that NADPH oxidase is the main source of ROS required for the induction of HR (9). Genetic experiments have shown that NADPH oxidase is essential for ROS production in the interaction between Arabidopsis and avirulent Pseudomonas syringae pv. tomato but also that the effect on the HR is modest (10). More recently, it was demonstrated that ROS generated by Arabidopsis NADPH oxidase can antagonize salicylic acid-dependent pro-death signals (11). It has been observed that the lipid signaling molecule phosphatidic acid (PA) is involved in the activation of NADPH oxidase in macrophages (12). In plants, PA has been shown to be able to trigger an oxidative burst suggesting a similar activation of NADPH oxidase (13–15). Protein kinase (PDK1) reported to be involved in oxidative burst-mediated signaling, has also been shown to bind PA and to be activated by PA (16, 17).

PA is produced in response to many different stresses including drought, pathogen attack, and oxidative stress (18, 19). It is generated via two distinct phospholipase pathways, either directly by phospholipase D (PLD) or by the sequential action of phospholipase C (PLC) and diacylglycerol (DAG) kinase (DGK). Both pathways have been shown to be involved in the generation of the oxidative burst in several pathosystems (13, 20–22). PLD hydrolyzes structural phospholipids, such as

HR, hypersensitive response; PR, pathogenesis-related; NADPH, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; SNAP, S-nitroso-N-acetylpenicillamine; cPTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide potassium; IP3, inositol 1,4,5-trisphosphate; PIP2, phosphatidylinositol 4,5-bisphosphate.
phosphatidylcholine (PC), to PA. PLC hydrolyzes phosphatidyl-
linositol (PI) 4,5-bisphosphate (PIP$_2$) into two different second
messengers, DAG and inositol 1,4,5-trisphosphate (IP$_3$). IP$_3$
is described in animals in the signaling of Ca$^{2+}$ release from inter-
nal stores, whereas in plants, DAG is rapidly phosphorylated by
DGK, generating PA (23). PA generated via the PLC/DGK-en-
zymatic pathway occurs in response to pathogen-derived mol-
cules, such as specific elicitors or pathogen-associated molec-
ular pattern (13, 20, 22, 24). A well studied pathogen-associated
molecular pattern is xylanase. Recently, a xylanase receptor was
identified in tomato (25). Xylanase perception triggers ethylene
biosynthesis, electrolyte leakage medium alkalinization, gene
expression, and HR (26–29). So far, the intermediate signals
have not been well defined. In cultured tomato cells treated
with xylanase, both PLD and PLC/DGK is activated (24, 29); in
addition, ROS production has been demonstrated (30). We
hypothesize a role for NO downstream of xylanase perception
and have investigated (i) NO production; (ii) the function of NO
in reported PA signaling; and (iii) the function of NO and PA in
the induction of ROS, gene expression, and cell death. Our
results indicate that xylanase treatment induces NO and that
NO is necessary for PA production. Moreover, NO and the
subsequent PA generation via the PLC/DGK pathway, are
required for xylanase-induced ROS production, gene expres-
sion, and cell death. These results provide evidence that xyla-
nase-induced NO activates PLC/DGK signaling during plant
defense.

**EXPERIMENTAL PROCEDURES**

**Material**—Xylanase from *Trichoderma viride* was purchased
from Fluka (Buchs, Switzerland). Tungstate, reagents for lipid
extractions and subsequent analysis and silica 60 thin-layer chro-
matography plates were purchased from Merck (Darmstadt, Ger-
many). The NO donor S-nitroso-N-acetylpenicillamine (SNAP);
the NO scavenger 2- (4-carboxyphenylalanine)4,4,5,5 tetra-
many). The NO donor

**Visualization of NO Using Fluorescence**—Endogenous NO
was monitored by incubating 100 μl of cells with 1 μM DAF-
2DA (or negative control with fluorophore 4-AF DA) for 30 min
at 25 °C in the dark. Thereafter, the cells were subjected to
different treatments (for the time periods indicated) and
mounted on microscope slides. Fluorescence of cells was visu-
alized by fluorescence microscopy with an excitation filter of
485 nm and a barrier filter of 515 nm and bright field micros-
copy in an Eclipse E200 (Nikon, Tokyo, Japan) microscope. The
production of green fluorescence under these conditions was
due to NO. Pictures show general phenomena representative of
at least six individual experiments.

**Quantification of NO and ROS Production by Fluorometry**—
Fluorometric measurements were performed in a Fluoroskan
Ascent microwell fluorometer (Thermo Electron Company,
Vantaa, Finland) using Chroma (Chroma Technology Corp.,
Rockingham, VT) filters D480-40 and D525-30 for excitation and
emission, respectively. Eighty-microliter batches of cul-
tured cells were carefully pipetted into wells of a Greiner
96-well plate containing 0.5 μM DAF-FM-DA or 0.025 μM
H$_2$DCF-DA and 20 μl of xylanase in the presence or absence
of inhibitors or scavengers, as indicated in Figs. 1 and 4. The
microwell plate was immediately transferred to the fluorom-
erometer for measurement. Fluorescence of each individual well
was measured every 2 min over 20 ms at 25 °C. In between
measurements, the temperature was maintained at 25 °C,
and cells were shaken at 120 revolutions per min with a 1-cm
rotation. All experiments were performed in triplicate.

**32P Phospholipid Labeling and Analyses**—Eighty-five micro-
liters of Msk8 cells were labeled for 3 h with 5 μCi of carrier-free
$^{32}$PO$_4$$^{3-}$ (Amersham Biosciences) prior to treatment with xyla-
nase or SNAP for the time periods and with the concentrations
indicated. Control treatments were performed by adding cell-
free medium. Incubations were stopped by adding 20 μl of 50%
perchloric acid and subsequently frozen in liquid nitrogen. For
short-labeling experiments, 85 μl of cell suspension was equil-
brated in a 2-ml reaction vial for 2 h. Two minutes prior to the
addition of xylanase or SNAP with the concentrations indi-
cated, 20 μl of cell-free medium containing 5 μCi of $^{32}$PO$_4$$^{3-}$
was added to the cells. Incubations were stopped as mentioned
above. The lipids were extracted by adding 3.75 volumes of
CHCl$_3$:MeOH:HCl (50:100:1, v/v) and processed as described
previously (32). The lipids were separated on Silica-60 thin-
layer chromatography (Merck) plates employing EtAc (EtAc/
iso-octane/formic acid/H$_2$O 13:2:3:10, v/v) as a mobile phase.
When indicated, alkaline solvent (CHCl$_3$:MeOH:H$_2$O (25%, w/v),
NH$_4$OH:H$_2$O (90:70:4:16, v/v)), heat-activated impregnated
thin-layer chromatography plates (1.2%, w/v), potassium oxal-
ate, and 2 mM EDTA in MeOH:H$_2$O (2.3, v/v) were used.
Radioactivity was visualized by autoradiograph. The autoradi-
ographs represent general phenomena, representative of at least
three individual experiments. Quantification of PA levels was
performed by plot analysis using Imagel (version 1.32) proc-
essing of non-overexposed autoradiographs. PA levels were
quantified against the standard structural phospholipid levels
(PS/SL) for Fig. 2. For Fig. 3, PA levels were quantified against
the total incorporation of $^{32}$P into phospholipids. The PA level of each sample was subsequently expressed as fold, taking the PA level of the control as 1.

RNA Blot Analysis—Total RNA from tomato cells was isolated using TRIzol as described by the manufacturer (Invitrogen). Fifteen micrograms of total RNA was denatured using glyoxal and subsequently separated on 1.5% agarose gel electrophoresis, and then transferred onto Hybond-XL (Amersham Biosciences). Membranes were hybridized in modified Church solution (0.5 M phosphate buffer, pH 7.2, 7% SDS, and 10 mM EDTA) (33) overnight at 65 °C with radiolabeled fragments of PR1 (34) or PLDβ1 (29). Fragments were radiolabeled with the Megaprime DNA labeling system according to the manufacturer's description (Amersham Biosciences) using $[^{32}$P]dCTP (Amersham Biosciences). The membranes were washed twice with $2\times$ SSC, 0.1% SDS for 15 min, $1\times$ SSC, 0.1% SDS for 30 min, and $0.2\times$ SSC, 0.1% SDS for 30 min at 65 °C. Hybridization patterns were visualized by autoradiograph. Before rehybridization, blots were stripped by washing the membrane with boiling in 0.5% SDS.

Cell Death—Cell death experiments were performed treating 100 μl of cells with 200 μg/ml xylanase, or 10 μM PA and PC in the presence or absence of inhibitors, as indicated in the figure 6 for 19 h at 25 °C. To quantify cell death, the cells were incubated for 10 min with 0.2 μM CytoxGreen and washed with cell-free medium. The fluorescence was then measured in a Fluoroskan Ascent microwell fluorometer using Chroma filters D480-40 and D525-30 for excitation and emission, respectively. Values are expressed as fold induction with respect to the non-treated cells. Four independent experiments were performed in triplicate. In addition, cells were evaluated by fluorescence microscopy with an excitation filter of 485 nm and a barrier filter of 515 nm and bright field microscopy in an Eclipse E 200 (Nikon, Tokyo, Japan) microscope. Pictures show general phenomena, representative of at least three individual experiments.

RESULTS

Xylanase Triggers NO Accumulation—To investigate whether the elicitor xylanase is able to trigger NO formation, tomato cell suspensions were pretreated for 30 min with the NO-specific fluorophore DAF-2DA (35) and subsequently incubated in the presence or absence of xylanase. Fig. 1A shows that xylanase induced high levels of NO (as observed by green fluorescence) as compared with the control cells. Fluorescence was abolished in the presence of the NO-specific scavenger cPTIO (Fig. 1A). No fluorescence was observed in the presence of the control fluorophore 4-AF DA (data not shown). This indicates that the fluorescence was caused by NO production. A qualitative dose-response experiment was performed using a fluorometer. NO-specific fluorescence obtained with the NO-specific probe DAF-FM-DA (36) was monitored for 30 min in cells treated with increasing concentrations of xylanase (Fig. 1B). Production of NO was observed from 1 μg/ml xylanase, increasing steadily until a maximum production was reached at 400 μg/ml xylanase. The production of NO was rapid and sustained over at least 30 min. The NO-specific scavenger cPTIO reduced NO levels (Fig. 1B). Several sources of NO, either non-enzymatic or enzymatic, have been described in plants. Enzymatic sources include a nitric-oxide synthase-like source and nitrate reductase, but evidence for other enzymatic NO sources exists (37). To identify the putative source of NO, inhibitors for NO-producing enzymes were applied. Nitric-oxide synthase inhibitors, such as S-ethyl-L-Itu (1 mM; (38)), L-NMMA (10 mM), and L-NAME (10 mM), were tested. NO levels were determined using a microwell fluorometer over a 30-min period and expressed as relative fluorescence units (RFU). Error bars represent S.E. of the means. A representative graph of three independent experiments is shown. Note that tomato cells treated in the presence of NO scavenger cPTIO (1 μM) showed no significant fluorescence increase.

FIGURE 1. Xylanase-treated tomato cells accumulate NO. A, NO detection by the fluorescent probe DAF-2DA. Cells were treated with cell-free medium (Control) or 200 μg/ml xylanase. Photos were taken 30 min after treatment. Note that tomato cells treated in the presence of NO scavenger cPTIO (1 μM) showed no fluorescence. A bright field image for each treatment is shown below the fluorescent image. A representative picture of six independent experiments is shown. Scale bar = 5 μm. B, dose- and time-response curves of NO production. Cells were treated with 0, 1, 10, 100, or 400 μg/ml xylanase in the presence of the NO-specific fluorescent probe DAF-FM-DA. Fluorescence was determined using a microwell fluorometer over a 30-min period and expressed as relative fluorescence units (RFU). Error bars represent S.E. of the means. A representative graph of three independent experiments is shown. Note that tomato cells treated in the presence of NO scavenger cPTIO (1 μM) showed no significant fluorescence increase.
or 1-NAME (10 mM) did not affect xylanase-induced NO production (data not shown). Inhibition of nitrate reductase by 1 mM tungstate did not result in a decrease of NO levels upon xylanase treatments (data not shown).

Xylanase-induced PA Formation Depends on NO—Fig. 1 shows that xylanase triggers NO production in tomato cells within minutes. Xylanase also triggers PA accumulation in tomato cells as shown previously (24). Therefore, we investigated whether NO is involved in PA production in xylanase-treated cells. Phospholipids were labeled by the incubation of cells with $^{32}$Pi for 3 h. The cells were subsequently treated for 10 min with xylanase in the presence or absence of cPTIO. Lipids were extracted and separated by EtAc thin-layer chromatography. Top panels show a representative autoradiograph. SL, structural phospholipids. The quantification of four independent experiments is plotted underneath. PA levels are expressed as fold increase in relation to control samples in the absence of cPTIO. Error bars indicate S.E. of the means. A, inhibition of PA formation was evaluated with different concentrations of cPTIO in the presence of 200 μg/ml xylanase. B, induction of PA formation was evaluated with different concentrations of xylanase in the presence of 1 mM cPTIO.

FIGURE 2. Xylanase-induced PA formation is inhibited by the NO scavenger cPTIO. Suspension-cultured tomato cells were labeled with $^{32}$P, for 3 h and then treated for 10 min with xylanase in the presence or absence of cPTIO. Lipids were extracted and separated by EtAc thin-layer chromatography. Top panels show a representative autoradiograph. SL, structural phospholipids. The quantification of four independent experiments is plotted underneath. PA levels are expressed as fold increase in relation to control samples in the absence of cPTIO. Error bars indicate S.E. of the means. A, inhibition of PA formation was evaluated with different concentrations of cPTIO in the presence of 200 μg/ml xylanase. B, induction of PA formation was evaluated with different concentrations of xylanase in the presence of 1 mM cPTIO.

The PLC/DGK Activation Is Responsible for the NO-mediated PA Accumulation—Xylanase-dependent PA increase is reported to occur via both PLC/DGK and PLD. To demonstrate which of the two PA sources NO modulates, experiments were performed directed at either source. The main PA source during xylanase treatments is via PLC/DGK (24). PLC hydrolyzes PIP$_2$, generating DAG, which is subsequently phosphorylated by DGK to produce PA. A short radiolabeling strategy (39) was applied to study whether NO mediates PA formation via PLC/DGK. The method is based on the fact that $^{32}$P$_i$ is rapidly incorporated into the ATP pool but slowly into structural phospholipids. AT$^{32}$P can subsequently be used rapidly by DGK to phosphorylate the PLC-derived DAG to $^{32}$PA. In contrast, $^{32}$PA derived from PLD activity is only detected upon slow labeling of its substrate, a structural phospholipid such as PC. The latter requires long $^{32}$Pi labeling times. Consequently, after a short labeling period, any $^{32}$PA produced results from DGK and, hence, PLC activity. Accordingly, tomato cells were labeled for 2 min and treated with xylanase for 30 min. Fig. 3 shows that xylanase induces PA production via PLC/DGK activation. To evaluate whether NO mediates PLC/DGK-dependent PA increase, we studied the effect of cPTIO on xylanase-treated cells. NO scavenger cPTIO did inhibit the xylanase-induced PA production from 2.6- to 1.3-fold (folds as compared with PA levels in control cells) (Fig. 3). Specific inhibitors of PLC (U73122) or DGK (R59022) were used to corroborate that xylanase induces PA formation via PLC/DGK. Inhibition of PLC reduced PA levels from 2.6- to 1.4-fold in xylanase-treated cells. The DGK inhibitor R59022 reduced the xylanase-induced PA levels from 2.6- to 1.7-fold (Fig. 3). PA levels in U73122-, R59022-, or cPTIO-treated cells were comparable with the control (Fig. 3). These studies indicate that xylanase-treated cells generate PA via the PLC/DGK pathway and that NO is required in this process.

Experiments were also performed to test whether PLD contributes to the NO-triggered PA formation. PLD has the ability to transfer the phosphatidyl group from its substrate to a primary alcohol, such as 1-butanol (for details, see Ref. 39). cPTIO could not inhibit the xylanase-induced PLD activation (data not shown). However, because the PLD activation during xylanase treatments is low, typically 1.25-fold after 30 min (24), it cannot
be discarded that, under our experimental conditions, we were not able to measure a significant effect of NO on PLD activation.

**ROS and NO Production in Xylanase-treated Cells**—ROS production is one of the first responses to pathogen attack. Studies have shown ROS to occur upon xylanase elicitation. PA is involved in the production of ROS in plants (13–15), supposedly by the activation of NADPH oxidase, as has been shown in neutrophils (40, 41). Our results (Figs. 2 and 3) demonstrate that xylanase induces PA via NO, suggesting that NO could act upstream of ROS production. Studies have shown that both NO and ROS are required for the induction of HR (7). This led us to investigate the relationship between PA, NO, and ROS in xylanase-treated cells. ROS generation was quantified in a fluorimeter using the ROS-specific fluorescent probe H$_2$DCF-DA (42, 43).

Fig. 4A shows the effects of PLC inhibition, DGK inhibition, and NO scavenging on ROS production in xylanase-treated cells. The NO scavenger cPTIO lowered xylanase-triggered ROS production from 100 to 73%. Similar levels of inhibition were found when either the PLC or the DGK inhibitor was used (from 100 to 67 and 63%, respectively). Inhibition of ROS formation is incomplete, which is correlated to the incomplete inhibition of PA formation as shown in Figs. 2 and 3. Hence, these results indicate that xylanase-induced ROS production is, at least partially, dependent on both full NO production and full PLC/DGK activation. If NO acts upstream of PLC/DGK-dependent ROS production, then PLC/DGK inhibitors should not affect xylanase-induced NO levels. NO production in the presence and absence of PLC/DGK inhibitors was measured using the DAF-FM-DA fluorescent probe. The DGK inhibitor did not reduce the xylanase-induced NO production, whereas the PLC inhibitor U73122 slightly reduced the NO levels (Fig. 4B). The NO and ROS levels in control treatments were not affected by the inhibitors (data not shown).

**NO and PA are Involved in Xylanase-elicited Gene Expression and Cell Death**—Xylanase is known to induce an increase of mRNA levels of, among others, PR1 and PLDβ1. PLDβ1 has been demonstrated to be responsible for xylanase-induced PLD activation (29, 30). Transcript levels were studied by mRNA blot analysis. Fig. 5 shows a xylanase-induced increase of PLDβ1 and PR1 transcript levels. Preincubations with NO scavenger cPTIO inhibited this response, indicating that NO is required for the increase of transcript levels. PLC inhibitor U73122 also inhibits the increase in the mRNA accumulation of PLDβ1 and PR1 upon xylanase treatments (Fig. 5). The use of the inhibitors did not alter cell viability (Fig. 6). These data indicate that NO and PLC activity are required for PLDβ1 and PR1 transcript accumulations during xylanase treatments. Xylanase induces cell death, which is correlated with the oxidative burst (28). Here we show that xylanase induces ROS production and that this accumulation requires NO and PLC/DGK activation (Fig. 4). Because NO and ROS are involved in the induction of cell death and because xylanase-induced ROS production partially depends on both NO and PA, we studied...
whether xylanase requires NO and PA to induce cell death. Cells were treated with xylanase in the presence or absence of NO scavenger cPTIO, PLC inhibitor U73122, or DGK inhibitor R59022. Cell death was determined 19 h after treatments by measuring the fluorescence of non-cell-permeating DNA-intercalating dye CytoxGreen (44). Cell death is expressed as fold induction with respect to non-treated cells (control, Fig. 6A). Application of NO scavenger or either PLC or DGK inhibitor diminished the fluorescence to levels slightly above control levels (Fig. 6A). Finally, synthetic, water-soluble PA C8:0 treatment induced cell death to levels similar as obtained with xylanase (Fig. 6). Experiments performed with naturally occurring PA showed similar results (data not shown).

DISCUSSION

In this report, we have shown that xylanase treatment of tomato cells results in NO production (Fig. 1). PA production following xylanase treatment has been reported previously by van der Luit et al. (24). Upon pretreatment with the NO scavenger cPTIO, NO was no longer detected, corresponding with an observed loss of PA production (Figs. 1 and 2). The enzymatic source of PA was further investigated. The xylanase-induced PLC/DGK activation required NO (Fig. 3). This is the first evidence showing that NO generates PA via activation of the PLC/DGK pathway. Here we discuss the signaling of NO and PA upon xylanase perception, as well as downstream events such as ROS production, gene expression, and cell death in tomato cells.

NO has been shown to be involved in the plant defense response of a growing list of plant-pathogen interactions (reviewed in Ref. 45). The exact function of NO is currently unknown. NO is likely to play various roles, this in analogy to what is found in animals. At this stage, the source(s) of xylanase-induced NO generation is/are unknown. Surprisingly, neither nitric-oxide synthase inhibitors nor the nitrate reductase inhibitor affect the xylanase-induced NO production, suggesting that these enzymes are not involved in the NO generation in our experimental system. Because several sources for NO production have been described in plants, such as xanthine oxidase, peroxidase, cytochrome P450, and heme proteins (37), genetic evidence will be necessary to assess this aspect. A number of mechanisms by which NO activates PLC/DGK can be envisaged. NO could (i) act directly on PLC or DGK enzymes by nitrosilation or nitration (46–48); (ii) act indirectly, e.g. via a mitogen-activated protein kinase signaling cascade (49–51) or by increasing cytosolic Ca²⁺ levels (52); (iii) change the redox potential affecting the signaling state of plants (53). At this point, it is not clear which of the above mechanisms are involved in NO-mediated PLC/DGK activation. This will be subject of future studies. In animals, NO modulates PIP2 hydrolysis and the subsequent generation of IP3 negatively (54). NO inhibition of PLC mediated via accumulation of cGMP and activation of G kinase is a widespread regulatory process that involves different types of PLC (54). Our results show, contrary to what is described in animals, that NO activates (via a so far
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unknown mechanism, see above) the PLC/DGK pathway during plant defense. However, the PLC pathway in plants has been suggested to be different from the "canonical" pathway described for mammalian cells (55). In addition, mammalian cells contain 5 classes of PLC isozymes \( \beta, \gamma, \delta, \epsilon \) and \( \zeta \), whereas Arabidopsis has 7 putative PLC isozymes that are closely related to the animal PLC\( \zeta \) (56).

PA is, similar to NO, emerging as a novel second messenger. PA is in animals involved in many processes including the regulation of oxidative stress, cell proliferation and cytoskeleton rearrangement. One of the responses downstream PA reported earlier is the activation of NADPH oxidase in animals (12). In plant-pathogen interactions, several reports indicate that the oxidative burst is due to an activation of membrane-bound NADPH oxidase. Correspondingly, the induction of oxidative stress in plants occurs downstream PA (13–15, 21). Our results suggest that PLC/DGK-generated PA is involved in the rapid accumulation of ROS in xylanase-treated cells, and that NO is required in this process (Fig. 4). The oxidative burst in plant-pathogen interactions mediated by specific elicitors occurs in two phases. The first phase shows a rapid (5 min onward), transient (60 min) and low ROS production whereas the second phase shows a prolonged (3–6 h) and massive ROS production (9). A biphasic ROS generation was observed and associated with the activation of PLC and PLD in rice cells induced by N-acetylcysteine. The activation of both enzymes was shown for the first phase of ROS generation, whereas for the second phase only the activation of PLD was evident (20). Recognition of P. syringae AvrRpm1 or AvrRpt2 in Arabidopsis induced a biphasic accumulation of PA. The first wave was attributed to the PLC/DGK pathway, the second to PLD; both phospholipase pathways acted upstream of ROS formation (22). PA accumulated rapidly via PLC/DGK in tobacco cells expressing the tomato Cf-4 resistance protein treated with Cladosporium fulvum Avr4 protein. PLC activity was required for the rapid ROS accumulation (13). Our results show that, NO-dependent, PLC/DGK-generated PA is involved in the induction of ROS production during the first peak of the oxidative burst in xylanase-treated cells. PLD activation during xylanase treatments could contribute to activation of NADPH oxidase, as suggested in rice cells, via an NO-independent pathway. A second phase of oxidative burst has so far not been studied following xylanase treatments.

Various PA target proteins in animals have been identified over the years, e.g. PA has been shown to activate a protein kinase, protein phosphatases, a lipid kinase, a phospholipase and a transcription factor (57, 58). Also in plants a number of PA targets have been identified suggesting PA is involved in many processes (59). PA has been shown to bind a protein kinase in Arabidopsis (PDK1) and to activate protein kinase AGC2-1 in a PDK-dependent manner (16, 60). PDK1 is specifically activated by PLD-generated PA in cell suspensions treated with xylanase (61). AGC2-1 is identical to OXI1, a protein kinase implicated in oxidative burst-mediated signaling in Arabidopsis (17). Studies show that PA activates a calcium-dependent protein kinase (CDPK) and a wound-related mitogen-activated protein kinase cascade (62, 63). NO activates a mitogen-activated protein kinase and a CDPK during adventitious root formation (50, 64). PLD\( \alpha \)-derived PA interacts with ABI1 phosphatase 2C and regulates abscisic acid (ABA) signaling during stomatal closure in Arabidopsis (65, 66). NO was shown to act upstream of ABI1 in ABA-induced stomatal closure (67). Future experiments will be performed to elucidate which of the PA downstream targets are related to NO-triggered cascade during xylanase treatments.

Xylanase is known to induce an increase of mRNA levels of, among others, PR1 and PLD\( \beta \). PLD\( \beta \)1 has been demonstrated to be responsible for xylanase-induced PLD activation (29, 30). Fig. 5 shows a xylanase-induced increase of PLD\( \beta \)1 and PR1 transcript levels. Preincubations with either NO scavenger cPTIO or the PLC inhibitor U73122 inhibited this response (Fig. 5). The use of the inhibitors did not alter cell viability (Fig. 6). These data indicate that NO and PLC activity are required for PLD\( \beta \)1 and PR1 transcript accumulations during xylanase treatments.

ROS and NO have been shown to be required for cell death during plant defense (7, 8, 68). We showed that NO and PLC/DGK-derived PA are required for xylanase-induced cell death. All of the xylanase-induced responses evaluated in this study (PLC/DGK-derived PA, ROS generation, induction of gene expression, and cell death) could be blocked by depletion of endogenous NO. This indicates NO is required for these xylanase-induced responses. This puts forward the question whether NO is sufficient for these xylanase-induced responses. We performed a series of experiments with NO donors to investigate this. Exogenously applied SNAP induces PA formation in a dose-dependent manner (supplemental Fig. S1A), but the concentrations required are high. One mM SNAP induces a 2–3-fold increase in PA formation via PLC/DGK within minutes (supplemental Fig. S1B), as was shown for xylanase previously (24). Inhibition of SNAP-induced PA formation was shown upon preincubation with inhibitors of PLC and DGK (supplemental Fig. S1C). Accordingly, PLC and the DGK inhibitors reduced SNAP-induced cell death (data not shown). Hence, it appears as if NO is also sufficient to induce PA formation via PLC/DGK. However, the concentration of SNAP that is required for the induction of significant levels of PA is high, which makes confirmation of the direct effect of NO uncertain. Scavenging the SNAP-released NO by cPTIO is required to unequivocally show that exogenously applied NO activates PA formation. The concentration of cPTIO required for the effective scavenging of NO released by 1 mM SNAP is 10–20 mM (50, 69–74), which is toxic to tomato cell suspensions. Hence, such scavenging experiments cannot be performed. Two opposite explanations should therefore be made. On one hand, xylanase-induced NO might be produced locally upon perception of xylanase by the receptor located at the plasma membrane. Given the short half-life of NO, this will result in a high local concentration but a more moderate global concentration. To mimic the local NO concentration that occurs upon xylanase, high doses of SNAP must be applied to mimic xylanase-induced NO. On the other hand, such high concentration of SNAP exceeds the global concentration as induced by xylanase, which might very well induce other pleiotropic or indirect effects. We can therefore not exclude that PA production induced by SNAP-released NO does not reflect the actual events following
xylanase treatment. Hence, the results shown in supplemental Fig. S1 suggest that NO solely induces the formation of PA, although the concentration of SNAP required could induce pleiotropic effects.

All together, our results unravel a signaling pathway that occurs after pathogen perception. Briefly, xylanase is perceived by the cell, triggering a rapid NO production (Fig. 1). NO then induces a PLC/DGK-dependent PA formation (Figs. 2 and 3). Finally, PA induces ROS production probably via the activation of the NADPH oxidase (13–15). Another source of PA in xylanase-treated cells occurs via PLD activation. SNAP treatments did not result in a detectable increase of PLD activity (see supplemental Fig. S2), and cPTIO could not inhibit the xylanase-induced PLD activation (data not shown). Thus, PLD activation is proposed as a NO-independent pathway. Genetic approaches are required to unequivocally demonstrate the initial order of events following xylanase perception. NO-null mutants should be used to prove its suggested function. Unfortunately, the simplicity of possible NO sources make straightforward genetic approaches to this problem in tomato unfeasible (37). Our data, but also data by many other authors, suggest that PA is upstream of ROS production. A model about PA and ROS signaling in (a)biotic stress was recently proposed (18). We cannot, however, exclude that ROS might also act upstream of PA or even upstream of NO. There are reports that show that (i) H$_2$O$_2$ induces PA formation in rice cells (75), (ii) H$_2$O$_2$ is required for NO production in ABA-treated Arabidopsis guard cells (42), and (iii) H$_2$O$_2$ triggers NO production in mung bean (76) and tobacco cells (68). NADPH oxidase-null mutants in tomato, as used by Torres et al. (10, 11) in Arabidopsis, might show whether ROS act upstream of NO and PA formation during xylanase treatments. Another interesting issue results from the fact that NO and ROS are both oxidizing agents. We can envisage functional overlap, but as was shown for HR in Arabidopsis (7), it might also be that both reactive species are required either in concert or in independent signaling pathways with or without feedback regulation, as was suggested earlier (77). It will be a challenge to elucidate exactly how NO, PA, and ROS are interrelated in stress signaling.

Another compound that is related to NO, PA, and ROS is Ca$^{2+}$. PLC activation also results in the formation of IP$_3$ that, either directly or indirectly via its metabolized form IP$_6$, may result in the release of Ca$^{2+}$ from internal stores (78, 79). Inhibition of PLC does also slightly affect NO production (Fig. 4B), suggesting a possible feedback mediated by either PA or IP$_3$. Inhibition of DGK (Fig. 4B) or reduction of PLD-produced PA does not affect NO production (see supplemental Fig. S2), suggesting IP$_3$ and Ca$^{2+}$ to be responsible for this putative feedback. Concordantly, it was shown that NO is required for the mobilization of Ca$^{2+}$ from internal stores (4, 52). Downstream, Ca$^{2+}$ might affect PLC, DGK, PLD (23), and NADPH oxidase (12), therewith constituting a feedback toward PA generation via PLC/DGK regulation and PLD. In animals, PLC activity has been shown to be part of the NO-dependent machinery that controls Ca$^{2+}$ homeostasis via IP$_3$ (5). Given the incomplete inhibition levels observed, genetic evidence will be required to unequivocally show whether and how this feedback occurs.

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